# Strain improvement for enhanced erythritol production by *Moniliella pollinis* using cost-effective substrate

by

**Mr. Anil Balasaheb Khatape**

**AcSIR Registration No. 10BB18J26023**

A thesis submitted to the
Academy of Scientific & innovative Research
for the award of the degree of
DOCTOR OF PHILOSOPHY
in
SCIENCE

Under the supervision of

**Dr. Syed G. Dastager**
**(Research Supervisor)**

**Dr. Vidhya Rangaswamy**
**(Research Co-supervisor)**

**CSIR-National Chemical Laboratory, Pune**

Academy of Scientific and Innovative Research
AcSIR Headquarters, CSIR-HRDC Campus
Sector 19, Kamla Nehru Nagar,
Ghaziabad, U.P. -201 002, India

**December, 2023**

## Certificate

This is to certify that the work incorporated in this Ph.D. thesis entitled, "*Strain improvement for enhanced erythritol production by Moniliella pollinis using cost-effective substrate*", submitted by *Mr. Anil Balasaheb Khatape* to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of *Doctor of Philosophy in Science,* embodies original research work carried-out by the student. We, further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material(s) obtained from other source(s) and used in this research work has/have been duly acknowledged in the thesis. Image(s), illustration(s), figure(s), table(s) etc., used in the thesis from other source(s), have also been duly cited and acknowledged.

| (Signature of Student) | (Signature of Co-supervisor) | (Signature of Supervisor) |
|---|---|---|
| Mr. Anil Balasaheb Khatape | Dr. Vidhya Rangaswamy | Dr. Syed G. Dastager |
| Date: 14/12/2023 | Date: 14/12/2023 | Date:14/12/2023 |

## STATEMENTS OF ACADEMIC INTEGRITY

I, Mr. Anil Balasaheb Khatape, a Ph.D. student of the Academy of Scientific and Innovative Research (AcSIR) with Registration No. 10BB18J26023 hereby undertake that, the thesis entitled "Strain improvement for enhanced erythritol production by *Moniliella pollinis* using cost-effective substrate" has been prepared by me and that the document reports original work carried out by me and is free of any plagiarism in compliance with the UGC Regulations on *"Promotion of Academic Integrity and Prevention of Plagiarism in Higher Educational Institutions (2018)"* and the CSIR Guidelines for "*Ethics in Research and in Governance (2020)*".

**Signature of the Student**

Date : 14/12/2023

Place : Pune

---

It is hereby certified that the work done by the student, under my/our supervision, is plagiarism-free in accordance with the UGC Regulations on *"Promotion of Academic Integrity and Prevention of Plagiarism in Higher Educational Institutions (2018)"* and the CSIR Guidelines for "*Ethics in Research and in Governance (2020)*".

**Name of Co-Supervisor**

Name: Dr. Vidhya Rangaswamy

Date: 14/12/2023

Place: Pune

**Signature of the Supervisor**

Name : Dr. Syed G. Dastager

Date : 14/12/2023

Place : Pune

# Acknowledgement

In this journey of Ph.D., I have come across a number of people who helped and walked with me to achieve this ambition of my life. It is my privilege to express my sincere gratitude for all those people who contributed and supported me during this work. I am grateful to God for blessing me and providing me power, energy, and patience to overcome all obstacles and achieve my goal.

First and foremost, I am grateful to my supervisor, Dr. Syed G. Dastager, for giving me the opportunity to work at this renowned institution. Throughout this journey, you have been an invaluable source of inspiration, providing valuable insights and constructive feedback that have significantly enhanced the quality and depth of this work.

I would like to express my heartfelt gratitude to my co-supervisor, Dr. Vidhya Rangaswamy. Your unwavering support, guidance, and expertise have played a pivotal role in shaping this research endeavour.

I would like to express my heartfelt gratitude to Dr. Sanjay Kamble, Dr. Mahesh Dharne, and Dr. Koteswara Rao for their constructive criticism, suggestions, and timely evaluation of my thesis work being members of the Doctoral Advisory Committee (DAC).

My heartwarming thanks to my labmates at NCL, Dr. Hari, Dr. Amit, Jini, Karthika, Vibha, Rachel, Sayali, Snehal, Pradeep, Dr. Ekramul, Amrapali, Vaishnavi and Sejal for always being helpful during my work at, NCL. Special thanks to Dr. Sneh, Dr. Mukesh, and Sambhaji for their cooperation regarding DOE and Fermenter-related work, and to Dr. Wasu, Dr. Bharat, Junaid, Vinay, Rakesh Pranav, and Aashish for their valuable inputs during course work presentations and while working at the NCL lab.

I am fortunate to have good colleagues at RIL lab who are continuously supporting, inspiring, and cheering me on in all situations. I would like to thank Dr. Shrikant, Dr. B.

Sivaraman, Harshvardhan, Dr. Piyush, Harikrishna, Dr. Jasmine, Dr. Santosh, and Dharmendra. I am truly thankful to the NCIM staff members Mrs. Sandhya Sudge, Mrs. Shivani Chaudhari, Mrs. Shalaka Gaikaiwari, Mrs. Ratnamala Vasave, Mrs. Meeta Patil, Dr. S. Kotwal, Mr. Ambadas, Kaustubh, Manoj, Shinde mama, Sagar, Mrs. Vrushali, and Bhavna. Thank you all for your cooperation and care during this work.

I would also like to acknowledge the Head of the NCIM Resource Centre, Division of Biochemical Science, and the Director of the CSIR-National Chemical Laboratory for providing all research facilities. I extend my heartfelt thanks to the High-Value Chemicals group at RTG, Reliance Industries Limited, Navi Mumbai, for generously providing the necessary infrastructure for conducting all experimental work and granting permission to pursue my doctoral research.

My gratitude is immeasurable for my family's sacrifices and patience, especially to my parents for their unwavering support, blessings, and well wishes. This thesis is dedicated to my parents, my wife, my in-laws, and other cherished family members whose unwavering support has been the cornerstone of my strength and determination throughout this academic pursuit. I would like to express my deep respect to my wife Snehal for her endless love, patience, emotional support, and cooperation. I extend heartfelt gratitude to my daughter, Shravika, whose arrival in the midst of my PhD journey has brought boundless joy and happiness into my life. I also want to thank my brother Sunil for always being there for me and cheering me up at the bad times.

Finally, I would like to give a big thanks to all those who helped me finish my Ph.D., either directly or indirectly. I apologize if I didn't mention anyone by name.

With heartfelt thanks

**Anil Khatape**

*This Thesis is dedicated to*

*My dear family*

*My parents,*

*My wife & My daughter*

# Table of contents

# List of Content

## Chapter 2. Screening of yeast cultures and optimization of culture conditions for erythritol production



## Chapter 3. Strain improvement using physical and chemical mutagenesis

## Chapter 6. Summary and Future perspectives

# List of Figures

**Chapter 5.**

# List of Tables

# Abbreviations

| | |
|---|---|
| AKR | Aldose ketose reductase |
| ALR | Aldose reductase |
| ANOVA | Analysis of Variance |
| °C | Degree centigrade |
| CBS | Central Bureau of Fungal Cultures |
| CCRD | Central composite rotatable design |
| DCW | Dry cell weight |
| DO | Dissolved oxygen |
| DoE | Design of Experiments |
| DTT | Dithiothreitol |
| EMS | Ethyl methyl sulphonate |
| ER | Erythrose reductase |
| FDH | Formate dehydrogenase |
| FDH | formate dehydrogenase |
| g | Gram |
| GRAS | Generally regarded as safe |
| h | Hour |
| HPLC | High performance liquid chromatography |
| Km | Michaelis constant (mM) |
| L | Liter |
| LDH | Lactate dehydrogenase |
| LFM | Liquid fermentation medium |
| m | Meter |

| | |
|---|---|
| min | Minute |
| MTCC | Microbial Type Culture Collection |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NCIM | National Collection of Industrial Microorganisms |
| NTG | N-methyl N-nitro N-nitroso guanidine |
| OD | Optical density |
| PMSF | Phenylmethanesulfonylfluoride |
| PPP | Pentose phosphate pathway |
| $R^2$ | Regression coefficient |
| RPM | Rotations per minute |
| RSM | Response Surface Methodology |
| sec | Second |
| SEM | Scanning electron microscopy |
| T | Temperature |
| TCA cycle | Tricarboxylic acid cycle |
| US FDA | United States Food and Drug Administration |
| UV | Ultra violate |
| v/v | Volume by volume |
| Vmax | Maximum reaction velocity |
| VVM | Volume per volume per minute |
| w/v | Weight by volume |
| w/w | Weight by weight |
| YPG | Yeast extract Peptone Glucose |

**Chapter. 1:**

# Introduction

**Abstract:**

Erythritol is a four-carbon polyol produced with the aid of microbes in the presence of hyper-osmotic stress. It is the most effective sugar alcohol that is produced predominantly by the fermentation. In comparison to various polyols, erythritol has many precise functions and is used as a flavour enhancer, sequestrant, humectant, nutritive sweetener, stabilizer, formulation aid, thickener and a texturizer. Erythritol production is a common trait in a number of the yeast genera viz., *Trigonopsis, Candida, Pichia, Moniliella, Yarrowia, Pseudozyma, Trichosporonoides, Aureobasidium* and *Trichoderma*. Extensive work has been carried out on the biological production of erythritol through *Yarrowia, Moniliella, Candida* and other yeast strains. Many strategies which have been used to improve the erythritol productivity through mutagenesis and genetic engineering are discussed. In this chapter, an overview on erythritol is presented. A literature survey on their physicochemical properties, functions, applications, methods of production and strategies used for enhanced biotechnological production are summarized.

## 1.1 Introduction

World Health Organization (WHO) information in 2016 on obesity declared that over 1.9 billion world's adult population is diagnosed to be overweight, and over 650 million have been obese. And also, 41 million kids were overweight worldwide (https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight). The contributing component to weight gain and lifetime physiological disorders includes obesity, heart infection, and diabetes is consumption of a diet that is high in added sugars, such as baked items, candy, sweetened beverages, and sugary cereals. Sweeteners that offer very less calories are known as non-nutritive sweeteners (Fig. 1.1) and have a poor glycemic response (Pepino, 2015). All artificial sweeteners fall under the category of artificial sweeteners without nutritional value. These substances provide a sweet taste without increasing blood sugar levels, making them advantageous for weight management. The United States Food and Drug Administration (US FDA) has authorized six artificial sweeteners inclusive of acesulfame K, aspartame, saccharin, sucralose, neotame, and stevioside (Kroger et al., 2006).

A large share of the non-nutrient food market is composed of sugar alcohols which are also known as polyols (Regnat et al., 2018). Polyols are carbohydrates characterized by reduction of the carbonyl group to either a primary or secondary hydroxyl group. Polyols are required for various functions involved in efficient carbon fixation, growth, carbon storage, and reductant recycling (Radeloff & Beck, 2013). Polyols are used as nutraceuticals with value-added properties and functional foods. At present, sorbitol, mannitol, xylitol, lactitol, maltitol, and erythritol are polyols that can be accessible commercially. The polyols are sweet and have an energy content that is approximately half that of sucrose. Though polyols can be found innately in foods, the amounts are insufficient for commercial exploitation. Hence, several of these are being produced using chemical or biotechnological approaches for commercial applications. Owing to the fact that polyols have no or little impact on glycemia, they may be a sugar

replacement for diabetics (Wölnerhanssen et al., 2016). However, the intake of polyols isn't limitless as excessive intake can cause diarrhea and symptoms including flatus, distention, and belly rumble (Oku & Nakamura, 2007). Chemical structures of commercially available polyols and non-nutritive sweeteners are presented in Fig. 1.1.

Among the various diverse polyols in the market, erythritol exhibits very unique capabilities. The caloric value of erythritol is zero, and its safe dosage is both well-tolerated and significantly higher than that of other polyols. The calories contributed per gram of different polyols and their approximate sweetness in relation to sucrose is presented in Table 1.1. However, because of its rather excessive price, the use of this sweetener isn't very widespread and is less than other polyols, despite having many benefits. Nonetheless, because of the rising need in the food sector, erythritol manufacturing by bio-based methods is gaining increasing significance. This chapter explores the present state of erythritol production and its use, with importance on the strain improvement strategies which include genetic engineering and mutagenesis in yeast for enhanced erythritol production in the industrial environment. Demand for high-intensity sweeteners has resulted in a stable development over the past five years and is anticipated to develop at a higher rate in the following five forecast years.

The market is driven by fitness consciousness and changes in lifestyle among consumers of emerging nations. Some key manufacturers of erythritol, operational across the value chain in the market, are Foodchem International Enterprise, Cargill Inc, Jungbunzlauer, and Shandong Sanyuan Biotechnology Co., Ltd (Erythritol marketplace - global enterprise evaluation 2015-2020 and possibility price 2020 - 2030).

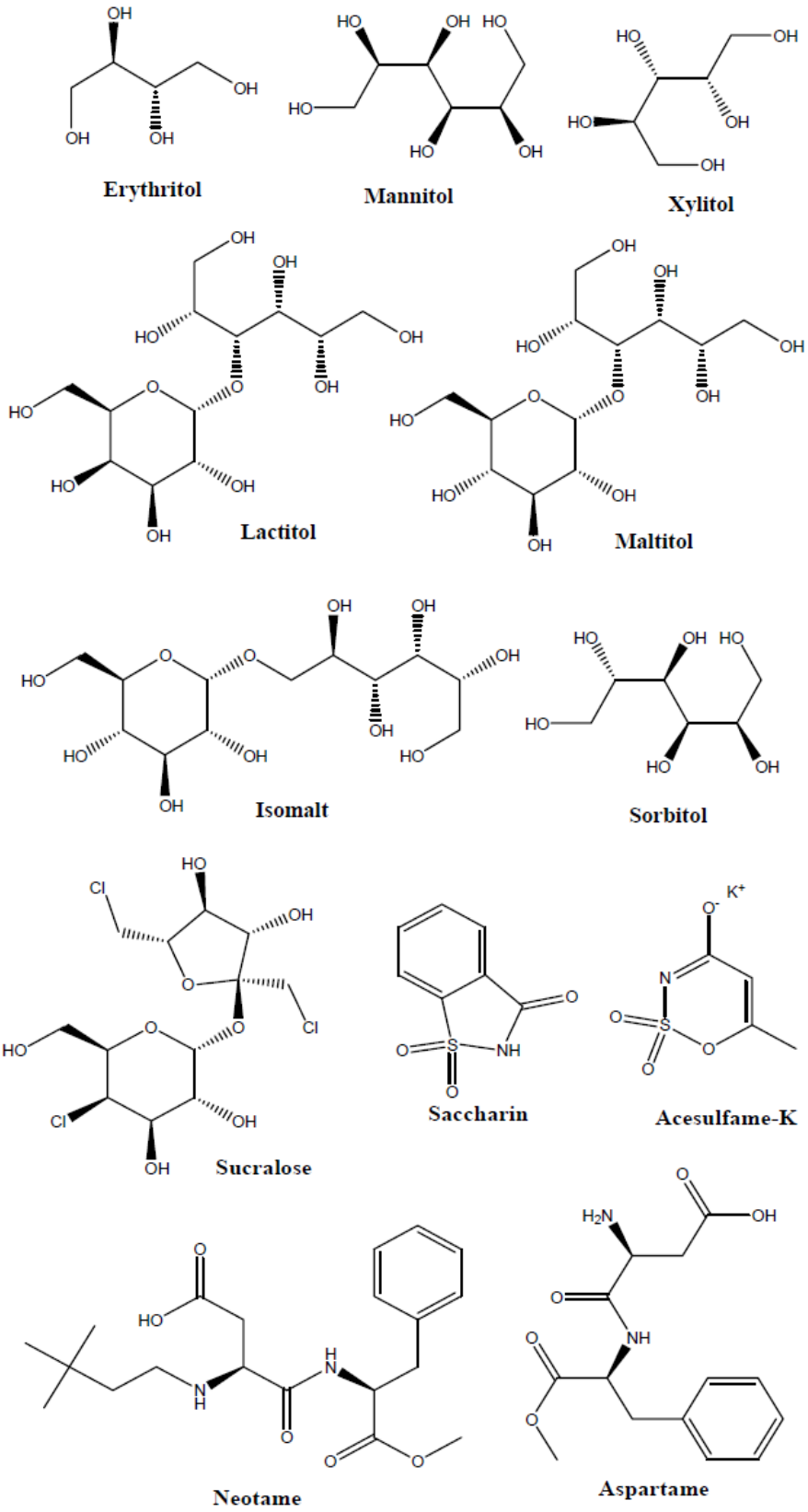



**Figure 1.1** Chemical structures of polyols and artificial non-nutritive sweeteners

## 1.2 Erythritol

Erythritol is a polyol with four carbon atoms, extensively widespread in the environment. Its chemical name is 1, 2, 3, 4-butanetetrol or meso-erythritol ($C_4H_{10}O_4$) (Krajangsod et al., 2018). It has been isolated from fruits (like pears, grapes, melons), mushrooms, alcoholic liquids (like beer, wine, sake), and fermented meals (like soy sauce, miso bean paste) (Yoshida & Sugahara, 1984; Shindou et al., 1988; Bernt et al., 1996). It is found in the biological fluids of humans and animals which include eye lens tissue, serum, plasma, fetal fluid, and urine (Horning et al., 1974; Roberts et al., 1976; Niwa et al., 1993). Erythritol is a symmetrical molecule, occurring as a white anhydrous crystalline powder having 70% of the relative sweetness of sucrose. Due to the negative heat of the solution, it provides a pleasing mouth feel after consumption. A unique characteristic of erythritol is its quick uptake in the proximal intestine followed by elimination through urine. (Lenhart & Chey, 2017). As the bulk of ingested erythritol is rapidly expelled, it is regarded as a zero-calorie sweetener (EU Commission directive 2008/100/EC). Therefore, erythritol does not have any effect on glycemia and is non-calorific (Hiele et al., 1993). At usual consumption levels, erythritol does not have any digestive distress, in contrast to other polyols that may have a laxative effect (Livesey, 2001). The study completed to date such as metabolic, toxicological, and medical effects on animals as well as humans has established that erythritol is safe for intake even if consumed in relatively higher amounts (approximately 0.7 mg/kg body weight/day for a 60-kg individual) (Munro et al., 1998).

Because of the low insulin index, erythritol has excellent potential as a perfect sugar alternative for people with diabetes (Livesey, 2003). Erythritol was initially introduced to the market commercially in Japan during the early 1990s. European Food Safety Regulatory Authority approved for the utilization of erythritol in beverages with no alcohol and a GRAS (generally regarded as safe) status was issued by US FDA in 2001. In 2003, the Scientific Committee on

Food (SCF) of the European Union decided that erythritol is safe for use in foods, and it was assigned the code E986.

**Table 1.1** The calories contributed per gram and the approximate sweetness compared to sucrose of different polyols (Adapted from International Food Information Council website www.foodinsight.org)

| **Sugar Alcohol** | **Calories/ gm (Sucrose = 4)** | **Approximate Sweetness (Sucrose = 100%)** |
|---|---|---|
| Sorbitol | 2.6 | 50-70% |
| Xylitol | 2.4 | 100% |
| Maltitol | 2.1 | 75% |
| Isomalt | 2.0 | 45-65% |
| Arabitol | 2.0 | 70% |
| Lactitol | 2.0 | 30-40% |
| Mannitol | 1.6 | 50-70% |
| Erythritol | 0.2 | 60-80% |

Erythritol was accepted in the EU as safe for consumption in 2006 (EU commission directive 2006/52/EC). Due to a disagreement about its laxative effect, the approval from the European Union did not extend to the use of erythritol in beverages. These constraints were revised in 2015 when the SCF projected an extreme level of 1.6% erythritol in non-alcoholic beverages ("Scientific Opinion on the Safety of the Proposed Extension of Use of Erythritol (E 968) as a Food Additive," 2015).

### 1.3 Production of erythritol

Erythritol can be formed through several chemical and biotechnological methods. From a chemical perspective, the synthesis of erythritol involves employing a nickel catalyst-driven method, utilizing dialdehyde starch, and subjecting it to elevated temperatures. (Otey et al., 1961). In an alternative approach, threitol and erythritol are formed through the hydrogenation of a dialkyl ester of tartaric acid in a solution of methanol or ethanol. The essential factors in methods based on chemistry involve elevated pressure (20 MPa) and temperature (125-200°C) (Trenner NR, 1951), and due to their low efficiency, these processes are no longer industrially used. Extraction of erythritol from vegetables and fruits is not an economically viable method even though it is present in them. In contrast, biosynthetic routes offer an environmentally friendly and probably the best erythritol synthesis process. At present, erythritol is produced commercially by fermentation strategies by using fungal strains. Various genera of yeast-like *Aureobasidium, Candida, Moniliella, Torula, Trichosporon, Trichosporonoides,* and *Yarrowia* are known to produce erythritol (Ishizuka et al., 1989; J. Park et al., 1998; Oh et al., 2001; Lin et al., 2010; Savergave et al., 2011; Tomaszewska, Rywińska, et al., 2014). The inability to implement erythritol production by *Pichia, Zygopichia, Candida, Torulopsis,* and *Trigonopsis* on a commercial scale is attributed to factors such as lower yield, productivity, and the formation of byproducts, including glycerol and ribitol. (Hirata et al., 1999).

### 1.4 Physicochemical properties of erythritol

Erythritol exhibits symmetry as a molecule, existing solely in the meso-form. Anhydrous crystals of erythritol possess a melting point of 122°C. Its lack of reducing end-groups contributes to exceptional heat and pH stability. In comparison to other polyols commonly employed as sugar substitutes, erythritol stands out with the lowest molecular weight of 122.12 g/mol. This characteristic imparts distinct properties, including higher

osmotic pressure and lower water activity in solution. Refer to Table 1.2 for technical data on erythritol.

**Table 1.2** Technical data on erythritol

| | |
|---|---|
| Systematic name | 1,2,3,4-Butanetetrol |
| C.A.S. number | 149-32-6 |
| Formula weight | 122.12 g/mol |
| Chemical formula | $C_4H_{10}O_4$ |
| Melting range | 119-123 ºC |
| Density | 1.45 g/cm$^3$ |
| Solubility | Freely soluble in water, slightly soluble in ethanol, insoluble in diethyl ether |

### 1.5 History

Stodola (1946), was the pioneer in documenting erythritol biosynthesis within *Aspergillus terreus*. Erythritol was effectively extracted from the ether extract of the concentrated culture broth on its limited solubility in methanol. The lack of a positive Fehling's test for reducing sugars suggested the polyhydric alcohol nature of the compound. The compound's identity as meso-erythritol was confirmed through melting point analysis, coupled with a comparison of X-ray diffraction patterns between samples from accepted and genuine specimens. Subsequently, trace amounts of erythritol were identified in the residue of fermented molasses, indicating yeast as a potential producer (Binkley & Wolfrom, 1950). This path was further explored, investigating the capacity of various osmophilic yeast to yield polyhydric alcohols, including glycerol, arabitol, erythritol, and mannitol in oxic environments (Spencer & Spencer, 1978). Peterson et al., (1958) extended this inquiry by examining osmophilic yeasts from various sources, such as brood comb honey, brood comb pollen, fresh pollen, and clover heads

near apiaries, for polyhydric alcohol production. Later, Hajny et al., (1964) dedicated their research to isolating osmophilic yeasts capable of exclusive erythritol production. Among these yeasts, a Torula-like fungus was identified, exhibiting erythritol yields of 35 to 40% of the consumed sugar. However, unfavorable fermentation conditions were shown to favor glycerol production at the expense of erythritol (Hajny et al., 1964). The utilization of a synthetic medium revealed that maintaining precise control over nitrogen and phosphorus concentrations in the medium was crucial for achieving adequate erythritol production.

Numerous scientists have also confirmed the presence of erythritol as a metabolite in seaweeds, as well as its existence in various fruits like melons, grapes, pears, and fermented products such as soy sauce, wines, and beers (Yoshida & Sugahara, 1984; Shindou et al., 1988; Goossens & Roper, 1994). The focus on screening and isolating microorganisms with high erythritol production capabilities gained momentum following the discovery (Hattori & Suzuki, 1974) during their studies on citric acid fermentation by *Candida zeylanoides*, where erythritol accumulation was observed as the medium pH dropped below 4.0. This initiated a broader interest in the exploration of microorganisms with enhanced erythritol production potential. (Table 1.3).

### 1.6 Erythritol biosynthetic pathway in yeast

The yeast strains of genera viz., *Yarrowia, Moniliella, Debaryomyces, Zygosaccharomyces, Hansenula,* and *Pichia* are capable of growing in an atmosphere of elevated salt or sugar levels. When these yeast strains encounter salt or osmotic stress, they accumulate compatible solutes. These solutes guard and support the enzymes, allowing cellular activities in osmotic surroundings. Among the yeasts, glycerol stands out as a predominant osmolyte; however, sugar alcohols such as erythritol, D-arabitol, and mannitol may also function as osmolytes. Other than serving as an osmolyte, these sugar alcohols could also play a vital element in

balancing the redox potential of the cells or as a storage compound (Brown, 1978; Kayingo et al., 2001). In yeast, the synthesis of erythritol is a multistep metabolic procedure, which operates commonly via the pentose phosphate pathway (PPP). In yeasts and other eukaryotic organisms, the role of the PPP is to yield NAD(P)H for cellular reactions and also to offer precursors for nucleotide and amino acid biosynthesis which includes D-ribose 5-phosphate and erythrose 4-phosphate (Nelson & Cox, 2017).

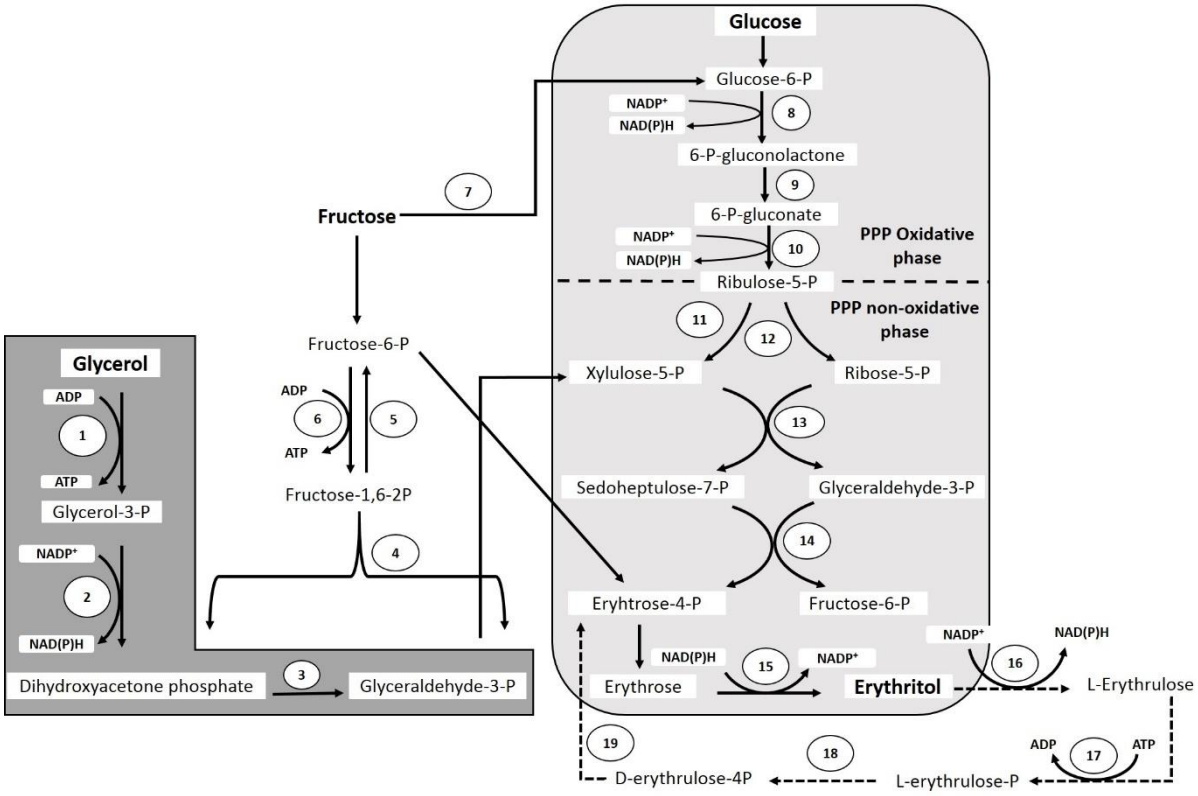



**Figure 1.2** Erythritol synthesis in yeast using glycerol, fructose, or glucose as carbon source. (Proposed catabolic pathway in *Y. lipolytica* (Mironczuk et al. 2017, Carly et al. 2018, Niang et al. 2020, Zhang et al. 2021) shown in dotted arrows) (1. Glycerol kinase, 2. Glycerol 3 phosphate dehydrogenase, 3. Triosephosphate isomerase, 4. Aldolase, 5. Fructose 1,6 bisphosphatase, 6.Phosphofructokinase, 7. Glucose-6-phosphate isomerase, 8. Glucose-6-phosphate dehydrogenase, 9. Gluconolactonase, 10. 6-phosphogluconate dehydrogenase, 11. Ribulose-5-phosphate 3-epimerase, 12.Ribulose-5-phosphate isomerase, 13. Transketolase, 14. Transaldolase, 15. Erythrose reductase, 16. Erythritol dehydrogenase, 17. L-erythrulose kinase, 18. D-erythrulose kinase, 19. D-rythrose-4P isomerase)

This biochemical route includes two different stages. NAD(P)H and ribulose-5P result from the initial oxidative stage, and erythrose-4P is the ultimate product of the non-oxidative phase.

When *Moniliella megachiliensis* was grown in glucose for erythritol synthesis, elevated activity of PPP important enzymes (glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, transketolase, and transaldolase) was observed (Sawada et al., 2009). Dephosphorylation of erythrose-4P to erythrose and finally reduction to erythritol with the utilization of one molecule of NAD(P)H constitute the final steps in the suggested pathway (D. H. Lee et al., 2010a Fig.1.2). As per the stoichiometry of erythritol production in microorganisms, one mole of glucose is converted into one mole of erythritol (Equation 1) (J. Park et al., 1998)

(1) Glucose + ATP $\xrightarrow{\text{Erythrose reductase}}$ Erythritol + $NADP^+$

Erythrose reductase (ER), a NAD(P) H dependent aldose reductase, is a crucial enzyme in the biological synthesis of erythritol (J. K. Lee et al., 2003). ER enzyme has been detected and reported in some erythritol-synthesizing yeast strains including *C. magnoliae* (D. H. Lee et al., 2010)**,** *M. megachiliensis* (Kobayashi et al., 2013), *Torula sp.* (J. K. Lee et al., 2002), and *Y. lipolytica* (Cheng et al., 2018; Szczepańczyk et al., 2021). *C. magnoliae* JH110 forms a considerable quantity of erythritol, signifying the occurrence of erythritol-synthesizing enzymes. Lee *et al*. (2010), studied the genetic code and functional traits of a unique NAD(P)H-dependent ER from *C. magnoliae* JH110 for the first time (Fig. 1.3). Purified ER was extensively inhibited non-competitively with the aid of increasing fumarate concentration

(Kobayashi et al., 2013). The gene responsible for the penultimate step in erythritol synthesis, specifically erythrose-4-phosphate dephosphorylation, has not been identified in any fungal species so far.

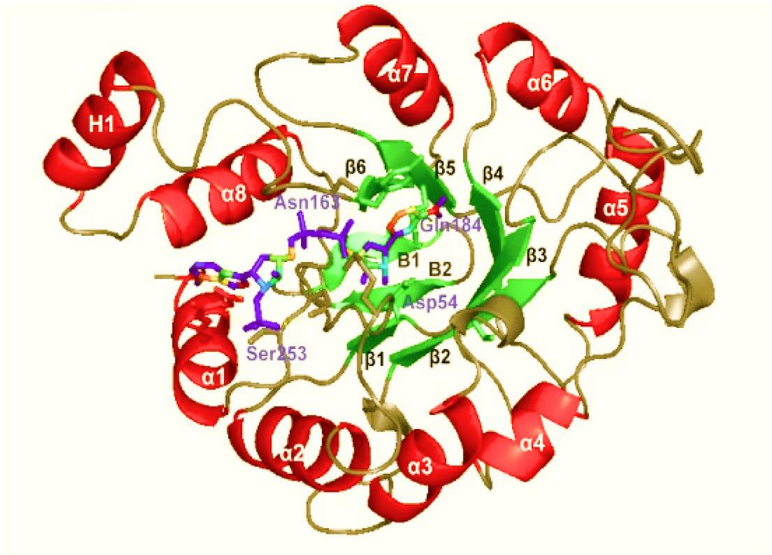



**Figure 1.3** Crystal structure of erythrose reductase characterized from *C. mangoliae*

Apart from this anabolic route, some yeast cultures also possess the capability to utilize erythritol as a carbon source. Within the erythritol catabolic route, it is initially oxidized into erythrulose by an erythritol dehydrogenase (EYD) enzyme and then phosphorylated into erythrulose-phosphate through an erythrulose kinase (EYK) (Carly et al., 2017a). The genes associated with these enzymes were recently discovered in *Y. lipolytica* (*YALI0F01650g* and *YALI0F01606g*, respectively), and it was observed that their expressions are induced by erythritol (Carly et al., 2018). *Y. lipolytica* is capable of both the synthesis (Rymowicz et al., 2009) and utilization of erythritol (Niang et al., 2020). It can produce erythritol from glycerol as well as assimilate it as a carbon source. This leads to a reduction in the productivity of polyol and signifies a major disadvantage in an effective erythritol production. The disruption of one

of the genes identified, *EYK1*, coding for an erythrulose kinase enzyme, led to 26 % (0.49 ± 0.02 g/g) higher erythritol yield and a 30 % higher specific productivity (0.052 ± 0.005 g/g) than the wild-type strain (Carly et al., 2017a).

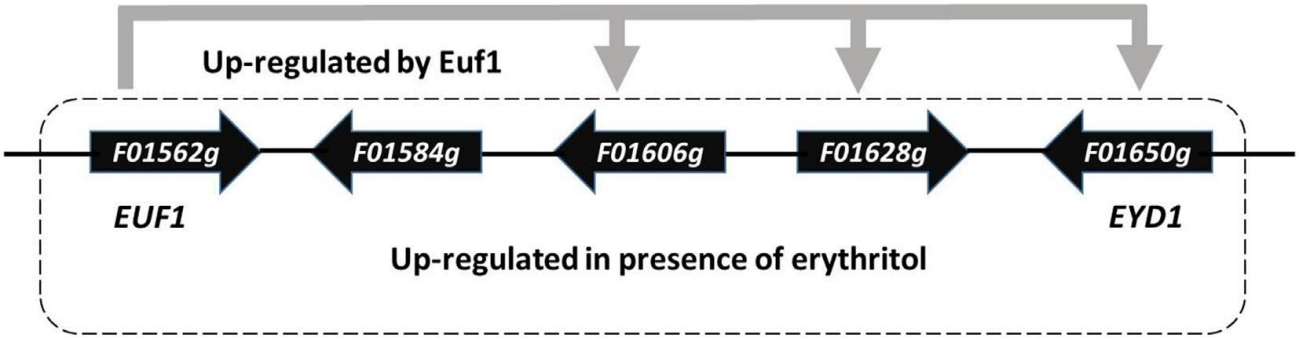



**Figure 1.4** Cluster of genes involved in erythritol utilization located on chromosome in *Y. lipolytica* CLIB122 genome (Adapted and modified from (Rzechonek et al., 2020a)

In a separate investigation, the analysis of the YALI0F01562g gene sequence uncovered the presence of domains typical of transcription factors. (Rzechonek et al., 2017). The authors have named this gene as erythritol utilization factor – EUF1. There are 4 enzymes identified in erythritol consumption in *Y. lipolytica* viz., erythritol dehydrogenase *Eyd1* (Carly et al., 2018), kinase *Eyk1* (Carly et al., 2017b), and isomerase, *Eyi1 and Eyi2* (Mirończuk et al., 2018). It was found that the regulation of genes encoding these proteins involves the aid of the transcription component EUF1 (Rzechonek et al., 2017). All of these, including EUF1, are organized in a cluster within the genome known as the erythritol utilization cluster (Fig. 1.4) (Rzechonek et al., 2020). Additionally, two new genes, *YALI0F01584g*, and *YALI0F1628g*, participating in erythritol catabolism were identified, which refers to a putative isomerase located in the same region as EUF1. Experimental results showed that *Y. lipolytica* displayed a noteworthy surge in *YALI0F01628g* expression in cell development using erythritol. Furthermore, the strain showed the deletion of *YALI0F01628g* considerably lessened erythritol utilization, while the erythritol yield remained unchanged (Mirończuk et al., 2018).

### 1.7 Substrates used for erythritol production

The utilization of disaccharides or complex sugars could be limited by the potential for hydrolysis of O-glycosidic bonds by different yeast strains. Apart from the most commonly used substrate glucose, other sugar substrates like fructose and sucrose can also be used for erythritol production. Interesting observations were made in yeast strain *C. magnoliae* which showed a choice for fructose as a carbon substrate over glucose (Yu et al., 2006).

Among other substrates, glycerol was found to be a good carbon source for erythritol synthesis, as observed in *Y. lipolytica* (Rymowicz et al., 2009). The glycerol assimilation proceeds through its phosphorylation by glycerol kinase (GK) to glycerol-3-phosphate, which is subsequently transformed to dihydroxyacetone phosphate by NAD+-glycerol-3-phosphate dehydrogenase (GPDH) (Fig. 1). Several strains of *Y. lipolytica* also possess a FAD- GPDH enzyme for the dehydration of glycerol phosphate to dihydroxyacetone phosphate. The enzyme activity of GK and GPDH through erythritol production using glycerol in *Y. lipolytica* Wratislavia K1 varied depending on NaCl concentration and pH values in the culture medium (Tomaszewska, et al., 2014). Raw glycerol was also exploited for erythritol formation by *Y. lipolytica* (Rymowicz et al., 2009) and *M. megachiliensis* (Kobayashi et al., 2015), achieving yields as significant as 0.56 g/g and 0.60 g/g, respectively. Higher erythritol yields on non-refined glycerol compared to purified glycerol indicates that impurities (NaCl, matter organic non-glycerol (MONG), methanol, metals like K, Na, Mg, Cu, Fe, Zn 2.0 and Ca) in the raw glycerol might have a positive effect on erythritol synthesis. Thus, raw glycerol could be a cost-effective alternative to glucose in erythritol production as demonstrated in various reports (Tomaszewska, Rywińska, et al., 2014; Mirończuk, Dobrowolski, et al., 2015; Carly et al., 2017b; Szczepańczyk et al., 2021).

Apart from glucose and glycerol, another study reported a five-time upsurge in erythritol synthesis from xylose as compared to glucose using *Y. lipolytica* (Sivaraman et al. 2016).

Sugarcane juice has also been used by *M. pollinis* (Deshpande et al., 2022) as a carbon substrate for erythritol production yielding a titer of 94.9 g/L with a 38 % erythritol yield.

### 1.8 Commercial production of erythritol

There are some establishments worldwide, manufacturing erythritol commercially, including Bolak Corporation (Seoul, South Korea), Cargill Food and Pharma Specialties (Minneapolis, MN), Baolingbao Biotechnology (Dezhou Shi, China), O'Laughlin Corporation (Tianjin, China), Jungbunzlauer (Basel, Switzerland), and Mitsubishi Chemical Corporation (Tokyo, Japan). Industrail-scale production of erythritol is mainly from glucose, sourced either chemically or from enzymatically hydrolyzed corn or wheat and using yeast strains viz., *Moniliella pollinis, Aureobasidium* sp., and *Torula* sp (Kim et al., 2000). Refinement of the process resulted in enhanced productivity and increased concentrations of erythritol (Table 1.3).

**Table 1.3** Erythritol production by yeast cultures using various carbon sources

| Yeast strain | Strain modification | Carbon source | Culture system | Yield % | Productivity g/L/h | Reference |
|---|---|---|---|---|---|---|
| *Trichosporon sp.* | - | Glucose | Batch | 47 | 1.96 | (Park et al. 1998b) |
| *Aureobasidium* sp. Mutant SN-124A | NTG + UV mutation | Glucose | Batch | 37.7 | 1.82 | (Ishizuka et al., 1989) |
| *C. magnoliae* | EMS mutation | Glucose | Two-stage | 41 | 2.8 | (Ryu et al., 2000) |
| *Torula* sp. | - | Glucose | Fed-batch | 48 | 2.26 | (Oh et al., 2001) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Moniliella tomentosa var. pollinis* | - | Glucose | Fed-batch | 40 | 1.61 | (Burschäpers et al., 2002) |
| *C. magnoliae* | EMS mutation | Glucose | Fed-batch | 43 | 1.2 | (Koh et al., 2003) |
| *Pseudozyma tsukubaensis* | - | Glucose | Batch | 61 | 1.65 | (Jeya et al., 2009) |
| | - | Glucose | Fed-batch | 60 | 2.84 | (Jeya et al., 2009) |
| *M. megachiliensis* sp. 440 mutants (mutant N61188-12) | NTG mutation | Glucose | Batch | 43 | 0.65 | (Lin et al., 2010) |
| *C. magnoliae* | UV, Chemical mutation | Glucose | Fed-batch | 36 | 0.52 | (Savergave et al., 2011) |
| *Y. lipolytica* CICC 1675 | - | Pure glycerol | Fed-batch | n.a. | 0.95 | (Yang et al., 2014) |
| *Y. lipolytica* K1 | - | Crude glycerol | Repeated batch culture | 56 | 0.3 | (Mirończuk et al., 2014) |
| *Y. lipolytica* | *SUC2* | Glycerol, Molasses | Batch | 26-57 | 0.58–1.04 | (Mirończuk, Rakicka, et al., 2015) |
| *Candida sorbosivorans* | - | Glucose | Batch | 38 | 0.50 | (Saran et al., 2015) |
| *Aureobasidium pullulans* | UV mutation | Xylose | Batch | 26 | 0.22 | (Guo et al., 2016) |
| *Trichosporonoides oedocephalis* | *Δhog1* | Glucose | Shake flask | 35 | 0.58 | (Li et al., 2016) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Fed-batch | | | |
| *Y. lipolytica* A101 | *GUT1, GUT2* | Glycerol | Batch | 43 | 1.08 | (Mirończuk et al., 2016) |
| *Y. lipolytica* | *SUC2, GUT1* | Glycerol, Molasses | Batch | 55 | 0.87 | (Rakicka et al., 2017) |
| *Y. lipolytica* MK1 | *TKL1* | Glycerol | Batch | 58 | 0.81 | (Mirończuk et al., 2017) |
| *Y. lipolytica* yliUA8 | UV and ARTP mutagenesis | Glucose | Batch culture | 53 | 1.55 | (Qiu et al., 2020) |
| *Y. lipolytica* | *ER(YALI0B07117g)* | Glycerol | Batch | 40 | 0.41 | (Szczepańczyk et al., 2021) |
| *Y. lipolytica* yliUA8 | UV and ARTP mutagenesis | Glucose | Batch culture | 53 | 1.55 | (Qiu et al., 2020) |
| *Y. lipolytica MY11* | *GUT1, TPI1, TKL1, TAL1; Δeyk1* | Glycerol | Shake flask Batch | 20 | 0.18 | (Zhang et al., 2021) |
| *Y. lipolytica MY12* | *RK11* | | | 52 | 0.24 | |
| *Y. lipolytica MY13* | *AMPD* | | | 46 | 0.21 | |
| *M.pollinis* | | Sugarcane juice | Fed-batch | 38 | 0.61 | (Deshpande et al., 2022) |

New biotechnological methods, mainly genetic modifications, in yeast strains are still being established and improved to further increase erythritol productivity, which will be discussed in the following sections.

### 1.8.1 *Yarrowia species*

*Yarrowia lipolytica* is one of the well-researched yeast strains for erythritol production. *Y. lipolytica* can utilize raw glycerol as an alternative to glucose as its primary carbon substrate and is highly capable of producing erythritol using glycerol (Rymowicz et al., 2009; Tomaszewska et al., 2014a, Kobayashi et al., 2015). In *Y. lipolytica,* two genes *GUT1* and *GUT2* encoding for GK and GPDH respectively, are involved in glycerol assimilation (Makri et al., 2010) and have been overexpressed in *Y. lipolytica* A101 (Mirończuk et al., 2016). The researchers have used a hybrid promoter containing sixteen upstream activating sequences enhancing the expression of the Translational Elongation Factor (TEF) promoter. Three strains were generated through the overexpression of both GK and GPDH or simultaneous expression of both enzymes. Erythritol productivity was enhanced by 24% while GK was overexpressed, whereas overexpression of GPDH did not have any impact. However, co-expression of both the genes improved erythritol productivity by 35% over the control. The overexpressed strain showed 1.08 g/L/h erythritol productivity from glycerol as opposed to 0.80 g/L/h in the parent strain (Mirończuk et al., 2016).

In a new approach for exploring the use of sucrose in molasses as substrate, the *SUC2* gene of *Saccharomyces cerevisiae,* coding for invertase (Nicaud et al., 1989; Xu et al., 2014), was overexpressed in *Y. lipolytica* Wratislavia K1 along with overexpression of the native *GUT1* gene (Rakicka et al., 2017). The *SUC2* gene was inserted inside the *ura* locus under the TEF promoter and CYC1 terminator (Blazeck et al., 2011) creating the strain *Y. lipolytica* AIB. Subsequently, the *GUT1* overexpressing cassette was introduced, leading to the creation of the

yeast strain AIB pAD-UTGut1. (Mirończuk et al. 2016). The engineered strain generated by this method was able to assimilate both sucrose and glycerol. This yeast culture synthesized 82.2 ± 3.0 g/L of erythritol with a yield of 0.55 ± 0.1 g/g and productivity of 0.87 ± 0.5 g/L/h. The first study that reports efficient polyol production by the modified *Y. lipolytica* strain from industrial raw molasses and crude glycerol was published by Mironczuk *et al*. (2015). In that study, researchers were able to produce 52–114 g/L of erythritol with 0.58–1.04 g/L/h productivity and 0.26–0.57 g/g of yield (Mirończuk et al., 2015).

In another study to assess their role in erythritol biosynthesis, transketolase (*TKL1*, *YALI0E06479g*), transaldolase (*TAL1*, *YALI0F15587g*), NADP+- dependent 6-phosphogluconate dehydrogenase (*GND1*, *YALI0B15598g*), and NADP+- dependent glucose-6-phosphate dehydrogenase (*ZWF1*, *YALI0E22649g*) genes of PPP, accountable for the formation of reducing agents in the cell were overexpressed. The study indicated that depending on the type of carbon source, erythritol levels produced by this engineered strain *Y. lipolytica* MK1 varied with maximum titers obtained on glycerol. Overexpression of *TKL1* led to an elevated level of *TKL1* transcription, resulting in considerably elevated erythritol production under oxygen scarcity. The engineered strain consistently produced two-fold higher erythritol than the control in shake-flask experiments. In a bioreactor study, it was observed that the AMM pAD-TKL1 strain exhibited a 67% increase in erythritol production compared to the control, and there was a notable inhibition of by-product formation. The engineered strain produced 62.5 g/L of erythritol with a productivity of 0.62 ± 0.05 g/L/h compared to 37.3 g/L of erythritol with a productivity of 0.42 ± 0.05 g/L h in the parent strain. (Mirończuk et al., 2017).

Overexpression of *GUT1* and *TKL1* and disruption of *EYK1* coding for erythrulose kinase that participates in an early step of erythritol catabolism were found to be beneficial for erythritol production. The genetically modified strain JCY218 demonstrated efficient erythritol

production in a brief timeframe under reduced oxygen concentration. Moreover, erythritol productivity was 75% higher (1.03 g/L/h) than the wild type (0.59 g/L/h) in glycerol, and the culturing period required to accomplish maximum concentration was reduced by 40%. The engineered strain formed 80.6 g/L of erythritol whereas in the parent strain, 55.8 g/L was noted. Additionally, the strain was incapable of consuming the erythritol it had produced thereby enhancing the efficiency of the process (Carly et al. 2017b).

Erythritol production has been identified as a completely distinctive reaction to hyperosmotic pressure in a group of yeasts inclusive of *Y. lipolytica*. The high osmolality glycerol (HOG) pathway gets activated by the mitogen-activated protein kinase (MAPK) pathway which controls the reaction to high osmotic stress conditions in the yeast strain *S. cerevisiae* (Smith et al., 2010). *Hog1* gene deletion resulted in a considerable fall in resistance to hyperosmotic stress and adversely hampered the formation of erythritol. In *Y. lipolytica*, the gene *YALI0E25135g* was detected as a homologue of *HOG1* gene and was phosphorylated in response to hyperosmotic pressure. On sequence comparison using the BLASTp with *S. cerevisiae* and *C. albicans HOG1*, the gene *YALI0E25135g* was identified as the *Y. lipolytica* homologue of *Hog1*. The flanking regions and gene sequence of *YALI0E25135g* were used for primer design to permit for its deletion and overexpression successfully. Two strains of *Y. lipolytica* were created in which either *yl-Hog1* was deleted (*yl-hog1*Δ) or over-expressed (*yl-HOG1*). Remarkably, the deletion strain (yl-hog1Δ) confirmed noteworthy morphological alterations, with the cells developing in a filamentous shape. Moreover, *yl-hog1Δ* cells were also immune to the cell wall destructive agents viz. Congo red and Calcofluor white. These observations indicated that *yl-Hog1* is vital for the cell-based reaction to hyperosmotic pressure, performs a part in the induction of erythritol synthesis, and probably thwarts crosstalk with distinctive MAPK signaling pathways within the cell. The *yl-hog1*Δ and *yl-HOG1* strains

synthesized 32 ± 4 g/L and 19.3 ± 3 g/L erythritol, respectively, whereas parent strain MK1 showed 36.6 ± 1 g/L erythritol production (Rzechonek et al., 2018).

Janek *et al*., (2017) identified an erythrose reductase (*ER*) gene (*YALI0F18590g*) from erythritol-synthesizing yeast *Y. lipolytica* MK1 for the first time. Overexpression of *ER* gene in the presence of $Zn^{+}$ resulted in an improved erythritol titer of 78.1 g/L, a yield of 0.52 ± 0.06 g/g, and productivity of 1.00 ± 0.12 g/L/h in glycerol-based medium as compared to 68.2 g/L of erythritol and 0.87 ± 0.05 g/L/h productivity in the control strain. However, deletion of *YALI0F18590g* gene still gave some erythritol formation in *Y. lipolytica* MK1 suggesting the existence of other ER genes in the genome of *Y. lipolytica* (Janek et al., 2017). Three isozymes (ER-1: MW 38,000 kDa; ER-2 and ER-31.2: MW 37,000 kDa) of ER were reported in *Aureobasidium sp*. SN-G42 (Tokuoka et al., 1992). Similarly, in *Y. lipolytica,* eight ERs were identified and overexpression of one of the ER homolog gene (*YALI0B07117g)* increased erythritol productivity (from 0.28 g/L/h to 0.41 g/L/h) for the modified A101 strain using glycerol as substrate. Moreover, knocking off this homolog gene (*YALI0B07117g*) did not abolish the production of erythritol in the strain AJD pAD B07117 reiterating the presence of other ER genes in the yeast *Y. lipolytica* (Szczepańczyk et al., 2021).

The significant homology among all ER homologs poses a challenge in determining which specific protein is responsible for erythritol synthesis in general. Overexpression of native GK and TKL and heterologous overexpression of sugar alcohol phosphatase (PYP) in *Y. lipolytica* Po1f (Madzak et al., 2000) showed improved erythritol production during batch and fed-batch fermentation using crude glycerol as substrate (Jagtap et al., 2021). The engineered strain gave 58.8 ± 1.7 g/L erythritol as compared to 30.52 g/L in *Y.lipolytica* Po1f. In both cases, productivity was very low as fermentation time lasted for 12 days.

Zhang *et al*., (2021) used several gene integration approaches to improve erythritol synthesis in *Y. lipolytica*. The collective expression of four genes, namely *GUT1*, *TPI1*, *TKL1*, *TAL1*

(coding for glycerol kinase, triosephosphate isomerase, transketolase, and transladolase ) and disruption of the *EYD1* gene (encoding erythritol dehydrogenase), showed enhanced erythritol production (20.0 g/L) than auxotrophic strain *Y. lipolytica* Po1f (17.0 g/L) from glycerol. Investigators have also demonstrated erythritol production by overexpressing the *RKI1* gene (encoding ribose 5-phosphate isomerase) and the *AMPD* gene (encoding AMP deaminase) in *Y. lipolytica*. This study suggested that RK11 possibly leads to an increased formation of erythrose-4P flux in *Y. lipolytica* which is subsequently utilized for enhanced erythritol production (Table 1.2).

Apart from the genetic modification approach for strain development in *Y. lipolytica*, Qiu *et al*., (2020) developed a high throughput screening method for rapid detection and screening of mutant strain libraries for enhanced erythritol production. In their study, they used an erythritol-responsive transcription factor called *EryD*, and built a sensor-regulator system for rapid screening and characterization of erythritol overproducing strain. With this method, they screened 1152 mutants resulting from mixed UV and atmospheric room temperature plasma (ARTP) mutagenesis, rapidly (1 week). One of the mutants, yliUA8s, was found to produce 103 g/L erythritol at the shake flask stage; a 2.4-fold upsurge in erythritol formation from glucose than the yeast culture used initially. In scale-up trails using 3.0 L bench-top reactors, mutant strain yliUA8 produced more than 148 g/L erythritol. This study enables fast improvement in strain efficacy and engineering of effective microbial cell factories for commercial uses (Qiu et al., 2020).

### 1.8.2 *Moniliella species*

The assessment of nucleotide sequences from the D1/D2 domains of the large subunit rDNA and phenotypic functions indicated that the *Moniliella* and *Trichosporonoides* genera are members of a sole, monophyletic clade that would be best- characterized by using a single

anamorphic genus (Rosa et al., 2009). A homologue of *Hog1* present in *Trichosporonoides (Moniliella) oedocephalis* was deleted by Li *et al*., (2016). In this study, an attempt was made to adopt the Cre/loxP system containing the KanMX gene disruption cassette for *HOG1* gene knockout in *T. oedocephalis*. The Cre/loxP cassette approach is widely utilized in yeast, often in conjunction with the heterologous dominant KanR, serving as a potent gene disruption technology. (Baudin et al., 1993). The newly constructed knockout mutants of *T. oedocephalis* HOG1 displayed slightly lower biomass in a liquid medium and impaired growth in a solid medium. However, mutant strain exhibited a 1.4-fold increase in erythritol synthesis (56.82 ± 0.31 g/L) with a concurrent decrease in glycerol production by 71.23 %. While the parent strain *T. oedocephalis* ATCC 16958 could only produce 39.37 ± 0.18 g/L erythritol with significantly high production of glycerol (36.22 ± 0.19 g/L).

Studies were also carried out on ERs of *M. megachiliensis* to detect hyperosmotic stress responses (Kobayashi et al., 2013a). *T. megachiliensis* has been documented to possess three ER isozymes, namely ER1, ER2, and ER3. (Ookura et al., 2005). The gene sequences of the *ER1*, *ER2*, and *ER3* loci together with sequences 2 kb upstream from each initiation codon and 1 kbp downstream from each termination codon were investigated. Upstream of the initiator methionine of *ER3*, two stress response elements (STREs) were identified—one at the 148 bp upstream position and another at the 80 bp upstream position. In contrast, *ER1* and *ER2* each had only one STRE. An examination of the genomic regions adjacent to the ER genes did not reveal any genes possible to be involved in sugar alcohol production (Fig. 1.5). The expression of three ER encoding genes (*ER1*, *ER2*, and *ER3*) was evaluated in cells cultivated on 20 % glucose. No significant changes were observed when compared to cells cultivated in 2% glucose. during the initial hours of treatment. However, there was a 60-fold surge in the expression of the glycerol-3-phosphate dehydrogenase gene at the same time, which shows that the principal response of cells was the production of glycerol. Intracellular erythritol

accumulation was observed after 12 h incubation and stayed high during the next five days. Only *ER3* expression rose significantly during extended osmotic stress and the level of its mRNA transcripts matched the erythritol formation profile. Hence, glycerol synthesis could be an initial response, while the production of erythritol may represent the reaction of the cell's stationary-phase to osmotic pressure. (Kobayashi et al., 2013).

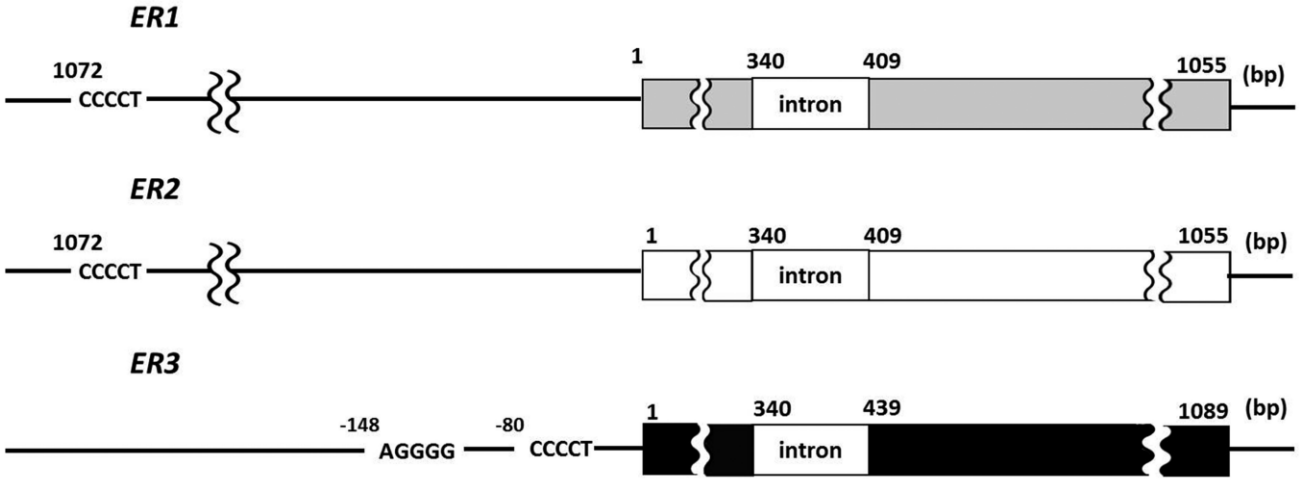



**Figure 1. 5** The structure of the ER1, ER2, and ER3 genes of *T. megachiliensis*. The sequence CCCCT or AGGGG represents a stress response element (adapted and modified from (Kobayashi et al., 2013)

Ookura and coworkers characterized the three isozymes of ERs viz. ER-I, ER-II, and ER-III biochemically from *T. megachiliensis* SNG-42 (previous name *Aureobasidium megachiliensis*), a strain employed in the industrial production of erythritol (Ookura et al., 2005; Tokuoka et al., 1992). Additionally, the researchers expressed the recombinant ER3 gene in *P. pastoris* and *S. cerevisiae* but did not observe erythritol synthesis in the growth medium. The authors it attributed to its low activity of erythrose reductase in each strain, necessitating a requirement for more efficient ERs to enhance erythritol synthesis. Moreover, for efficient erythritol production, an increased availability of cofactors (NADPH) or D-erythrose is essential. It is believed that while D-erythrose resulted from erythrose-4P in the pentose

phosphate shunt, the specific enzymes like phosphatases required for this reaction remain unidentified (Ookura et al., 2005).

In another study, *Moniliella sp*. 440 isolated from honey was sequentially exposed to N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for iterative rounds of mutation and selection (Lin et al., 2010). Six strains were tested for erythritol synthesis in 40 % glucose and 1% yeast extract in a shake flask and all of them produced more erythritol than the parent strain. Mutant strain N61188-12 produced the best erythritol (237.8 g/L) than wild type (113 g/L) strain. Mutagenesis not only enhanced the yield of erythritol but also resulted in a mutated strain that exhibited accelerated glycerol utilization. Additionally, this mutated strain displayed advantages such as minimal foaming, reduced byproduct concentration, and enhanced resistance to osmotic stress. Upon scaling up the fermentation process to a 2000 L reactor, the mutant N61188-12 successfully generated 152.4 g/L of erythritol within a 10-day period.

### 1.8.3 Candida species

*Candida magnoliae*, an osmophilic yeast is one of the strains capable of biological erythritol production. *C. magnoliae* was isolated from honeycombs and mutated using 0.8% ethyl methane sulfonate (EMS) to improve erythritol yield from glucose. The *C. magnoliae* mutant confirmed the over-production of erythritol with a productivity of 1.2 g/L/h, a titer of 200 g/L, and a yield of 0.43 g/g of glucose by sustaining the precise glucose consumption during the fed-batch fermentation. Erythritol productivity increased 5-fold in the fed-batch fermentation in comparison with the batch fermentation (Koh et al., 2003).

A mutant of C. magnoliae DSM70638, obtained through the combined use of UV and EMS mutagenesis (designated as mutant 12–2), exhibited a 2.4–fold increase in erythritol production, reaching 20.32 g/L. In contrast, the wild-type strain yielded 8.54 g/L of erythritol. Additionally, the mutant showed a 5.5–fold reduction in glycerol production, with a measured

quantity of 2.36 g/L, in comparison to the wild strain that produced 12.93 g/L of glycerol. The gene encoding for ER enzyme of this mutant, when subjected to a sequence-based mapping, exhibited a replacement of the A321 by G321, and this replacement did not cause any amino acid exchange in protein structure. The heightened erythritol production observed in the C. magnoliae mutant 12–2 strain was suggested to be primarily attributed to an elevated expression of the open reading frame within the gene. Consequently, a mutation or subtle alteration in the sequence of genes associated with the erythritol production pathway can result in a significant increase in protein translation. (Ghezelbash et al., 2014).

Among the mutants of *C. magnoliae* NCIM 3470 created through UV and chemical mutagenesis, the mutant R23 demonstrated a four-fold increase in erythritol production, reaching 60.3 g/L, in comparison to the wild strain's production of 14 g/L. Additionally, the erythritol production pathway's enzyme (ER) activity in the R23 mutant was found to be two-fold higher than that of the parent strain (Savergave et al., 2011). Glucose and yeast extract have been known as key growth additives that decide the ratio of production of polyols including erythritol, mannitol, and glycerol. To boost erythritol formation while reducing the production of mannitol and glycerol, a four-factor, five-stage-three reaction, relevant-composite-rotatable-design (CCRD) of response surface methodology (RSM) model was employed. In fed-batch fermentation, with statistically optimized media composition, 87.8 g/L erythritol was formed with 31.1% yield.

*Candida magnoliae* parent and mutants underwent proteomic analyses employing two-dimensional gel electrophoresis and nano electrospray mass spectrometry. This approach was employed to investigate intracellular proteins and evaluate the impact of newly characterized metabolic enzymes on yeast cell growth and erythritol formation. A high growth of the *C. magnoliae* mutant strain was observed when enzymes associated with the citric acid cycle were up-regulated. The mutant strain also showed increased NADH and ATP concentration. It was

also discovered that mutations downregulated the gene encoding for enolase and upregulation of fumarase encoding gene. In comparison with wild-type strain, this regulation plays an important part in enhanced bioconversion of erythrose-4P to erythritol (Lee et al. 2003a). It was evident that overexpression of fumarase permits faster fumarate degradation and subsequently inhibits the activity of ER (Lee et al. 2002). Downregulation of enolase is supposed to decrease phosphoenolpyruvate concentration. Phosphoenolpyruvate and erythrose-4P are starting materials for shikimate synthesis. Therefore, by reducing phosphoenolpyruvate levels, erythrose-4P can be channeled towards erythritol synthesis rather than shikimate synthesis.

### 1.9 Erythritol production by other yeast and fungal species

Besides *Moniliella*, *Candida* and *Yarrowia*, various yeast species have been employed for the erythritol synthesis, particularly *Aureobasidium* (Ishizuka et al., 1989), *Trichosporonoides* (Aoki et al., 1993), *Trichosporon* (Park et al., 1998), *Torula* (Oh et al., 2001), and *Pseudozyma* (Jeya et al., 2009). The biological transformation of xylose, the second highest copious sugar, has additionally been studied in the last few years.

*Aureobasidium pullulans* CGMCC3.0837 strain was mutated using UV to enhance its erythritol synthesizing capability from xylose. In comparison with wild-type strain, mutant strain ER35 produced erythritol which was 50.92 % (17.28 g/L) higher. The erythritol concentration produced by mutant ER35 in shake flasks and a 5-L fermentor were similar (31 g/L). Therefore, this study offered the likelihood of employing xylose as a carbon substrate for the synthesis of erythritol using *A. pullulans* (Guo et al., 2016). *Aureobasidium sp*. SN-124A was repeatedly mutated with NTG treatment and UV irradiation for improved erythritol production. The mutant strain SN-G42 exhibited superior characteristics by maintaining a non-foaming state during cultivation, whereas the wild strain foamed severely. The erythritol production yield in

the mutant was 47.6 % in a medium containing 22.5 % glucose as compared to that of 41.8% yield in the wild strain. Furthermore, erythritol productivity was not affected in the mutant culture even when the glucose concentration was amplified from 22.5 % to 47 % (Ishizuka et al., 1989).

A well-studied filamentous fungus *Fusarium graminearum* known for its relevance as a plant pathogen affecting several plant such as cereals and dicotyledons (Jovanović et al., 2013) was also explored for erythritol production. Filamentous fungi use plant biomass like lignocellulose as a substrate in contrast to yeast. These fungi secrete xylanolytic enzymes which hydrolyze xylans into their key monomers L-arabinose and D-xylose. The monomers produced by these fungi can be metabolized to D-xylose-5-phosphate to supplement the PPP, the source of erythritol. In one study (Jovanović et al., 2013), the investigators identified ER by *in silico* analysis of proteins in *Trichoderma reesei, Aspergillus niger,* and *F. graminearum* which displayed high sequence similarity to the ER (*ER1*) from *T. megachiliensis* (Ookura et al., 2005). *ER1* genes from *T. reesei* were cloned and their protein products were expressed heterologously in *E. coli* and purified. Enzyme assay parameters were optimized for ten possible substrates including D-erythrose. *In vitro* analysis of these proteins by an enzyme assay confirmed high substrate specificity and the turnover rate for D-erythrose amongst all the ten substrates. The *ER1* from *T. reesei* showed a higher turnover number for D-erythrose substrate than *ER1* from *A. niger* and *F. graminearum*. On overexpression of the ER enzyme in *T. reesei,* erythritol production increased in the overexpressed strain was compared to the parental strain *in vivo.* After 24 h of culture time, the erythritol concentration in the modified strain was 1.6-fold higher than in the parental strain, and after 30 h it was 3.2-fold higher (Jovanović et al., 2013).

Furthermore, the same research group overexpressed the *ER1* gene in the *T. reesei* wild-type and the cellulase hyper-producing, carbon catabolite-repressed strain Rut-C30 to evaluate

erythritol production. ER1 overexpression was facilitated in both strains using two distinct promoters: a constitutive one (the native pyruvate kinase promoter) and an inducible one (the native β-xylosidase promoter). Recombinant strains were pre-characterized by analysis of *ER1* transcript formation on xylan and D-xylose and pre-treated wheat straw. In shake flasks and fermenter experiments. it was found that overexpression of *ER1* in Rut-C30 led to a higher erythritol formation on pre-treated wheat straw (Jovanović et al., 2014).

### 1.10 Efficient erythritol recovery

Depending on the strain and substrate used throughout erythritol synthesis, some by-products such as glycerol, ribitol, mannitol, fumarate, D-arabitol, and citrate are produced (Lin et al., 2001; Mirończuk et al., 2017). The typical erythritol formation process initiates with fermentation, followed by the key phases of downstream process. This includes biomass clarification to remove cells, separation of insoluble compounds, purification of clarified erythritol from impurities and byproducts, and ultimately concentration to yield erythritol crystals. (Fig. 1.6). (Troostembergh et al., 2002; Saran et al., 2015; Rakicka et al., 2016; Deshpande et al., 2022).

Various approaches have been reported for biomass removal like cell separation (Maeda et al., 1997; Deshpande et al., 2022), biomass inactivation (Sasman & Alan, 2007), and vacuum filtration (Saran et al., 2015). Warming of the culture broth after fermentation at an elevated temperature of up to 70°C to inactivate the cells followed by separation of the cells using centrifugation are performed (Sasman, and Alan, 2007). Some researchers have used techniques such as ultrafiltration at pH range between 3.5-5.5 to avoid the foaming and subsequent intrusion of intracellular proteins in ion exchange chromatography (Kresnowati et al., 2019).

The evaporation stage caters to the elimination of non-soluble portions, and color impurities from the cell-free fermented broth and to separate the erythritol portion for purification in the chromatography step. To perform these operations, different processes like ultrafiltration, ion exchange, and concentration have been used. At this stage, the fermented broth primarily has a residual carbon source, erythritol, and by-products. For enhanced erythritol purification, nanofiltration was included after the microfiltration as an added step to eliminate leftover macromolecules before the ensuing ion exchange step (Li et al., 2020; Zhao et al., 2020). In the report by Troostembergh et al. (2002), the cell-free fermented broth underwent treatment with activated charcoal to remove both dissolved and colored impurities. The solution, obtained after filtration, underwent concentration in a rotary vacuum evaporator at 55°C and 72 psi to yield a condensed viscous liquid. This liquid was further subjected to desalination through precipitation. The filtrate containing erythritol was processed by passing it through an ion exchange column, which was packed (18.15 x 2.0 cm) with cationic exchange resin (Diaion SK116, BF 100 INDION). Subsequently, the elution was carried out using water, as described in the studies by Saran et al. (2015) and Deshpande et al. (2022).

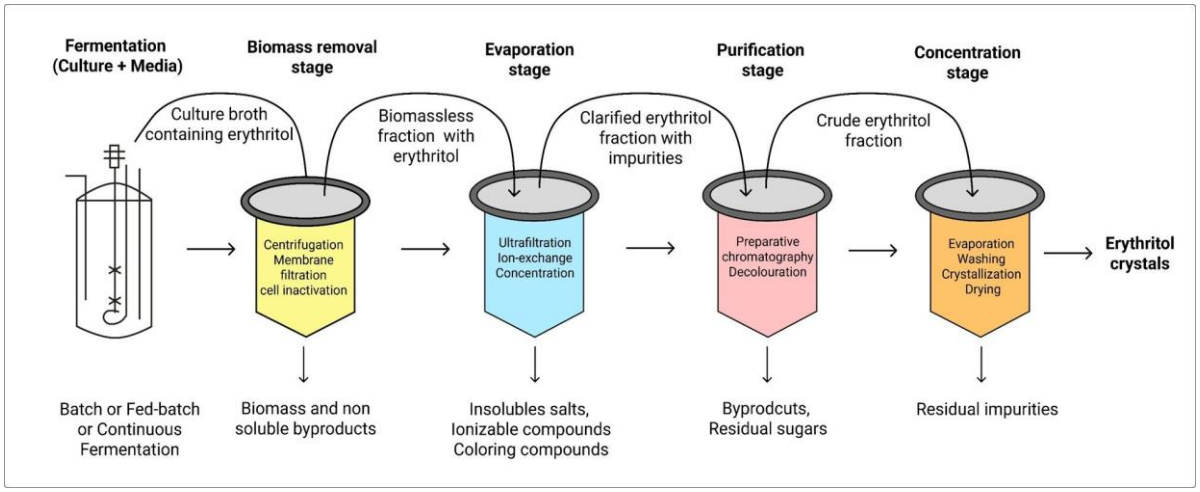



**Figure 1.6**. Downstream process for erythritol recovery (adapted and modified from (Daza-Serna et al., 2021)

The major byproducts in erythritol production are glycerol, ethanol, and other polyols. The subsequent step in this process is to separate the erythritol fraction from these byproducts. The purification of erythritol fraction relies on charge (chemical sites on the exterior of the molecules) based separation on ion exchange chromatographic resins. A cationic acid resin (Purolite PCR-821) was used for erythritol purification by batch preparative chromatography (Sasman and Alan, 2007).

The final step of the purification stage consists of various processes like evaporation, crystallization, crystal separation, washing, and drying to obtain the highly purified erythritol. In a method previously outlined by Saran et al. (2015), various fractions containing erythritol were combined and subjected to concentration once more using a rotary evaporator (rotavapor). The concentrated filtrate was subsequently set aside for the crystallization process at 4°C. The erythritol recovery achieved was approximately 52.24%, and the purity of the obtained erythritol in its crystallized form was reported to be 98.99%, as detailed in the study by Saran et al. (2015). In another study, 43 % erythritol recovery from sugarcane-based feedstock with 96.34 % erythritol purity (Deshpande et al., 2022) was obtained. Morioka et al. (2000) achieved erythritol crystals with a purity of 99.9% by adjusting the cooling rate, specifically employing a rate of 7.5 °C/h from 70 to 15 °C. Similarly, Toshihiro et al. (1993) reported comparable results when lowering the temperature from 60°C to 20°C with a consistent cooling rate. The residual impurities fraction contained 26.3 % of erythritol and other byproducts. Isolation of crystals from the liquid solution is the last step in a downstream process where cold water spray was applied to achieve crystal separation. A rotary drum dryer can be used for drying the washed crystals as has been the practice for common sugars (Morioka et al., 2000).

The sticky and reddish-brown liquor called waste erythritol mother liquor (WEML) is the waste product left over after the purification process. The WEML contains erythritol along with other waste polyols and is problematic to deal with. Various methods, such as bio-removal and

biotransformation, have been employed for treating waste effluents from molasses (WEML) due to their high efficiency and specificity. These approaches are especially notable in the fields of environmental remediation and the recovery of high-value chemicals from crude sugar feedstocks. In a separate study, researchers sought to establish an efficient procedure for purifying erythritol from waste effluents of molasses (WEML) using yeast. Following the identification of polyol impurities in waste effluents of molasses (WEML), researchers employed the yeast strain *Candida maltosa* to biologically enrich erythritol in WEML by reducing the polyol impurities. The newly developed bio-removal approach was studied both in shake flasks and at the fermenter level, as detailed in the work by Wang et al. (2017).

### 1.11 Summary

In recent decades, significant attention has been devoted to the biological production of erythritol, primarily from yeast, due to the rising demand. Numerous studies have been conducted to enhance the erythritol yield in yeast strains, highlighting their potential as promising organisms for erythritol production. In the literature, certain yeast cultures have been demonstrated to exhibit high productivity, and researchers have meticulously optimized the medium composition and culture conditions to maximize production titers. Furthermore, genetic engineering and proteomic studies have been conducted, yielding promising results that not only enhance efficiency but also contribute to a deeper understanding of erythritol metabolism, its regulation, and the limiting steps in its production. Additionally, the development of robust microbial systems by genetic engineering, targeting the key enzyme, i.e., erythrose reductase could improve erythritol production. Application of the latest techniques such as the Cas9/ CRISPR genome editing in erythritol-producing strains would allow improving the highly efficient microbial systems. The use of renewable carbon sources

as feedstock for erythritol production and innovative approaches in the erythritol purification process will have a great impact on the economics of the process.

### 1.12 Statement of the problem

Erythritol is a 4-carbon polyol produced with the aid of microbes in the presence of hyper-osmotic stress. It is the most effective sugar alcohol that is produced predominantly by fermentation. In comparison to various polyols, it has many precise functions and is used as a flavor enhancer, sequestrant, humectant, nutritive sweetener, stabilizer, formulation aid, thickener, and texturizer. Due to the low insulin index, erythritol has the potential as a sugar replacement for people with diabetes. Among various polyols in the market, erythritol shows numerous distinctive features. The calorific value of erythritol is near zero, and when consumed within the safe dosage limit, it does not lead to side effects compared to other polyols. Its chemical structure enables it to resist changes in insulin levels rendering it safe for diabetic patients. Erythritol can be produced through several chemical and biotechnological methods. Due to their low efficiency, chemical processes have no longer been industrially used. Extraction of erythritol from vegetables and fruits is not an economically viable method even though it is present in them. In contrast, biosynthetic routes offer an environment-friendly and probably the best erythritol synthesis process. Because of the drawbacks of the chemical synthesis approach, at present, on a commercial scale, erythritol is produced by fermentation strategies by the use of fungal strains. Various genera of yeast-like *Aureobasidium, Candida, Moniliella, Torula, Trichosporon, Trichosporonoides,* and *Yarrowia* are known to produce erythritol. Because of lower yield, productivity, or byproducts formation including glycerol and ribitol, erythritol production by *Pichia, Zygopichia, Candida, Torulopsis,* and *Trigonopsis* could not be implemented on a commercial scale. Despite having several advantages, the high price of erythritol results in scarce usage. A cost-effective production regime using cheap carbon and nitrogen substrates that support erythritol production in yeast is essential. The present study aims at strain improvement for erythritol production using cheap cost-effective carbon substrate.

**1.13 Objectives:**

1. Screening of yeast cultures and optimization of culture conditions for erythritol production
2. Strain improvement using chemical and physical mutagenesis
3. Statistical medium optimization for enhanced erythritol production
4. Scale up of erythritol production in a laboratory fermentor

## 1.14 References

Aoki, M. A. Y., Pastore, G. M., & Park, Y. K. (1993). Microbial transformation of sucrose and glucose to erythritol. *Biotechnology Letters*, 15, 383–388.

Baudin, A., Ozier-kalogeropoulos, O., Denouel, A., Lacroute, F., & Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. In *Nucleic Acids Research,* 21, 3329–3330.

Bernt, W. O., Borzelleca, J. F., Flamm, G., & Munro, I. C. (1996). Erythritol: A review of biological and toxicological studies. *Regulatory Toxicology and Pharmacology*, 24, 24:S191-S197.

Binkley, W. W., & Wolfrom, M. L. (1950). Chromatographic fractionation of cane blackstrap molasses and of its fermentation residue. *Journal of the American Chemical Society*, 72 4778–4782.

Blazeck, J., Liu, L., Redden, H., & Alper, H. (2011). Tuning gene expression in *yarrowia lipolytica* by a hybrid promoter approach. *Applied and Environmental Microbiology*, 77, 7905-7914.

Brown, A. D. (1978). Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Advances in Microbial Physiology*, 1, 181-242.

Burschäpers, J., Schustolla, D., Schügerl, K., Röper, H., & De Troostembergh, J. C. (2002). Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa var pollinis:* Part 2. Batch and fed-batch operation in bubble column and airlift tower loop if reactors. *Process Biochemistry*, 38, 559–570.

Carly, F., Gamboa-Melendez, H., Vandermies, M., Damblon, C., Nicaud, J. M., & Fickers, P. (2017a). Identification and characterization of EYK1, a key gene for erythritol catabolism in *Yarrowia lipolytica*. *Applied Microbiology and Biotechnology*, 101, 6587-6596.

Carly, F., Vandermies, M., Telek, S., Steels, S., Thomas, S., Nicaud, J. M., & Fickers, P. (2017b). Enhancing erythritol productivity in *Yarrowia lipolytica* using metabolic engineering. *Metabolic Engineering*, 42, 19–24.

Carly, F., Steels, S., Telek, S., Vandermies, M., Nicaud, J. M., & Fickers, P. (2018). Identification and characterization of EYD1, encoding an erythritol dehydrogenase in *Yarrowia lipolytica* and its application to bioconvert erythritol into erythrulose. *Bioresource Technology*, 247, 963–969.

Cheng, H., Wang, S., Bilal, M., Ge, X., Zhang, C., Fickers, P., & Cheng, H. (2018). Identification, characterization of two NADPH-dependent erythrose reductases in the yeast *Yarrowia lipolytica* and improvement of erythritol productivity using metabolic engineering. *Microbial Cell Factories*, 17, 1–12.

Daza-Serna, L., Serna-Loaiza, S., Masi, A., Mach, R. L., Mach-Aigner, A. R., & Friedl, A. (2021). From the culture broth to the erythritol crystals: an opportunity for circular economy. In *Applied Microbiology and Biotechnology*, 105, 4467-4486.

Deshpande, M. S., Kulkarni, P. P., Kumbhar, P. S., & Ghosalkar, A. R. (2022). Erythritol production from sugar based feedstocks by *Moniliella pollinis* using lysate of recycled cells as nutrients source. *Process Biochemistry*, 112, 45-52

Ghezelbash, G. R., Nahvi, I., & Malekpour, A. (2014). Erythritol production with minimum By-product using *Candida magnoliae* mutant. *Applied Biochemistry and Microbiology*, *50*, 292–296.

Goossens, J., & Roper, H. (1994). Erythritol: a new sweetener. *Confectionery Production, 60,* 182-184.

Guo, J., Li, J., Chen, Y., Guo, X., & Xiao, D. (2016). Improving erythritol production of *Aureobasidium pullulans* from xylose by mutagenesis and medium optimization. *Applied Biochemistry and Biotechnology,* 180, 717–727.

Hajny, G. J., Smith, J. H., & Garver, J. C. (1964). Erythritol production by a yeast like fungus. *Applied Microbiology*, 12, 240-246.

Hattori, K., & Suzuki, T. (1974). Production of erythritol by n-alkane-grown yeasts. *Agricultural and Biological Chemistry*, 38, 581-586.

Hiele, M., Ghoos, Y., Rutgeerts, P., & Vantrappen, G. (1993). Metabolism of erythritol in humans: Comparison with glucose and lactitol. *British Journal of Nutrition, 69*, 169–176.

Hirata, Y., Igarashi, K., Ezaki, S., Atomi, H., & Imanaka, T. (1999). High-level production of erythritol by strain 618A-01 isolated from pollen. *Journal of Bioscience and Bioengineering, 87*, 630–635.

Horning, E. C., Horning, M. G., Szafranek, J., Van Hout, P., German, A. L., Thenot, J. P., & Pfaffenberger, C. D. (1974). Gas phase analytical methods for the study of human metabolites. *Journal of Chromatography A,* 91, 367-378.

Ishizuka, H., Wako, K., Kasumi, T., & Sasaki, T. (1989). Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. *Journal of Fermentation and Bioengineering, 68,* 310–314.

Jagtap, S. S., Bedekar, A. A., Singh, V., Jin, Y. S., & Rao, C. V. (2021). Metabolic engineering of the oleaginous yeast *Yarrowia lipolytica* PO1f for production of erythritol from glycerol. *Biotechnology for Biofuels*, 14, 188-205.

Janek, T., Dobrowolski, A., Biegalska, A., & Mirończuk, A. M. (2017). Characterization of erythrose reductase from *Yarrowia lipolytica* and its influence on erythritol synthesis. *Microbial Cell Factories*, 16, 118-201

Jeya, M., Lee, K. M., Tiwari, M. K., Kim, J. S., Gunasekaran, P., Kim, S. Y., Kim, I. W., & Lee, J. K. (2009). Isolation of a novel high erythritol-producing *Pseudozyma tsukubaensis* and scale-up of erythritol fermentation to industrial level. *Applied Microbiology and Biotechnology*, *83*, 225–231.

Jovanović, B., Mach, R. L., & Mach-Aigner, A. R. (2013). Characterization of erythrose reductases from filamentous fungi. *AMB Express*, *3*, 1–11.

Jovanović, B., Mach, R. L., & Mach-Aigner, A. R. (2014). Erythritol production on wheat straw using *Trichoderma reesei*. *AMB Express*, 4, 1–12.

Kayingo, G., Kilian, S. G., & Prior, B. A. (2001). Conservation and release of osmolytes by yeasts during hypo-osmotic stress. *Archives of Microbiology*, 177, 29-35.

Kim, K. A., Noh, B. S., Lee, J. K., Kim, S. Y., Park, Y. C., & Oh, D. K. (2000). Optimization of culture conditions for erythritol production by *Torula* sp. *Journal of Microbiology and Biotechnology*, *10*, 69–74.

Kobayashi, Y., Iwata, H., Mizushima, D., Ogihara, J., & Kasumi, T. (2015). Erythritol production by *Moniliella megachiliensis* using nonrefined glycerol waste as carbon source. *Letters in Applied Microbiology*, *60*, 475–480.

Kobayashi, Y., Yoshida, J., Iwata, H., Koyama, Y., Kato, J., Ogihara, J., & Kasumi, T. (2013). Gene expression and function involved in polyol biosynthesis of *Trichosporonoides megachiliensis* under hyper-osmotic stress. *Journal of Bioscience and Bioengineering*, 115, 645–650.

Koh, E. S., Lee, T. H., Lee, D. Y., Kim, H. J., Ryu, Y. W., & Seo, J. H. (2003). Scale-up of erythritol production by an osmophilic mutant of *Candida magnoliae*. *Biotechnology Letters*, *25*, 2103–2105.

Krajangsod, S., Chotikamas, S., Tawai, A., & Sriariyanun, M. (2018). Measurement and thermodynamic modelling of erythritol solubility in aqueous solvents. *Oriental Journal of Chemistry*, 34, 265–275.

Kresnowati, M. T. A. P., Regina, D., Bella, C., Wardani, A. K., & Wenten, I. G. (2019). Combined ultrafiltration and electrode ionization techniques for microbial xylitol purification. *Food and Bioproducts Processing*, 114, 245-252

Kroger, M., Meister, K., & Kava, R. (2006). Low-calorie sweeteners and other sugar substitutes: A review of the safety issues. In *Comprehensive Reviews in Food Science and Food Safety*, 5, 35–47.

Lee, D. H., Lee, Y. J., Ryu, Y. W., & Seo, J. H. (2010). Molecular cloning and biochemical characterization of a novel erythrose reductase from *Candida magnoliae* JH110. *Microbial Cell Factories*, 9, 43-55

Lee, D. Y., Park, Y. C., Kim, H. J., Ryu, Y. W., & Seo, J. H. (2003). Proteomic analysis of *Candida magnoliae* strains by two-dimensional gel electrophoresis and mass spectrometry. *Proteomics*, *3*, 2330–2338.

Lee, J. K., Kim, S. Y., Ryu, Y. W., Seo, J. H., & Kim, J. H. (2003). Purification and characterization of a novel erythrose reductase from *Candida magnoliae*. *Applied and Environmental Microbiology*, 69, 3710–3718.

Lee, J. K., Koo, B. S., & Kim, S. Y. (2002). Erratum: Fumarate-mediated inhibition of erythrose reductase, a key enzyme for erythritol production by *Torula corallina*. In *Applied and Environmental Microbiology*, 68, 5788-5793.

Lenhart, A., & Chey, W. D. (2017). A systematic review of the effects of polyols on gastrointestinal health and irritable bowel syndrome. In *Advances in Nutrition*, 8, 587–596.

Li K, Luan Q, Jia H, Xiong X, Zhang L, Li Q, Gao L, H. W. (2020). *High-yield preparation method of erythritol* (Patent CN110903165).

Li, L., Yang, T., Guo, W., Ju, X., Hu, C., Tang, B., Fu, J., Gu, J., & Zhang, H. (2016). Construction of an efficient mutant strain of *trichosporonoides oedocephalis* with HOG1 gene deletion for production of erythritol. *Journal of Microbiology and Biotechnology*, *26*, 700–709.

Lin, S. J., Wen, C. Y., Liau, J. C., & Chu, W. S. (2001). Screening and production of erythritol by newly isolated osmophilic yeast-like fungi. *Process Biochemistry*, *36*, 1249–1258.

Lin, S. J., Wen, C. Y., Wang, P. M., Huang, J. C., Wei, C. L., Chang, J. W., & Chu, W. S. (2010). High-level production of erythritol by mutants of osmophilic *Moniliella* sp. *Process Biochemistry*, *45*, 973–979.

Livesey, G. (2001). Tolerance of low-digestible carbohydrates: a general view. *British Journal of Nutrition*, *85*, S7–S16.

Livesey, G. (2003). Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. *Nutrition Research Reviews*, *16*, 163–191.

Madzak, C., Tréton, B., & Blanchin-Roland, S. (2000). Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *Journal of Molecular Microbiology and Biotechnology*, 2, 207-216

Maeda, T., Shida, M., Ohshima, Y., Yamada, T., & Fujimura, K. (1997). Process for preparing erythritol crystals - Patent EP0525659B1. *European Patents*.

Makri, A., Fakas, S., & Aggelis, G. (2010). Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. *Bioresource Technology*, 101, 2351–2358.

Mirończuk, A. M., Biegalska, A., & Dobrowolski, A. (2017). Functional overexpression of genes involved in erythritol synthesis in the yeast *Yarrowia lipolytica*. *Biotechnology for Biofuels*, 10, 77-89.

Mirończuk, A. M., Biegalska, A., Zugaj, K., Rzechonek, D. A., & Dobrowolski, A. (2018). A role of a newly identified isomerase from *Yarrowia lipolytica* in erythritol catabolism. *Frontiers in Microbiology*, 9, 1122-1134.

Mirończuk, A. M., Dobrowolski, A., Rakicka, M., Rywińska, A., & Rymowicz, W. (2015). Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol. *Process Biochemistry*, 50, 61–68.

Mirończuk, A. M., Furgała, J., Rakicka, M., & Rymowicz, W. (2014). Enhanced production of erythritol by *Yarrowia lipolytica* on glycerol in repeated batch cultures. *Journal of Industrial Microbiology and Biotechnology*, *41*, 57–64

Mirończuk, A. M., Rakicka, M., Biegalska, A., Rymowicz, W., & Dobrowolski, A. (2015a). A two-stage fermentation process of erythritol production by yeast *Y. lipolytica* from molasses and glycerol. *Bioresource Technology*, 198, 445–455.

Mirończuk, A. M., Rzechonek, D. A., Biegalska, A., Rakicka, M., & Dobrowolski, A. (2016). A novel strain of *Yarrowia lipolytica* as a platform for value-added product synthesis from glycerol. *Biotechnology for Biofuels*, 9, 180-193.

Morioka S, Abe T, Maeda T, Taki A, Sawada K, I. H. (2000). *Process for producing high-purity erythritol crystal.* (Patent US6030820.).

Munro, I. C., Bernt, W. O., Borzelleca, J. F., Flamm, G., Lynch, B. S., Kennepohl, E., Bär, E. A., & Modderman, J. (1998). Erythritol: An interpretive summary of biochemical, metabolic, toxicological and clinical data. In *Food and Chemical Toxicology*, 36, 1139–1174.

Nelson, D. L., & Cox, M. M. (2017). Lehninger Principles of Biochemistry 7th. *W.H. Freeman and Company*, *2*.

Niang, P. M., Arguelles-Arias, A., Steels, S., Denies, O., Nicaud, J. M., & Fickers, P. (2020). In *Yarrowia lipolytica* erythritol catabolism ends with erythrose phosphate. *Cell Biology International*, 44, 651-660.

Nicaud, J. M., Fabre, E., & Gaillardin, C. (1989). Expression of invertase activity in *Yarrowia lipolytica* and its use as a selective marker. *Current Genetics*, *16*, 253–260.

Niwa, T., Tohyama, K., & Kato, Y. (1993). Analysis of polyols in uremic serum by liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications*, *613*, 9–14.

Oh, D. K., Cho, C. H., Lee, J. K., & Kim, S. Y. (2001). Increased erythritol production in fed-batch cultures of *Torula* sp. by controlling glucose concentration. *Journal of Industrial Microbiology and Biotechnology*, 26, 248–252.

Oku, T., & Nakamura, S. (2007). Threshold for transitory diarrhea induced by ingestion of xylitol and lactitol in young male and female adults. *Journal of Nutritional Science and Vitaminology*, 53, 13–20.

Ookura, T., Azuma, K., Isshiki, K., Taniguchi, H., Kasumi, T., & Kawamura, Y. (2005). Primary structure analysis and functional expression of erythrose reductases from erythritol-

producing fungi (*Trichosporonoides megachiliensis* SNG-42). *Bioscience, Biotechnology and Biochemistry*, *69*, 944–951.

Otey, F. H., Sloan, J. W., Wilham, C. A., & Mehltretter, C. L. (1961). Erythritol and ethylene glycol from dialdehyde starch. *Industrial and Engineering Chemistry*, 53, 267–268.

Park, J. B., Yook, C., & Park, Y. K. (1998). Production of erythritol by newly isolated osmophilic *Trichosporon* sp. *Starch/Staerke*, 50, 120–123.

Park, J., Seo, B., Kim, J., & Park, Y. (1998b). Production of erythritol in fed-batch cultures of *Trichosporon* sp. *Journal of Fermentation and Bioengineering*, *86*, 577–580.

Pepino, M. Y. (2015). Metabolic effects of non-nutritive sweeteners. In *Physiology and Behavior*, 152, 450–455.

Peterson, W. H., Hendershot, W. F., & Hajny, G. J. (1958). Factors affecting production of glycerol and D-arabitol by representative yeasts of the genus *Zygosaccharomyces* . *Applied Microbiology*, *6.349-357*.

Qiu, X., Xu, P., Zhao, X., Du, G., Zhang, J., & Li, J. (2020). Combining genetically-encoded biosensors with high throughput strain screening to maximize erythritol production in *Yarrowia lipolytica*. *Metabolic Engineering*, 60, 66–76.

Radeloff, M. A., & Beck, R. H. F. (2013). Polyols – more than sweeteners. *Sugar Industry*, 138, 226–234.

Rakicka, M., Biegalska, A., Rymowicz, W., Dobrowolski, A., & Mirończuk, A. M. (2017). Polyol production from waste materials by genetically modified *Yarrowia lipolytica*. *Bioresource Technology*, 243, 393–399.

Rakicka, M., Rukowicz, B., Rywińska, A., Lazar, Z., & Rymowicz, W. (2016). Technology of efficient continuous erythritol production from glycerol. *Journal of Cleaner Production*, 139, 905-913.

Regnat, K., Mach, R. L., & Mach-Aigner, A. R. (2018). Erythritol as sweetener—wherefrom and whereto? In *Applied Microbiology and Biotechnology*, 102, 587-595.

Roberts, G. P., McDiarmid, A., & Gleed, P. (1976). The presence of erythritol in the fetal fluids of fallow deer (Dama dama). *Research in Veterinary Science*, 20, 254–256.

Rosa, C. A., Jindamorakot, S., Limtong, S., Nakase, T., Lachance, M. A., Fidalgo-Jiménez, A., Daniel, H. M., Pagnocca, F. C., Inácio, J., & Morais, P. B. (2009). Synonymy of the yeast genera *Moniliella and Trichosporonoides* and proposal of *Moniliella fonsecae* sp. nov. and five new species combinations. *International Journal of Systematic and Evolutionary Microbiology*, *59*, 425–429.

Rymowicz, W., Rywińska, A., & Marcinkiewicz, M. (2009). High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*. *Biotechnology Letters*, 31, 377–380.

Ryu, Y. W., Park, C. Y., Park, J. B., Kim, S. Y., & Seo, J. H. (2000). Optimization of erythritol production by *Candida magnoliae* in fed-batch culture. *Journal of Industrial Microbiology and Biotechnology*, *25*(2), 100–103.

Rzechonek, D. A., Day, A. M., Quinn, J., & Mirończuk, A. M. (2018). Influence of ylHog1 MAPK kinase on *Yarrowia lipolytica* stress response and erythritol production. *Scientific Reports*, 8, 14735-14747.

Rzechonek, D. A., Neuvéglise, C., Devillers, H., Rymowicz, W., & Mirończuk, A. M. (2017). EUF1-A newly identified gene involved in erythritol utilization in *Yarrowia lipolytica*. *Scientific Reports*, 7, 12507-12516.

Rzechonek, D. A., Szczepańczyk, M., Wang, G., Borodina, I., & Mirończuk, A. M. (2020). Hog-independent osmoprotection by erythritol in yeast yarrowia lipolytica. *Genes*, 11, 1–15.

Saran, S., Mukherjee, S., Dalal, J., & Saxena, R. K. (2015). High production of erythritol from *Candida sorbosivorans* SSE-24 and its inhibitory effect on biofilm formation of *Streptococcus mutans. Bioresource Technology*, 198, 31–38.

Sasman T, Alan W, C. C. (2007). *Process for producing erythritol. 4. Pate* (Patent nt US20070037266A1).

Savergave, L. S., Gadre, R. V., Vaidya, B. K., & Narayanan, K. (2011). Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochemical Engineering Journal, 55*, 92–100.

Sawada, K., Taki, A., Yamakawa, T., & Seki, M. (2009). Key role for transketolase activity in erythritol production by *Trichosporonoides megachiliensis* SN-G42. *Journal of Bioscience and Bioengineering*, 108, 385–390.

Scientific Opinion on the safety of the proposed extension of use of erythritol (E 968) as a food additive. (2015). *EFSA Journal*, *13*(3).

Shindou, T., Sasaki, Y., Miki, H., Eguchi, T., Hagiwara, K., & Ichikawa, T. (1988). Determination of erythritol in fermented foods by high performance liquid chromatography. *Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi), 29*, 419.

Sivaraman, B., Badle, S. S., & Rangaswamy, V. (2016). Production of 1,3-butadiene from erythritol. *ChemistrySelect*, 1, 4630-4632.

Smith, D. A., Morgan, B. A., & Quinn, J. (2010). Stress signalling to fungal stress-activated protein kinase pathways. In *FEMS Microbiology Letters*, 306, 1-8.

Spencer, J. F. T., & Spencer, D. M. (1978). Production of polyhydroxy alcohols by osmotolerant yeasts. In *Economic Microbiology: Primary Products of Metabolism*, 393-425.

Stodola, F. H. (1946). A note on meso-erythritol, a metabolic product of *Aspergillus terreus*. *The Journal of Biological Chemistry*, *166*, *79*

Szczepańczyk, M., Rzechonek, D. A., Dobrowolski, A., & Mirończuk, A. M. (2021). The overexpression of yali0b07117g results in enhanced erythritol synthesis from glycerol by the yeast *yarrowia lipolytica*. *Molecules*, 26, 7549-7561.

Tokuoka, K., Sasaki, T., Taniguchi, H., & Ishizuka, H. (1992). Purification and Some Properties of an erythrose reductase from an *Aureobasidium* sp. mutant. *Bioscience, Biotechnology and Biochemistry*, 56, 941–945.

Tomaszewska, L., Rakicka, M., Rymowicz, W., & Rywińska, A. (2014). A comparative study on glycerol metabolism to erythritol and citric acid in *Yarrowia lipolytica* yeast cells. *FEMS Yeast Research*, 14, 966-976.

Tomaszewska, L., Rywińska, A., & Rymowicz, W. (2014a). High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*. *Biomass and Bioenergy*, *64*, 309–320.

Toshihiro M, Makoto S, Yoshikazu O, Tetsuo Y, K. F. (1993). *Process for preparing erythritol crystals.* (Patent EP 0 525 659 B1).

Trenner NR, B. F. (1951). Hydrogenation of tartaricacid esters to erythritol. *US2571967A*.

Troostembergh, Ignace, A.D, Willy, R. O. (2002). Process for producing and recovering erythritol from the culture medium containing the same (Patent US 2002/0127667 A1).

Wang, S., Wang, H., Lv, J., Deng, Z., & Cheng, H. (2017). Highly efficient erythritol recovery from waste erythritol mother liquor by a yeast-mediated biorefinery process. *Journal of Agricultural and Food Chemistry*, *65*, 11020–11028.

Wölnerhanssen, B. K., Cajacob, L., Keller, N., Doody, A., Rehfeld, J. F., Drewe, J., Peterli, R., Beglinger, C., & Meyer-Gerspach, A. C. (2016). Gut hormone secretion, gastric emptying, and glycemic responses to erythritol and xylitol in lean and obese subjects. *American Journal of Physiology - Endocrinology and Metabolism*, 310, E1053–E1061.

Xu, W., Liang, L., Song, Z., & Zhu, M. (2014). Continuous ethanol production from sugarcane molasses using a newly designed combined bioreactor system by immobilized *Saccharomyces cerevisiae*. *Biotechnology and Applied Biochemistry*, 61, 289–296.

Yang, L. B., Zhan, X. B., Zheng, Z. Y., Wu, J. R., Gao, M. J., & Lin, C. C. (2014). A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by *Yarrowia lipolytica* from glycerol. *Bioresource Technology*, 151, 120–127.

Yoshida H, Sugahara, T, H. J. (1984a). Studies on free sugars and free sugar alcohols of mushrooms. *Nippon Shokuhin Koyko Gakkaishi*, 31, 765–771.

Yu, J. H., Lee, D. H., Oh, Y. J., Han, K. C., Ryu, Y. W., & Seo, J. H. (2006). Selective utilization of fructose to glucose by *Candida magnoliae*, an erythritol producer. *Applied Biochemistry and Biotechnology*, 131, 870–879.

Zhang, L., Nie, M. Y., Liu, F., Chen, J., Wei, L. J., & Hua, Q. (2021). Multiple gene integration to promote erythritol production on glycerol in *Yarrowia lipolytica*. *Biotechnology Letters*, 43, 1277–87.

Zhao X, Zhang J, Liu L, Tian Y, Liu J, Han Y, H. M. (2020). *Process method for producing erythritol from co-produced wheat starch in wheat gluten production* (Patent CN111454996A).

**Chapter. 2:**

# Screening of yeast cultures and optimization of culture conditions for erythritol production

**Abstract**

The present chapter discusses an investigation into the screening of osmotolerant yeast strains for erythritol production. For screening, a liquid fermentation medium (LFM) was utilized with the subsequent composition (g/L): glucose 200, yeast extract (YE) 10, $KH_2PO_4$ 5, and $MgSO_4$ 0.25. Subsequently, *Moniliella pollinis* CBS 461.67 showed significant erythritol production than the other screened yeast cultures. *M. pollinis* was selected further to enhance erythritol production through the optimization of both medium components and environmental factors. *M. pollinis* exhibited a distinctive trait by producing erythritol as the sole metabolite, irrespective of variations in the composition of the growth medium or alterations in the culture conditions. In contrast, other yeast strains produced a limited amount of erythritol along with a spectrum of other metabolites. The primary determinants governing erythritol production in *M. pollinis* were identified as oxygen availability and nitrogen content within the medium. Given the exceptional erythritol-producing capacity observed in *M. pollinis*, the strain was further selected for a strain improvement by mutagenesis.

## 2.1 Introduction

Numerous yeast strains from genera including *Candida, Debaromyces, Hanseniaspora, Hansenula, Issatchenkia, Metschnikowia, Pichia, Trigonopsis, Yarrowia*, and *Zygosaccharomyces* have demonstrated the ability to thrive in environments which are characterized by reduced water content, usually occurring during elevated levels of sugar or salt. To cope with the challenges posed by osmotic stress, these sugar or salt-tolerant yeasts employ a strategy of accumulating compatible solutes. In addition to their function in osmoregulation, these organisms employ osmolytes for various purposes, including acting as reservoirs for energy sources (Shen et al., 1999; Diano et al., 2006). While glycerol stands as the most prevalent osmolyte produced by yeasts, there is substantial evidence supporting the production of various sugar alcohols, including arabitol, erythritol, mannitol, and xylitol, as alternative osmolytes (Kogej et al., 2007).

The predominant focus of research endeavors in erythritol production has primarily revolved around the selection of microbes capable of achieving high yields and productivity in erythritol synthesis. Subsequently, these studies have aimed to optimize process parameters in both batch and fed-batch fermentation systems. Only a limited number of researchers have achieved success in isolating microorganisms from their respective environmental niches, including pollen, honeycomb, high-sugar-containing fruits, and various food products. These microorganisms exhibit the unique ability to produce erythritol as the exclusive polyol at significantly higher concentrations. The application of these exceptional strains in industrial erythritol production has been realized through the optimization of strain cultivation and fermentation conditions, as demonstrated by Jeya et al. (2009). In an another study, an erythritol-producing yeast-like fungus was isolated from pollen in an extremely osmotic environment containing 35% glucose (Hajny et al., 1964). Subsequently, researchers determined that this yeast-like fungus *Moniliella tomentosa var. pollinis*. Likewise, Ishizuka et

al. (1989) conducted thorough research into the isolation of erythritol-synthesizing yeast strains. They examined an extensive assortment of 1700 isolates derived from Okinawa, Japan's soil, and effectively-identified *Aureobasidium* SN 124. This strain showcased a noteworthy ability to produce erythritol. This yeast strain was then subjected to physical and chemical mutagenesis, and it is currently employed for commercial erythritol production in Japan. In large-scale 100,000 L fermentors, the mutant *Aureobasidium* sp. SN-G42 has been shown to achieve remarkable yields and production rates of erythritol, reaching 47% and 2.0 g/L/h, respectively (Sawada et al., 2009). Since these seminal findings, numerous researchers have embarked on extensive searches for microorganisms capable of producing erythritol, further advancing the understanding and application of erythritol-producing microorganisms.

Hirata et al. (1999), conducted an extensive screening effort encompassing over 2000 isolates derived from diverse sources, including soil, seawater, plants, and fermented foods. From this collection, they identified 20 erythritol-producing isolates, with one specific isolate (designated as strain 618A-01) standing out by producing 75 g/l of erythritol over a period of 930 hours, utilizing a substrate of 200 g/l glucose. Subsequent analysis of the 18S rRNA sequence of this strain verified its classification within the genus *Ustilago* in the class *Ustilaginomycetes*. In a separate study, 1753 strains were isolated from honey and pollen samples, among these strains, only three exhibited erythritol-producing capabilities, consisting of one *Trichosporon* strain and two strains belonging to the *Trichosporonoides* genus (Park et al., 1996). Meanwhile, Lin et al., (2001) investigated the samples from various origins, including honey, beehives, pollens, preserved fruits, fresh fruits, and wastewater from sugar manufacturing processes in Taiwan for erythritol producing yeasts. From a pool of 658 isolates, they identified six strains that displayed notable erythritol production abilities. Of these six strains, *Moniliella* sp. 440 exhibited the highest erythritol production of 116 g/l of erythritol with an 39% yield in a medium containing 30% glucose. This particular strain underwent mutagenesis via multiple

rounds of NTG treatment, leading to the development of a mutant designated as N61188-12, which demonstrated remarkable erythritol production capabilities achieving 237.8 g/l of erythritol in a medium containing 400 g/l glucose (Lin et al., 2010).

The literature extensively documents the synthesis of erythritol in complex media utilizing glucose as the primary carbon substrate. Commonly investigated carbon sources include glucose, sugarcane juice, molasses, glycerol, and xylose.

In a separate study, Jeya et al., (2009), isolated a new erythritol-synthesizing yeast strain, *Pichia tsukubaensis* KN75. Under aerobic conditions in a fed-batch culture employing glucose as the carbon substrate, this strain demonstrated much better erythritol production, yielding 245 g/l with a productivity of 2.86 g/l/h and a yield of 61%. This achievement represents the highest documented erythritol production by any microorganism involved in erythritol synthesis to date. The scale-up of erythritol production was successfully realized, progressing from a lab-scale 7L fermenter to a pilot-scale 300 L system and ultimately to a large-scale 50,000L plant, employing dissolved oxygen as a key scale-up parameter. The yeast culture was officially deposited as *Pichia tsukubaensis* KCCM 10356 in the Korean Culture Collection of Microorganisms (KCCM). While the precise regulation of the pentose phosphate pathway (PP pathway) in osmophilic yeasts remains an area of ongoing research, it is understood that the fermentative production of erythritol is influenced by various factors, including the osmotic pressure of the medium, pH, dissolved oxygen levels, nitrogen sources, and the type of carbohydrates employed (Burschäpers et al., 2002).

The majority of scientific publications and patents have focused on the isolation of various osmophilic yeast strains or mutants with enhanced erythritol yield and productivity. An essential prerequisite for the establishment of an economically feasible way is the identification of strains that exhibit product output and efficiency while reducing the generation of interfering

metabolites. To optimize erythritol production through fermentation, an extensive investigation was conducted into the constituents of the growth medium. It was determined that a high carbon-to-nitrogen (C: N) ratio is a critical parameter for achieving elevated erythritol yields (Kim et al., 2000; Ghezelbash et al., 2014a). Researchers commonly employed elevated glucose concentrations within the range of 100-300 g/L as the primary carbon source. In this context, yeast extract emerged as the frequently utilized nitrogen source. In addition to yeast extract, researchers explored diverse mixtures of organic and inorganic nitrogen substrates to enhance erythritol production. It is noteworthy that the initiation of erythritol biosynthesis generally takes place towards the conclusion of the growth phase, coinciding with the depletion of the nitrogen source in the medium, emphasizing the essentiality of nitrogen limitation for this process. However, it should be noted that nitrogen limitation often leads to the challenge of foam formation, which can be difficult to control. Certain publications have discussed strategies for carefully managing carbon and nitrogen content to mitigate foam formation (Burschäpers et al., 2002).

An intriguing approach to erythritol production involves the use of cost-effective carbon sources such as jaggery, molasses, and sugarcane juice in fermentation media. These alternative carbon sources offer distinct advantages, including their ready availability and affordability compared to conventional sugars like glucose or sucrose. Moreover, these carbon sources encompass a diverse array of nutrients and organic compounds that can facilitate microbial growth and support product formation during the fermentation process. Exploring the potential of jaggery, molasses, and sugar cane juice as a carbon source can contribute to the development of sustainable and economically viable fermentation strategies. (Savergave et al., 2011; Kobayashi et al., 2015; Mirończuk, Dobrowolski, et al., 2015; Mirończuk, Rakicka, et al., 2015; Sivaraman et al., 2016; Deshpande et al., 2022). Sugarcane juice, apart from containing sucrose, glucose, and fructose, is abundant in diverse nutrients that facilitate microbial growth.

These nutrients include magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), iron (Fe), and phosphorus (P), in concentrations ranging from 5 to 500 ppm levels. Furthermore, sugarcane juice contains essential vitamins, phenolic compounds, and enzymes, which contribute to its nutritional value and potential benefits in fermentation processes. Molasses, on the other hand, is a thick, viscous syrup with a dark colour and distinctive taste. It is a secondary product acquired through the sugar refining process from the juice of sugarcane or sugar beet. Molasses is well-known for its high concentration of non-crystallizable sugars, such as sucrose, glucose, and fructose. Additionally, molasses contains organic acids, minerals, vitamins, and antioxidant compounds, which contribute to its composition and potential utilization in fermentation. The precise composition of molasses can vary depending on factors such as the method of sugar processing and the source of the raw material used. Jaggery, a product of the sugarcane industry is another carbon source broadly utilized in the Indian context as a replacement for sucrose. Besides sucrose, it also contains protein, vitamins (A, B1, B2, B5, B6, C, D2, E), and minerals (iron, copper, magnesium, potassium, phosphorous, and sodium). Jaggery is available in solid (lumped), liquid, and granular forms (Nath, 2015). Over 70% of the world's jaggery is produced in India (Jagannadha Rao et al., 2007), with sucrose concentrations varying from 75-85% (Vijayendra et al. 2001). The analysis of jaggery, prepared from sugar cane (100 g), revealed its nutritional composition. The carbohydrate content was found to be range from 72 to 78 g, with sucrose being the predominant component and fructose and glucose present at 1.5 to 7 g. The mineral composition included calcium (40-100 mg), magnesium (70-90 mg), phosphorus (20-90 mg), sodium (19-30 mg), iron (10-13 mg), manganese (0.2-0.4 mg), zinc (0.2-0.4 mg), and chloride (5.3-0.0 mg), along with trace amounts of other minerals (0.1-0.9 mg). The vitamin content of jaggery comprised vitamin A (3.8 mg), vitamin B2 (0.06 mg), vitamins B1, B5, and B6 (0.01 mg each), vitamin C (7.00 mg), vitamin D2 (650 mg), and vitamin E (111.30 mg). Furthermore, jaggery contained a significant

amount of protein, approximately 280 mg (Singh J 2013). Reports on the use of jaggery for erythritol production in commercial-scale fermentation processes are scarce.

Using information gathered from a review of the literature, a liquid fermentation medium was formulated for the assessment of cultures in terms of erythritol production. This chapter outlines the evaluation of yeasts with high sugar tolerance from a culture collection for their erythritol-producing capabilities, the identification of medium components (with a focus on affordable carbon substrates other than glucose), and the determination of optimal environmental conditions to enhance erythritol production.

## 2.2 Materials and methods

### 2.2.1 Microorganisms

The production of erythritol by yeast involves a multi-step metabolic pathway, primarily advancing through the pentose phosphate pathway. Thirty yeast cultures, sourced from various genera including *Candida, Debaromyces, Hanseniaspora, Hansenula, Issatchenkia, Metschnikowia, Trigonopsis, Moniliella,* and *Yarrowia*, were acquired from esteemed repositories such as the National Collection of Industrial Microorganisms (NCIM) and the Microbial Type Culture Collection (MTCC) in India, the American Type Culture Collection in the USA, and the Central Bureau of Fungal Cultures (CBS) in the Netherlands.

### 2.2.2 Culture conditions

The liquid fermentation medium (LFM) described in the literature consists of the following components (g/L): Yeast extract, 10; $K_2HPO_4$, 5; $MgSO_4.7H_2O$, 0.25, final pH 7.5 supplemented with 200 g/L glucose used for shake flask experiment using 10% v/v inoculum (Savergave et al., 2011). All 30 cultures were inoculated into YPD media, 50 ml Erlenmeyer flasks with 12.5 ml YPD media. Flasks were incubated for 24hrs at 28°C, 200RPM. Following

a 24 h incubation period, all cultures were assessed for the production of polyols. This was achieved by inoculating each culture with a 10% inoculum ($OD_{A600nm}$: ~ 12 to 15 and CC: ~ 40 to 60 million cells/ ml) in 250 ml Erlenmeyer flasks containing 22.5 ml of the liquid fermentation medium (LFM). The flasks were then incubated at 28°C, with continuous shaking at 200 RPM, for a duration of 72 h. At different intervals, samples were taken, and their biomass and erythritol concentrations were analyzed. The schematic diagram (Fig. 2.1) represents the experimental protocol that is followed throughout the work. All the components of the culture medium were procured from HiMedia, located in Mumbai, India.

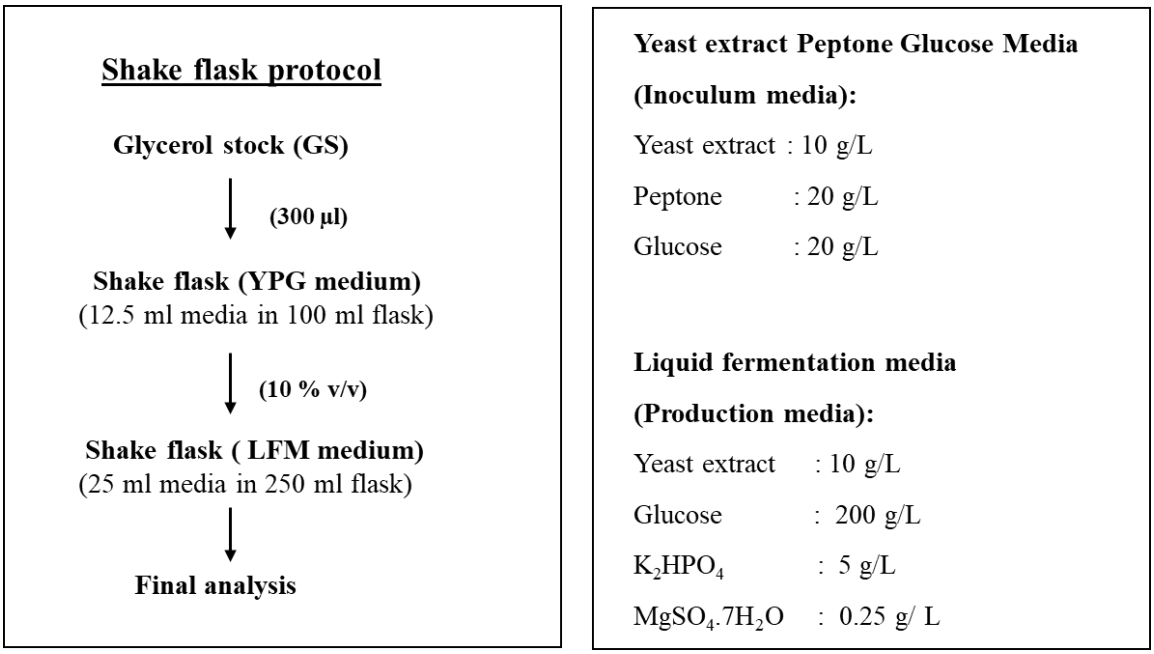



**Figure 2.1** Experimental protocol and media composition

### 2.2.3 Analysis

A spectrophotometer was used for optical denisity (OD) measurement at 600nm. Quantification of glucose, fructose, sucrose, erythritol, glycerol and ethanol was carried out by HPLC with an Aminex HPX-87H, 300 mm × 7.8 mm column (Bio-Rad, USA).

The elution process employed a mobile phase of 5 mM $H_2SO_4$ flowing at a rate of 0.6 m/min, and the temperature was maintained at 35°C. Samples were introduced into the system via an autosampler injector with a fixed volume of 20 µl. Detection of sugars and other metabolites was performed using a refractive index detector (Agilent HPLC 1260 series). A mixed standard curve was prepared using all sugars and polyols from 125, 250, 500, and 1000 ppm concentrations. To obtain erythritol yield ($Y_{ERY}$ %), the following formula was used:

$$\text{YERY yield (\%)} = \frac{\text{Erythritol produced (g/L)}}{\text{Carbon source consumed (g/L)}} \text{ X (100)}$$

### 2.2.4 Screening of cultures for the production of erythritol

Cultures obtained from diverse culture collections underwent screening for erythritol production. This screening involved inoculating the respective cultures into 250 ml Erlenmeyer flasks containing 25 ml of liquid fermentation medium (LFM), followed by incubation at 28°C for 72 h. Samples were withdrawn at the end of fermentation (72 h) and analyzed for biomass, pH levels, and erythritol concentration.

### 2.2.5 Screening of media components for erythritol production for selected strain

Shake flask experiments were conducted to assess the impact of various media components on erythritol production by *M. pollinis*. For inoculum preparation, the wild-type strain *M. pollinis* was first grown in YPG liquid media, following the previously described methodology. Following an 18 h incubation period, a 2.5 mL inoculum (10% v/v) was transferred into a 250 ml flask, which contained 22.5 ml of LFM. The nature and concentration of the nitrogen source are crucial parameters, although there are inconsistencies in the results obtained regarding the

optimal conditions. In the case of *M. pollinis*, certain studies identified organic nitrogen sources, such as yeast extract, as the most suitable for erythritol production. The influence of YE on erythritol production by *M. pollinis* was evaluated in shake flasks. The influence of various pH (3.5, 4.5, 5.5, 6.0, and 6.5) on erythritol production was observed by inoculating cultures in media having different pH values. The pH of the medium was adjusted using 5N NaOH. Additionally, the mutant strain was subjected to incubation at various temperatures (25°C, 28°C, 30°C, and 37°C) to evaluate the potential improvement in erythritol yield. After screening media components yeast strain was used for erythritol production using a cost-effective carbon substrate. The effect of metal ions on erythritol production was checked by using various metal ions (Fe, Mn, Zn, cu, ca, and Co) at different concentrations (10, 50, and 100ppm) using *M. pollinis* strain. In the LFM media, glucose was replaced with other inexpensive carbon substrates *viz* jaggery and sugarcane juice to study the effect on erythritol production by *M. pollinis* mutant-58. Sugarcane juice was obtained from a local market (Pune) and evaporated in a rotary vacuum evaporator. The concentrated syrup was utilized in the experiment as per requirement. Molasses was obtained from a sugar cane factory near Pune. The carbon sources were used in the range of 200 g/L. The detailed composition of sugar-based substrates (quantified using HPLC) is provided in Table 2.1.

**Table 2.1** Sugar composition of carbon substrates

| Carbon | Sucrose (%) | Glucose (%) | Fructose (%) | Total sugar (%) |
|---|---|---|---|---|
| **Glucose** | Nil | 99.8±0.8 | Nil | 99.8±0.8 |
| **Jaggery** | 78.5±3.8 | 11.2±3.1 | 5.8±2.1 | 95.5±9 |
| **Sugarcane Juice** | 10.2 ±1.2 | 2.27±0.8 | 0.55±0.5 | 13.02±2.5 |

Results are means ± S.D. for triplicate experiments

Respective media were inoculated with 18h seed culture into 250 mL Erlenmeyer flasks with 22.5 mL LFM medium with various carbon sources having 6.67 moles carbon concentration (6.67 mole which is equivalent to 200g/L glucose) and incubated at 28°C, 180 RPM in the shaking incubator. Samples were examined at 72h for optical (OD) density, pH, residual glucose, and polyol concentrations. Each test was performed in triplicates. Sugarcane juice first concentrated in a rotary vacuum evaporator to make the total sugar concentration of 50%. Then it was autoclaved for further use. Three different substrates were used for erythritol production in LFM medium as mentioned below,

**Liquid fermentation media (LFM-I):** Yeast extract, 10 g/L; **Glucose,** 200 g/L; $K_2HPO_4$, 5 g/L; $MgSO_4.7H_2O$, 0.25 g/ L

**Liquid fermentation media (LFM-II):** Yeast extract, 10 g/L; **Sugarcane juice** (Sucrose+ Glucose+ Fructose) 200 g/L; $K_2HPO_4$, 5 g/L; $MgSO_4.7H_2O$, 0.25 g/ L

**Liquid fermentation media (LFM-III):** Yeast extract, 10 g/L; **Jaggery** (Sucrose+ Glucose+ Fructose) 200 g/L; $K_2HPO_4$, 5 g/L; $MgSO_4.7H_2O$, 0.25 g/ L

A 300 µl aliquot of the culture glycerol stock was seeded into 12.5 ml of YPD medium within a 100 ml Erlenmeyer flask. Flasks were incubated for 24 hrs at 28°C, 200 RPM. After 24 hrs, 2.5 ml of inoculum was used to inoculate 22.5 ml (SF-2) LFM in 250 ml Erlenmeyer flask. Flasks were incubated for 72 hrs at 28°C, 200 RPM. Samples were withdrawn at various time points and analyzed for biomass, and erythritol concentration.

## 2.3 Results and discussion

### 2.3.1 Screening of cultures for erythritol production

Screening of microbial strains for their performance in erythritol production and identifying the most suitable environmental conditions was carried out. This thorough evaluation is crucial

in shaping the advancement of an integrated technological solution for erythritol production through biological processes. The primary goals were to isolate and characterize erythritol-synthesizing microorganisms, subjecting these strains to diverse culture conditions, and subsequently choosing the most promising strains for the mutation process to achieve preferred enhancement. The High-Performance Liquid Chromatography (HPLC) analysis method employed in this study demonstrated its ability to effectively separate and quantify key metabolites, including sucrose, fructose, erythritol, ethanol, glucose, and glycerol. All the metabolites generated during the fermentation process were separated and quantified with baseline resolution. The chromatograms presented in Fig. 2.2 include those of reference compounds as well as a representative sample of the fermentation broth, clearly illustrating the separation of sugars and erythritol.

One of the pioneering studies in the field of microbial erythritol production was conducted by Hajny et al. (1064). Subsequently, research has expanded to encompass a wide range of microbial strains evaluated for their erythritol-producing capabilities across diverse cultures and environments. Although the research on microbial erythritol production remains relatively limited, it is gaining significance due to the escalating demand for erythritol within the food industry. Newly isolated strains and their derivatives have the potential to compete effectively with existing industrial production processes. Furthermore, the optimization of various fermentation techniques holds promise for enhancing productivity and yield, as demonstrated by Jeya et al. ( 2009).

In a majority of prior screening investigations, the rate of success in isolating an erythritol-producing strain from the overall microbial isolates was notably low, typically falling within the range of 0.6-6% of the total osmophilic yeasts isolated. Aoki et al. (1993) identified 402 strains of osmophilic yeasts, among which 5.47% of the isolates demonstrated erythritol production. In contrast, Park et al. (1996) isolated 1752 osmophilic yeasts from honey and

pollens, with only 11 of the isolates (0.6%) displaying the ability to synthesize erythritol. Considering the low likelihood of acquiring erythritol-producing strains from their natural habitat, a decision was made to screen yeast strains with sugar and salt tolerance available at different culture collection centers.

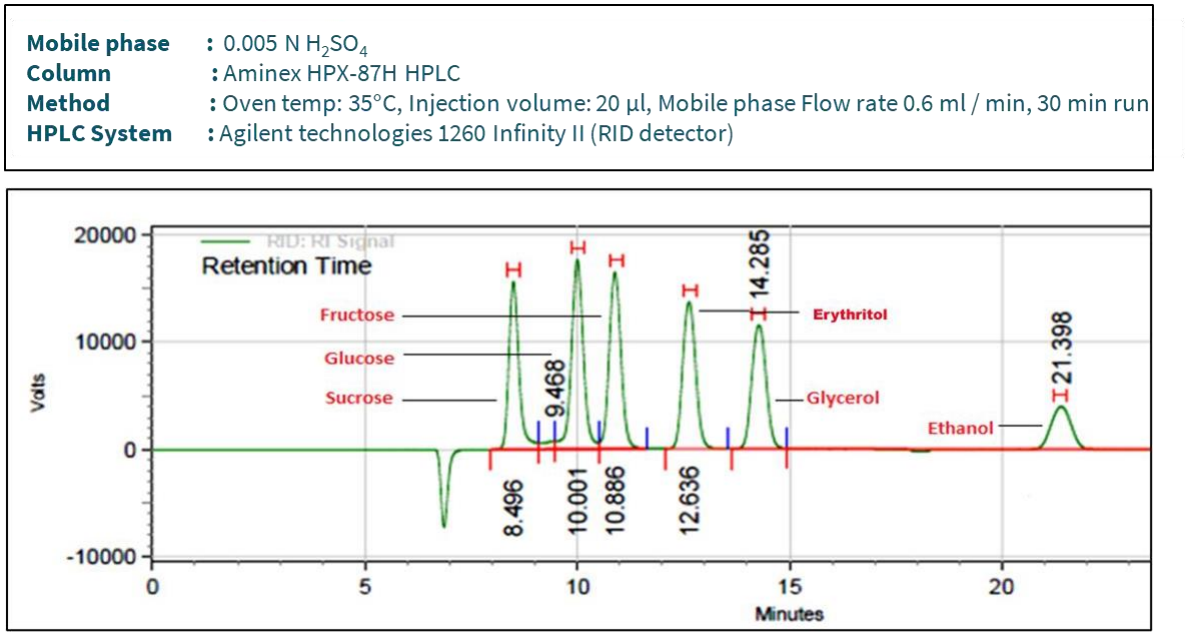



**Figure 2.2** Ion exclusion HPLC chromatogram of standard compound mixture

The selection of cultures was based on their documented capacity to thrive in media with elevated salt or sugar concentrations, as reported in the existing literature. Upon screening thirty osmophilic yeast strains obtained from NCIM, CBS, and MTCC, four yeast strains were identified as erythritol producers in the liquid fermentation medium (LFM) (Table 2.2).

**Table 2.2** Screening of yeast cultures from collection centers and isolates for erythritol production in LFM

| Sr. No. | Yeast cultures and culture isolates | Erythritol (g/L) |
|---|---|---|
| **1** | *Candida shehatae ATCC 34887* | ND |

| | | |
|---|---|---|
| **2** | *Candida tropicalis NCIM 3119* | 0.5±0.2 |
| **3** | *Pachysolen tannophilus ATCC 32691* | ND |
| **4** | *Saccharomyces cerevisiae NCIM 3090* | ND |
| **5** | *Saccharomyces cerevisiae NCIM 3204* | ND |
| **6** | *Saccharomyces cerevisiae NCIM 3095* | ND |
| **7** | *Pichia kudriavzevii MTCC 10344* | ND |
| **8** | *Yamadazyma farinosa ATCC 2021* | ND |
| **9** | *Cryptococcus curvatus ATCC 20508* | ND |
| **10** | *Yarrowia lipolytica ATCC 2613* | 7.6±0.8 |
| **11** | *Moniliella pollinis CBS 461.67* | 30.1±2.8 |
| **12** | *Candida magnoliae ATCC 56463* | 12.1±1.5 |
| **13** | *Yarrowia lipolytica NCIM 3472* | 9.8±0.9 |
| **14** | *Cybealindnera sp.* | ND |
| **15** | *Saccharomyces sp.* | ND |
| **16** | *Meyeaozyma sp.* | ND |
| **17** | *Toaulaspoda sp* | ND |
| **18** | *Stadmeua sp.* | ND |
| **19** | *Saccharomyces sp.* | ND |
| **20** | *Papilioteama sp.* | ND |
| **21** | *Hanseniaspora sp.* | ND |
| **22** | *Hanseniaspora sp.* | ND |
| **23** | *Cyberlindnera sp.* | ND |
| **24** | *Pichia sp.* | ND |
| **25** | *Pichia sp.* | ND |

| | | |
|---|---|---|
| **26** | *Pichia sp.* | ND |
| **27** | *Pichia sp.* | ND |
| **28** | *Zygoasas sp.* | ND |
| **29** | *Candida sp.* | ND |
| **30** | *Pichia sp.* | ND |

*ND: Not detected

**Table 2.3** Screening of selected erythritol-producing yeast cultures

| **Strain \Time (h)** | **24** | **48** | **72** | **96** | **120** | **144** | **168** |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* ATCC 2613 | 0 | 2.7±0.8 | 7.4±0.9 | 7.5±0.5 | 7.5±0.7 | 7.4±1.0 | 7.2±0.8 |
| *Y. lipolytica* NCIM 3472 | 0.7±0.2 | 2.2±0.5 | 8.6±1.1 | 8.6±1.3 | 8.4±1.1 | 8.3±0.6 | 8.3±1.1 |
| *M. pollinis* CBS 461.67 | 8.8±0.3 | 16.6±0.9 | 23.9±2.5 | 24±0.8 | 24.1±0.8 | 24.6±1.4 | 24.6±2.7 |
| *C. magnoliae* ATCC 56463 | 0 | 0 | 0.3±0.1 | 0.6±0.3 | 1.4±1.1 | 4.2±0.9 | 4.3±0.9 |
| **Repeat experiment** | | | | | | | |
| **Strain \Time (h)** | **24** | **48** | **72** | **96** | **120** | **144** | **168** |
| *Y. lipolytica* ATCC 2613 | 0.3±0.1 | 2.1±0.5 | 7.2±0.5 | 7.8±1.1 | 7.6±1.1 | 7.4±0.8 | 7.4±1.3 |
| *Y. lipolytica* NCIM 3472 | 0.4±0.2 | 1.9±0.4 | 7.9±1.2 | 8.1±1.6 | 8.0±2.5 | 8.0±1.1 | 7.9±1.8 |
| *M. pollinis* CBS 461.67 | 9.4±0.1 | 17.6±0.7 | 28.8±2.6 | 30.1±2.3 | 27.2±2.0 | 27.2±2.8 | 27.2±3.1 |
| *C. magnoliae* ATCC 56463 | 0.3±0.1 | 0.7±0.2 | 0.8±0.5 | 0.9±0.4 | 0.9±0.4 | 0.9±0.4 | 0.9±0.6 |

**Note:** Erythritol production presented in g/L unit

Hence, the strategy of choosing yeast strains from culture collections based on their osmotolerance proved to be effective. Within the subset of erythritol producers, *Y. lipolytica* NCIM 3472, *Yarrowia lipolytica ATCC 2613, C. magnoliae* NCIM 3470, and *C. tropicalis NCIM 3119* produced 9.8, 8.2, 4.3 and 1.1 g/l erythritol, respectively. These four yeast cultures

were subsequently employed to investigate erythritol production. Among 30 cultures tested for erythritol production, *M. pollinis* CBS 461.67 gave the highest erythritol production after 168 hrs of incubation (30.1 g/L). Moreover, four cultures namely, *Y. lipolytica* NCIM 3472, *Yarrowia lipolytica ATCC 2613, C. magnoliae* NCIM 3470, and *M. pollinis* CBS 461.67 gave prominent erythritol production further tested for erythritol production experiment in shake flak for confirmation (Table 2.3). The results showed *M. pollinis* CBS 461.67 to be the highest erythritol-producing strain amongst the screened cultures and was selected for further studies. It was also observed that the fermentation time required for *Moniliella pollinis* CBS 461.67 to ferment 20 % glucose into products was 72h.

The findings from this study unequivocally identified *Moniliella pollinis* CBS 461.67 as the most proficient erythritol-producing strain among the various cultures assessed. Given its excellent performance, it was chosen for further in-depth analysis. Furthermore, it was observed that *Moniliella pollinis* CBS 461.67 displayed a remarkable efficiency in fermenting 20% glucose into its corresponding products. Specifically, this yeast strain achieved the conversion of 20% glucose to desired products within a relatively short fermentation period of 72 h. These initial findings emphasize the potential of the strain as a promising candidate for erythritol production.

### 2.3.2 Screening of media components for erythritol production for selected strain

#### 2.3.2.1 Effect of YE concentration

In addition to the choice of carbon source, it is widely acknowledged that the origin and concentration of nitrogen in the growth medium typically exert the most significant influence on both yeast growth and erythritol production. A fundamental requirement for erythritol biosynthesis is the limitation of nitrogen availability, as the onset of erythritol production generally coincides with the exhaustion of nitrogen sources within the medium. Within the

context of *M. pollinis*, previous studies have indicated that organic nitrogen source, such as yeast extract (YE), is particularly conducive to erythritol production. Therefore, in this study, the impact of varying concentrations of yeast extract (YE) on erythritol formation by *M. pollinis* through shake flask experiments was investigated. This exploration is aimed at shedding light on the optimal conditions for erythritol production. The impact of yeast extract (YE) was assessed at varying concentrations, specifically 2, 4, 6, 8, and 10 g/L YE, followed by a subsequent assessment covering a range of concentrations from 1 to 10 g/L.

Among the various concentrations of YE screened, *M. pollinis* exhibited robust growth and erythritol production with all nitrogen sources, as detailed in Table 2.4. In particular, the medium containing 4 g/L of yeast extract (YE) yielded the highest erythritol production at 78.95 g/L, surpassing the outcomes obtained with other YE concentrations. In *Y. lipolytica*, research has demonstrated varying preferences for nitrogen sources in erythritol production. Some studies, such those by Tomaszewska et al. (2014), identified organic nitrogen sources like yeast extract as the most suitable. In contrast, others, including Ghezelbash et al. (2014) and Rakicka et al. (2016), reported better results with inorganic nitrogen sources such as ammonium sulfate.

**Table 2.4 Optimization of yeast extract concentrations on erythritol production**

| YE (g/L) | Substrate | Substrate (%) | Culture system | Volume (ml) | Erythritol (g/L) | Erythritol (%) | $Y_{ERY}$ (%) | Glycerol (g/L) | Ethanol (g/L) |
|---|---|---|---|---|---|---|---|---|---|
| **2** | Glucose | 19.4 | Batch | 25 | 49.8±1.5 | 5.0 | 25.5 | 6.40±.7 | 10.0±1.5 |
| **4** | Glucose | 17.9 | Batch | 25 | 78.9±2.1 | 7.9 | 43.9 | 3.85±0.4 | 11.1±2.2 |
| **6** | Glucose | 20.3 | Batch | 25 | 77.5±1.1 | 7.8 | 38.1 | 3.80±0.5 | 27.7±1.3 |
| **8** | Glucose | 18.5 | Batch | 25 | 71.2±3.2 | 7.1 | 38.3 | 2.20±0.4 | 28.5±1.2 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **10** | Glucose | 18.0 | Batch | 25 | 68.3±1.4 | 6.8 | 37.8 | 2.25±0.2 | 25.6±1.0 |

**Note:** Ery: Erythritol, $Y_{ERY}$: yield, $Q_{ERY}$: productivity

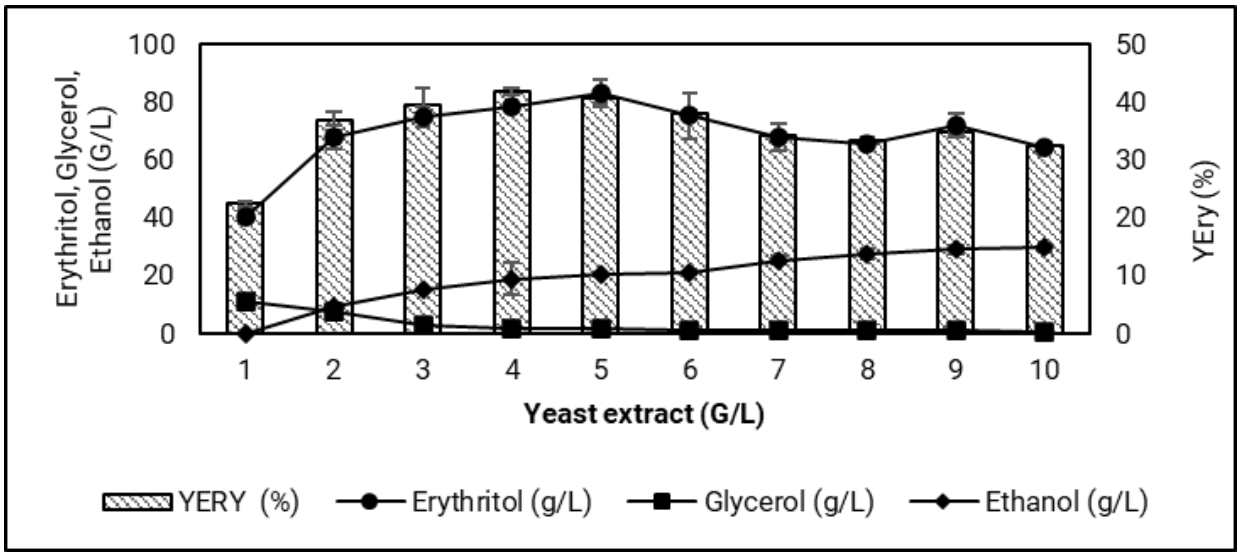


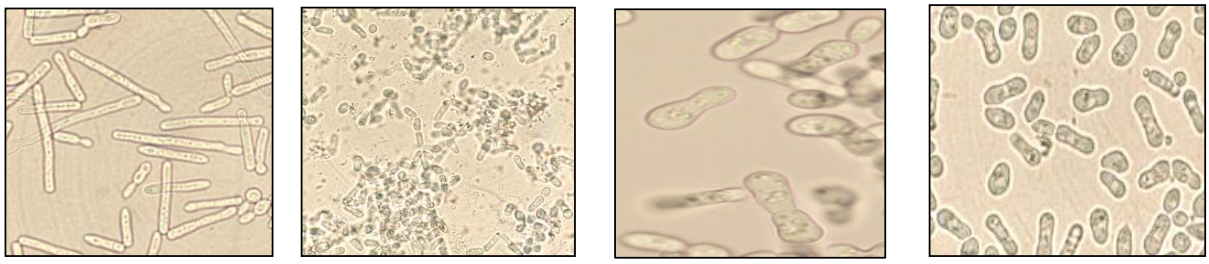

**Figure 2.3** Optimization of yeast extract concentrations on erythritol production

The investigation into the impact of yeast extract (YE) concentrations on the fermentation process revealed intriguing findings. Specifically, when higher YE concentrations ranging from 6 to 8 g/L were employed, a substantial quantity of ethanol production, typically falling within the range of 25-27 g/L, was observed. However, a noteworthy reduction in ethanol production was observed when the YE concentration was decreased from 6 g/L to 4 g/L. To ascertain the optimal YE concentration conducive to erythritol production, an additional experiments were conduscted, covering a broader range of YE concentrations ranging from 1 to 10 g/L. The outcomes of these supplementary experiments are presented in Fig. 2.3. Upon repeating the experiments with varying YE concentrations, it became evident that the YE concentration of 4 g/L showed the optimum level for the highest erythritol yield. This observation underscores the critical role of YE concentration in influencing the erythritol

production process, and it emphasizes the significance of precise optimization in biotechnological studies.

### 2.3.2.2 Screening of alternative low-cost substrates for erythritol production

The synthesis of erythritol from glucose is a process that has already been firmly established. However, the high cost of glucose is a deterrent to commercialization. A decrease in manufacturing expenses could be attained by using alternative substrates like molasses, crude glycerol, agriculture waste, etc. For evaluating the alternative low-cost substrates for erythritol production, jaggery, and sugar cane juice were tested. Both substrates contain high sugar concentrations.



**a)** **b)** **c)** **d)**

**Figure 2.4** Change in the morphology with respect to media change after 48 h, a) Inoculum (glucose), b) Sugarcane juice, c) Jaggery, d) Glucose

Microscopic examination of *M. pollinis* provided intriguing insights into its morphology and its correlation with erythritol production. Notably, *M. pollinis* exhibited a dimorphic nature, and there appeared to be a discernible relationship between the size and shape of the cells and their erythritol production capabilities. When *M. pollinis* was cultured in a YPG medium, it adopted a filamentous shape, but as it transitioned into a glucose-rich environment for erythritol production, a notable transformation occurred, leading to a shift towards an oval-shaped morphology. This dynamic alteration in yeast cell shape and size during the transition from YPG to glucose-rich conditions was a striking observation (Fig. 2.4 a, d).

Furthermore, a substantial contrast in cell morphology was observed between cells cultivated in glucose, jaggery, and sugarcane juice media. In the case of jaggery, yeast cells assumed smaller dimensions and exhibited a distinct shape, whereas cells grown in glucose and jaggery displayed similar morphological characteristics (Fig. 2.4 a, b). This intriguing observation of differential cell morphology coincided with the observed variations in erythritol production. Specifically, enhanced erythritol production was evident in both glucose and jaggery media compared to sugarcane juice. These findings suggest that sugarcane juice may contain inhibitory constituents that hinder optimal yeast cell growth, consequently impacting erythritol production. This multifaceted relationship between yeast morphology, carbon source, and erythritol production underscores the complex interplay of factors influencing microbial bioprocesses and warrants further investigation to elucidate the underlying mechanisms.

Our research encompassed a series of experiments aimed at exploring cost-effective carbon sources for erythritol production utilizing the *M. pollinis* CBS 461.67 strain. A comprehensive summary of the outcomes obtained from these experiments is presented in Table 2.5, providing valuable insights into the performance of *M. pollinis* under varying carbon sources.

**Table 2.5** Summary of erythritol production from different sugars using *Moniliella pollinis*

| Exp. No. | Substrate | Substrate (%) | Culture system | Erythritol (g/L) | Erythritol (%) | $Y_{ERY}$ (%) | $Q_{ERY}$ (g/L/h) | Byproducts | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Glycerol (g/L) | Ethanol (g/L) |
| E-1 | Glucose | 19.25 | Batch | 24.48±1.5 | 2.4 | 12.7 | 0.34 | 7.7±1.1 | 79.4±3.5 |
| E-2 | Glucose | 20.75 | Batch | 20.95±1.8 | 2.1 | 10.1 | 0.29 | 6.0±0.5 | 55.7±2.8 |
| E-3 | Glucose | 23.09 | Batch | 68.70±3.2 | 6.9 | 29.8 | 0.95 | 4.7±0.4 | 42.0±1.2 |
| | | 24.00 | Fed-Batch | 70.20±1.9 | 7.0 | 29.3 | 0.98 | 4.3±0.5 | 35.2±1.9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| E-4 | Glucose | 21.4 | Batch | 69.1±1.8 | 6.9 | 32.3 | 0.96 | 2.8±0..5 | 40.2±2.5 |
| | Sucrose (Sugarcane juice) | 27.5 | Batch | 57.0±2.2 | 5.7 | 20.7 | 0.79 | 1.3±0.2 | 54.1±3.8 |
| | Sucrose (Jaggery) | 25.9 | Batch | 58.5±3.1 | 5.9 | 22.6 | 0.81 | 2.4±1.0 | 52.8±3.5 |
| E-5 | Glucose | 23.11 | Batch | 89.8±3.2 | 9.0 | 38.9 | 1.25 | 2.8±0.3 | 37.2±2.2 |
| | Sucrose (Jaggery) | 19.34 | Batch | 76.4±2.5 | 7.6 | 39.5 | 1.06 | 1.5±20.4 | 33.1±2.1 |
| E-6 | Glucose | 17.63 | Batch | 64.5±1.9 | 6.5 | 36.6 | 0.90 | 2.1±0.6 | 25.2±2.8 |
| | Sucrose (Jaggery) | 19.88 | Batch | 56.1±2.0 | 5.6 | 28.2 | 0.78 | 1.3±0.8 | 21.5±2.5 |
| E-7 | Glucose | 18.44 | Batch | 66.6±1.8 | 6.66 | 36.1 | 0.93 | 2.9±0.6 | 32.3±1.3 |
| | Sucrose (Jaggery) | 19.48 | Batch | 66.4±2.1 | 6.6 | 34.1 | 0.92 | 1.3±0.3 | 32.4±1.2 |

**Note:** Ery: Erythritol, $Y_{ERY}$: yield, $Q_{ERY}$: productivity

Notably, the results consistently revealed that *M. pollinis* strain exhibited a consistent fermentation time of 72 hours for the conversion of 20% glucose into erythritol, as explained earlier. Furthermore, we explored the feasibility of employing jaggery as a viable and cost-effective alternative substrate to glucose. Our findings, as illustrated in Table 2.5, indicate that jaggery indeed serves as a promising substitute, with erythritol production levels comparable to those achieved with glucose. This observation underscores the potential use of jaggery as a readily available and economical carbon source for erythritol production, offering economic advantages in biotechnological processes.

To investigate the potential impact of glucose pulsing on erythritol production, the experiment was conducted for a comparative study using the *M. pollinis* strain. In this experimental design,

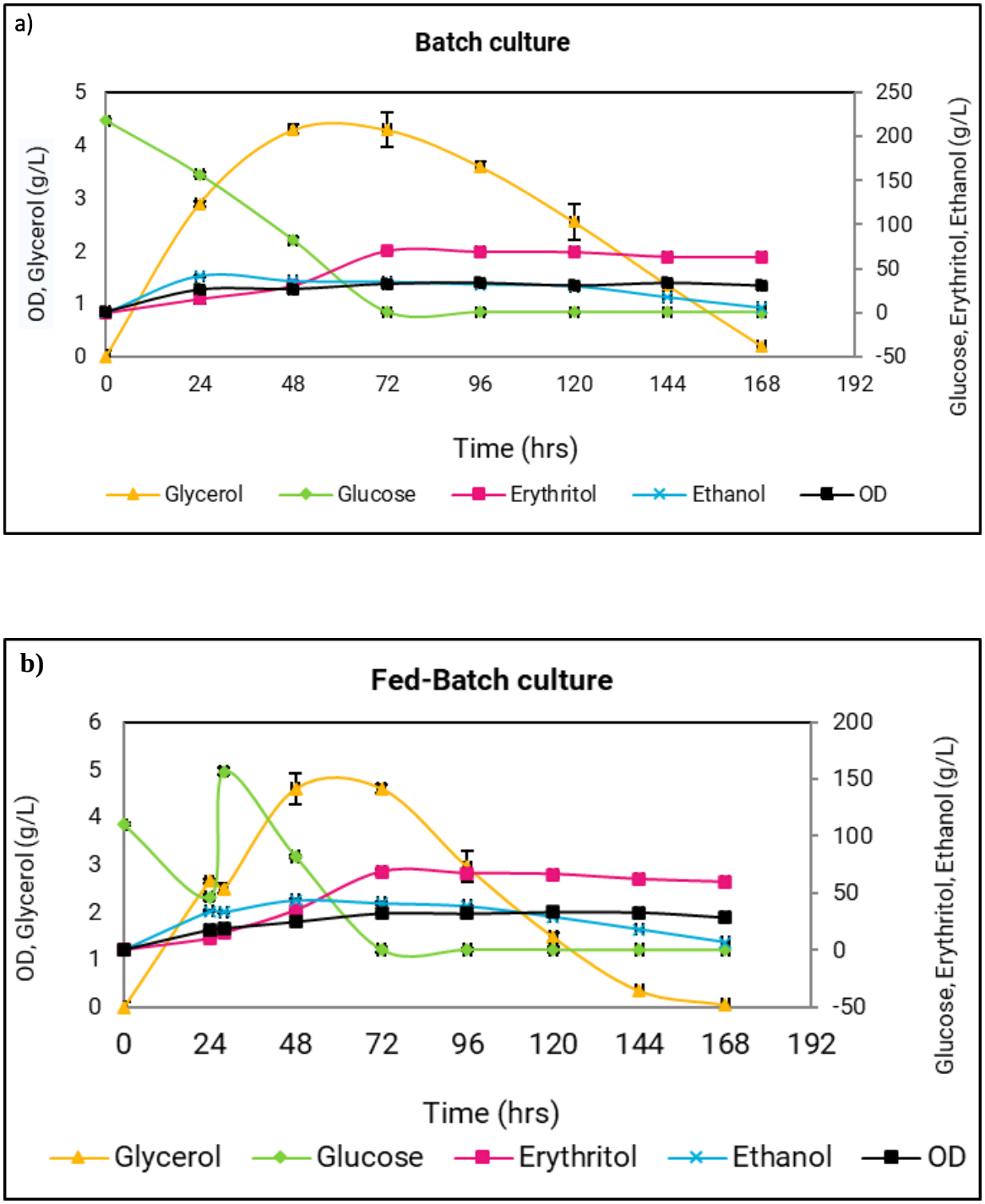



**Figure 2.5** Effect of glucose pulsing on erythritol production. **a)** Fed-batch and **b)** Batch fermentation

an initial glucose concentration of 100 g/L was maintained, and at the 24$^{th}$ hour of fermentation, an additional 100 g/L glucose pulsing was introduced, simulating a fed-batch fermentation approach. This was juxtaposed with a control scenario where a total glucose concentration of

200 g/L was provided at the commencement of the experiment, representing a conventional batch fermentation setup. Surprisingly, the results, depicted in Fig. 2.5, revealed that the application of glucose pulsing, simulating a fed-batch strategy, did not yield a discernible positive effect on erythritol production.

In both scenarios, where glucose was introduced initially or via pulsing, similar erythritol yields were achieved. These findings suggest that the *M. pollinis* strain exhibits consistent erythritol production capabilities under the tested conditions, regardless of the glucose-feeding strategy employed. This insight holds significance for the design and optimization of fermentation processes for erythritol production, indicating that traditional batch approaches may suffice in this context. It was also observed that batch fermentation yielded erythritol at 1.3±0.25 g/g biomass, whereas the fed-batch yielded 0.9±0.48 g/g biomass. However, for a deeper understanding, experiments could be conducted at the laboratory-scale fermentation, allowing for the monitoring of nitrogen source utilization and biomass growth to evaluate carbon flux towards biomass. Subsequently, conclusions can be drawn regarding the effect of fed-batch fermentation on erythritol production.

#### 2.3.2.3 Effect of various pH and Temperature

To investigate the influence of pH on erythritol production, we conducted experiments with *M. pollinis* cultivated in 20% glucose media, spanning a pH range from 3.0 to 7.0 (Fig. 2.6 a). Our observations revealed that the parent strain consistently generated equivalent quantities of erythritol within the pH range of 5.5–6.5. Notably, it was observed that both maximal growth (OD660nm) and erythritol yield (26.8±2.3%) were attained at pH 6.0. Conversely, erythritol yield exhibited a diminishing trend as the pH deviated further from 6.0, either towards higher or lower pH values.

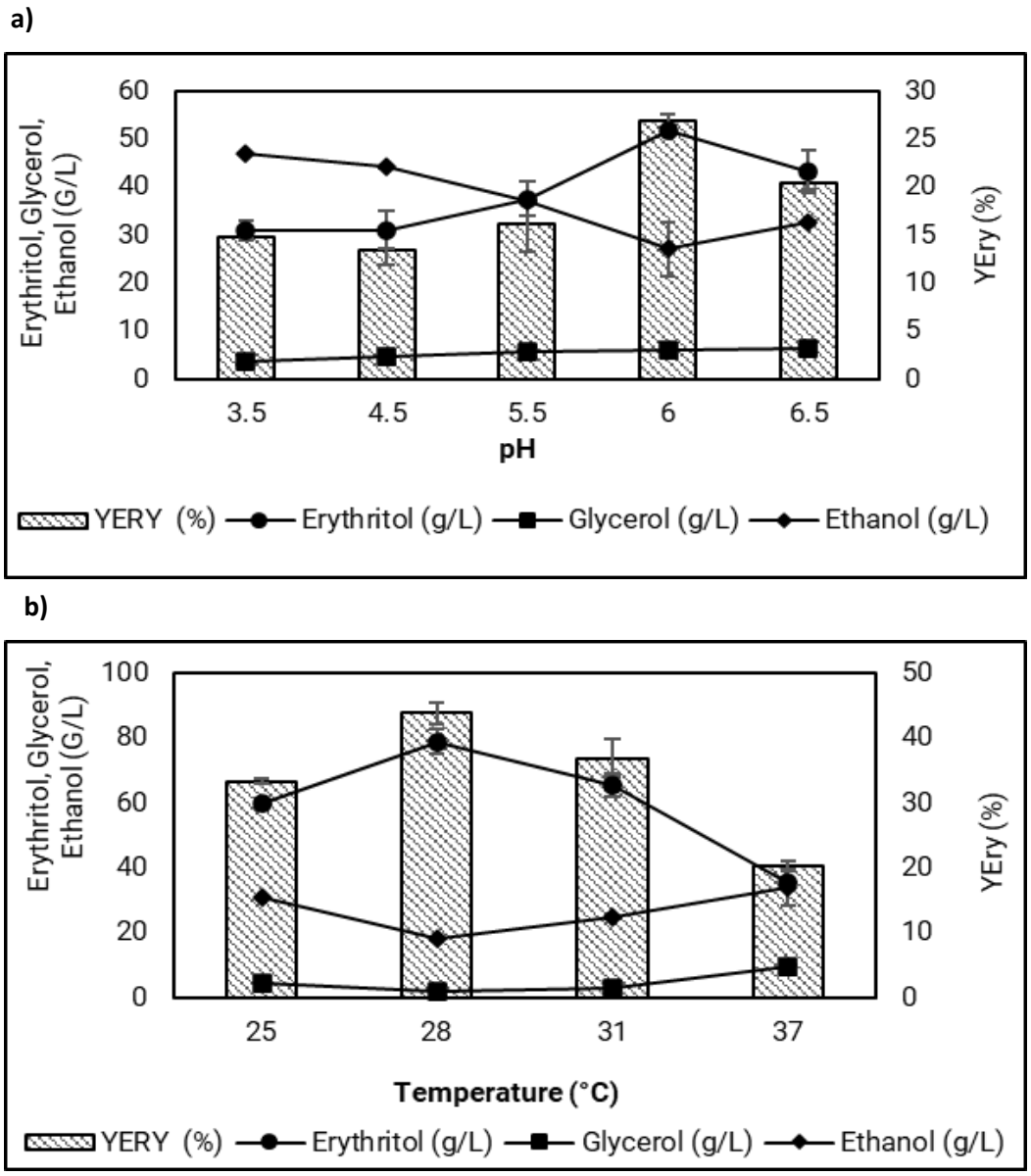



**Figure 2.6** Effect of various **a)** pH, and **b)** Temperature on erythritol production

The research work also explored the impact of different temperatures on erythritol production, subjecting the *M. pollinis* strains to temperatures ranging from 25°C to 37°C. As depicted in Fig. 2.6 b, the highest erythritol yield (43.7±0.4%) was achieved at 28°C. Furthermore, it was noticed a gradual decline in erythritol yield as the temperature increased from 28°C to 37°C. These results conclusively identify the optimal temperature and pH conditions for achieving

the highest erythritol production, namely 28°C and pH 6.0, respectively. Notably, the pH was observed to have a noteworthy effect on the synthesis of erythritol in *Y. lipolytica* as well. When utilizing glycerol as the carbon source, a pH of 3.0 was determined to be most effective, resulting in both higher erythritol productivity and reduced byproduct synthesis, particularly citric acid (Rymowicz et al., 2009; Tomaszewska et al., 2014).

Conversely, *Y. lipolytica* strain DSM70562, cultivated in a glucose-based medium, exhibited enhanced productivity at a pH of 5.5 (Ghezelbash et al., 2014b). In the case of *Moniliella* sp., the optimal conditions for temperature and pH were identified as 30°C and within the range of 4.0 to 5.0, respectively (Lin et al., 2001, 2010). These findings underscore the nuanced influence of pH and temperature on erythritol production, emphasizing the importance of tailored environmental conditions in biotechnological processes.

#### 2.3.2.4 Effect of metal ions

Various investigations have assessed the advantages of incorporating metal ions. This study demonstrated that Fe, Mn Zn, Cu, and Co ions at lower concentrations (10-50 ppm) have a beneficial impact on erythritol formation while ca ions at higher concentrations (100 ppm) show increased erythritol production (Table 2.6). Manganese ions ($Mn^{2+}$) consistently exhibited the most notable impact across various strains, significantly enhancing erythritol production in *Y. lipolytica*, *C. magnoliae*, and *Torula* sp. (Lee et al., 2000; Savergave et al., 2011a; Tomaszewska et al., 2014). In the case of *C. magnoliae*, $Fe^{2+}$ and $Ca^{2+}$ demonstrated positive effects. Different theories were proposed to elucidate the favorable influence of metal ions. The suggested explanation in this instance was that $Mn^{2+}$ heightened cell permeability, resulting in an augmented extracellular erythritol quantity. (Lee et al., 2000).

**Table 2.6** Screening of metal ions for erythritol production

| Metal ions | Concentration (ppm) | Erythritol (g/L) | $Y_{ERY}$ (%) | Byproducts | |
|---|---|---|---|---|---|
| | | | | Glycerol (g/L) | Ethanol (g/L) |
| $Fe^{2+}$ | 10 | 60.4±1.3 | 36.0 | 2.9±0.4 | 24.0±2.5 |
| | 50 | 66.5±2.3 | 38.0 | 2.1±0.2 | 26.0±1.8 |
| | 100 | 65.2±1.5 | 37.1 | 1.9±0.2 | 20.4±1.3 |
| $Mn^{2+}$ | 10 | 60.2±2.2 | 35.8 | 2.3±.4 | 21.2±2.1 |
| | 50 | 68.7±2.3 | 38.4 | 2.2±0.3 | 21.1±2.5 |
| | 100 | 72.5±1.9 | 39.2 | 2.7±0.6 | 22.6±2.8 |
| $Zn^{2+}$ | 10 | 80.3±2.5 | 47.7 | 5.1±0.2 | 27.2±1.5 |
| | 50 | 61.1±1.7 | 36.3 | 6.4±0.2 | 2.6±1.8 |
| | 100 | 0.00±2.0 | 0.0 | 0.0 | 0.0 |
| $Cu^{2+}$ | 10 | 72.2±2.1 | 40.7 | 3.3±0.3 | 18.5±1.2 |
| | 50 | 69.1±2.5 | 39.8 | 4.6±0.4 | 15.1±1.3 |
| | 100 | 47.4±2.6 | 29.4 | 11.0±0.8 | 17.5±1.5 |
| $Ca^{2+}$ | 10 | 66.1±2.2 | 36.7 | 2.8±0.4 | 22.7±1.6 |
| | 50 | 66.4±2.3 | 38.5 | 1.7±0.3 | 20.9±1.7 |
| | 100 | 76.5±1.9 | 39.9 | 2.7±0.3 | 20.6±2.1 |
| | 10 | 23.4±2.2 | 13.1 | 6.1±0.4 | 14.8±0.8 |

| | | | | | |
|---|---|---|---|---|---|
| **$Co^{2+}$** | 50 | 9.00±1.1 | 5.2 | 5.9±0.4 | 10.2±1.2 |
| | 100 | 4.5±0.5 | 2.7 | 4.0±0.7 | 7.4±1.0 |
| **No metal ions** | NA | 60.2±2.4 | 36 | 2.3±0.3 | 21.1±1.2 |

Another experiment was carried out after initial screening at various concentrations. The findings are depicted in Fig. 2.7. The highest erythritol production was observed in media supplemented with 10 ppm cu concentration. Additionally, it was also reported that $Cu^{2+}$ positively influenced erythritol production in *Y. lipolytica* and *Torula* sp., but had a detrimental effect in *C. magnoliae*. $Zn^{2+}$ enhanced production in *C. magnoliae* and *Y. lipolytica*, but not in *Torula* sp. Except Fe, all other metal ions showed a slight increase in erythritol yield than the control (without metal ions). A combination of $Fe^{2+}$ and Ca ions is to be tested since all other metal ions are toxic for yeast and may cause problems in downstream processing.

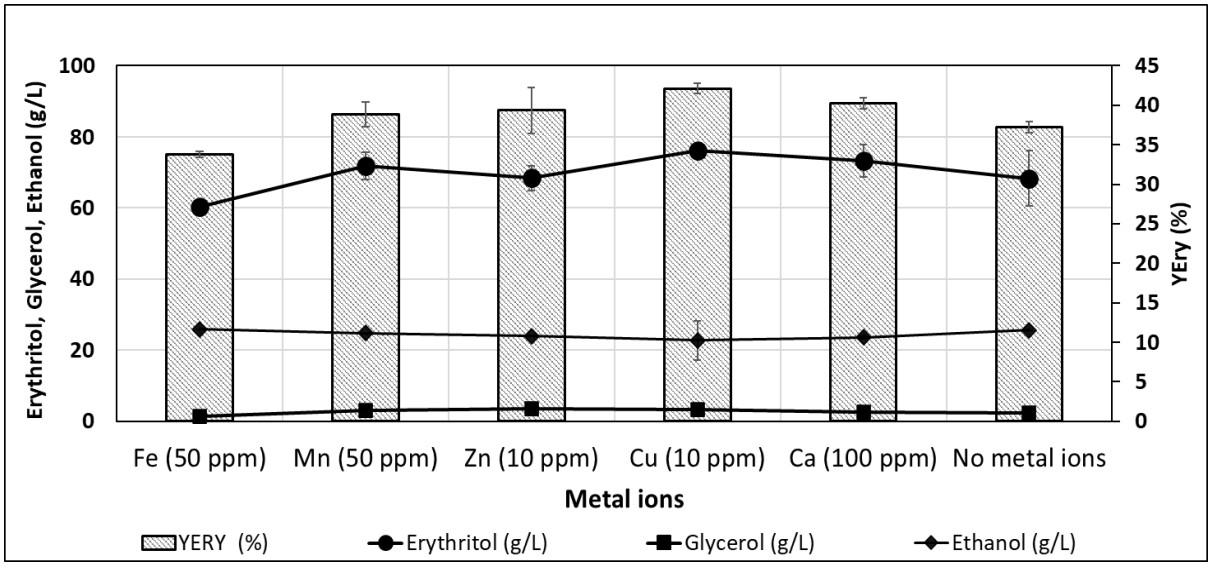



**Figure 2.7** Screening of metal ions for erythritol production

## 2.4 Conclusions

Evaluation of yeast strains from various culture collections revealed that *M. pollinis* which produced the highest 30.1 g/L from 20% glucose was selected for further investigations. Microscopic observation showed that *M. pollinis* appears to be dimorphic yeast and there seems to be a positive correlation between the size and shape of the yeast and erythritol production. Examinations of initial culture conditions indicate that glucose served as the most favorable carbon source for *M. pollinis* while media with jaggery also produced erythritol yield as high as glucose. There was no positive effect observed by glucose pulsing (fed-batch) on erythritol production as in both cases similar erythritol yield was achieved. In the case of the nitrogen source, various YE concentrations were tested for screening. The findings suggested that erythritol production was primarily contingent upon the availability of oxygen and nitrogen content in the medium. The addition of yeast extract above 4.0 g/L drastically decreased the erythritol production and favored ethanol production. Further, optimum temperature and pH values for the highest erythritol production were found to be 28 °C and 6.0 respectively. Except for $Fe^{2+}$, all other metal ions showed a slight increase in erythritol yield in comparison with the control (without metal ions). It is necessary to assess the compatibility of a mixture of $Fe^{2+}$ and $Ca^{2+}$ ions, as other metallic ions are harmful to yeast and could potentially introduce complications in subsequent downstream processing stages. An additional improvement in erythritol production can be attained through the modification of organisms involved in biosynthesis via mutagenesis or genetic engineering, along with statistical optimization of the medium. Thus, *M. pollinis CBS 461.67* was selected for strain development using mutagenesis techniques for further enhancement in erythritol production.

## 2.5 References

Aoki, M. A. Y., Pastore, G. M., & Park, Y. K. (1993). Microbial transformation of sucrose and glucose to erythritol. *Biotechnology Letters*, *15*, 383–388.

Burschäpers, J., Schustolla, D., Schügerl, K., Röper, H., & De Troostembergh, J. C. (2002). Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa var pollinis*: Part 2. Batch and fed-batch operation in bubble column and airlift tower loop if reactors. *Process Biochemistry*, *38*, 559–570.

Deshpande, M. S., Kulkarni, P. P., Kumbhar, P. S., & Ghosalkar, A. R. (2022). Erythritol production from sugar based feedstocks by *Moniliella pollinis* using lysate of recycled cells as nutrients source. *Process Biochemistry*, *112*, 45-52

Diano, A., Bekker-Jensen, S., Dynesen, J., & Nielsen, J. (2006). Polyol synthesis in *Aspergillus niger*: Influence of oxygen availability, carbon and nitrogen sources on the metabolism. *Biotechnology and Bioengineering*, *94*, 899-908.

Ghezelbash, G. R., Nahvi, I., & Emamzadeh, R. (2014a). Improvement of erythrose reductase activity, deletion of by-products and statistical media optimization for enhanced erythritol production from *Yarrowia lipolytica* mutant 49. *Current Microbiology*, *69*, 149-57.

Ghezelbash, G. R., Nahvi, I., & Malekpour, A. (2014b). Erythritol production with minimum By-product using *Candida magnoliae* mutant. *Applied Biochemistry and Microbiology*, *50*, 292–296.

Hajny, G. J., Smith, J. H., & Garver, J. C. (1964). Erythritol Production by a Yeastlike Fungus. *Applied Microbiology*, *12*, 240–246.

Hirata, Y., Igarashi, K., Ezaki, S., Atomi, H., & Imanaka, T. (1999). High-level production of erythritol by strain 618A-01 isolated from pollen. *Journal of Bioscience and Bioengineering*, *87*, 630–635.

Ishizuka, H., Wako, K., Kasumi, T., & Sasaki, T. (1989). Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. *Journal of Fermentation and Bioengineering*, *68*, 310–314.

Jagannadha Rao, P., Das, M., & Das, S. (2007). Jaggery – A Traditional Indian Sweetener. *Indian Journal of Traditional Knowledge*, *6*, 95-102.

Jeya, M., Lee, K. M., Tiwari, M. K., Kim, J. S., Gunasekaran, P., Kim, S. Y., Kim, I. W., & Lee, J. K. (2009). Isolation of a novel high erythritol-producing *Pseudozyma tsukubaensis* and scale-up of erythritol fermentation to industrial level. *Applied Microbiology and Biotechnology*, *83*, 225–231.

Kim, K. A., Noh, B. S., Lee, J. K., Kim, S. Y., Park, Y. C., & Oh, D. K. (2000). Optimization of culture conditions for erythritol production by *Torula* sp. *Journal of Microbiology and Biotechnology*, *10*, 69–74.

Kobayashi, Y., Iwata, H., Mizushima, D., Ogihara, J., & Kasumi, T. (2015). Erythritol production by *Moniliella megachiliensis* using nonrefined glycerol waste as carbon source. *Letters in Applied Microbiology*, *60*, 475–480.

Kogej, T., Stein, M., Volkmann, M., Gorbushina, A. A., Galinski, E. A., & Gunde-Cimerman, N. (2007). Osmotic adaptation of the halophilic fungus *Hortaea werneckii*: Role of osmolytes and melanization. *Microbiology*, *153*, 4261-4273.

Lee, J. K., Ha, S. J., Kim, S. Y., & Oh, D. K. (2000). Increased erythritol production in *Torula* sp. by $Mn^{2+}$ and $Cu^{2+}$. *Biotechnology Letters*, *22*, 983–986.

Lin, S. J., Wen, C. Y., Liau, J. C., & Chu, W. S. (2001). Screening and production of erythritol by newly isolated osmophilic yeast-like fungi. *Process Biochemistry, 36,* 1249–1258.

Lin, S. J., Wen, C. Y., Wang, P. M., Huang, J. C., Wei, C. L., Chang, J. W., & Chu, W. S. (2010). High-level production of erythritol by mutants of osmophilic *Moniliella* sp. *Process Biochemistry, 45,* 973–979.

Mirończuk, A. M., Dobrowolski, A., Rakicka, M., Rywińska, A., & Rymowicz, W. (2015). Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol. *Process Biochemistry, 50,* 61–68.

Mirończuk, A. M., Rakicka, M., Biegalska, A., Rymowicz, W., & Dobrowolski, A. (2015). A two-stage fermentation process of erythritol production by yeast *Y. lipolytica* from molasses and glycerol. *Bioresource Technology, 198,* 445–455.

Nath A, D. D. (2015). Review on recent advances in value addition of jaggery based products. *Journal of Food Processing & Technology, 06,* 4.

Park, Y. K., Koo, M. H., & De Aguiar Oliveira, I. M. (1996). Biochemical characteristics of osmophilic yeasts isolated from pollens and honey. *Bioscience, Biotechnology and Biochemistry, 60,* 1872–1873.

Rakicka, M., Rukowicz, B., Rywińska, A., Lazar, Z., & Rymowicz, W. (2016). Technology of efficient continuous erythritol production from glycerol. *Journal of Cleaner Production, 139,* 905-913.

Rymowicz, W., Rywińska, A., & Marcinkiewicz, M. (2009). High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*. *Biotechnology Letters, 31,* 377–380.

Savergave, L. S., Gadre, R. V., Vaidya, B. K., & Narayanan, K. (2011b). Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochemical Engineering Journal*, *55*, 92–100.

Sawada, K., Taki, A., Yamakawa, T., & Seki, M. (2009). Key role for transketolase activity in erythritol production by *Trichosporonoides megachiliensis* SN-G42. *Journal of Bioscience and Bioengineering*, *108*, 385–390.

Shen, B., Hohmann, S., Jensen, R. G., & Bohnert, H. J. (1999). Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. *Plant Physiology*, *121*, 45-52.

Singh, J. (2013). Manufacturing jaggery, a product of sugarcane, as health food. *Agrotechnology*, *01*, S11.

Sivaraman, B., Badle, S. S., & Rangaswamy, V. (2016). Production of 1,3-butadiene from erythritol. *ChemistrySelect*, *1*, 4630-4632.

Tomaszewska, L., Rywińska, A., & Rymowicz, W. (2014). High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*. *Biomass and Bioenergy*, *64*, 309–320.

Vijayendra, S. V. N., Bansal, D., Prasad, M. S., & Nand, K. (2001). Jaggery: A novel substrate for pullulan production by *Aureobasidium pullulans* CFR-77. *Process Biochemistry*, *37*, 359-364.

**Chapter. 3**

# Strain improvement using physical and chemical mutagenesis

**Abstract**

Mutants of *M. pollinis* were created using a combination of UV, EMS, and NTG treatments. A total of 198 mutants were screened for increased erythritol production of which Mutant-58, generated through EMS treatment, was singled out due to its better erythritol production, and selected for further detailed investigation. The mutant cells exhibited an oval shape in contrast to the wild-type cells, which displayed a long filamentous morphology. Additionally, the mutant cells demonstrated the anintriguing capability to produce a yellow pigment over time, a phenomenon which was not observed in the parent strain. In batch culture supplemented with 20% glucose, the *M. pollinis* Mutant-58 strain demonstrated remarkable erythritol production, reaching a substantial concentration of 92.2±2.3 g/L, accompanied by a better yield of 0.46 ± 0.11 g/g, with a productivity rate of 1.28 ± 0.02 g/L/h. Notably, in shake-flask culture, Mutant-58 exhibited a 30% increase in the erythritol production compared to wild-type strains, while concurrently reducing the ethanol production by 27%. Further investigation into optimal conditions for enhanced erythritol production revealed that Mutant-58 strain thrived at a temperature of 28°C, a pH of 6.0, and with the addition of 4.0 g/L of yeast extract. Moreover, Mutant-58 strain, when cultured in jaggery-containing media, yielded a higher erythritol concentration (37.6 ± 0.5 g/g) compared to the erythritol production by the wild-type strain in glucose-containing media (36 ± 1.3 g/g). In summary, the present study highlights the successful improvement of the strain through chemical mutagenesis, followed by media optimization, resulting in a 30% increase in erythritol production and a significant 27% reduction in ethanol production. It also demonstrated that jaggery could be used as a possible alternative to glucose for industrial-scale production.

## 3.1 Introduction

Microorganisms have a tendency to generate a variety of valuable metabolites, albeit often in smaller quantities. Comprehensive strain improvement initiatives are typically essential to obtain a superior strain with a high yield of the desired product. Throughout history, industrial strain development programs have played a pivotal role in significantly amplifying the production of microbial metabolites. Enhancing the productivity of specific microbial cultures has remained a crucial element within the fermentation industry. A substantial reduction in the cost of fermentation products has predominantly been achieved through strain improvement techniques, involving mutagenesis or the utilization of recombinant DNA technology (Parekh et al., 2000; Adrio & Demain, 2006; Demain & Adrio, 2008). The routine approach for obtaining high-yield mutants involves subjecting cells of the optimal culture to a mutagenic compound until the desired level of mutation is attained. Subsequently, the survivors are cultivated on appropriate, preferably discriminating media, and each resultant colony or a randomly selected grouping of colonies is examined for product generation in the shake flasks. Mutagens used in this process are N-methyl N-nitro N-nitroso guanidine (NTG), methyl methane sulfonate (MMS), ethyl methyl sulphonate (EMS), hydroxylamine (HA), and exposure to ultraviolet light (UV)(Adrio & Demain, 2006; Demain & Adrio, 2008)

The production of erythritol is a multifaceted process, involving numerous genes. Consequently, traditional mutagenesis is a favored approach for enhancing erythritol production by obtaining better mutant strains. Beyond augmenting erythritol production, classical mutagenesis has proven to be effective in addressing undesirable characteristics. These include inadequate osmo-tolerance in media, the formation of undesirable metabolites, and excessive foaming during aerobic culture conditions. Ishizuka et al., (1989) obtained a high erythritol-producing mutant of *Aureobasidium* sp. SN124A with 47.6% yield by UV irradiation and NTG treatment. Strain KJUV29 originating from *Penicillium* sp. KJ81, displayed a notable

enhancement in erythritol synthesis while concurrently showing decreased glycerol levels and reduced foam formation in comparison to the parent strain (Lee & Lim, 2003).

Qiu et al., (2020) introduced a high-throughput screening method for enhancing erythritol production in *Y. lipolytica*, distinct from genetic modification. They harnessed the erythritol-responsive transcription factor EryD to create a sensor-regulator system, allowing rapid screening of mutant strains. This approach swiftly evaluated 1152 mutants derived from UV and ARTP mutagenesis within a week. Notably, one mutant, yliUA8s, produced 103 g/L erythritol, a 2.4-fold increase over the initial strain, and scaled up to yield over 148 g/L in 3.0 L bench-top reactors. This methodology offers a rapid means to enhance strain performance and engineer efficient microbial cell factories for industrial applications. *Moniliella* sp. 440, isolated from honey, underwent iterative mutation and selection with N-methyl-N'-nitro-N-nitrosoguanidine. Six strains, including mutant N61188-12, outperformed the parent strain in erythritol production, with N61188-12 reaching 237.8 g/L compared to the wild type's 113 g/L. Mutagenesis improved erythritol production, enhanced glycerol utilization, reduced foaming, minimized byproducts, and increased osmotic stress resistance. At a 2000L scale, mutant N61188-12 produced 152.4 g/L erythritol in 10 days (Lin et al., 2010).

A mutant strain (12-2) of *C. magnoliae* DSM70638, generated through a combination of UV and EMS mutagenesis, exhibited a 2.4-fold boost in erythritol production (20.32 g/L) in comparison to the parent strain (8.54 g/L). Additionally, glycerol production in this mutant decreased by 5.5-fold (2.36 g/L), contrasting with the wild strain's production of 12.93 g/L (Ghezelbash et al., 2014). One of the mutants (R23) derived from *C. magnoliae* NCIM 3470 using a combination of UV and chemical mutagenesis exhibited a significant increase in erythritol production, achieving 4-fold higher levels (60.3 g/L) compared to the parent strain (14.0 g/L). Additionally, the ER activity in this mutant was 2-fold higher than the parent (Savergave et al., 2011).

A yeast strain with a notable erythritol production capacity, *C. magnoliae*, was initially isolated from honeycombs and further improved through mutagenesis using UV and NTG. This resulted in the creation of an osmotolerant mutant called M2, which exhibited a 25% upsurge in erythritol production (25 g/l) and a 30% increase in productivity (0.54 g/L/h) compared to the wild strain. Subsequent optimization of fed-batch fermentation enabled the production of 200 g/L erythritol with a productivity of 1.2 g/L/h and a yield of 43%. It is noteworthy that this procedure also resulted in the generation of substantial quantities of organic acids, including gluconic, citric, and butyric acid (Ryu et al., 2000; Koh et al., 2003). Likewise, the erythritol yield of *Torula* sp., as isolated by Kim et al. (2000), was enhanced to 48.9% through the optimization of both composition of the medium and operational conditions. As of now, there is a limited number of microorganisms recognized for industrial-scale erythritol production. In these processes, glucose sourced from wheat and corn starch serves as the primary carbon substrate for erythritol synthesis. This is typically achieved through fermentation, employing yeast-like fungi like *Aureobasidium* sp. SN-G42, *Torula* sp., and *M. pollinis* (Moon et al., 2010).

Based on the results the preceding chapter, the *M. pollinis* yeast culture was chosen for strain development by mutagenesis. The goal of this current segment is to elevate erythritol production and reduce undesirable byproducts through conventional mutagenesis techniques and to fine-tune the culture conditions for the preferred mutants.

## 3.2 Materials and methods

### 3.2.1 Microorganisms and media

The wild-type culture *M. pollinis* CBS 461.67 and Mutant-58 were grown in Yeast extract glucose peptone (YPG) liquid medium, and incubated for 18h at 28°C and 180 RPM (MaxQ 8000 incubator shaker, Thermo Scientific).

N-methyl N-nitro N-nitroso guanidine (NTG), ethyl methyl sulphonates (EMS), and sodium thiosulphate were purchased from Sigma-Aldrich, USA. All medium ingredients were procured from Difco Thermo Scientific, USA. All other chemicals obtained were reagent grade.

### 3.2.2 Mutagenesis and mutant selection

The procedure used for mutagenesis was adapted from various literature reports earlier (Ishizuka et al., 1989b; Winston, 2008; Lin et al., 2010a; Ghezelbash et al., 2014). A flow diagram illustrating the general protocol employed throughout the strain improvement program for *M. pollinis* is presented and adhered to until the desired mutant is successfully obtained. (Fig. 3.1). The wild-type strain *M. pollinis* was inoculated into a sterile 250 mL flask having 50 mL YPG medium and incubated at 28˚C, 180 RPM for 18 h. Post incubation, cell count was checked using Neubauer improved cell counting chamber (Marienfeld, Germany) under the light microscope (Thesseling et al. 2019). The well-grown culture broth was centrifuged at 10000 RPM for 4 minutes. The cells were washed thrice with 10 mL of 0.1 M phosphate buffer (pH 6.5) and again mixed in the same buffer (30 mL) to adjust the final cell count to about 300 x $10^6$ cells/mL. For UV irradiation, 2.0 mL of cell suspension was used in sterile petri plates and exposed to UV light (254nm) under a laminar flow hood at 10 cm and 20 cm distances for various time period up to 60 minutes (1, 5, 10, 15, 30, and 60 minutes).

For chemical mutagenesis, EMS treatment was given to cell suspension at varying concentrations (25, 50, 75, 100, and 150µl/mL) for 60 minutes. Post-exposure, the reaction was stopped by adding 1mL sterile 10% $Na_2O_2S_3$ (sodium thiosulphate). NTG treatment was given to the cell suspension at varying concentrations (50, 100, 150, 200, and 250 µg/mL) for 60 minutes. After completion of exposure time, the reaction was stopped by adding 1mL sterile 10% $Na_2O_2S_3$ (sodium thiosulphate) and incubated at 28˚C for 3 h in the dark.

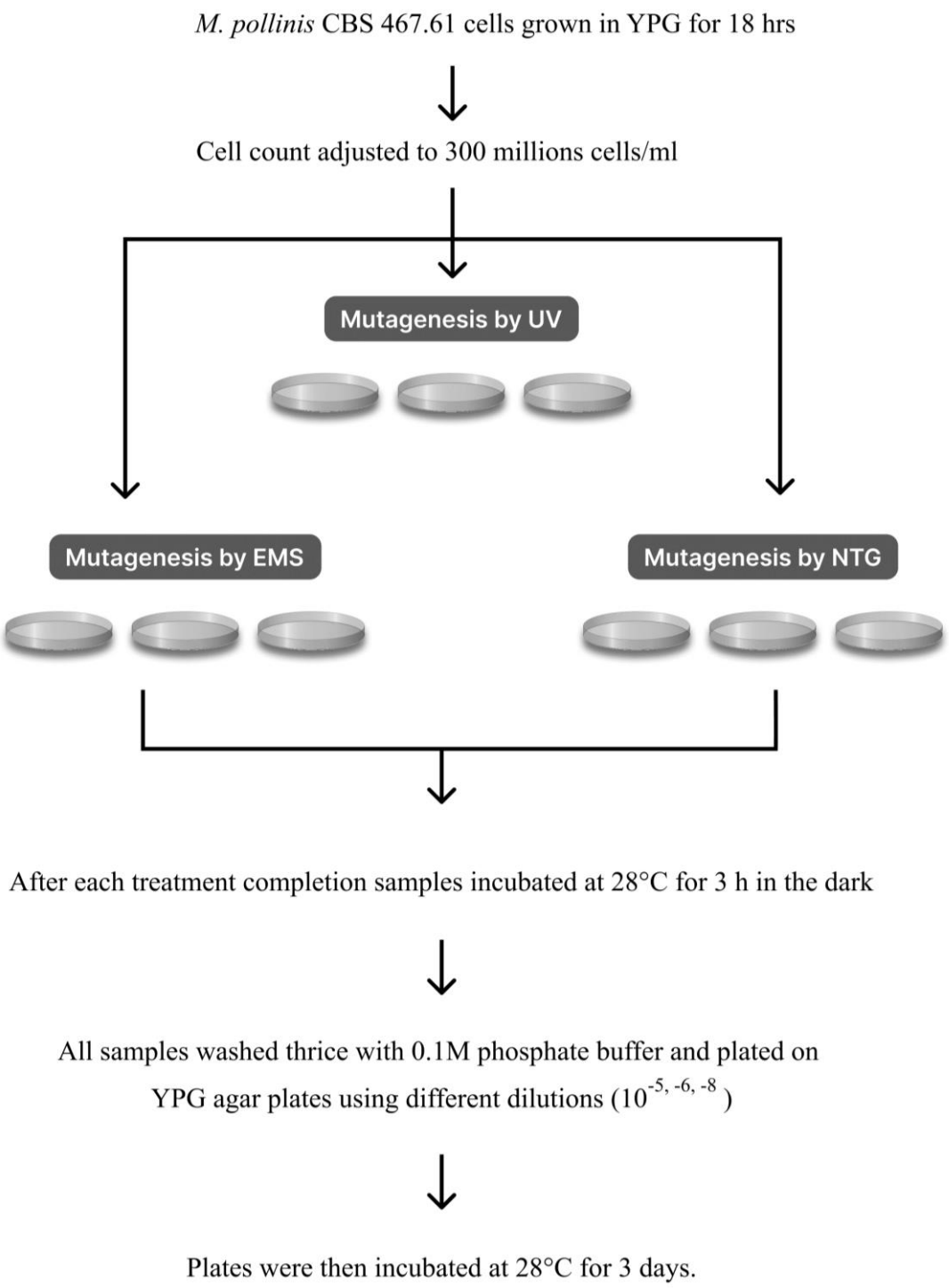



**Figure 3.1** Methodology used for strain improvement by random mutagenesis

All treated samples (UV, EMS, and NTG) were then centrifuged at 10000 RPM for 4 minutes. The cells were washed twice with 2.0 mL of 0.1M phosphate buffer (pH 6.5) and again mixed in the same buffer (2.0 mL). For each treatment, the cell suspension was diluted appropriately and spread on plates containing 1% YE, 2 % bacteriological peptone, 20 % glucose, and 2 %

agar. All plates were incubated at 28°C for 3 days. Plates showing a 90-95 % kill rate were chosen for mutant selection. The colonies were picked and inoculated in 50 mL glass tubes with 5 mL liquid fermentation medium (LFM) (Savergave et al., 2011). The LFM contained the following components (g/L): YE, 10; Glucose, 200; $K_2HPO_4$, 5; $MgSO_4.7H_2O$, 0.25. The tubes were incubated in the shaker at 180 RPM, 28°C for 3 days and monitored for erythritol production by high-performance liquid chromatography (HPLC) along with quantification of glucose, fructose, sucrose, glycerol, and ethanol using an Aminex HPX-87H, 300 mm × 7.8 mm column (Bio-Rad, USA). The mobile phase used for elution was 5 mM $H_2SO_4$ at 0.6 mL/min flow rate at 35°C. Samples were injected using an autosampler injector with a 20 µL fixed volume. A refractive index detector (Agilent HPLC 1260 series), was used for the detection of sugars and other metabolites. The mutant strains selected for further evaluation were grown in a YPG medium containing 20% glucose. Among all the mutant strains, mutant no.58 (Mutant-58) showed the highest erythritol production and hence was selected for further analysis.

### 3.2.3 Morphology

Morphological observations of wild-type strain *M. pollinis* and Mutant-58 were made by observing colony size, shape, appearance, and cell shape under the light microscope (Lin et al. 2010). Cell morphology, cell biomass, and fermented broth color were observed after 72 h incubation of the wild-type and mutant strains in the LFM medium.

### 3.2.4 Screening of medium components for erythritol production by *M. pollinis* CBS 461.67 Mutant-58

For inoculum preparation, wild-type yeast *M. pollinis* and Mutant-58 were first cultivated in YPG liquid medium, as described earlier. After 18h of incubation, 2.5 mL inoculum (10% v/v)

was transferred into a 250 mL flask containing 22.5 ml LFM medium. The influence of varying YE concentrations (1.0 to 10 g/L), pH values (3.5, 4.5, 5.5, 6.0, and 6.5), and temperature (25°C, 28°C, 30°C, and 37°C) on erythritol production by *M. pollinis* Mutant-58 was evaluated in shake flasks. For screening of cost-effective carbon substrate for erythritol production, glucose in the LFM medium was replaced with other inexpensive carbon substrates viz jaggery, molasses, sugarcane juice, and glycerol. Sugarcane juice was obtained from a local market and evaporated in a rotary vacuum evaporator. The concentrated syrup was utilized in the experiment as per requirement. Molasses was obtained from a sugar cane factory near Pune. The molasses exhibited the following sugar composition: sucrose (42.3±4.2%), glucose (2.9±1.2%), fructose (1.6±1%), with a total sugar concentration of 46.9±6.4%. The carbon sources were used in the range of 200 g/L.

An 8 h old seed culture was inoculated in 250 mL Erlenmeyer flasks containing 22.5 mL of LFM medium with varying carbon sources. The concentration of the carbon sources used was kept equivalent to carbon content present in 200 g/L glucose, which was calculated to be 6.67 M. Thus, the final concentrations of the different carbon sources used were – a) 200 g/L glucose; b) 202 g/L jaggery (containing 132 g/L sucrose, 36.0 g/L glucose, and 25.0 g/L fructose); c) 480 g/L molasses (containing 128 g/L sucrose, 35.0 g/L glucose, and 31.0 g/L fructose); d) 205 g/L glycerol; and e) 202 g/L concentrated sugarcane juice (containing 128 g/L sucrose, 30.0 g/L glucose, and 26.0 g/L fructose).

The inoculated flasks were kept for incubation at 28°C, 180 RPM in the shaking incubator. Samples were examined at 72h for optical (OD) density, pH, residual glucose, and polyol concentrations. Each test was performed in triplicates.

### 3.2.5 Measurement of residual sugar and intracellular protein concentration of parent strain and Mutant-58

*M. pollinis* wild type and Mutant-58 were grown in LFM for 72 h and the cell pellet was collected by centrifugation. The pellet was washed twice with 200 mM phosphate buffer (pH 6) containing 20 % glycerol. Glycerol was used to stabilize the protein content in the crude cell lysate (Vagenende et al., 2009). After washing, the pellet was suspended in 300 µL crushing buffer (10 mL, 200 mM phosphate buffer with 20 % glycerol, pH 6.0, 1 mM dithiothreitol, and 1 mM PMSF) and crushed by mixing with 0.5 mm glass beads (Sigma) in a 2 mL microfuge tube on a vortex mixture for 3 cycles of 5 min with cooling on ice for 5 min between the cycles. After completing the crushing process, 700 µL crushing buffer was added to the microfuge tube, of which 0.1 mL sample was used for cell counting, and the remaining crushing mixture was centrifuged at 10,000×g for 5 min, at 4°C. The aliquot removed for cell counting was diluted to 1mL with sterile distilled water. To perform a viability count, one drop of 0.1% (w/v) methylene blue solution was added to the 1 mL sample. The sample was mixed well, allowed to react for 1 min, and processed for cell counting using a counting Neubauer's chamber. The method reported earlier in the literature (Shukla, 2015) was used for the protein analysis of the samples. Initial and 72h fermented broth samples of both parent and mutant cultures were analyzed for metabolite and glucose analysis by HPLC (discussed earlier) to check the residual sugar. The estimation of residual sugar from metabolite analysis is explained in the supplementary information. Protein concentration was checked using a standard curve prepared using BSA (Fig. 3.2 a). DCW of both strains was calculated using a standard graph between DCW and OD600nm (Fig. 3.2 b). Where 1.0 $OD_{600nm}$ was found to be equivalent to 0.58 and 0.44 g/L DCW for parent and mutant strains respectively. Samples at the end of the fermentation (72h) were checked for metabolite as well as protein concentration determination.

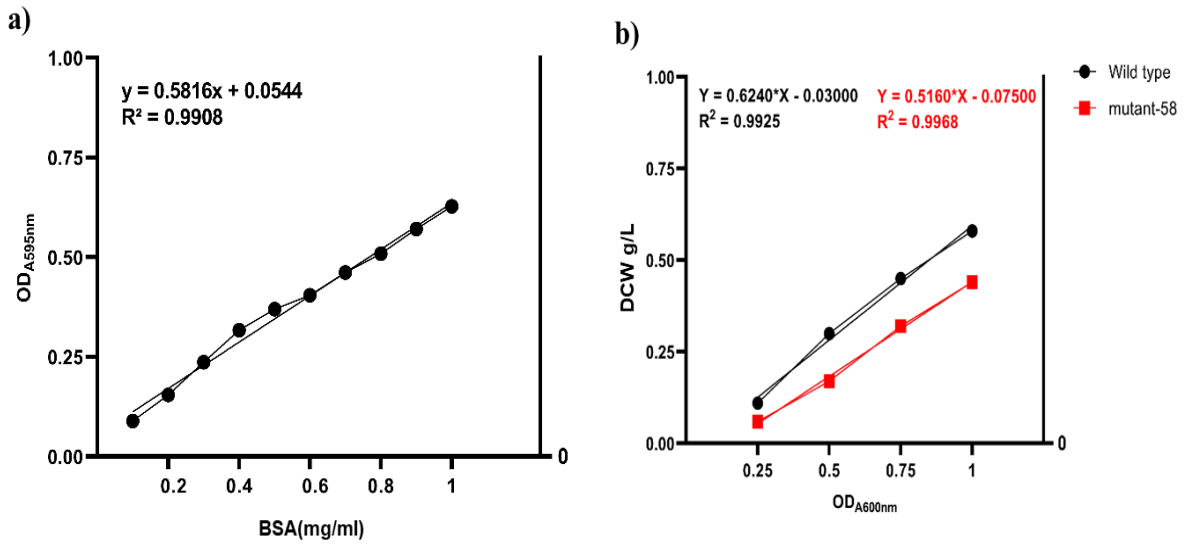



**Figure 3.2** Standard graph illustrating the **a)** Dry Cell Weight (DCW) and **b)** Protein estimation for mutant-58 and the parent strain

### 3.2.6 Analytical methods

OD was measured using a spectrophotometer at 600 nm. The HPLC method described earlier was used to quantify the concentration of the metabolite produced during fermentation. To obtain erythritol yield (YERY %), the following formula was used:

$$\text{YERY yield (\%)} = \frac{\text{Erythritol produced (g/L)}}{\text{Carbon source consumed g/L}} \text{ X (100)}$$

Respective Y values for the standard curves were obtained using a mixed standard curve prepared from standard chemicals, and these values were used for the quantification of all the metabolites (Fig.3.3).

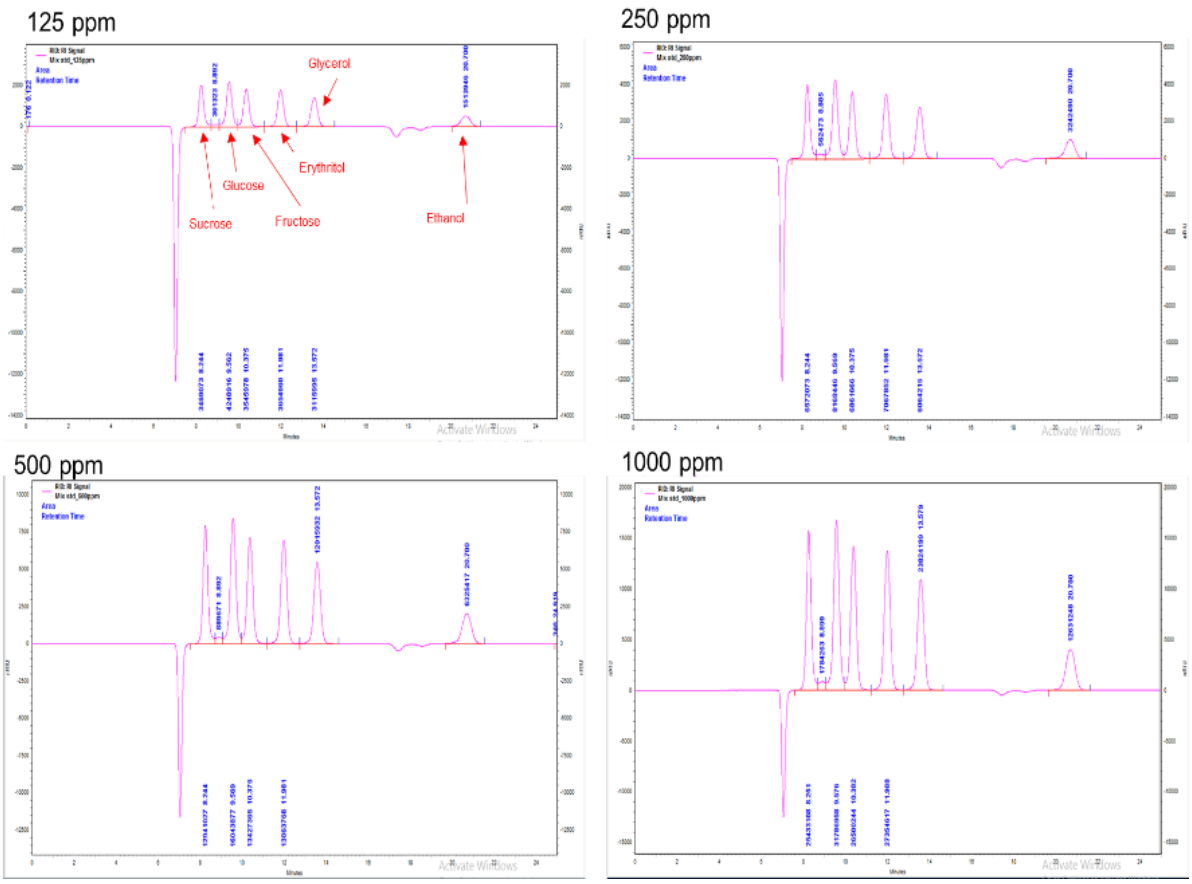



**Figure 3.3** Mix standard curve of metabolites for HPLC a) 125 ppm, b) 250 ppm, c) 500 ppm, d)1000 ppm

## 3.3 Results and discussions

### 3.3.1 Mutagenesis

Currently, large-scale production of erythritol primarily depend on fermentation methods using fungal strains. Genetic modifications through UV or chemical mutagenesis have been explored to enhance erythritol production and reduce unwanted byproducts in various yeast strains, such as osmotolerant yeast strain *Moniliella* sp. 440, which exhibited increased erythritol formation, improved glycerol consumption, reduced byproduct production, and enhanced resistance to osmotic pressure (Lin et al., 2010). Using *Moniliella pollinis* cells lysate-based media in sugarcane juice fermentation led to a two-fold enhancement in both erythritol yield and

productivity when compared to a yeast extract-based medium. In a fed-batch fermentation using sugarcane juice, a peak erythritol titer of 94.9 g/L was reached, accompanied by a yield of 0.38 g/g total sugar. (Deshpande et al., 2022).

Strain improvement by mutagenesis is a well-known strategy to improve microorganisms' ability to produce the desired product. Various research studies have been carried out on improvement in erythritol production in new strains of yeasts that have been modified by physical and chemical mutagenesis (Hirata et al., 1999; Savergave et al., 2011; Ghezelbash et al., 2014).

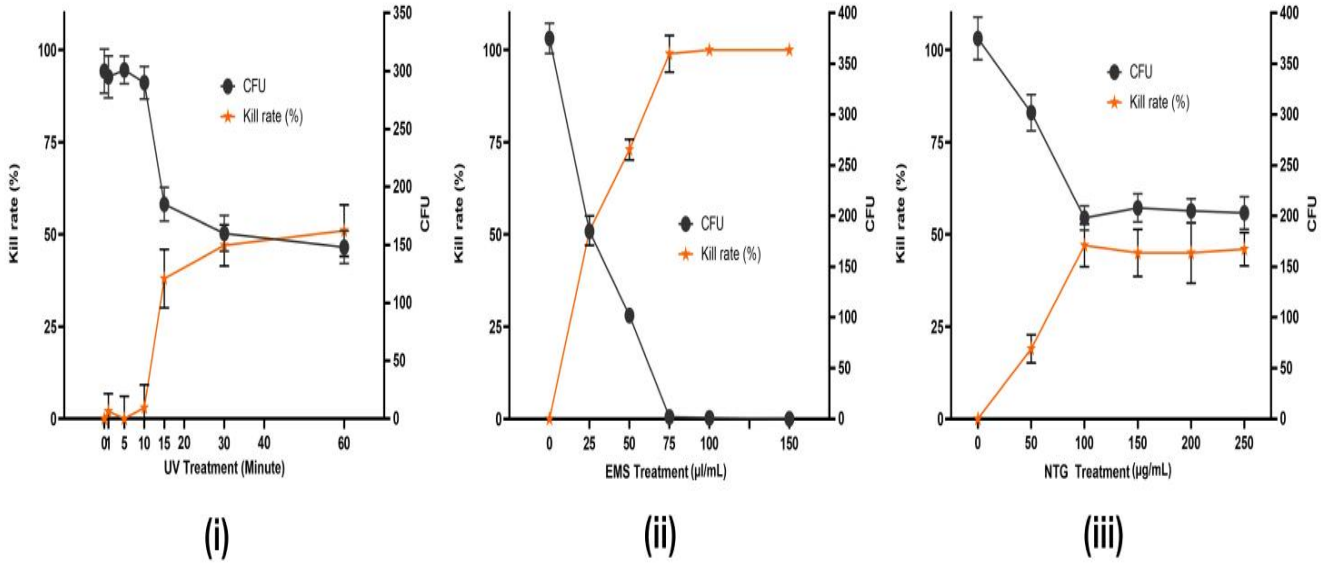



**Figure 3.4** Mutagenesis survival curve results (i) UV irradiation treatment and survival curve. (ii) EMS irradiation treatments and survival curve. (iii) NTG irradiation treatment and survival curve

Survival curves of *M. pollinis* after the mutagen treatments are presented in Fig. 3.4. The graph in Fig 3.4 (i) illustrates that a 50-60 % kill rate was achieved by exposing the cells to UV for 60 min.


**Figure 3.5** Mutagenesis results **a)** EMS, b**)** NTG and **c)** UV

For obtaining a 90-95% kill rate in EMS mutagenesis, the time required was between 30 to 40 min with 75µL/mL concentration (Fig. 3.4 (ii)). EMS mutagenesis at 75µL/mL concentration and above showed around a 100 % kill rate. While 51 and 69 % kill rates were observed at 25 and 50 µL/mL EMS concentration, respectively. In comparison with wild-type colonies,

mutants were smaller in shape (Fig. 3.5 a). In the case of NTG treatment, the highest kill rate (~ 40 %,) was observed by treating the cells for 60 min at 100 µL/mL (Fig. 3.4 (iii)).

Overall, the highest kill rate and changes in the morphology of cells were observed in cells treated with EMS at 50µL/mL concentrations and above in 60 min (Fig. 3.5 b). For subsequent mutagenesis treatment, 50 and 75µL/mL EMS concentrations were chosen and more mutants were generated.

### 3.3.2 Screening of high erythritol-producing mutants

A total of 198 mutants chosen from all mutagenesis treatments were evaluated for erythritol production. Mutants generated from EMS mutagenesis exhibited a significant increase in erythritol production. (Table 3.1).

**Table 3.1** Evaluation of selected mutants along with parent strain for erythritol and by-product formation in 50mL glass tubes with 5.0mL liquid fermentation medium (LFM), batch culture. Samples were replicated in triplicate, and all values are expressed as mean ± SD.

| Culture ID | Mutagenesis method | Erythritol (g/L) | Glycerol (g/L) | Ethanol (g/L) | Increase/decrease in erythritol yield (%) |
|---|---|---|---|---|---|
| **Wild type** | NA | 68.1±10 | 4.1±11 | 23.1±54 | NA |
| **Mutant-1** | **UV** | **77.6±17** | **4.6±4** | **27.6±14** | **13.0** |
| **Mutant-2** | UV | 63.2±23 | 3.2±9 | 26.2±70 | -8.2 |
| **Mutant-3** | UV | 63.5±22 | 2.5±32 | 25.5±40 | -7.5 |
| **Mutant-4** | UV | 68.4±10 | 2.4±11 | 27.4±80 | -0.6 |
| **Mutant-5** | UV | 69.1±17 | 3.1±4 | 27.1±4 | 0.5 |
| **Mutant-6** | UV | 72.9±27 | 2.9±9 | 21.9±20 | 5.88 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-7** | UV | 66.8±22 | 2.8±32 | 18.8±2 | -2.6 |
| **Mutant-8** | UV | 54.4±10 | 2.2±10 | 26.4±10 | -20.3 |
| **Mutant-9** | UV | 65.2±60 | 3.4±13 | 23.2±20 | -5.3 |
| **Mutant-10** | UV | 63.1±40 | 2.3±14 | 30.1±30 | -7.8 |
| **Mutant-11** | UV | 64.6±40 | 2.1±70 | 27.6±15 | -6.5 |
| **Mutant-12** | UV | 48.2±10 | 2.9±40 | 27.2±18 | -29.9 |
| **Mutant-13** | UV | 62.5±40 | 2.8±80 | 18.5±18 | -8.4 |
| **Mutant-14** | UV | 62.4±23 | 1.4±4 | 28.4±24 | -8.6 |
| **Mutant-15** | UV | 52.1±50 | 2.2±20 | 24.1±80 | -24.1 |
| **Mutant-16** | UV | 57.9±30 | 1.4±2 | 27.9±20 | -15.9 |
| **Mutant-17** | UV | 62.8±80 | 2.3±10 | 23.8±14 | -8.8 |
| **Mutant-18** | UV | 61.4±24 | 2.5±20 | 25.4±14 | -10.1 |
| **Mutant-19** | UV | 64.2±38 | 2.4±30 | 22.2±12 | -6.0 |
| **Mutant-20** | UV | 61.9±18 | 2.1±10 | 26.9±28 | -11.1 |
| **Mutant-21** | UV | 55.8±25 | 2.9±20 | 23.8±16 | -18.8 |
| **Mutant-22** | UV | 62.4±15 | 2.8±50 | 24.4±12 | -8.3 |
| **Mutant-23** | UV | 61.2±21 | 2.1±30 | 21.2±15 | -11.1 |
| **Mutant-24** | UV | 64.4±15 | 2.6±40 | 22.4±29 | -6.2 |
| **Mutant-25** | UV | 63.3±15 | 2.1±10 | 25.3±15 | -8.3 |
| **Mutant-26** | UV | 61.8±18 | 2.6±30 | 27.8±18 | -10.4 |
| **Mutant-27** | UV | 51.4±17 | 1.2±20 | 27.4±18 | -25.7 |
| **Mutant-28** | UV | 60.2±90 | 2.4±60 | 27.2±24 | -11.7 |
| **Mutant-29** | UV | 67.4±21 | 3.3±10 | 39.1±80 | -2.1 |
| **Mutant-30** | UV | 65.3±29 | 2.1±50 | 33.6±20 | -5.3 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-31** | UV | 62.1±45 | 2.9±20 | 24.2±14 | -8.4 |
| **Mutant-32** | UV | 55.9±24 | 2.8±12 | 27.5±14 | -19.4 |
| **Mutant-33** | UV | 61.8±15 | 2.4±11 | 26.8±12 | -11.1 |
| **Mutant-34** | UV | 60.1±20 | 2.2±28 | 26.1±12 | -12.2 |
| **Mutant-35** | UV | 61.6±13 | 2.4±42 | 24.6±15 | -9.9 |
| **Mutant-36** | UV | 61.2±21 | 2.3±19 | 24.2±29 | -10.1 |
| **Mutant-37** | UV | 58.5±28 | 1.4±10 | 27.5±15 | -14.3 |
| **Mutant-38** | UV | 51.4±16 | 1.2±40 | 29.4±18 | -24.8 |
| **Mutant-39** | UV | 57.1±12 | 2.4±40 | 25.1±18 | -15.8 |
| **Mutant-40** | UV | 61.9±15 | 3.3±40 | 29.9±24 | -11.0 |
| **Mutant-41** | UV | 65.8±29 | 2.5±21 | 26.8±80 | -5.2 |
| **Mutant-42** | UV | 62.4±15 | 2.4±10 | 24.4±20 | -8.9 |
| **Mutant-43** | UV | 63.2±18 | 2.1±40 | 20.2±14 | -7.6 |
| **Mutant-44** | EMS | 63.4±18 | 2.9±50 | 25.1±14 | -7.3 |
| **Mutant-45** | EMS | 63.3±24 | 2.8±41 | 24.6±12 | -7.6 |
| **Mutant-46** | EMS | 64.1±80 | 3.1±10 | 21.2±8 | -5.5 |
| **Mutant-47** | EMS | 61.8±28 | 2.2±10 | 32.2±40 | -10.9 |
| **Mutant-48** | EMS | 59.4±16 | 3.4±40 | 23.1±51 | -13.9 |
| **Mutant-49** | EMS | 72.4±14 | 4.6±49 | 30.4±87 | 5.5 |
| **Mutant-50** | **EMS** | **76.2±12** | **3.2±24** | **25.2±38** | **10.6** |
| **Mutant-51** | EMS | 51.6±17 | 1.2±20 | 27.4±18 | -25.5 |
| **Mutant-52** | EMS | 60.4±90 | 2.4±60 | 27.2±24 | -11.6 |
| **Mutant-53** | EMS | 59.8±12 | 2.9±25 | 20.8±25 | -13.1 |
| **Mutant-54** | EMS | 65.4±17 | 3.9±5 | 23.4±17 | -5.4 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-55** | EMS | 57.1±29 | 2.2±51 | 21.8±21 | -16.4 |
| **Mutant-56** | EMS | 60.6±45 | 2.5±20 | 22.4±29 | -11.8 |
| **Mutant-57** | **EMS** | **74.8±11** | **2.2±11** | **26.8±29** | **8.0** |
| **Mutant-58** | **EMS** | **93.1±17** | **5.4±53** | **24.1±45** | **36.1** |
| **Mutant-59** | EMS | 67.6±24 | 2.3±93 | 24.6±24 | -1.6 |
| **Mutant-60** | EMS | 64.1±23 | 4.5±33 | 22.1±66 | -6.0 |
| **Mutant-61** | EMS | 73.6±11 | 4.4±10 | 26.6±67 | 6.2 |
| **Mutant-62** | EMS | 59.2±61 | 3.1±13 | 25.2±71 | -13.7 |
| **Mutant-63** | EMS | 59.4±40 | 2.9±14 | 24.4±15 | -13.0 |
| **Mutant-64** | EMS | 64.3±40 | 2.8±71 | 26.2±18 | -6.6 |
| **Mutant-65** | EMS | 64.1±10 | 3.4±40 | 27.9±17 | -5.5 |
| **Mutant-66** | EMS | 73.9±40 | 2.2±81 | 20.8±91 | 2.71 |
| **Mutant-67** | EMS | 62.8±23 | 2.9±45 | 18.4±21 | -8.4 |
| **Mutant-68** | EMS | 51.4±35 | 2.8±20 | 26.9±29 | -25.1 |
| **Mutant-69** | EMS | 61.2±30 | 3.4±22 | 23.8±45 | -10.9 |
| **Mutant-70** | EMS | 59.4±81 | 2.2±10 | 30.4±24 | -13.4 |
| **Mutant-71** | EMS | 60.3±24 | 2.4±20 | 26.2±15 | -12.1 |
| **Mutant-72** | EMS | 45.4±39 | 2.3±30 | 27.1±18 | -34.1 |
| **Mutant-73** | EMS | 59.2±18 | 2.8±10 | 18.6±17 | -13.9 |
| **Mutant-74** | EMS | 59.4±25 | 1.4±20 | 27.2±91 | -14.1 |
| **Mutant-75** | EMS | 49.3±15 | 2.2±51 | 23.5±21 | -28.7 |
| **Mutant-76** | EMS | 54.5±21 | 1.4±30 | 27.4±29 | -20.9 |
| **Mutant-77** | EMS | 58.4±15 | 2.3±40 | 23.1±45 | -14.3 |
| **Mutant-78** | EMS | 58.1±15 | 2.1±10 | 24.9±24 | -15.5 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-79** | EMS | 60.9±18 | 2.9±30 | 22.8±15 | -11.7 |
| **Mutant-80** | EMS | 57.8±17 | 2.8±20 | 25.4±18 | -16.5 |
| **Mutant-81** | EMS | 52.1±91 | 2.1±61 | 23.2±17 | -23.7 |
| **Mutant-82** | EMS | 59.6±21 | 2.6±10 | 23.9±91 | -13.8 |
| **Mutant-83** | EMS | 57.1±29 | 2.2±51 | 21.8±21 | -16.4 |
| **Mutant-84** | EMS | 60.6±45 | 2.5±20 | 22.4±29 | -11.8 |
| **Mutant-85** | EMS | 59.2±24 | 2.4±12 | 24.2±45 | -13.8 |
| **Mutant-86** | EMS | 57.4±15 | 2.1±11 | 26.4±24 | -15.7 |
| **Mutant-87** | EMS | 47.3±20 | 1.9±28 | 27.3±40 | -30.2 |
| **Mutant-88** | EMS | 57.1±13 | 2.8±42 | 27.8±21 | -17.0 |
| **Mutant-89** | EMS | 63.9±21 | 3.4±19 | 39.4±10 | -8.0 |
| **Mutant-90** | EMS | 61.8±28 | 2.2±10 | 32.2±40 | -10.9 |
| **Mutant-91** | EMS | 59.4±16 | 3.4±40 | 23.1±51 | -13.9 |
| **Mutant-92** | EMS | 52.2±12 | 2.3±40 | 26.6±42 | -24.3 |
| **Mutant-93** | EMS | 57.4±15 | 2.1±40 | 25.2±91 | -16.4 |
| **Mutant-94** | EMS | 56.3±29 | 2.9±21 | 26.5±22 | -17.5 |
| **Mutant-95** | EMS | 58.5±15 | 2.8±10 | 24.8±56 | -15.3 |
| **Mutant-96** | EMS | 58.4±18 | 2.4±40 | 24.1±51 | -15.5 |
| **Mutant-97** | EMS | 55.1±18 | 1.2±51 | 26.6±10 | -19.4 |
| **Mutant-98** | **EMS** | **73.9±25** | **3.4±42** | **27.2±11** | **7.5** |
| **Mutant-99** | EMS | 54.8±81 | 2.3±10 | 24.5±13 | -20.8 |
| **Mutant-100** | EMS | 57.4±20 | 3.5±6 | 29.4±14 | -16.3 |
| **Mutant-101** | EMS | 61.2±14 | 2.4±50 | 26.1±11 | -10.9 |
| **Mutant-102** | EMS | 58.9±14 | 2.1±50 | 24.9±14 | -14.4 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-103** | EMS | 59.8±12 | 2.9±25 | 20.8±25 | -13.1 |
| **Mutant-104** | EMS | 59.4±70 | 2.8±25 | 24.4±14 | -12.8 |
| **Mutant-105** | EMS | 59.2±43 | 3.4±10 | 24.2±15 | -13.2 |
| **Mutant-106** | EMS | 61.4±11 | 4.2±11 | 20.1±12 | -11.2 |
| **Mutant-107** | EMS | 68.3±17 | 3.4±52 | 20.3±11 | 0.3 |
| **Mutant-108** | EMS | 73.8±24 | 4.3±9 | 26.8±95 | 7.1 |
| **Mutant-109** | EMS | 68.4±23 | 4.1±34 | 30.4±63 | -0.8 |
| **Mutant-110** | EMS | 71.2±11 | 3.9±11 | 25.2±18 | 4.0 |
| **Mutant-111** | EMS | 68.4±17 | 4.8±5 | 22.4±16 | -0.2 |
| **Mutant-112** | EMS | 76.3±24 | 1.1±9 | 18.3±42 | 10.8 |
| **Mutant-113** | EMS | 85.1±23 | 3.9±34 | 30.1±25 | 24.4 |
| **Mutant-114** | EMS | 61.9±11 | 3.8±10 | 23.9±28 | -11.1 |
| **Mutant-115** | EMS | 80.8±62 | 2.4±13 | 24.8±46 | 16.6 |
| **Mutant-116** | EMS | 71.1±41 | 3.2±14 | 24.1±13 | 3.5 |
| **Mutant-117** | EMS | 69.6±41 | 2.4±72 | 25.6±14 | 1.5 |
| **Mutant-118** | EMS | 68.2±10 | 5.2±41 | 23.2±11 | 0.3 |
| **Mutant-119** | EMS | 65.5±41 | 2.5±83 | 23.5±14 | -4.1 |
| **Mutant-120** | EMS | 73.4±23 | 4.4±41 | 22.4±25 | 6.8 |
| **Mutant-121** | EMS | 60.1±36 | 4.1±20 | 26.1±14 | -12.0 |
| **Mutant-122** | EMS | 60.9±31 | 3.9±22 | 25.9±15 | -11.3 |
| **Mutant-123** | EMS | 65.8±83 | 2.8±10 | 24.8±12 | -4.7 |
| **Mutant-124** | EMS | 66.4±24 | 2.4±20 | 26.4±11 | -3.6 |
| **Mutant-125** | EMS | 75.2±40 | 3.2±31 | 26.2±95 | 9.2 |
| **Mutant-126** | EMS | 64.4±18 | 3.4±10 | 20.4±63 | -6.6 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-127** | EMS | 52.3±26 | 2.3±20 | 17.3±18 | -23.6 |
| **Mutant-128** | EMS | 62.1±15 | 2.1±52 | 25.1±16 | -9.2 |
| **Mutant-129** | EMS | 60.9±21 | 3.9±31 | 22.9±42 | -11.6 |
| **Mutant-130** | EMS | 61.8±15 | 2.8±41 | 29.4±25 | -10.3 |
| **Mutant-131** | EMS | 46.4±15 | 2.4±10 | 25.1±28 | -32.8 |
| **Mutant-132** | EMS | 69.2±18 | 2.2±31 | 26.9±46 | 1.0 |
| **Mutant-133** | EMS | 68.4±17 | 2.4±20 | 17.8±14 | -0.5 |
| **Mutant-134** | EMS | 60.3±93 | 1.3±62 | 27.4±11 | -12.0 |
| **Mutant-135** | EMS | 68.5±21 | 2.5±10 | 23.2±14 | -0.5 |
| **Mutant-136** | EMS | 67.4±30 | 1.4±52 | 26.1±25 | -1.9 |
| **Mutant-137** | EMS | 66.1±46 | 2.1±20 | 22.3±14 | -2.7 |
| **Mutant-138** | EMS | 70.9±24 | 2.9±12 | 24.8±15 | 2.3 |
| **Mutant-139** | EMS | 58.8±15 | 2.8±11 | 21.4±12 | -14.8 |
| **Mutant-140** | EMS | 72.1±20 | 2.4±29 | 25.2±11 | 5.3 |
| **Mutant-141** | EMS | 70.6±13 | 2.2±43 | 23.4±95 | 2.4 |
| **Mutant-142** | EMS | 68.1±21 | 2.4±197 | 23.3±63 | -0.8 |
| **Mutant-143** | EMS | 61.6±29 | 2.3±10 | 21.1±18 | -10.0 |
| **Mutant-144** | EMS | 60.2±16 | 2.4±41 | 21.9±16 | -12.1 |
| **Mutant-145** | EMS | 65.5±12 | 2.2±41 | 24.8±42 | -4.8 |
| **Mutant-146** | EMS | 64.4±15 | 2.9±43 | 26.4±25 | -6.2 |
| **Mutant-147** | EMS | 83.1±30 | 1.8±21 | 26.1±28 | 21.5 |
| **Mutant-148** | EMS | 64.9±15 | 2.4±10 | 26.9±46 | -6.1 |
| **Mutant-149** | EMS | 62.8±18 | 3.2±41 | 38.8±52 | -9.2 |
| **Mutant-150** | EMS | 60.4±18 | 2.4±52 | 31.4±10 | -11.4 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-151** | EMS | 53.2±25 | 3.3±43 | 23.2±12 | -22.8 |
| **Mutant-152** | EMS | 58.4±83 | 2.8±11 | 26.4±8 | -14.7 |
| **Mutant-153** | EMS | 57.3±21 | 2.4±6 | 25.3±7 | -15.8 |
| **Mutant-154** | EMS | 59.4±14 | 2.2±51 | 25.4±11 | -13.6 |
| **Mutant-155** | EMS | 59.2±148 | 2.4±51 | 23.2±13 | -13.8 |
| **Mutant-156** | EMS | 56.4±12 | 2.3±25 | 23.4±14 | -17.8 |
| **Mutant-157** | EMS | 72.3±71 | 1.1±26 | 26.1±11 | 5.3 |
| **Mutant-158** | EMS | 55.1±44 | 3.9±10 | 26.9±14 | -19.2 |
| **Mutant-159** | EMS | 58.9±11 | 2.9±12 | 24.8±25 | -14.6 |
| **Mutant-160** | **EMS** | **74.8±18** | **2.8±5** | **31.4±14** | **8.1** |
| **Mutant-161** | EMS | 60.1±25 | 3.1±9 | 25.2±15 | -12.7 |
| **Mutant-162** | EMS | 60.6±24 | 2.6±34 | 23.1±12 | -11.4 |
| **Mutant-163** | EMS | 61.2±11 | 3.2±12 | 19.3±11 | -11.1 |
| **Mutant-164** | EMS | 60.5±18 | 2.5±5 | 24.8±95 | -11.4 |
| **Mutant-165** | EMS | 62.4±25 | 3.4±9 | 23.4±63 | -9.4 |
| **Mutant-166** | EMS | 70.1±24 | 4.1±34 | 20.2±18 | 2.3 |
| **Mutant-167** | EMS | 72.9±11 | 3.9±10 | 20.4±16 | 5.2 |
| **Mutant-168** | EMS | 69.8±63 | 4.8±13 | 25.3±42 | 1.1 |
| **Mutant-169** | EMS | 72.4±42 | 4.4±14 | 29.4±25 | 6.1 |
| **Mutant-170** | EMS | 69.2±42 | 3.2±74 | 24.2±28 | 1.8 |
| **Mutant-171** | NTG | 72.4±10 | 4.4±42 | 22.4±46 | 5.3 |
| **Mutant-172** | NTG | 67.3±42 | 1.3±84 | 18.3±24 | -1.8 |
| **Mutant-173** | NTG | 62.1±24 | 3.1±4 | 29.4±1 | -9.3 |
| **Mutant-174** | NTG | 71.9±37 | 3.9±21 | 23.2±106 | 3.7 |

| | | | | | |
|---|---|---|---|---|---|
| **Mutant-175** | NTG | 72.8±31 | 3.8±2 | 24.4±7 | 5.6 |
| **Mutant-176** | NTG | 71.4±84 | 3.1±10 | 23.1±11 | 3.5 |
| **Mutant-177** | NTG | 70.2±25 | 3.9±21 | 23.9±13 | 2.3 |
| **Mutant-178** | NTG | 67.4±40 | 2.8±31 | 22.8±14 | -2.2 |
| **Mutant-179** | NTG | 72.3±19 | 2.4±10 | 23.4±11 | 5.72 |
| **Mutant-180** | NTG | 61.5±26 | 4.2±21 | 22.2±14 | -10.3 |
| **Mutant-181** | NTG | 62.4±15 | 4.4±53 | 25.1±25 | -9.5 |
| **Mutant-182** | NTG | 66.1±22 | 3.3±31 | 24.3±14 | -2.8 |
| **Mutant-183** | NTG | 67.9±15 | 2.4±42 | 23.8±15 | -1.7 |
| **Mutant-184** | NTG | 72.1±15 | 3.2±10 | 25.4±12 | 4.31 |
| **Mutant-185** | NTG | 65.1±19 | 3.9±31 | 25.2±11 | -4.7 |
| **Mutant-186** | NTG | 53.6±18 | 3.8±21 | 19.4±95 | -22.0 |
| **Mutant-187** | NTG | 63.1±95 | 3.4±63 | 17.3±63 | -7.3 |
| **Mutant-188** | NTG | 61.6±22 | 2.2±10 | 25.1±18 | -9.9 |
| **Mutant-189** | NTG | 62.2±30 | 3.4±53 | 22.9±16 | -8.6 |
| **Mutant-190** | NTG | 47.5±47 | 2.3±21 | 29.8±42 | -31.4 |
| **Mutant-191** | NTG | 70.4±25 | 2.8±12 | 25.3±25 | 3.0 |
| **Mutant-192** | **NTG** | **76.1±15** | **2.4±11** | **27.1±28** | **11.0** |
| **Mutant-193** | NTG | 70.9±21 | 2.2±29 | 17.9±46 | 1.9 |
| **Mutant-194** | NTG | 70.8±13 | 2.4±44 | 26.8±84 | 2.1 |
| **Mutant-195** | NTG | 68.4±22 | 2.3±20 | 22.4±11 | -0.9 |
| **Mutant-196** | NTG | 66.2±29 | 1.1±10 | 26.2±16 | -3.5 |
| **Mutant-197** | NTG | 67.4±16 | 2.9±42 | 22.4±42 | -2.2 |
| **Mutant-198** | NTG | 68.3±12 | 3.3±42 | 23.3±10 | -0.8 |

The mutants, which exhibited significantly higher erythritol production than the parent strain, were reevaluated in a shake flask experiment. Significant changes in erythritol and other metabolite profiles were observed in the comparison of the parent and some of the screened mutants as shown in Fig. 3.6.

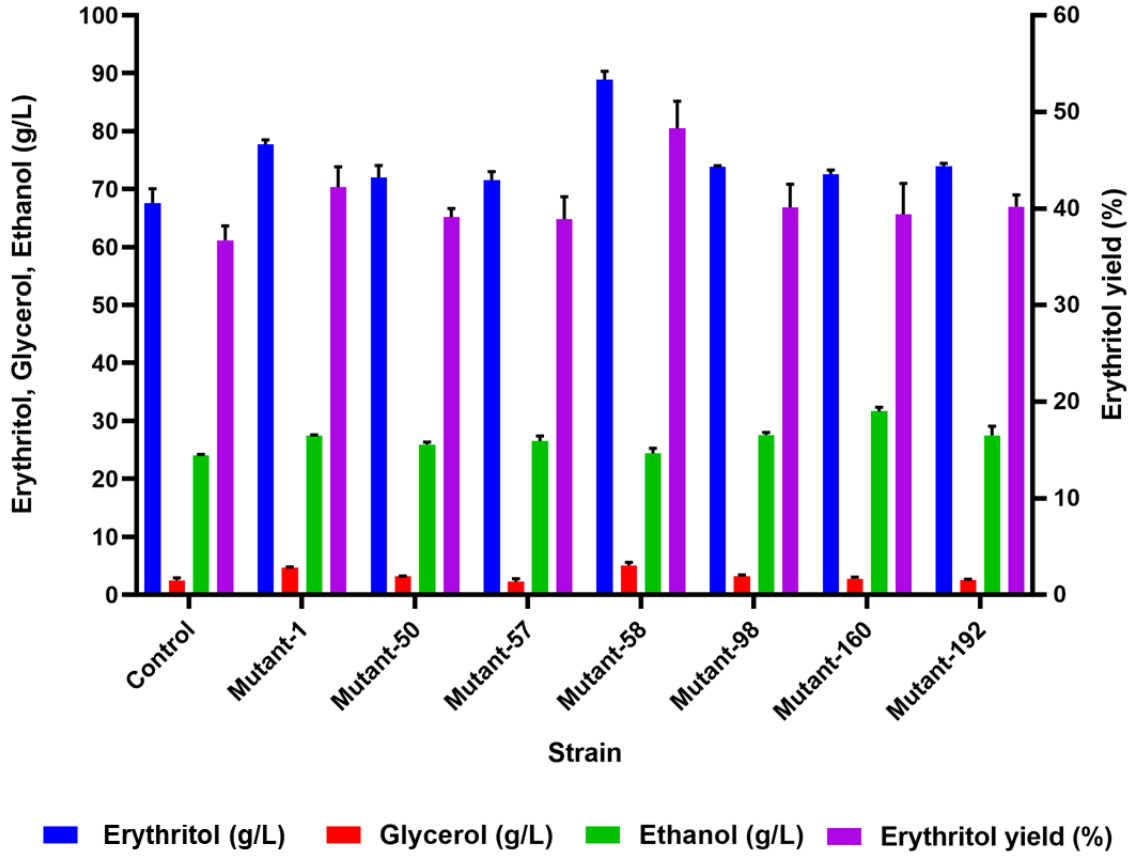



**Figure 3.6** Evaluation of selected mutants along with parent strain for erythritol and by-product formation in the shake-flask experiment, batch culture. Samples were replicated in triplicate, and all values are expressed as mean ± SD

The Mutant-58 showed the highest production of erythritol (88.9±1.4 g/L) as compared to the parent strain (67.6±2.4 g/L) and other mutant strains with erythritol productivity of 1.23 g/L/h. There was no significant difference in glycerol and ethanol yield in parent and mutant cultures except Mutant-1 and 58, where a slight increase in glycerol production was observed (5.0±0.5 g/L). However, a significant increase in erythritol production was seen in Mutant-58 than in

Mutant-1. The total sugar carbon percentage transformed into the product for the wild type was 48%, while 59% of the carbon was transformed to product formation in the case of mutant-58. Therefore, the Mutant-58 strain was selected for further evaluation for erythritol production. The stability of the mutant was confirmed by subculturing the strain for 20 generations and at each generation, metabolite production, and cell morphology were monitored and were found to be similar.

### 3.3.3 Morphology of parent strain and Mutant-58

Apart from higher erythritol production ability, Mutant-58 strain showed unique morphological traits, which differed from the parent strain. Mutant-58 colonies appeared to be rough-surfaced compared to the smoother colonies of the parent strain (Fig. 3.7 a, e). Wild-type cells were observed as elongated rods from the colony to the inoculum stage and later formed an oval shape at 72 h when grown in LFM medium (Fig. 3.7 b, c, d).

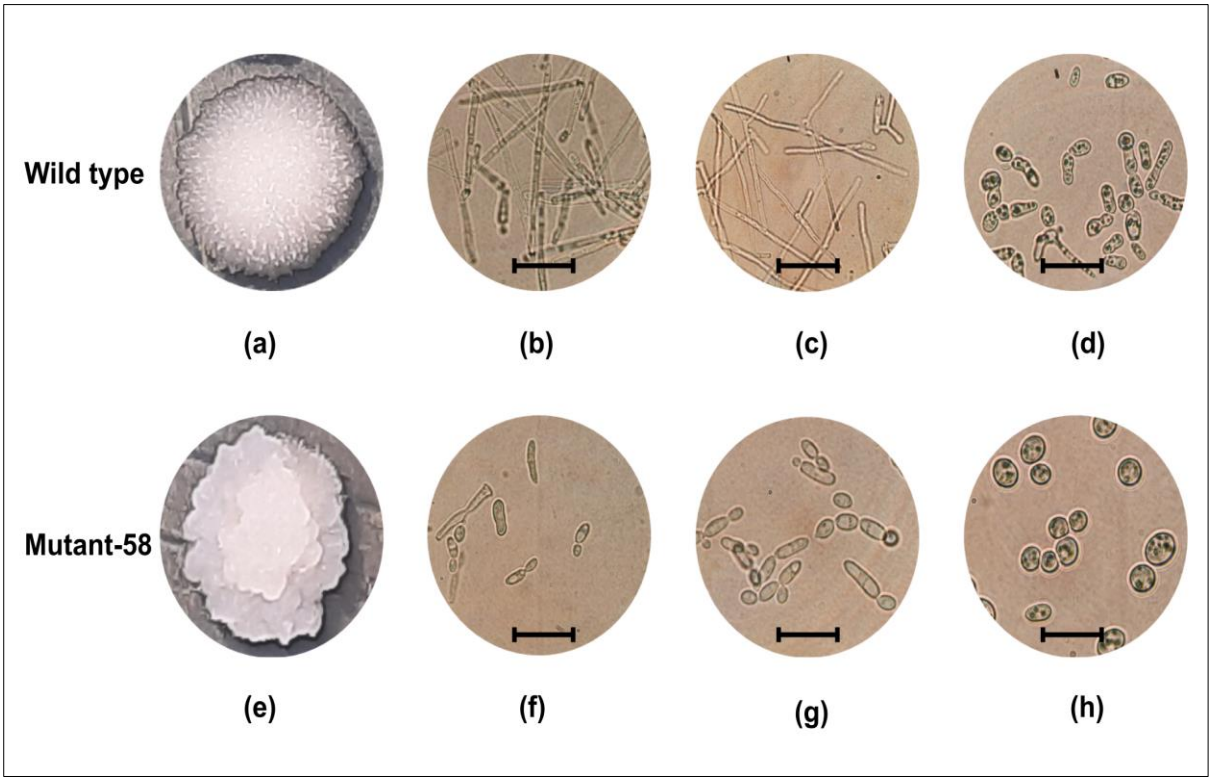


**Figure 3.7** Morphological difference between parent and mutant strain. Parent strain (a) Single colony on YPG plate, (b) Microscopy of a single colony from a YPG plate, (c) 18 h grown inoculum in YPG, (d) 72 h old culture in LFM. Mutant-58 strain (e) Single colony on YPG plate, (f) Microscopy of a single colony from YPG plate, (g) 18 h grown inoculum in YPG, (h) 72 h old culture in LFM. Bar denotes 50 µm

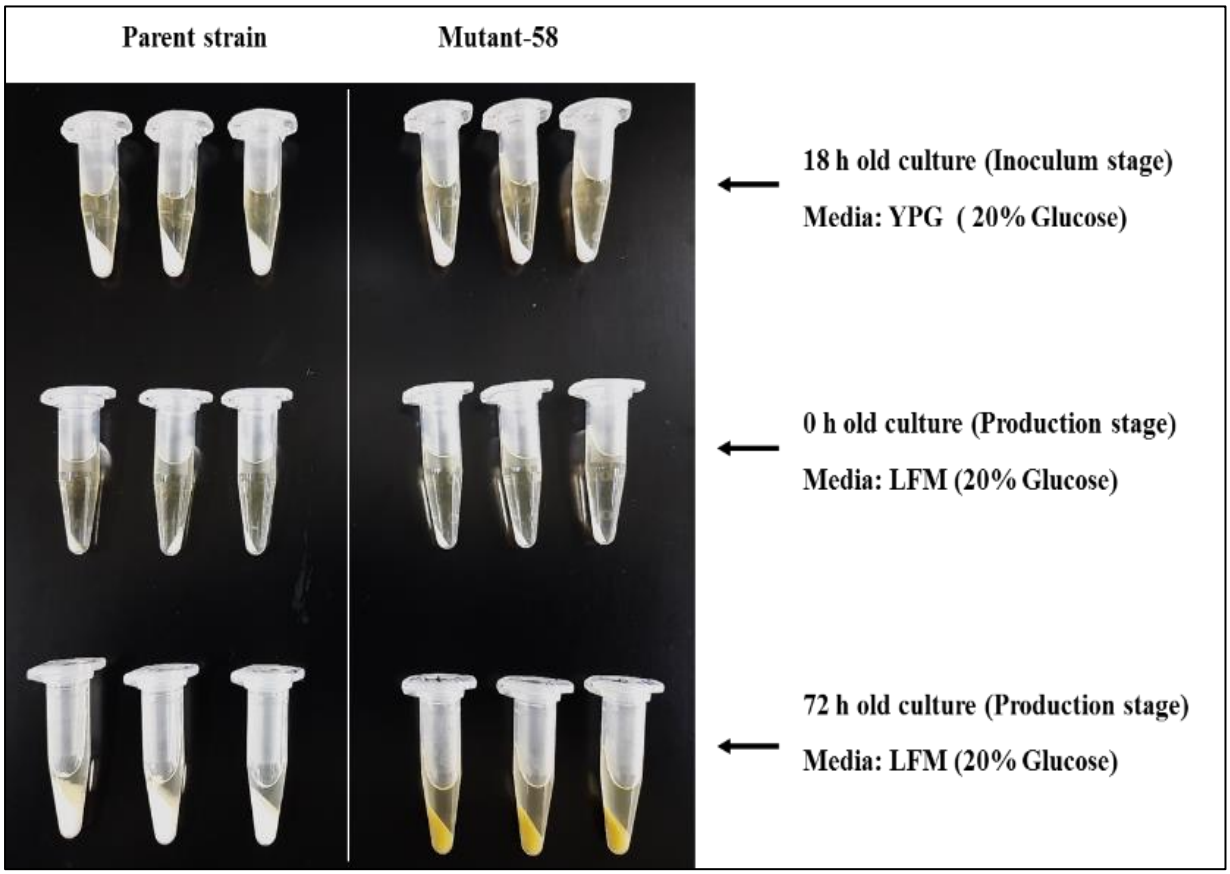



**Figure 3.8** Change in biomass colour with incubation time in parent and mutant strain

In contrast, Mutant-58 cells' size and structure appeared to have an oval shape right from the colony, and inoculum to the big circular-shaped cells in the production medium (Fig. 3.7 f, g, h). Thus, the morphological differences concerning size and shape in mutant cells might be suggestive of their resistance to osmotic stress environments. Furthermore, the color of the mutant biomass started to turn pale yellow at the end of the fermentation (72h) (Fig. 3.8).

Similar observations were made when *Yarrowia lipolytica* Wratislavia K1 wild-type strain was subjected to UV mutagenesis and one of the mutants had shown changed colony morphology compared with wild-type yeast (Mirończuk et al., 2015). It was reported in the literature that the mutant cells of *Moniliella* sp. appeared to be a short rod-like shape, while the parent cells have a long rod-like shape (Lin et al., 2010). A similar type of exterior change in cell shape and size was also detected in *Aureobasidium* sp. and its mutant (Ishizuka et al., 1989).

There are reports where microbes have been reported to exhibit multiple phenotypes in response to mutagenesis viz. producing numerous compounds including carotenoids, monascins, phenazines, melanin's, violacein, flavins, quinones, indigo, etc. In fungi, these pigments produced by fungal strains have been known to act as "fungal armor" shielding them from stressful environments, and counterbalancing oxidants produced in reply to the stress (Pombeiro-Sponchiado et al., 2017).

### 3.3.4 Optimization of medium components for erythritol production

#### 3.3.4.1 Nitrogen source, pH, and temperature

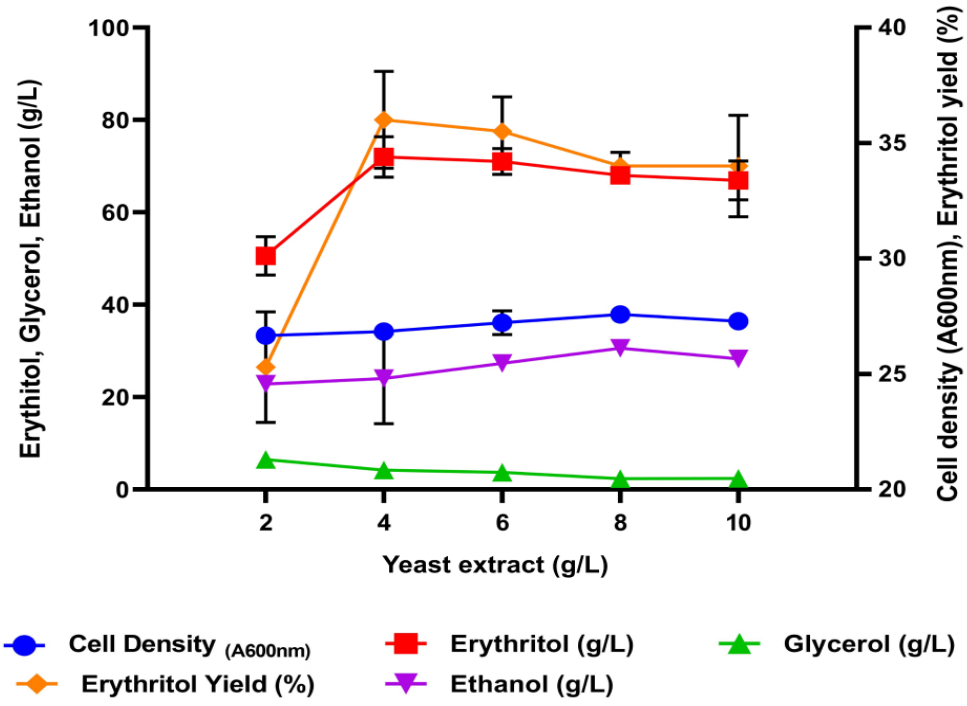


**Figure 3.9** Effect of YE concentrations on erythritol yield (Parent strain). The strain was cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

Optimization studies revealed that maximum erythritol production was observed in the mutant strain (94.3±1.9g/L) at 4 g/L YE concentration instead of 10 g/L YE present in LFM medium. An increase in YE concentration increases ethanol production in both the parent (30.6±0.4g/L) as well as in the mutant culture (21.4±0.5g/L) (Fig. 3.9, 3.10).

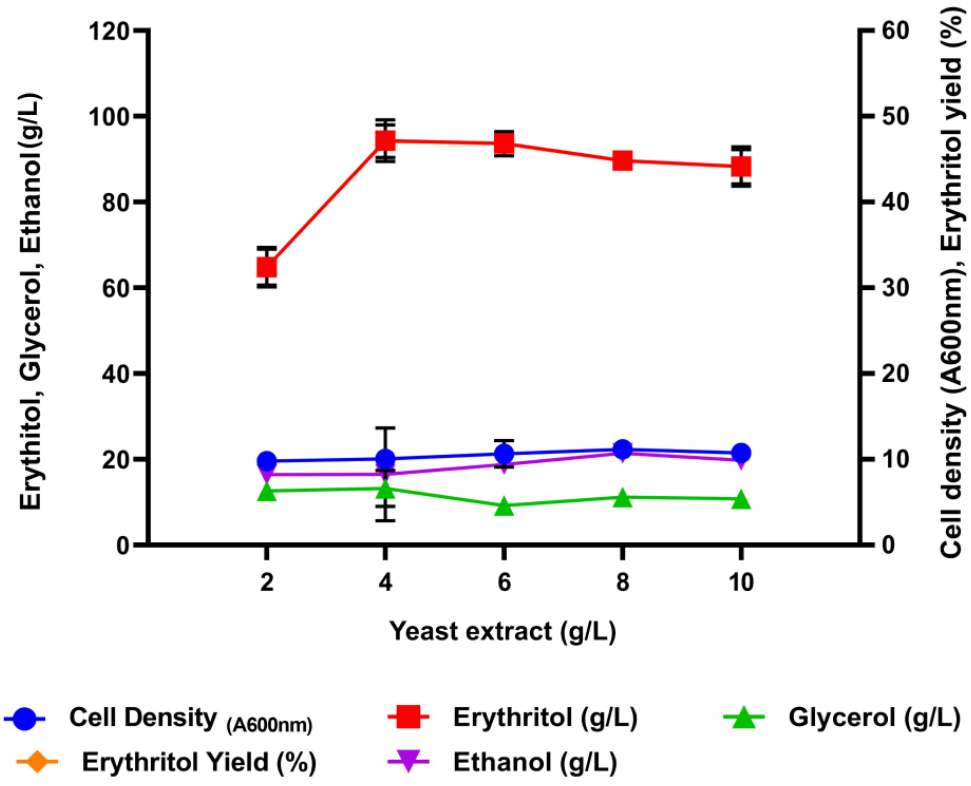



**Figure 3.10** Effect of YE concentrations on erythritol yield (Mutant-58 strain). The strain was cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

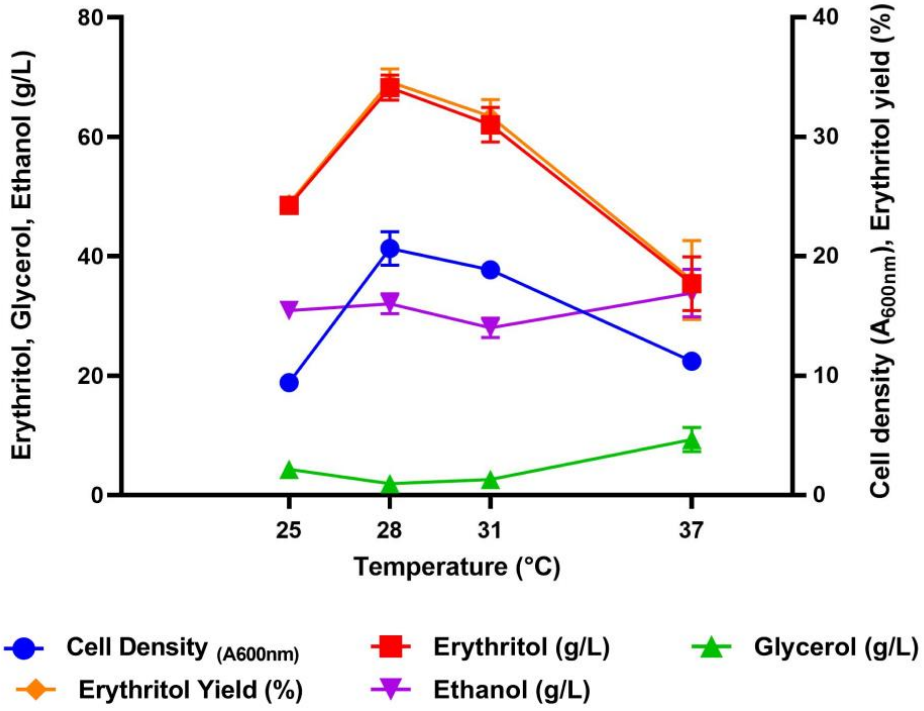



**Figure 3.11** Effect of temperature on erythritol yield (Parent strain). The strain was cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

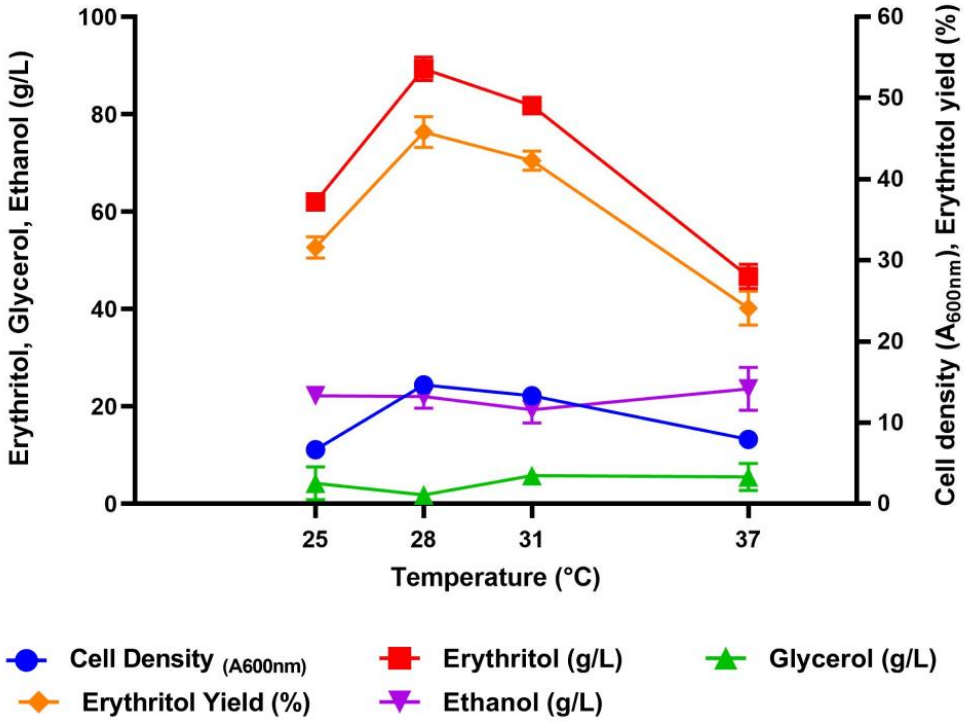


**Figure 3.12** Effect of temperature on erythritol yield (Mutant strain). The strain was cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

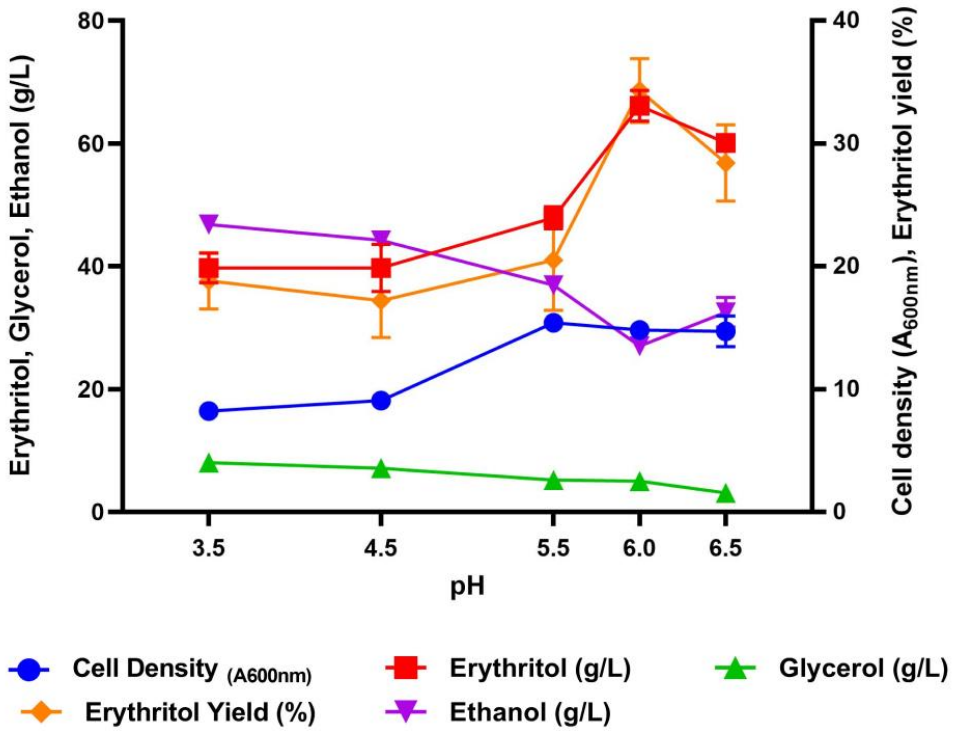



**Figure 3.13** Effect of pH on erythritol yield (Parent strain). The strain was cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

Studies on the effect of temperature on erythritol production revealed that both strains had a similar erythritol production trend, with the highest erythritol yield (45.8±1.9 %) obtained in Mutant-58 than in the parent strain (34.6±1.1%) at 28°C (Fig. 3.11, 3.12). At 37°C, a significant decline in erythritol yield (24.1±2.1 %) was observed along with growth ($OD_{600nm}$ 13.2± 0.7). Furthermore, in the parent strain, a reduction in the erythritol yield from 34.6± 1.1 % to 18± 3.3 % was noted when the temperature was raised from 28°C to 37°C.

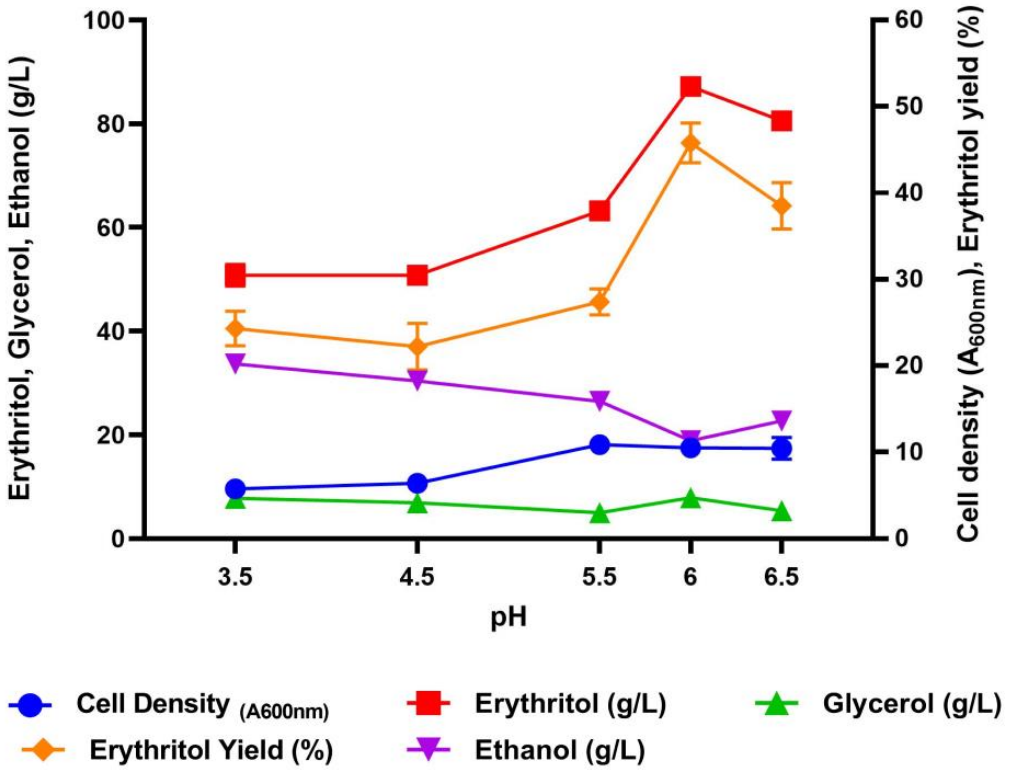



**Figure 3.14** Effect of pH on erythritol yield (Mutant strain). The strain was cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

Studies on the effect of pH on erythritol production revealed that the parent strain formed equivalent amounts of erythritol in the pH range of 5.5–6.5. Both the maximal growth (OD660nm) and erythritol yield (34.3±2.6 %) were achieved at pH 6.0. Notably, the erythritol yield tends to decrease as the pH value fluctuates from pH 6.0. Similarly in Mutant-58, erythritol yield (45.8±2.3 %) increased at pH 6.0., There was a reduction in ethanol concentration (18.9±1.1 g/L) in the Mutant-58 strain by ~33 % as compared to the parent strain (27.0±1.4 g/L) (Fig 3.13, 3.14).

### 3.3.4.2 Screening of cost-effective carbon source

Various carbon sources (glucose, xylose, molasses, sugarcane juice, sucrose, and glycerol) have been used previously for erythritol production using different yeast cultures (*M. pollinis, C. magnoliae*, *Moniliella megachiliensis*, *Aureobasidium pullulans*, and *Y. lipolytica*) (Savergave et al., 2011; Kobayashi et al., 2015; Mirończuk, et al., 2015; Sivaraman et al., 2016; Deshpande et al., 2022). Kobayashi *et al*., (2015) reported that *M. megachiliensis* can use inexpensive glycerol, a byproduct from the bio-diesel industry as a carbon source. In the most recent report, *M. pollinis* strain displayed 0.29 and 0.12g/L erythritol yield on sugarcane juice with MCL (*Moniliella* culture lysate) medium and molasses with MCL medium, respectively (Deshpande et al., 2022).

Jaggery, another cost-effective sugar source explored, contains minerals like Ca, Fe, Mn, Zn, and Cu which could be beneficial for cell growth and metabolism (Hirpara et al., 2020). In this study, cost-effective high sugar-based substrates were evaluated for erythritol production along with glycerol Fig. 3.15 a. The results indicate that glucose was the preferred carbon source for cell growth and erythritol metabolism. In the preliminary experiment, it was noted that the glycerol produced at the fermentation's start was slightly decreased. However, glycerol was poorly utilized by *M. pollinis* Mutant-58 as 184.9±0.4 g/L residual glycerol was detected in the broth after 72h. The highest erythritol was produced when jaggery (63.6±0.6 g/L) was used as a carbon source as compared to molasses, glycerol, and sugar cane juice. Cell biomass in glucose and the jaggery-based medium was observed to be 19.8±0.02 and 22.8±0.05 g/L respectively. Ethanol production in jaggery (25.3±0.70 g/L) is comparable with glucose (27.2±2.70 g/L), whereas, in molasses, ethanol production was higher compared to the other carbon substrates (52.7±1.1 g/L). An additional experiment was performed for erythritol production using Mutant-58 and parent strain for confirmation and comparisons of their productivity in a medium comprising jaggery and glucose.

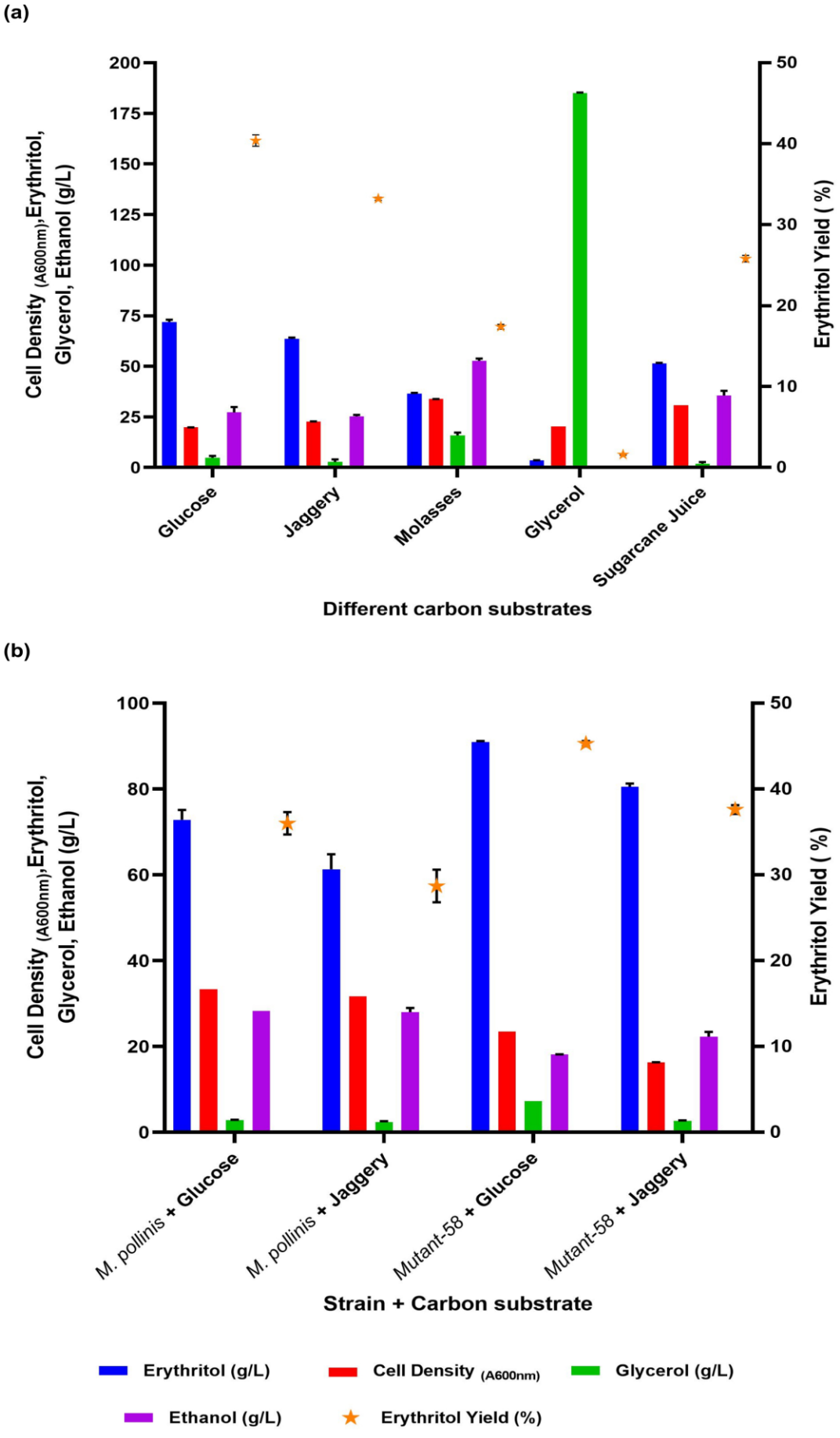

(a)
Cell Density (A600nm),Erythritol, Glycerol, Ethanol (g/L)
Erythritol Yield ( %)
200
175
150
125
100
75
50
25
0
50
40
30
20
10
0
Glucose
Jaggery
Molasses
Glycerol
Sugarcane Juice
Different carbon substrates
(b)
Cell Density (A600nm),Erythritol, Glycerol, Ethanol (g/L)
Erythritol Yield ( %)
100
80
60
40
20
0
50
40
30
20
10
0
M. pollinis + Glucose
M. pollinis + Jaggery
Mutant-58 + Glucose
Mutant-58 + Jaggery
Strain + Carbon substrate
Erythritol (g/L)
Cell Density (A600nm)
Glycerol (g/L)
Ethanol (g/L)
Erythritol Yield (%)

**Figure 3.15 a)** Evaluation of cost-effective carbon source using Mutant-58. The strain was cultured at 1% YE, pH-6.5, 28°C, and 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments). **b)** Erythritol production from parent and Mutant-58 strain using glucose and jaggery as carbon substrate. Strains were cultured at 1% YE, pH-6.5, 28°C, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

The repeat experiment confirmed that the mutant strain displays a significant surge in erythritol yield (45.3±0.3 %) and a decrease in ethanol production (18.1±0.07) than the wild-type strain when cultured in a medium having glucose as a carbon source (Fig. 3.15 b).

In jaggery, erythritol yield was lowered and 37.6±0.5 % erythritol yield achieved with Mutant-58 strain was more than the erythritol yield (36±1.3 %) attained by wild-type strain in glucose. The Mutant-58 produced 80.5±0.8 g/L erythritol as compared to the parent strain (61.3±3.5 g/L) in a jaggery-based medium, which was 31% higher than the parent strain. In this study, 37 % erythritol yield was attained using jaggery as a cost-effective carbon source with the mutant strain as compared to 30.5 % with the parent strain. Yang et al. (1999) isolated an osmotolerant strain of *C. magnoliae* and improved it with EMS treatment (Ghezelbash et al., 2014). In the present study, Mutant-58, obtained by EMS mutagenesis of *M. pollinis* produced enhanced erythritol (88.9±1.0g/L) than the parent strain (67.6±2.4 g/L) from glucose. This is the first report on the production of erythritol using jaggery as a carbon substrate. Therefore, the outcomes of this work offer a basis for the application of jaggery as an alternative to glucose for the synthesis of erythritol on an industrial scale.

Erythritol is manufactured commercially by Bolak Corporation in Whasung, Kyungki-do, Korea, Cargill Food & Pharma Specialties in Blair, Nebraska, USA, and Mitsubishi Chemical Corporation in Tokyo, Japan. These companies employ fermentation processes using *Aureobasidium* sp, *Torula* sp, and *Moniliella pollinis* to synthesize erythritol employing glucose as a carbon source (Savergave et al., 2011). The current study holds significant

importance in the field of erythritol production, especially considering recent research by Rakicka-Pustułka et al. (2020) for upscaling erythritol production from glycerol employing the *Y. lipolytica* MK1. This study highlights a novel aspect where a *Moniliella* mutant strain is utilized to produce erythritol using jaggery as a cost-effective substrate. Jaggery, known for its high sucrose content, becomes particularly valuable in the context of erythritol production. The fact that some erythritol-producing yeasts, like *Yarrowia*, encounter difficulties in accessing sucrose as a carbon source further emphasizes the intriguing advantage of sucrose utilization by *Moniliella*. In the context of this study, it was observed that the cost of commercially available glucose and pure sucrose is higher compared to locally produced jaggery (Sugarcane Gud. Available from: *https://dir.indiamart.com/impcat/sugarcane-jaggery.html*). Furthermore, pure sucrose lacks certain micronutrients that are naturally present in jaggery, which has been found to benefit yeast performance and enhance erythritol production. In the present investigation, comparable erythritol production was achieved using different types of jaggery, sourced either from the market or from local manufacturers. These findings contribute significantly to the understanding and potential advancement of erythritol production.

### 3.3.5 Measurement of residual carbon and protein concentration of parent and Mutant-58

To evaluate if there is any correlation between the rate of erythritol production and the protein content, the protein concentration in the parent and mutant strains was analyzed at the end of fermentation. The protein analysis could support the improved erythritol production in the mutant strain in comparison with the parent strain. As depicted in Table 3.2, protein concentration almost doubled (140.7±0.18mg/g DCW) in the case of Mutant-58 with a concurrent 34% rise in erythritol production as compared to the parent strain. Table 3.4 provides a detailed breakdown of the carbon balance for the Wild type and Mutant-58 strains,

offering insights into the utilization of sugar and the production of different carbon-containing compounds. Sucrose (moles), Glucose (moles), Fructose (moles), Total Sugar (moles), Erythritol (moles), Glycerol (moles), and Ethanol (moles) specify the moles of each component produced or consumed by the strains during fermentation. The total amount of carbon input into the system, originating from sugar sources specified as the total carbon input (Carbon moles). Total carbon output (Sum of Erythritol, Glycerol, and Ethanol Carbon moles) explains the total carbon output, combining the carbon in erythritol, glycerol, and ethanol. Carbon yield (%) represents the percentage of carbon output from the total input, categorized by the individual products (erythritol, glycerol, ethanol). In both the Wild type and Mutant-58, 6.7 moles of glucose are consumed during fermentation, leading to the generation of diverse compounds, including erythritol, glycerol, and ethanol. The carbon yield percentages indicate the efficiency of carbon utilization for each strain. For the Wild type, the carbon output is distributed as follows: 4.2% in erythritol, 33.83% in glycerol, and 28.52% in ethanol. The total carbon output is 63.34%. In Mutant-58 under the specified conditions (GS-4), the carbon output is distributed differently: 4.6% in erythritol, 45.35% in glycerol, and 20.62% in ethanol. The total carbon output is 68.64%. Furthermore, Table 3.3 clearly shows that in Mutant-58, more carbon flow goes towards erythritol production and less towards cell mass (CC-258 x $10^6$) and ethanol production. Whereas reverse observations were noted in the parent strain.

**Table 3.2** Measurement of protein concentration of parent and mutant strain

| Organism | DCW (g/L) | Cell count ($10^6$) | Yield (%) | Protein mg/ g DCW |
|---|---|---|---|---|
| Parent Strain | 41.6 ± 0.01 | 625 | 34.4±0.2 | 67.4 ± 0.16 |
| Mutant-58 | 15.7 ± 0.03 | 258 | 46.1±1.1 | 140.7 ± 0.18 |

Strains were cultivated in 20% glucose, 1% YE at 28°C, pH 6.5, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

**Table 3.3** Carbon balance of parent and mutant strain

| Organism | Carbon Input (100 %) | % Carbon Erythritol | % Carbon Glycerol | % Carbon Ethanol | Total carbon output (moles) | Total % carbon output |
|---|---|---|---|---|---|---|
| Parent strain | 6.7 moles | 33.8 | 1.0 | 28.5 | 4.2 | 63.3 |
| Mutant-58 | 6.7 moles | 45.4 | 2.7 | 20.6 | 4.6 | 68.6 |

Strains were cultivated in 20% glucose, 1% YE at 28°C, pH 6.5, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

**Table 3.4** Calculation of carbon balance (for Table 3.3)

| **Schemes** | **Sucrose (moles)** | **Glucose (moles)** | **Fructose (moles)** | **Total Sugar (moles)** | **Erythritol (moles)** | **Glycerol (moles)** | **Ethanol (moles)** |
|---|---|---|---|---|---|---|---|
| **Wild type** | 0.0 | 6.7 | 0.0 | 6.67 | 2.25 | 0.066 | 1.901 |
| **Mutant-58** | 0.0 | 6.7 | 0.0 | 6.67 | 3.02 | 0.18 | 1.37 |
| **Carbon balance** | **Total carbon input (Carbon moles)** | **Total carbon output (Sum of Erythritol, Glycerol and Ethanol Carbon moles)** | | **Carbon yield (%)** | | | **Total carbon output (%)** |
| | | | | Erythritol | Glycerol | Ethanol | |
| **Wild type MP** | 6.7 | 4.2 | | 33.83 | 0.98 | 28.52 | 63.34 |
| **Mut-58, GS-4** | 6.7 | 4.6 | | 45.35 | 2.66 | 20.62 | 68.64 |

Interestingly, a 2.7-fold increase in glycerol production was seen in the mutant strain than in the wild-type strain. The results of this study have revealed substantial disparities between the parent and mutant strains, particularly in terms of morphology and total dry cell weight. Notably, although the mutant strain exhibited a lower total cell mass compared to the parent strain, it displayed a higher protein content, which directly influenced metabolite production. This augmented protein concentration observed in the mutant strain could potentially be attributed to the upregulation of specific genes responsible for improving erythritol synthesis. The higher expression levels of these genes in the mutant strain likely contributed to the increased protein content, subsequently influencing the synthesis of metabolites. Furthermore, another interesting observation noted during the shake flask study was that in the mutant strain, glycerol production was slightly higher than in the parent strain. In yeast, the synthesis of erythritol is a multistep metabolic procedure, which operates commonly via the pentose phosphate pathway (PPP). In yeasts and other eukaryotic organisms, the role of the PPP is to yield NAD(P)H for cellular reactions and also to offer precursors for nucleotide and amino acid biosynthesis which includes D-ribose 5-phosphate and erythrose 4-phosphate. Erythrose reductase (ER), a NAD(P) H-dependent aldose reductase, is a crucial enzyme in the biological synthesis of erythritol (Lee et al., 2003). Dephosphorylation of erythrose-4P to erythrose and finally reduction to erythritol with the utilization of one molecule of NAD(P)H constitute the final steps in the suggested pathway. It was reported earlier that three ER isozymes; ER1, ER2, and ER3 were present in *M. megachiliensis* (Ookura et al., 2005). The expression of three ER-encoding genes (ER1, ER2, and ER3) was analyzed in a culture cultivated in 20% glucose and compared with a culture grown in 2 % glucose. Although no notable change was observed in ER expression levels during the initial hours of treatment, there was a 60-fold surge in the expression of the glycerol-3-phosphate dehydrogenase gene during the same time period. This shows that the principal response of cells was the production of glycerol. Intracellular erythritol

accumulation was observed after 12 h incubation and stayed higher during the next five days. Only ER3 expression rose remarkably during extended osmotic stress, and the level of its mRNA transcripts matched the erythritol production profile. Therefore, glycerol synthesis might be an initial response, whereas the cell's stationary phase response to osmotic stress was the production of erythritol (Kobayashi et al., 2013).

These findings highlight the intricate relationship between genetic modifications, protein synthesis, and metabolic pathways in the context of erythritol production. Elucidating the underlying mechanisms responsible for the observed phenotypic changes in the mutant strain can provide valuable insights into optimizing erythritol production through genetic manipulation. Furthermore, understanding the genetic factors involved with increased protein content and enhanced erythritol production can potentially facilitate the development of targeted strategies for improving erythritol yields in industrial applications.

### 3.4 Conclusion

Mutants of *M. pollinis* were generated through multiple methods, including UV irradiation, EMS, and NTG treatment, to achieve specific improvements in their characteristics. Among the pool of 198 mutants selected from these various mutagenesis treatments, a distinct trend emerged: those derived from EMS mutagenesis displayed a noteworthy enhancement in erythritol production. Following a rigorous screening process, one particular mutant, known as Mutant-58, was singled out for its exceptional capacity to produce significantly higher quantities of erythritol while concurrently minimizing the production of unwanted byproducts. Notably, Mutant-58 exhibited not only heightened erythritol production but also distinctive morphological traits, setting it apart from its parental counterpart. The colonies of Mutant-58 displayed a rough surface texture, contrasting with the smoother appearance of the parental strain. In terms of cellular structure, observations revealed a pronounced distinction between

the two. Wild-type cells exhibited an elongated, filamentous shape from the colony stage to the inoculum stage, later transitioning to an oval shape in the production medium. In stark contrast, Mutant-58 cells exhibited an oval shape right from the colony and inoculum stages to their final form as large, circular cells within the production medium.

In batch culture supplemented with 20% glucose, *M. Pollinis* Mutant-58 displayed an impressive capability to produce up to 92.2 ± 2.3g/L of erythritol, yielding 0.46 ± 0.11g/g, and achieving a productivity rate of 1.28 ± 0.02g/L/h. Notably, this led to a 30% increase in erythritol production while simultaneously reducing ethanol production by 27%. The optimal conditions for achieving high erythritol production were identified as follows: a temperature of 28°C, a pH of 6.0, and the addition of 4.0 g/L of yeast extract. Additionally, a comparative analysis revealed that the protein content per cell in the mutant strain was twice that of the parental strain. A metabolite profile analysis indicated that in the culture of Mutant-58, a greater portion of carbon flux was directed towards erythritol production, whereas in the parental strain, a larger proportion was allocated to cell biomass production. Furthermore, it is noteworthy that the erythritol concentration produced by the mutant strain (37.6 ± 0.5 g/g) in a jaggery-based medium exceeds the erythritol yield achieved by the Wild-type strain using glucose (36.0 ± 1.3 g/g). The erythritol yield of the mutant strain in jaggery is significantly higher (30%) than that of the wild-type strain in jaggery. This study presents an innovative approach by demonstrating the potential use of jaggery as a viable alternative to glucose for industrial-scale erythritol production.

Thus, the Mutant-58 strain used medium, and process optimization experiments were used to achieve optimum parameters such as nitrogen source (EY), pH, temperature, $KH_2PO_4$, $MgSO_4$, and carbon source (Jaggery).

### 3.5 References

Adrio, J. L., & Demain, A. L. (2006). Genetic improvement of processes yielding microbial products. In *FEMS Microbiology Reviews*, 30, 187–214.

Demain, A. L., & Adrio, J. L. (2008). Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation. In *Progress in Drug Research*, 65, 251–289.

Deshpande, M. S., Kulkarni, P. P., Kumbhar, P. S., & Ghosalkar, A. R. (2022b). Erythritol production from sugar based feedstocks by *Moniliella pollinis* using lysate of recycled cells as nutrients source. *Process Biochemistry, 112, 45-52*.

Ghezelbash, G. R., Nahvi, I., & Malekpour, A. (2014). Erythritol production with minimum by-product using *Candida magnoliae* mutant. *Applied Biochemistry and Microbiology, 50*, 292–296.

Hirata, Y., Igarashi, K., Ezaki, S., Atomi, H., & Imanaka, T. (1999). High-level production of erythritol by strain 618A-01 isolated from pollen. *Journal of Bioscience and Bioengineering, 87*, 630–635.

Hirpara, P., Thakare, N., Patel, D., & Kele, V. D. (2020). Jaggery: A natural sweetener. *Journal of Pharmacognosy and Phytochemistry*, *9*, 3145-3148.

Ishizuka, H., Wako, K., Kasumi, T., & Sasaki, T. (1989a). Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. *Journal of Fermentation and Bioengineering*, *68*, 310–314.

Kim, K. A.; Lee, J. K.; Kim, S. Y.; Oh, & D. K. (2000) Optimization of culture conditions for erythritol production by *Torula* sp. J.*Microbiology. Biotechnology,* 10, 69-74.

Kobayashi, Y., Iwata, H., Mizushima, D., Ogihara, J., & Kasumi, T. (2015). Erythritol production by *Moniliella megachiliensis* using nonrefined glycerol waste as carbon source. *Letters in Applied Microbiology*, *60*, 475–480.

Kobayashi, Y., Yoshida, J., Iwata, H., Koyama, Y., Kato, J., Ogihara, J., & Kasumi, T. (2013). Gene expression and function involved in polyol biosynthesis of *Trichosporonoides megachiliensis* under hyper-osmotic stress. *Journal of Bioscience and Bioengineering*, *115*, 645–650.

Koh, E. S., Lee, T. H., Lee, D. Y., Kim, H. J., Ryu, Y. W., & Seo, J. H. (2003). Scale-up of erythritol production by an osmophilic mutant of *Candida magnoliae*. *Biotechnology Letters*, *25*, 2103–2105.

Lee, J. K., Kim, S. Y., Ryu, Y. W., Seo, J. H., & Kim, J. H. (2003). Purification and characterization of a novel erythrose reductase from *Candida magnoliae*. *Applied and Environmental Microbiology*, *69*, 3710–3718.

Lee, K. J., & Lim, J. Y. (2003). Optimized conditions for high erythritol production by *Penicillium* sp. KJ-UV29, mutant of *Penicillium* sp. KJ81. *Biotechnology and Bioprocess Engineering*, *8*, 173–178.

Lin, S. J., Wen, C. Y., Wang, P. M., Huang, J. C., Wei, C. L., Chang, J. W., & Chu, W. S. (2010). High-level production of erythritol by mutants of osmophilic *Moniliella* sp. *Process Biochemistry*, *45*, 973–979.

Mirończuk, A. M., Dobrowolski, A., Rakicka, M., Rywińska, A., & Rymowicz, W. (2015). Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol. *Process Biochemistry*, *50*, 61–68.

Mirończuk, A. M., Rakicka, M., Biegalska, A., Rymowicz, W., & Dobrowolski, A. (2015). A two-stage fermentation process of erythritol production by yeast *Y. lipolytica* from molasses and glycerol. *Bioresource Technology, 198*, 445–455.

Moon, H. J., Jeya, M., Kim, I. W., & Lee, J. K. (2010). Biotechnological production of erythritol and its applications. *Applied Microbiology and Biotechnology*, 86, 1017–1025.

Ookura, T., Azuma, K., Isshiki, K., Taniguchi, H., Kasumi, T., & Kawamura, Y. (2005). Primary structure analysis and functional expression of erythrose reductases from erythritol-producing fungi (*Trichosporonoides megachiliensis* SNG-42). *Bioscience, Biotechnology and Biochemistry*, *69*, 944–951.

Parekh, S., Vinci, V. A., & Strobel, R. J. (2000). Improvement of microbial strains and fermentation processes. In *Applied Microbiology and Biotechnology*, 54, 287-301.

Pombeiro-Sponchiado, S. R., Sousa, G. S., Andrade, J. C. R., Lisboa, H. F., & Gonçalves, R. C. R. (2017). Production of melanin pigment by fungi and its biotechnological applications. In *Melanin*. InTech.

Qiu, X., Xu, P., Zhao, X., Du, G., Zhang, J., & Li, J. (2020). Combining genetically-encoded biosensors with high throughput strain screening to maximize erythritol production in *Yarrowia lipolytica*. *Metabolic Engineering*, *60*, 66–76.

Rakicka-Pustułka M, Mirończuk A, Celińska E, Białas W, & Rymowicz W (2020). Scale-up of the erythritol production technology process simulation and techno-economic analysis. J Clean Prod, 257,1–11

Ryu, Y. W., Park, C. Y., Park, J. B., Kim, S. Y., & Seo, J. H. (2000). Optimization of erythritol production by *Candida magnoliae* in fed-batch culture. *Journal of Industrial Microbiology and Biotechnology*, *25*, 100–103.

Savergave, L. S., Gadre, R. V., Vaidya, B. K., & Narayanan, K. (2011). Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochemical Engineering Journal*, *55*, 92–100.

Shukla, S. (2015). A Bradford multiplexing method for protein estimation in fermented foods: Soy sauces. *Bangladesh Journal of Pharmacology*, *11*, 24-27.

Sivaraman, B., Badle, S. S., & Rangaswamy, V. (2016). Production of 1,3-butadiene from erythritol. *ChemistrySelect*, *1*, 4630-4632.

Vagenende, V., Yap, M. G. S., & Trout, B. L. (2009). Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. *Biochemistry*, *48*, 11084–11096.

Winston F (2008). EMS and UV mutagenesis in yeast. In, Curr Protoc Mol Biol Wiley, New York City, US, pp 133B1–133B5

Yang SW, Park JB, & Soo Han N (1999). Production of erythritol from glucose by an osmophilic mutant of Candida magnoliae. Biotechnoly Letters, 21, 887–890

**Chapter. 4**

# Statistical medium optimization for enhanced erythritol production

**Abstract**

The optimization of both the composition of the medium and culturing conditions for Mutant-58 strain was systematically conducted using a two-fold approach. Initially, a "one factor at a time" method was employed in shake flask experiments, which allowed for the individual adjustment of specific parameters to identify their effects on erythritol production. Subsequently, to further enhance erythritol yield while concurrently reducing byproduct formation, a more comprehensive statistical media optimization was implemented. This advanced technique utilized a central composite design (CCD). The key variables subject to optimization included the nitrogen source, pH levels, temperature, concentrations of $KH_2PO_4$, $MgSO_4$, and the choice of carbon sources. These parameters were fine-tuned to achieve their respective optimum values. The optimized medium composition, as determined by the RSM model and expressed in grams per liter (g/L), included jaggery at 226.26, yeast extract at 4.36, $KH_2PO_4$ at 4.34, $MgSO_4$ at 0.31, and pH at 5.42. The statistically optimized medium achieved the highest erythritol production (91.2±3.4 g/L) and a 10% increase in yield (40.7±3.4%) compared to the unoptimized medium (37%) in a 25 mL shake flask using jaggery as a carbon substrate. This not only underscores the effectiveness of the optimization process but also holds significant promise for the large-scale production of erythritol.

## 4.1 Introduction

The traditional approach of media optimization, which involves adjusting varying one parameter individually while maintaining others constant, is known for being a laborious and time-consuming method. Additionally, due to its linear nature, it doesn't consider the potential synergistic effects among operating parameters throughout the fermentation procedure (Lotfy et al., 2007; Choudhari & Singhal, 2008). As a result, this conventional method often falls short in predicting the 'true' optimum conditions. To overcome these limitations, statistical optimization techniques have gained prominence in medium optimization (Tanyildizi et al., 2005; Pal et al., 2009). Design of Experiments (DoE) can effectively understand the interactions among the various physiological parameters to optimize media conditions. Response Surface Methodology (RSM) has been widely employed to optimize responses by modifying the composition of the media. (Kekez et al., 2015). Since the production of polyols is intricately tied to the environmental conditions created by various medium components, the application of statistical methods, such as RSM, became the method of choice for optimizing the conditions for Mutant-58. Recently, RSM has become one of the most widely utilized statistical optimization techniques, finding applications across a spectrum of compounds synthsized by microbes, including enzymes (Vohra & Satyanarayana, 2002), acids (Bustos et al., 2004), terpenoids (Choudhari & Singhal, 2008), polyols ( (Ghezelbash et al., 2014; Savergave et al., 2011), exopolysaccharides (Wagh et al., 2022) and more.

In the previous chapter, we identified *M. pollinis* yeast culture as the focal point for a strain improvement initiative involving mutagenesis. Among 198 isolated mutants, Mutant-58 exhibited significantly higher erythritol production compared to other mutant strains. Moreover, it was noted that jaggery, as a carbon source, ranked second only to glucose in terms of its efficacy for erythritol production. Previous experimental data of Mutant-58 strain indicated that the concentration of erythritol produced was profoundly influenced by the

composition of the growth medium. Consequently, the primary objective of this section is to elevate erythritol production by statistically optimizing the constituents and parameters of the growth medium. This strategic optimization process holds promise for significantly enhancing the efficiency of erythritol production.

## 4.2 Materials and methods

### 4.2.1 Media optimization for maximum erythritol formation from *M. pollinis* Mutant-58 using Response Surface Methodology (RSM)

For the optimization of the medium by RSM, 5 factors viz. jaggery, YE, $KH_2PO_4$, $MgSO_4$, and pH were chosen. These parameters are critical in the erythritol production process and can significantly impact the rate and final yield of erythritol production. Central composite design (CCD) was used to study the effect of optimized parameters. A two-level factorial: Half fraction factorial design leading to a total of 32 experiments were performed in triplicate (cube points 16 plus 6 center points in cube plus 10 axial). In all flasks (250ml), 10% (v/v) inoculum was added and incubated for 3 days and HPLC was used to analyze the concentration of sugar alcohols as explained previously. As specified in Table 4.1, higher and lower values for every single variable were used in the design.

**Table 4.1** Coded values of independent variables

| Coded values | Jaggery (g/L) | YE (g/L) | $KH_2PO_4$ (g/L) | $MgSO_4$ (g/L) | pH |
|---|---|---|---|---|---|
| -1 | 100 | 2.0 | 2.5 | 0.2 | 5.5 |
| 0 | 200 | 4.0 | 5.0 | 0.25 | 6.0 |
| 1 | 300 | 6.0 | 7.2 | 0.5 | 6.5 |

The results of erythritol productivity were used to form the RSM model. To obtain the optimized concentrations of the medium compositions and experimental parameters, response

surface graphs are generated, and statistical analysis of the data is performed using the Minitab 19 software (Version 19.1). The correctness of the optimization of the process parameters of the model was studied by analysis of variance (ANOVA) and the coefficient of $R^2$ (Abdelgalil et al., 2018). The value of $R^2$ shows whether the model would be a good fit for the given data set. The statistical significance of the fit of the polynomial model equation was examined by the F test. Optimized medium constituents and process parameters (attained by CCD trials) were validated at the shake flask and the bioreactor scale for erythritol production.

### 4.2.2 Validation of the model

The validation of the experimental model involved testing the selected optimum parameters determined by the Central Composite Design (CCD) in Erlenmeyer flasks of varying sizes-250 ml, 2000 ml and a 3.0L fermentor. The respective medium volumes were 25 mL, 200 mL, and 1.3 L. The results obtained from the 1.3 L fermentor are detailed in the upcoming chapter. This chapter focuses on the validation of the model conducted in 25 ml and 200 ml shake flasks.

For the 20 ml and 200 ml shake flask studies, experiments were performed in 250 ml and 2-liter flasks, respectively. The optimal medium composition utilized for these experiments included 225 g/L jaggery, 4.4 g/L yeast extract (YE), a pH of 5.5, 4.4 g/L $KH_2PO_4$, and 0.31 g/L $MgSO_4$. The inoculated flasks were placed in a shaking incubator set at 28°C and 180 RPM for incubation. Sampling was performed at 72 hours to measure optical density (OD), pH, residual glucose, and polyol concentrations. Each test was conducted in triplicate to ensure the reliability and consistency of the results.

## 4.3 Results and discussions

### 4.3.1 Media optimization for maximum erythritol formation from *M. pollinis* Mutant-58 using RSM

The medium and process optimization experiments aimed to fine-tune various key parameters critical for erythritol production. These parameters encompassed the nitrogen source, pH levels, temperature, $KH_2PO_4$ and $MgSO_4$ concentrations, as well as the choice of the carbon source. To achieve precise optimization, the study employed a Central Composite Design (CCD), focusing on factors that required minute adjustments. A total of 32 distinct experimental setups were meticulously designed, incorporating various combinations of jaggery (A), yeast extract (YE) (B), $KH_2PO_4$ (C), $MgSO_4$ (D), and pH (E). These experimental designs encompassed a range of factorial, axial, and center points, ensuring comprehensive coverage of potential variations. The CCD experimental runs and their corresponding observed responses are systematically documented in Table 4.2. Each response was calculated through the utilization of a regression equation, thereby providing a clear and quantitative evaluation of the experimental outcomes. This systematic approach not only allowed for a thorough exploration of optimal conditions but also facilitated precise adjustments in response to the specific needs of the production process.

**Table 4.2** CCD designs run with their observed responses

| Run Order | Jaggery (g/L) | YE (g/L) | pH | $KH_2PO_4$ (g/L) | $MgSO_4.7H_2O$ (g/L) | Observed erythritol yield (%) |
|---|---|---|---|---|---|---|
| 1 | 100 | 2.0 | 5.5 | 2.5 | 0.5 | 16.9±1.2 |
| 2 | 300 | 2.0 | 5.5 | 2.5 | 0.2 | 20.2±2.3 |
| 3 | 100 | 6.0 | 5.5 | 2.5 | 0.2 | 24.5±1.2 |
| 4 | 300 | 6.0 | 5.5 | 2.5 | 0.5 | 24.5±1.7 |
| 5 | 100 | 2.0 | 6.5 | 2.5 | 0.2 | 18.2±2.1 |
| 6 | 300 | 2.0 | 6.5 | 2.5 | 0.5 | 9.0±0.9 |
| 7 | 100 | 6.0 | 6.5 | 2.5 | 0.5 | 17.0±0.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8 | 300 | 6.0 | 6.5 | 2.5 | 0.2 | 23.4±1.5 |
| 9 | 100 | 2.0 | 5.5 | 7.5 | 0.2 | 17.8±1.1 |
| 10 | 300 | 2.0 | 5.5 | 7.5 | 0.5 | 22.4±1.0 |
| 11 | 100 | 6.0 | 5.5 | 7.5 | 0.5 | 16.5±2.3 |
| 12 | 300 | 6.0 | 5.5 | 7.5 | 0.2 | 21.8±2.1 |
| 13 | 100 | 2.0 | 6.5 | 7.5 | 0.5 | 18.5±0.8 |
| 14 | 300 | 2.0 | 6.5 | 7.5 | 0.2 | 12.5±0.6 |
| 15 | 100 | 6.0 | 6.5 | 7.5 | 0.2 | 19.9±1.8 |
| 16 | 300 | 6.0 | 6.5 | 7.5 | 0.5 | 20.8±2.0 |
| 17 | 00 | 4.0 | 6.0 | 5.0 | 0.35 | 3.2±0.2 |
| 18 | 400 | 4.0 | 6.0 | 5.0 | 0.35 | 20.2±1.3 |
| 19 | 200 | 0.0 | 6.0 | 5.0 | 0.35 | 12.97±1.3 |
| 20 | 200 | 8.0 | 6.0 | 5.0 | 0.35 | 22.5±2.2 |
| 21 | 200 | 4.0 | 5.0 | 5.0 | 0.35 | 40.0±2.4 |
| 22 | 200 | 4.0 | 7.0 | 5.0 | 0.35 | 26.4±1.8 |
| 23 | 200 | 4.0 | 6.0 | 0.0 | 0.35 | 24.5±2.0 |
| 24 | 200 | 4.0 | 6.0 | 10.0 | 0.35 | 22.1±1.8 |
| 25 | 200 | 4.0 | 6.0 | 5.0 | 0.05 | 32.8±3.0 |
| 26 | 200 | 4.0 | 6.0 | 5.0 | 0.65 | 30.0±2.1 |
| 27 | 200 | 4.0 | 6.0 | 5.0 | 0.35 | 35.0±1.4 |
| 28 | 200 | 4.0 | 6.0 | 5.0 | 0.35 | 35.9±1.2 |
| 29 | 200 | 4.0 | 6.0 | 5.0 | 0.35 | 36.0±0.8 |
| 30 | 200 | 4.0 | 6.0 | 5.0 | 0.35 | 37.2±0.9 |
| 31 | 200 | 4.0 | 6.0 | 5.0 | 0.35 | 37.0±0.9 |
| 32 | 200 | 4.0 | 6.0 | 5.0 | 0.35 | 37.8±1.4 |

To establish a robust connection between the independent process variables and erythritol production, a second-order polynomial equation was employed. This equation encapsulated the

interplay of these variables, and the coefficients for each term within the equation were meticulously determined through a comprehensive multiple regression analysis. By employing this regression equation, each response could be accurately calculated, ensuring a precise evaluation of the impact of these variables on erythritol production. This method allowed for a deeper understanding of the complex relationships and facilitated the prediction and optimization of erythritol production.

$$Y = -153.3 + 0.386\,(A) + 6.55\,(B) + 46.2\,(E) + 1.17\,(C) + 42.8\,(D) - 0.000642\,(A)*(A) - 1.227\,(B)*(B) - 4.17\,(E)*(E) - 0.5628\,(C)*(C) - 66.3\,(D)*(D) + 0.00622\,(A)*(B) - 0.0264\,(A)*(E) + 0.00107\,(A)*(B) + 0.0429\,(A)*(D) + 0.806\,(B)*(E) - 0.216\,(B)*(C) - 1.85\,(B)*(D) + 0.585\,(E)*(C) - 3.9\,(E)*(D) + 4.18(C)*(D)$$

where Y is the response variable (Erythritol g/L), A= Jaggery, B=YE, C= $KH_2PO_4$, D= $MgSO_4.7H_2O$ and E=pH

The exploration of optimal parameter concentrations and their combined effects on the response variable was conducted through the utilization of 2D contour plots and 3D scattered plots. In the initial experiments, where parameters were adjusted individually using a one-factor-at-a-time approach, it was established that the ideal conditions for erythritol production entailed 200 g/L of glucose, 4 g/L of yeast extract (YE), a temperature of 28°C, and a pH of 6.0. Subsequent in-depth analyses underscored the significance of carbon and nitrogen sources, as well as pH, in erythritol production. This was confirmed by their respective P values (0.029, 0.007, and 0.007), as indicated in Table 4.3. Furthermore, it was evident that jaggery, particularly at a higher concentration of 225 g/L, exerted the most substantial influence on erythritol production, as depicted in Fig. 4.1.

**Table 4.3** Analysis of variance for anticipation of erythritol (%) yields influenced by variables in screening design of experiment.

| **Source** | **DF*** | **Adj SS**** | **Adj MS***** | **F-Value** | **P-Value** |
|---|---|---|---|---|---|
| Model | 20 | 2439.16 | 121.96 | 11.90 | 0.001 |
| Linear | 5 | 308.52 | 61.70 | 6.02 | 0.006 |
| Jaggery (g $L^{-1}$) | 1 | 64.35 | 64.35 | 6.28 | 0.029 |
| YE (g $L^{-1}$) | 1 | 112.49 | 112.49 | 10.98 | 0.007 |
| KH2PO4 (g $L^{-1}$) | 1 | 2.87 | 2.87 | 0.28 | 0.607 |
| MgSO4.7H2O (g $L^{-1}$) | 1 | 13.95 | 13.95 | 1.36 | 0.268 |
| pH | 1 | 114.84 | 114.84 | 11.21 | 0.007 |
| Square | 5 | 1986.91 | 397.38 | 38.78 | 0.000 |
| A*A | 1 | 1208.07 | 1208.07 | 117.90 | 0.000 |
| B*B | 1 | 706.81 | 706.81 | 68.98 | 0.000 |
| C*C | 1 | 362.94 | 362.94 | 35.42 | 0.000 |
| D*D | 1 | 65.34 | 65.34 | 6.38 | 0.028 |
| E*E | 1 | 31.88 | 31.88 | 3.11 | 0.105 |
| 2-Way Interaction | 10 | 143.73 | 14.37 | 1.40 | 0.293 |
| A*B | 1 | 24.75 | 24.75 | 2.42 | 0.048 |
| A*E | 1 | 27.83 | 27.83 | 2.72 | 0.128 |
| A*C | 1 | 1.16 | 1.16 | 0.11 | 0.743 |
| A*D | 1 | 6.63 | 6.63 | 0.65 | 0.438 |
| B*E | 1 | 10.40 | 10.40 | 1.02 | 0.335 |
| B*C | 1 | 18.71 | 18.71 | 1.83 | 0.204 |
| B*D | 1 | 4.95 | 4.95 | 0.48 | 0.501 |

| | | | | | |
|---|---|---|---|---|---|
| E*C | 1 | 8.56 | 8.56 | 0.84 | 0.380 |
| E*D | 1 | 1.38 | 1.38 | 0.13 | 0.721 |
| C*D | 1 | 39.38 | 39.38 | 3.84 | 0.076 |
| Error | 11 | 112.71 | 10.25 | | |
| Lack-of-Fit | 6 | 107.42 | 17.90 | 16.93 | 0.004 |
| Pure Error | 5 | 5.29 | 1.06 | | |
| Total | 31 | 2551.87 | | | |

*DF- degree of freedom; **SS- sum of squares; and ***MS- mean square.

$R^2$(Coefficient of determination in regression model)-95.58%

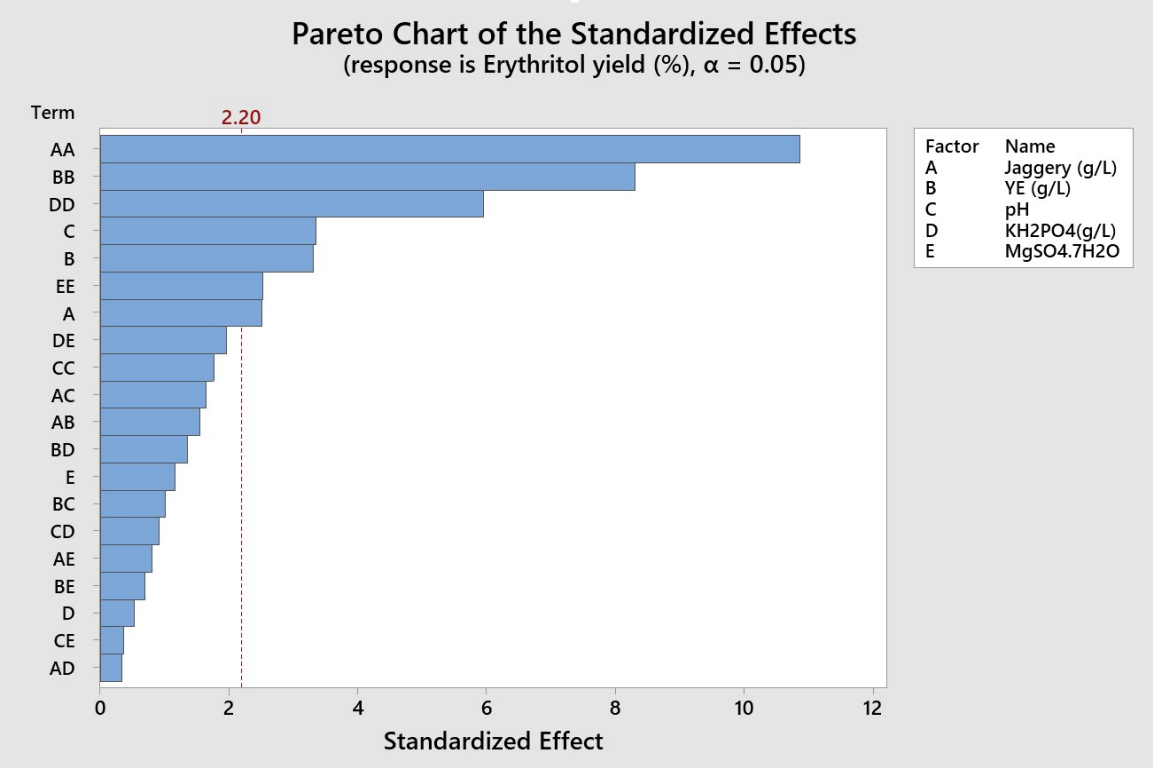



**Figure 4.1** Main effect of a variable on erythritol production

Conversely, the P values for $KH_2PO_4$ and $MgSO_4$ suggested that these factors held less significance in the context of erythritol production. To gain a more comprehensive

understanding, a RSM model was employed to investigate a three-component, three-level setup with a single response variable. This focused on three key medium constituents: jaggery, YE, and pH, while keeping other variables at their central points. This approach allowed for a thorough examination of the intricate relationships among these parameters, thereby enabling the precise optimization of conditions for erythritol production.

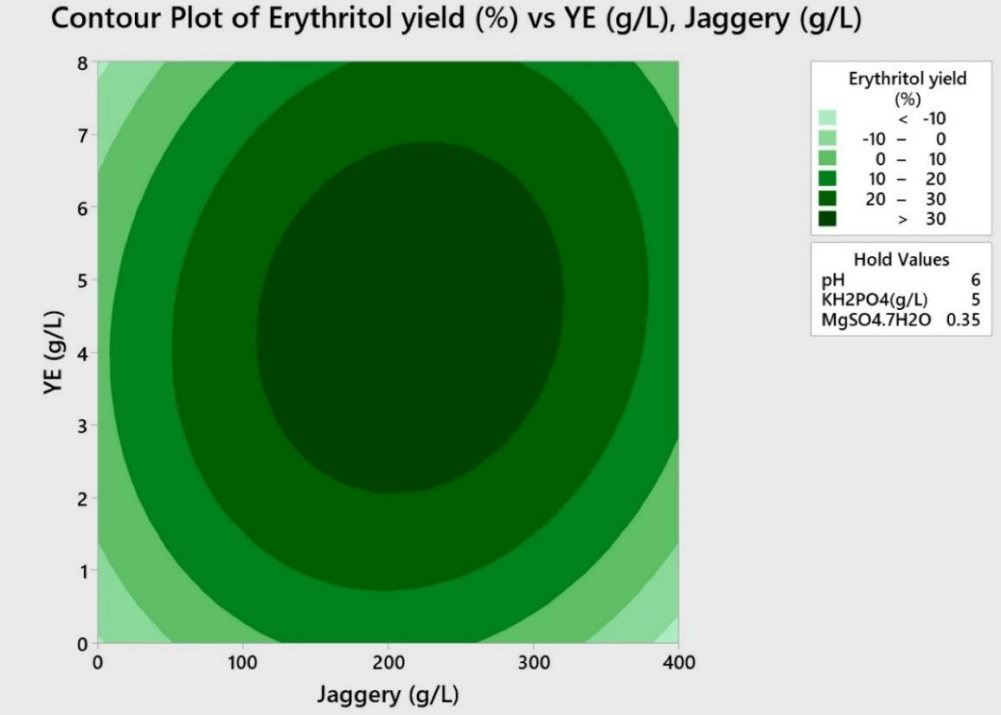



**Figure 4.2** Counter plot of a statistically significant interaction for the erythritol production (YE, Jaggery Vs Erythritol yield)

In Fig. 4.2, we can observe the impact of varying concentrations of jaggery and yeast extract (YE) on erythritol production. The plot reveals that the optimal concentrations tend to be situated around the mid-range of the jaggery and YE concentrations. Notably, there is a linear increase in erythritol synthesis as the concentrations of jaggery and YE are raised, up to approximately 215g/L for jaggery and 4.6g/L for YE.

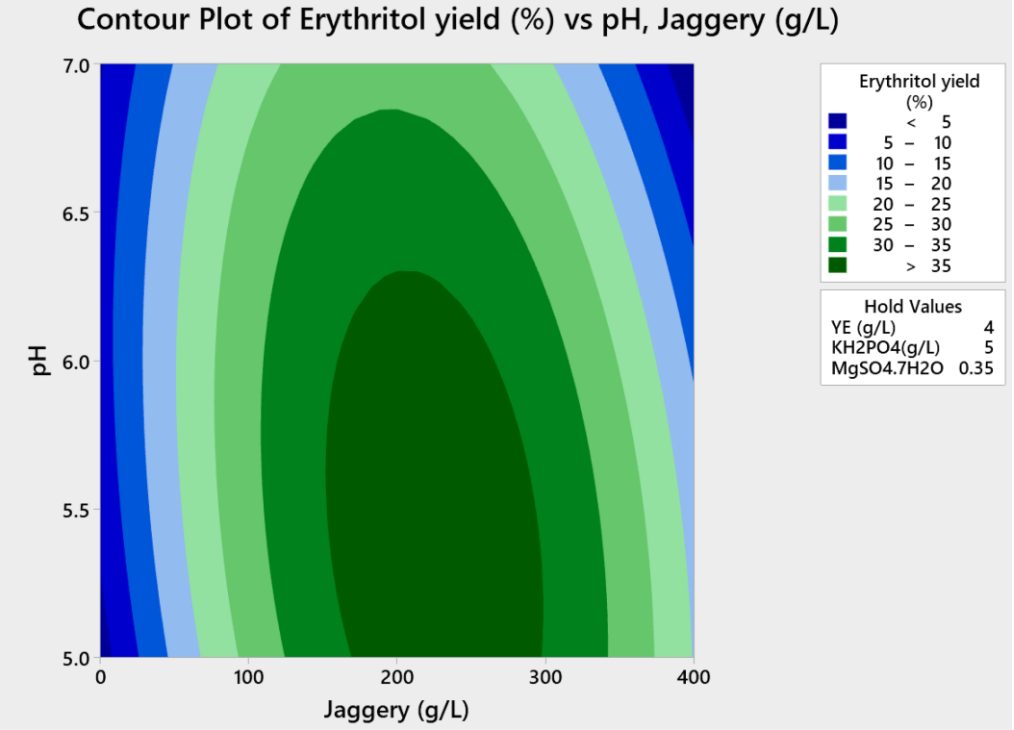



**Figure 4.3** Counter plot of a statistically significant interaction for the erythritol production (pH, Jaggery Vs Erythritol yield)

Beyond these thresholds, we observed a decline in erythritol production. This graphical representation highlights the interplay between jaggery and YE concentrations, shedding light on the conditions that yield the highest erythritol production while also providing insight into the range within which these factors exert their positive influence before encountering diminishing returns. These findings have significant implications for the optimization of erythritol production processes. Fig. 4.3 serves as a visual representation of the influence of jaggery concentration and pH on erythritol productivity, showcasing their interaction. This graph distinctly illustrates the highest erythritol yield, reaching an impressive 38.22%. This optimal yield was achieved at a specific pH level of 5.36 in conjunction with a jaggery concentration of 225 g/L. The figure demonstrates the dynamic relationship between these two vital variables in the production of erythritol.

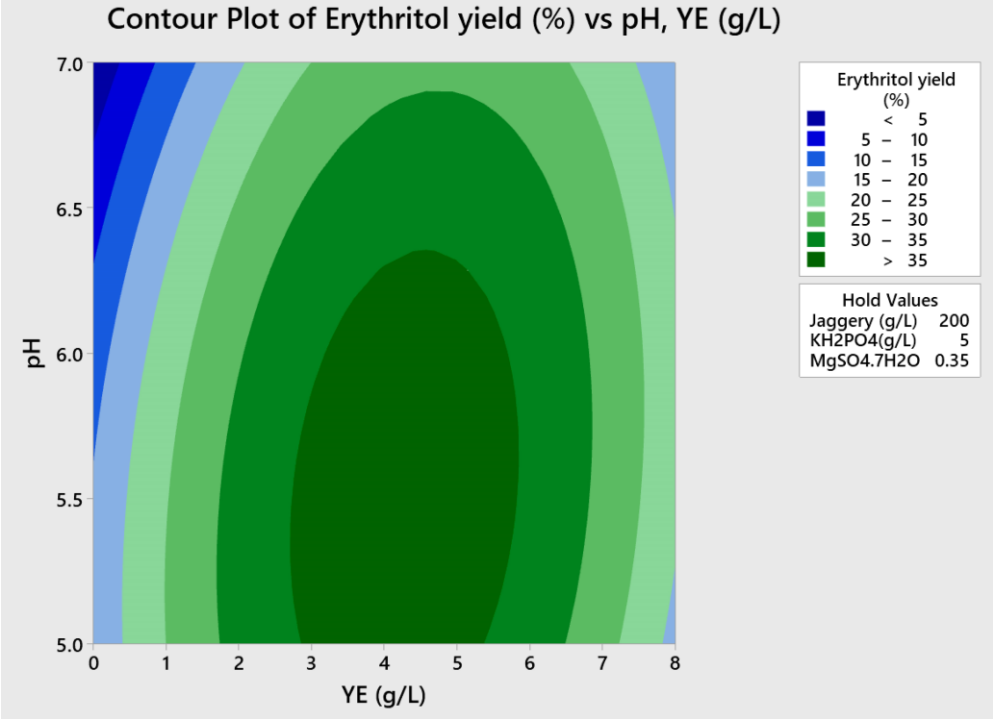



**Figure 4.4** Counter plot of a statistically significant interaction for the erythritol production (pH, YE Vs erythritol yield)

By pinpointing the conditions that result in the highest erythritol yield, it offers essential insights for the optimization of the production process. This knowledge aids in fine-tuning the production parameters to achieve maximum efficiency in erythritol production.

Fig. 4.4 provides an illustrative representation of erythritol productivity concerning the interplay between pH and yeast extract (YE) concentrations. The figure offers valuable insights into the optimal conditions, which tend to converge around the mid-range values of YE, approximately 4.26, and the initial pH levels. Furthermore, it is evident from the graph that the interaction between pH and jaggery concentration, at a level of 5.47, significantly influences erythritol production.

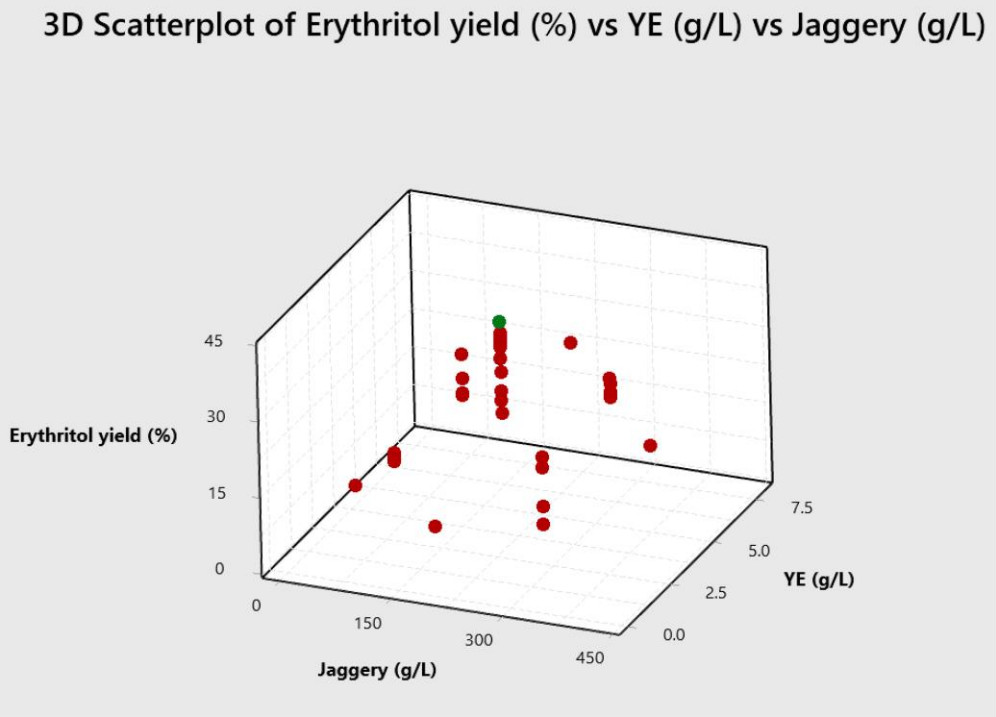



**Figure 4.5** 3D scatter plot of central composite design data points

Additionally, Fig. 4.5 delves deeper into the significance of these factors. The interaction between jaggery and YE emphasizes their collective role in maximizing erythritol production, highlighting the synergy between these variables. Furthermore, the influence of factor E, represented by pH, is shown to be equally vital in determining erythritol production. The combined information from these figures paints a comprehensive picture of the intricate relationship between pH, jaggery concentration, and yeast extract in the context of erythritol production. These insights are indispensable for optimizing production parameters and ensuring the highest efficiency in erythritol synthesis processes.

The analysis of variance (ANOVA) conducted in this study revealed that factors A (Jaggery concentration), B (YE), and C (pH) exerted the most significant influence on erythritol production. In contrast, the interactions between these factors, specifically AB, AC, and BC, were found to be nonsignificant in the context of the model. The significance of each factor was assessed using the P-value, which served as a valuable tool for evaluating their impact.

The responses extracted from Table 4.3 indicated that the linear coefficients of factors A, B, and C did not display statistically significant differences ($P > 0.05$).

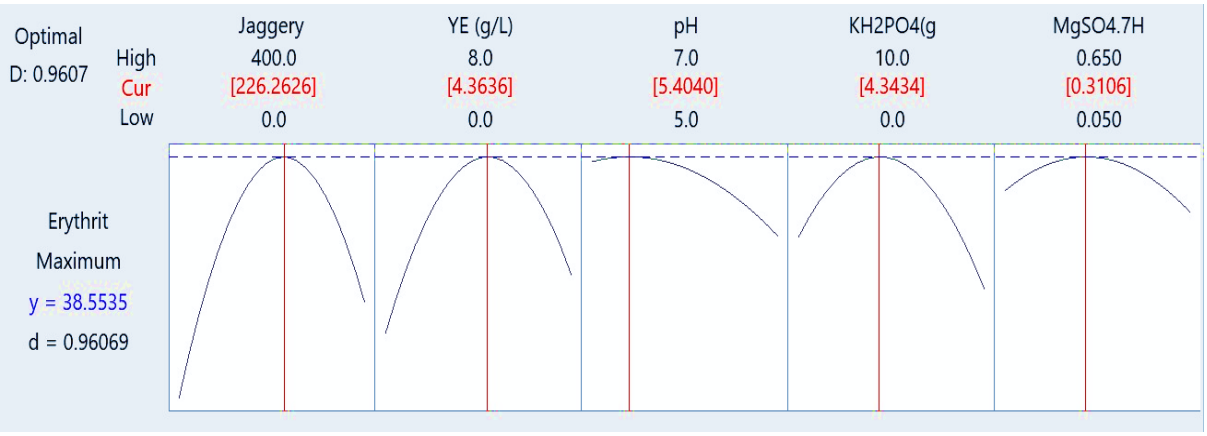



**Figure 4.6** Media optimization curve for maximum erythritol yield

The optimal composition of the growth medium for achieving high erythritol yields was determined, resulting in a formulation consisting of 226.26 g/L of jaggery, 4.36 g/L of YE, a pH of 5.42, 4.34 g/L of KH2PO4, and 0.31 g/L of MgSO4, as illustrated in Fig. 4.6. This comprehensive analysis not only identifies the most influential factors but also provides a precise recipe for optimizing the medium to achieve maximum erythritol production, thereby contributing to the efficiency and yield of the process.

### 4.3.2 Validation of the model

A thorough examination of the model's effectiveness was conducted through erythritol production experiments, comparing erythritol production in a non-optimized medium against media constituents optimized through statistical methods, incorporating optimized process conditions. The outcomes, as depicted in Fig. 4.7, showed that employing the statistically optimized medium composition led to the maximum erythritol production (91.2±3.4 g/L) with a yield of 40.7±3.4% in a 25 mL shake flask, utilizing jaggery as a carbon substrate.

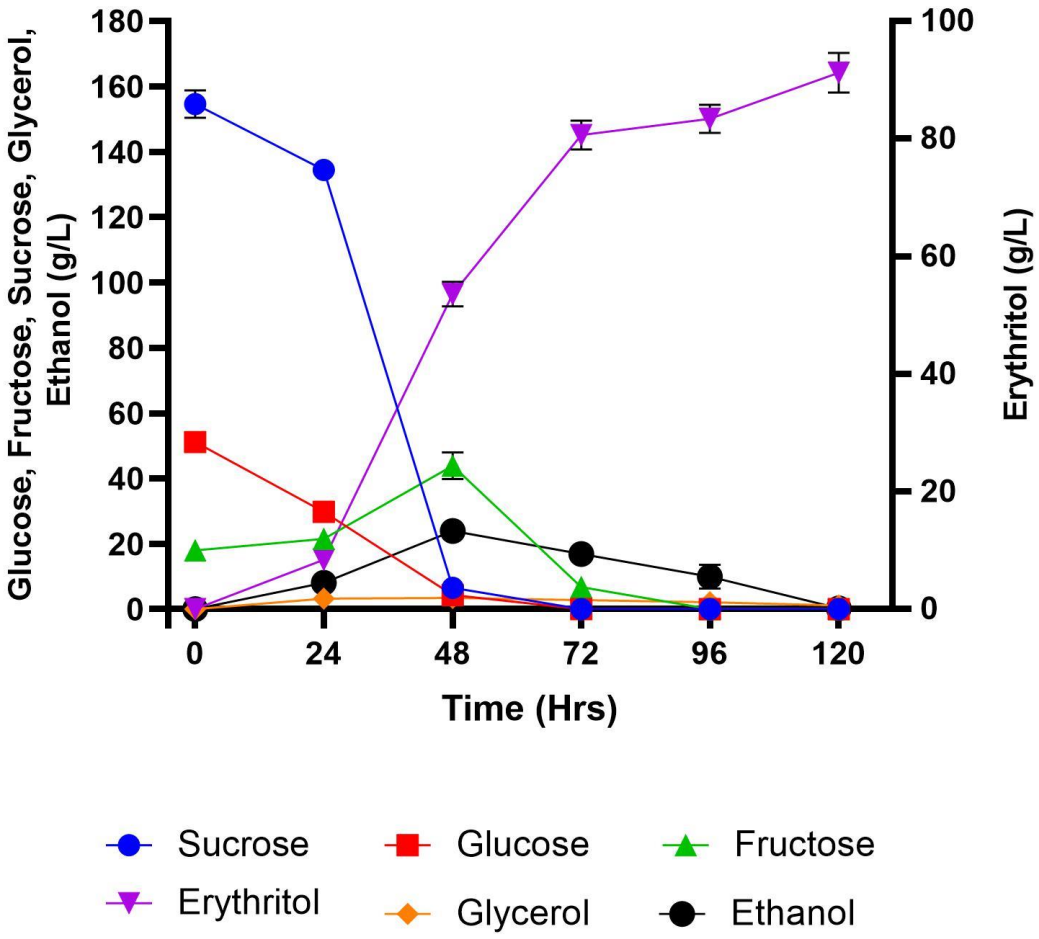



**Figure 4.7** Validation of erythritol production using Mutant-58 strain in shake flask (25 mL)

Notably, in the unoptimized medium with jaggery, erythritol production registered at 37 %, while the statistically optimized medium exhibited a 10% increase in erythritol yield (40.7±3.4 %). This substantiates the acceptability of the model, as evidenced by the experimental runs. The application of RSM optimization provided insights into the intricate interactions among various media components, enhancing the production of diverse industrially significant microbial products. Additionally, the findings from RSM optimization align with previous results in the literature for erythritol production, further corroborating the validity and reliability of the obtained results (Savargave et al., 2011). This validation process was executed

in triplicates, ensuring robustness and consistency in the results, thus reinforcing the credibility of the established model. Validation experiments once confirmed the significance of the model. The strong alignment between the anticipated figures and the actual values for erythritol underscored the importance of the model.

### 4.4 Conclusions

The strain improvement program for *M. pollinis* Mutant-58 employed a Statistical Response Surface Methodology (RSM) approach to optimize the growth medium for maximizing erythritol production while minimizing mannitol and glycerol formation. This comprehensive optimization involved fine-tuning the concentrations of key media components, including jaggery, yeast extract, potassium dihydrogen phosphate, magnesium sulfate, and pH. The utilization of a Central Composite Rotatable Design (CCRD) within the RSM framework facilitated the optimization process. The effectiveness of the RSM model in explaining the variations in the response was underscored by the coefficient of regression $R^2$, which was calculated to be 0.9558. This high $R^2$ value signifies that approximately 95.58% of the variability in the erythritol production response could be accounted for by the model. The counter plots revealed that jaggery and yeast extract and pH were the most influential factors in the medium composition, while $KH_2PO_4$ and $MgSO_4$ had comparatively lesser influence on erythritol production. The optimized medium composition, as determined by the RSM model and expressed in grams per liter (g/L), included jaggery at 226.26 g/L, yeast extract at 4.36 g/L, $KH_2PO_4$ at 4.34 g/L, $MgSO_4$ at 0.31 g/L, and pH maintained at 5.42. The statistically optimized medium achieved the highest erythritol production (91.2±3.4 g/L) and a 10% increase in yield (40.7±3.4%) compared to the unoptimized medium (37%) in a 25 mL shake flask using jaggery as a carbon substrate. These optimized conditions showcased the potential of *M. pollinis* Mutant-58 for erythritol production, characterized by high yield and productivity, and minimal interference from undesirable by-products, as observed in shake flask experiments. The

validation experiments confirmed the significance and credibility of the established model. The validation of this model through the application of statistically optimized media in improving erythritol production, at the laboratory-scale fermentation level, will be discussed in the subsequent chapter, further confirming the practical utility and relevance of these findings.

## 4.5 References

Abdelgalil, S. A., Attia, A. M., Reyed, R. M., Soliman, N. A., & El Enshasy, H. A. (2018). Application of experimental designs for optimization the production of *alcaligenes faecalis* nyso laccase. *Journal of Scientific and Industrial Research*, *77*, 713-722.

Bustos, G., Moldes, A. B., Alonso, J. L., & Vázquez, M. (2004). Optimization of D-lactic acid production by *Lactobacillus coryniformis* using response surface methodology. *Food Microbiology*, *21*, 143-148.

Choudhari, S., & Singhal, R. (2008). Media optimization for the production of β-carotene by *Blakeslea trispora*: A statistical approach. *Bioresource Technology*, *99*, 722-730.

Ghezelbash, G. R., Nahvi, I., & Emamzadeh, R. (2014). Improvement of erythrose reductase activity, deletion of by-products and statistical media optimization for enhanced erythritol production from *Yarrowia lipolytica* mutant 49. *Current Microbiology*, *69*, 149-157.

Kekez, B. D., Gojgic-Cvijovic, G. D., Jakovljevic, D. M., Stefanovic Kojic, J. R., Markovic, M. D., Beskoski, V. P., & Vrvic, M. M. (2015). High levan production by *bacillus licheniformis* NS032 using ammonium chloride as the sole nitrogen source. *Applied Biochemistry and Biotechnology*, *175*, 3068-3083.

Lotfy, W. A., Ghanem, K. M., & El-Helow, E. R. (2007). Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. *Bioresource Technology*, *98*, 3470-3477.

Pal, M. P., Vaidya, B. K., Desai, K. M., Joshi, R. M., Nene, S. N., & Kulkarni, B. D. (2009). Media optimization for biosurfactant production by *Rhodococcus erythropolis* MTCC 2794: Artificial intelligence versus a statistical approach. *Journal of Industrial Microbiology and Biotechnology*, *36*, 747-756.

Savergave, L. S., Gadre, R. V., Vaidya, B. K., & Narayanan, K. (2011). Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochemical Engineering Journal*, *55*(2), 92–100.

Tanyildizi, M. S., Özer, D., & Elibol, M. (2005). Optimization of α-amylase production by *Bacillus* sp. using response surface methodology. *Process Biochemistry*, *40*, 2291-2296.

Vohra, A., & Satyanarayana, T. (2002). Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. *Process Biochemistry*, *37*, 999–1004.

Wagh, V. S., Said, M. S., Bennale, J. S., & Dastager, S. G. (2022). Isolation and structural characterization of exopolysaccharide from marine *Bacillus* sp. and its optimization by microbioreactor. *Carbohydrate Polymers*, *285*, 119241.

**Chapter. 5**

# Scale up of erythritol production in the laboratory fermentor

**Abstract**

This chapter outlines a) the assessment of a statistically-enhanced medium in a laboratory-scale fermenter b) the formulation of an efficient strategy for erythritol production through the optimization of diverse fermentation modes c) the purification and characterization of erythritol derived from the fermentation broth. During batch fermentation, foaming, and flocculation emerged as pivotal factors influencing erythritol production among the tested culture conditions. It was found that foaming and flocculation were associated with Mutant strain rather than media with jaggery. It was observed that proper mixing of cells within the fermentation media was required for effective erythritol production. By developing various strategies to mitigate foaming and flocculation problems. The medium optimized through RSM yielded 58.5±0.09 g/L of erythritol with a 26.35% yield. A simple purification procedure involving activated carbon treatment, concentration, and crystallization was employed to obtain erythritol crystals with approximately 96.2% purity.

## 5.1 Introduction

Shake flask experiments revealed that the production of erythritol utilizing both *M. pollinis* and the Mutant-58 strain was significantly impacted by the composition of the medium and process parameters. Therefore, it is essential to investigate the process by manipulating the parameters in a laboratory-scale fermenter, where precise control over process variables can be achieved. As larger samples can be withdrawn from the fermentor, both growth and production can be more comprehensively observed. The preceding chapter encompassed the enhancement of *M. pollinis* strain, succeeded by statistical optimization of the medium specifically for the chosen Mutant-58. Jaggery, yeast extract, and pH were recognized as pivotal medium constituents that indirectly influence the availability of dissolved oxygen in the shake flasks. The erythritol produced by Mutant-58 primarily relied on the percentage of oxygen within the fermentation medium. Enhancements in strain and statistical optimization of the medium led to a 10% increase in erythritol production by the Mutant-58 strain. The investigation delved into the reasons for the elevated erythritol production in the mutant compared to the parent strain. The higher erythritol production in Mutant-58 was ascribed to a greater protein concentration than the parent strain. In Mutant-58, protein concentration was doubled (140.7±0.18 mg/g DCW), leading to a 30% increase in erythritol production compared to the parent one. Metabolite analysis revealed that Mutant-58 directed more carbon toward erythritol (68.6%) and less towards the cell mass and ethanol, as discussed previously.

In addition to strain isolation and enhancement through chemical and physical mutagenesis, erythritol production research has primarily concentrated on optimizing batch and fed-batch fermentation processes (refer to Table 1.3). Some strains have demonstrated a two to threefold increase in the erythritol yield and productivity through fermentation optimization. The attainment of elevated erythritol production values has been linked to the high cell density of the cultures. This is achieved with precise control over crucial process parameters, especially

dissolved oxygen and substrate concentration—a circumstance that is not feasible in shake flasks. Glucose, derived from the chemical or enzymatic hydrolysis of wheat and corn starch, is a common choice as the primary carbon substrate for erythritol synthesis, yielding between 0.15 to 0.6 g/g of glucose utilized in erythritol fermentation (Kim et al., 1997; Jeya et al., 2009).

On an industrial scale, at 100,000 L fermenter utilizing *Aureobasidium* sp. SN-G42 reported an erythritol yield of 47% at a production rate of 2.0 g/L/h. Through the utilization of an optimized medium composition and regulating the rate of oxygen transfer, the generation of byproducts was reduced (Moon et al., 2010). Recently, Sawada et al., (2009) successfully scaled up erythritol production to 200,000 L for industrial purposes. Jeya et al. (2009) provided detailed insights into the isolation of the erythritol-producing yeast *Pseudozyma tsukubaensis*. They also discussed the optimization of culture conditions at both flask and fermenter levels, along with scale-up investigations from a laboratory scale (7.0 L fermenter) to a production plant scale (50,000 L), highlighting the significance of dissolved oxygen as a critical scale-up parameter. In this investigation, the plant-scale production of erythritol reached the highest reported concentration of 245 g/L, accompanied by an high yield of 61%. The research delved into the intricacies of batch and fed-batch fermentations conducted in bubble column and airlift tower loop reactors ranging from 30 to 500 L, as extensively studied by Burschäpers et al., (2002). In the initial exploration, nitrogen limitation led to foam development, while anoxic conditions increased ethanol production. Employing appropriate aeration and a fed-batch regimen with a glucose and nitrogen source mixture effectively minimized ethanol and foam generation. A collaborative effort between Cerestar R&D Centre, Belgium, and Cargill Corn Milling USA resulted in the establishment of a commercial process. This approach, featuring optimal medium components and a feeding tactic, achieved an impressive erythritol concentration of up to 175 g/L in a 500 $m^3$ reactor (Schügerl, 2005). In the fed-batch

fermentation, conducted by Savergave et al., (2011) they attained an erythritol concentration of 87.8 g/L with a yield of 31.1%, and notably, no other polyols were formed.

Notably, there exists a singular process utilizing *C. magnoliae* KFCC 11023 for erythritol production, originating from honeycombs and subjected to EMS mutagenesis. The mutant M2 exhibited an output of 25 g/L of erythritol with a 25% yield in a medium comprising 100 g/L of glucose. Enhancing a fed-batch approach, which involved concurrent feeding of glucose and yeast extract, attained 75 g/L of dry cell weight (DCW), yielding 200 g/L of erythritol, with a productivity of 1.2 g/L/h and a 43% yield. Nonetheless, this method produced significant quantities of organic acids, such as gluconic acid, citric acid, and butyric acid. (Yang et al., 1999; Ryu et al., 2000; Koh et al., 2003).

The objectives of the research delineated in this chapter were: a) assessing the laboratory fermenters for statistically-optimized medium b) formulating an effective fermentation approach for the production of erythritol c) purifying erythritol from the fermentation broth, and d) conducting a chemical characterization of the erythritol produced in the fermentation medium.

## 5.2 Materials and methods

All components of the medium, the strain, and the analytical methods employed remained consistent with those detailed in Chapter 3 unless specified otherwise. The majority of the chemicals and solvents were procured from Hi-media, Mumbai, and Sigma Aldrich, as mentioned earlier.

### 5.2.1 Evaluation of Mutant-58 for erythritol production in 2.0-liter shake flask and 3.0-liter fermentor

Three consecutive fermentation batches at fixed RPM (180) were completed using RSM model-optimized medium, in a 2.0 L Erlenmeyer flask with 0.2L working volume. The

agitation was maintained at 180 RPM. Validation in a shake flask done in triplicates with a fermentation time is 120h.

*Moniliella pollinis* Mutant-58 culture (130 µL glycerol stock) was inoculated into a multiple 250 ml Erlenmeyer flask containing 25 ml Yeast extract glucose peptone (YPG) liquid medium, and incubated at 28°C and 180 RPM. Following an 18-hour incubation period, 10% (v/v) of the seed culture was then introduced into a fermenter. Fermentor batches were conducted in a 3.0 L Bioflo (Eppendorf) 115 fermentor with a 1.3 L working volume. The initial sugar concentration maintained was 225 g/L using jaggery as a carbon source. The YE was used as a nitrogen source at a concentration of 4.36 g/L, $KH_2PO_4$ 4.34 g/L, $MgSO_4.7H_2O$: 0.45 g/L, and the pH of the fermentation medium was adjusted and maintained at 5.4 throughout fermentation using 5 N NaOH. The temperature was regulated at 28°C through automated heating or the circulation of chilled water. The jaggery and LFM medium were sterilized at 121°C for 20 min before inoculation separately. The agitation and aeration were kept at 200 RPM and 1 VVM respectively. The dissolved oxygen was not maintained in the medium during fermentation. The total time for batch fermentation was 120h and, samples were taken for metabolite analysis every 24h for OD and microscopy. HPLC sampling for metabolite quantification analysis was conducted at 24h intervals.

### 5.2.2 Optimization batch fermentation for erythritol production

When operating at the fermentor level, numerous challenges were encountered, particularly issues related to foaming and flocculation that adversely affected the erythritol productivity. Various strategies were employed to address these challenges, including the implementation of diverse aeration techniques, jaggery feeding, baffle modifications, and media circulation using a peristaltic pump. To improve yield and productivity, a series of fermentation experiments

(R1, R2, R3, R4, R5 and R6) were conducted using a statistically-optimized medium with Mutant-58 (Table 5.1).

**Table 5.1** Fermentor runs demonstrating protocol modifications throughout fermentation

| No | Strain | Fermentation mode | Changes made in the protocol |
|---|---|---|---|
| R1 | Mutant-58 | Batch fermentation,<br>Agitation- 200 RPM,<br>Aeration- 1 VVM | LFM medium with 222 g/L<br>Jaggery added at 0 h |
| R2 | Mutant-58 | Fed-Batch fermentation<br>Agitation- 200 RPM,<br>Aeration- 1 VVM | At 0 h 50%, Jaggery (113 g/L) added and after 24 h 50% jaggery (113 g/L) pulsing started at 0.5 g/minute flowrate |
| R3 | Mutant-58 | Batch fermentation,<br>Agitation- 200 RPM,<br>Aeration- 1 VVM | LFM medium with 224 g/L<br>glucose added at 0 h |
| R4 | Wild type | Batch fermentation,<br>Agitation- 200 RPM,<br>Aeration- 1 VVM | LFM medium with 200 g/L<br>glucose added at 0 h |
| R5 | Mutant-58 | Batch fermentation,<br>Agitation- 200 RPM,<br>Aeration- 1 VVM | LFM medium with 222 g/L<br>jaggery added at 0 h<br>Changes made with the baffles<br>Media recirculation using a peristaltic pump |

The initial reactor operation focused on optimizing the nutrient-feeding approach, specifically addressing jaggery and refining aeration and agitation parameters. However, the initial run revealed significant issues such as heavy flocculation and foaming. In response, new

experiments were designed, incorporating nutrient feeding at specific intervals (R2, Fed-batch). Subsequent R3 runs, using glucose, were conducted to investigate whether foaming and flocculation were linked to the jaggery-based media, which contains additional nutrients that are absent in the pure glucose-based media. The fourth run (R4) introduced a wild-type strain with jaggery to discern whether flocculation resulted from strain-specific or medium-specific factors. The fifth and final reactor run (R5) implemented various strategies to address the flocculation and foaming challenges associated with the mutant strain. The changes made during fermentation are outlined in Table 5.1, illustrating the evolving protocol adjustments throughout the reactor runs.

### 5.2.3 Purification and characterization of erythritol from the fermentation broth

Depending on the specific strain and substrate employed in erythritol production, by-products such as glycerol, ribitol, mannitol, fumarate, D-arabitol, and citrate can be generated (Lin et al., 2001; Mirończuk et al., 2017). The typical process for erythritol production initiates with fermentation, followed by key stages in the downstream process. These stages encompass biomass clarification, removal of cells, separation of insoluble compounds, purification of erythritol from impurities and by-products, and ultimately concentration to obtain erythritol crystals (Troostembergh et al., 2002; Saran et al., 2015; Rakicka et al., 2016; Deshpande et al., 2022).

In the previously described fed-batch experiment, fermentation was continued until all remaining glucose was utilized. Subsequently, 500ml of the fermentation medium was centrifuged at 10000g for 10 minutes, and the resulting supernatant was treated with 2 g/L activated charcoal at 90°C for 20 minutes with gentle mixing. Following charcoal treatment, the supernatant was filtered using Whatman filter paper. Post-charcoal treatment, the filtrate was passed through a cation exchange resin (190 INDION, opaque grey to dark grey beads

with a sulphonic acid functional group and particle size of 0.42–1.2 mm, possessing a minimum total exchange capacity of 4.7 meq/dry gm) preactivated with 5% HCl. The eluate from the cation exchange column was concentrated using a rotary vacuum evaporator (Rotavapor - KV600 IKA digital) at 15 mbar vacuum and 30°C, followed by crystallization on a glass plate at room temperature. Due to erythritol's low solubility in acetone, the crystals were washed with acetone (1:10) to enhance purity. The quality of erythritol was assessed using HPLC.

### 5.2.4 Identification of the erythritol in the fermented broth by HPLC

The concentrations of initial and intermediate metabolites, in addition to erythritol, were assessed using HPLC. A 500 ppm erythritol standard was prepared in distilled water. Another 500 ppm erythritol standard was created using a 72h old LFM medium initially supplemented with 20% glucose and inoculated with the *M. pollinis* strain. The resulting broth contained a mixture of metabolites, including erythritol, sugars, glycerol, and ethanol. Each of the three samples underwent separate analysis, and chromatograms and area under the curve (AUC) were observed for all samples. This procedure aimed to validate that the polyol produced in the fermentation process was indeed erythritol, achieved by comparing it with the erythritol standard procured from Sigma.

## 5.3 Results and discussions

### 5.3.1 Evaluation of Mutant-58 for erythritol production in 2.0-liter shake flask

The outcomes indicated that the utilization of the statistically optimized medium composition led to the highest erythritol production (91.2±3.4 g/L) with a yield of 40.7±3.4% in a 25 mL shake flask by *M. pollinis* Mutant-58 (Fig. 4.7). However, the same high levels of erythritol production were not reproducible in a 200 mL setup (Fig. 5.1).

Although there was relatively less flocculation and foaming observed in the 200 ml shake flask compared to the fermentor, the erythritol yield was lower (32.9±1.2%) than in the 25 ml shake

flask experiment. The 200 ml shake flask showed higher glycerol production (6.1±0.4 g/L) compared to the 25 ml shake flask.

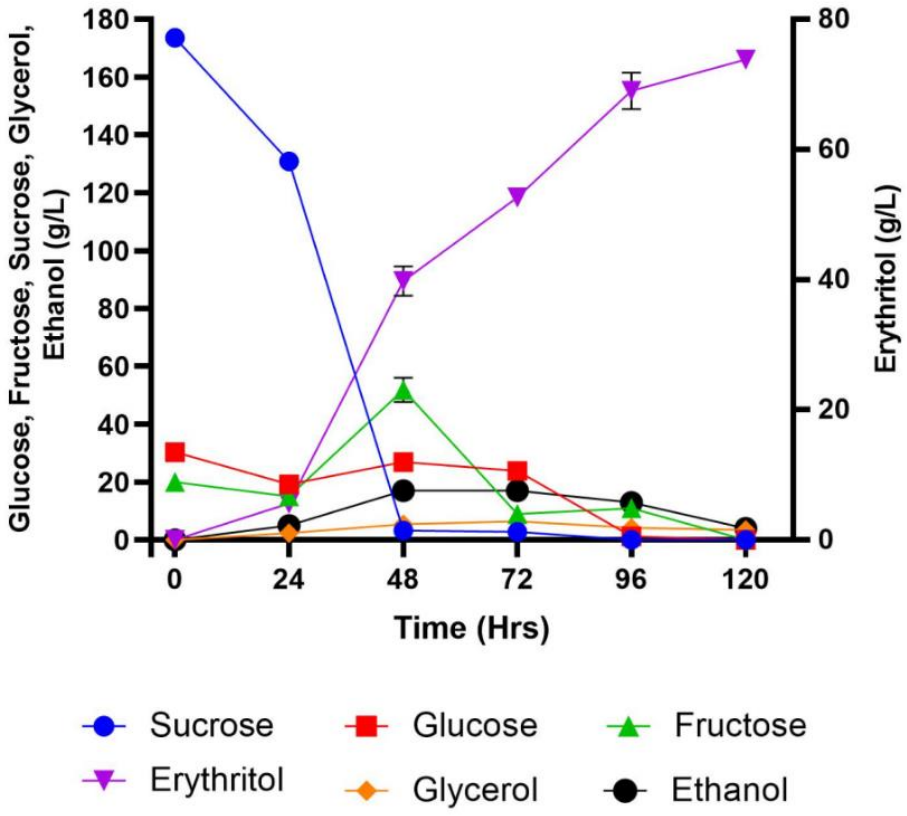



**Figure 5.1** Validation of erythritol production using Mutant-58 strain in shake flask (200 mL)

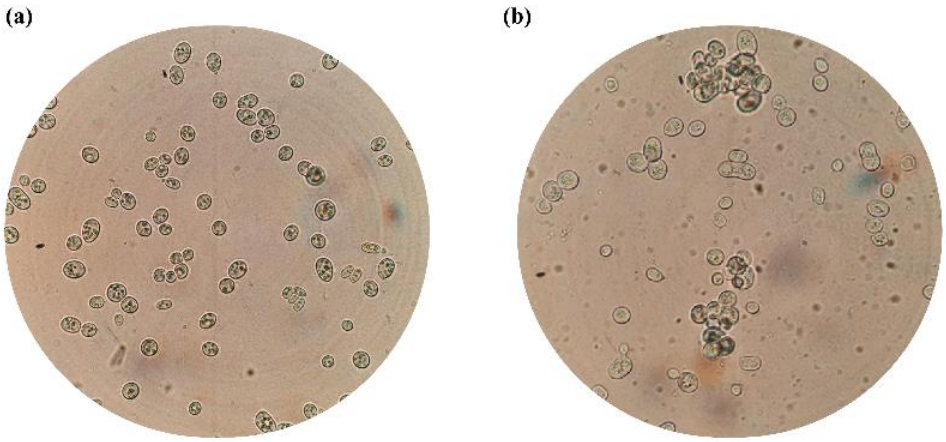



**Figure 5.2** *Moniliella pollinis* Mutant-58 cells microscopic observation, a) 25 Shake flask and b) 200 ml shake flask (photographs obtained by: optical Bright field microscopy 100X)

However, despite the increased volume, the 200 ml shake flask experiment achieved a 32.9±1.2% erythritol yield, which surpassed the erythritol yield of the parent strain with glucose. The reduced erythritol yield is attributed to the lower aeration available in the 2.0 L flask compared to the 250 ml flask due to the higher volume. Microscopic observations also indicated that in the 25 ml shake flask experiment cells were isolated, single state, whereas clumps of yeast cells were observed in the 200 ml shake flask (Fig. 5.2).

The mutant strain exhibited a 34% increase in erythritol production and a 27% decrease in ethanol compared to the parent strain. Additionally, an intriguing finding from the shake flask study revealed that the mutant strain had slightly higher glycerol production than the parent strain. In yeast, the synthesis of erythritol involves a multistep metabolic process, typically operating through the pentose phosphate pathway (PPP). Previous reports indicated the presence of three ER isozymes (ER1, ER2, and ER3) in *M. megachiliensis*. (Ookura et al., 2005). The analysis of three ER-encoding genes (ER1, ER2, and ER3) expression was conducted in a culture cultivated with 20% glucose, and the findings were compared with a culture grown with 2% glucose. Although no significant change in ER expression levels was observed during the initial hours of treatment, there was a substantial 60-fold increase in the expression of the glycerol-3-phosphate dehydrogenase gene within the same time frame. This indicates that the primary cellular response was the production of glycerol. Intracellular erythritol accumulation became evident after 12h of incubation and remained elevated over the next five days. Notably, only ER3 expression showed a significant increase during extended osmotic stress, and the level of its mRNA transcripts mirrored the erythritol production profile. Thus, it suggests that glycerol synthesis might be an initial response, whereas the cell's stationary phase response to osmotic stress involves the production of erythritol (Y. Kobayashi et al., 2013). In the 200ml shake flask, there was a reduction in erythritol concentration

accompanied by an increase in glycerol production, suggesting that the mutant cells encountered stress due to reduced agitation and aeration at this scale. This elevated glycerol synthesis in comparison to the 25 ml shake flask, ultimately impacting the erythritol yield.

### 5.3.2 Evaluation of Mutant-58 for erythritol production in 3.0-liter fermentor

Upon inoculating the fermenter with a 10% v/v Mutant-58 inoculum, a significant amount of foaming and flocculation was observed within the first 12 hours of the growth phase. To address these issues, antifoam was added, and aeration was reduced after 12 hours to control the observed problems. The aeration of the fermentor was lowered from 1.3 Liters per minute (LPM) to 0.5 LPM after 24h to prevent medium overflow and foam generation during batch fermentation. Despite these measures, the challenges of foaming and flocculation persisted in the fermentor.

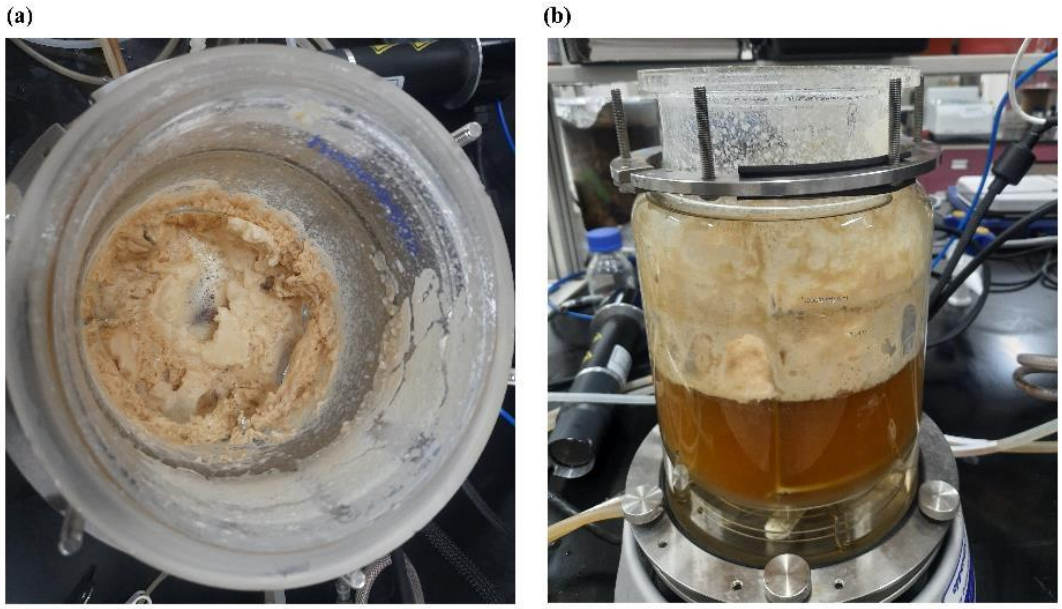



**Figure 5.3** Foaming and flocculation in fermentor (R1), **a)** Top view of the fermentor, **b)** Front view of the fermentor

After 24h of fermentation, the reduction in aeration was reflected in higher ethanol production (12.1±0.6 g/L) compared to erythritol (2.5±0.2 g/L). While 1 vvm aeration would be optimal for erythritol production, excessive aeration led to increased flocculation and foam generation. This, in turn, caused cell biomass to float on the medium surface, resulting in decreased productivity. The R1 batch exhibited a significantly lower erythritol yield (6.2±0.4 g/L), and post-fermentation, a substantial amount of biomass adhered to the fermentor wall and impeller shaft was observed (Fig. 5.3).

The hypothesis was that jaggery might contain nutrients contributing to the foaming problem. Consequently, the subsequent fermentor batch (R2) was initiated with a modification in the initial sugar concentration, reducing it to 50%, with the plan to add the remaining 50% sugar concentration after 24 hours. Despite implementing these adjustments, the same foaming issue persisted, emerging after 24 hours. Modifications were made to aeration and agitation during this period in an attempt to address the foaming problem. However, the outcomes resembled those of the R1 run, indicating that the issue remained unresolved.

In the subsequent fermentor run (R3), jaggery was substituted with glucose to eliminate the assumption that the foaming issue was related to the media rather than the strain. Surprisingly, even with the replacement of the medium's carbon source with glucose, a less complex substrate than jaggery, the problems of foaming and flocculation persisted from 12 hours to the conclusion of the fermentation. This run partially confirmed that the foaming issue is associated with the strain rather than the jaggery-based media. However, fermentation could not proceed beyond 120 hours because the substrate was unable to be converted by yeast cells into the product, as yeast biomass consistently remained on the top of the fermentation media due to flocculation and foaming. The maximum erythritol produced in this run was 14±1.2 g/L, and the residual sugar remaining after 120 hours was 102±2.3 g/L.

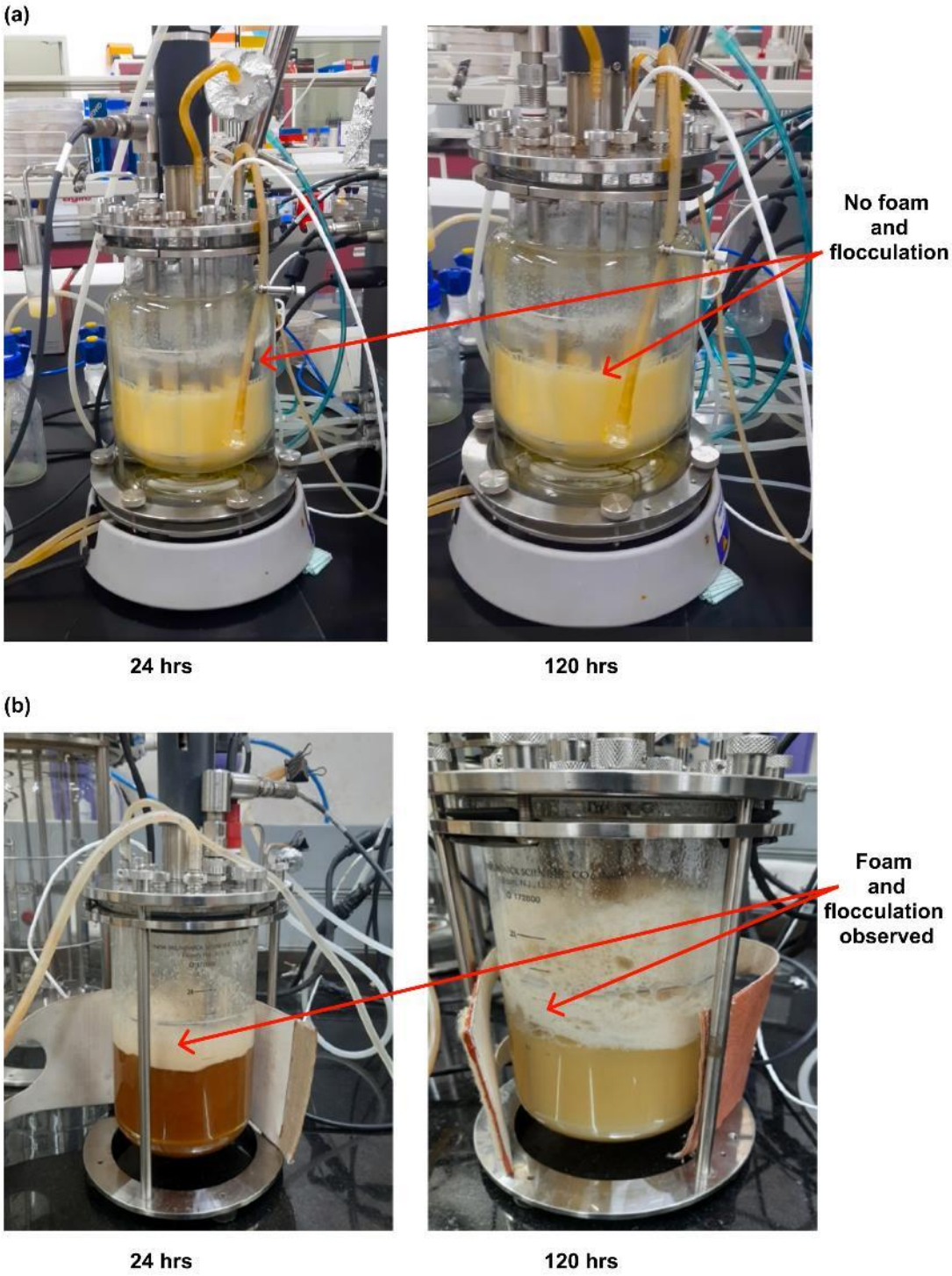



**Figure 5.4** Erythritol fermentation using a**)** Wild type, and **b)** Mutant-58 strain with jaggery as carbon substrate

Flocculation and subsequent foaming emerged as prominent challenges during the scale-up process in the fermentor. To explore a potential link between the foaming issue and the mutant

strain obtained through EMS mutagenesis, erythritol production experiments were conducted in a 3.0 L bioreactor using the parent strain under conditions similar to those applied for Mutant-58. The findings are depicted in Fig. 5.4. Strikingly, the wild-type strain exhibited lower erythritol production at 46.8±2.1 g/L, indicating a 26% reduction compared to the mutant strain. Additionally, higher ethanol production was observed in the parent strain (25.8±2.7 g/L) compared to the mutant culture (17.6±2.1 g/L) during fermentation in the fermentor (Fig. 5.5). Notably, the issues of flocculation and foaming, prevalent in the mutant strain, were conspicuously absent when the wild-type strain was utilized in the fermentor studies (Fig. 5.4).

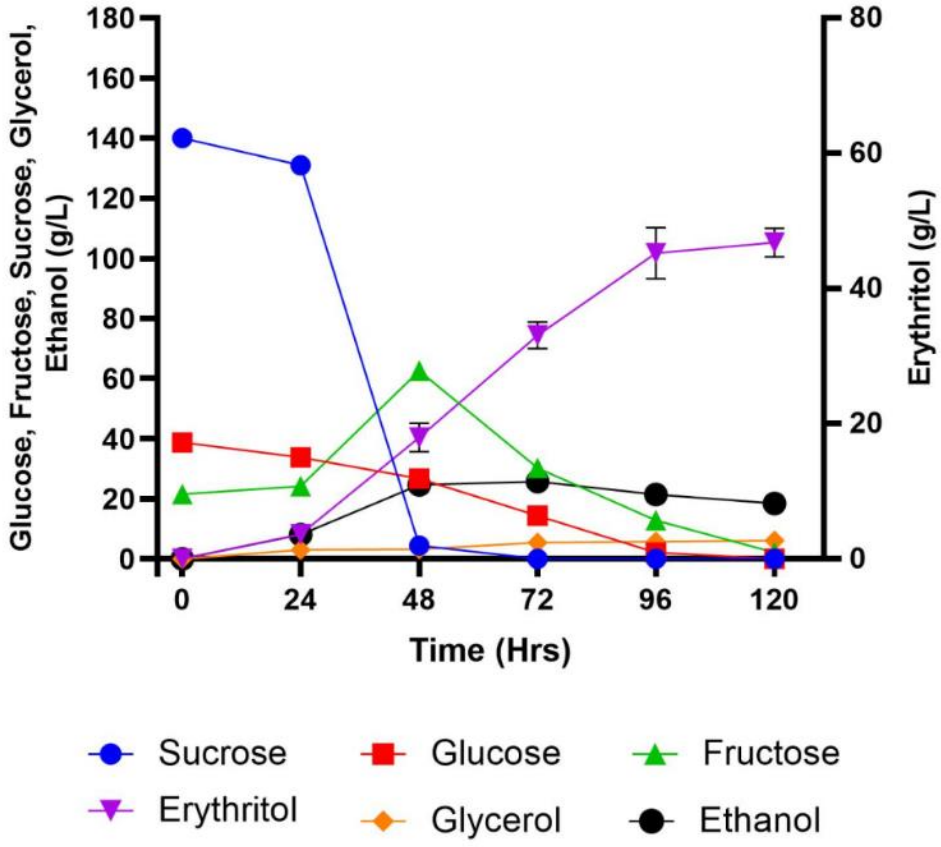



**Figure 5.5** Erythritol production from glucose in a 1.3-L fermentor using the wild-type strain

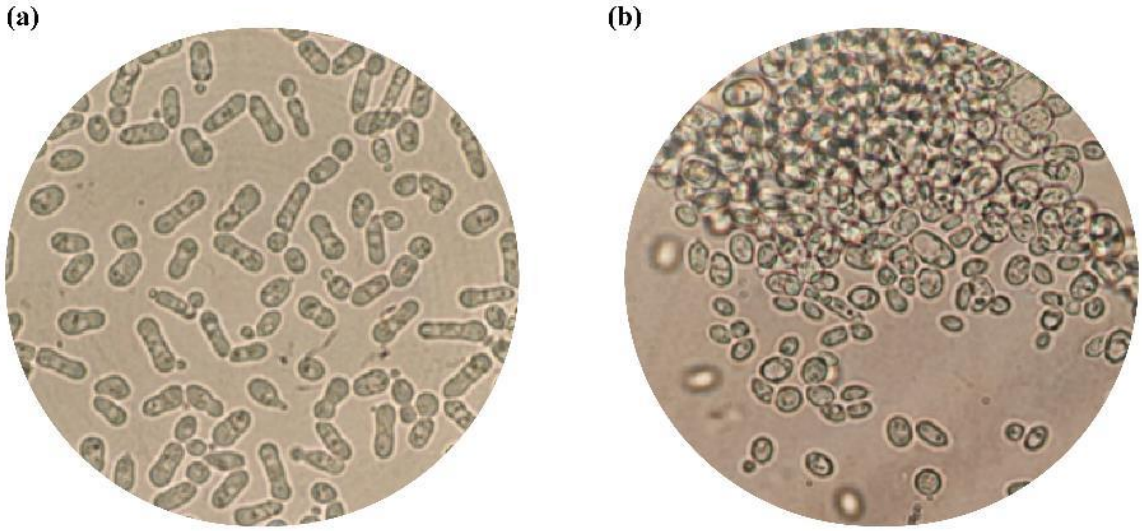



**Figure 5.6** Microscopic observation, **a)** *M. pollinis* 24 h sample of fermentor medium, **b)** *M. pollinis* Mutant-58 24 h sample of fermentor medium (Optical Bright field microscopy 100X)

In Fig. 5.6, a photomicroscopic examination of both mutant and wild cells is presented. It is evident that the wild-type cells exist in a single, isolated state, whereas the mutant cells, form clumps of cell aggregates. This aggregation is identified as the primary cause of foaming and flocculation within the fermentation media. Similar observations were reported by Ishizuka et al., (1989) when mutants of *Aureobasidium* sp. displayed noteworthy changes in morphology and foaming after mutation.

### 5.3.3 Optimization of batch fermentation for erythritol production

To address the challenges encountered during fermentation studies, several new strategies were employed. The issue of biomass floating on the surface of the fermentation medium after 24 hours prompted the implementation of three combined strategies to mitigate flocculation and foaming problems. These strategies encompassed: 1) Continuous circulation of the fermentation broth using a peristaltic pump, 2) Utilizing a sprinkler system over the impeller, and 3) Intermittent circulation of the fermentation broth to the inner fermentation walls using

a baffle. The depicted illustrations and actual set up of these strategies can be found in Fig. 5.7 and 5.8. The fermentor run commenced with a 10% (v/v) seed culture, and starting from the 12th hour, media circulation was initiated to counteract flocculation and foaming. While the flocculation issue was not entirely resolved, there was notable improvement compared to previous batches. A high-capacity peristaltic pump (Watson Marlow- 520L) was employed, operating at a circulation rate of 1.8 L/min, as shown in Fig. 5.8.

Another implemented strategy involved connecting one end of the circulation tubing to an additional port adjusted just above the impeller inside the fermentor. This setup facilitated continuous broth flow over the impeller, acting as a sprinkler with a speed of 200 RPM. This arrangement aided in detaching the biomass adhered to the inner wall of the fermentor, as depicted in Fig. 5.9a.

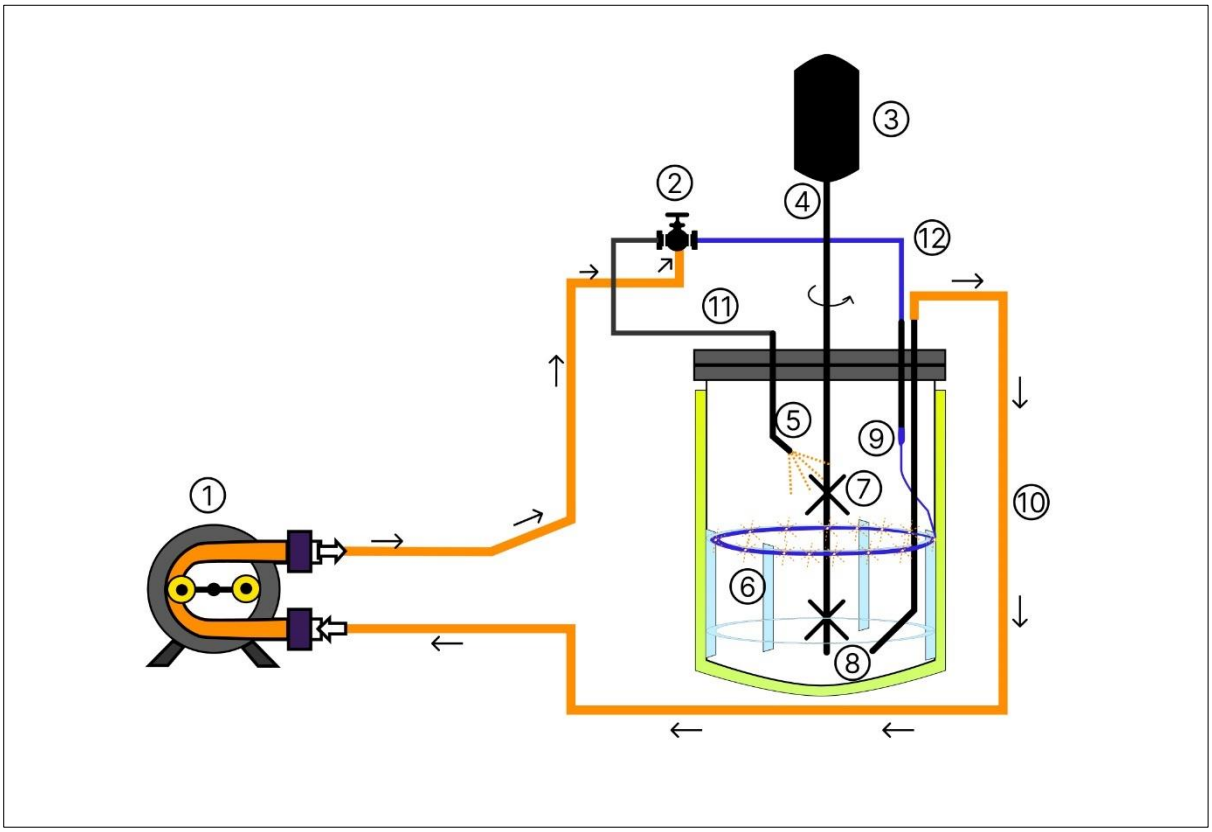



**Figure 5.7** Strategies to mitigate the flocculation and foaming problem

**1.** Peristaltic pump, **2.** T Switch valve, **3.** Motor, **4.** Impeller shaft, **5**. Port over the impeller, **6.** Baffle, **7.** Impeller, **8.** Sampling port, **9.** An additional port connected to a silicon tube bonded with the baffle, **10.** A Silicon tube connected to the sampling port for recirculation through a peristaltic pump, **11.** A silicon tube connected to an additional port for sprinkling action, **12.** A silicon tube connected to an addition port which connected to the baffle

A third strategy focused on ensuring proper mixing of the biomass within the fermentation media. A silicon tube, fastened over the baffle using cable ties, featured random holes. An additional port in the fermentor connected to this silicon tubing allowed media circulation through the peristaltic pump over the impeller. The tube was utilized to divert the media flow by incorporating a T switch. Although not applied continuously, an intermittent flow of media through this port assisted in detaching the biomass adhered to the fermentor's side wall, as shown in Fig. 5.9b.

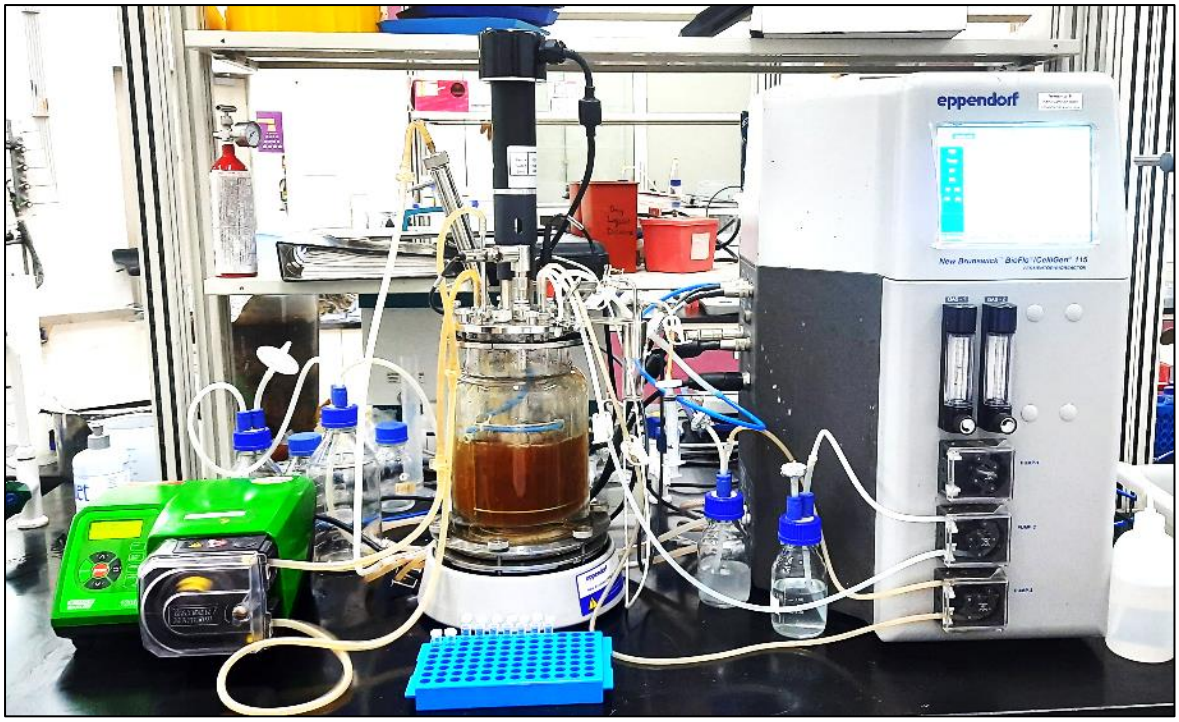



**Figure 5.8** Fermntor setup illustrated in fig. 5.7

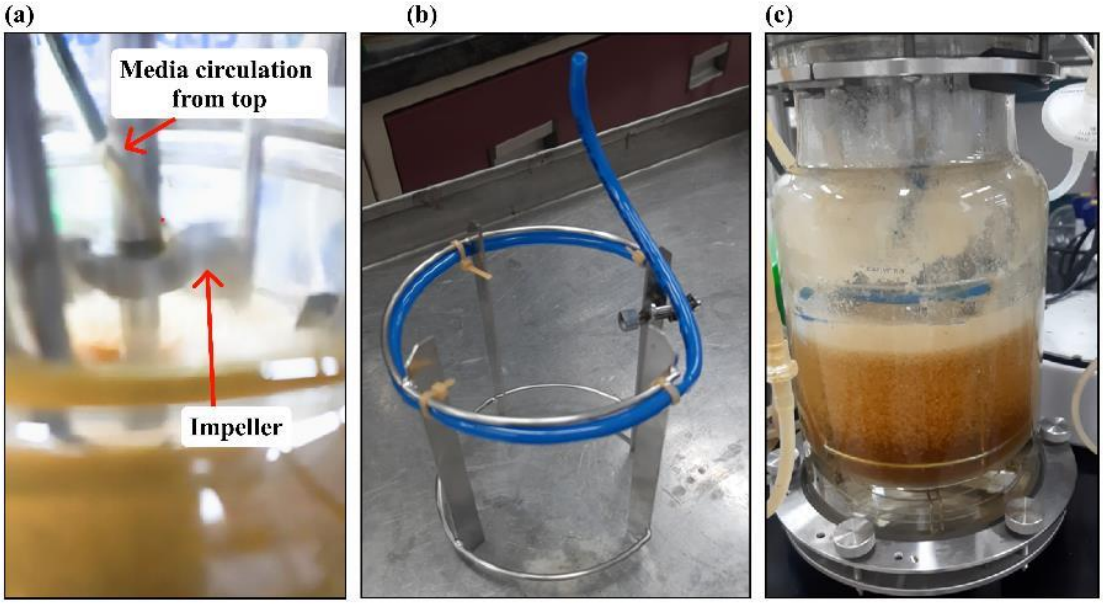



**Figure 5.9** Strategies to mitigate foaming and flocculation by using a) Impeller, b) Baffle, c) Both impeller, peristaltic pump, and baffle

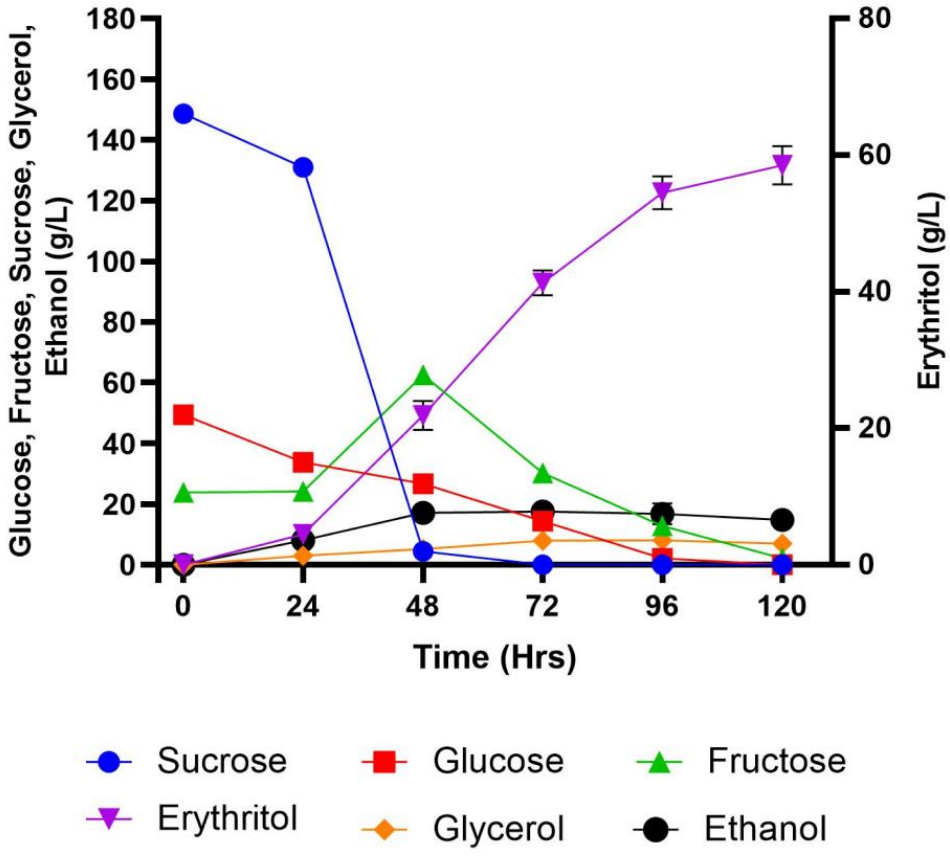


**Figure 5.10** Erythritol production from glucose in a 1.3-L fermentor using the wild-type strain

By implementing all three methods (Fig. 5.9c), we achieved a production of 58.5±2.8 g/L erythritol using the Mutant-58 strain in a jaggery-based medium. This result was significantly higher than in previous batches where fermentation could not be completed due to flocculation and foaming issues. Despite the challenges of uncontrolled optimal conditions, the erythritol yield was 26% higher than that achieved with the wild-type strain (Fig. 5.10).

The findings from the experiments conducted in a 3.0 L bioreactor, using jaggery and glucose as substrates with the wild-type strain, have revealed important insights into the relationship between microbial culture, media components, and the issues of foam and flocculation. The results indicate that the occurrence of foam and flocculation is primarily attributed to the microbial culture itself rather than the specific components of the media. Based on these observations, a hypothesis was formulated suggesting that the phenotypic changes in the mutant cells had a substantial impact on the occurrence of flocculation and foaming. This hypothesis was further supported by the absence of both flocculation and foam formation when the parent strain was cultivated with either glucose or jaggery in the bioreactor during fermentation. The comparison of phenotypic characteristics between the parent and mutant strains as well as the metabolite production served to strengthen the hypothesis. Overall, these results shed light on the underlying factors contributing to foam and flocculation problems, highlighting the importance of phenotypic variations in understanding and addressing these issues. The insights gained from this study can contribute to the development of strategies aimed at mitigating foam and flocculation challenges in bioreactor-based fermentations.

The complication and the highly culture-dependent trait of flocculation make it problematic to regulate fermentation (Verstrepen et al., 2003). Flocculation of yeast is a very composite process that is determined by various flocculation gene expressions (*FLO1*, *FLO5*, *FLO8*, and

*FLO11*) (Kobayashi et al., 1999; Russell et al., 1980). As reported in the literature, yeast with flocculation properties can be operated in airlift bioreactors. Operating flocculating yeast cells in the bioreactors have specific features, mostly related to a high solid-phase hold-up (Domingues et al., 2000). Various bioreactors can be used for handling flocculating yeast cultures but bubble columns and especially airlift reactors are generally preferred over other bioreactors (Kennard & Janekeh, 1991). On a large scale, except for beer manufacturers, no one has taken the advantage of yeast flocculation ability by using this feature to clarify the fermented must. This is a somewhat incomplete application of flocculating yeast cells as the benefits of airlift bioreactors for ethanol production have been established (Restiawaty et al., 2020; Vicente et al., 1999). There is a requirement for additional investigation on flocculent yeast cultures and using appropriate bioreactors, to gather the critical data to enable a successful process.

### 5.3.4 Purification and characterization of erythritol from the fermentation broth

Many downstream processing methods for erythritol purification involve centrifugation to separate cells from the fermentation broth, removal of colored impurities through adsorption on activated carbon, and subsequent use of ion-exchange resins, along with steps such as concentration, crystallization, re-crystallization, and drying. In this research, subjecting the fermentation mixture to two rounds of activated carbon treatment resulted in a clear and colorless aqueous solution.

The utilization of charcoal treatment efficiently diminished color-causing impurities, specifically phenolic compounds, leading to a partial decolorization of the fermentation mixture. The partially decolorized broth was then concentrated and crystallized. Additional use of cation exchange resins further enhanced decolorization, yielding a clear erythritol syrup.

During the downstream process, the concentration step successfully removed all residual ethanol, and washing erythritol crystals with acetone reduced glycerol content (a byproduct of

erythritol production) to 8 g/L. The recovered crystals exhibited a purity of 99%, as confirmed by HPLC analysis. The overall erythritol recovery in this unoptimized process was around 27%, with a percentage purity of 96.2%. In comparison, a study on erythritol production from pure glucose by *Candida sorobosivorans* achieved a purity of up to 98.99%, albeit with a loss of 52.24% (Saran et al., 2015). Another study using *M. pollinis* and sugar cane syrup achieved a 43% erythritol yield with 96.34% purity (Deshpande et al., 2022). Acknowledging the relatively poor overall recovery in the current unoptimized process, potential enhancements include substituting various stages of anion or cation exchange with versatile resins in a singular step and fine-tuning each step to minimize losses.

### 5.3.5 Identification of the metabolites in the fermented broth by HPLC

The analysis of the initial and intermediate metabolites, along with polyol, was conducted using HPLC. Sucrose, glucose, fructose, erythritol, glycerol, and ethanol were identified at retention times of 8.25 min, 9.57 min, 10.38 min, 11.98 min, 13.57 min, and 20.7 min, respectively. These retention times align with those of analytical grade products under the given conditions.

**Table 5.2** Area under the curve (AUC) of erythritol standard and fermented broth

| Sr. No. | Samples ID | Erythritol | Glycerol | Ethanol |
|---|---|---|---|---|
| 1 | 72h fermentation broth | 46892270±134 | 1773278±148 | 4314742±275 |
| 2 | Erythritol-500ppm | 14093433±418 | 0 | 0 |
| 3 | 72h fermentation broth + Erythritol 500ppm | 60765318±248 | 1847725±310 | 4411826±218 |
| | AUC of 1+2 | 60985703 | 1773278 | 4314742 |

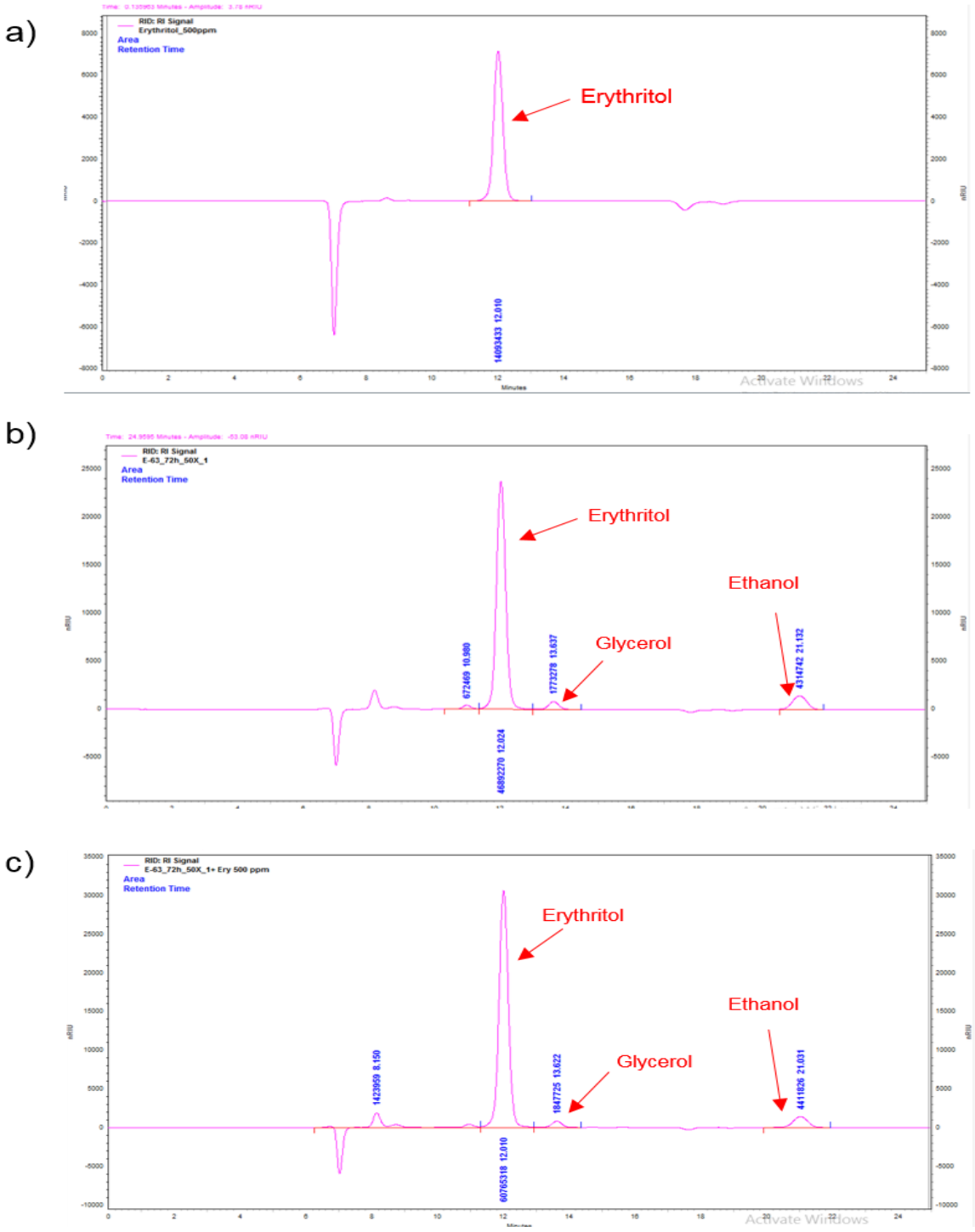



**Figure 5.11** Spike-in experiment for identification of erythritol in the fermentation broth, **a)** Erythritol 500ppm standard in DW, **b)** 72 h fermentation broth, **c)** Erythritol 500ppm standard spiked in the centrifuged fermentation broth

For standardization, a 500 ppm erythritol solution was prepared in distilled water. Another standard, also at 500 ppm, was created using a 72h old LFM medium initially supplemented with 20% glucose and inoculated with the *M. pollinis* strain. This broth contained all the relevant metabolites, including erythritol, sugars, glycerol, and ethanol. Each of the three samples underwent separate analysis, and chromatograms and area under the curve (AUC) were observed for all the samples (Fig. 5.11).

As indicated in Table 5.2, close to 100% recovery of spiked erythritol was achieved from the fermented broth with the same retention time (RT). The HPLC chromatography results confirm that the polyol produced at 11.98 RT by the *M. pollinis* Mutant-58 strain in the fermentation broth is indeed erythritol.

## 5.4 Conclusions

The highest erythritol production in a shake flask by Mutant-58, utilizing a statistically optimized medium, reached 58.5 g/L. This mutant's performance was further assessed in a 3.0 L fermentation batch mode using the same optimized medium. Within the batch process, foaming and flocculation, associated with the strain and aeration, emerged as pivotal factors impacting erythritol production. While the foaming issue was not entirely resolved, diverse strategies were implemented to optimize erythritol production in the fermentor. Results suggest that both foam and flocculation primarily stem from the microbial culture itself rather than specific components in the media. Observations led to the formulation of a hypothesis positing that phenotypic changes in the mutant cells significantly contributed to flocculation and foaming. This hypothesis found support in the absence of both flocculation and foam formation when the parent strain was cultivated with either glucose or jaggery in the bioreactor during fermentation.

Moreover, a straightforward purification protocol involving activated carbon treatment, concentration, and crystallization yielded erythritol crystals with a purity of 96.2%, as

confirmed by HPLC. The isolated Mutant-58 of *M. pollinis*, optimized for key process parameters in a laboratory fermenter, represents a notable achievement. This study reports the highest erythritol production (91.2±3.4 g/L) using jaggery as a cost-effective carbon substrate at the shake flask level. Consequently, Mutant-58 demonstrates significant potential for economical jaggery-based fermentation and could be a strong contender for industrial-scale erythritol production.

## 5.5 References

Burschäpers, J., Schustolla, D., Schügerl, K., Röper, H., & De Troostembergh, J. C. (2002). Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa* var pollinis: Part 2. Batch and fed-batch operation in bubble column and airlift tower loop if reactors. *Process Biochemistry*, *38*, 559–570.

Deshpande, M. S., Kulkarni, P. P., Kumbhar, P. S., & Ghosalkar, A. R. (2022b). Erythritol production from sugar based feedstocks by *Moniliella pollinis* using lysate of recycled cells as nutrients source. *Process Biochemistry*, *112*, *45-52*.

Domingues, L., Vicente, A. A., Lima, N., & Teixeira, J. A. (2000). Applications of yeast flocculation in biotechnological processes. In *Biotechnology and Bioprocess Engineering*, *5*, 288–305.

Ishizuka, H., Wako, K., Kasumi, T., & Sasaki, T. (1989). Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. *Journal of Fermentation and Bioengineering*, *68*, 310–314.

Jeya, M., Lee, K. M., Tiwari, M. K., Kim, J. S., Gunasekaran, P., Kim, S. Y., Kim, I. W., & Lee, J. K. (2009). Isolation of a novel high erythritol-producing *Pseudozyma tsukubaensis* and scale-up of erythritol fermentation to industrial level. *Applied Microbiology and Biotechnology*, *83*, 225–231.

Kennard, M., & Janekeh, M. (1991). Two- and three-phase mixing in a concentric draft tube gas-lift fermentor. *Biotechnology and Bioengineering*, *38*, 1261-1270.

Kim, S. Y., Lee, K. H., Kim, J. H., & Oh, D. K. (1997). Erythritol production by controlling osmotic pressure in *Trigonopsis variabilis*. *Biotechnology Letters*, *19*, 727–729.

Kobayashi, O., Yoshimoto, H., & Sone, H. (1999). Analysis of the genes activated by the FLO8 gene in *Saccharomyces cerevisiae*. *Current Genetics*, *36*, 256–261.

Kobayashi, Y., Yoshida, J., Iwata, H., Koyama, Y., Kato, J., Ogihara, J., & Kasumi, T. (2013). Gene expression and function involved in polyol biosynthesis of *Trichosporonoides megachiliensis* under hyper-osmotic stress. *Journal of Bioscience and Bioengineering*, *115*, 645–650.

Koh, E. S., Lee, T. H., Lee, D. Y., Kim, H. J., Ryu, Y. W., & Seo, J. H. (2003). Scale-up of erythritol production by an osmophilic mutant of *Candida magnoliae*. *Biotechnology Letters*, *25*, 2103–2105.

Lin, S. J., Wen, C. Y., Liau, J. C., & Chu, W. S. (2001). Screening and production of erythritol by newly isolated osmophilic yeast-like fungi. *Process Biochemistry*, *36*, 1249–1258.

Mirończuk, A. M., Biegalska, A., & Dobrowolski, A. (2017). Functional overexpression of genes involved in erythritol synthesis in the yeast *Yarrowia lipolytica. Biotechnology for Biofuels*, 10, 77-89.

Moon HJ, Jeya M, Kim IW, & Lee JK. (2010). Biotechnological production of erythritol and its applications. Applied Microbiology Biotechnology. 86,1017-1025.

Ookura, T., Azuma, K., Isshiki, K., Taniguchi, H., Kasumi, T., & Kawamura, Y. (2005). Primary structure analysis and functional expression of erythrose reductases from erythritol-producing fungi (*Trichosporonoides megachiliensis* SNG-42). *Bioscience, Biotechnology and Biochemistry*, *69*, 944–951.

Rakicka, M., Rukowicz, B., Rywińska, A., Lazar, Z., & Rymowicz, W. (2016). Technology of efficient continuous erythritol production from glycerol. Journal of Cleaner Production, 139, 905-913.

Restiawaty, E., Gani, K. P., Dewi, A., Arina, L. A., Kurniawati, K. I., Budhi, Y. W., & Akhmaloka. (2020). Bioethanol production from sugarcane bagasse using *neurospora intermedia* in an airlift bioreactor. *International Journal of Renewable Energy Development*, *9*, 247-253.

Russell, I., Stewart, G. G., Reader, H. P., Johnston, J. R., & Martin, P. A. (1980). Revised nomenclature of genes that control yeast flocculation. *Journal of the Institute of Brewing*, *86*, 120–121.

Ryu, Y. W., Park, C. Y., Park, J. B., Kim, S. Y., & Seo, J. H. (2000). Optimization of erythritol production by *Candida magnoliae* in fed-batch culture. *Journal of Industrial Microbiology and Biotechnology*, *25*, 100–103.

Saran, S., Mukherjee, S., Dalal, J., & Saxena, R. K. (2015). High production of erythritol from Candida sorbosivorans SSE-24 and its inhibitory effect on biofilm formation of *Streptococcus mutans. Bioresource Technology*, *198*, 31–38.

Savergave, L. S., Gadre, R. V., Vaidya, B. K., & Narayanan, K. (2011). Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochemical Engineering Journal*, *55*, 92–100.

Sawada, K., Taki, A., Yamakawa, T., & Seki, M. (2009). Key role for transketolase activity in erythritol production by *Trichosporonoides megachiliensis* SN-G42. *Journal of Bioscience and Bioengineering*, *108*, 385–390.

Schügerl, K. 2005. Process development in biotechnology-A re-evaluation. *Engineering in Life Sciences*, 5, 15-28.

Thesseling FA, Bircham PW, Mertens S, Voordeckers K, & Verstrepen KJ (2019). A hands-on guide to brewing and analyzing beer in the laboratory. *Current Protocols in Microbiology* 54, 91-122.

Troostembergh, Ignace, A.D, Willy, R. O. (2002). Process for producing and recovering erythritol from the culture medium containing the same (Patent US 2002/0127667 A1). In *US Patent* (US 2002/0127667 A1).

Verstrepen, K. J., Derdelinckx, G., Verachtert, H., & Delvaux, F. R. (2003). Yeast flocculation: What brewers should know. *Applied Microbiology and Biotechnology*, 61, 197-205.

Vicente, A. A., Dluhý, M., & Teixeira, J. A. (1999). Increase of ethanol productivity in an airlift reactor with a modified draught tube. *Canadian Journal of Chemical Engineering*, 77, 497–502.

Yang, S. W., Park, J. B., Han, N. S., Ryu, Y. W., & Seo, J. H. (1999). Production of erythritol from glucose by an osmophilic mutant of *Candida magnoliae*. *Biotechnology Letters, 21*, 887–890.

**Chapter. 6**

# Summary and Future Perspectives

**Overall summary**

Erythritol, a four-carbon polyol, is biosynthesized by microbes under hyper-osmotic stress, making it a prominent sugar alcohol primarily derived through fermentation. Distinguished among polyols, erythritol serves multiple roles as a flavor enhancer, sequestrant, humectant, nutritive sweetener, stabilizer, formulation aid, thickener, and texturizer. Notably, its low insulin index positions erythritol as a potential sugar substitute for individuals with diabetes. Erythritol stands out with unique features compared to other polyols; it boasts nearly zero calorific value and, within safe dosage limits, is associated with fewer side effects. Its chemical structure imparts resistance to insulin level fluctuations, enhancing its safety for diabetic patients. Several chemical and biotechnological methods exist for erythritol production, with biosynthetic routes recognized as environmentally friendly and efficient. Currently, on an industrial scale, fermentation strategies employing fungal strains, particularly yeast-like genera such as *Aureobasidium, Candida, Moniliella, Torula, Trichosporon, Trichosporonoides,* and *Yarrowia*, are preferred. Despite the advantages, the high cost of erythritol hinders widespread usage. Addressing this, a cost-effective production approach utilizing economical carbon and nitrogen substrates to support yeast-mediated erythritol production becomes imperative. The present study focuses on strain improvement for erythritol production, specifically targeting the use of cost-effective carbon substrates.

The current study explores the screening of osmotolerant yeast strains for erythritol production. For screening, a liquid fermentation medium (LFM) was used with the following composition (g/L): glucose 200, yeast extract (YE) 10, $KH_2PO_4$ 5, and $MgSO_4$ 0.25. Subsequently, *Moniliella pollinis* CBS 461.67 showed more significant erythritol production than the other screened yeast cultures. *M. pollinis* was selected further to enhance erythritol production through the optimization of both medium components and environmental factors. *M. pollinis* exhibited a distinctive trait by producing erythritol as the sole metabolite than other byproducts,

irrespective of variations in the composition of the growth medium or alterations in the culture conditions. The primary determinants governing erythritol production in *M. pollinis* were identified as oxygen availability and nitrogen content within the medium. Given the exceptional erythritol-producing capacity observed in *M. pollinis*, the strain was further selected for strain improvement by mutagenesis.

Furthermore, mutants of *M. pollinis* were created using a combination of UV, EMS, and NTG treatments. A total of 198 mutants screened for increased erythritol production, a Mutant-58, generated through EMS treatment, was singled out due to its better erythritol production, and selected for further detailed investigation. The mutant cells exhibited an oval shape in contrast to the wild-type cells, which displayed a long filamentous morphology. Additionally, the mutant cells demonstrated the intriguing capability to produce a yellow pigment over time, this phenomenon was not observed in the parent strain. In batch culture supplemented with 20% glucose, the *M. pollinis* Mutant-58 strain demonstrated remarkable erythritol production, reaching a substantial concentration of 92.2±2.3 g/L, accompanied by a better yield of 0.46 ± 0.11 g/g, with a productivity rate of 1.28 ± 0.02 g/L/h. Notably, in shake-flask culture, Mutant-58 exhibited a 30% increase in the erythritol production compared to wild-type strains, while concurrently reducing the ethanol production by 27%. Further investigation into optimal conditions for enhanced erythritol production revealed that the Mutant-58 strain thrived at a temperature of 28°C, a pH of 6.0, and with the addition of 4.0 g/L of yeast extract. Moreover, the mutant strain, when cultured in jaggery-containing media, yielded a higher erythritol concentration (37.6 ± 0.5 g/g) compared to the erythritol production by the wild-type strain in glucose-containing media (36 ± 1.3 g/g).

The optimization of both the medium compositions and culture conditions for the Mutant-58 strain was systematically conducted using a two-fold approach. Initially, a "one factor at a time" method was employed in shake flask experiments, which allowed for the individual

adjustment of specific parameters to identify their effects on erythritol production. Subsequently, to further enhance erythritol yield while concurrently reducing byproduct formation, a more comprehensive statistical media optimization was implemented. This advanced technique utilized a central composite design (CCD). The key variables subject to optimization included the nitrogen source, pH levels, temperature, concentrations of $KH_2PO_4$, and $MgSO_4$, and the choice of carbon sources. These parameters were fine-tuned to achieve their respective optimum values. The optimized medium composition, as determined by the RSM model and expressed in grams per liter (g/L), included jaggery at 226.26, yeast extract at 4.36, $KH_2PO_4$ at 4.34, $MgSO_4$ at 0.31, and pH at 5.42. The statistically optimized medium achieved the highest erythritol production (91.2±3.4 g/L) and a 10% increase in yield (40.7±3.4%) compared to the unoptimized medium (37%) in a 25 mL shake flask using jaggery as a carbon substrate.

Ultimately, the statistically-enhanced medium underwent assessment in a laboratory-scale fermenter, concurrently with the formulation of an efficient strategy for erythritol production through the optimization of diverse fermentation modes. Key factors influencing erythritol production during batch fermentation included foaming and flocculation, among the culture conditions investigated. It was found that foaming and flocculation were associated with mutant strain rather than media with jaggery. It was found that proper mixing of cells within the fermentation media was required for effective erythritol production. By developing various strategies to mitigate foaming and flocculation problems, the medium optimized through Response Surface Methodology (RSM) yielded 58.5±0.09 g/L of erythritol with a 26.35% yield. Erythritol crystals with approximately 96.2% purity were acquired using a straightforward purification protocol involving activated carbon treatment, concentration, and crystallization.

In conclusion, this study underscores the effective enhancement of the *M. pollinis* strain via chemical mutagenesis, coupled with statistical media optimization. This intervention led to a notable 30% upsurge in erythritol production and a substantial 27% decrease in ethanol production compared to the parent strain. Additionally, it marks the first demonstration of jaggery as a viable alternative to glucose for large-scale industrial production.

### 6.2. Future Perspective

In recent decades, there has been considerable focus on the biological production of erythritol, primarily from yeast. This heightened attention is attributed to the growing demand for this product. The Mutant-58 strain of *M. pollinis*, derived in this investigation, holds the potential to emerge as a robust candidate for industrial-scale erythritol production through an economically viable jaggery-based fermentation process. Moreover, genetic engineering and proteomic investigations have been conducted, yielding promising results. These advancements not only enhance efficiency but also contribute to a deeper understanding of erythritol metabolism, its regulation, and the limiting steps in production. Mutant-58 can be used for comprehensive gene expression and transcriptome analysis to give a snapshot of actively expressed genes and transcripts under various conditions. Additionally, the development of robust microbial systems by genetic engineering, targeting the key enzyme, i.e. erythrose reductase could improve erythritol production. The scale-up studies faced significant challenges primarily due to flocculation and foaming, both of which were linked to the genetic characteristics of Mutant-58. Yeast with flocculation properties can be operated in airlift bioreactors. Various bioreactors can be used for handling flocculating yeast cultures but bubble columns and especially airlift reactors are generally preferred over other bioreactors. This is a somewhat incomplete application of flocculating yeast cells as the benefits of airlift bioreactors for ethanol production have been established. There is a requirement for additional

investigation on Muatnt-58 strain and using appropriate bioreactors, to gather the critical data to enable a successful process. Optimizing the scale-up process with the suitable reactor holds significant potential for enhancing erythritol yield with the *M. pollinis* Mutant-58 strain.

## ABSTRACT

**Name of the Student:** Anil Balasaheb Khatape
**Registration No:** 10BB18J26023
**Faculty of Study:** Biological Science
**Year of Submission:** 2023
**AcSIR Academic Centre/CSIR Lab:**CSIR-NCL
**Name of the Supervisor(s):**
Dr. Syed G. Dastager**,**
Dr. Vidhya Rangaswamy

**Thesis Title:** Strain improvement for enhanced erythritol production by *Moniliella pollinis* using cost-effective substrate

Erythritol has been produced by various microorganisms including *Yarrowia, Moniliella, Aureobasidium,* and *Candida strains*. Due to its relatively high price, erythritol sweetener is used less than other polyols despite having many advantages. In this study, the *Moniliella pollinis* strain was enhanced for erythritol production through chemical mutagenesis, including N-methyl N-nitro N-nitroso guanidine (NTG), ethyl methyl sulphonate (EMS), and UV mutagenesis, followed by screening for cost-effective carbon sources to optimize erythritol yield. The mutant strains were evaluated for enhanced erythritol production and medium optimization by using different carbon substrates at the shake flask level. To enhance the production of erythritol, statistical media optimization was carried out using a central composite design (CCD). Among 198 isolated mutants, the Mutant-58 strain generated by EMS mutagenesis was selected for further assessment. The Mutant-58 strain showed significant morphological changes as compared to the parent strain. Furthermore, statistically optimized media composition resulted in the higher production of erythritol (91.2±3.4 g/L) with a yield of 40.7±3.4 % in shake flask experiments. The optimized medium composition for erythritol production constitutes (g/L) 225 jaggery, 4.4 yeast extract (YE), 4.4 $KH_2PO_4$, 0.31 $MgSO_4$, and pH 5.5. The present study demonstrated strain improvement, media, and process optimization resulting in a 30% increase in the erythritol production in the Mutant-58 as compared to the parent strain. This is also the first instance where jaggery has been used as a cost-effective carbon source alternative to glucose for industrial-scale erythritol production.

## List of Publications

1. **Anil B. Khatape,** Syed G. Dastager, Vidhya Rangaswamy. (**2022**). An overview of erythritol production by yeast strains. ***FEMS Microbiology Letters,*** 369, 1–12.

2. **Anil B. Khatape,** Vidhya Rangaswamy, Syed G. Dastager. **(2023).** Strain improvement for enhanced erythritol production by *Moniliella pollinis* Mutant-58 using jaggery as a cost-effective substrate, ***International Microbiology*** **(**In press).

# Conference

**Third place in the Oral Presentation Competition**

**Conference:** National Conference on Recent Advances in Biotechnology organized by Manipal Institute of Technology at Manipal, India.

**Conference date:** 31st October, 2023

**Title:** Strain improvement for enhanced erythritol production by *Moniliella pollinis* Mutant-58 using jaggery as a cost-effective substrate.

**Authors:** Anil B. Khatape*, Vidhya Rangaswamy, Syed G. Dastager

**Abstract:** Various microorganisms, including *Yarrowia*, *Moniliella*, *Aureobasidium*, and *Candida* strains, can produce erythritol. Despite its advantages, erythritol sweetener is used in smaller amounts than other polyols due to its comparatively high cost. This research focused on improving erythritol synthesis by exposing *Moniliella pollinis* strain to N-methyl N-nitro N-nitroso guanidine (NTG), Ethyl methyl sulphonate (EMS), and UV mutagenesis. The mutant cultures were then assessed for improved production of erythritol using different carbon substrates at the shake flask level. Among 150 selected mutants, the mutant-58 strain was selected for further assessment which was generated by EMS mutagenesis. The mutant cells were found to have an oval shape, while the wild-type has long elongated rod-like cells. Furthermore, mutant cells produce yellow pigment at the end of 72h incubation time which was not observed in the parent strain. In batch culture supplemented with 20% glucose, *M. Pollinis* Mutant-58 strain was able to produce up to 90.2 ± 2.3 g/L erythritol with 0.39 ± 0.11 g/g yield and productivity of 1.18 ± 0.02 g/L/h. Interestingly, compared with the wild-type strains, the results of the shake-flask culture showed that Mutant-58 increased erythritol production by 30 % while decreasing ethanol production by 22%. The optimal conditions for high erythritol production are Temperature- 28°C, pH- 6.0, and Yeast extract- 4 g/L. The economical substrate, jaggery, was assessed for erythritol production, revealing its potential as a cost-effective alternative to glucose, given the comparable erythritol production values. Therefore, current research deals with strain improvement by EMS mutagenesis followed by media optimization leading to a 30% increase in erythritol production and a 25% reduction in ethanol production when cultivated in glucose. This is also the first instance where jaggery has been used as a cost-effective carbon source alternative to glucose for erythritol production.

FEMS Microbiology Letters, 2022, 369, 1–12
DOI: 10.1093/femsle/fnac107
Advance access publication date: 10 November 2022
Minireview – Biotechnology & Synthetic Biology

# An overview of erythritol production by yeast strains

Anil B. Khatape[1,2,3], Syed G. Dastager[1,2,*], Vidhya Rangaswamy[3,*]

[1]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad- 201002, India
[2]NCIM-Resource Center, Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune-411008, India
[3]High Value Chemicals group, Reliance Industries Limited, Ghansoli, Navi Mumbai 400701, India
*Corresponding author: Academy of Scientific and Innovative Research (AcSIR), Ghaziabad- 201002, India. E-mail: sg.dastager@ncl.res.in; High Value Chemicals group, Reliance Industries Limited, Ghansoli, Navi Mumbai 400701, India. E-mail: vidhya.rangaswamy@ril.com
**Editor:** Paul Christakopoulos

## Abstract

Erythritol is a 4-carbon polyol produced with the aid of microbes in presence of hyper-osmotic stress. It is the most effective sugar alcohol that is produced predominantly by fermentation. In comparison to various polyols, it has many precise functions and is used as a flavor enhancer, sequestrant, humectant, nutritive sweetener, stabilizer, formulation aid, thickener, and a texturizer. Erythritol production is a common trait in a number of the yeast genera viz., *Trigonopsis, Candida, Pichia, Moniliella, Yarrowia, Pseudozyma, Trichosporonoides, Aureobasidium*, and *Trichoderma*. Extensive work has been carried out on the biological production of erythritol through *Yarrowia, Moniliella, Candida*, and other yeast strains, and numerous strategies used to improve erythritol productivity through mutagenesis and genetic engineering are discussed in this review.

**Keywords:** erythritol, metabolic pathways, yeast, erythrose reductase, hyperosmotic stress response, yeast expression

## Introduction

WHO information in 2016 on obesity declared that of over 1.9 billion world's adult population diagnosed to be overweight, over 650 million had been obese. Also, 41 million kids were overweight worldwide (World Health Organization. Obesity and overweight fact Sheet; 2016. Available from: https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight). The contributing component to mass gain and lifetime fitness troubles which include obesity, heart infection, and diabetes is consuming a diet high in added sugars, inclusive of those observed in baked items, candy, sweetened beverages, and sugary cereals. Sweeteners that offer very less calories are known as non-nutritive sweeteners and have a poor glycemic response (Pepino 2015). All synthetic sweeteners are classified in a single cluster called non-nutritive sweeteners. They offer a sugary feeling to the taste buds, without levitating sugar concentration in the blood and are beneficial for weight reduction. The United States Food and Drug Administration (US FDA) has authorized six non-nutritive sweeteners inclusive of acesulfame K, aspartame, saccharin, sucralose, neotame, and stevioside (Kroger et al. 2006).

A large share of the non-nutrient food market composes of sugar alcohols also known as polyols (Regnat et al. 2018). Polyols can be defined as the carbohydrates whose carbonyl group has been reduced to a primary or secondary hydroxyl group. Polyols are required for various functions involved in efficient carbon fixation, growth, carbon storage, and reductant recycling (Radeloff and Beck 2013). Polyols are applied as nutraceuticals with value-added properties and functional foods. At present, sorbitol, mannitol, xylitol, lactitol, maltitol, and erythritol are polyols that can be accessible commercially. The polyols are sweet and have an energy content that is approximately half that of sucrose. Though polyols can be found naturally in foods, the amounts are too little for industrial exploitation. Hence, several of these are being produced using chemical or biotechnological approaches for commercial applications. Owing to the fact that polyols have no or little impact on glycemia, they may be a sugar replacement for diabetics (Wölnerhanssen et al. 2016). However, the intake of polyols is not limitless as excessive intake can cause diarrhea and symptoms including flatus, distention, and belly rumble (Oku and Nakamura 2007).

Among the various diverse polyols in the market, erythritol exhibits numerous unique capabilities. Erythritol's calorific value is close to zero, and the safe dosage that does not cause side effects is quite higher than other polyols. However, because of its rather excessive price, the use of this sweetener is not very widespread and is lesser than other polyols despite having many benefits. Nonetheless, due to its rising demand in the food enterprise, erythritol manufacturing by biological methods is turning progressively more important. This review discusses the current status of the production and applications of erythritol, with importance on the strain improvement strategies which include genetic engineering and mutagenesis in yeast for enhanced erythritol production in the industrial environment. Demand for high-intensity sweeteners has resulted in a stable development over the past five years and is anticipated to develop at a higher rate in the following five forecast years.

The market is driven by fitness consciousness and change in lifestyle among consumers of emerging nations. Some key manufacturers of erythritol, operational across the value chain in the market, are Foodchem international enterprise, Cargill Inc, Jungbunzlauer, and Shandong Sanyuan Biotechnology Co., Ltd (Erythritol marketplace—global enterprise evaluation 2015–2020 and possibility price 2020–2030).

**Received:** July 21, 2022. **Revised:** September 26, 2022. **Accepted:** November 7, 2022

## Erythritol

Erythritol is a 4-carbon polyol extensively found in the environment. Its chemical name is 1, 2, 3, 4-butanetetrol or meso-erythritol (C4H10O4) (Krajangsod et al. 2018). It has been isolated from fruits (like pears, grapes, melons), mushrooms, alcoholic liquids (like beer, wine, sake), and fermented meals (like soy sauce, miso bean paste) (Yoshida and Sugahara 1984, Shindou et al. 1988, Bernt et al. 1996). It could also be found in the biological fluids of human beings and animals which include eye lens tissue, serum, plasma, fetal fluid, and urine (Horning et al. 1974, Roberts et al. 1976, Niwa et al. 1993). Erythritol is a symmetrical molecule, occurring as a white anhydrous crystalline powder having 70% of the relative sweetness of sucrose. Due to the negative heat of the solution, it provides a pleasing mouth feel after consumption. A unique characteristic of erythritol is its quick absorption in the proximal intestine followed by excretion in the urine (Lenhart and Chey 2017). As the majority of consumed erythritol is rapidly expelled, it is considered as a zero-calorie sweetener (EU Commission directive 2008/100/EC). Therefore, erythritol does not have any effect on glycemia and is non-calorific (Hiele et al. 1993). At usual consumption levels, erythritol does not have any digestive distress, in contrast to other polyols that may have a laxative effect (Livesey 2001). The study completed to date such as metabolic, toxicological, and medical effects on animals and humans has established that erythritol is safe for intake even if consumed in relatively higher quantities (Munro et al. 1998).

Because of the low insulin index, erythritol has excellent potential as a perfect sugar alternative for people with diabetes (Livesey 2003). Erythritol was first commercialized in Japan in the early 1990s. European Food Safety Authority gave positive notice for erythritol use in non-alcoholic drinks and a GRAS (generally regarded as safe) status was issued by US FDA in 2001. In 2003, the European Union Scientific Committee on Food (SCF) concluded that erythritol is safe for utilization in foods, and it was given the code E986. Erythritol was approved in the EU as safe for intake in 2006 (EU commission directive 2006/52/EC). Due to a disagreement about its laxative effect, EU approval did not cover erythritol utilization in beverages. These constraints were revised in 2015 when the SCF projected a maximum level of 1.6% erythritol in nonalcoholic beverages (Scientific Opinion on the safety of the proposed extension of use of erythritol (E 968) as a food additive 2015).

## Production of erythritol

Erythritol can be produced through several chemical and biotechnological methods. Chemically, a nickel catalyst-based process is used for the production of erythritol from dialdehyde starch at elevated temperatures (Otey et al. 1961). In another method, the hydrogenation of a dialkyl ester of tartaric acid in methanol or ethanol solution leads to the formation of threitol and erythritol. The crucial parameters in chemical-based methods are high pressure (20 MPa) and temperature (125°C–200°C) (Trenner NR 1951) and due to their low efficiency, these processes have no longer been industrially used. Extraction of erythritol from vegetables and fruits is not an economically viable method even though it is present in them. In contrast, biosynthetic routes offer an environment-friendly and probably the best erythritol synthesis process. Because of the drawbacks of the chemical synthesis approach, at present, on a commercial scale, erythritol is produced by fermentation strategies by the use of fungal strains. Various genera of yeast-like *Aureobasidium, Candida, Moniliella, Torula, Trichosporon, Trichosporonoides,* and *Yarrowia* are known to produce erythritol (Ishizuka et al. 1989, Park et al. 1998, Oh et al. 2001, Lin et al. 2010, Savergave et al. 2011, Tomaszewska et al. 2014). Of these, because of lower yield, productivity, or byproducts formation including glycerol and ribitol, erythritol production by *Pichia, Zygopichia, Candida, Torulopsis,* and *Trigonopsis* could not be implemented on a commercial scale (Hirata et al. 1999).

## Erythritol biosynthetic pathway in yeast

The yeast strains of genera viz., *Yarrowia, Moniliella, Debaryomyces, Zygosaccharomyces, Hansenula*, and *Pichia* are capable of growing in an atmosphere of high salt or sugar concentrations. When these osmotolerant yeasts encounter salt or osmotic stress, they tend to accumulate compatible solutes. These solutes guard and stabilize enzymes, permitting cellular functions in osmotic surroundings. In yeasts, glycerol is the most common osmolyte but sugar alcohols which include erythritol, D-arabitol, and mannitol may also function as osmolytes. Other than serving as an osmolyte, these sugar alcohols could also play a vital element in balancing the redox potential of the cells or as a storage compound (Brown 1978, Kayingo et al. 2001). In yeast, the synthesis of erythritol is a multistep metabolic procedure, which operates commonly via the pentose phosphate pathway (PPP). In yeasts and other eukaryotic organisms, the role of the PPP is to yield NAD(P)H for cellular reactions and also to offer precursors for nucleotide and amino acid biosynthesis which includes D-ribose 5-phosphate and erythrose 4-phosphate (Nelson and Cox 2017).

This metabolic pathway includes two distinct phases. NAD(P)H and Ribulose-5P are the products of the primary oxidative phase and the last product of the non-oxidative phase is erythrose-4P. When *Moniliella megachiliensis* was grown in glucose for erythritol synthesis, high activity of PPP key enzymes (glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, transketolase, and transaldolase) was observed (Sawada et al. 2009). Dephosphorylation of erythrose-4P to erythrose and finally reduction to erythritol with the utilization of one molecule of NAD(P)H constitute the final steps in the suggested pathway (Lee et al. 2010, Fig. 1). As per the stoichiometry of erythritol biosynthesis within microbes, one mole of glucose is converted into one mole of erythritol (Equation 1) (Park et al. 1998).

$$\text{Glucose} + \text{ATP} \xrightarrow{\textit{Erythrose reductase}} \text{Erythritol} + \text{NADP}^{+} \qquad (1)$$

Erythrose reductase (ER), a NAD(P)H dependent aldose reductase, is a crucial enzyme in the biological synthesis of erythritol (Lee et al. 2003b). ER enzyme has been detected and reported in some erythritol-producing yeasts including *C. magnoliae* (Lee et al. 2010), *M. megachiliensis* (Kobayashi et al. 2013), *Torula sp.* (Lee et al. 2002), and *Y. lipolytica* (Cheng et al. 2018, Szczepańczyk et al. 2021). *C. magnoliae* JH110 produces a considerable amount of erythritol, signifying the presence of erythrose metabolizing enzymes. Lee et al. (2010), studied the genetic sequence and functional characteristics of a novel NAD(P)H-dependent ER from *C. magnoliae* JH110 for the first time (Fig. 2). Purified ER was extensively inhibited non-competitively with the aid of increasing fumarate concentration (Kobayashi et al. 2013). The gene encoding the enzyme accountable for the penultimate step in erythritol synthesis viz., erythrose-4-phosphate dephosphorylation has not yet been detected in any fungal species.

Other than this anabolic pathway, a few yeasts are also capable of using erythritol as a carbon source. Within the erythritol catabolic pathway, it is initially oxidized into erythrulose by

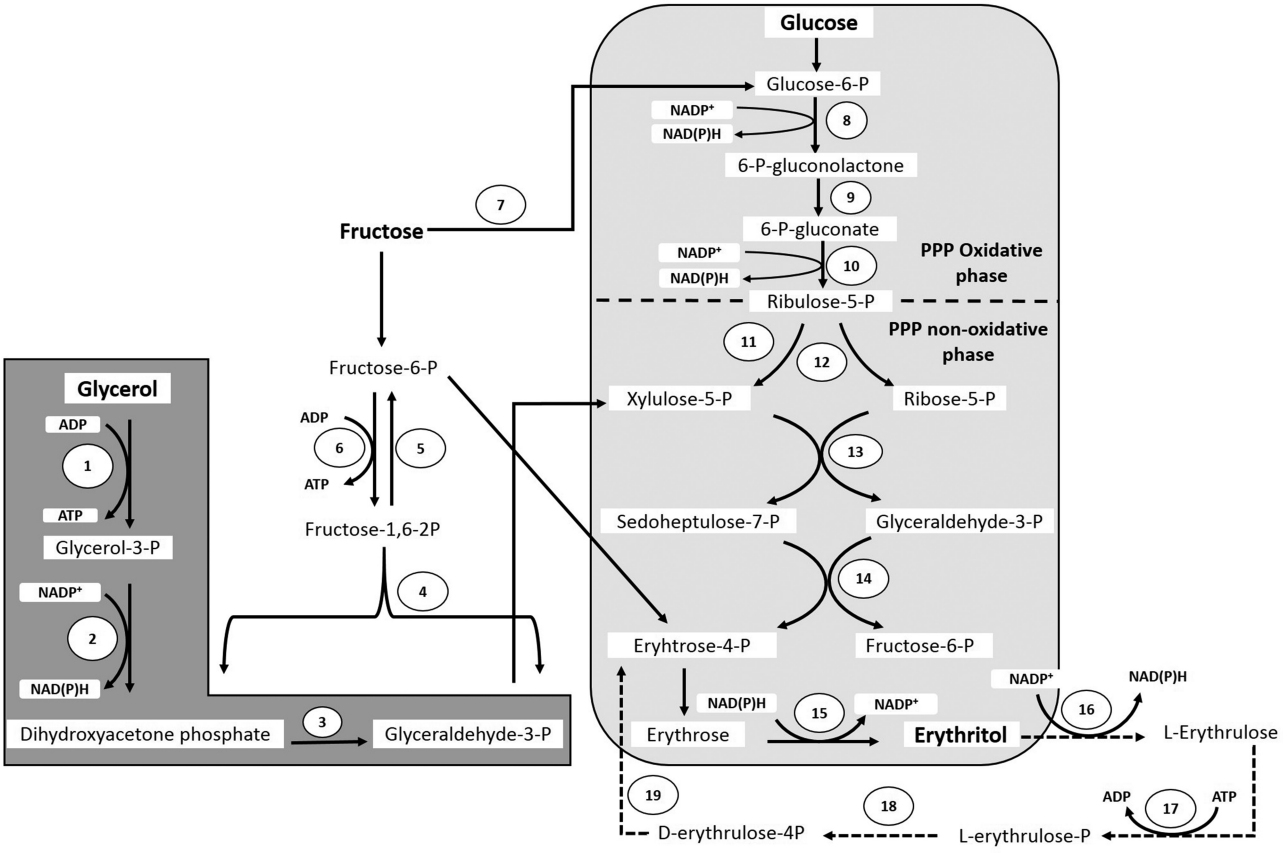



**Figure 1.** Erythritol synthesis in yeast using glycerol, fructose, or glucose as carbon source. (Proposed catabolic pathway in *Y. lipolytica* (Mironczuk et al. 2017, Carly et al. 2018, Niang et al. 2020, Zhang et al. 2021) shown in dotted arrows) (1. Glycerol kinase, 2. Glycerol 3 phosphate dehydrogenase, 3. Triosephosphate isomerase, 4. Aldolase, 5. Fructose 1,6 bisphosphatase, 6. Phosphofructokinase, 7. Glucose-6-phosphate isomerase, 8. Glucose-6-phosphate dehydrogenase, 9. Gluconolactonase, 10. 6-phosphogluconate dehydrogenase, 11. Ribulose-5-phosphate 3-epimerase, 12. Ribulose-5-phosphate isomerase, 13. Transketolase, 14. Transaldolase, 15. Erythrose reductase, 16. Erythritol dehydrogenase, 17. L-erythrulose kinase, 18. D-erythrulose kinase, 19. D-erythrose-4P isomerase).

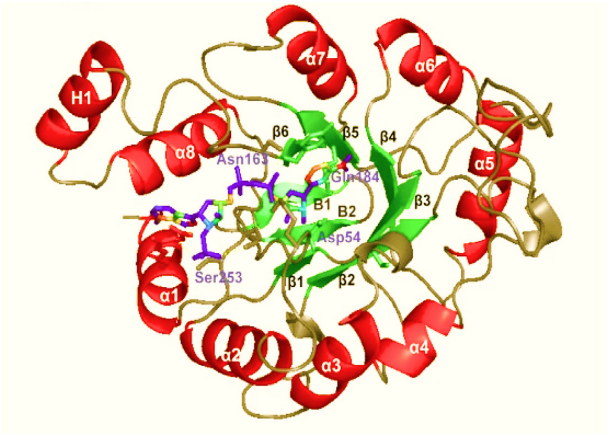



**Figure 2.** Crystal structure of erythrose reductase characterized from *C. magnoliae* (Lee et al. 2010).

an erythritol dehydrogenase (EYD) enzyme and then phosphorylated into erythrulose-phosphate through an erythrulose kinase (EYK) (Carly et al. 2017a). The genes corresponding to those enzymes were recently identified in *Y. lipolytica* (*YALI0F01650 g* and *YALI0F01606g*, respectively), and it was observed that their expressions are induced by erythritol (Carly et al. 2018). *Y. lipolytica* is capable of both the synthesis (Rymowicz et al. 2009) and utilization (Niang et al. 2020) of erythritol. It can produce erythritol from glycerol as well as assimilate it as a carbon source. This leads to a decrease in the productivity of erythritol and signifies a major disadvantage for the development of an efficient erythritol production process. The disruption of one of the genes identified, EYK1, coding for an erythrulose kinase enzyme, led to 26% (0.49 ± 0.02 g/g) higher erythritol yield and a 30% higher specific productivity (0.052 ± 0.005 g/g) than the wild-type strain (Carly et al. 2017a).

In another study, characterization of the *YALI0F01562 g* gene sequence revealed the existence of domains characteristic of transcription factors (Rzechonek et al. 2017). The authors have named this gene as erythritol utilization factor—EUF1. There are 4 enzymes identified in erythritol consumption in *Y. lipolytica* viz., erythritol dehydrogenase *Eyd1* (Carly et al. 2018), kinase *Eyk1* (Carly et al. 2017a), and isomerase, *Eyi1* and *Eyi2* (Mirończuk et al. 2018). It was found that the genes encoding these proteins are regulated with the aid of the transcription component EUF1 (Rzechonek et al. 2017). All of them, including EUF1, are arranged in a cluster inside the genome called the erythritol utilization cluster (Fig. 3) (Rzechonek et al. 2020). Additionally, two new genes, *YALI0F01584 g* and *YALI0F1628g*, participating in erythritol catabolism were identified, which is a putative isomerase and is localized in the same region as EUF1. Experimental results showed that *Y. lipolytica* displayed a noteworthy surge in *YALI0F01628 g*

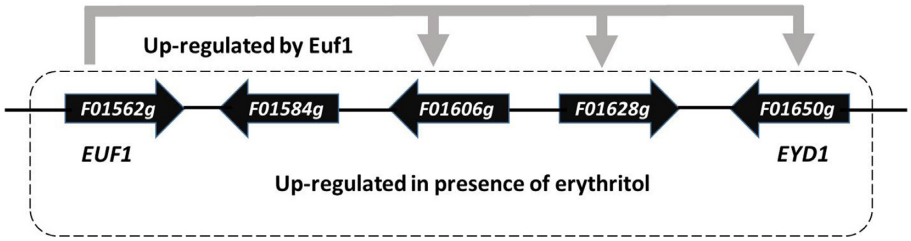



**Figure 3.** Cluster of genes involved in erythritol utilization located on chromosome in *Y. lipolytica* CLIB122 genome (Adapted and modified from Rzechonek et al. 2020).

expression in growth on erythritol. Furthermore, the strain showed the deletion of *YALI0F01628 g* revealed considerably lessened erythritol utilization, while production of erythritol not changed (Mirończuk et al. 2018).

## Substrates used for erythritol production

The usage of disaccharides or complex sugars might be restricted by the potential for hydrolysis of O-glycosidic bonds by different yeast species. Apart from the most commonly used substrate glucose, other sugar substrates like fructose and sucrose can also be used for erythritol production. Interesting observations were made in yeast strain *C. magnoliae* which showed a choice for fructose as a carbon substrate over glucose (Yu et al. 2006).

Among other substrates, glycerol was found to be a good carbon source for erythritol synthesis, as observed in *Y. lipolytica* (Rymowicz et al. 2009). The glycerol assimilation proceeds through its phosphorylation by glycerol kinase (GK) to glycerol-3-phosphate, which is subsequently converted to dihydroxyacetone phosphate by NAD+-glycerol-3-phosphate dehydrogenase (GPDH) (Fig. 1). Several strains of *Y. lipolytica* also possess a FAD- GPDH enzyme for the dehydration of glycerol phosphate to dihydroxyacetone phosphate. The enzyme activity of GK and GPDH during erythritol production from glycerol in *Y. lipolytica* Wratislavia K1 varied depending on NaCl concentration and pH values in the culture medium (Tomaszewska et al. 2014). Raw glycerol can also be utilized for erythritol production by *Y. lipolytica* (Rymowicz et al. 2009) and *M. megachiliensis* (Kobayashi et al. 2015), with yields as high as 0.56 g/g and 0.60 g/g, respectively. Higher erythritol yields on non-refined glycerol compared to purified glycerol, indicates that impurities in the raw glycerol might have a positive effect on erythritol synthesis. Thus, raw glycerol could be a cost-effective alternative to glucose in erythritol production as demonstrated in various reports (Mirończuk et al. 2015a, Carly et al. 2017b, Tomaszewska et al. 2014, Szczepańczyk et al. 2021).

Apart from glucose and glycerol, another study reported a five-fold increase in erythritol production from xylose as compared to glucose using *Y. lipolytica* (Sivaraman et al. 2016). Sugarcane juice has also been used by *M. pollinis* (Deshpande et al. 2022) as carbon substrate for erythritol production yielding a titer of 94.9 g/L with a 38% erythritol yield.

## Commercial production of erythritol

There are some establishments worldwide, manufacturing erythritol commercially, including Bolak Corporation (Seoul, South Korea), Cargill Food and Pharma Specialties (Minneapolis, MN), Baolingbao Biotechnology (Dezhou Shi, China), O'Laughlin Corporation (Tianjin, China), Jungbunzlauer (Basel, Switzerland), and Mitsubishi Chemical Corporation (Tokyo, Japan). Large-scale erythritol production is mainly from glucose, sourced either chemically or from enzymatically hydrolyzed corn or wheat and using yeast strains viz., *Moniliella pollinis, Aureobasidium* sp., and *Torula* sp (Kim et al. 2000). Optimization of the process led to improved productivity and concentrations of erythritol (Table 1).

New biotechnological methods, mainly genetic modifications, in yeast strains are still being established and improved to further increase erythritol productivity, which will be discussed in the following sections.

### Yarrowia species

*Yarrowia lipolytica* is one of the well-researched yeast strains for erythritol production. *Yarrowia lipolytica* can use raw glycerol instead of glucose as its main carbon source and is highly capable of producing erythritol using glycerol (Rymowicz et al. 2009, Kobayashi et al. 2015). In *Y. lipolytica,* two genes *GUT1* and *GUT2* encoding for GK and GPDH respectively, are involved in glycerol assimilation (Makri et al. 2010) and have been overexpressed in *Y. lipolytica* A101 (Mirończuk et al. 2016). The researchers have used a hybrid promoter containing sixteen upstream activating sequences enhancing the expression of the Translational Elongation Factor (TEF) promoter. Three strains were created with overexpression of both GK and GPDH or co-expression of both enzymes. Erythritol productivity was enhanced by 24% while GK was overexpressed, whereas overexpression of GPDH did not have any impact. However, co-expression of both the genes improved erythritol productivity by 35% over the control. The overexpressed strain showed 1.08 g/L/h erythritol productivity from glycerol whereas in the parent strain it was 0.80 g/L/h (Mirończuk et al. 2016).

In a new approach to exploring the use of sucrose in molasses as substrate, the *SUC2* gene of *Saccharomyces cerevisiae,* coding for invertase (Nicaud et al. 1989, Xu et al. 2014), was overexpressed in the yeast strain *Y. lipolytica* Wratislavia K1 along with overexpression of the native *GUT1* gene (Rakicka et al. 2017). The *SUC2* gene was inserted inside the *ura* locus under the TEF promoter and CYC1 terminator (Blazeck et al. 2011) creating the strain *Y. lipolytica* AIB. This was followed by an introduction of the *GUT1* overexpressing cassette, resulting in the yeast strain AIB pAD-UTGut1 (Rakicka et al. 2017). The engineered strain generated by this method was able to assimilate both sucrose and glycerol. This strain produced 82.2 ± 3.0 g/L of erythritol with a yield of 0.55 ± 0.1 g/g and productivity of 0.87 ± 0.5 g/L/h. The first study that reports efficient polyol production by the modified *Y. lipolytica* strain from industrial raw molasses and crude glycerol was published by Mironczuk et al. 2015bIn that study researchers were able to produce 52–114 g/L of erythritol with 0.58–1.04 g/L/h productivity and 0.26–0.57 g/g of yield (Mironczuk et al. 2015b).

In another study to assess their role in erythritol biosynthesis, transketolase (*TKL1*, *YALI0E06479g*), transaldolase (*TAL1*, *YALI0F15587g*), NADP+- dependent 6-phosphogluconate

**Table 1.** Erythritol production by yeast cultures using various carbon sources.

| Yeast strain | Strain modification | Carbon source | Culture system | Yield% | Productivity g/L/h | Reference |
|---|---|---|---|---|---|---|
| *Aureobasidium* sp. Mutant SN-124A | NTG + UV mutation | Glucose | Batch | 37.7 | 1.82 | (Ishizuka et al. 1989) |
| *Aureobasidium pullulans* | UV mutation | Xylose | Batch | 26 | 0.22 | (Guo et al. 2016) |
| *Torula* sp. | - | Glucose | Fed-batch | 48 | 2.26 | (Oh et al. 2001) |
| *Moniliella tomentosa var. pollinis* | - | Glucose | Fed-batch | 40 | 1.61 | (Burschäpers et al. 2002) |
| *M.pollinis* | | Sugarcane juice | Fed-batch | 38 | 0.61 | (Deshpande et al. 2022) |
| *M. megachiliensis* sp. 440 mutants (mutant N61188-12) | NTG mutation | Glucose | Batch | 43 | 0.65 | (Lin et al. 2010) |
| *Trichosporon sp.* | - | Glucose | Batch | 47 | 1.96 | (Park et al. 1998) |
| *Trichosporonoides oedocephalis* | Δ*hog1* | Glucose | Shake flask Fed-batch | 35 | 0.58 | (Li et al. 2016) |
| *C. magnoliae* | EMS mutation | Glucose | Two-stage | 41 | 2.8 | (Ryu et al. 2000) |
| *C. magnoliae* | EMS mutation | Glucose | Fed-batch | 43 | 1.2 | (Koh et al. 2003) |
| *C. magnoliae* | UV, Chemical mutation | Glucose | Fed-batch | 36 | 0.52 | (Savergave et al. 2011) |
| *Candida sorbosivorans* | - | Glucose | Batch | 38 | 0.50 | (Saran et al. 2015) |
| *Pseudozyma tsukubaensis* | - | Glucose | Batch | 61 | 1.65 | (Jeya et al. 2009) |
| | - | Glucose | Fed-batch | 60 | 2.84 | (Jeya et al. 2009) |
| *Y. lipolytica* CICC 1675 | - | Pure glycerol | Fed-batch | n.a. | 0.95 | (Yang et al. 2014) |
| *Y. lipolytica* K1 | - | Crude glycerol | Repeated batch culture | 56 | 0.3 | (Mirończuk et al. 2014) |
| *Y. lipolytica* | *SUC2* | Glycerol, Molasses | Batch | 26–57 | 0.58–1.04 | (Mirończuk et al. 2015b) |
| *Y. lipolytica* A101 | *GUT1, GUT2* | Glycerol | Batch | 43 | 1.08 | (Mirończuk et al. 2016) |
| *Y. lipolytica* | *SUC2, GUT1* | Glycerol, Molasses | Batch | 55 | 0.87 | (Rakicka et al. 2017) |
| *Y. lipolytica* MK1 | *TKL1* | Glycerol | Batch | 58 | 0.81 | (Mirończuk et al. 2017) |
| *Y. lipolytica* | *GUT1, TKL1,* Δ*eyk1* | Glycerol | Batch | 53 | 1.03 | (Carly et al. 2017a) |
| *Y. lipolytica* | *ER(ALI0F18590g)* | Glycerol | Batch | 44 | 0.77 | (Janek et al. 2017) |
| *Y. lipolytica* yliUA8 | UV and ARTP mutagenesis | Glucose | Batch culture | 53 | 1.55 | (Qiu et al. 2020) |
| *Y. lipolytica* | *ER(YALI0B07117g)* | Glycerol | Batch | 40 | 0.41 | (Szczepańczyk et al. 2021) |
| *Y. lipolytica MY11* | *GUT1, TPI1, TKL1, TAL1;* Δ*eyk1* | Glycerol | Shake flask Batch | 20 | 0.18 | (Zhang et al. 2021) |
| *Y. lipolytica MY12* | *RK11* | | | 52 | 0.24 | |
| *Y. lipolytica MY13* | *AMPD* | | | 46 | 0.21 | |

dehydrogenase (*GND1, YALI0B15598g*), and NADP+- dependent glucose-6-phosphate dehydrogenase (*ZWF1, YALI0E22649g*) genes of PPP, responsible for the production of reducing agents in the cell were overexpressed. The study indicated that depending on the type of carbon source, erythritol levels produced by this engineered strain *Y. lipolytica* MK1 varied with maximum titers obtained on glycerol. Overexpression of *TKL1* led to an increased level of *TKL1* transcription, resulting in considerably higher erythritol synthesis under oxygen limitation. The engineered strain consistently produced two-fold higher erythritol than the control in shake-flask experiments. A bioreactor study showed that the strain AMM pAD-*TKL1* gave 67% more erythritol production than the control and by-product formation was significantly inhibited. The engineered strain produced 62.5 g/L of erythritol with a productivity of 0.62 ± 0.05 g/L/h compare to 37.3 g/L of erythritol with a productivity of 0.42 ± 0.05 g/L/h in the parent strain. (Mirończuk et al. 2017).

Overexpression of *GUT1* and *TKL1* and disruption of *EYK1* coding for erythrulose kinase that participates in an early step of erythritol catabolism were found to be beneficial for erythritol production. The engineered strain JCY218 synthesized erythritol proficiently in a short period at a lower oxygen concentration. Moreover, erythritol productivity was 75% higher (1.03 g/L/h) than the wild type (0.59 g/L/h) in glycerol, and the culturing period required to accomplish maximum concentration was reduced by 40%. Engineered strain produced 80.6 g/L erythritol whereas in parent strain 55.8 g/L was observed. Additionally, the strain was incapable of consuming the erythritol it had produced thereby further increasing the productivity of the process (Carly et al. 2017a).

Erythritol production has been identified as a completely distinctive reaction to hyperosmotic pressure in a group of yeasts inclusive of *Y. lipolytica*. High osmolality glycerol (HOG) pathway gets activated by mitogen-activated protein kinase (MAPK) pathway which regulates the response to high osmotic stress-conditions in the yeast strain *S. cerevisiae* (Smith et al. 2010). *Hog1* gene deletion resulted in a considerable fall in resistance to hyperosmotic stress and adversely hampered the production of erythritol. In *Y. lipolytica*, the gene *YALI0E25135 g* was detected as a homologue

of *HOG1* gene and was phosphorylated in response to hyperosmotic pressure. On sequence comparison using the BLASTp with *S. cerevisiae* and *C. albicans HOG1*, the gene *YALI0E25135 g* was identified as the *Y. lipolytica* homologue of *Hog1*. The flanking regions and gene sequence of *YALI0E25135 g* were used for primer design to allow for its deletion and overexpression successfully. Two strains of *Y. lipolytica* were created in which either *yl-Hog1* was deleted (*yl-hog1*Δ) or over-expressed (*yl-HOG1*). Interestingly, the deletion strain (*yl-hog1*Δ) confirmed noteworthy morphological alterations, with the cells developing in a filamentous shape. Moreover, *yl-hog1*Δ cells were also immune to the cell wall destructive agents viz. Congo red and Calcofluor white. These observations indicated that *yl-Hog1* is vital for the cellular reaction to hyperosmotic pressure, performs a role in the induction of erythritol production, and probably prevents crosstalk with distinctive MAPK signaling pathways within the cell. The *yl-hog1*Δ and *yl-HOG1* strains synthesized 32 ± 4 g/L and 19.3 ± 3 g/L erythritol respectively whereas parent strain MK1 showed 36.6 ± 1 g/L erythritol production (Rzechonek et al. 2018).

Janek et al. (2017) identified an erythrose reductase (ER) gene (*YALI0F18590g*) from erythritol-producing yeast *Y. lipolytica* MK1 for the first time. Overexpression of *ER* gene in presence of $Zn^{+}$ resulted in an improved erythritol titer of 78.1 g/L, a yield of 0.52 ± 0.06 g/g, and productivity of 1.00 ± 0.12 g/L/h in glycerol-based medium as compared to 68.2 g/L of erythritol and 87 ± 0.05 g/L/h productivity in the control strain. However, deletion of *YALI0F18590 g* gene still gave some erythritol production in *Y. lipolytica* MK1 suggesting the existence of other ER genes in the genome of *Y. lipolytica* (Janek et al. 2017). Three isozymes (ER-1: MW 38 000 Da; ER-2 and ER-3: MW 37 000 Da) of ER were reported in *Aureobasidium sp*. SN-G42 (Tokuoka et al. 1992). Similarly, in *Y. lipolytica*, eight ERs were identified and overexpression of one of the ER homolog gene (*YALI0B07117g*) caused increased erythritol productivity (from 0.28 g/L/h to 0.41 g/L/h) for the modified A101 strain using glycerol as substrate. Moreover, knocking off this homolog gene (*YALI0B07117g*) did not abolish the production of erythritol in the strain AJD pAD B07117 reiterating the presence of other ER genes in the yeast *Y. lipolytica* (Szczepańczyk et al. 2021). The high percentage of homology between all of the ER homologs causes a problem in establishing which one of these proteins is responsible for erythritol synthesis in general.

Overexpression of native GK and TKL and heterologous overexpression of sugar alcohol phosphatase (PYP) in *Y. lipolytica* Po1f (Madzak et al. 2000) showed improved erythritol production during batch and fed-batch fermentation using crude glycerol as substrate (Jagtap et al. 2021). The engineered strain gave 58.8 ± 1.7 g/L erythritol as compared to 30.52 g/L in *Y.lipolytica* Po1f. In both the cases, productivity was very low as fermentation time lasted for 12 days.

Zhang et al. (2021) used a multiple gene integration approach to improve erythritol synthesis in *Y. lipolytica*. The collective expression of four genes, namely *GUT1, TPI1, TKL1, TAL1* (coding for glycerol kinase, triosephosphate isomerase, transketolase, and transladolase) and disruption of the *EYD1* gene (encoding erythritol dehydrogenase), showed enhanced erythritol production (20 g/L) than auxotrophic strain *Y. lipolytica* Po1f (17.0 g/L) from glycerol. Authors have also demonstrated erythritol production by overexpressing the *RKI1* gene (encoding ribose 5-phosphate isomerase) and the *AMPD* gene (encoding AMP deaminase) in *Y. lipolytica*. This study suggested that RKI1 possibly leads to an increased formation of erythrose-4P flux in *Y. lipolytica* which is subsequently utilized for enhanced erythritol production (Table 1).

Apart from the genetic modification approach for strain development in *Y. lipolytica*, Qiu et al. (2020) developed a high throughput screening method for rapid detection and screening of mutant strain libraries for enhanced erythritol production. In their study, they used an erythritol-responsive transcription factor called *EryD*, and built a sensor-regulator system for rapid screening and characterization of erythritol overproducing strain. With this method, they screened 1152 mutants derived from mixed UV and atmospheric room temperature plasma (ARTP) mutagenesis, rapidly (1 week). One of the mutants, yliUA8s, was found to produce 103 g/L erythritol at the shake flask stage; a 2.4-fold increase in erythritol production from glucose than the strain used initially. In scale-up trails using 3.0 L bench-top reactors, mutant strain yliUA8 produced more than 148 g/L erythritol. This study enables fast improvement in strain performance and engineering effective microbial cell factories for industrial applications (Qiu et al. 2020).

## Moniliella species

The assessment of nucleotide sequences from the D1/D2 domains of the large subunit rDNA and phenotypic functions indicated that the *Moniliella* and *Trichosporonoides* genera are members of a sole, monophyletic clade that would be best characterized by using a single anamorphic genus (Rosa et al. 2009). A homologue of *Hog1* present in *Trichosporonoides (Moniliella) oedocephalis* was deleted by Li et al., (Li et al. 2016). In this study, an attempt was made to adopt the Cre/loxP system containing the KanMX gene disruption cassette for *HOG1* gene knockout in *T. oedocephalis*. The Cre/loxP cassette approach is substantially applied to yeast in combination with the heterologous dominant KanR, as a powerful gene disruption technology (Baudin et al. 1993). The newly constructed knockout mutants of *T. oedocephalis* HOG1 displayed slightly lower biomass in a liquid medium and impaired growth in a solid medium. However, mutant strain exhibited a 1.4-fold increase in erythritol synthesis (56.82 ± 0.31 g/L) with a concurrent decrease in glycerol production by 71.23%. While the parent strain *T. oedocephalis* ATCC 16958 could only produce 39.37 ± 0.18 g/L erythritol with significantly high production of glycerol (36.22 ± 0.19 g/L).

Studies were also carried out on ERs of *M. megachiliensis* to detect hyperosmotic stress responses (Kobayashi et al. 2013). *T. megachiliensis* has been reported to have three ER isozymes; ER1, ER2, and ER3 (Ookura et al. 2005). The gene sequences of the *ER1*, *ER2*, and *ER3* loci together with sequences 2 kb upstream from each initiation codon and 1 kbp downstream from each termination codon were investigated. There were two stress response elements (STREs) upstream of the initiator methionine of *ER3* (one at 148 bp upstream position and the other at 80 bp upstream position) whereas, *ER1* and *ER2* had only one STRE each. An exploration of the genomic regions near the *ER* genes did not disclose any gene likely to have participated in polyol production (Fig. 4). The expression of three ER encoding genes (*ER1, ER2,* and *ER3*) was evaluated in cells cultivated on 20% glucose. There was no noteworthy change observed compared to cells grown on 2% glucose, during the initial hours of treatment. However, there was a 60-fold surge in the expression of the glycerol-3-phosphate dehydrogenase gene at the same time, which shows that the principal response of cells was the production of glycerol. Intracellular erythritol accumulation was observed after 12 h incubation and stayed high during the next five days. Only *ER3* expression rose significantly during extended osmotic stress and the level of its mRNA transcripts matched the erythritol formation profile.

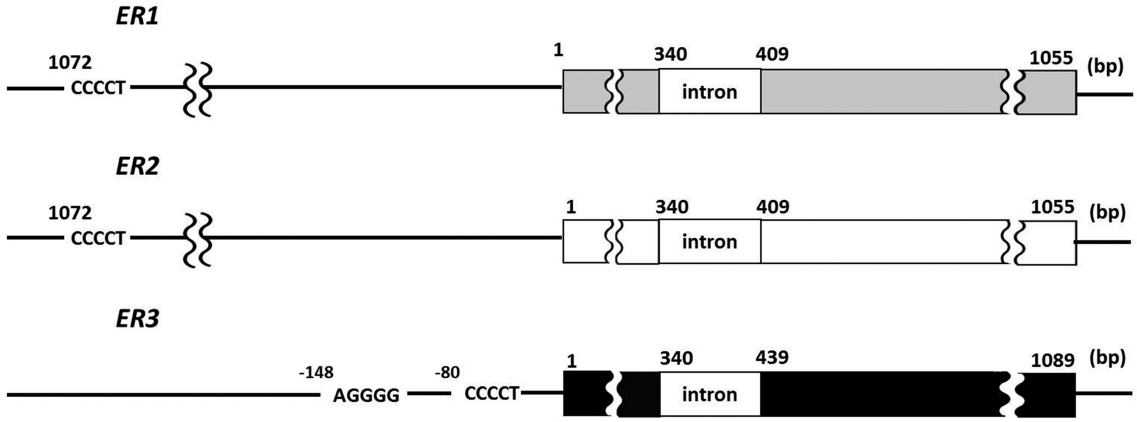



**Figure 4.** The structure of the *ER1*, *ER2*, and *ER3* genes of *T. megachiliensis*. The sequence CCCCT or AGGGG represents a stress response element (adapted and modified from Kobayashi et al. 2013).

Therefore, glycerol synthesis might be an initial reaction, while the production of erythritol is the cell's stationary phase reaction to osmotic stress (Kobayashi et al. 2013).

Tetsuya Ookura and coworkers characterized the three isozymes of ERs viz. ER-I, ER-II, and ER-III biochemically from *T. megachiliensis* SNG-42 (previous name *Aureobasidium megachiliensis*), one of the strains used for commercial production of erythritol (Tokuoka et al. 1992, Ookura et al. 2005). Furthermore, the authors have expressed recombinant *ER3* gene in *P. pastoris* and *S. cerevisiae* but were not able to observe erythritol production in the culture medium. Authors have claimed that this is due to the low yield of erythrose reductase in each strain mandating demand for more functional ERs for improved erythritol production. Secondly, for proper erythritol production, a greater supply of cofactors (NADPH) or D-erythrose is required. It is believed that while D-erythrose resulted from erythrose-4P in the pentose phosphate shunt, the specific enzymes like phosphatases required for this reaction have not been identified. (Ookura et al. 2005).

In another study, *Moniliella sp*. 440 isolated from honey was sequentially treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for iterative rounds of mutation and selection (Lin et al. 2010). Six strains were tested for erythritol synthesis in 40% glucose and 1% yeast extract in a shake flask and all of them produced more erythritol than the parent strain. Mutant strain N61188-12 produced the best erythritol (237.8 g/L) than wild type (113 g/L) strain. Mutagenesis not only improved erythritol production, but mutated strain showed faster utilization of glycerol along with no foaming, lower concentration of byproducts, and greater resistance to osmotic stress. When the fermentation was scaled up to a 2000 L reactor, the mutant N61188-12 produced 152.4 g/L erythritol within 10 days.

## Candida species

*Candida magnoliae*, an osmophilic yeast is one of the strains capable of biological erythritol production. *Candida magnoliae* was isolated from honeycombs and mutated using 0.8% ethyl methane sulfonate (EMS) to improve erythritol yield from glucose. The mutant strain of *C. magnoliae* confirmed over-production of erythritol with a productivity of 1.2 g/L/h, a titer of 200 g/L, and a yield of 0.43 g erythritol per g of glucose by sustaining the precise glucose consumption rate in the fed-batch phase. Erythritol productivity increased 5-fold in the fed-batch fermentation in comparison with the batch fermentation (Koh et al. 2003).

One of the mutants of *C. magnoliae* DSM70638 created by the combination of UV and EMS mutagenesis (mutant 12–2) gave a 2.4–fold increase in erythritol (20.32 g/L) than wild type (8.54 g/L) and a 5.5–fold decrease in glycerol production (2.36 g/L) compared to the wild strain (12.93 g/L). Gene encoding for ER enzyme of this mutant, when subjected to a sequence-based mapping, showed a replacement of the A321 by G321, and this replacement did not cause any amino acid exchange in protein structure. The cause for enhanced erythritol production in *C. magnoliae* mutant 12–2 strain was proposed to be most likely due to the increase in expression of the open reading frame of the gene. Thus, a mutation or slight change in the sequence of genes involved in an erythritol production pathway can lead to a noteworthy upsurge in protein translation (Ghezelbash et al. 2014).

Among *C. magnoliae* NCIM 3470 mutants generated by UV and chemical mutagenesis, the mutant R23 produced 4-fold more erythritol (60.3 g/L) than the wild strain (14 g/L) and ER activity was 2-fold higher (Savergave et al. 2011). Glucose and yeast extract have been identified as key medium additives that decided the ratio of production of polyols including erythritol, mannitol, and glycerol. To enhance the formation of erythritol and decrease the production of mannitol and glycerol, a four factor-five stage-three reaction relevant-composite-rotatable-design (CCRD) of response surface methodology (RSM) model was used. In fed-batch fermentation, with statistically optimized media composition, 87.8 g/L erythritol was formed with 31.1% yield.

*Candida magnoliae* wild and mutant strains were subjected to proteomic analyses using two-dimensional gel electrophoresis and nano electrospray mass spectrometry to explore intracellular proteins and to assess the influence of newly characterized metabolic enzymes on the yeast cell growth and erythritol production. The high growth of the *C. magnoliae* mutant strain was observed when enzymes involved in the citric acid cycle were up-regulated. Mutant strain also showed increased NADH and ATP concentration. It was also discovered that mutations caused downregulation of gene encoding for enolase and upregulation of fumarase encoding gene. In comparison with wild-type strain, this regulation plays an important role in improved bioconversion of erythrose-4P to erythritol (Lee et al. 2003a). It was evident that overexpression of fumarase permits faster fumarate degradation and subsequently inhibits the activity of ER (Lee et al. 2002). Downregulation of enolase is supposed to decrease phosphoenolpyruvate concentration. Phosphoenolpyruvate and erythrose-4P are starting materials for shikimate

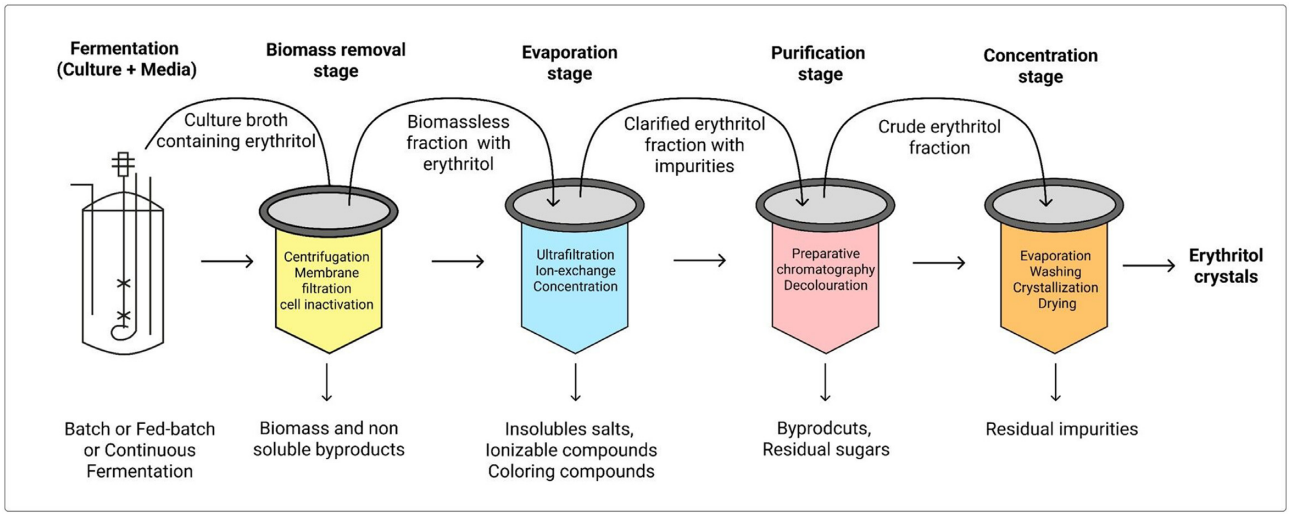



**Figure 5.** Downstream process for erythritol recovery (adapted and modified from Daza-Serna et al. 2021).

synthesis. Hence, erythrose-4P could be directed towards erythritol synthesis rather than shikimate synthesis by decreasing phosphoenolpyruvate levels.

## Erythritol production by other yeast and fungal species

Besides *Moniliella*, *Candida*, and *Yarrowia*, several different yeast genera have been used for erythritol production, particularly *Aureobasidium* (Ishizuka et al. 1989), *Trichosporonoides* (Aoki et al. 1993), *Trichosporon* (Park et al. 1998), *Torula* (Oh et al. 2001), and *Pseudozyma* (Jeya et al. 2009). The biotransformation of xylose, the second most copious sugar has additionally been studied in recent years.

*A. pullulans* CGMCC3.0837 strain was mutated using UV to improve its erythritol production from xylose. In comparison with wild-type strain, mutant strain ER35 produced erythritol which was 50.92% (17.28 g/L) higher. The erythritol concentration produced by mutant ER35 in shake flasks and a 5-L fermentor were similar (~31 g/L). Therefore, this study offered the possibility of using xylose as a carbon source for the production of erythritol using *A. pullulans* (Guo et al. 2016). Yeast strain *Aureobasidium sp.* SN-124A was repeatedly mutated with NTG treatment and UV irradiation for improved erythritol production. Mutant strain SN-G42 showed superior properties by not foaming during cultivation while the wild strain foamed severely. The erythritol production yield in the mutant was 47.6% in a medium containing 22.5% glucose as compared to that of 41.8% yield in the wild strain. Furthermore, erythritol productivity was not affected in the mutant culture even when the glucose concentration was increased from 22.5% to 47% (Ishizuka et al. 1989).

A well-studied filamentous fungus *Fusarium graminearum* known for its relevance as a plant pathogen affecting several plant such as cereals and dicotyledons (Jovanović et al. 2013) was also explored for erythritol production. Filamentous fungi use plant biomass like lignocellulose as a substrate in contrast to yeast. These fungi secrete xylanolytic enzymes which hydrolyze xylans into their key monomers L-arabinose and D-xylose. The monomers produced by these fungi can be metabolized to D-xylose-5-phosphate to supplement the PPP, the source of erythritol. In one study (Jovanović et al. 2013), the investigators identified ER by in silico analysis of proteins in *Trichoderma reesei, Aspergillus niger*, and *F. graminearum* which displayed high sequence similarity to the ER (*ER1*) from *T. megachiliensis* (Ookura et al. 2005). *ER1* genes from *T. reesei* were cloned and their protein products were expressed heterologously in *E. coli* and purified. Enzyme assay parameters were optimized for ten possible substrates including D-erythrose. *In vitro* analysis of these proteins by an enzyme assay confirmed high substrate specificity and the turnover rate for D-erythrose amongst all the ten substrates. The *ER1* from *T. reesei* showed a higher turnover number for D-erythrose substrate than *ER1* from *A. niger* and *F. graminearum*. On overexpression of the ER enzyme in *T. reesei*, erythritol production increased in the overexpressed strain as compared to the parental strain *in vivo*. After 24 h of culture time, the erythritol concentration in the modified strain was 1.6-fold higher than in the parental strain, and after 30 h it was 3.2-fold higher (Jovanović et al. 2013).

Furthermore, the same research group overexpressed the *ER1* gene in the *T. reesei* wild-type and in the cellulase hyper-producing, carbon catabolite repressed strain Rut-C30 to evaluate erythritol production. Two different promoters were used for *ER1* overexpression in both strains, a constitutive (the native pyruvate kinase promoter) and an inducible one (the native $\beta$-xylosidase promoter). Recombinant strains were pre-characterized by analysis of *ER1* transcript formation on xylan and D-xylose and pre-treated wheat straw. In shake flasks and fermenter experiments, it was found that overexpression of *ER1* in Rut-C30 led to a higher erythritol formation on pre-treated wheat straw (Jovanović et al. 2014).

## Efficient erythritol recovery

Depending on the strain and substrate used during erythritol production, some by-products such as glycerol, ribitol, mannitol, fumarate, D-arabitol, and citrate are produced (Lin et al. 2001, Mirończuk et al. 2017). A typical erythritol production process begins with the fermentation followed by the main phases of the downstream process, including clarification of biomass by removing cells, separation of compounds that are insoluble, purification of clarified erythritol from impurities and byproducts, and finally concentration to obtain erythritol crystals (Fig. 5) (Troostembergh et al. 2002, Saran et al. 2015, Rakicka et al. 2016, Deshpande et al. 2022).

Various approaches have been reported for biomass removal like cell separation (Maeda et al. 1997, Deshpande et al. 2022), biomass inactivation (Sasman et al. 2007), and vacuum filtration (Saran et al. 2015). Warming of the culture broth after fermentation at elevated temperature up to 70°C to inactivate the cells followed by separation of the cells using centrifugation are performed (Sasman et al. 2007). Some researchers have used techniques such as ultrafiltration at pH range between 3.5 and 5.5 to avoid the foaming and subsequent intrusion of intracellular proteins in ion exchange chromatography (Kresnowati et al. 2019).

The evaporation stage caters to elimination of non-soluble portions, and color impurities from the cell-free fermented broth and to separate the erythritol portion for purification in the chromatography step. To perform these operations different processes like ultrafiltration, ion exchange, and concentration have been used. At this stage, the fermented broth primarily has residual carbon source, erythritol, and by-products. For enhanced erythritol purification, nanofiltration was included after the microfiltration as an added step to eliminate leftover macromolecules before the ensuing ion exchange step (Li et al. 2020, Zhao et al. 2020). In the report by Troostembergh et al. (2002) the cell-free fermented broth was treated with activated charcoal to eliminate the dissolved as well as colored impurities. The solution obtained after filtration was concentrated in a rotatory vacuum evaporator at 55°C and 72 psi to acquire condensed viscous liquid which was desalinated by precipitation. The filtrate having erythritol was passed through an ion exchange column packed (18.15 × 2.0 cm) with cationic exchange resin (Diaion SK116, BF 100 INDION) and then eluted with water. (Saran et al. 2015, Deshpande et al. 2022). The major byproducts in erythritol production are glycerol, ethanol, and other polyols. The subsequent step in this process is to separate the erythritol fraction from these byproducts. The purification of erythritol fraction relies on charge (chemical sites on the exterior of the molecules) based separation on ion exchange chromatographic resins. A cationic acid resin (Purolite PCR-821) was used for erythritol purification by batch preparative chromatography (Sasman et al. 2007).

The final step of the purification stage consists of various processes like evaporation, crystallization, crystals separation, washing, and drying.to obtain the highly purified erythritol. In one of the methods described earlier (Saran et al. 2015), different fractions containing erythritol were pooled and again concentrated in rota vapor. The concentrated filtrate was then kept for the crystallization process at 4°C. Erythritol recovery was around 52.24% with a purity of 98.99% in a pure crystallized form (Saran et al. 2015). In another study, 43% erythritol recovery from sugarcane-based feedstock with 96.34% erythritol purity (Deshpande et al. 2022) was obtained. By adjusting the cooling rate, erythritol crystals with 99.9% purity were obtained (cooling rate of 7.5°C/h from 70°C to 15°C) by Morioka et al. (2000). Similar results were observed when the temperature was lowered from 60°C to 20°C with steady cooling rate in another study by Toshihiro et al. (1993). The residual impurities fraction contained 26.3% of erythritol and other byproducts. Isolation of crystals from the liquid solution is the last step in downstream process where cold water spray was applied to achieve crystal separation. A rotary drum dryer can be used for drying the washed crystals as has been the practice for common sugars (Morioka et al. 2000).

The sticky and reddish-brown liquor called waste erythritol mother liquor (WEML) is the waste product left over after the purification process. The WEML contains erythritol along with other waste polyols and is problematic to deal with. Various approaches like bio-removal and biotransformation have been used to treat the WEML due to their high efficiency and specificity; particularly, in the area of environmental remediation and retrieval of high-value chemicals from crude sugar feedstocks. In another study, researchers aimed to develop an efficient procedure to purify erythritol from WEML using yeast. After identifying the impurities of polyol in WEML, a yeast strain, *Candida maltosa* was used to biologically enrich erythritol in WEML by reducing the polyol impurities. They also studied the newly developed bio-removal approach in shake flasks and at the fermenter level (Wang et al. 2017).

## Conclusions and future perspectives

The biological production of erythritol mainly from yeast has been given much attention in the past decades owing to the increasing demand for this product. Several studies have been demonstrated to improve the yield of erythritol in yeast strains, which proved to be promising organisms for erythritol production. In the literature, some of the yeast cultures were shown to give high productivity, and the medium composition along with culture conditions has been fine-tuned to maximize the production titer. Further, genetic engineering and proteomic studies have been carried out with promising results, leading not only to greater efficiency but also to a better understanding of the erythritol metabolism, its regulation, and its limiting steps in production. Additionally, the development of robust microbial systems by genetic engineering, targeting the key enzyme, i.e. erythrose reductase could improve erythritol production. Application of the latest techniques such as the Cas9/CRISPR genome editing in erythritol producing strains would allow improving the highly efficient microbial systems. The use of renewable carbon sources as a feedstock for erythritol production and innovative approaches in the erythritol purification process will have a great impact on the economics of the process.

## Author contributions

Anil Khatape: Conceptualization, writing original draft preparation, visualization, and editing. Syed Dastager: Reviewing, visualisation; Vidhya Rangaswamy: Editing, reviewing, visualization, and supervision.

All authors have approved the final version of the manuscript.

## Ethical statement

Not applicable.

## Acknowledgements

The authors are thankful to the Directors of CSIR-NCL, Pune, Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India, and High Value Chemicals group, Reliance Industries Limited, Ghansoli, Navi Mumbai, India, for infrastructure and support.

***Conflict of interest statement.*** The authors declare that there is no conflict of interest.

## Funding information

Not applicable.

## References


Aoki MAY, Pastore GM, Park YK. Microbial transformation of sucrose and glucose to erythritol. *Biotechnol Lett* 1993;**15**:383–8.

Baudin A, Ozier-kalogeropoulos O, Denouel A *et al.* A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 1993;**21**:3329–30.

Bernt WO, Borzelleca JF, Flamm G *et al.* Erythritol: a review of biological and toxicological studies. *Regul Toxicol Pharmacol* 1996;**24**:S191–7.

Blazeck J, Liu L, Redden H *et al.* Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. *Appl Environ Microbiol* 2011;**77**:7905–14.

Brown AD. Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Adv Microb Physiol* 1978;**17**:181–242

Burschäpers J, Schustolla D, Schügerl K *et al.* Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa var pollinis*: part 2. Batch and fed-batch operation in bubble column and airlift tower loop if reactors. *Process Biochem* 2002;**38**:559–70.

Carly F, Gamboa-Melendez H, Vandermies M *et al.* Identification and characterization of EYK1, a key gene for erythritol catabolism in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 2017a;**101**:6587–96.

Carly F, Steels S, Telek S *et al.* Identification and characterization of EYD1, encoding an erythritol dehydrogenase in *Yarrowia lipolytica* and its application to bioconvert erythritol into erythrulose. *Bioresour Technol* 2018;**247**:963–9.

Carly F, Vandermies M, Telek S *et al.* Enhancing erythritol productivity in *Yarrowia lipolytica* using metabolic engineering. *Metab Eng* 2017b;**42**:19–24.

Cheng H, Wang S, Bilal M *et al.* Identification, characterization of two NADPH-dependent erythrose reductases in the yeast *Yarrowia lipolytica* and improvement of erythritol productivity using metabolic engineering. *Microb Cell Fact* 2018;**17**:1–12.

Daza-Serna L, Serna-Loaiza S, Masi A *et al.* From the culture broth to the erythritol crystals: an opportunity for circular economy. *Appl Microbiol Biotechnol* 2021;**105**:4467–86.

Deshpande MS, Kulkarni PP, Kumbhar PS *et al.* Erythritol production from sugar based feedstocks by *Moniliella pollinis* using lysate of recycled cells as nutrients source. *Process Biochem* 2022;**112**:45–52.

Ghezelbash GR, Nahvi I, Malekpour A. Erythritol production with minimum By-product using *Candida magnoliae* mutant. *Appl Biochem Microbiol* 2014;**50**:292–6.

Guo J, Li J, Chen Y *et al.* Improving erythritol production of *Aureobasidium pullulans* from xylose by mutagenesis and medium optimization. *Appl Biochem Biotechnol* 2016;**180**:717–27.

Hiele M, Ghoos Y, Rutgeerts P *et al.* Metabolism of erythritol in humans: comparison with glucose and lactitol. *Br J Nutr* 1993;**69**:169–76.

Hirata Y, Igarashi K, Ezaki S *et al.* High-level production of erythritol by strain 618A-01 isolated from pollen. *J Biosci Bioeng* 1999;**87**:630–5.

Horning EC, Horning MG, Szafranek J *et al.* Gas phase analytical methods for the study of human metabolites. *J Chromatogr A* 1974;**91**:367–78**.**

Ishizuka H, Wako K, Kasumi T *et al.* Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. *J Ferment Bioeng* 1989;**68**:310–4.

Jagtap SS, Bedekar AA, Singh V *et al.* Metabolic engineering of the oleaginous yeast *Y arrowia lipolytica* PO1f for production of erythritol from glycerol. *Biotechnol Biofuels* 2021;**14**:188–205.

Janek T, Dobrowolski A, Biegalska A *et al.* Characterization of erythrose reductase from *Yarrowia lipolytica* and its influence on erythritol synthesis. *Microb Cell Fact* 2017;**16**:118–201.

Jeya M, Lee KM, Tiwari MK *et al.* Isolation of a novel high erythritol-producing *Pseudozyma tsukubaensis* and scale-up of erythritol fermentation to industrial level. *Appl Microbiol Biotechnol* 2009;**83**:225–31.

Jovanović B, Mach RL, Mach-Aigner AR. Characterization of erythrose reductases from filamentous fungi. *AMB Express* 2013;**3**:1–11.

Jovanović B, Mach RL, Mach-Aigner AR. Erythritol production on wheat straw using *Trichoderma reesei*. *AMB Express* 2014;**4**:1–12.

Kayingo G, Kilian SG, Prior BA. Conservation and release of osmolytes by yeasts during hypo-osmotic stress. *Arch Microbiol* 2001;**177**:29–35.

Kim KA, Noh BS, Lee JK *et al.* Optimization of culture conditions for erythritol production by *Torula* sp. *J Microbiol Biotechnol* 2000;**10**:69–74.

Kobayashi Y, Iwata H, Mizushima D *et al.* Erythritol production by *Moniliella megachiliensis* using nonrefined glycerol waste as carbon source. *Lett Appl Microbiol* 2015;**60**:475–80.

Kobayashi Y, Yoshida J, Iwata H *et al.* Gene expression and function involved in polyol biosynthesis of *Trichosporonoides megachiliensis* under hyper-osmotic stress. *J Biosci Bioeng* 2013;**115**:645–50.

Koh ES, Lee TH, Lee DY *et al.* Scale-up of erythritol production by an osmophilic mutant of *Candida magnoliae*. *Biotechnol Lett* 2003;**25**:2103–5.

Krajangsod S, Chotikamas S, Tawai A *et al.* Measurement and thermodynamic modelling of erythritol solubility in aqueous solvents. *Orient J Chem* 2018;**34**:265–75.

Kresnowati MTAP, Regina D, Bella C *et al.* Combined ultrafiltration and electrodeionization techniques for microbial xylitol purification. *Food Bioprod Process* 2019;**114**:245–52.

Kroger M, Meister K, Kava R. Low-calorie sweeteners and other sugar substitutes: a review of the safety issues. *Compr Rev Food Sci Food Saf* 2006;**5**:35–47.

Lee DH, Lee YJ, Ryu YW *et al.* Molecular cloning and biochemical characterization of a novel erythrose reductase from *Candida magnoliae* JH110. *Microb Cell Fact* 2010;**9**:43–55**.**

Lee DY, Park YC, Kim HJ *et al.* Proteomic analysis of *Candida magnoliae* strains by two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* 2003a;**3**:2330–8.

Lee JK, Kim SY, Ryu YW *et al.* Purification and characterization of a novel erythrose reductase from *Candida magnoliae*. *Appl Environ Microbiol* 2003b;**69**:3710–8.

Lee JK, Koo BS, Kim SY. Erratum: fumarate-mediated inhibition of erythrose reductase, a key enzyme for erythritol production by *Torula corallina* (Applied and environmental microbiology (2002) 68:9 (4534-4538)). *Appl Environ Microbiol* 2002;**68**:5788–93.

Lenhart A, Chey WD. A systematic review of the effects of polyols on gastrointestinal health and irritable bowel syndrome. *Adv Nutr* 2017;**8**:587–96.

Li K, Luan Q, Jia H *et al.* High-yield preparation method of erythritol. 2020. *Patent CN110903165(A)*.

Li L, Yang T, Guo W *et al.* Construction of an efficient mutant strain of *Trichosporonoides oedocephalis* with HOG1 gene deletion for production of erythritol. *J Microbiol Biotechnol* 2016;**26**:700–9.

Lin SJ, Wen CY, Liau JC *et al.* Screening and production of erythritol by newly isolated osmophilic yeast-like fungi. *Process Biochem* 2001;**36**:1249–58.

Lin SJ, Wen CY, Wang PM *et al.* High-level production of erythritol by mutants of osmophilic *Moniliella* sp. *Process Biochem* 2010;**45**:973–9.

Livesey G. Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. *Nutr Res Rev* 2003;**16**:163–91.
Livesey G. Tolerance of low-digestible carbohydrates: a general view. *Br J Nutr* 2001;**85**:S7–16.
Madzak C, Tréton B, Blanchin-Roland S. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J Mol Microbiol Biotechnol* 2000;**2**:207–16.
Maeda T, Shida M, Ohshima Y *et al.* Process for preparing erythritol crystals.1997. *Patent EP0525659B1*.
Makri A, Fakas S, Aggelis G. Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. *Bioresour Technol* 2010;**101**:2351–8.
Mirończuk AM, Biegalska A, Dobrowolski A. Functional overexpression of genes involved in erythritol synthesis in the yeast *Yarrowia lipolytica*. *Biotechnol Biofuels* 2017;**10**:77–89.
Mirończuk AM, Biegalska A, Zugaj K *et al.* A role of a newly identified isomerase from *Yarrowia lipolytica* in erythritol catabolism. *Front Microbiol* 2018;**9**:1122–34**.**
Mirończuk AM, Dobrowolski A, Rakicka M *et al.* Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol. *Process Biochem* 2015a;**50**:61–8.
Mirończuk AM, Furgała J, Rakicka M *et al.* Enhanced production of erythritol by *Yarrowia lipolytica* on glycerol in repeated batch cultures. *J Ind Microbiol Biotechnol* 2014;**41**:57–64.
Mirończuk AM, Rakicka M, Biegalska A *et al.* A two-stage fermentation process of erythritol production by yeast *Y . lipolytica* from molasses and glycerol. *Bioresour Technol* 2015b;**198**:445–55.
Mirończuk AM, Rzechonek DA, Biegalska A *et al.* A novel strain of *Yarrowia lipolytica* as a platform for value-added product synthesis from glycerol. *Biotechnol Biofuels* 2016;**9**:180–93.
Morioka S, Abe T, Maeda T *et al.* Process for producing high-purity erythritol crystal. 2000:1–19. *Patent US6030820*
Munro IC, Bernt WO, Borzelleca JF *et al.* Erythritol: an interpretive summary of biochemical, metabolic, toxicological and clinical data. *Food Chem Toxicol* 1998;**36**:1139–74.
Nelson DL, Cox MM. *Lehninger Principles of Biochemistry 7th*. WH Free Co 2017;**2**.
Niang PM, Arguelles-Arias A, Steels S *et al.* In *Yarrowia lipolytica* erythritol catabolism ends with erythrose phosphate. *Cell Biol Int* 2020;**44**:651–60.
Nicaud JM, Fabre E, Gaillardin C. Expression of invertase activity in *Yarrowia lipolytica* and its use as a selective marker. *Curr Genet* 1989;**16**:253–60.
Niwa T, Tohyama K, Kato Y. Analysis of polyols in uremic serum by liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry. *J Chromatogr B Biomed Sci Appl* 1993;**613**:9–14.
Oh DK, Cho CH, Lee JK *et al.* Increased erythritol production in fed-batch cultures of *Torula* sp. by controlling glucose concentration. *J Ind Microbiol Biotechnol* 2001;**26**:248–52.
Oku T, Nakamura S. Threshold for transitory diarrhea induced by ingestion of xylitol and lactitol in young male and female adults. *J Nutr Sci Vitaminol (Tokyo)* 2007;**53**:13–20.
Ookura T, Azuma K, Isshiki K *et al.* Primary structure analysis and functional expression of erythrose reductases from erythritol-producing fungi (*Trichosporonoides megachiliensis* SNG-42). *Biosci Biotechnol Biochem* 2005;**69**:944–51.
Otey FH, Sloan JW, Wilham CA *et al.* Erythritol and ethylene glycol from dialdehyde starch. *Ind Eng Chem* 1961;**53**:267–8.
Park J, Seo B, Kim J *et al.* Production of erythritol in fed-batch cultures of *Trichosporon* sp. *J Ferment Bioeng* 1998;**86**:577–80.
Pepino MY. Metabolic effects of non-nutritive sweeteners. *Physiol Behav* 2015;**152**:450–5.
Qiu X, Xu P, Zhao X *et al.* Combining genetically-encoded biosensors with high throughput strain screening to maximize erythritol production in *Yarrowia lipolytica*. *Metab Eng* 2020;**60**:66–76.
Radeloff MA, Beck RHF. Polyols – more than sweeteners. *Sugar Ind* 2013;**138**:226–34.
Rakicka M, Biegalska A, Rymowicz W *et al.* Polyol production from waste materials by genetically modified *Yarrowia lipolytica*. *Bioresour Technol* 2017;**243**:393–9.
Rakicka M, Rukowicz B, Rywińska A *et al.* Technology of efficient continuous erythritol production from glycerol. *J Clean Prod* 2016;**139**:905–13.
Regnat K, Mach RL, Mach-Aigner AR. Erythritol as sweetener—wherefrom and whereto? *Appl Microbiol Biotechnol* 2018;**102**:587–95.
Roberts GP, McDiarmid A, Gleed P. The presence of erythritol in the fetal fluids of fallow deer (Dama dama). *Res Vet Sci* 1976;**20**:254–6.
Rosa CA, Jindamorakot S, Limtong S *et al.* Synonymy of the yeast genera *Moniliella* and *Trichosporonoides* and proposal of *Moniliella fonsecae* sp. nov. and five new species combinations. *Int J Syst Evol Microbiol* 2009;**59**:425–9.
Rymowicz W, Rywińska A, Marcinkiewicz M. High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*. *Biotechnol Lett* 2009;**31**:377–80.
Ryu YW, Park CY, Park JB *et al.* Optimization of erythritol production by *Candida magnoliae* in fed-batch culture. *J Ind Microbiol Biotechnol* 2000;**25**:100–3.
Rzechonek DA, Day AM, Quinn J *et al.* Influence of ylHog1 MAPK kinase on *Yarrowia lipolytica* stress response and erythritol production. *Sci Rep* 2018;**8**:14735–47
Rzechonek DA, Neuvéglise C, Devillers H *et al.* EUF1-A newly identified gene involved in erythritol utilization in *Yarrowia lipolytica*. *Sci Rep* 2017;**7**:12507–16.
Rzechonek DA, Szczepańczyk M, Wang G *et al.* Hog-independent osmoprotection by erythritol in yeast *Yarrowia lipolytica*. *Genes (Basel)* 2020;**11**:1–15.
Saran S, Mukherjee S, Dalal J *et al.* High production of erythritol from *Candida sorbosivorans* SSE-24 and its inhibitory effect on biofilm formation of *Streptococcus* mutans. *Bioresour Technol* 2015;**198**:31–8.
Sasman T, Alan W, Cameron C. Process for producing erythritol. 4. Pate. 2007. *Patent US20070037266A1*
Savergave LS, Gadre RV, Vaidya BK *et al.* Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochem Eng J* 2011;**55**:92–100.
Sawada K, Taki A, Yamakawa T *et al.* Key role for transketolase activity in erythritol production by *Trichosporonoides megachiliensis* SN-G42. *J Biosci Bioeng* 2009;**108**:385–90.
Scientific opinion on the safety of the proposed extension of use of erythritol (E 968) as a food additive. *EFSA J* 2015;**13**, DOI: 10.2903/j.efsa.2015.4033.
Shindou T, Sasaki Y, Miki H *et al.* Determination of erythritol in fermented foods by high performance liquid chromatography. *Food Hyg Saf Sci (Shokuhin Eiseigaku Zasshi)* 1988;**29**:419–22.
Sivaraman B, Badle SS, Rangaswamy V. Production of 1,3-Butadiene from erythritol. *ChemistrySelect* 2016;**1**:4630–2.
Smith DA, Morgan BA, Quinn J. Stress signalling to fungal stress-activated protein kinase pathways. *FEMS Microbiol Lett* 2010;**306**:1–8.

Szczepańczyk M, Rzechonek DA, Dobrowolski A *et al.* The overexpression of *YALI0B07117g* results in enhanced erythritol synthesis from glycerol by the yeast *Yarrowia lipolytica*. *Molecules* 2021;**26**:7549–61.

Tokuoka K, Sasaki T, Taniguchi H *et al.* Purification and some properties of an erythrose reductase from an *Aureobasidium* sp. Mutant. *Biosci Biotechnol Biochem* 1992;**56**:941–5.

Tomaszewska L, Rakicka M, Rymowicz W *et al.* A comparative study on glycerol metabolism to erythritol and citric acid in *Yarrowia lipolytica* yeast cells. *FEMS Yeast Res* 2014;**14**:966–76.

Tomaszewska L, Rywińska A, Rymowicz W. High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*. *Biomass Bioenergy* 2014;**64**:309–20.

Toshihiro M, Makoto S, Yoshikazu O *et al.* Process for preparing erythritol crystals. 1993. *Patent EP0525659B1*.

Trenner NR BF. Hydrogenation of tartaricacid esters to erythritol. 1951. *Patent US2571967A*.

Troostembergh, Ignace AD, Willy R. Process for producing and recovering erythritol from the culture medium containing the same. 2002. *Patent US6440712B12*.

Wang S, Wang H, Lv J *et al.* Highly efficient erythritol recovery from waste erythritol mother liquor by a yeast-mediated biorefinery process. *J Agric Food Chem* 2017;**65**:11020–8.

Wölnerhanssen BK, Cajacob L, Keller N *et al.* Gut hormone secretion, gastric emptying, and glycemic responses to erythritol and xylitol in lean and obese subjects. *Am J Physiol - Endocrinol Metab* 2016;**310**:E1053–61.

World Health Organization. Obesity and overweight fact sheet; 2016. Available from: https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight.

Xu W, Liang L, Song Z *et al.* Continuous ethanol production from sugarcane molasses using a newly designed combined bioreactor system by immobilized *Saccharomyces cerevisiae*. *Biotechnol Appl Biochem* 2014;**61**:289–96.

Yang LB, Zhan XB, Zheng ZY *et al.* A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by *Yarrowia lipolytica* from glycerol. *Bioresour Technol* 2014;**151**:120–7.

Yoshida H, Sugahara THJ. Studies on free sugars and free sugar alcohols of mushrooms. *Nippon Shokuhin Koyko Gakkaishi* 1984;**31**:765–71.

Yu JH, Lee DH, Oh YJ *et al.* Selective utilization of fructose to glucose by *Candida magnoliae*, an erythritol producer. *Appl Biochem Biotechnol* 2006;**131**:870–9.

Zhang L, Nie MY, Liu F *et al.* Multiple gene integration to promote erythritol production on glycerol in *Yarrowia lipolytica*. *Biotechnol Lett* 2021;**43**:1277–87

Zhao X, Zhang J, Liu L *et al.* Process method for producing erythritol from co-produced wheat starch in wheat gluten production. 2020. *Patent CN111454996A*.

International Microbiology
https://doi.org/10.1007/s10123-023-00411-8

RESEARCH

# Strain improvement for enhanced erythritol production by *Moniliella pollinis* Mutant-58 using jaggery as a cost-effective substrate

**Anil B. Khatape[1,2,3] · Vidhya Rangaswamy[3] · Syed G. Dastager[1,2]**

Received: 15 May 2023 / Revised: 23 June 2023 / Accepted: 21 July 2023


## Abstract

Erythritol has been produced by various microorganisms including *Yarrowia*, *Moniliella*, *Aureobasidium*, and *Candida* strains. Due to its relatively high price, erythritol sweetener is used lesser than other polyols despite having many advantages. Therefore, in this study, *Moniliella pollinis* strain was improved for erythritol production by chemical mutagenesis and subsequently screening for cost-effective carbon sources for the enhanced erythritol yield. *M. pollinis* was subjected to *N*-methyl *N*-nitro *N*-nitroso guanidine (NTG), ethyl methyl sulfonate (EMS), and UV mutagenesis for improved erythritol production. The fmutant strains were evaluated for enhanced erythritol production medium optimization by using different carbon substrates at the shake flask level. To enhance the production of erythritol and statistical media, optimization was carried out using a central composite design (CCD). Among 198 isolated mutants, Mutant-58 strain generated by EMS mutagenesis was selected for further assessment. The Mutant-58 strain showed significant morphological changes as compared to the parent strain. Furthermore, statistically optimized media composition resulted in the higher production of erythritol (91.2 ± 3.4 g/L) with a yield of 40.7 ± 3.4 % in shake flask experiments. The optimized medium composition for erythritol production constitutes (g/L) 225 jaggery, 4.4 yeast extract (YE), 4.4 $KH_2PO_4$, 0.31 $MgSO_4$, and pH 5.5. The present study demonstrated strain improvement, media, and process optimization resulting in a 30% increase in the erythritol production in the Mutant-58 as compared to the parent strain. This is also the first instance where jaggery has been used as a cost-effective carbon source alternative to glucose for industrial-scale erythritol production.

**Keywords** Erythritol · *Moniliella pollinis* · Mutation · Fermentation · Renewable resource · Optimization

## Introduction

Erythritol, a four-carbon polyol(chemical nomenclature is 1, 2, 3, 4-butanetetrol or meso-erythritol ($C_4H_{10}O_4$)), is extensively found in the environment (Krajangsod et al. 2018). Erythritol has been found in various fruits (pears, grapes, and melons), alcoholic drinks (wine and beer), and fermented foods (soy sauce, and miso bean paste) (Yoshida and Sugahara 1984; Shindou et al. 1988; Bernt et al. 1996). Erythritol is a symmetrical molecule, occurring as a white anhydrous crystalline powder having 70% of the relative sweetness of sucrose. Due to the low insulin index, erythritol has the potential as a sugar replacement for people with diabetes (Livesey 2003). Among various polyols in the market, erythritol shows numerous distinctive features. The calorific value of erythritol is near zero, and when consumed within the safe dosage limit, it does not lead to side effects compared to other polyols. Its chemical structure enables it to resist changes in insulin levels rendering it as a safe for diabetic patients (Rzechonek et al. 2017).

Despite having several advantages, the high price of erythritol results in scarce usage. A cost-effective production regime using cheap carbon and nitrogen substrates that supports the erythritol production in yeast is essential. The most commonly used carbon sources explored are glucose, sugarcane juice, molasses, glycerol, and xylose. Utilizing jaggery, molasses, and sugar cane juice as inexpensive carbon sources in fermentation media offers several advantages. These carbon sources provide a readily available and

✉ Syed G. Dastager
sg.dastager@ncl.res.in

1 NCIM-Resource Center, Biochemical Sciences Division, CSIR-National Chemical Laboratory, -411008, Pune, India

2 Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India

3 High Value Chemicals group, Reliance Industries Limited, Ghansoli, Navi Mumbai 400701, India

Published online: 31 July 2023

cost-effective alternative to conventional sugars such as glucose or sucrose. Additionally, their composition includes various nutrients and organic compounds that can support microbial growth and product formation during fermentation processes. Exploring the potential of jaggery, molasses, and sugar cane juice as carbon sources can contribute to the development of sustainable and economically viable fermentation strategies (Savergave et al. 2011; Mirończuk et al. 2015a, b; Kobayashi et al. 2015; Sivaraman et al. 2016; Deshpande et al. 2022). Sugarcane juice, in addition to sucrose, glucose, and fructose, is rich in various nutrients that support microbial growth. These nutrients include magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), iron (Fe), and phosphorus (P), present in concentrations ranging from 5 to 500 ppm levels. Furthermore, sugarcane juice contains essential vitamins, phenolic compounds, and enzymes, which contribute to its nutritional value and potential benefits in fermentation processes. Molasses, on the other hand, is a thick, viscous syrup with a dark color and distinctive taste. It is a byproduct obtained during the sugar-refining process from sugarcane or sugar beet juice. Molasses is well known for its high concentration of non-crystallizable sugars, such as sucrose, glucose, and fructose. Additionally, molasses contain organic acids, minerals, vitamins, and antioxidant compounds, which contribute to its composition and potential utilization in fermentation. The precise composition of molasses can vary depending on factors such as the method of sugar processing and the source of the raw material used. Jaggery, a product of the sugarcane industry, is another carbon source broadly utilized in the Indian context as a replacement for sucrose. Besides sucrose, it also contains protein, vitamins (A, B1, B2, B5, B6, C, D2, E), and minerals (iron, copper, magnesium, potassium, phosphorous, and sodium). Jaggery is available in solid (lumped), liquid, and granular forms (Nath et al. 2015). Over 70% of the world's jaggery is produced in India (Jagannadha et al. 2007) with sucrose concentrations varying from 75 to 85% (Vijayendra et al. 2001). The analysis of jaggery, prepared from sugar cane (100 g), revealed its nutritional composition. The carbohydrate content was found to range from 72 to 78 g, with sucrose being the predominant component and fructose and glucose present at 1.5 to 7 g. The mineral composition included calcium (40–100 mg), magnesium (70–90 mg), phosphorus (20–90 mg), sodium (19–30 mg), iron (10–13 mg), manganese (0.2–0.4 mg), zinc (0.2–0.4 mg), and chloride (5.3–0.0 mg), along with trace amounts of other minerals (0.1–0.9 mg). The vitamin content of jaggery comprised vitamin A (3.8 mg); vitamin B2 (0.06 mg); vitamins B1, B5, and B6 (0.01 mg each); vitamin C (7.00 mg); vitamin D2 (650 mg); and vitamin E (111.30 mg). Furthermore, jaggery contained a significant amount of protein, approximately 280 mg (Singh J 2013). Reports on the use of jaggery for erythritol production in commercial-scale fermentation processes are scarce. The present study aims at improving erythritol production by the yeast *Moniliella pollinis* mutant using jaggery as a carbon source. Mutagenesis studies were also carried out to decrease the byproduct formation besides screening cost-effective carbon sources and optimization of fermentation parameters using response surface methodology (RSM). Validation of the model at shake flask (SF) and fermentor scale was also carried out.

## Materials and methods

### Microorganisms and media

A wild-type yeast *M. pollinis* CBS 461.67 (Westerdijk Fungal Biodiversity Institute, Netherland) strain was used in this study. The culture was grown in yeast extract glucose peptone (YPG) liquid medium, containing the following components (g/L): yeast extract (YE), 10; peptone, 20; glucose 200 and incubated for 18 h at 28 °C and 180 rpm (MaxQ 8000 incubator shaker, Thermo Scientific). The culture was first streaked on YPG agar plates, and after 24 h, a single isolated colony was selected. After microscopic examination, the colony was inoculated into a 12.5-mL YPG medium in a 100-mL Erlenmeyer flask. The flasks were then incubated under the same conditions. Glycerol stocks were prepared using an 18-h old culture. For all fermentation trials, 300 µL of the glycerol stock was used to inoculate 12.5 mL YPG medium under the same conditions, and after 18 h, it was used as the inoculum.

### Materials

*N*-methyl *N*-nitro *N*-nitroso guanidine (NTG), ethyl methyl sulphonates (EMS), and sodium thiosulfate were purchased from Sigma-Aldrich, USA. All medium ingredients were procured from Difco Thermo Scientific, USA. All other chemicals used obtained were reagent grade.

### Mutagenesis and mutant selection

The procedure used for mutagenesis was adapted from various works of literature reported earlier (Ishizuka et al. 1989; Winston 2008; Lin et al. 2010; Ghezelbash, Nahvi, and Malekpour. 2014b). The wild-type strain *M. pollinis* was inoculated into a sterile 250-mL flask having 50-mL YPG medium and incubated at 28 °C, 180 rpm for 18 h. Post incubation, cell count was checked using Neubauer improved cell counting chamber (Marienfeld, Germany) under the light microscope (Thesseling et al. 2019). The well-grown culture broth was centrifuged at 10,000 rpm for 4 min. The cells were washed thrice with 10 mL of 0.1 M phosphate buffer (pH 6.5) and again mixed in the

same buffer (30 mL) to adjust the final cell count to about $300 \times 10^6$ cells/mL. For UV irradiation, 2.0 mL of cell suspension was used in sterile petri plates and exposed to UV light (254 nm) under a laminar flow hood at 10-cm and 20-cm distances for various time periods up to 60 min (1, 5, 10, 15, 30, and 60 min).

For chemical mutagenesis, EMS treatment was given to cell suspension at varying concentrations (25, 50, 75, 100, and 150 µL/mL) for 60 min. Post-exposure, the reaction was stopped by adding 1-mL sterile 10% $Na_2O_2S_3$ (sodium thiosulfate). NTG treatment was given to the cell suspension at varying concentrations (50, 100, 150, 200, and 250 µg/mL) for 60 min. After completion of exposure time, the reaction was stopped by adding 1-mL sterile 10% $Na_2O_2S_3$ (Sodium thiosulphate) and incubated at 28 °C for 3 h in the dark. All treated samples (UV, EMS, and NTG) were then centrifuged at 10,000 rpm for 4 min. The cells were washed twice with 2.0 mL of 0.1 M phosphate buffer (pH 6.5) and again mixed in the same buffer (2.0 mL). For each treatment, the cell suspension was diluted appropriately and spread on plates containing 1% YE, 2% bacteriological peptone, 20% glucose, and 2% agar. All plates were incubated at 28 °C for 3 days. Plates showing a 90–95 % kill rate were chosen for mutant selection. The colonies were picked and inoculated in 50-mL glass tubes with 5-mL liquid fermentation medium (LFM) (Savergave *et al*. 2011). The LFM contained the following components (g/L): YE, 10; glucose, 200; $K_2HPO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.25. The tubes were incubated in the shaker at 180 rpm, 28 °C for 3 days, and monitored for erythritol production by high-performance liquid chromatography (HPLC) along with quantification of glucose, fructose, sucrose, glycerol, and ethanol using an Aminex HPX-87H, 300 mm × 7.8 mm column (Bio-Rad, USA). The mobile phase used for elution was 5 mM $H_2SO_4$ at 0.6 mL/min flow rate at 35 °C. Samples were injected using an autosampler injector with a 20-µL fixed volume. A refractive index detector (Agilent HPLC 1260 series) was used for the detection of sugars and other metabolites. The mutant strains selected for further evaluation were grown in YPG medium containing 20% glucose. Among all the mutant strains, mutant no. 58 (Mutant-58) showed the highest erythritol production and hence was selected for further analysis.

## Morphology

Morphological observations of wild-type strain *M. pollinis* and Mutant-58 were made by observing colony size, shape, appearance, and cell shape under the light microscope (Lin et al. 2010). Cell morphology, cell biomass, and fermented broth color were observed after 72-h incubation of the wild-type and mutant strains in LFM medium.

## Screening of medium components for erythritol production by *M. pollinis* CBS 461.67 Mutant-58

For inoculum preparation, wild-type yeast *M. pollinis* and Mutant-58 were first cultivated in YPG liquid medium, as described earlier. After 18 h of incubation, 2.5 mL inoculum (10% v/v) was transferred into a 250-mL flask containing 22.5 mL LFM medium. The influence of varying YE concentrations (1 to 10 g/L), pH values (3.5, 4.5, 5.5, 6.0, and 6.5), and temperature (25 °C, 28 °C, 30 °C, and 37 °C) on erythritol production by *M. pollinis* Mutant-58 was evaluated in shake flasks. For screening of cost-effective carbon substrate for erythritol production, glucose in the LFM medium was replaced with other inexpensive carbon substrates viz jaggery, molasses, sugarcane juice, and glycerol. Sugarcane juice was obtained from a local market and evaporated in a rotary vacuum evaporator. The concentrated syrup was utilized in the experiment as per requirement. Molasses were obtained from a sugar cane factory near Pune. The carbon sources were used in the range of 200 g/L. The detailed composition of sugar-based substrates has been mentioned in Table 1.

Eighteen-hour old seed culture was inoculated in 250-mL Erlenmeyer flasks containing 22.5 mL of LFM medium with varying carbon sources. The concentration of the carbon sources used was kept equivalent to carbon content present in 200 g/L glucose, which was calculated to be 6.67 M. Thus, the final concentrations of the different carbon sources used were as follows: (a) 200 g/L glucose, (b) 202 g/L jaggery (containing 132 g/L sucrose, 36 g/L glucose and 25 g/L fructose), (c) 480 g/L molasses (containing 128 g/L sucrose, 35 g/L glucose and 31 g/L fructose), (d) 205 g/L glycerol, and (e) 202 g/L concentrated sugarcane juice (containing 128 g/L sucrose, 30 g/L glucose, and 26 g/L fructose).

The inoculated flasks were kept for incubation at 28 °C, 180 rpm in the shaking incubator. Samples were examined

**Table 1** Sugar composition of carbon substrates

| Carbon | Sucrose (%) | Glucose (%) | Fructose (%) | Total sugar (%) |
|---|---|---|---|---|
| Glucose | Nil | 99.8 ± 0.8 | Nil | 99.8 ± 0.8 |
| Jaggery | 78.5 ± 3.8 | 11.2 ± 3.1 | 5.8 ± 2.1 | 95.5 ± 9 |
| Molasses | 42.3 ± 4.2 | 2.9 ± 1.2 | 1.6 ± 1 | 46.9 ± 6.4 |
| Sugarcane Juice | 10.2 ± 1.2 | 2.27 ± 0.8 | 0.55 ± 0.5 | 13.02 ± 2.5 |

at 72 h for optical (OD) density, pH, residual glucose, and polyol concentrations. Each test was performed in triplicates.

## Measurement of residual sugar and intracellular protein concentration of parent and Mutant-58

*Moniliella pollinis* wild type and Mutant-58 were grown in LFM for 72 h, and the cell pellet was collected by centrifugation. The pellet was washed twice with 200 mM phosphate buffer (pH 6) containing 20% glycerol. Glycerol was used to stabilize the protein content in the crude cell lysate (Vagenende et al. 2009). After washing, the pellet was suspended in 300 µL crushing buffer (10 mL, 200 mM phosphate buffer with 20 % glycerol, pH 6.0, 1 mM dithiothreitol, and 1 mM PMSF) and crushed by mixing with 0.5-mm glass beads (Sigma) in a 2 mL microfuge tube on a vortex mixture for 3 cycles of 5 min with cooling on ice for 5 min between the cycles. After completing the crushing process, 700 µL crushing buffer was added to the microfuge tube, of which 0.1 mL sample was used for cell counting, and the remaining crushing mixture was centrifuged at 10,000×g for 5 min, at 4 °C. The aliquot removed for cell counting was diluted to 1 mL with sterile distilled water. To perform a viability count, one drop of 0.1% (w/v) methylene blue solution was added to the 1-mL sample. The sample was mixed well, allowed to react for 1 min, and processed for cell counting using a counting Neubauer's chamber. The method reported earlier in the literature (Shukla 2015) was used for the protein analysis of the samples. Initial and 72-h fermented broth samples of both parent and mutant cultures were analyzed for metabolite and glucose analysis by HPLC (discussed earlier) to check the residual sugar. The estimation of residual sugar from metabolite analysis is explained in the supplementary information. Protein concentration was checked using a standard curve prepared using BSA (Supplementary Fig. S2). DCW of both strains was calculated using a standard graph between DCW and OD600nm (Supplementary Fig. S3), where 1.0 $OD_{600nm}$ was found to be equivalent to 0.58 and 0.44 g/L DCW for parent and mutant strains respectively. Samples at end of the fermentation (72 h) were checked for metabolite as well as protein concentration determination.

## Optimization of medium: design of experiment and statistical analysis

The optimization of the medium by RSM, 5 factors viz. jaggery, YE, $KH_2PO_4$, $MgSO_4$, and pH was chosen. These parameters are critical in the erythritol production process and can significantly impact the rate and final yield of erythritol production. Central composite design (CCD) was used to study the effect of optimized parameters. A two-level factorial: half fraction factorial design leading to a total of 32 experiments was performed in triplicate (cube points 16 plus 6 center points in cube plus 10 axial). In all flasks, 10% (v/v) inoculum was added and incubated for 3 days, and HPLC was used to analyze the concentration of sugar alcohols as explained previously. As specified in supplementary Table S2, higher and lower values for every single variable were used in the design. The results of erythritol productivity were used to form the RSM model. To obtain the optimized concentrations of the medium compositions and experimental parameters, generate response surface graphs, and perform statistical analysis of the data, Minitab 19 software was used (Version 19.1). Process parameter optimization of the correctness of the model was studied by analysis of variance (ANOVA) and the coefficient of $R^2$ (Abdelgalil et al. 2018). The value of $R^2$ shows whether the model would be a good fit for the given data set. The statistical significance of the fit of the polynomial model equation was examined by the $F$ test. Optimized medium constituents and process parameters (attained by CCD trials) were validated at the bioreactor scale for erythritol production.

## Validation of erythritol production at shake flask and fermentor level

Fermentor batches were conducted in 3.0 L Bioflo (Eppendorf) 115 fermentor with a 1.3-L working volume. The initial sugar concentration maintained was 225 g/L using jaggery as a carbon source. The YE was used as a nitrogen source at a concentration of 4.36 g/L, $KH_2PO_4$ 4.34 g/L, $MgSO_4 \cdot 7H_2O$: 0.45 g/L, and the pH of the fermentation medium was adjusted and maintained at 5.4 throughout fermentation using 5 N NaOH. The jaggery and LFM medium were sterilized at 121 °C for 20 min before inoculation separately. The agitation and aeration were kept at 200 rpm and 1 vvm respectively. The dissolved oxygen was not maintained in the medium during fermentation. The total time for batch fermentation was 120 h, and samples were taken for metabolite analysis every 24 h. Three consecutive fermentation batches at fixed rpm (180) were completed using RSM model-optimized medium, in a 2.0-L Erlenmeyer flask with 0.2 L working volume. The agitation was maintained at 180 rpm. Validation in shake flask was done in triplicates with fermentation time 120 h. Sampling for HPLC analysis was done every 24 h.

## Analytical methods

OD was measured using a spectrophotometer at 600 nm. The HPLC method described earlier was used to quantify the concentration of the metabolite produced during

fermentation. To obtain erythritol yield (YERY %), the following formula was used:

$$YERY\ yield\ (\%) = \frac{Erythritol\ produced\ (g/L)}{Carbon\ source\ consumed\ g/L} \times (100)$$

Respective standard curve *Y* values were used for all the metabolite's quantification

## Results

### Mutagenesis

Survival curves of *M. pollinis* after the mutagen treatments are presented in Fig. S1. The graph in Fig S1(i) illustrates that a 50–60% kill rate was achieved by exposing the cells to UV for 60 min. For obtaining a 90–95% kill rate in EMS mutagenesis, the time required was between 30 and 40 min 75 µL/mL concentration (Fig. S1(ii)). EMS mutagenesis at 75 µL/mL concentration and above showed around a 100% kill rate. While 51 and 69% kill rates were observed at 25 and 50 µL/mL EMS concentration, respectively. In comparison with wild-type colonies, mutants were smaller in shape (Supplementary Fig. S4a). In the case of NTG treatment, the highest kill rate (~ 40 %,) was observed by treating the cells for 60 min at 100 µL/mL (Fig. S1(iii)). Overall, the highest kill rate and changes in the morphology of cells were observed in cells treated with EMS at 50 µL/mL concentrations and above in 60 min (Supplementary Fig. S4a). For subsequent mutagenesis treatment, 50 and 75 µL/mL EMS concentrations were chosen, and more mutants were generated.

### Screening of high erythritol-producing mutants

A total of 198 mutants chosen from all mutagenesis treatments were evaluated for erythritol production. Mutants generated from EMS mutagenesis exhibited a significant increase in erythritol production. Significant changes in erythritol and other metabolite profile were observed in the comparison of the parent and some of the screened mutants as shown in Fig. 1. The Mutant-58 showed the highest production of erythritol (88.9 ± 1.4 g/L) as compared to the parent strain (67.6 ± 2.4 g/L) and other mutant strains with erythritol productivity of 1.23 g/L/h. There was no significant difference in glycerol and ethanol yield in parent and mutant cultures except Mutant-1 and 58, where a slight increase in glycerol production was observed (5.0 ± 0.5 g/L). However, a significant increase in erythritol production was seen in Mutant-58 than in Mutant-1. Therefore, the Mutant-58 strain was selected for further evaluation for erythritol production. The stability of the mutant was confirmed by subculturing the strain for 20 generations, and at each generation, metabolite production and cell morphology were monitored and were found to be similar.

### Morphology

Apart from higher erythritol production ability, the Mutant-58 strain has shown unique morphological traits,

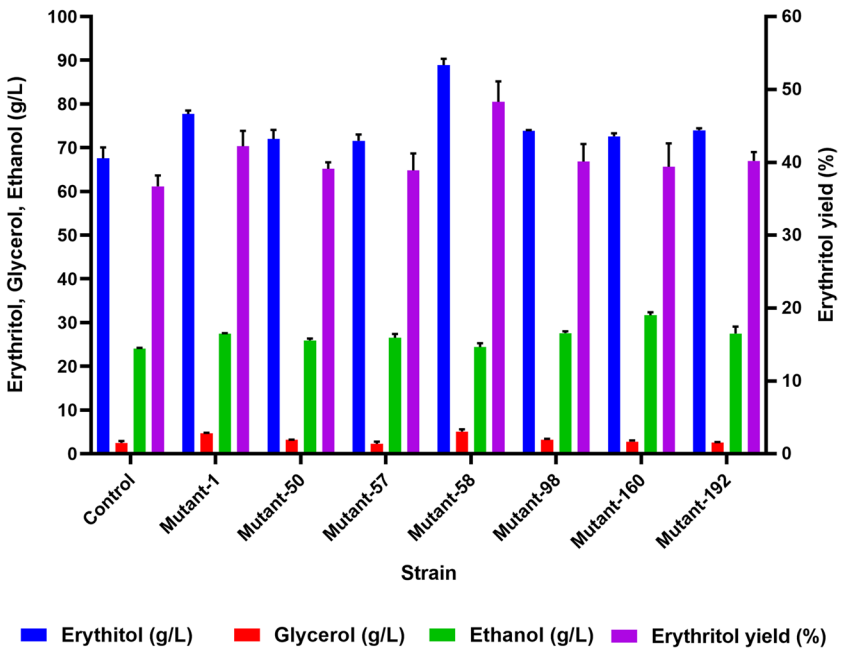



**Fig. 1** Evaluation of selected mutants along with parent strain for erythritol and by-product formation in the shake-flask experiment, batch culture. Samples were replicated in triplicate, and all values are expressed as mean ± SD

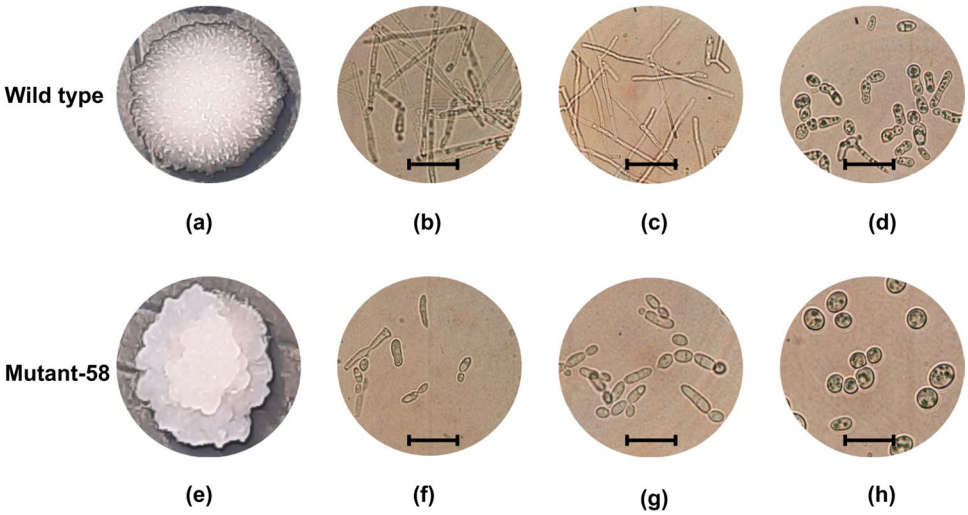



**Fig. 2** Morphological difference between parent and mutant strain. Parent strain: **a** Single colony on YPG plate, **b** Microscopy of a single colony from a YPG plate, **c** 18-h grown inoculum in YPG, **d** 72-h old culture in LFM. Mutant-58 strain: **e** single colony on YPG plate, **f** microscopy of a single colony from YPG plate, **g** 18-h grown inoculum in YPG, **h** 72-h-old culture in LFM. Bar denotes 50 µm

which differed from the parent strain. Mutant-58 colonies appeared to be rough-surfaced compared to the smoother colonies of the parent strain (Fig. 2a, e). Wild-type cells were observed as elongated rods from the colony to the inoculum stage and later formed an oval shape at 72 h when grown in LFM medium (Fig. 2b, c, d). In contrast, Mutant-58 cells' size and structure appeared to have an oval shape right from the colony and inoculum to the big circular-shaped cells in the production medium (Fig. 2f, g, h). Thus, the morphological differences concerning size and shape in mutant cells might be suggestive of their resistance to osmotic stress environments. Furthermore, the color of the mutant biomass started to turn pale yellow at the end of the fermentation (72 h) (Supplementary Fig. S5).

## Optimization of medium components for erythritol production

### Nitrogen source, pH, and temperature

YE is the only major nitrogen component in the LFM medium. Optimization studies revealed that maximum erythritol production was observed in the mutant strain (94.3 ± 1.9 g/L) at 4 g/L YE concentration instead of 10 g/L YE present in LFM medium. Increase in YE concentration increases ethanol production in both the parent (30.6 ± 0.4 g/L) and the mutant culture (21.4 ± 0.5 g/L) (Fig. 3(a, b)). Studies on the effect of temperature on erythritol production revealed that both strains had a similar erythritol production trend, with the highest erythritol yield (45.8 ± 1.9 %) obtained in Mutant-58 than in the parent strain (34.6 ± 1.1%) at 28 °C (Fig. 3(c, d)). At 37 °C, a significant decline in erythritol yield (24.1 ± 2.1 %) was observed along with growth ($OD_{600nm}$ 13.2 ± 0.7). Furthermore, in the parent strain, a reduction in the erythritol yield from 34.6 ± 1.1 to 18 ± 3.3% was noted when the temperature was raised from 28 to 37 °C. Studies on the effect of pH on erythritol production revealed that (Fig. 3(e, f)).

Parent strain formed equivalent amounts of erythritol in the pH range of 5.5–6.5. Both the maximal growth (OD 660 nm) and erythritol yield (34.3 ± 2.6 %) were achieved at pH 6.0. Notably, the erythritol yield tends to decrease as the pH value fluctuates from pH 6.0. Similarly in Mutant-58, erythritol yield (45.8 ± 2.3 %) increased at pH 6.0. There was a reduction in ethanol concentration (18.9 ± 1.1 g/L) in the Mutant-58 strain by ~ 33% as compared to the parent strain (27.0 ± 1.4 g/L).

### Screening of cost-effective carbon source

In this study, cost-effective high sugar-based substrates were evaluated for erythritol production along with glycerol Fig. 4(a). The results indicate that glucose was the preferred carbon source for cell growth and erythritol metabolism. In the preliminary experiment, it was noted that the glycerol produced at the fermentation's start was slightly decreased. However, glycerol was poorly utilized by *M. pollinis* Mutant-58 as 184.9 ± 0.4 g/L residual glycerol was detected in the broth after 72 h. The highest erythritol was produced when jaggery (63.6 ± 0.6 g/L) was used as a carbon source as compared to molasses, glycerol, and sugar cane juice. Cell biomass in glucose and the jaggery-based medium was observed to be 19.8 ± 0.02 and 22.8 ± 0.05 respectively. Ethanol production in jaggery (25.3 ± 0.70 g/L) is comparable with glucose (27.2 ± 2.70 g/L), whereas, in molasses, ethanol production was higher compared to the other carbon substrates (52.7 ± 1.1 g/L).

An additional experiment was performed for erythritol production using Mutant-58 and parent strain for confirmation and comparisons of their productivity in a medium comprising jaggery and glucose. The repeat experiment confirmed that the mutant strain displays a significant surge

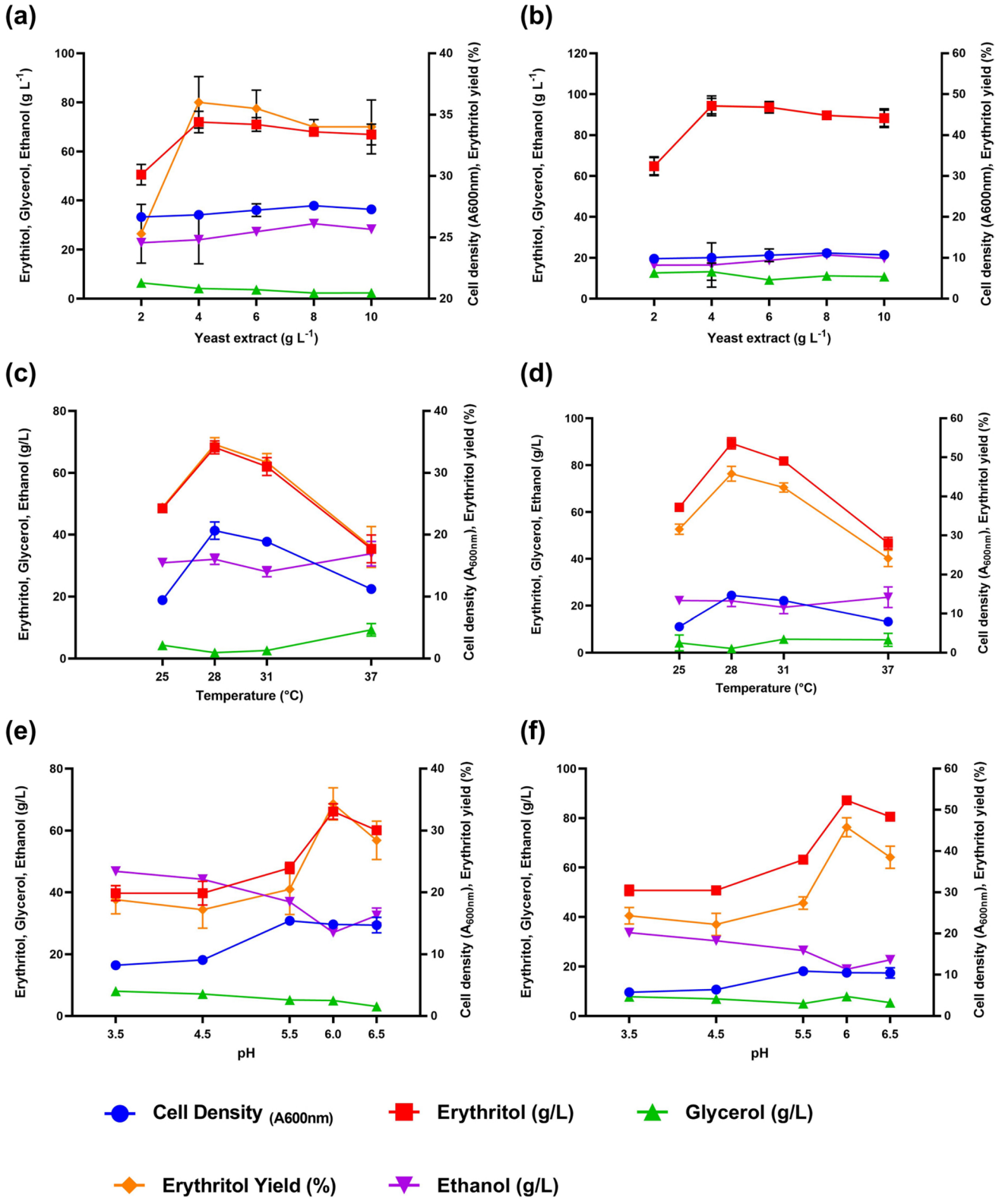



**Fig. 3** Effect of YE concentrations on erythritol yield. (**a**) Parent and (**b**) Mutant-58 strain. Effect of temperature on erythritol yield, (**c**) parent and (**d**) Mutant-58 strain. Effect of pH on erythritol yield, (**e**) parent and (**f**) Mutant-58 strain. Strains were cultivated in 20% glucose, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

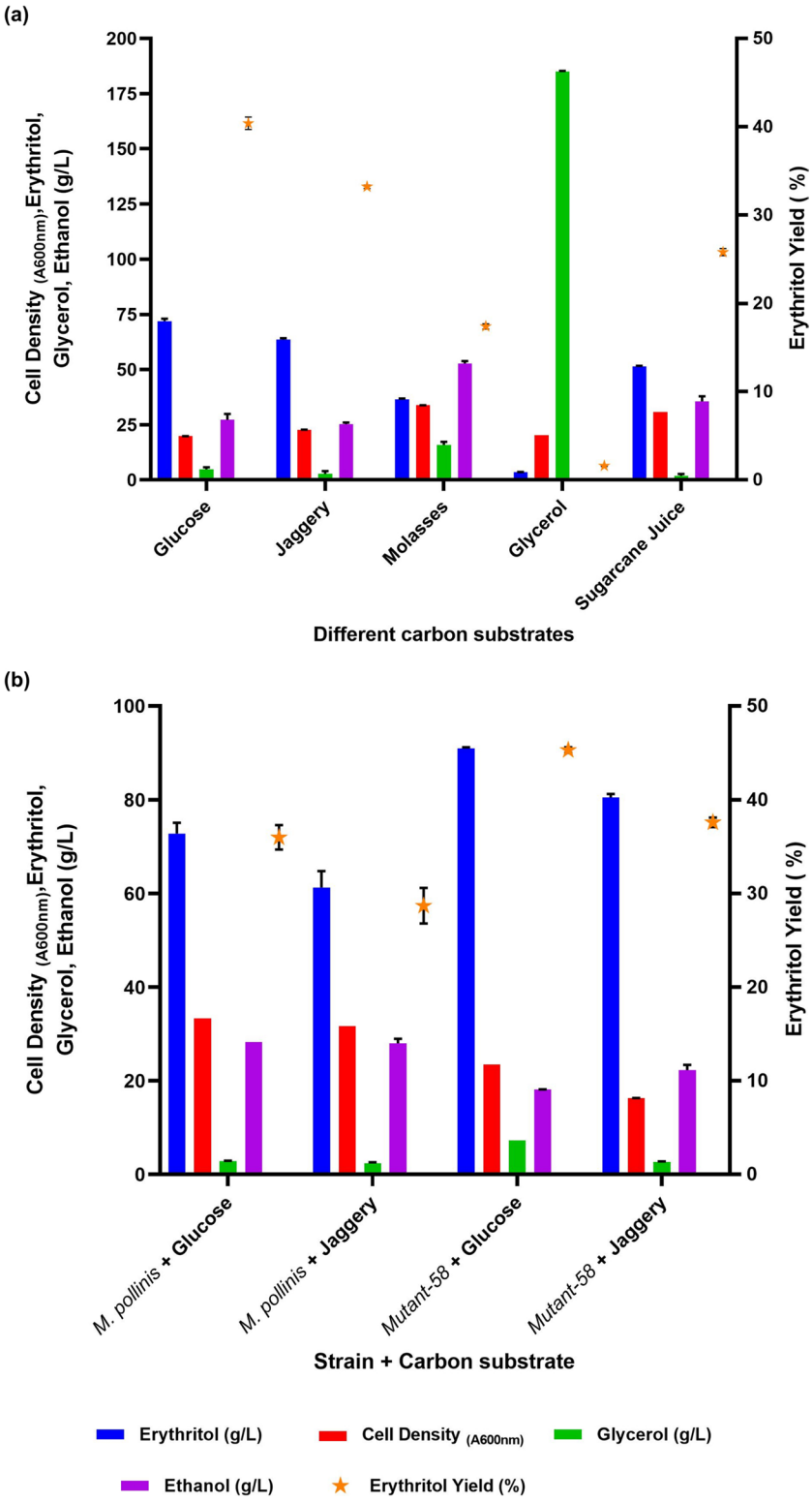



**Fig. 4** (**a**) Evaluation of cost-effective carbon source using Mutant-58. The strain was cultured at 1% YE, pH-6.5, 28 °C, and 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments. (**b**) Erythritol production from parent and Mutant-58 strain using glucose and jaggery as carbon substrate. Strains were cultured at 1% YE, pH-6.5, 28 °C, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

in erythritol yield (45.3 ± 0.3 %) and a decrease in ethanol production (18.1 ± 0.07) than the wild-type strain when cultured in a medium having glucose as a carbon source (Fig. 4(b)). In jaggery, erythritol yield was lowered and 37.6 ± 0.5% erythritol yield achieved with Mutant-58 strain was more than the erythritol yield (36 ± 1.3%) attained by wild-type strain in glucose. The Mutant-58 produced 80.5 ± 0.8 g/L erythritol as compared to the parent strain (61.3 ± 3.5 g/L) in a jaggery-based medium, which was 31% higher than the parent strain. In this study, 37% erythritol yield

was attained using jaggery as a cost-effective carbon source with the mutant strain as compared to 30.5% with the parent strain. This is the first report on the production of erythritol using jaggery as a carbon substrate. Therefore, the outcomes of this work offer a basis for the application of jaggery as an alternative to glucose for the synthesis of erythritol on an industrial scale.

### Measurement of residual carbon and protein concentration of parent and Mutant-58

To evaluate if there is any correlation between the rate of erythritol production and the protein content, the protein concentration in the parent and mutant strains was analyzed at the end of fermentation. The protein analysis could support the improved erythritol production in the mutant strain in comparison with the parent strain.

As depicted in Table 2, protein concentration almost doubled (140.7 ± 0.18 mg/g DCW) in the case of Mutant-58 with a concurrent 34% rise in erythritol production as compared to the parent strain. The metabolite profile analysis showed that 63.3% of total carbon output was obtained in the case of parent culture whereas 68.6% of total carbon output was in Mutant-58. Furthermore, supplementary Table S2 clearly shows that in Mutant-58, more carbon flow goes towards erythritol production and less towards cell mass (CC-258 × 106) and ethanol production, whereas reverse observations were noted in the parent strain. Interestingly, a 2.7-fold increase in glycerol production was seen in the mutant strain than in the wild-type strain. The results of this study have revealed substantial disparities between the parent and mutant strains, particularly in terms of morphology and total dry cell weight. Notably, although the mutant strain exhibited a lower total cell mass compared to the parent strain, it displayed a higher protein content, which directly influenced metabolite production. This augmented protein concentration observed in the mutant strain could potentially be attributed to the upregulation of specific genes responsible for enhancing erythritol production. The higher expression levels of these genes in the mutant strain likely contributed to the increased protein content, subsequently influencing the synthesis of metabolites. These findings highlight the intricate relationship between genetic modifications, protein synthesis, and metabolic pathways in the context of erythritol production. Elucidating the underlying mechanisms responsible for the observed phenotypic changes in the mutant strain can provide valuable insights into optimizing erythritol production through genetic manipulation. Furthermore, understanding the genetic factors involved with increased protein content and enhanced erythritol production can potentially facilitate the development of targeted strategies for improving erythritol yields in industrial applications.

## Optimization of medium: design of experiment and statistical analysis

The medium and process optimization experiments were used to achieve optimum parameters such as nitrogen source, pH, temperature, $KH_2PO_4$, $MgSO_4$, and carbon source. In this study, the optimized concentrations of a factor with minute alterations were further optimized by CCD. Overall, 32 different schemes were designed with several combinations of jaggery (A), YE (B), $KH_2PO_4$ (C), $MgSO_4$ (D), and pH (E) in a set of factorials, axial, and center points. The CCD experimental runs and the observed response are shown in supplementary Table S3. The result of every response was calculated using the following regression equation:

$$\begin{aligned} Y = {} & -153.3 + 0.386\,(A) + 6.55\,(B) + 46.2\,(E) + 1.17\,(C) \\ & + 42.8\,(D) - 0.000642\,(A) * (A) - 1.227\,(B) * (B) \\ & - 4.17\,(E) * (E) - 0.5628\,(C) * (C) - 66.3\,(D) * (D) \\ & + 0.00622\,(A) * (B) - 0.0264\,(A) * (E) \\ & + 0.00107\,(A) * (B) + 0.0429\,(A) * (D) + 0.806\,(B) * (E) \\ & - 0.216\,(B) * (C) - 1.85\,(B) * (D) + 0.585\,(E) * (C) \\ & - 3.9\,(E) * (D) + 4.18\,(C) * (D) \end{aligned}$$

where $Y$ is the response variable (Erythritol g/L), $A$ = Jaggery, $B$ = YE, $C = KH_2PO_4$, $D = MgSO_4 \cdot 7H_2O$ and $E$ = pH.

The optimal concentrations of each parameter and the outcome of their interactivity on response were examined by the 2D contour plot counter and 3D scattered plot. Initial experiments, one factor at a time, showed that 200 g/L glucose, 4 g/L YE, 28 °C temperature, and pH 6.0, were the best conditions for erythritol production. The carbon and nitrogen sources and pH are the most important variables in erythritol production were indicated in further analysis. These factors were selected refereeing to their $P$ values

**Table 2** Measurement of protein concentration of parent and mutant strain

| Organism | DCW (g/L) | Cell count ($10^6$) | Yield (%) | Protein mg/g DCW |
|---|---|---|---|---|
| Parent strain | 41.6 ± 0.01 | 625 | 34.4 ± 0.2 | 67.4 ± 0.16 |
| Mutant-58 | 15.7 ± 0.03 | 258 | 46.1 ± 1.1 | 140.7 ± 0.18 |

Strains were cultivated in 20% glucose, 1% YE at 28°C, pH 6.5, 180 RPM for 3 days. Results are means ± S.D. for triplicate experiments

**Table 3** Analysis of variance for anticipation of erythritol (%) yields influenced by variables in screening design of experiment

| Source | DF | Adj SS | Adj MS | *F*-value | *p* value |
|---|---|---|---|---|---|
| Model | 20 | 2439.16 | 121.96 | 11.90 | 0.001 |
| Linear | 5 | 308.52 | 61.70 | 6.02 | 0.006 |
| A. Jaggery (g $L^{-1}$) | 1 | 64.35 | 64.35 | 6.28 | 0.029 |
| B. YE (g $L^{-1}$) | 1 | 112.49 | 112.49 | 10.98 | 0.007 |
| C. KH2PO4 (g $L^{-1}$) | 1 | 2.87 | 2.87 | 0.28 | 0.607 |
| D. MgSO4.7H2O (g $L^{-1}$) | 1 | 13.95 | 13.95 | 1.36 | 0.268 |
| E. pH | 1 | 114.84 | 114.84 | 11.21 | 0.007 |
| Square | 5 | 1986.91 | 397.38 | 38.78 | 0.000 |
| A*A | 1 | 1208.07 | 1208.07 | 117.90 | 0.000 |
| B*B | 1 | 706.81 | 706.81 | 68.98 | 0.000 |
| C*C | 1 | 362.94 | 362.94 | 35.42 | 0.000 |
| D*D | 1 | 65.34 | 65.34 | 6.38 | 0.028 |
| E*E | 1 | 31.88 | 31.88 | 3.11 | 0.105 |
| 2-Way Interaction | 10 | 143.73 | 14.37 | 1.40 | 0.293 |
| A*B | 1 | 24.75 | 24.75 | 2.42 | 0.048 |
| A*E | 1 | 27.83 | 27.83 | 2.72 | 0.128 |
| A*C | 1 | 1.16 | 1.16 | 0.11 | 0.743 |
| A*D | 1 | 6.63 | 6.63 | 0.65 | 0.438 |
| B*E | 1 | 10.40 | 10.40 | 1.02 | 0.335 |
| B*C | 1 | 18.71 | 18.71 | 1.83 | 0.204 |
| B*D | 1 | 4.95 | 4.95 | 0.48 | 0.501 |
| E*C | 1 | 8.56 | 8.56 | 0.84 | 0.380 |
| E*D | 1 | 1.38 | 1.38 | 0.13 | 0.721 |
| C*D | 1 | 39.38 | 39.38 | 3.84 | 0.076 |
| Error | 11 | 112.71 | 10.25 | | |
| Lack-of-fit | 6 | 107.42 | 17.90 | 16.93 | 0.004 |
| Pure error | 5 | 5.29 | 1.06 | | |
| Total | 31 | 2551.87 | | | |

*DF* degree of freedom, *SS* sum of squares, *MS* mean square, $R^2$ (coefficient of determination in regression model) 95.58%

(0.029, 0.007, and 0.007) respectively (Table 3), and jaggery with a higher concentration (225 g/L) has the main effect on erythritol production (Supplementary Fig. S6).

The *P* value for $KH_2PO_4$ and $MgSO_4$ suggests that these are not very significant factors for erythritol production. The RSM model was used to inspect three components—one response for three medium constituents (jaggery, YE, and pH) while other variables were kept at their central points. Figure 5a displays the effect of different concentrations of jaggery and YE on erythritol production. Based on the plot, the optimum concentrations lie near the mid-range of the jaggery and YE. A rectilinear rise in erythritol synthesis was noted with increasing jaggery and YE concentrations up to 215 and 4.6 g/L, respectively, and then dropped. Figure 5b signifies erythritol productivity as a result of the interaction between jaggery concentration and pH.

The highest erythritol yield (38.22 %) was observed at pH 5.36 and 225 g/L jaggery concentration. Figure 5c exemplifies erythritol productivity as a function of pH and YE. As per the plot, the optimum value lies near the mid-range of the YE (4.26) and initial values of pH. Similarly, the interaction between pH and jaggery is 5.47. The interaction of jaggery and YE showed that it is important to maximize erythritol production, while factor *E* (pH) revealed also plays a vital part in erythritol production (Fig. 5d).

ANOVA indicated that the factors *A* (Jaggery concentration), *B* (YE), and *C* (pH) were the most significant factors for erythritol production, whereas the no significant factors in model terms were *AB*, *AC*, and *BC*. The *P* value helps as a tool for analyzing the significance of every factor. The responses taken from Table 3 show the linear coefficient of *A*, *B*, and *C* ($P > 0.05$). The optimized medium for producing high erythritol yields comprises 226.26 g/L jaggery, 4.36 g/L YE, 5.42 pH, 4.34 g/L $KH_2PO_4$, and 0.31 g/L $MgSO_4$ (Supplementary Fig. S7).

## Validation of the model

The experimental model was validated with the selected optimum parameters of the CCD in 250-mL, 2000-mL Erlenmeyer flask, and 3.0 L fermentor containing 25 mL, 200 mL, and 1.3 L of medium respectively. The optimal medium composition is as follows: 225 g/L jaggery, 4.4 g/L YE, 5.5 pH, 4.4 g/L $KH_2PO_4$, and 0.31 g/L $MgSO_4$.

The results obtained (Fig. 6(a)) showed that erythritol production by *M. pollinis* Mutant-58 using the statistically optimized medium composition resulted in the highest production of erythritol (91.2 ± 3.4 g/L) with a yield of 40.7 ± 3.4 % in 25-mL shake flask.

However, similar results of high erythritol production could not be replicated in 200 mL and 1.3 L fermentor (Fig. 6(b, c)). In the 1.3-L fermentor, after 24 h, foaming and flocculation were observed, and it was controlled using antifoam addition and lowering the aeration. Aeration of the fermentor was reduced from 1.3 L per minute (LPM) to 0.5 LPM after 24 h to avoid overflow of the medium and foam generation in the batch fermentation. After 24 h of fermentation, aeration was reduced which is reflected in the higher ethanol production (8.1 ± 0.9 g/L) than erythritol (4.5 ± 0.8 g/L). Aeration (1 vvm) would be optimum for erythritol production; however, high aeration causes more flocculation and generation of foam. Since cell biomass floated on the surface of the medium, it resulted in low productivity (Supplementary Fig. S8a). Though comparatively less flocculation and foaming were observed in the 200-mL shake flask, the erythritol yield (32.9 ± 1.2%) was less than the 25-mL shake flask experiment. Higher glycerol production was observed in 200 mL SF and 1.3 L fermetor experiments as compared to that in 25 mL SF. Flocculation and subsequent foaming were the major issues witnessed throughout the scale-up at the fermentor level. To investigate the potential association between the foaming problem and the mutant strain derived from EMS mutagenesis, erythritol production experiments were conducted in a 3.0-L bioreactor under conditions similar

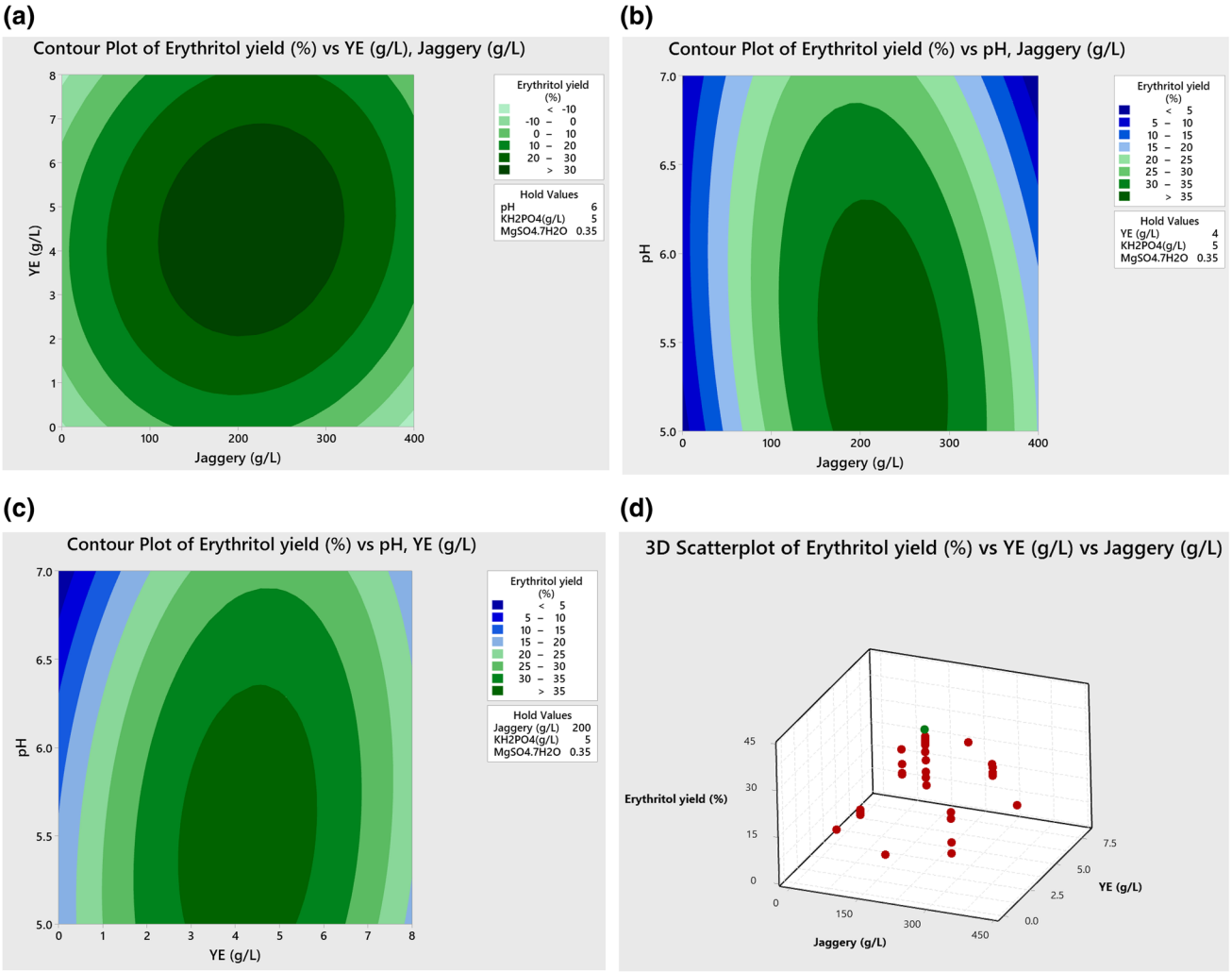



**Fig. 5** Response of variables and 3D scatter plots on erythritol yield (%) production. **a** YE, jaggery vs erythritol yield, **b** pH, jaggery vs erythritol yield, **c** pH, YE vs erythritol yield, and **d** 3D scatter plot of central composite design data points

to those employed for Mutant-58. The results are presented in Fig. 6(d). Remarkably, the wild-type strain exhibited a lower erythritol production of 46.8 ± 2.1 g/L, representing a 26% reduction compared to the mutant strain. Additionally, it was observed higher ethanol production in the parent strain (25.8 ± 2.7 g/L) compared to the mutant culture (17.6 ± 2.1 g/L) during fermentation in the fermentor. Furthermore, it is noteworthy that the issues of flocculation and foaming, which were observed in the mutant strain, were notably absent when the wild-type strain was utilized in the fermentor studies (Fig. S8a).

## Discussion

Erythritol can be made via numerous chemical methods besides biological routes. Chemically synthesizing erythritol from dialdehyde starch with a nickel catalyst (Otey et al. 1961) or through the hydrogenation of a dialkyl ester of tartaric acid using methanol or ethanol under high pressure and temperature (Trenner. 1951) is limited in efficiency, hindering its implementation at a larger scale. Their low efficiency precludes these procedures from implementation at a higher scale. In contrast to chemical synthesis methods, biosynthetic routes offer environmentally friendly and potentially safer processes for erythritol synthesis. Currently, industrial-scale production of erythritol primarily relies on fermentation methods using fungal strains. Genetic modifications through UV or chemical mutagenesis have been explored to enhance erythritol production and reduce unwanted byproducts in various yeast strains, such as osmotolerant yeast strain *Moniliella* sp. 440, which exhibited increased erythritol formation, improved glycerol consumption, reduced byproduct production, and enhanced resistance to osmotic pressure (Lin et al. 2010). Using *Moniliella pollinis* cells, lysate-based media

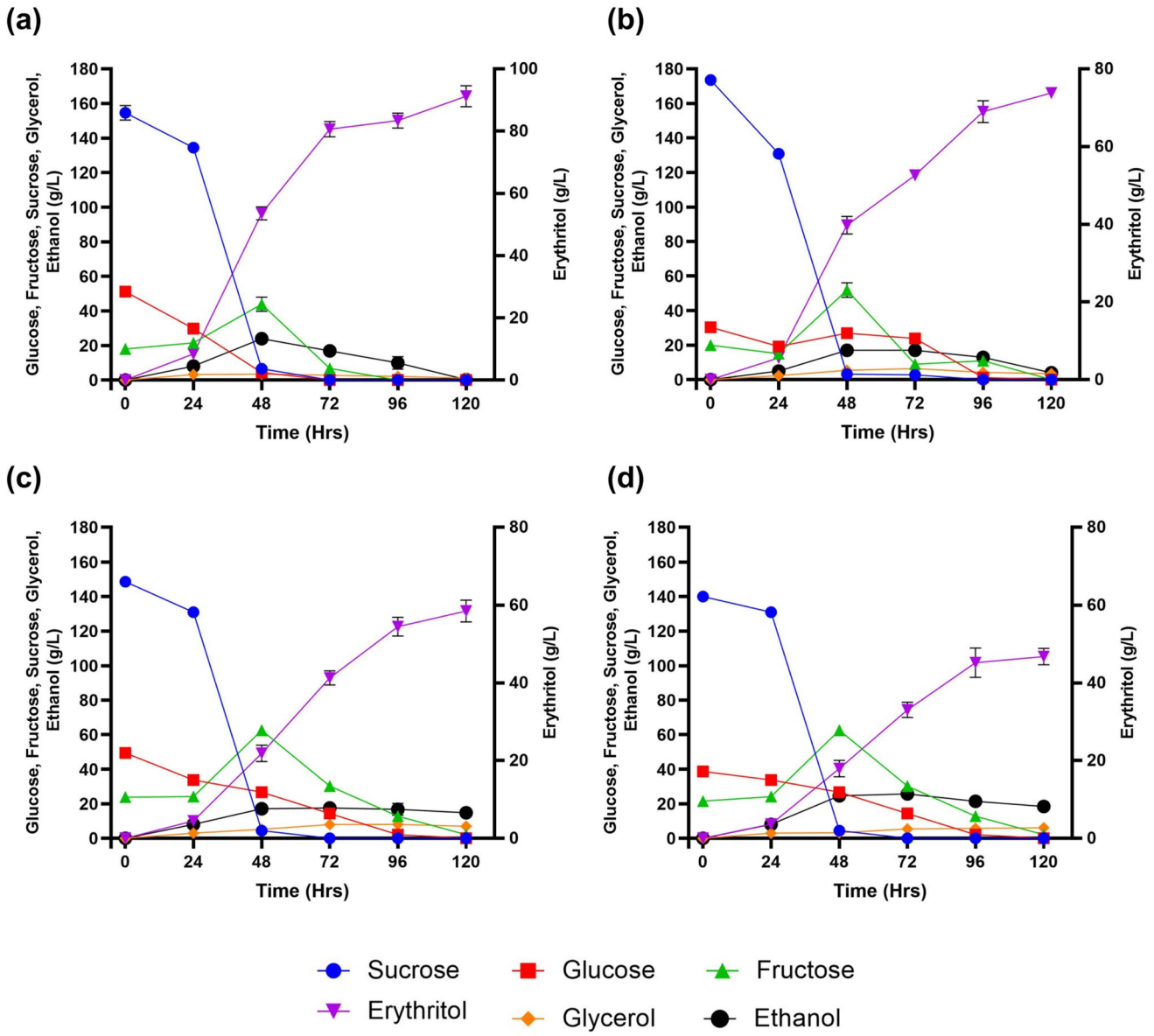



**Fig. 6** Validation of erythritol production using Mutant-58 strain in different cultivation scales. (**a**) Shake flask (25 mL), (**b**) shake flask (200 mL), and (**c**) fermentor (1.3 L). Additionally, (**d**) depicts erythritol production in a 1.3-L Fermentor using the wild-type strain. The data presented are means ± S.D. from triplicate experiments

**Table 4** Erythritol production by yeast cultures using various carbon sources

| Yeast strain | Strain modification | Carbon source | Culture system | ERY yield %[b] | Productivity g/L/h | Reference |
|---|---|---|---|---|---|---|
| *Moniliella tomentosa* var. *pollinis* | –[a] | Glucose | Fed-batch | 40 | 1.61 | Burschäpers et al. (2002) |
| *M. megachiliensis* sp. 440 mutants (mutant N61188-12) | NTG mutation | Glucose | Batch | 43 | 0.65 | Lin et al. (2010) |
| *M. pollinis* | –[a] | Sugarcane juice | Fed-batch | 38 | 0.61 | Deshpande et al. (2022) |
| *M. pollinis* Mut-58 | EMS mutation | Jaggery | Shake flask | 40.7 | 1.27 | This study |

[a]No strain modification

[b]ERY yield (%) $= \frac{\text{Erythritol produced (g/L)}}{\text{Carbon source consumed g/L}} \times (100)$

in sugarcane juice fermentation led to a two-fold increase in erythritol yield and productivity compared to yeast extract–based medium. In fed-batch fermentation with sugarcane juice, a maximum erythritol titer of 94.9 g/L with a yield of 0.38 g/g total sugar was achieved (Deshpande et al. 2022).

Strain improvement by mutagenesis is a well-known strategy to improve microorganisms' ability to produce the desired product. Various research studies have been carried out on improvement in erythritol production in new strains of yeasts that have been modified by physical and chemical mutagenesis (Hirata et al. 1999; Savergave et al. 2011; Ghezelbash GR, Nahvi I and Malekpour A 2014b).

Erythritol production by *Moniliella* cultures using different carbon sources is presented in Table 4. Yang et al. (1999) isolated an osmotolerant strain of *C. magnoliae* and improved it with EMS treatment (Ghezelbash et al. 2014b). In the present study, Mutant-58, obtained by EMS mutagenesis of *M. pollinis* produced enhanced erythritol (88.9 ± 1. g/L) than the parent strain (67.6 ± 2.4 g/L) from glucose.

Apart from higher erythritol production ability, Mutant-58 strain has shown unique morphological traits which differed from the parent strain. Similar observations were made when *Yarrowia lipolytica* Wratislavia K1 wild-type strain was subjected to UV mutagenesis, and one of the mutants had shown changed colony morphology compared with wild-type yeast (Mirończuk et al. 2015a). It was reported in the literature that the mutant cells of *Moniliella* sp. appeared to be a short rod-like shape, while the parent cells have a long rod-like shape (Lin et al. 2010). A similar type of exterior change in cell shape and size was also detected in *Aureobasidium* sp. and its mutant (Ishizuka et al. 1989).

There are reports where microbes have been reported to exhibit multiple phenotypes in response to mutagenesis viz. producing numerous compounds including carotenoids, monascins, phenazines, melanins, violacein, flavins, quinones, and indigo. In fungi, these pigments produced by fungal strains have been known to act as "fungal armor" shielding them from stressful environments and counterbalancing oxidants produced in reply to the stress (Sandra et al. 2017).

Optimization of physiochemical parameters to achieve higher erythritol production is a very crucial step. Previous studies using *Moniliella tomentosa* var. *pollinis* strain observed that YE is a more appropriate nitrogen substrate than corn steep liquor, ammonium salts, and urea (Burschäpers et al. 2002). In the current study, YE optimization studies revealed that the highest erythritol production was observed in the mutant strain (94.3 ± 1.9 g/L) than the parent strain at 4 g/L YE concentration. It was observed that higher YE concentrations reduced the production of erythritol with higher ethanol production. The optimum temperature and pH for both strains were found to be 28 °C and 6.0 respectively.

Various carbon sources (glucose, xylose, molasses, sugarcane juice, sucrose, and glycerol) have been used previously for erythritol production using different yeast cultures (*M. pollinis*, *C. magnoliae*, *Moniliella megachiliensis*, *Aureobasidium pullulans*, and *Y. lipolytica*) (Savergave et al. 2011; Mirończuk et al. 2015a; Kobayashi et al. 2015; Mirończuk et al. 2015b; Sivaraman et al. 2016; Deshpande et al. 2022). Also, Y. Kobayashi et al. (Kobayashi et al. 2015) reported that *M. megachiliensis* can use inexpensive glycerol, a byproduct from the bio-diesel industry as a carbon source. In the most recent report, *M. pollinis* strain displayed 0.29 and 0.12 g/L erythritol yield on sugarcane juice with MCL (*Moniliella* culture lysate) medium and molasses with MCL medium respectively (Deshpande et al. 2022). Jaggery, another cost-effective sugar source explored, contains minerals like Ca FeIron, Mn, Znc, and Cu which could be beneficial for cell growth and metabolism (Hirpara et al. 2020). In the present study, 37% erythritol yield was attained using jaggery in the mutant strain as compared to 30.5% with the parent strain. This is the first report on the production of erythritol using jaggery as a carbon substrate. Erythritol is manufactured commercially by Bolak Corporation in Whasung, Kyungki-do, Korea, Cargill Food & Pharma Specialties in Blair, Nebraska, USA, and Mitsubishi Chemical Corporation in Tokyo, Japan. These companies employ fermentation processes using *Aureobasidium* sp., *Torula* sp., and *Moniliella pollinis* to produce erythritol using glucose as a carbon substrate (Savergave et al. 2011). The current study holds significant importance in the field of erythritol production, especially considering recent research by Rakicka-Pustułka et al. (2020) regarding the scale-up of erythritol production from glycerol using the *Yarrowia lipolytica* strain MK1. This study highlights a novel aspect where a *Moniliella* mutant strain is utilized to produce erythritol using jaggery as a cost-effective substrate. Jaggery, known for its high sucrose content, becomes particularly valuable in the context of erythritol production. The fact that some erythritol-producing yeasts, like *Yarrowia*, encounter difficulties in accessing sucrose as a carbon source further emphasizes the intriguing advantage of sucrose utilization by *Moniliella*. In the context of this study, it was observed that the cost of commercially available glucose and pure sucrose is higher compared to locally produced jaggery (Sugarcane Gud. Available from: https://dir.indiamart.com/impcat/sugarcane-jaggery.html). Furthermore, pure sucrose lacks certain micronutrients that are naturally present in jaggery, which have been found to benefit yeast performance and enhance erythritol production. In the present investigation, comparable erythritol production was achieved using different types of jaggery, sourced either from the market or from local manufacturers. These findings contribute significantly to the understanding and potential advancement of erythritol production.

A 34% increase in erythritol and a 27% decrease in ethanol were noted in the mutant strain. Furthermore, another

interesting observation noted during the shake flask study was that in the mutant strain, glycerol production was slightly higher than in the parent strain. In yeast, the synthesis of erythritol is a multistep metabolic procedure, which operates commonly via the pentose phosphate pathway (PPP). In yeasts and other eukaryotic organisms, the role of the PPP is to yield NAD(P)H for cellular reactions and also to offer precursors for nucleotide and amino acid biosynthesis which includes D-ribose 5-phosphate and erythrose 4-phosphate. Erythrose reductase (ER), a NAD(P)H-dependent aldose reductase, is a crucial enzyme in the biological synthesis of erythritol (Lee et al. 2003). Dephosphorylation of erythrose-4P to erythrose and finally reduction to erythritol with the utilization of one molecule of NAD(P)H constitute the final steps in the suggested pathway. It was reported earlier that three ER isozymes, ER1, ER2, and ER3, were present in *M. megachiliensis* (Ookura et al. 2005). The expression of three ER-encoding genes (ER1, ER2, and ER3) was analyzed in a culture cultivated in 20% glucose and compared with a culture grown in 2% glucose. Although no notable change was observed in ER expression levels during the initial hours of treatment, there was a 60-fold surge in the expression of the glycerol-3-phosphate dehydrogenase gene during the same time period. This shows that the principal response of cells was the production of glycerol. Intracellular erythritol accumulation was observed after 12-h incubation and stayed higher during the next 5 days. Only ER3 expression rose remarkably during extended osmotic stress, and the level of its mRNA transcripts matched the erythritol production profile. Therefore, glycerol synthesis might be an initial response, whereas the cell's stationary phase response to osmotic stress was the production of erythritol (Kobayashi et al. 2013).

In biotechnology, a statistical technique like response surface methodology (RSM) has been applied for a wide range of metabolites produced by microorganisms like enzymes (Vohra and Satyanarayana. 2002), acids (Bustos et al. 2004), terpenoids (Choudhari and Singhal 2008), and erythritol (Savergave et al. 2011; Ghezelbash et al. 2014a). To validate the summary equation of the model and statistical output, ANOVA test was performed, the model coefficient of determination $R^2$ value is 95.58%, demonstrating that experimental results fitted well with predicted values. The model's $F$ values of 11.90 and the $p$ values of $< 0.001$ indicate the model is statistically significant. When the statistically optimized medium composition was validated in a shake flask at 25 mL scale, it produced the highest erythritol (91.2 ± 3.4 g/L) with a yield of 40.7 ± 3.4 %. Nevertheless, in 200 mL shake flask experiment, 32.9 ± 1.2 % erythritol yield was achieved which is more than the erythritol yield of the parent strain with glucose. However, similar results were not replicated in 1.3-L fermentor studies due to flocculation and subsequent foaming throughout the fermentor run in the case of the Mutant-58 strain as against the parent strain (Supplementary Fig. S9). The findings from the experiments conducted in a 3.0-L bioreactor, using jaggery and glucose as substrates with the wild-type strain, have revealed important insights into the relationship between microbial culture, media components, and the issues of foam and flocculation. The results indicate that the occurrence of foam and flocculation is primarily attributed to the microbial culture itself rather than the specific components of the media. Based on these observations, a hypothesis was formulated suggesting that the phenotypic changes in the mutant cells played a significant role in the occurrence of flocculation and foaming. This hypothesis was further supported by the absence of both flocculation and foam formation when the parent strain was cultivated with either glucose or jaggery in the bioreactor during fermentation. The comparison of phenotypic characteristics between the parent and mutant strains as well as the metabolite production served to strengthen the hypothesis. Overall, these results shed light on the underlying factors contributing to foam and flocculation problems, highlighting the importance of phenotypic variations in understanding and addressing these issues. The insights gained from this study can contribute to the development of strategies aimed at mitigating foam and flocculation challenges in bioreactor-based fermentations.

The complication and the highly culture-dependent trait of flocculation make it problematic to regulate fermentation (Verstrepen et al. 2003). Flocculation of yeast is a very composite process that is determined by various flocculation gene expressions (FLO1, FLO5, FLO8, and FLO11) (Russell et al. 1980; Kobayashi et al. 1999). As reported in the literature, yeast with flocculation properties can be operated in airlift bioreactors. Operating flocculating yeast cells in the bioreactors has specific features, mostly related to a high solid-phase hold-up (Domingues et al. 2000). Various bioreactors can be used for handling flocculating yeast cultures, but bubble columns and especially airlift reactors are generally preferred over other bioreactors (Kennard and Janekeh. 1991). On a large scale, except for beer manufacturers, no one has taken the advantage of yeast flocculation ability by using this feature to clarify the fermented must. This is a somewhat incomplete application of flocculating yeast cells as the benefits of airlift bioreactors for ethanol production have been established (Vicente et al. 1999; Restiawaty et al. 2020). There is a requirement for additional investigation on flocculent yeast cultures and using appropriate bioreactors, to gather the critical data to enable a successful process.

## Conclusion

The present study involved strain improvement using EMS mutagenesis followed by medium and process optimization resulting in a 30% increase in erythritol production in

comparison to the parent strain. The changes in the mutant strain at morphological and genetic levels toadied in enhancing the erythritol production with a significant concomitant reduction in ethanol production, a major by-product of the erythritol biosynthesis pathway. Further enhancement in the erythritol production was achieved by statistical medium optimization using Mutant-58 strain by the RSM model where jaggery, YE, and pH were found to be significant variables that affect erythritol production. This is the first report where the highest erythritol production (91.2 ± 3.4 g/L) was achieved using jaggery as a cost-effective carbon substrate at the SF level. Therefore, Mutant-58 obtained in this study has the potential to be a strong contender for erythritol production at the industrial level in an economical jaggery-based fermentation.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10123-023-00411-8.

**Acknowledgements** The authors are thankful to the Directors of CSIR-NCL, Pune., Academy of Scientific and Innovative Research (AcSIR), Ghaziabad- 201002, India, and High Value Chemicals group, Reliance Industries Limited, Ghansoli, Navi Mumbai, India, 400701 for infrastructure and support.

**Author contributions** Anil Khatape designed, carried out the experiments, and wrote the original draft. Vidhya Rangaswamy visualized and supervised the experiments and reviewed and edited the manuscript. Syed Dastager visualized and supervised the experiments and reviewed and edited the manuscript. All authors have approved the final version of the manuscript.

## Declarations

**Ethical approval** This article does not contain any studies with human participants or vertebrates.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interest** The authors declare no competing interests.

## References

Abdelgalil SA, Attia AM, Reyed RM, Soliman NA, El Enashasy AH (2018) Application of experimental designs for optimization the production of *Alcaligenes faecalis* nyso laccase. *J Sci Ind Res* 77:713–722

Bernt WO, Borzelleca JF, Flamm G, Munro IC (1996) Erythritol, a review of biological and toxicological studies. *Regul Toxicol Pharmacol* 24:S191–S197

Burschäpers J, Schustolla D, Schügerl K, Röper H, De Troostembergh JC (2002) Engineering aspects of the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa* var pollinis, Part 2 batch and fed-batch operation in bubble column and airlift tower loop if reactors. *Process Biochem* 38:559–570

Bustos G, Moldes AB, Alonso JL, Vázquez M (2004) Optimization of D-lactic acid production by *Lactobacillus coryniformis* using response surface methodology. *Food Microbiol* 21:143–148

Choudhari S, Singhal R (2008) Medium optimization for the production of β-carotene by *Blakeslea trispora*, a statistical approach. *Bioresour Technol* 99:722–730

Deshpande MS, Kulkarni PP, Kumbhar PS, Ghosalkar AR (2022) Erythritol production from sugar based feedstocks by *Moniliella pollinis* using lysate of recycled cells as nutrients source. *Process Biochem* 112:45–52

Domingues L, Vicente AA, Lima N, Teixeira JA (2000) Applications of yeast flocculation in biotechnological processes. *Biotechnol Bioprocess Eng* 5:288–305

Ghezelbash GR, Nahvi I, Emamzadeh R (2014a) Improvement of erythrose reductase activity, deletion of by-products and statistical medium optimization for enhanced erythritol production from *Yarrowia lipolytica* mutant. *Curr Microbiol* 49:149–157

Ghezelbash GR, Nahvi I, Malekpour A (2014b) Erythritol production with minimum By-product using *Candida magnoliae* mutant. *Appl Biochem Microbiol* 50:292–296

Hirata Y, Igarashi K, Ezaki S, Atomi H, Imanaka T (1999) High-level production of erythritol by strain 618A-01 isolated from pollen. *J Biosci Bioeng* 87:630–635

Hirpara P, Thakare N, Patel D, Kele VD (2020) Jaggery, a natural sweetener. *J Pharmacogn Phytochem.* 9:3145–3148

Ishizuka H, Wako K, Kasumi T, Sasaki T (1989) Breeding of a mutant of *Aureobasidium* sp with high erythritol production. *J Ferment Bioeng* 68:310–314

Jagannadha Rao PVK, Das M, Das SK (2007) Jaggery-a traditional Indian sweetner. *Ind J Trad Knowl* 6:95–102

Kennard M, Janekeh M (1991) Two- and three-phase mixing in a concentric draft tube gas-lift fermentor. *Biotechnol Bioeng* 38:261–1270

Kobayashi O, Yoshimoto H, Sone H (1999) Analysis of the genes activated by the *FLO8* gene in *Saccharomyces cerevisiae*. *Curr Genet* 36:256–261

Kobayashi Y, Iwata H, Mizushima D, Ogihara J, Kasumi T (2015) Erythritol production by *Moniliella megachiliensis* using non-refined glycerol waste as carbon source. *Lett Appl Microbiol* 60:475–480

Kobayashi Y, Yoshida J, Iwata H, Koyama Y, Kato J, Ogihara J, Kasumi T (2013) Gene expression and function involved in polyol biosynthesis of *Trichosporonoides megachiliensis* under hyper-osmotic stress. *J Biosci Bioeng* 115:645–650

Krajangsod S, Chotikamas S, Tawai A, Sriariyanun M (2018) Measurement and thermodynamic modelling of erythritol solubility in aqueous solvents. *Orient J Chem* 34:265–275

Lee K, Kim Y, Ryu W, Seo H, Kim H (2003) Purification and characterization of a novel erythrose reductase from Candida magnoliae. *Appl Environ Microbiol* 69:3710–3718

Lin SJ, Wen CY, Wang PM, Huang JC, Wei CL, Chang JW, Chu WS (2010) High-level production of erythritol by mutants of osmophilic *Moniliella* sp. *Process Biochem* 45:973–979

Livesey G (2003) Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. *Nutr Res Rev* 16:163–191

Mirończuk AM, Dobrowolski A, Rakicka M, Rywińska A, Rymowicz W (2015a) Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol. *Process Biochem* 50:61–68

Mirończuk AM, Rakicka M, Biegalska A, Rymowicz W, Dobrowolski A (2015b) A two-stage fermentation process of erythritol production by yeast *Y lipolytica* from molasses and glycerol. *Bioresour Technol* 198:445–455

Nath A, Dutta D, Kumar P, Singh JP (2015) Review on recent advances in value addition of jaggery based products. *J Food Proc and Technol* 6:1–2

Ookura T, Azuma K, Isshiki K, Taniguchi H, Kasumi T, Kawamura Y (2005) Primary structure analysis and functional expression of erythrose reductases from erythritol-producing fungi (*Trichosporonoides megachiliensis* SNG-42). *Biosci Biotechnol Biochem* 69:944–951

Otey FH, Sloan JW, Wilham CA, Mehltretter CL (1961) Erythritol and ethylene glycol from dialdehyde starch. *Ind Eng Chem* 5:267–268

Rakicka-Pustułka M, Mirończuk A, Celińska E, Białas W, Rymowicz W (2020) Scale-up of the erythritol production technology – process simulation and techno-economic analysis. *J Clean Prod* 257:1–11

Restiawaty E, Gani KP, Dewi A, Arina LA, Kurniawati KI, Budhi YW, Akhmaloka (2020) Bioethanol production from sugarcane bagasse using *Neurospora intermedium* in an airlift bioreactor. *Int J Renew Energy Dev* 9:247–253

Russell I, Stewart GG, Reader HP, Johnston JR, Martin PA (1980) Revised nomenclature of genes that control yeast flocculation. *J Inst Brew* 86:120–121

Rzechonek DA, Dobrowolski A, Rymowicz W, Mirończuk AM (2017) Recent advances in biological production of erythritol. *Crit Rev Biotechnol* 38:620–633

Sandra RP, Gabriela SS, Jazmina CRA, Helen FL, Rita CRG (2017) Production of melanin pigment by fungi and its biotechnological applications, 1st edn. InTech, Melanin, London, UK, pp 47–75

Savergave LS, Gadre RV, Vaidya BK, Narayanan K (2011) Strain improvement and statistical medium optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochem Eng J* 55:92–100

Shindou T, Sasaki Y, Miki H, Eguchi T, Hagiwara K, Ichikawa T (1988) Determination of erythritol in fermented foods by high performance liquid chromatography. Food Hyg Saf Sci (Shokuhin Eiseigaku Zasshi) 29:419

Shukla SA (2015) Bradford multiplexing method for protein estimation in fermented foods, Soy sauces. *Bangladesh J Pharmacol* 11:24–27

Singh J (2013) Manufacturing jaggery, a product of sugarcane, as health food. *Agrotechnology* 1:1–3

Sivaraman B, Badle S, Rangaswamy V (2016) Production of 1,3-butadiene from erythritol. Chemistry Select 1:4630–4632

Thesseling FA, Bircham PW, Mertens S, Voordeckers K, Verstrepen KJ (2019) A hands-on guide to brewing and analyzing beer in the laboratory. *Curr Prot Microbiol* 54:91

Trenner NR BF (1951) Hydrogenation of tartaricacid esters to erythritol. 1951, US2571967A.

Vagenende V, Yap MGS, Trout BL (2009) Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. *Biochemistry* 48:11084–11096

Verstrepen KJ, Derdelinckx G, Verachtert H, Delvaux FR (2003) Yeast flocculation, what brewers should know. *Appl Microbiol Biotechnol* 61:197–205

Vicente AA, Dluhý M, Teixeira JA (1999) Increase of ethanol productivity in an airlift reactor with a modified draught tube. *Can J Chem Eng* 77:497–502

Vijayendra SVN, Bansal D, Prasad MS, Nand K (2001) Jaggery, a novel substrate for pullulan production by *Aureobasidium pullulans*. CFR-77. *Process Biochem* 37:359–364

Vohra A, Satyanarayana T (2002) Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomal*a. *Process Biochem* 37:999–1004

Winston F (2008) EMS and UV mutagenesis in yeast. In, Curr Protoc Mol Biol Wiley, New York City, US, pp 133B1–133B5

Yang SW, Park JB, Soo Han N (1999) Production of erythritol from glucose by an osmophilic mutant of Candida magnoliae. Biotechnol Lett 21:887–890

Yoshida H, Sugahara THJ (1984) Studies on free sugars and free sugar alcohols of mushrooms. *Nippon Shokuhin Koyko Gakkaishi* 31:765–771