MICROBIAL PRODUCTION OF ERYTHRITOL AND MANNITOL: STRAIN IMPROVEMENT AND PROCESS OPTIMIZATION

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BY

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UNDER THE GUIDANCE OF

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AT

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Dedicated to my teachers & parents...

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled '**MICROBIAL PRODUCTION OF ERYTHRITOL AND MANNITOL: STRAIN IMPROVEMENT AND PROCESS OPTIMIZATION**' submitted by **Mr. Laxman S. Savergave** was carried out by the candidate under my supervision at Chemical Engineering and Process Development Division, National Chemical Laboratory, Pune 411008 (India). Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION BY THE CANDIDATE

I hereby declare that the work incorporated in the thesis entitled '**MICROBIAL PRODUCTION OF ERYTHRITOL AND MANNITOL: STRAIN IMPROVEMENT AND PROCESS OPTIMIZATION**' is my own work conducted under the supervision of **Dr. Ramchandra V. Gadre**, at Chemical Engineering and Process Development Division, National Chemical Laboratory, Pune 411008 (India). I further declare that to the best of my knowledge, this thesis does not contain any part of work, which has been submitted for the award of any degree either of this University or any other University without proper citation.

Mr. Laxman S. Savergave (*Research student*)

Dr. Ramchandra V. Gadre (*Research guide*)

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ABBREVIATIONS, ACRONYMS AND SYMBOLS

AKR	Aldose ketose reductase
ALR	Aldose reductase
ANOVA	ANalysis Of VAriance
°C	Degree centigrade
CCRD	Central composite rotatable design
DCW	Dry cell weight
DHN	Dihydroxynaphthalene
DO	Dissolved oxygen
DoE	Design of Experiments
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methyl sulphonate
ER	Erythrose reductase
FDH	Formate dehydrogenase
FDH	formate dehydrogenase
FID	Flame ionization detector
g	Gram
GC	Gas chromatography
GFP	Glucose facilitator protein
GI	Glucose isomerase
GRAS	Generally regarded as safe
h	Hour
HA	Hydroxylamine
HPLC	High performance liquid chromatography
K_{cat}	Turnover number (s^{-1})
K_{cat}/K_m	Specificity constant ($mM^{-1} \cdot s^{-1}$)
KCCM	Korean Culture Collection of Microorganisms
*K*m	Michaelis constant (mM)
L or l	Liter
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
LFM	Liquid fermentation medium
m	Meter
M1Pase	Mannitol-1-phosphatase
M1P-DH	Mannitol-1-phosphate dehydrogenase
M572	Erythritol producing mutant of *Candida magnoliae* NCIM 3470
MCBR	Membrane cell recycle bioreactor
MDH	Mannitol dehydrogenase
MDRs	Medium-chain dehydrogenase reductases
MDRs	Long-chain dehydrogenase reductases

MGYP	Malt extract glucose yeast extract peptone
min	Minute
MMS	Methyl methane sulfonate
MTCC	Microbial Type Culture Collection
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	Nikken Fine Chemicals
NCIM	National Collection of Industrial Microorganisms
NCYC	National Collection of Yeast Cultures
NTG	*N*-methyl *N*-nitro *N*-nitroso guanidine
OD	Optical density
PDMS	Polydimethylsiloxane
PMSF	Phenylmethanesulfonylfluoride
PP pathway	Pentose phosphate pathway
PTS	Phosphotransferase system
R1	Mannitol producing mutant of *Candida magnoliae* NCIM 3470
R^2	Regression coefficient
R23	Erythritol producing mutant of *Candida magnoliae* NCIM 3470
R9	Mannitol producing mutant of *Candida magnoliae* NCIM 3470
rpm	Rotations per minute
RSM	Response Surface Methodology
[S]	Substrate concentration (mM)
SDRs	Short-chain dehydrogenase reductases
sec	Second
SEM	Scanning electron microscopy
SSF	Simultaneous Saccharification and fermentation
t	Time
T	Temperature
TCA cycle	Tricarboxylic acid cycle
TTC	2, 4, 5-triphenyl tetrazolium chloride
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
WCW	Wet cell weight
YNB	Yeast Nitrogen Base

Chapter 1

General Introduction

Abstract:

In this chapter, an overview about erythritol and mannitol is presented. A literature survey on their physicochemical properties, functions, applications, methods of production and strategies used for enhanced biotechnological production is summarized.

INTRODUCTION

Growing focus on maintaining physical fitness by avoiding high-calorie food has increased the demand of “sugar free” food products. "Sugar free" food products are sweetened by sugar substitutes, which are known by many names: non-nutritive sweeteners, low calorie sweeteners, no-calorie sweeteners, artificial sweeteners and alternative sweeteners. They all contain little to no calories and have little glycemic response. Some sugar substitutes are natural and some are synthetic. Synthetic sugar substitutes are also called as artificial sweeteners.

Artificial sweeteners as a group are classed as non-nutritive sweeteners. They provide a sweet sensation to the taste buds, without raising blood sugar levels and are useful for weight-loss. United States Food and Drug Administration (US FDA) has approved six non-nutritive sweeteners such as saccharin, aspartame, acesulfame K, sucralose, neotame and stevioside (Kroger et al., 2006).

1) Saccharin has been used around the world since the turn of the century. It is 300 times sweeter than sucrose. It is very stable in foods, but has a bitter aftertaste. The common brand name is ‘Sweet’N Low’. 2) Aspartame is 200 times sweeter than sucrose. It is composed of two amino acids, phenylalanine and aspartic acid. It is an excellent sweetener with no aftertaste. Aspartame cannot be used in baking, because it breaks down with heat. It is not suitable for patients with phenylketonuria. The common brand names are ‘Nutra-Sweet’ and ‘Equal’. 3) Acesulfame-K is 200 times sweeter than sugar. Acesulfame K is stable and does not break down in cooking. The common brand names are ‘Ace-K’, ‘Sweet One’ and ‘Sunette’. 4) Sucralose is about 600 times sweeter than sucrose. Sucralose is extremely stable and does not break down during cooking. Johnson & Johnson markets sucralose under the brand name ‘Splenda’ as being made from sugar. 5) Neotame is 8000 times sweeter than sucrose. Although neotame contains phenylalanine it is not degraded during digestion. 6) Stevioside is 250 times sweeter than sugar and is a natural sweetener extracted from leaves of plant named *Stevia rebaudiana* (Mitchell, 2006).

Although the zero calorie content of non-nutritive sweeteners provide obvious advantages when replacing sugar, these are also reported to have drawbacks. They do a good job in sweetening, but their inability to provide bulk to food products limits

their applicability. Moreover, non-nutritive sweeteners are often accompanied by a bitter and metallic aftertaste and does not provide the 'realistic' and 'voluminous' mouth feel of sucrose.

Polyols are all natural and considered as sugar-free sweeteners. Chemically, polyols are polyhydric alcohols or sugar alcohols. They are derived from carbohydrates whose carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group. Unlike high-intensity artificial sweeteners, which are used in very small amounts, polyols are used in the same quantity as sucrose. Some of the commercially available polyols are erythritol, isomalt, lactitol, maltitol, mannitol, sorbitol and xylitol.

Sugar alcohols provide the bulk and sweetness of sugar, and are incompletely absorbed in the intestine. Products containing sugar alcohol may claim to be "sugar free" but this does not mean they are "calorie free". In fact, they provide fewer calories and result in a much slower and minor rise in blood sugar level. They are considered as safe for diabetic patients and therefore the products sweetened with these products may legally be labelled "sugar-free". These health benefits have made polyols a popular choice as sugar replacers in a variety of products. Polyols are now widely used in baked food. Chemical structures of commercially available polyols and non-nutritive sweeteners are presented in Fig. 1.1. The calories contributed per gram of different polyols and their approximate sweetness in relation to sucrose is presented in Table 1.1

Polyols are predominantly consumed in the confectionery, food, drink and pharmaceutical industries. Polyols serve as humectants, bulking agents, and freeze-point depressants. They are multi-functional ingredients, used in a variety of applications to provide value-adding properties. Polyols function well in fillings and frostings, canned fruits, beverages, yogurt and table-top sweeteners. In United States, polyols are used in a wide range of products including chewing gums, candies, ice cream, baked goods and fruit spreads. They are also incorporated in toothpastes, mouthwashes, breath mints and pharmaceuticals such as cough syrups or drops and throat lozenges. Polyols have received generally regarded as safe (GRAS) status from the US FDA (Frost&Sullivan, 2007).

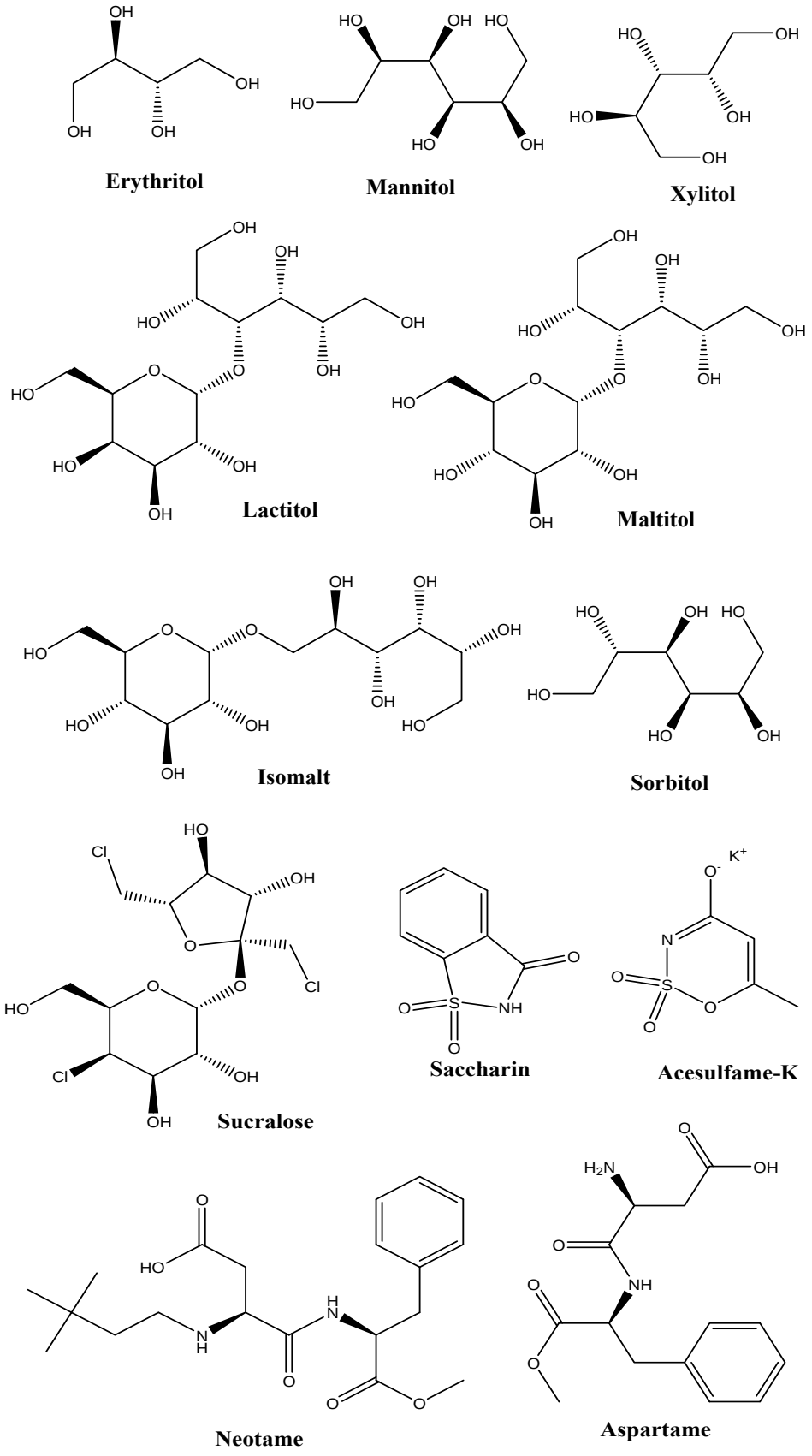

Figure 1.1 Chemical structures of polyols and artificial non-nutritive sweeteners

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%

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

1.1 Erythritol

"Erythritol" has ability to completely substitute sugar and can meet most of the criteria required to produce sugar free food products. Erythritol is a four-carbon sugar alcohol that is used as food grade sweetener. It is naturally present in small quantities in fruits such as pears, melons and grapes. It is also present in mushrooms and fermented foods like soy sauce, cheese and wine (de Cock & Bechert, 2002; Park et al., 1998a). Its sweet taste is very close to sucrose, while its sweetness is about 70% of the sweetness of sucrose with a very low caloric value (de Cock, 1999). It is used as bulk sweetener with mouth feel enhancing properties. Since erythritol does not have an aftertaste, it is used in combination with intense sweeteners that have a bitter aftertaste like aspartame and stevioside to mask the off taste and mimic the texture and mouth feel of sucrose (de Cock & Bechert, 2002). More than 90% of ingested erythritol is not metabolized by the human body and is excreted unchanged in the urine without changing blood glucose and insulin levels (Lin et al., 2010). Therefore, it could be used advantageously in special foods for people with diabetes and obesity. It is non-cariogenic because it cannot be metabolized by oral bacteria. Unlike other sugar alcohols, it does not exert laxative effect and has the highest digestive tolerance among all polyols (de Cock, 1999). Erythritol has been approved as food additive in Japan in 1990, then in USA in 2001 and in Europe in 2006. It can be used as a flavor

enhancer, formulation aid, humectants, non-nutritive sweetener, stabilizer, thickener, sequestrant and texturizer at maximum levels of 100% in sugar substitutes (Park et al., 2005).

1.2 Physicochemical properties of erythritol

Erythritol is a symmetrical molecule and exists only in one form, the meso-form. It forms anhydrous crystals and has a melting point of 122°C. It does not have reducing end-groups due to which it has excellent heat and pH stability. Compared with the group of polyols presently used as sugar substitutes, erythritol has the lowest molecular weight 122.12 g/mol, which gives it different properties, such as higher osmotic pressure and lower water activity in solution. Technical data of erythritol is given in Table 1.2

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 °C
Density:	1.45 g/cm^3
Solubility:	Freely soluble in water, slightly soluble in ethanol, insoluble in diethyl ether

Table 1.2 Technical data on erythritol

1.3 Functions

Erythritol is a natural product also produced by fermentation process. It has virtually no calorie, 0.2 kcal/g, which is 5% of the caloric value of sucrose.

- Has a zero glycemic index, it does not raise plasma glucose or insulin levels.
- Has a high digestive tolerance and it is non-laxative. Adults ingesting up to 1 g erythritol per day, per kg body weight, do not show any gastrointestinal effects.
- Is resistant to metabolism by oral bacteria and is tooth-friendly.
- Has a clean sweet taste with no aftertaste and is approximately 70% as sweet as sucrose. It improves mouth feel, provides body and masks off-tastes.

- Exhibits interesting qualitative and quantitative synergies with intense sweeteners (e.g. sucralose, aspartame, acesulfame-K) or stevia.
- Shows a cooling effect on dissolution due to its high negative heat of solution.
- Is heat stable up to 180 °C and exhibits no Maillard browning reaction.
- Has a very low hygroscopicity.

1.4 History

Stodola (1946) was first to report erythritol production in *Aspergillus terreus*. He separated erythritol from ether extract of concentrated culture liquor, based on its insolubility in methanol. Negative Fehling's test for reducing sugars suggested that the compound was a polyhydric alcohol. The melting point analysis specified the compound to be meso-erythritol and was confirmed by comparison of X-ray diffraction patterns of the natural and authentic samples.

Based on traces of erythritol detected in the residue of fermented molasses Binkley & Wolfrom (1950) suggested that erythritol could be produced by yeast. Subsequently, Spencer et al., (1957) investigated that many of the osmophilic yeasts were capable of producing the polyhydric alcohols such as glycerol, arabitol, erythritol and mannitol in good yields under aerobic conditions. Peterson et al., (1958) and Hajny et al., (1960) surveyed osmophilic yeasts for their ability to produce the polyhydric alcohols isolated from brood comb honey, brood comb pollen, fresh pollen and fresh clover heads growing near the apiary. Subsequently, Hajny et al., (1964) examined these osmophilic yeasts for erythritol production as sole polyhydric alcohol. Yeast like fungus, probably belonging to the genus *Torula*, was identified to produce erythritol in yields of 35 to 40% of the sugar utilized. The ability to produce erythritol was an inherent characteristic of the isolate, but unfavorable fermentation conditions resulted production of glycerol at the expense of erythritol (Hajny et al., 1964). By the use of a synthetic medium, it was shown that the concentrations of both nitrogen and phosphorous in the medium must be carefully controlled to obtain satisfactory erythritol yields. Furthermore, occurrence of erythritol as a metabolite or storage compound in seaweeds and as a component of a number of fruits such as melons, grapes, pears and fermented foods like soy sauce, wines and beers was confirmed by a number of researchers (Röper & Goossens, 1993; Shindou et al., 1989; Yoshida et al., 1986).

In the course of study on citric acid fermentation by *Candida zeylanoides*, in which n-alkane was used as the sole source of carbon, Hattori & Suzuki, (1974) found that erythritol accumulated when the medium-pH decreased below 4.0. Since then, emphasis was given by many researchers on the screening and isolation of microorganisms which produce erythritol in high yield (Table 1.3).

1.5 Biotechnological production of erythritol

Chemical and biotechnological processes have been employed for the production of erythritol. Chemically, erythritol can be synthesized from dialdehyde starch at high-temperature in the presence of a nickel catalyst (Otey et al., 1961). This process has not been industrially used because of its low efficiency. Although erythritol is present in fruits and vegetables, it is not economic to extract it from them. In contrast, biosynthetic routes offer potentially safe and environment friendly erythritol production.

Owing to its increasing demand in the food industry, erythritol production using biological processes had turn out to be significant area of research in food industry. Erythritol had world market of 20,000 metric tons in 2006, which is expected to grow to 23,000 metric tons by 2011 (Frost&Sullivan, 2007). The price of erythritol is currently about $ 4.5/kg.

Large-scale production of erythritol uses fermentative processes with sucrose or glucose from chemically or enzymatically hydrolyzed wheat and corn starches. Erythritol can be produced using osmophilic yeasts belonging to the genus *Aureobasidium, Candida, Moniliella, Pichia, Pseudozyma, Trigonopsis, Trichosporon, Trichosporonoides and Yarrowia* (Jeya et al., 2009; Lin et al., 2001; Moon et al., 2010; Rymowicz et al., 2009).

Erythritol production using *Pichia, Zygopichia, Candida, Torulopsis* and *Trigonopsis* could not be applied on an industrial scale due to lower yield, productivity or by-products formation such as glycerol and ribitol. Hirata et al., (1999) isolated *Ustilaginomycetes sp*. 618A-01 from pollen that did not produce any by-product. However, the culture time was 500-1,000 h. An erythritol producing *Candida magnoliae* was isolated from honeycombs by Yang et al., (1999) and it was

mutatagenized using UV irradiation and *N*-methyl *N*-nitro *N*-nitroso guanidine (NTG) treatment for higher erythritol yield and productivity. Various biological processes using the mutant strain have been optimized for maximizing erythritol production. The optimized fed-batch fermentation resulted in 200 g/l erythritol concentration. However, the said organism produced high amount of organic acids as by-products (Koh et al., 2003; Ryu et al., 2000). Recently, Jeya et al., (2009) had studied optimization of erythritol production from *Pseudozyma tsukubaensis* at 50,000L pilot plant using dissolved oxygen as a scale-up parameter.

Similarly, a large-scale screening study was conducted by Lin et al., (2001) for isolation of erythritol producing microorganisms from sugar-containing samples collected from Taiwan. Out of 658 osmophilic yeast isolates, twenty-eight isolates produced erythritol, among which eight strains gave a yield higher than 10%. Further, one of the selected osmophilic yeast-like fungus, *Moniliella sp*. 440, was then successively modified by NTG treatment (Lin et al., 2010). The most efficient erythritol producer mutant N61188-12 produced 151.4 g/l and 152.4 g/l erythritol, respectively, in 250-L and 2000-L pilot-scale fermenter. A simple fed-batch culture, of strain N61188-12 in a 2000-L fermenter, increased erythritol production to 189.4 g/l in 10-day fermentation (Lin et al., 2010). Comprehensive comparison of erythritol concentration, productivity and yield by all reported microbial strains till date is presented in Table 1.3

1.6 Industrial production of erythritol

Erythritol is commercially produced by Bolak Corporation (Whasung, Kyungki-do, Korea), Cargill Food & Pharm Specialties (Blair, Nebraska, USA), and Mitsubishi Chemical Corporation (Tokyo, Japan) by the fermentation of glucose using *Aureobasidium* sp, *Torula* sp and *Moniliella pollinis* (Moon et al., 2010).

In the early 1980s when natural food sweetener started to grow in popularity, Cerestar, from Italy a leading European manufacturer of starch, initiated a research project aiming at the production of different types of polyols using a fermentation process. In the frame of this research, *Moniliella pollinis* a yeast strain was found to produce significant amounts of erythritol (de Cock, 1999). Cerestar developed a fermentation process using natural raw materials, improving yield and fermentation

efficiencies and designing better purification steps leading to high-purity erythritol. Cerestar successfully commercialized erythritol the under brand name ‘Eridex’ in 1993.

Erythritol was first introduced into the Japanese market in 1990 by Mitsubishi Chemical Foods Co., Ltd. (wholly owned by Mitsubishi Chemical Corp. and Nikken Chemicals Co., Ltd). Later Mitsubishi obtained manufacturing patent for the sweetener erythritol from Nikken Chemicals, since Nikken Chemicals planned to withdraw from erythritol operations, in order to concentrate on its pharmaceutical business. This made Mitsubishi Chemical the sole erythritol producer in Japan.

To meet the rapid domestic demand of erythritol in Japan, in 1997, Mitsubishi Chemical Corporation made agreement to establish a joint venture in USA with Cargill, one of the largest US manufacturers of glucose, for manufacturing and marketing of erythritol, with the aspect of securing a stable supply source of glucose the major starting material for erythritol. But when the plant was completed in 1999, with 20,000 metric tons of capacity, the market had not developed as quickly as anticipated and the unit was never fully commissioned. Cargill acquired full ownership of the joint venture in 2002.

Cerestar has been producing erythritol in Italy, Cargill purchased 56 % of shares from Cerestar and since Cargill had made a significant investment in the US, the most economic option was to consolidate erythritol production only at the Blair site in USA. Commercial production at the Blair facility has enabled a new agency agreement in 2004 between Cargill and Mitsui for distribution of Cargill's erythritol to food company customers in Japan. Cargill subsidiary, Cerestar imports ‘Eridex’ into Japan and Mitsui-owned Nikken Fine Chemicals (NEC) is responsible for managing stock inventory as a sales agent for Cerestar.

1.7 Erythritol biosynthesis pathway

Osmotolerant yeasts accumulate compatible solutes on encountering salt or osmotic stress. Compatible solutes protect and stabilize enzymes, enabling the cellular functions in osmotic conditions. Glycerol is the most common osmolyte in yeasts, whereas sugar alcohols such as arabitol, erythritol and mannitol may also serve as

osmolytes (Diano et al., 2006). The sugar alcohols produced by osmotolerent microorganisms may also have a role in redox balancing or as storage compounds (Shen et al., 1999).

Erythritol produced by yeast and fungus species is synthesized via pentose phosphate pathway (PP pathway). The role of the pentose phosphate pathway in yeasts and other eukaryotic organisms is to produce NADPH for the cellular reactions and also to produce precursors such as D-ribose 5-phosphate and erythrose 4-phosphate for nucleotide and amino acid biosynthesis (Lehninger et al., 1982). Biochemical pathway of polyol synthesis is presented in Fig 1.2

The sum of erythritol biosynthesis within microbes is suggested to be as follows: One mole of glucose is converted into two mole of carbon dioxide and one mole of erythrose-4-phosphate in the pentose phosphate pathway. Then one mole of erythrose-4-phosphate is converted into one mole of erythritol (Park et al., 1998a). It can be visualized in Fig 1.2 that erythritol is synthesized from erythrose 4-phosphate by dephosphorylation, followed by reduction (Lee et al., 2002).

In *Aspergillus nidulans*, the synthesis of erythritol via PP pathway is favored by growth under glycolytic rather than gluconeogenic condition (Dijkema et al., 1985). Under these conditions, the high carbon fluxes through both the PP pathway and the glycolysis pathway generate sufficient reduction capacity that leads to the overflow of the carbon into various polyols.

Eukaryotes possess erythrose reductase enzyme to catalyze the hydrogenation of erythrose. Erythrose reductase (ER) that catalyses this last step, is a key enzyme in erythritol biosynthesis (Lee et al., 2010; Lee et al., 2003d; Lee et al., 2003e; Lee et al., 2003f). Regulation of PP pathway in osmophilic yeasts is not yet fully understood but it is known that fermentative production of erythritol is influenced by factors like osmotic pressure of the medium, pH, dissolved oxygen, nitrogen sources and type of carbohydrates used as substrate (Burschäpers et al., 2002a). Citric acid, ethanol, gluconic acid, glycerol, mannitol and ribitol are reported to be the additional metabolites produced during erythritol production by different microorganisms (Moon et al., 2010; Rymowicz et al., 2008; Ryu et al., 2000).

Culture	*Flask medium g/l*	*Fermenter medium g/l*	*Other metabolites*	*PH*	*Tem p ºC*	*DO%*	*Aeration VVM Agitation rpm*	*Biom ass g/l*	*Eryt hritol g/l*	*Y% P/S*	*Q_P g $l^{-1}h^{-1}$*	*Reference*
Torula sp	Glucose 357, CSL 1, Urea 1.7 50 ml in 500 ml flask		Glycerol		30	42 mM O_2 l^{-1} h^{-1}		24	130	45.6	0.77	(Hajny et al., 1964)
Aureobasidium sp Mutant SN-G42		Glucose 400, YE 20, 3L Batch fermenter	Glycerol during growth phase	Initial 5.4	35		2 VVM 100 rpm	10.7 x 10^8 cells ml^{-1}	175	43.7	1.82	(Ishizuka et al., 1989)
Trichosporonoides sp	Glucose 100, YE 5, Urea 1 50 ml medium in 250 ml flask		Glycerol 2.7 g/l	Initial 6-6.4	30		200 rpm		43	43	0.29	(Aoki et al., 1993)
Trichosporon sp 832-1	Sucrose 10, YE 5, Urea 1 10 ml in 50 ml flask				30				40.7	40.7	0.55	(Park et al., 1996)
Trigonopsis variabilis KCCM 35523		Glucose 300, YE 5, 5L batch		Controlled 6.5	30		1 VVM 300-1200 rpm	18	24	8	0.1	(Kim et al., 1997)
		Glucose 100+200, YE 5 5L Two stage fed-batch		Controlled 6.5	30		1 VVM 300-1200 rpm	34	46	15	0.19	
Trichosporon sp		Glucose 220, CSL 40,	Glycerol	Initial 3.5	35		1 VVM 600 rpm	63	149	45	1.86	(Park et al., 1998a)

Culture	***Flask medium g/l***	***Fermenter medium g/l***	***Other metabolites***	***PH***	***Tem p ºC***	***DO%***	***Aeration VVM Agitation rpm***	***Biom ass g/l***	***Eryt hritol g/l***	***$Y_{P/S}$%***	***Q_P g $l^{-1}h^{-1}$***	***Reference***
		Repeated fed-batch										
Trichosporon sp		Glucose 300, CSL 40, 500L Batch fermenter	Glycerol 18	Initial 3.5	35		1 VVM 160 rpm	OD (660) 62	142	47.3	1.97	(Park et al., 1998b)
Ustelaginomycetes 618A-01		Glucose 200, Peptone 10, YE 5, NaCl 5 3 L Fed batch fermenter		Controlled 4.9-5.1	30			80 OD (660)	100	39.3	0.18	(Hirata et al., 1999)
Candida magnoliae Mutant M2	Glucose 100, YE 5, KH_2PO_4 5, $(NH_4)_2SO_4$ 2, $MgSO_4$ 0.4, 200 ml in 500 ml flask		Glycerol	Initial 7	28		200 rpm	22	23	25	0.3	(Yang et al., 1999)
		Glucose 200, YE 5, KH_2PO_4 5, $(NH_4)_2SO_4$ 2, $MgSO_4$ 0.4 3 L Batch		Initial 7	28		0.5 VVM 700 rpm	25	85	43	0.54	
Torula sp	Sucrose 300, YE10, KH_2PO_4 3, $CuSO_4$ 0.01 100 ml in 500 ml baffle flask			Initial 5.5	34		250 rpm	10.4	166	53.3	1.11	(Kim et al., 2000)

Culture	*Flask medium g/l*	*Fermenter medium g/l*	*Other metabolites*	*PH*	*Temp °C*	*DO%*	*Aeration VVM Agitation rpm*	*Biomass g/l*	*Erythritol g/l*	*$Y_{P/S}$%*	*Q_P g $l^{-1}h^{-1}$*	*Reference*
		Sucrose 400, YE20, KH_2PO_4 3, $CuSO_4$ 0.01 5L Batch		Initial 5.5	34	5-10%	0.5 VVM 600 rpm	30.4	200	50	1.67	
Candida magnoliae Mutant M2		Glucose 10+316-400, YE 6.7+212 3.3 L Fed batch	137 Gluconic, butyric and citric	Initial 7	28	20%		75	187	41	2.8	(Ryu et al., 2000)
Torula sp		Glucose 300 + 100, YE 20, Phytic acid 2, 5 L fed batch		Controlled 5.5	34	Above 20 % rpm	0.5 VVM 500-800	24.1	192	48	2.26	(Oh et al., 2001)
Moniliella sp 440		Glucose 300, YE 10, 5 L Batch	Glycerol , Ribitol	Initial 5.3	30		1 VVM 900 rpm		111	37	0.77	(Lin et al., 2001)
Moniliella tomentosa var pollinis		Glucose 352, YE 5, urea 1, SAG 0.3, xanthan0.3 20 L Batch reactor	60 g/l (Glycerol, Ribitol) 16 g/l polysaccharides	Initial 5.5	30	Above 20%	0.3 VVM 400 rpm	65	90	25.5	0.58	(Burschäpers et al., 2002a)
		Glucose 352, YE 5, urea 1, SAG 0.3, xanthan0.3 Feeding sol. Glucose and $(NH_4)_2SO_4$ 20 L Fed batch	18 g/l polyols Glycerol 1-14, Ethanol 7	Initial 5.5	30	Above 20%	0.3 VVM 400 rpm	116	170	38	1.58	
Moniliella tomentosa var		Glucose 352, CSL 20, urea 1, SAG	45 g/l (Glycerol,	Initial 5.5	30	Above 20%	0.4-1.4 400 rpm	65	105	30	2.05	(Burschäpers et al.,

Culture	*Flask medium g/l*	*Fermenter medium g/l*	*Other metabolites*	*PH*	*Temp °C*	*DO%*	*Aeration VVM Agitation rpm*	*Biomass g/l*	*Erythritol g/l*	*$Y_{P/S}$%*	*Q_P g $l^{-1}h^{-1}$*	*Reference*
pollinis Mutant HAT 01		0.3, xanthan 0.3 60L Batch Air-lift fermenter	Ribitol) 16 g/l polysaccharides									2002b)
		Glucose 352, CSL 20, urea 1, SAG 0.3, xanthan 0.3, Feed sol. glucose 50% and urea 1% 60 L Fed-batch in air-lift fermenter Total 500 g/l glucose	65 g/l (Glycerol, Ribitol and polysaccharides)	Initial 5.5	30	Above 20%	0.4-1.4 400 rpm	35	175	35	0.85	
Candida magnoliae Mutant M2		Glucose 300,Peptone 20, YE 10, Phytic acid 2, Feed sol. 700 g/l glucose, 100 g/l YE 50L Fed batch		Initial 7	28		1 VVM 500 rpm	76	200	43	1.2	(Koh et al., 2003)
Penicillium sp KJ-UV29 Mutant		Sucrose 300, YE 5, $(NH_4)_2C2O_4$ 5, $NaNO_3$ 1, KNO_3 1, 5 L Batch fermenter	Glycerol 20.7	Controlled 7	37		1 VVM 200	35	45.2	15	0.28	(Lee & Lim, 2003)
Yarrowia lipolytica Wratislavia K1 Mutant		Glycerol 180+70, YE 1, NH_4Cl 3, KH_2PO_4 0.3, $MgSO_4$ 1, 5L Fed-	Citric acid 110	Controlled at 5.5	30		600 rpm	21	32.4	81	0.48	(Rymowicz et al., 2008)

Culture	*Flask medium g/l*	*Fermenter medium g/l*	*Other metabolites*	*PH*	*Tem p ºC*	*DO%*	*Aeration VVM Agitation rpm*	*Biom ass g/l*	*Eryt hritol g/l*	*$Y_{P/S}$%*	*Q_P $g\ l^{-1}h^{-1}$*	*Reference*
		batch										
Pseudozyma tsukubaensis KN75	Glucose 300, CSL 15, 100 ml in 500 ml baffle flask,			Initial 5.5	34		250 rpm	12.8	149	49.7	1.65	(Jeya et al., 2009)
		Glucose 400, CSL 15 50,000 L Batch		Initial 5.5	34	Above 5%	1 VVM 170 rpm	23.5	243	61	1.65	
		Glucose 400, CSL 15 50,000 L Fed-batch		Initial 5.5	34	Above 20-30%	1 VVM	22.8	241	60	2.84	
Yarrowia lipolytica Wratislavia K1 Mutant		Glycerol 150+150, YE 1 NH_4Cl $_3$, KH_2PO_4 0.3, $MgSO_4$ 1 5L Fed-batch fermentation	Mannitol 12	Controlled 2.5-3	30		800 rpm	22	170	56	1	(Rymowicz et al., 2009)
Moniliella sp mutant N61188-12		Glucose 350, YE 10, 2000 L Batch fermenter	Glycerol 30 g/l	Initial 4.5	30		0.2-0.5 VVM 150-180 rpm		152	43.4	0.64	(Lin et al., 2010)
Moniliella sp mutant N61188-12		Glucose 350, YE 10, 2000 L Fed-batch fermenter	Glycerol	Initial 4.5	30		0.2-0.5 VVM 150-180 rpm		189	48	0.8	

Table 1.3 Details of erythritol producing microorganisms

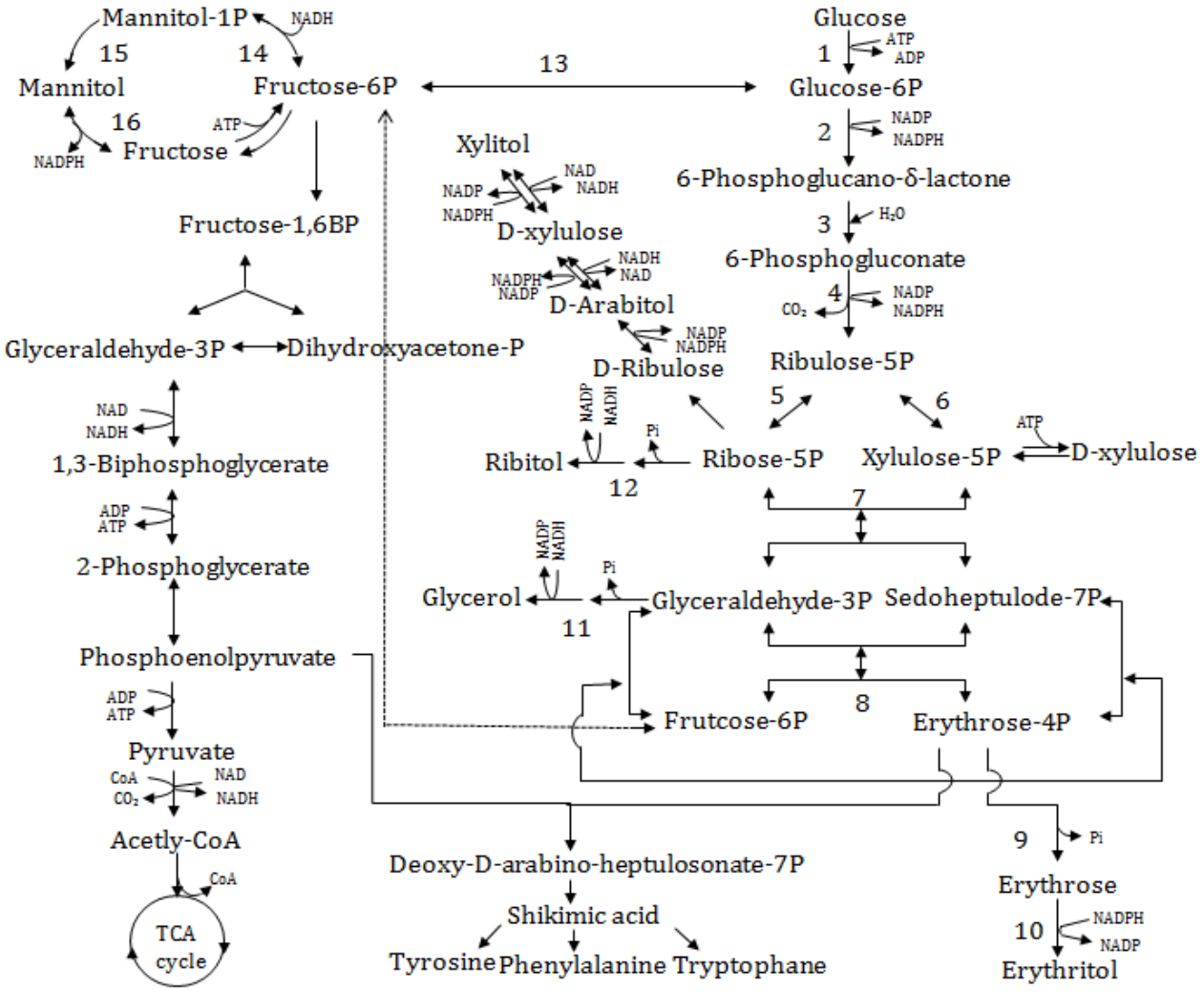
Mannitol-1P
NADH
15
14
Mannitol
Fructose-6P
13
Glucose
1
ATP
ADP
Glucose-6P
16
ATP
NADPH
Fructose
2
NADP
NADPH
6-Phosphoglucano-δ-lactone
Xylitol
NAD
NADH
NADP
NADPH
D-xylulose
3
H2O
Fructose-1,6BP
6-Phosphogluconate
NADH
NAD
NADPH
NADP
D-Arabitol
4
CO2
NADP
NADPH
NADP
NADPH
D-Ribulose
Ribulose-5P
Glyceraldehyde-3P
Dihydroxyacetone-P
5
6
NAD
NADH
NADP
NADH
Pi
ATP
Ribitol
Ribose-5P
Xylulose-5P
D-xylulose
1,3-Biphosphoglycerate
12
7
ADP
ATP
NADP
NADH
Pi
2-Phosphoglycerate
Glycerol
Glyceraldehyde-3P
Sedoheptulode-7P
11
Phosphoenolpyruvate
8
ADP
ATP
Frutcose-6P
Erythrose-4P
Pyruvate
CoA
CO2
NAD
NADH
9
Pi
Acetly-CoA
Deoxy-D-arabino-heptulosonate-7P
CoA
Erythrose
Shikimic acid
10
NADPH
NADP
TCA
cycle
Tyrosine
Phenylalanine
Tryptophane
Erythritol

Figure 1.2 Biochemical pathway of polyols synthesis. Enzymes involved 1) hexokinase, 2) glucose 6-phosphate dehydrogenase, 3) 6-phosphogluconolactonase, 4) 6-phosphogluconate dehydrogenase, 5) ribulose 5-phosphate isomerase, 6) ribulose 5-phosphate 3-epimerase, 6,7,8) transketolase, 9) erythrose-4-phosphate phosphatase, 10) erythrose reductase, 11) glyceraldehydes-3-phosphate dehydrogenase, 12) ribulose-5-phosphate-dehydrogenase, 13) glucose-6-phosphate isomerase, 14) mannitol-1-phosphate dehydrogenase, 15) mannitol 1-phosphatase and 16) mannitol dehydrogenase.

1.8 Strategies for enhancement in erythritol production

Majority of the research efforts of enhancement in erythritol production through biological processes have been focused on the selection of microbial strains able to produce erythritol with high yield and the optimization of operation strategies in batch and fed-batch fermentation processes. Comprehensive comparison of media used, erythritol concentration, productivity and yield by all reported microbial strains till date is presented in Table 1.3.

Although microbes are extremely good at producing an amazing array of valuable products, they usually produce these compounds in small amounts. When a suitable strain is found, a strain improvement program is initiated by modification of culture conditions using mutagenesis and recombinant DNA techniques in order to increase the desired product concentration. Ease of increasing production by manipulation of environmental and genetic conditions is the main reason for the use of microorganisms to produce compounds. Over 1000-fold enhancement in production level have been recorded in literature for some of the small metabolites (Demain, 2000). Following strategies have been employed for increasing the production level and yield of erythritol.

1.8.1 Strain improvement by mutagenesis

Several microorganisms have been reported to produce erythritol, most of which are yeasts that can tolerate high osmotic pressure. *Leuconostoc oenos,* a lactic acid bacterium has also been reported to produces erythritol, under anaerobic conditions (Veiga-Da-Cunha et al., 1993). Apart from improvement in erythritol titer, some of

the undesirable properties of wild strains, especially formation of undesirable co-metabolites, insufficient osmo-tolerance in media and vigorous foaming under aerobic culturing conditions were eliminated by UV irradiation and chemical mutagen treatment. Erythritol is the first sugar alcohol to be produced commercially by fermentation (Röper & Goossens, 1993).

There are several reports on use of mutants with higher erythritol production and lower undesirable properties or unwanted metabolites as discussed below.

- Ishizuka et al., (1989) generated a high erythritol-producing nonfoaming mutant of *Aureobasidium sp.* SN124A by UV irradiation and NTG treatment. The mutant gave 47.6% yield of erythritol as compared to 41.8 % of the wild type.
- *Penicillium sp*. KJUV29, a mutant derived from *Penicillium sp*. KJ81, exhibited a significantly improved erythritol production (15 g/l) and reduced glycerol and foam production compared with the wild-type strain (Lee & Lim, 2003).
- A mutant of *Torula sp*. produced erythritol at a high concentration of 196 g/l with a high yield of 49% without any byproducts formation such as glycerol and ribitol, resulting in application on an industrial scale (Kim et al., 2000; Lee et al., 2000).
- *C. magnoliae* was mutagenized using UV irradiation and NTG treatment, to improve erythritol-producing ability. An osmophilic mutant M2 of *C. magnoliae* showed 25% increase in erythritol yield and 30% increase in productivity than the wild strain (Yang et al., 1999). Optimum biological process using the mutant strain of *C. magnoliae* was developed which resulted in 200 g/l erythritol, with 1.2 $g\ l^{-1}\ h^{-1}$ productivity and 43% yield (Koh et al., 2003; Ryu et al., 2000).
- Lin et al., (2001) screened 658 strains from various sources, such as pollen, honey, and high sugar foods, and were able to isolate high erythritol-producing microorganisms identified as *Moniliella sp*. 440. Recently, a mutant of this strain named N61188-12 obtained by NTG treatment showed significantly higher erythritol productivity. Under the optimal culture conditions in a 2000 L fed-batch fermentation the mutant N61188-12 produced 189.4 g/l (Lin et al., 2010). Because of the occurrence of foaming during fermentation, the mutant N61188-12 was simultaneously improved further by performing more set of mutagenesis by NTG and Co^{60} irradiation and a promising mutant N133058-5 was obtained with an even higher erythritol production (261.8 g/l) with very little foam formation.

1.8.2 Optimization of culture conditions

In order to increase yield and productivity, erythritol producing strains have also been studied in the context of bioprocess engineering. It has been found that a high initial concentration of glucose favors erythritol production by osmophilic microorganisms (Ishizuka et al., 1989; Lin et al., 2010). If the microorganisms can tolerate a higher osmotic pressure, increase in initial glucose concentration was found to increase the production rate and yield of erythritol in a batch process. Erythritol has been produced commercially using a mutant of *Aureobasidium* in a medium containing 40% glucose (Ishizuka et al., 1989).

Maximum erythritol productivity with *C. magnoliae* mutant was achieved in a two-stage fed-batch process by optimization of cell growth and erythritol production conditions. When the initial glucose concentration was adjusted to 400 g/l by adding glucose powder during the production phase, maximum erythritol productivity of 2.8 g l^{-1} h^{-1} with 41% yield was achieved which was around fivefold higher as compared to those in the simple batch fermentation (Ryu et al., 2000).

In contrast to this, *Trichosporon sp*. produced maximum erythritol at 220 g/l glucose concentration but the production decreased at 300 g/l (Park et al., 1998a), which may be due to an osmotic effect on cells or substrate repression of glucose-metabolizing enzymes.

In *Torula sp* maximum volumetric productivity of erythritol was obtained at an initial glucose concentration of 300 g/l in batch culture. In a fed-batch culture of *Torula sp* erythritol production was improved by controlling glucose concentration (Oh et al. 2001). In the fed-batch culture, the volumetric productivity was maximal at a controlled glucose concentration around 225 g/l and reduced the lag time of erythritol production. In the optimized fed-batch process, with an initial glucose concentration of 300 g/l, which was later maintained at 225 g/l, 192 g erythritol was obtained from 400 g glucose in 88 h which corresponds to 48% yield and a volumetric productivity of 2.26 g l^{-1} h^{-1} (Oh et al., 2001). Recently, Jeya et al., (2009) reported that when *P. tsukubaensis* KN75 was grown aerobically in a fed-batch culture with glucose as a carbon source, it produced 241 g/l erythritol with 60% yield, the highest erythritol yield, ever reported through microbial fermentation process.

Although erythritol production using glucose has been extensively studied, only a few reports discuss about its production with carbon sources other than glucose. The ability of *Yarrowia lipolytica* to grow and to produce erythritol from glycerol, a renewable low cost product generated in industrial biodiesel processes, has been reported. An acetate-negative mutant of *Y. lipolytica* Wratislavia K1 showed the ability of simultaneously producing high amounts of erythritol and citric acid in glycerol-containing media (Rymowicz et al., 2008). Further, an acetate-negative mutant of *Y. lipolytica* when grown at pH 3 with 300 g/l raw glycerol produced 170 g/l erythritol after 7 days, with 56% yield and 1 $gl^{-1}h^{-1}$ productivity without citric acid production (Rymowicz et al., 2009).

Apart from the carbon source, the type and concentration nitrogen source strongly influences erythritol production. Nitrogen limitation in the active culture is a prerequisite for erythritol formation, because production of erythritol starts upon exhaustion of the nitrogen source from the medium. Yeast extract is a rich source of amino acids, polypeptides as well as vitamins and has been used by several investigators as a nitrogen source for erythritol production (Kim et al., 1999a; Ryu et al., 2000). Although yeast extract is a favourite nitrogen source in fermentation, it is expensive and also at times enhances production of undesirable compounds. An attempt was made by some researchers to minimize the yeast extract requirement in the fermentation medium by supplementation with corn steep liquor or other cheap inorganic nitrogen sources.

Media constituents used for erythritol production in flask and fermenter by reported microorganisms is presented in Table 1.3. A process for erythritol production by *Moniliella sp* has been recently patented by Edlaur et al. (2009) which uses at least one inorganic nitrogen source, either potassium nitrate or sodium nitrate, in amount of 45 to 65% of the total nitrogen source, as a pH regulator in the culture medium without formation of undesirable by-products.

1.8.3 Supplementation with vitamins and trace metals

Inositol was found to be the most effective vitamin for erythritol production in *Torula sp.* which acted as stimulator of cell growth and erythritol production (Lee et al., 2001). Erythritol production in the presence of inositol and phytic acid was greater

than that in the presence of inositol alone, due to the synergistic effect of phosphate and inositol. Supplementation of medium with phosphate and inositol increased cell growth, erythritol production and the activity of erythrose reductase in cells (Lee et al., 2001). Fermentations for erythritol production in a complete synthetic medium using yeast-like fungus were carried out by Hajny et al., (1964). Additional studies were performed to determine necessity of essential vitamins. When thiamine was omitted from the synthetic medium, resulted in poor yeast growth and lower sugar uptake and there was hardly any erythritol production. Fermentations trials using thiamine supplementation confirmed that thiamine is the essential vitamin in the synthetic medium to obtain satisfactory erythritol yields.

Torula sp. isolated from a 400 g/l sucrose solution showed improved erythritol production when the medium was supplemented with Mn^{+2} and Cu^{+2} (Kim et al., 2000; Lee et al., 2000). Cu^{+2} increased the activity of erythrose reductase in cells, whereas Mn^{+2} increased the permeation of erythritol. The synergistic effect of the increased ER activity because of Cu^{+2} and the increased cell permeability because of Mn^{+2} resulted in enhanced erythritol production (Lee et al., 2000).

1.8.4 Removal of inhibitors and byproducts

Torula corallina is a potent erythritol producer used in industrial production of erythritol (Kim et al., 2000; Lee et al., 2000; Lee et al., 2001; Oh et al., 2001). However, melanin accumulation during culture represents a serious problem for the purification of erythritol from the fermentation broth. The 1,8-dihydroxynaphthalene (DHN)-melanin pathway in *T. corallina* is responsible for cell wall melanization and the inhibition of erythrose reductase. Supplementation of tricyclazole, inhibitor of DHN melanin biosynthesis to the culture medium increased the erythritol production by *T. corallina* (Lee et al., 2003e). Tricyclazole inhibits the production of DHN-melanin, an inhibitor of erythrose reductase. As a result, erythrose reductase activity may be less repressed, which results in a higher yield of erythritol (Fig. 1.3).

Erythrose reductase purified from *C. magnoliae* showed unusual dual coenzyme specificity (Lee et al., 2003f). Fumarate and DHN-melanin inhibited the activity of erythrose reductase in an uncompetitive and non-competitive type, respectively. Erythritol production could be improved in *C. magnoliae* by inhibiting the synthesis

of byproducts thereby enhancing the erythrose reductase activity (Lee et al., 2003e; Lee et al., 2002). Correspondingly, supplementation with Cu^{+2} in cultures of *T. coralline* reduced the production of fumarate, a strong inhibitor of erythrose reductase. Hence, erythrose reductase activity became less inhibited and a high yield of erythritol was produced (Lee et al., 2002).

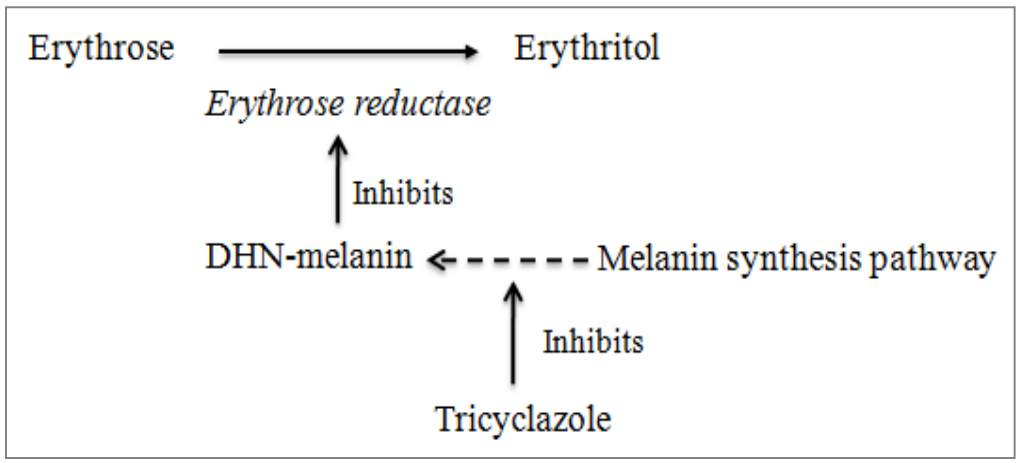

Figure 1.3 Schematic representation of enhanced erythritol production in *Torula sp*

Proteome analysis of *C. magnoliae* and its mutant derivative having enhanced growth and erythritol production from D-glucose showed altered expression levels of some of the genes encoding TCA cycle and glycolytic enzymes. The levels of the TCA cycle enzymes (citrate synthase, succinyl-CoA ligase and fumarase) and the glycolytic pyruvate decarboxylase were elevated in the mutant strain, whereas the level of enolase was diminished compared with the wild-type strain (Lee et al., 2003a). Down-regulation of enolase in the mutant probably directed the flux of erythrose-4-phosphate to erythritol production rather than shikimate biosynthesis. Up-regulation of fumarase in the mutant coincided with the result in the inhibitory effect of fumarate on erythritol production (Park et al., 2005).

Trichosporonoides megachiliensis SN-G42 is used for commercial production of erythritol. Activities of PP pathway enzymes of this organism were measured under various culture conditions to examine the production mechanism and the key enzymes in erythritol biosynthesis by Sawada et al., (2009). The activities of hexokinase, glucose-6-phosphate dehydrogenase, gluconate dehydrogenase, transketolase, transaldolase and erythrose reductase were evaluated. In the cultures, in which

erythritol was produced after completion of cell growth, activities of PP pathway enzymes were higher than those of the TCA cycle. In particular, transketolase activity was correlated with erythritol productivity and a high transketolase activity is required to produce abundant intermediates, which results in high erythritol productivity. Therefore, transketolase appears to be a key enzyme for erythritol production in the said microorganism (Sawada et al., 2009).

1.9 Erythrose reductase (EC 1.1.21)

Erythrose reductase, an NAD(P)H dependant aldose reductase, is a key enzyme in the erythritol biosynthesis (Ishizuka et al., 1989; Lee et al., 2003f; Tokuoka et al., 1992). Erythrose reductase is present in a number of organisms and catalyzes the reduction of erythrose into erythritol. Tokuoka et al., (1992) and Lee et al., (2003d) purified ER from *Aureobasidium sp*. SN-G42 mutant and *T. corallina*, respectively. Tokuoka et al., (1992) reported the three isozymes (ER-1: MW 38,000; ER-2 and ER-3: MW 37,000) of erythrose reductase in *Aureobasidium sp*. SN-G42. The complete gene sequences of the three isozymes of ER were determined by Ookura et al., (2005). *T. corallina* expressed an erythrose reductase with 7.12 mM *Km* and 26 μmol min^{-1} mg-$protein^{-1}$ of *Vmax* (Lee et al., 2003d).

Although there are reports related to the purification and characterization of erythrose reductase (ER) from microbial sources (Lee et al., 2003d; Lee et al., 2003f), the primary structure and the corresponding DNA for ER are not known in most of the erythritol-producing yeasts. ER-encoding genes were identified only recently in *T. megachiliensis* SNG-42 by Ookura et al., (2005), which is one of the yeasts used for commercial production of erythritol.

Recently, ER from *C. magnoliae* JH110 and *C. magnoliae* mutant KFCC-11023 were studied. ER from this mutant has enzymatic properties different from the previously reported ER of *C. magnoliae* JH110 (Lee et al., 2010). The ER gene from *C. magnoliae* JH110 is composed of 849 nucleotides which encodes a polypeptide with molecular mass of 31.4 kDa. The amino acid sequence of ER has a high degree of similarity to other members of the aldo-keto reductase super family including three ER isozymes from *Trichosporonoides megachiliensis* SNG-42. The intact coding region of ER from *C. magnoliae* JH110 was cloned, functionally expressed in

Escherichia coli using a combined approach of gene fusion and molecular chaperone co-expression, and subsequently purified to homogeneity. Molecular basis of erythrose reduction with NADPH, studied by homology structural modeling suggested that NADPH binding partners are completely conserved in the *C. magnoliae* JH110 ER. Furthermore, NADPH interacts with the amino acids Lys252, Thr255, and Arg258, responsible for the enzyme's absolute requirement of NADPH over NADH (Lin et al., 2010).

A comparison of kinetic characteristics of purified ERs from *C. magnoliae* JH 110*, C. magnoliae* KFCC-11023, *S. cerevisiae*, *Torula corallina, T. megachiliensis* SNG-42, *Schizophyllum commune* and *Hordeum vulgare* (Barley) is presented in Table 1.4. *C. magnoliae* ER had a higher k_{cat} and higher catalytic efficiencies (k_{cat}/Km) with respect to erythrose and NADPH. The *Km* value for D-erythrose (8.5 mM and 7.9) was comparable to the values for the ERs from *T. megachiliensis* SNG-42, *T. corallina*, and *S. commune*, which had *Km* of 8.2, 7.1, and 5.0 mM, respectively. There were several key differences between two ERs from *C. magnoliae* KFCC- 11023, a mutant of *C. magnoliae* JH110. The molecular weights and apparent kinetic constants were also different. The two enzymes differed in their pH optima and *Km* values (Table 1.4).

A homology model of *C. magnoliae* JH110 erythrose reductase docked with NADPH was created by (Lin et al., 2010). Unlike other aldose reductase (ALR) enzymes, the resulting *C. magnoliae* ER model is an (α8/β6) barrel oxidoreductase with only one α-helix (H1). A hairpin (B1 + B2) covers one end of the barrel while a NAPDH binding site is sealing the opposite end. The H1-helix represents a junction between strand β6 and helix α7. However, the overall folding and binding of coenzyme were similar to the barley ALR crystal structure. Comparison of homology model crystal structure of *C. magnoliae* JH110 ER (A) and barley ALR1 (B) is presented in (Fig. 1.4). The conserved catalytic residues, Trp31, Tyr60, His121, and Trp122 in *C. magnoliae* ER as well as from barley ALR have similar orientations and locations in the *C magnoliae* ER model. Structures of other aldose ketose reductase (AKR) super family members display similar interactions with NADPH.

ER Source	**Molecular mass**		**Optimum**		**Erythrose**				**NADPH**		*Reference*
	Subunit (kDa)	***Native (kDa)***	***pH***	***Temp. (°C)***	***Km (mM)***	k_{cat} (s^{-1})	***kcat/Km*** $(mM^{-1}\ s^{-1})$	k_{cat} (s^{-1})	***Km (mM)***	k_{cat}/Km $(mM^{-1}\ s^{1})$	
Sccharomyces cerevisiae	35	N.R.	N.R.	N.R.	3.4	N.R.	N.R.	N.R.	N.R.	N.R.	(Kataoka et al., 1992)
Trichosporonoides megachiliensis	38	38	6.5	45	7.1	N.R.	N.R.	N.R.	N.R.	N.R.	(Tokuoka et al., 1992)
Trichosporonoides megachiliensis	37	37	6.5	45	7.6	N.R.	N.R.	N.R.	N.R.	N.R.	(Tokuoka et al., 1992)
Trichosporonoides megachiliensis	37	37	6.5	45	8.2	N.R.	N.R.	N.R.	N.R.	N.R.	(Tokuoka et al., 1992)
Hordeum vulgare	34	34	N.R.	N.R.	45	2.3	51	1.5	0.2	9.0	(Roncarati et al., 1995)
Torulla coralline	35.4	71.0	6.0	40	7.12	N.R.	N.R.	N.R.	N.R.	N.R.	(Lee et al., 2003d)
Candida magnoliae KFCC-11023	38.8	79.0	7.0	N.R.	7.9	5.7	0.73	0.013	450	0.66	(Lee et al., 2003f)
Candida magnoliae JH110	31	N.R.	5.5	42	8.5	7.6	0.89	48	0.016	3000	(Lee et al., 2010)

Table 1.4 Properties of ER from various organisms [a]NR = Not reported

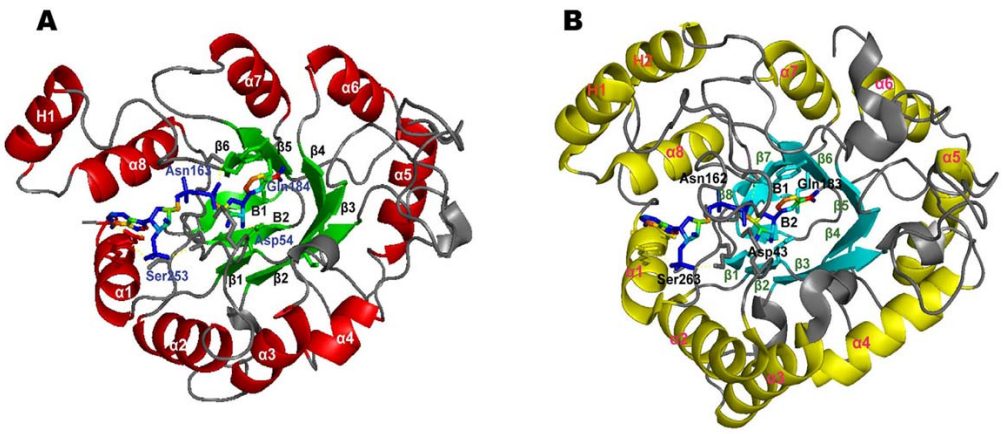

Figure 1.4 Comparison of the crystal structure of homology model for *C. magnoliae* JH110 ER (A) with barley ALR1 (B); adopted from (Lin et al., 2010). Helices are red (*C. magnoliae* ER) and yellow (barley ALR), and sheets are green (*C. magnoliae* ER) and cyan (barley ALR). The cofactor is included in sticks. Amino acids bound to the NADPH were colored by blue (*C. magnoliae* ER) and black (barley ALR).

1.10 Uses of erythritol

Erythritol has been part of the human diet for thousands of years. It is used as a flavor enhancer, formulation aid, humectants, nutritive sweetener, stabilizer, thickener, sequestrant and texturizer at maximum level of 100% in sugar substitutes; 60% in fat-based cream for use in cookies, cakes and pastries; 50% in hard candies; 40% in soft candies; 1.5% in reduced and low-calorie beverages (Park et al., 2005). Erythritol is preferred over other polyols because (1) It evokes a very less or no glycemic response. (2) It is produced by natural processes and can well be incorporated into foods that claim to be "natural". (3) It yields 0-0.2 cal/g while the other polyols yield approximately 2 cal/g. (4) From the digestive point of view, it is well tolerated and results in minimum or no gastric discomfort (Goossens & Gonze, 1996). Erythritol is intended for use as a bulk sweetener in confectionery, chewing gum, beverages, and bakery products. Because it does not affect glucose and insulin levels, it is safe for diabetic patients.

Erythritol acts as an antioxidant *in-vivo* and may help protect against hyperglycemia induced vascular damage (den Hartog et al., 2010). While all other polyols have the distinct disadvantage of causing digestive distress, erythritol is well-tolerated.

Scientific reports show that at typical consumption levels, erythritol has no laxative effect. The key factor in erythritol high digestive tolerance is its small molecular size (Harald & Jozef, 1993). This allows it to be absorbed quickly in the upper digestive tract, so that very small amount reaches the lower tract-where degradation of polyols and osmotic effects would normally cause gastric distress.

India is the second largest sugar producer in the world and produces over 20 million tonnes of sugar a year. India is the world's largest consumer of sugar. The country also hosts the highest number of diabetic patients in a country. The number of diabetics' patients has doubled in the last decade and is likely to touch 70 million in the next fifteen years. It has been known for a long time that calorie intake of urban population is excessive. Modernization of lifestyle which has transformed our eating habits, consumption of fast-food rich in starch and oil and use of "sweets" to celebrate each and every occasion are the main reasons for increasing number of diabetic patients.

The market for low-calorie sweeteners in India is currently valued at around Rs 600 million. The market is growing at a rate of around 28 percent per year. In the Indian low- calorie sweetener market, the `Sugar Free' brand (aspartame) from Zydus Cadila has a 72 percent share, while `Equal' from Merisant has 14 percent share. Alembic has launched the only zero- calorie sweetener sucralose in India under the brand name of `Zero' (http://www.ibid.informindia.co.in/results.asp).

1.11 Mannitol

D-Mannitol is the most abundant polyol in nature. It is produced by bacteria, yeasts, fungi, algae, lichens and many plants (Wisselink et al., 2002). Mannitol is about half as sweet as sucrose, mostly not metabolized by humans and therefore considered as a low-calorie sweetener (Soetaert et al., 1999). D-Mannitol and D-sorbitol, both isomers are produced by hydrogenation of second carbonyl carbon atom of D-fructose (Fig.1.5).

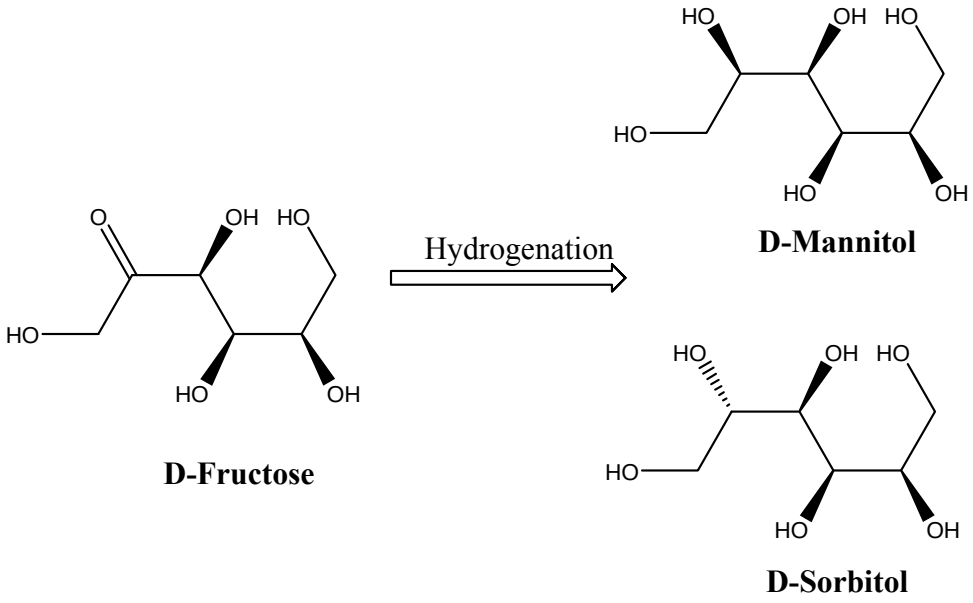

Figure 1.5 Chemical structures of D-fructose, D-mannitol and D-sorbitol

Mannitol forms white, needle-like crystals and has a melting point 165-170 ˚C. Due to its high negative heat of solution (–121 kJ/kg) it has a sweet cool taste. The solubility of mannitol in water is significantly lower than that of sorbitol and most of the other sugar alcohols. At 25 **°**C the solubility of mannitol in water is approximately 180 g/l (Perry et al., 1997). Mannitol is sparingly soluble in organic solvents unlike ethanol and glycerol and practically insoluble in ether, ketones and hydrocarbons (Patra et al., 2009).

Its sweetness is approximately half as compared to sucrose. However, like other sugar alcohols, mannitol exhibits reduced caloric values compared to that of sucrose, making it applicable as sweeteners in so-called “light” foods. Chemical synthesis is the only strategy that is able to meet the present mannitol market demand.

Biotechnological production of mannitol has been implemented using yeasts, fungi and bacteria in recent years (Saha & Racine, 2011). Lactic acid bacteria (LAB) are particularly used for mannitol production as they display a fermentative metabolism associated with an important redox modulation and has a limited biosynthetic capacity (Monedero et al., 2010).

1.12 Chemical process for mannitol production

Mannitol is produced industrially by high pressure hydrogenation of fructose and glucose- fructose mixtures in aqueous solution at high temperature (120–160 °C) with Raney nickel catalyst. α-Fructose gets converted to mannitol and β-fructose is converted to sorbitol where as glucose is exclusively hydrogenated to sorbitol. Due to poor selectivity of the nickel catalyst, hydrogenation of 50:50 fructose:glucose mixture results in an approximately 25:75 mixture of mannitol and sorbitol (Saha & Racine, 2011). Even when pure fructose is catalytically hydrogenated, only about 50% of it is converted into mannitol, whereas the rest is converted into sorbitol.

Chemical hydrogenation process has several limitations, including the need for highly purified substrates, high reaction temperatures and pressures, costly purification steps and a poor mannitol yield. For pharmaceutical use metallic impurities coming from catalyst needs to be removed by chromatographic methods. This process produces mostly sorbitol and mannitol is produced as the side product. This situation makes mannitol production dependent on the market and price of sorbitol. Because of the drawbacks associated with the current mannitol production process and increasing market demand, biological mannitol production processes have been actively investigated predominantly in the last decade (Table 1.5).

Makkee et al. (1985) developed a process involving both bio- and chemo catalysts for the conversion of glucose and fructose mixture into mannitol. Good yield (62–66%) was obtained by using glucose isomerase (GI) immobilized on silica in combination with a copper-on-silica catalyst (Makkee et al., 1985). In another method, mannitol was produced from mannose by hydrogenation with 100% stoichiometric yield by Devos (1995). Mannose can be obtained from glucose by chemical epimerization with a yield of 30–36% (w/w). Thus, the mannitol yield from glucose can be as high as 36%. However, the total cost of using the multi-step process is not economical.

1.13 Mannitol production by lactic acid bacteria (LABs)

Several lactic acid bacterial strains are known to produce mannitol (Table 1.5). Its production by 'food-grade' lactic acid bacteria offers important advantages. Firstly, their products are directly applicable in food products, without any restriction. Secondly, there is no need for a careful separation of products and microorganisms. Thirdly, some LABs are claimed as beneficial in the gastrointestinal tract. Mannitol production by those bacteria may strengthen their health-promoting ability. Mannitol production in LAB is strongly dependent on the pathway of carbohydrate fermentation. LABs are grouped either as homofermentative or heterofermentative based on their metabolic pathways and end products synthesized (Wisselink et al., 2002). Both pathways are briefly described below in relation to mannitol production.

1.13.1 Mannitol production by homofermentative LABs

Homofermentative LABs are widely used in dairy industries. Lactic acid is the main product of sugar fermentation in these bacteria and they do not normally produce mannitol. In homofermentative LAB, the carbon flow from carbohydrates is directed mainly to lactate production, formation of other fermentation products like mannitol is possible only when strains are more or less hampered in the lactate production.

Some homofermentative LABs such as *Lactobacillus leichmanii* and *Streptococcus mutans* were found to produce small amounts of mannitol (Wisselink et al., 2002). When homofermentative lactic acid bacteria grow on substrates such as glucose, a typical homolactic fermentation pattern can be observed. Pyruvate is reduced to lactic acid by a NADH-dependent lactate dehydrogenase (LDH), thereby reoxidizing the NADH formed during the early glycolytic steps. Under certain conditions, glycolysis may also lead to a mixed acid fermentation, leading to end products such as acetate, ethanol, 2,3-butanediol (Fig. 1.6a).

In homofermentative LABs, sugars such as glucose and fructose are phosphorylated by phosphotransferase system (PTS) before entering glycolysis and then translocated into the cell. In homofermentative LABs, mannitol synthesis starts from fructose-6-phosphate. Mannitol-1-phosphate dehydrogenase (M1P-DH, EC 1.1.1.17) reduces fructose-6-phosphate into mannitol-1-phosphate, which in turn, is dephosphorylated to mannitol by mannitol-1-phosphatase (M1Pase, EC 3.1.3.22).

1.13.2 Mannitol production by heterofermentative LABs

The natural mannitol-producing lactic acid bacteria show a heterofermentative metabolism. Heterofermentative lactic acid bacteria use a combination of the hexose monophosphate pathway and the phosphoketolase pathway for carbohydrate dissimilation. Under anaerobic condition, glucose is converted to equimolar amount of lactic acid, ethanol and carbon dioxide and one mol of ATP is produced per mol of glucose fermented (Soetaert et al., 1999). With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Then the regeneration of NAD^+ is achieved by using an alternative electron acceptor. Under anaerobic conditions, compounds like pyruvate and fructose are reduced. Mannitol is the end product of fructose reduction, which is catalyzed by the enzyme mannitol dehydrogenase (Fig. 1.6b). In heterofermentative LABs mmannitol production was found in concentrations up to 200 g/l at 37 °C, more than its solubility limit of 180 g/l at 25°C, indicating that mannitol has no toxic effects on the organism.

The enzyme mannitol dehydrogenase plays a key role in this conversion (Soetaert et al., 1999; von Weymarn et al., 2002a). When heterofermentative LABs were grown on glucose plus fructose mixture, they preferentially used glucose as carbon source for metabolism and to produce ATP, while fructose was reduced to mannitol to replenish the cell's NAD(P) pool. In these conditions, the fermentation follows *Eq. 1.1* (Grobben et al., 2001).

1 Glucose + 2 Fructose → 2 Mannitol + 1 Lactic acid +1 Acetic acid + CO_2 ……. Eq 1.1

Heterofermentative species do not use the PTS for hexose uptake. Glucose and fructose enter the cell through permeases, before being phosphorylated by glucokinase and fructokinase, respectively. In heterofermentative LABs, reduction of fructose to mannitol is catalyzed by an NADH-dependent mannitol dehydrogenase (MDH, EC 1.1.1.67), which reduces fructose exclusively to mannitol (Wisselink et al., 2002). This enzyme is also able to oxidize mannitol to fructose, but with a fourfold lower activity (Table 1.5).

Culture	***Mode of operation***	***Substrate (g/l)***	***Yield% (g/g fruc)***	***$Q_{(p)}$ ($g\ l^{-1}h^{-1}$)***	***Mannitol (g/l)***	***Reference***
Lactobacillus sp KY-107	Batch	Fructose 100	70	0.87	70	(Yun et al., 1996)
Lactobacillus fermentum	Batch	Glu 50+Fru 100	89.6	7.6	83	(von Weymarn et al., 2002a)
Leuconostoc mesenteroides	Fed-batch	Fru 50 +150	89.9	1.79	179	(Kim et al., 2002)
Leuconostoc mesenteroides	Resting cells MCRB	Glu 50+Fru 100	97	26.2	98	(Von Weymarn et al., 2002b)
Lactobacillus intermedius	Batch	Fructose 300	66.1	1.45	198.3	(Saha & Nakamura,
	Fed-batch	Glu/fru ratio 1:2	67.5	2.2	202.5	2003)
Leuconostoc mesenteroides	Resting cells MCRB	Glu 50+Fru 100	87	18.8	87	(von Weymarn et al., 2003)
C. magnoliae HH-01	Fed-batch	Glu 50 fru 250	84	1.94	213	(Lee et al., 2003c)
L. lactis, Δldh ΔmtlF	Batch, Resting cells	Glu 7.2	32.8	2.7	2.4	(Gaspar et al., 2004)
E. coli overexpressing *MDH, FDH* and *GLF*	Batch, Resting cells	Fru 90 NaFormate17	84	8.2	66	(Kaup et al., 2004)
*L. lactis Δldh,*overexpressing *M1PDH* and *M1Pase*	Batch	Glu 20	50	0.16	9	(Wisselink et al., 2005)
E. coli overexpressing	Batch, Resting cells	Glu 180	80	3.6	146	(Kaup et al., 2005)

Culture	***Mode of operation***	***Substrate (g/l)***	***Yield% (g/g fruc)***	***$Q_{(p)}$ ($g\ l^{-1}h^{-1}$)***	***Mannitol (g/l)***	***Reference***
MDH, FDH and *GLF*		NaFormate34				
Lactobacillus mesenteroides	Batch	Fru/sucrose 1:1 (150)	87.5	4.7	105	(Saha, 2006a)
Lactobacillus mesenteroides	SSF	Inulin 300	69	2.88	207	(Saha, 2006b)
		Fru 150+Inulin 250	57	2.1	227	
Lactobacillus intermedius	Fed-batch MCRB	Glu 33.5+Fru 67	94.6	5.9	176	(Racine & Saha,
		Glu 50+Fru 100	93	28.4	95	2007)
C. glutamicum overexpressing *MDH, FDH* and *GLF*	Fed-batch, Resting cells	Fructose 94 NaFormate17	91	2.7	87	(Bäumchen & Bringer-Meyer, 2007)
Bacillus megaterium overexpressing *MDH* and *FDH*	Resting cells	Fructose 90 NaFormate17	91	0.92	22	(Bäumchen et al., 2007)
C. magnoliae HH-01	Fed-batch	Glu 30 fru 250	88	1.72	223	(Lee et al., 2007)
Lactobacillus mesenteroides	Batch	Cashu apple juice Glu/fru ratio 1:2	66.2	1.8	18	(Fontes et al., 2009)
C. magnoliae NCIM 3470	Resting cells	Glycerol 100	50	0.53	51	(Khan et al., 2009)

Table 1.5 Biotechnological production of mannitol, [a] Membrane cell recycle bioreactor

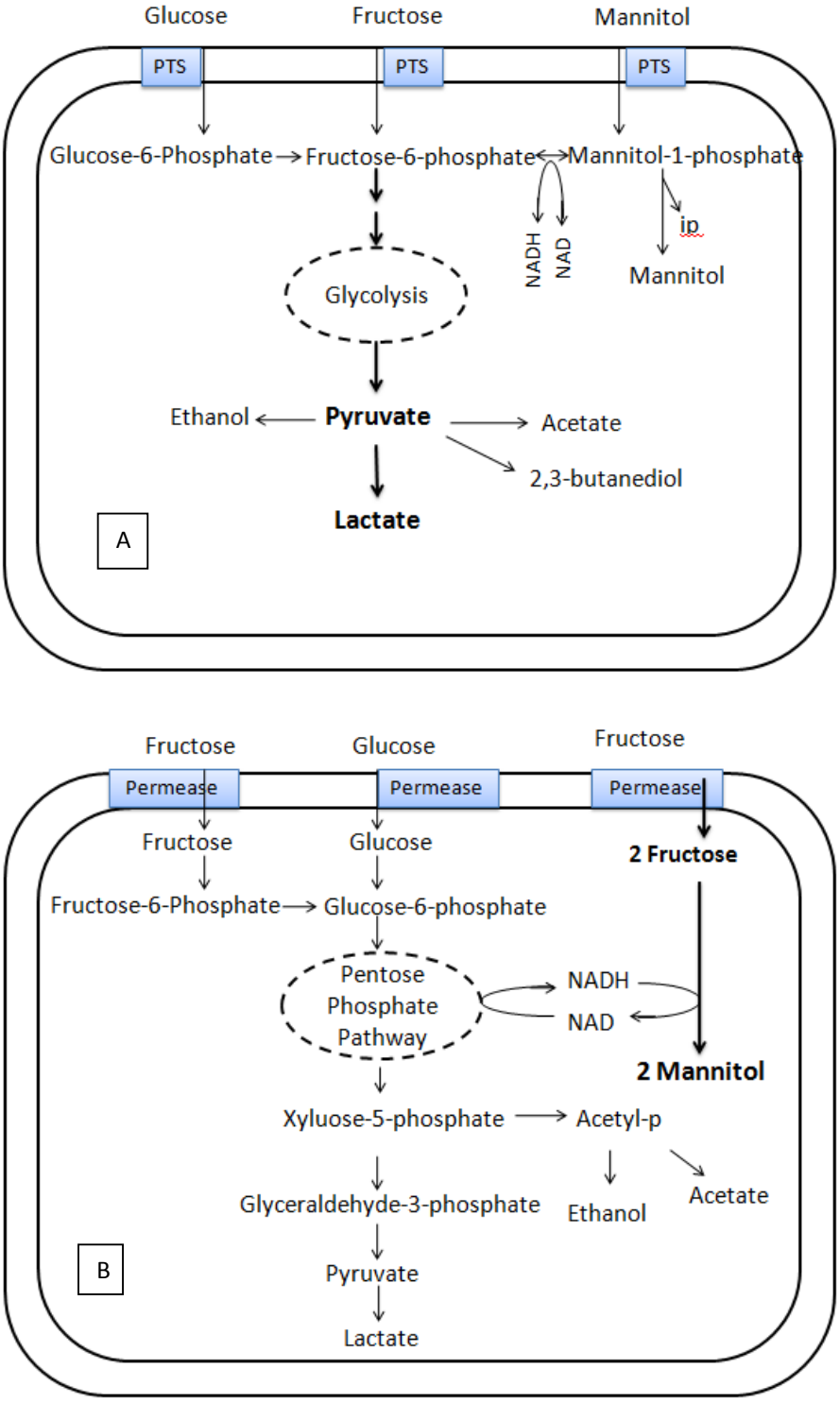

Figure 1.6a, b Biosynthetic pathways of mannitol in LABs, (A) homofermentative LABs and (B) heterofermentative LABs

Thus, mannitol biosynthesis in LABs is achieved via two main routes. 1) In heterofermentative LABs direct reduction of fructose to mannitol by a NADH-dependent MDH. 2) In homofermentative which includes the formation of fructose 6-phosphate from fructose by fructokinase, the reduction to mannitol 1-phosphate by a NAD(P)H-linked mannitol-1-phosphate dehydrogenase, and the dephosphorylation of mannitol 1-phosphate into mannitol by mannitol 1-phosphatase. Schematic overview of mannitol cycle in homofermentative and heterofermentative LABs is represented in Fig. 1.7. Heterofermentative LABs do not express a M1P-DH and their MDH is inactive on mannitol-1-phosphate, fructose-1-phosphate and fructose-6-phosphate (Grobben et al., 2001). Thus, homo- and heterofermentative LABs distinctly differ in their mannitol production pathways.

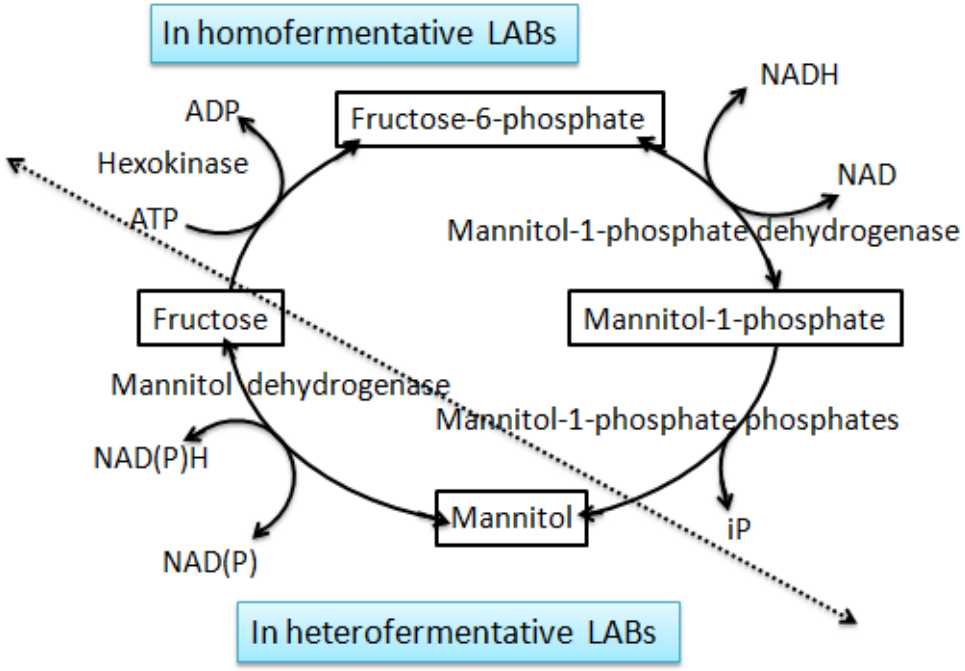

Figure 1.7 Schematic overview of the mannitol cycle in homofermentative and heterofermentative LABs

The knowledge of mannitol formation in LABs dates back to late 1930's (Onishi & Suzuki, 1968). Research relating to the mannitol-producing ability of these bacteria was re-awakened by studies with *Leuconostoc pseudomesenteroides* ATCC 12291 (Soetaert, 1990). Trying to produce sucrose phosphorylase with this species, the group at Ghent University, Belgium, observed that the fructose moiety was efficiently converted into mannitol. Moreover, they found that low pH (4.5) increases the yield

of mannitol from fructose. Soetaert (1990) reported average volumetric mannitol productivity of 6.3 g $l^{-1}h^{-1}$ and a yield of 94 mol%, using a fed-batch cultivation protocol with *L. pseudomesenteroides*. Based on continuous cultivation studies it was also observed that the yield of mannitol from fructose was strongly correlated to the substrate concentration in the growth medium. At a low fructose concentration (3 g/l) only 48 mol% yield was obtained, while 99 mol% yield was obtained at 120 g/l (Soetaert, 1990). Grobben et al., (2001) reported spontaneous formation of a mannitol-producing variant of *Leuconostoc pseudomesenteroides* grown in the presence of fructose. The mannitol producing variant differed from the mannitol-negative original strain in two physiological aspects: presence of MDH activity and the simultaneous utilization of fructose and glucose.

Heterofermentative LABs have been extensively studied for mannitol production from fructose. Most of the studies of mannitol production by heterofermentative LABs have been focused on optimizing mannitol conversion processes using wild-type strains. Optimization of mannitol production in heterofermentative LABs has been usually carried out by a classical methodology which included changes in medium composition, the use of different glucose:fructose ratios and cell-recycle bioreactors. Volumetric productivities up to 26 g $l^{-1}h^{-1}$ with 100 to 200 g/l of mannitol have been reported by many researchers (Table 1.5). Inspite of the high mannitol yield, mannitol synthesis was always dependent on the presence of fructose. Moreover, LABs are fastidious organisms, unable to synthesize many of their essential building blocks and typically grow on expensive yeast extract and peptone based medium. For cost-effective industrial production of mannitol by fermentation process, economical carbon and nitrogen sources are required to replace fructose syrup, peptone and yeast extract. Saha (2006a) investigated the effects of molasses, various inorganic, organic and complex nitrogen sources such as corn steep liquor on mannitol and lactic acid production by *Lactobacillus intermedius* NRRL B-3693. Soy peptone and corn steep liquor, cheaper nitrogen sources could support proper growth and mannitol production which could replace Bacto-peptone and Bacto-yeast extract in batch fermentations. *L. intermedius* was also shown to produce mannitol in high yields from inulin in a simultaneous saccharification and fermentation (SSF) process. Inulin is a polymer of β-2,1-linked fructose with a glucose residue at its reducing end and is found as a storage polymer in a variety of tubers and roots. In optimized

conditions (with a mix of endo- and exo-inulinases and at 37°C), the SSF process yielded final mannitol concentrations over 200 g/l (Saha, 2006b). Cashew apple juice a cheap and abundant agriculture by-product of cashew nuts production that was also investigated as a feedstock for mannitol production with *Leuconostoc mesenteroides*. The results suggest that this carbon source, which naturally contains ~ 55% fructose and ~ 45% glucose, is a suitable feedstock for mannitol production (Fontes et al., 2009). Another approach was investigated by Von Weymarn et al., (2002b) for decreasing the cost of LAB-based mannitol production processes by growing the bacterial biomass first, and then repeatedly using the resting cells in membrane cell recycle bioreactors (MCRB). They could obtain a stable, high-level mannitol production in 14 successive bioconversion batches without decrease in cell viability between the batches. The concentrations of by-products lactate and acetate did not vary from batch to batch. A significantly high volumetric productivity of 26 g l^{-1} h^{-1} was obtained in this MCRB process. The scalability of this MCRB mannitol production process was also investigated at a 100-l pilot plant scale by von Weymarn et al., (2003). Volumetric productivity up to 40 g l^{-1} h^{-1} was obtained by overcoming the limitation caused by high substrate concentration with continuous feeding of fructose syrup in a continuous cell-recycle fermentation process (Racine & Saha, 2007).

1.14 Mannitol production by yeast and filamentous fungi

A mannitol-producing Yeast *Candida magnoliae* HH-01 was isolated from fermentation sludge during a screening of over 1,000 microorganisms by Song et al., (2002). In an optimized fed-batch fermentation process, *C. magnoliae* produced 208 g/l mannitol from fructose. Improvements in the fed-batch process included co-feeding glucose with fructose and supplementing the culture with Ca^{+2} and Cu^{+2} (Baek et al., 2003; Lee et al., 2007; Lee et al., 2003c). Although the final mannitol concentrations using *C. magnoliae* were among the highest reported, the volumetric productivity was comparatively low (Table 1.5). Production of mannitol in *C. magnoliae*, from fructose is achieved by an NADP-dependent MDH. The co-feeding of glucose was used for cell maintenance and NADPH regeneration (Baek et al., 2003). Lee et al. (2007) investigated that addition of Ca^{+2} to the medium increased cell permeability and mannitol secretion, while Cu^{+2} increased the activity of MDH.

Khan et al., (2009) reported that the resting cells of *C. magnoliae* produced mannitol from glycerol with a yield of 45%. Apart from mannitol, *C. magnoliae* is also reported to produce glycerol (Sahoo & Agarwal, 2002), erythritol (Koh et al., 2003) and xylitol (Tada et al., 2004), using different substrates and fermentation conditions.

Several filamentous fungi produce mannitol from variety carbohydrates. *Aspergillus candidus* was found to produce mannitol from glucose (Smiley et al., 1967). Enzymatic studies of the metabolic route confirmed that in *A. candidus* mannitol is formed from glucose via fructose 6-phosphate and mannitol 1- phosphate (Strandberg, 1969). Consequently, a process was developed from glucose which gave 31.0% yield and 0.15 g l^{-1} h^{-1} volumetric productivity. The same metabolic route (glucose to mannitol) seemed to be active in *Penicillium sp* (Boonsaeng et al., 1976). Hendriksen et al., (1988) found *Penicillium scabrosum* produced high concentration of mannitol and glycerol from sucrose. The volumetric mannitol productivity (0.14 g $l^{-1}h^{-1}$) was similar to that reported for *A. candidus*. However, the yield (56.7 mol %) was clearly better. Both Smiley *et al*. (1969) and Hendriksen *et al*. (1988) noted that when the sugar in the medium was exhausted, the cells started to reutilize sugar alcohols.

1.15 Mannitol production by recombinant microorganisms

Biocatalytic conversion of fructose to mannitol, without formation of sorbitol, has been accomplished with genetically engineered *Escherichia coli*. The constructed recombinant *E.coli* co-expressed *L. pseudomesenteroides mdh* gene, encoding MDH; the *Mycobacterium vaccae fdh* gene, encoding formate dehydrogenase (FDH) and the *Zymomonas mobilis glf* gene, encoding the glucose facilitator protein (GLF). The engineered strain was able to take up fructose independent of PTS and stoichiometrically couple fructose reduction to mannitol with formate oxidation to CO_2 (Fig. 1.8). This bioconversion produced mannitol essentially free of by-products with a good volumetric productivity of 8.2 g l^{-1} h^{-1} (Kaup et al., 2004). Supplementing the recombinant strain expressing MDH, FDH and GLF with extracellular glucose isomerase resulted in production of 146 g/l mannitol from 180 g/l glucose. Overexpressing glucose isomerase intracellularly rather than providing the enzyme extracellularly led to a less efficient conversion of glucose to mannitol and only 76 g mannitol was produced from 180 g/l glucose (Kaup et al., 2005). A similar approach was also followed using *Bacillus megaterium* and *Corynebacterium glutamicum*

expressing MDH and FDH. The processes had mannitol yields over 90 mol% from fructose with no accumulation of by-products and the biomass could be used in repeated bioconversions. However, the volumetric productivity was low, 2.7 g $l^{-1}h^{-1}$ (Bäumchen & Bringer-Meyer, 2007; Bäumchen et al., 2007).

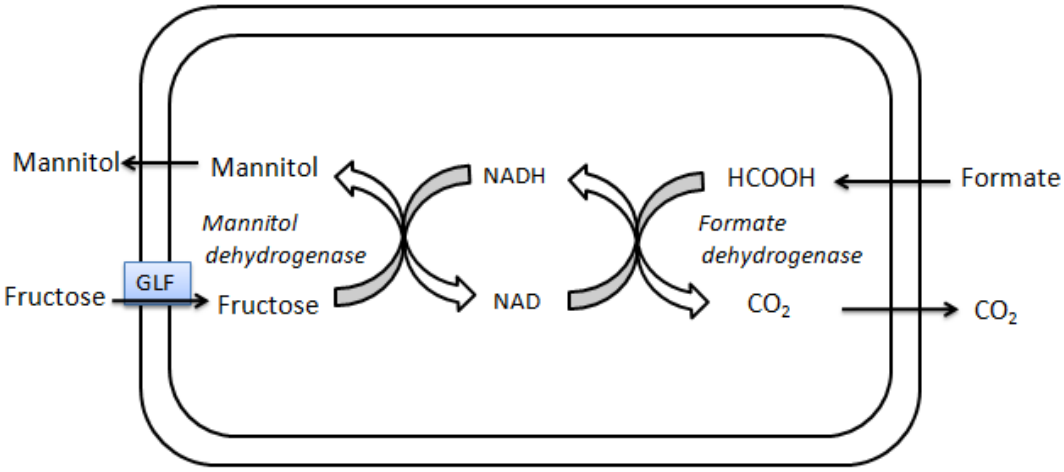

Figure 1.8 D-Mannitol production from D-fructose using a recombinant redox cycle in *Escherichia coli*

Gaspar et al., (2004) and Wisselink et al., (2005) engineered *L. lactis* for mannitol production. A mannitol-producing *L. lactis* was developed by deleting mannitol-specific PTS gene mtlF in a food grade *Δldh L. lactis* strain (Gaspar et al., 2004). Glucose metabolism by non-growing cells of the *Δldh, ΔmtlF* strain produced mannitol as a major end product. In a different approach, Wisselink et al., (2005) co-expressed *L. plantarum mtlD* gene (encoding M1P-DH) and the *Eimeria tenella* mannitol 1-phosphatase (*M1Pase*) gene in *Δldh L. lactis*. Optimizing induction conditions and substrate concentrations resulted in glucose to mannitol conversion up to 50 mol% (Wisselink et al., 2005). In both these studies, by-products such as formate, lactate, ethanol and 2,3-butanediol were also produced in significant quantities.

1.16 Mannitol dehydrogenase (EC 1.1.67)

MDH catalyzes the oxidation of D-mannitol and reduction of D-fructose and is found in a number of organisms. Lee et al., (2003b) purified and characterized a novel NAD(P)H dependent MDH from industrially mannitol-producing *Candida magnoliae* HH-01. This MDH was distinguished from the MDHs of other microorganisms by its

high substrate specificity and high catalytic efficiency only for fructose. These characteristics reflected the high yield and productivity of mannitol from fructose by *C. magnoliae* HH-1. Comparison of properties of NAD(P)H dependent MDH from various sources is accessible in Table 1.6

MDH	***Sub unit (kDa)***	***Native (kDa)***	***PI***	***Tem (°C)***	***pH Red***	***pH Oxy***	***Km Fru (µM)***	***Co-factor***	***Ref***
Leuconostoc mesenteroides	38	137	3.9		5.3	8.0	35	NADH	(Yamanaka, 1975)
Pseudomonas fluorescens	Monomer	54	5.6		7.3	NA	25	NADH	(Brünker et al., 1997)
Gluconobacter suboxidans	Monomer	50	NA		6-8	9-10	12	NADPH	(Adachi et al., 1999)
Fusarium graminearum	29	110	NA		7	9	290	NADPH	(Trail & Xu, 2002)
Lactobacillus sanfranciscensis	Monomer	53	NA	35	5.8	8	24	NADPH	(Korakli & Vogel, 2003)
Candida magnoliae	35	142	NA	37	7.5	10	28	NADPH	(Lee et al., 2003b)
Leuconostoc pseudomesenteroides	43	155	4.4			5.4	44	NADH	(Hahn et al., 2003)
Lactobacillus intermedius	43, 34.5	171.	4.7	35	5.5	7.0	25	NADPH	(Saha, 2004a)

Table 1.6 Properties of MDH from various organisms

Dehydrogenase reductases fall into three main groups based on the sequence analysis, protein size and coenzyme- binding motifs 1) Short-chain dehydrogenase reductases (SDRs), 2) Medium-chain dehydrogenase reductases (MDRs) and 3) Long-chain dehydrogenase reductases (LDRs). The SDR (with subunits typically of 250 residues) enzymes have a typical coenzyme-binding site, GXXXGXG, and an active site, YXXXK. The MDR (with subunits typically of 350 residues) enzymes are zinc dependent and have the N-terminal coenzyme-binding motif GXGXXG. In spite of an overall sequence identity that can be as low as 10%, the LDR (350 to 560 residues) enzymes have a KXXXXNXXG motif (Lee et al., 2003b).

Several research groups have reported the purification and characterization of MDH from plants and microbial sources (Adachi et al., 1999; Brünker et al., 1997; Lee et

al., 2003b; Nüss et al., 2010; Ruffner et al., 1978; Saha, 2004b; Yamanaka, 1975). MDH from plants have been characterized as members of the MDR family. Other MDHs from fungi are members of the SDR family. Often, bacterial MDHs do not share significant similarity with either of these families but instead belong to a family of LDRs that includes 66 recognized members (Lee et al., 2003b).

1.17 Enzymatic production of mannitol

One of the major concern with the fermentative production of mannitol is the formation of by-products in large amount sometimes equal to the main product, mannitol. Even if mannitol is easily crystallized, by-products such as lactic or gluconic acid complicate downstream processing of mannitol. By-product-free enzymatic approach of mannitol synthesis was proposed by Kulbe et al., (1987). In this process relatively cheap fructose/glucose mixtures was converted into mannitol and gluconic acid. They combined purified enzymes in a single membrane reactor to produce mannitol. In this process, the NADH consumed in mannitol production by *S. cerevisiae* MDH was regenerated during gluconic acid production by glucose dehydrogenase from *B. megaterium*. They obtained turnover numbers over 100,000 for NADH regeneration and 88% of fructose was conversion to mannitol. Limiting factors in such process included poor enzyme stability and substrate inhibition. To produce mannitol, free from gluconic acid, mannitol production was investigated with *Candida boidinii* formate dehydrogenase as the second enzyme. In this system, the second product, CO_2, was easily removed from the reaction solution, and it did not inhibited the MDH reaction. In the batch reactor, 2.2 g $l^{-1}h^{-1}$ productivity was achieved by Slatner et al., (1998). A similar conversion was performed using the *L. mesenteroides* MDH and *C. boidinii* FDH that reached over 95 mol % conversion in 70 h (Parmentier et al., 2003).

In conclusion, even though biological processes for mannitol production are still being optimized, they already compete favourably with the current chemical process (Table 1.7). Mannitol can be also produced using feedstock cheaper than fructose. Whole-cell bio-transformations with engineered microorganisms or enzymatic conversions have potential to produce mannitol without by-products. More research is needed to evaluate commercial viability of whole-cell transformations or enzymatic conversions.

Factor	*Catalytic hydrogenation*	*Biological conversion*
Catalyst	Non-specific	Specific
Substrate(s)	High purity needed	Low-purity can be used
Process conditions	High pressure and temperature	30–37 °C, 1 atm
Mannitol	Side product	Main product
Theoretical yield from fructose	50%	100%
Theoretical yield from total sugar	25%	67% (LAB-based fermentation), 100% (resting cells or enzymatic)
Other products and impurities	Sorbitol and Ni catalyst, both difficult to separate	Organic acids, ethanol, and sugars, all easy to separate
Yield of crystalline mannitol	≤39% mol/mol initial sugar	52% mol/mol initial sugar

Table 1.7 Comparison of the catalytic hydrogenation process with microbial and enzymatic conversion methods (Adapted from von Weymarn et al., (2003))

1.18 Uses of mannitol

Major applications of mannitol are in food industry as a food additive. It is used as a sweet-tasting, bodying and texturing agent (Soetaert et al., 1999). Mannitol reduces the crystallization tendency of sugars and is used as such to increase the shelf-life of foodstuffs. Since the metabolism of this sugar alcohol is not dependant on insulin, it can be safely consumed by the diabetic patients. As it does not cause dental caries, it is commonly used in confectionary, cold drinks, chocolates and sweets (von Weymarn et al., 2002a; Wisselink et al., 2002). In clinical study on rats and humans it was found that mannitol did not significantly influence either the blood sugar or respiratory quotient and is non carcinogenic. It was found that 75% of ingested mannitol got fermented by the intestinal flora. The remaining 25% was absorbed before being excreted in urine (Livesey, 2003).

Besides these applications in the food industry, mannitol is used in the pharmaceutical industry as a base in tablets of vitamins, antacids, aspirin and other pharmaceuticals. Crystalline mannitol exhibits a very low hygroscopicity, prevents moisture absorption from the air, exhibits excellent mechanical compressing properties, does not interact with the active components in the drugs and its sweet cool taste masks the unpleasant

taste of many drugs (Ghoreishi & Shahrestani, 2009b). In medicine, mannitol is used as a powerful osmotic diuretic for intoxication therapy by increasing the urinary output. In many types of surgeries, mannitol solutions are applied to prevent kidney failure. Mannitol is also used in brain surgery to reduce cerebral edema (Soetaert et al., 1999). Mannitol hexanitrate is a well-known vasodilator, used in the treatment of hypertension. It is used as cryoprotectant during freezing, thawing and storage of blood (Ghoreishi & Shahrestani, 2009a). It is used during the treatment of cystic fibrosis for thinning the thick sticky mucous in the lungs of the patient (Jaques et al., 2008). Mannitol is used extensively in scientific research, especially in microbiological media, where it is a classical carbon source for determinative purposes. Mannitol protects freeze-dried bacterial cultures during storage (Soetaert et al., 1999). The complex of boric acid with mannitol is used in the production of dry electrolytic capacitors. It is an extensively used polyol for production of resins and surfactants (Soetaert et al., 1999).

The global market for sugar alcohols in 2000 was $1.3 billion. Sorbitol has the largest share in sugar alcohol market in terms of volume and dollar sales. It is mostly sold as a 70% (w/v) aqueous solution. The market of all other sugar alcohols is relatively small in volume and they were typically developed and introduced into the market in the 1990s (von Weymarn, 2002). Mannitol has a steady market, but the methods for producing mannitol are still being evaluated, developed and optimized. Over 70% of the mannitol produced in China in 2005 was extracted as a by-product of alginate and iodine production from seaweeds (Song & Vieille, 2009), the rest of the world produces mannitol (50,000 tons/year) by catalytic hydrogenation of 1:1 mixture of glucose-fructose syrups at high pressures. American company, zuChem Inc., had commercialized mannitol production by *L. intermedius* NRRL B-3693 in 2005. After 2000 the global market sales of mannitol reached to 30 million lb/year at the price of 3.32 USD/lb (Saha & Nakamura, 2003). Presently, bulk price of mannitol is 7.32 USD/kg (Saha & Racine, 2011). For most of the sugar alcohols, the market is mature and volume growths are expected to follow the trends of the large-scale consumer products which contain sugar alcohols (von Weymarn, 2002). A few selected patents on erythritol and mannitol production by various microorganisms are presented in Table 1.8.

Patent No.	*Grant date*	*Title*	*Reference*
US 4939091	3 Jul 1990	Novel *Auerobasidium sp*. microorganisms, method for obtaining the same and method for preparing erythritol with the same	(Sasaki et al., 1990)
US 5036011	30 Jul 1991	Novel *Aureobasidium sp*. microorganisms and method for obtaining the same, and method for preparing erythritol with the same	(Sasaki et al., 1991)
US 5902739	11 May 1999	Method of producing erythritol	(Abe & Morioka, 1999)
US 5962287	5 Oct 1999	Process for producing erythritol using mutant *Trichosporonoides*	(Suh et al., 1999)
US 5981241	9 Nov 1999	Method of producing erythritol	(Cho et al., 1999)
US 5989878	23 Nov 1999	Fermentation process for preparing erythritol using mutant cells by controlling osmotic pressure	(Kim et al., 1999b)
US 6060291	9 May 2000	Fermentation process for preparing erythritol using *Trichosporonoides madida* DS 911	(Park et al., 2000)
US 6074857	13 Jun 2000	Method of producing erythritol	(Chida & Ochiai, 2000)
EP 1151128	14 June 2001	A fermentation process for preparing erythritol using mother liquor produced from purification process of palatinose	(Kim et al., 2001)
US 0055796	Dec 27 2001	Fermentation process for preparing erythritol by a high salt tolerant mutant of *Candida sp*	(Seo et al., 2001)
US 6365383	2 Apr 2002	Method of producing erythritol by repeated fed-batch fermentation	(Segueilha, 2002)
US 6448053	10 Sep 2002	Erythritol-producing yeast strains	(Lin et al., 2002)
US 6602691	5 Aug 2003	Process for the production of mannitol by immobilized micro-organisms	(Ojamo et al., 2003)
US 6855526	15 Feb 2005	Method for making mannitol with *Lactobacillus intermedius*	(Saha, 2005)
US 6916639	12 Jul 2005	Erythritol-producing *Moniliella* strains	(Lin et al., 2005)
EP 1088893	3 Jan 2006	Fermentation process for preparing erythritol using novel cell of *Pichia*	(Kim et al., 2006)
EP 1092781	1 Mar 2006	Fermentation process for preparing erythritol by a high salt tolerant mutant of *Candida sp.*	(Seo et al., 2006)
US 0037266	21 Jun 2006	Process for producing erythritol	(Sasman et al.,

			2006)
EP 0940471	17 Sept 2008	Method of producing erythritol	(Cho et al., 2008)
US 0246843	1 Oct 2009	Process for producing erythritol using *Moniliella tomentosa* strains in the presence of neutral inorganic nitrates, such as potassium nitrate, ammonium nitrate or sodium nitrate, as nitrogen source	(Edlauer & Trimmel, 2009)
EP 1352079	18 Aug 2010	Process for producing d-mannitol	(Von Weymarn & Airaksinen, 2010)

Table 1.8 A few selected patents on erythritol and mannitol production by various microorganisms

There are hardly any efforts made in India to produce erythritol, irrespective of the requirement. Although other sweeteners are in the market and being accepted, erythritol is not yet available in the country. Production of polyols in specialized microbes is governed by a variety of factors like osmotolerance, substrate concentration and type, medium composition and environmental factors like pH, temperature and dissolved oxygen. Production of erythritol and mannitol is thus a scientifically challenging topic and has an industrial importance because of its increasing demand throughout the world.

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Chapter 2

Screening of Yeast Cultures and Culture Conditions for Erythritol Production

Abstract:

This chapter describes screening of osmotolerant yeasts, for their ability to produce erythritol. A liquid fermentation medium (LFM) composed of (g/l) glucose 250, yeast extract 10, KH_2PO_4 5 and $MgSO_4$ 0.25 was used for screening. Candida magnoliae NCIM 3470 and Yarrowia lipolytica NCIM 3472 were chosen and investigated further to enhance erythritol production by optimizing medium constituents and environmental conditions. Y. lipolytica produced erythritol as the only metabolite irrespective of the media constituents and culture conditions, whereas C. magnoliae produced small amount of mannitol and glycerol as co-metabolites. Erythritol production from C. magnoliae was mainly dependent on oxygen availability and nitrogen content in the medium. These two cultures were selected for strain improvement programme through mutagenesis.

2.1 INTRODUCTION

Several yeast species of genera *Candida, Debaromyces, Hanseniaspora, Hansenula, Issatchenkia, Metschnikowia, Pichia*, *Trigonopsis, Yarrowia* and *Zygosaccharomyces* are able to grow in environment with low water activity *i.e.* in the presence of high sugar or salt concentrations. Sugar or salt-tolerant yeasts accumulate compatible solutes to encounter osmotic stress. Apart from osmo-regulation, role of osmolites in such organisms could be as carbohydrate reserves or storing reducing power (Diano et al., 2006; Hohmann, 2002). Glycerol is the most frequent osmolyte produced by yeasts, but it is also well documented that several sugar alcohols such as arabitol, erythritol, mannitol and xylitol can also serve as osmolytes (Bilanx et al., 1991; Kogej et al., 2007).

Majority of the published reports on erythritol biosynthesis have been focused on the screening of microbial strains which produce erythritol with high yield and productivity followed by the optimization of process parameters in batch and fed-batch fermentation. Very few researchers were successful in isolation of high erythritol-producing microorganisms from their environmental niche (*viz* pollen, honey comb, high sugar containing fruits and food) which produces erythritol as the sole polyol at higher concentration. These organisms were employed for erythritol production in industries by optimizing culture and fermentation conditions (Jeya et al., 2009).

In one of the early report an erythritol producing yeast like fungus was isolated from pollen in a highly osmotic medium containing 35% glucose (Hajny et al., 1964). Later on, this yeast like fungus was identified as *Moniliella tomentosa var pollinis.* Similarly, Ishizuka et al., (1989) carried out extensive investigations on isolation of erythritol producing yeasts and on screening 1700 isolates from the soil of Okinawa, Japan, isolated *Aureobasidium* SN 124 which produced considerable high amount of erythritol. This yeast was subsequently modified by physical and chemical mutagenesis, which is being used to produce erythritol commercially in Japan wherein the yield and production rate of erythritol by mutant *Aureobasidium* sp. SN-G42 have been found to reach 47% and 2.0 $gl^{-1}h^{-1}$ respectively, in 100,000 L fermentor (Sawada

et al., 2009). Since then, several researchers have carried out extensive search for erythritol producing microorganisms which are discussed in detail underneath.

Hirata et al., (1999) screened over 2000 isolates from sources such as soil, seawater, plants and fermented foods. Among the 20 erythritol-producing isolates, one of the isolate (strain 618A-01) produced 75 g/l erythritol in 950 h, from 200 g/l glucose. Later, 18s rRNA sequence of this strain confirmed that it belonged to the genus *Ustilago* of the class *Ustilaginomycetes*. Park et al., (1996) isolated 1753 strains from honey and pollen, out of which only three strains (one *Trichosporon* and two strains of *Trichosporonoides*) produced erythritol.

Lin et al., (2001) investigated samples of honey, beehives, pollens, preserved fruits, fresh fruits and sugar-manufacture waste water from Taiwan, in search of erythritol-producing microorganisms. Among 658 isolates, six high erythritol-producing strains were selected. Among these six erythritol-producing strains, *Moniliella sp.* 440 produced highest (116 g/l) erythritol with 39% yield in medium containing 30% glucose. This strain was recently mutagenized through several rounds of NTG treatment and one of the mutant N61188-12, produced 237.8 g/l erythritol in medium containing 400 g/l glucose (Lin et al., 2010).

A selected mutant of *Torulla sp*, isolated from 400 g/l sucrose solution at R&D center of Bolak Corporation, Osan, Korea produced 192 g/l erythritol, without formation of by-products such as glycerol and ribitol. In a batch culture a higher volumetric productivity was observed when an initial glucose concentration was 300 g/l. Subsequently a fed-batch fermentation process was optimized by Lee et al., (2003) with 300 g/l initial glucose and phytic acid as a phosphate source. During the production phase, glucose concentration was controlled around 225 g/l by feeding sterile glucose solution. A final erythritol concentration of 192 g/l was obtained from 400 g/l glucose with a volumetric productivity of 2.26 g l^{-1} h^{-1} and 48% yield (Kim et al., 2000; Lee et al., 2000; Lee et al., 2001; Lee et al., 2002; Oh et al., 2001).

Yang et al., (1999) isolated erythritol-producing yeast from honeycombs which was identified as *Candida magnoliae*. To improve the erythritol-producing ability, the parent strain was mutagenized by UV irradiation and NTG treatment. The selected mutant ‘M2’ showed higher erythritol yield and productivity compared to the wild

strain. The optimized fed-batch fermentation by this strain resulted in 200 g/l erythritol with 1.2 g l^{-1} h^{-1} productivity and 43% yield (Koh et al., 2003; Koh et al., 2000; Ryu et al., 2000).

Erythritol production in complex media containing glucose as carbon source has been well documented. However, there are a few reports on erythritol production with alternative carbon sources like glycerol which is presently available as a cheap raw material generated during biodiesel production. Rymowicz et al., (2008) isolated an acetate-negative mutant *Y. lipolytica* Wratislavia K1 of which had the ability of simultaneously producing high amounts of erythritol and citric acid in medium containing glycerol. At lower pH (2.5-3), this strain produced erythritol as major product whereas with the increase in pH up to 5.5, the content of citric acid increased to 106-108 g/l. In fed-batch cultivation at pH 3, mutant Wratislavia K1 was able to produce 170 g/l from glycerol which was the highest concentration reported from glycerol (Rymowicz et al., 2009).

Recently, Jeya et al., (2009) isolated novel erythritol-producing yeast *P. tsukubaensis* KN75. When this strain was cultured aerobically in a fed-batch culture with glucose as a carbon source, it produced 245 g/l of erythritol, with 2.86 g l^{-1} h^{-1} productivity and 61% yield. This is the highest erythritol yield ever reported by an erythritol-producing microorganism. Erythritol production was scaled up from a laboratory scale 7 L fermenter to pilot 300 L and then to 50,000 L plant scale using the dissolved oxygen as a scale-up parameter. This strain was deposited as *P. tsukubaensis* KCCM 10356 in the Korean Culture Collection of Microorganisms (KCCM).

The biosynthetic mechanism of erythritol within microbes is suggested to be as follows: One mole of glucose is converted into two moles of carbon dioxide and one mole of erythrose-4-phosphate in the PP pathway. Then one mole of erythrose-4-phosphate is reduced to one mole of erythritol (Fig. 1.2). Regulation of PP pathway in osmophilic yeasts is not yet fully understood but it is known that fermentative production of erythritol is influenced by factors like osmotic pressure of the medium, pH, dissolved oxygen, nitrogen sources and type of carbohydrates (Burschäpers et al., 2002a).

Several osmotolerant yeasts are known to produce erythritol, a detailed list of erythritol producing microorganisms, medium components and culture conditions at flask and fermentor is presented in Table 1.3. Majority of the publications and patents claim isolates or mutants of diverse osmophilic yeasts with relatively higher erythritol yield and productivity. A strain with high yield and productivity with minimal amount of interfering by-products is the prime requirement for the development of a commercially viable process.

A exhaustive search was carried out for the medium constituents for erythritol production by fermentation and it was found high C:N ratio is vital parameter for its production. Majority of the researchers have used high glucose concentration ranging from 100-300 g/l. Yeast extract was found to be the commonly used nitrogen source. Along with yeast extract, various combinations of organic and inorganic nitrogen sources were also screened for erythritol production. Nitrogen limitation is prerequisite for erythritol formation because erythritol production starts towards the end of the growth phase with the exhaustion of nitrogen source from the medium. Nitrogen limitation is associated with problem of foam formation, which is difficult to suppress. Careful control of carbon and nitrogen content is required to avoid foam formation are discussed in some publications (Burschäpers et al., 2002a; Burschäpers et al., 2002b). Based on literature survey, a liquid fermentation medium was constituted to screen the cultures for erythritol production.

The present chapter describes screening of high sugar or salt tolerant yeasts available in culture collection centers for their ability to produce erythritol and identification of medium constituents and environmental conditions for effective production of erythritol.

2.2 MATERIALS AND METHODS

2.2.1 Microorganisms

Yeast cultures belonging to genera *Candida, Debaromyces, Hanseniaspora, Hansenula, Issatchenkia, Metschnikowia, Trigonopsis* and *Yarrowia* were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune and Microbial Type Culture Collection (MTCC), Chandigarh, India,

based on their reported ability to grow in medium with high osmotic pressure. The obtained cultures were grown on Petri plates by streak plate technique using dilute suspensions and pure colonies were transferred to malt extract glucose yeast extract peptone (MGYP) agar slants for maintenance. All media ingredients were purchased from HiMedia, Mumbai, India

2.2.2 Culture conditions

The liquid fermentation medium (LFM) was composed of (g/l) glucose 250, yeast extract 10, KH_2PO_4 5 and $MgSO_4 \cdot 7H_2O$ 0.25. Initial pH of the medium was adjusted to 6. Carbon sources were autoclaved separately for all the experiments and later mixed with other media components. A loop-full of respective cultures from freshly prepared slants were inoculated into 5 ml LFM in 150 × 25 mm test tubes and incubated at 28 °C, 210 rpm, for 48 h. Two and a half ml of these seed cultures were aseptically transferred to 250 ml Erlenmeyer flasks containing 22.5 ml LFM. All flask experiments were performed in duplicate, with 250 ml Erlenmeyer flasks containing 25 ml fermentation broth incubated at 28 °C, 210 rpm for 168 h, unless otherwise mentioned. At the end of the incubation period, final volume of the culture broth was adjusted to 25 ml by sterile distilled water to compensate for evaporative loss of water. Samples were withdrawn at successive interval and analyzed for optical density at 600 nm (OD), pH, residual sugars and polyol concentration by high performance liquid chromatography (HPLC).

2.2.3 Analysis

Optical density was measured at 600 nm using a spectrophotometer. The samples were diluted in such a manner that the absorbance values were in the range of 0.1 to 0.9 absorbance unit. Dry cell weight (DCW) of *C. magnoliae* was estimated from a calibration curve derived from the relationship between OD and DCW wherein 1.0 OD was found to be equivalent to 0.37 g/l DCW. Residual glucose from fermentation broth was analyzed by LIQUIZONE Glucose-MR GOD-POD kit (Medsource Ozone Biochemical's Pvt. Ltd., Delhi, India). Concentrations of glucose, fructose, mannitol, erythritol, glycerol and other co-metabolites were determined using HPLC equipped with an Aminex HPX-87H, 300 × 7.8 mm column (Bio-Rad) at 50 °C. Mobile phase used was 0.01 N H_2SO_4 at 0.5 ml/min flow rate. A refractive index detector, Shodex

RI-71, was used for detection of sugars, acids and polyols. The quantification was done by external standard technique using peak area of reference compounds. Multiple injections of standard solutions with varying concentrations were done for preparing of standard curves of the compound of interest.

2.2.4 Screening of cultures for production of erythritol

The cultures obtained from NCIM and MTCC were screened for polyols production by inoculating respective cultures in 250 ml Erlenmeyer flasks with 25 ml LFM and incubating the flasks at 28 °C, for 168 h. Samples were withdrawn at 96 and 168 h and analyzed for biomass, pH and polyols concentration.

2.2.5 Screening of nitrogen sources for selected erythritol producing cultures

The shake flask experiments were done to evaluate the nitrogen sources for erythritol production by *C. magnoliae* and *Y. lipolytica*. The effect of nitrogen source was studied in LFM with 2 g/l yeast extract and different inorganic and organic nitrogen sources equivalent to 0.8 g/l "N". The nitrogen sources were used on equal "N" basis. Respective seed cultures were inoculated in 250 ml Erlenmeyer flasks with 22.5 ml medium and incubating at 28 °C, 210 rpm for, 168h. Samples were analyzed for optical density at 600 nm, pH, residual glucose and polyol concentration.

2.2.6 Choice of carbon source

Utilization of carbon sources namely glucose, sucrose and fructose was investigated for growth and polyol production by *Y. lipolytica* and *C. magnoliae*. The carbon sources (250 g/l) ware autoclaved separately and later added to remaining constituents of LFM. Flasks were inoculated with respective cultures and incubated at 28 °C, 210 rpm. Samples were analysed after 168h as described earlier.

2.2.7 Evaluation of combinations of yeast extract and inorganic nitrogen sources for erythritol production by *Y. lipolytica* and *C. magnoliae*

The inorganic nitrogen sources surveyed for *Y. lipolytica* and *C. magnoliae* on which they could grow and produce erythritol were ammonium acetate, ammonium nitrate, ammonium phosphate, ammonium sulphate, sodium nitrate and urea. From these

inorganic nitrogen sources, taking into account cost and regulatory affairs to prepare food grade product, di-ammonium phosphate for *Y. lipolytica* and di-ammonium phosphate and sodium nitrate for *C. magnoliae* were used in combination with yeast extract on "N" basis, with glucose or sucrose as carbon source. Samples were analyzed after 168 h of incubation as described earlier.

2.2.8 Effect of medium volume in shake flask for erythritol production

Erlenmeyer flasks containing different volumes of LFM were inoculated with 48 h old 5% (v/v) inoculum of *C. magnoliae* and *Y. lipolytica* and incubated for 168 h at 28°C, 210 rpm. The volumes of the cultures were corrected with distilled water and then analyzed for OD, residual sugars and products formed.

2.2.9 Effect of pH and on erythritol production by *Y. lipolytica and C. magnoliae*

During the experiments described above, the rate of erythritol production was found to decline as the age of culture progressed. During this period, pH of the broth also decreased to 2.5. Therefore possibility of decrease in erythritol due to decrease in pH was investigated by addition of 4 g/l calcium carbonate to the flask at the time of inoculation to avoid lowering of pH. Calcium carbonate powder was weighed in Erlenmeyer flask and autoclaved separately.

2.2.10 Evaluation of growth and erythritol production by *C. magnoliae* in medium with different yeast extract concentrations

In the preliminary experiments it was observed that a substantial quantity of sugar was leftover unutilized when 250 g/l sugar (glucose or sucrose) was used in the medium. This was thought to be due to insufficient nitrogen source for the growth and fermentation. *C. magnoliae* was grown in 25 ml LFM with different quantities of yeast extract between 10 and 50 g/l, in 250 ml Erlenmeyer flasks. Samples were withdrawn after 120 and 168 h and analyzed for OD, residual sugar and erythritol production.

2.2.11 Effect of initial sucrose concentration on erythritol production by *C. magnoliae*

Effect of initial sucrose concentration on growth and erythritol production by *C. magnoliae* was investigated in LFM with different initial sucrose concentrations between 150 and 300 g/l. Two and half ml of 48 h old seed culture was inoculated to respective flasks containing 22.5 ml LFM and incubated at 28°C, 210 rpm. Samples were analyzed as above after 168 h of incubation.

2.2.12 Requirement of phosphate for erythritol production by *C. magnoliae*

Effect of phosphate on growth and polyol production was investigated in LFM by addition of different quantities potassium di-hydrogen phosphate in shake flasks. Five sets of flasks with 25 ml medium containing 1 to 5 g/l phosphate were inoculated with *C. magnoliae* and analyzed as described earlier after 168 h of incubation.

2.2.13 Time course of erythritol production by *C. magnoliae* using sucrose as carbon source

C. magnoliae was grown in identical shake flasks in LFM. One flask was harvested every 24 h, the volume was corrected to 25 ml and analyzed for OD as well as residual glucose, fructose, erythritol, glycerol and mannitol using HPLC. Glucose and fructose uptake rates and erythritol production rate were calculated.

2.2.14 Effect of sucrose and glucose feeding on erythritol production by *C. magnoliae*

Effect of sucrose and glucose feeding on erythritol production for *C. magnoliae* was investigated by inoculating 48 h old seed culture to 25 ml LFM with initial 100 g/l sucrose/glucose. After 24 h of growth period, sterile glucose solution was fed at a concentration 30, 20, 20, 20, 15, 15, 15 and 15 g $l^{-1}d^{-1}$ from 24 to 192 h. Samples were analyzed periodically as described earlier.

2.2.15 Production of polyols by resting cells of *C. magnolia*

Erlenmeyer flasks of 250 ml capacity containing 25 ml LFM with sucrose were inoculated with two and half ml inoculum of *C. magnoliae* and incubated at 28 °C, on

a rotary shaker, for 48 h. The broth was aseptically centrifuged at 10,000 g for 10 min. The supernatant was discarded and the cell pellet was suspended in 25 ml sterile physiological saline and the cells were recovered by centrifugation. The cells were then suspended in 25 ml sterile solutions of glucose, fructose, sucrose and glycerol (100 g/l individually), in 250 ml Erlenmeyer flasks and incubated on a rotary shaker. Samples were withdrawn at regular interval and analyzed as above. In a separate experiment, sterile yeast extract to get 1 g/l final concentration was added to the flasks in order to maintain cells metabolically active during production phase.

2.3 RESULTS AND DISCUSSION

2.3.1 Screening of cultures for erythritol production

This chapter is focused on objective of screening microbial strains, their performance and suitable environment for erythritol production which is the most essential aspect to develop the integrated technological solution for erythritol production via biotechnological process. The endeavor was to identify erythritol producing microbes, to study the strains using various culture conditions and consequently select the best strain/s for mutagenesis for further desired improvements.

The HPLC analysis method used could well resolve acetic acid, citric acid, erythritol, ethanol, fructose, glucose, glycerol and mannitol. Fructose and mannitol eluted rather close to each other but the rest of the compounds showed almost baseline separation. Chromatograms of the reference compounds and a sample of fermentation broth showing sugars and polyols are presented in Fig. 2.1.

One of the early reports on microbial production of erythritol was published by Hajny et al., (1964). Since then, diversity of microbial strains has been studied for erythritol production under various culture and environmental conditions. Although the research on microbial erythritol production is rather limited, erythritol production using biological processes is becoming more important because of the growing demand of erythritol in food industry. Newly isolated strains and their mutants can be competitive to the current industrial production process, additionally productivity and yield could be further increased by optimizing different fermentation methods (Jeya et al., 2009).

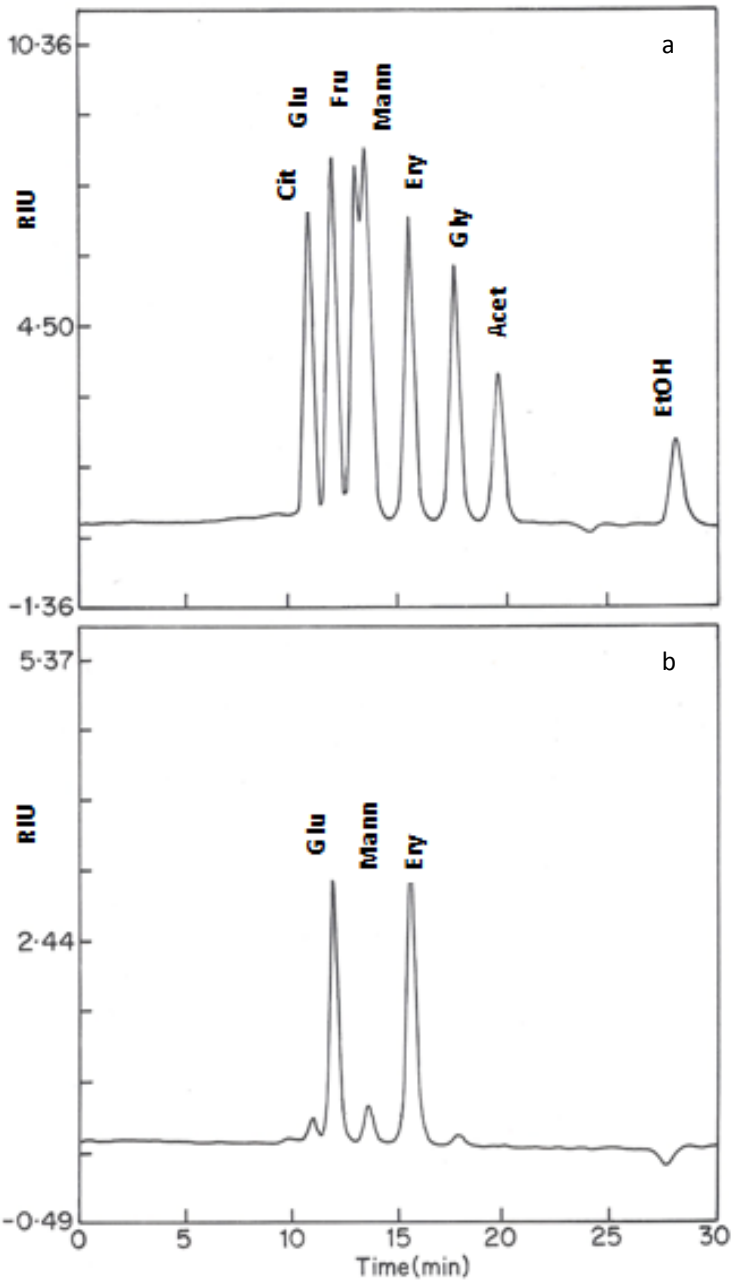

Figure 2.1 Ion exclusion HPLC chromatogram of standard compound mixture (a) and a sample of fermentation broth (b)

In most of the previous screening studies, the success rate of obtaining an erythritol-producing strain among the total microbial isolates was much less and observed in the range of 0.6-6% of the total osmophilic yeasts isolated. Aoki et al., (1993) isolated 402 strains of osmophilic yeasts out of which 5.47% isolates produced erythritol. Whereas Park et al., (1996) isolated 1752 osmophilic yeasts from honey and pollens

but only 11 of the isolates (0.6%) could synthesize erythritol. Similarly, Hirata et al., (1999) selected 20 erythritol-producing microorganisms after evaluating over 2000 isolates. Lin et al., (2001) isolated 658 strains of osmophilic yeasts from sugar-containing samples collected from Taiwan, out of which 28 isolates produced erythritol accounting for 4.3% of total isolates. Taking into account low probability of obtaining erythritol producing strains from its natural environment, it was decided to screen the sugar and salt tolerant yeast strains which are available at various culture collection centers. The cultures were selected on the basis of their ability to grow in high salt/ sugar containing media from the reports in literature.

On screening twenty-six osmophilic yeast strains belonging to the genera *Candida, Debaromyces, Hanseniaspora, Hansenula, Issatchenkia, Metschnikowia, Trigonopsis* and *Yarrowia* from NCIM and MTCC, seven yeast strains were found to produce erythritol in LFM (Table 2.1). Thus, the idea of selecting cultures from culture collections on the basis of their osmotolerance worked reasonably well. Among the erythritol producers, *Y. lipolytica* NCIM 3472, *C. magnoliae* NCIM 3470 and *T. variabilis* NCIM 3344 produced 17.1 and 13.9 and 8.9 g/l erythritol, respectively, with a yield of 12.4, 9.5 and 6.1%. These three strains were used further to study erythritol production using various organic and inorganic nitrogen sources.

Culture	***OD 600***	***pH***	***Glucose utilized g/l***	***Erythritol g/l***	***Yield %***
Candida bombicola MTCC 1910	54	1.8	192	0	0.0
Candida magnoliae MTCC 3602	59	1.4	104	4.4	4.2
Candida magnoliae NCIM 3470	57	3	146	13.9	9.5
Debaromyces polymorphus NCIM 3419	7.4	3.4	26.6	0.0	0.0
Debaryomyces castellii NCIM 3410	24	2.6	235.5	0.0	0.0
Debaryomyces hansenii NCIM 3441	33.4	3.9	38.4	0.0	0.0
Debaryomyces hansenii NCIM 3446	26	3.8	93.0	0.0	0.0
Debaryomyces hansenii NCIM 3447	21.2	3.8	148.8	0.0	0.0
Debaryomyces hansenii NCIM 3448	23.4	3.7	124.9	0.0	0.0
Debaryomyces merma NCIM 3410	41.4	3.3	152.2	0.0	0.0
Debaryomyces vanriji NCIM 3356	13.1	3.2	92.2	0.0	0.0

Hanseniaspora osmophila MTCC 650	10.8	3.6	60	0.0	0.0
Hanseniaspora vineae NCIM 3356	17.2	3.4	85.3	0.0	0.0
Hansenula anmola NCIM 3341	12	3.5	124.9	0.0	0.0
Hansenula anmola NCIM 3342	18	3.7	143.4	0.0	0.0
Hansenula polymorpha NCIM 3377	13.8	3.1	113.8	0.0	0.0
Issatchenkia orientalis NCIM 3621	20.8	3.6	250.0	0.0	0.0
Metschnikowia refaufii NCIM 3324	49.8	3	143.6	0.0	0.0
Metschnikowia refaufii NCIM 3335	36.2	5.3	32.5	0.0	0.0
Trigonopsis variabilis NCIM 3344	74	5.1	147	8.9	6.1
Trigonopsis variabilis NCIM 3409	46	3.1	157	0.0	0.0
Trigonopsis variabilis MTCC 1354	89	4.4	96	5.3	5.5
Trigonopsis variabilis MTCC 3389	61	2.9	109	3.4	3.1
Yarrowia lipolytica NCIM 3229	44	3.9	93	3.5	3.8
Yarrowia lipolytica NCIM 3472	40	2.8	138	17.1	12.4
Yarrowia lipolytica NCIM 3589	58	3.8	85	0	0.0

Table 2.1 Screening of osmo-tolerant yeasts for erythritol production from NCIM and MTCC in LFM

2.3.2 Screening of nitrogen sources for selected erythritol producing cultures

Apart from the carbon source, it was assumed that the source and concentration of the nitrogen, in the media would normally have the greatest effect on yeast growth and erythritol production. Nitrogen limitation is the prerequisite for erythritol formation as production of erythritol starts towards the exhaustion of nitrogen source from the medium. Therefore, C: N of 100:1 was maintained in all the experiments.

Among the various organic and inorganic nitrogen sources screened, *C. magnoliae* showed good growth with all nitrogen sources as presented in Table 2.2 except malt extract. Yeast extract based medium resulted in production of 13.9 g/l erythritol which was more or less similar to all complex organic nitrogen sources used. Among the inorganic nitrogen source based media, the use of nitrates resulted in equivalent amount of erythritol to yeast extract based medium. There was decline in erythritol concentration when ammonium chloride, sulphate and phosphate were used. This

could be either due to lowering of pH in the fermentation medium in the flask when chlorides and sulphates were used or because of oxygen limitation.

However with *Y. lipolytica*, ammonium acetate, phosphate, nitrate and urea were as good as yeast extract based medium (Table 2.3). Complex organic nitrogen sources Casein hydosylate, Soya peptone, Meat extract, Beef extract, Peptone type 1 and Meat peptone resulted in better erythritol production as compared to yeast extract.

C. magnoliae NCIN 3470	***OD 600 nm***	***pH***	***Glucose utilized g/l***	***Mannitol g/l***	***Erythritol g/l***	***Glycerol g/l***	***Yield %***
$(NH_4)H_2PO_4$	60	1.3	145.1	2.8	9.7	7.1	6.7
$(NH_4)_2HPO_4$	65	1.8	128.7	2.3	10.7	8.6	8.3
$(NH_4)_2SO_4$	54	1.3	115.6	1.3	6.3	8.0	5.5
NH_4Cl	56	0.9	117.0	1.3	3.5	11.4	3.0
NH_4NO_3	67	2.6	140.8	4.2	14.5	8.6	10.3
$NaNO_3$	63	3.7	128.8	1.3	14.9	4.4	11.6
CH_3COONH_4	65	2.3	147.5	4.0	12.3	5.4	8.3
NH_2CONH_2	66	2.9	110.8	2.9	7.1	0.0	6.4
Malt extract	26	2.9	70.4	0.8	9.1	2.3	12.9
Liver extract	60	3.0	137.4	3.3	12.8	7.0	9.3
Casein enzyme hydolysate	63	3.2	142.0	2.5	10.2	6.3	7.2
Casein acid hydrolysate	59	3.6	124.0	2.0	10.0	7.6	8.1
Soya peptone	58	3.0	153.2	4.1	15.6	6.6	10.2
Meat extract	61	3.3	139.2	3.0	14.5	6.2	10.4
Beef extract	64	3.0	151.1	5.3	15.4	6.0	10.2
Peptone type 1	63	3.0	134.8	3.3	10.6	7.8	7.9
Meat peptone	67	3.1	125.7	1.0	10.9	4.9	8.7
Yeast extract	57	3.0	146.1	4.6	13.9	6.8	9.5

Table 2.2 Comparison of nitrogen sources for erythritol production by *C. magnoliae.* The media contained 2 g/l Yeast extract uniformly. Remaining 8 g/l yeast extract was replaced with different nitrogen source, on equal 'N' basis

Y. lipolytica NCIM 3472	*OD 600*	*pH*	*Glucose utilized g/l*	*Erythritol g/l*	*Yield %*
$(NH_4)H_2PO_4$	30	2.1	139.5	13.2	9.5
$(NH_4)_2HPO_4$	40	2.5	130.8	14.8	11.3
$(NH_4)_2SO_4$	30	2.0	88.9	9.2	10.4
NH_4Cl	28	1.6	92.5	6.6	7.1
NH_4NO_3	29	2.2	127.6	16.3	12.8
$NaNO_3$	14	2.4	81.9	8.8	10.7
CH_3COONH_4	42	2.6	160.1	19.7	12.3
NH_2CONH_2	43	2.8	128.0	19.5	15.2
Malt extract	15	2.4	94.2	7.7	8.2
Liver extract	36	2.9	132.3	13.1	9.9
Casein enzyme hydolysate	42	3.1	144.6	19.0	13.2
Casein acid hydrolysate	45	2.5	142.2	16.9	11.9
Soya peptone	42	2.9	134.4	20.5	15.3
Meat extract	39	2.9	148.2	19.7	13.3
Beef extract	41	2.8	145.9	20.2	13.8
Peptone type 1	39	2.9	139.8	23.5	16.8
Meat peptone	37	2.8	147.2	24.1	16.4
Yeast extract	40	2.8	138.1	17.1	12.4

Table 2.3 Comparison of nitrogen sources for erythritol production by *Y. lipolytica*. The media contained 2 g/l Yeast extract uniformly. Remaining 8 g/l yeast extract was replaced with different nitrogen source, on equal 'N' basis

2.3.3 Choice of carbon sources

C. magnoliae was able to utilize sucrose and produced highest erythritol (23 g/l) as compared to glucose and fructose containing medium, but it produced other undesirable polyols, mainly mannitol and glycerol, 11 and 14 g/l, respectively (Fig. 2.2). Likewise, same results were obtained when fructose was used as carbon source. There was comparatively very low amount of mannitol and glycerol produced from

glucose as carbon source. Sucrose/glucose was used as carbon source for *C. magnoliae* in successive experiments. *Y. lipolytica* produced 15.2 g/l erythritol from glucose and 21.2 g/l from fructose as carbon source in LFM (Fig. 2.3).

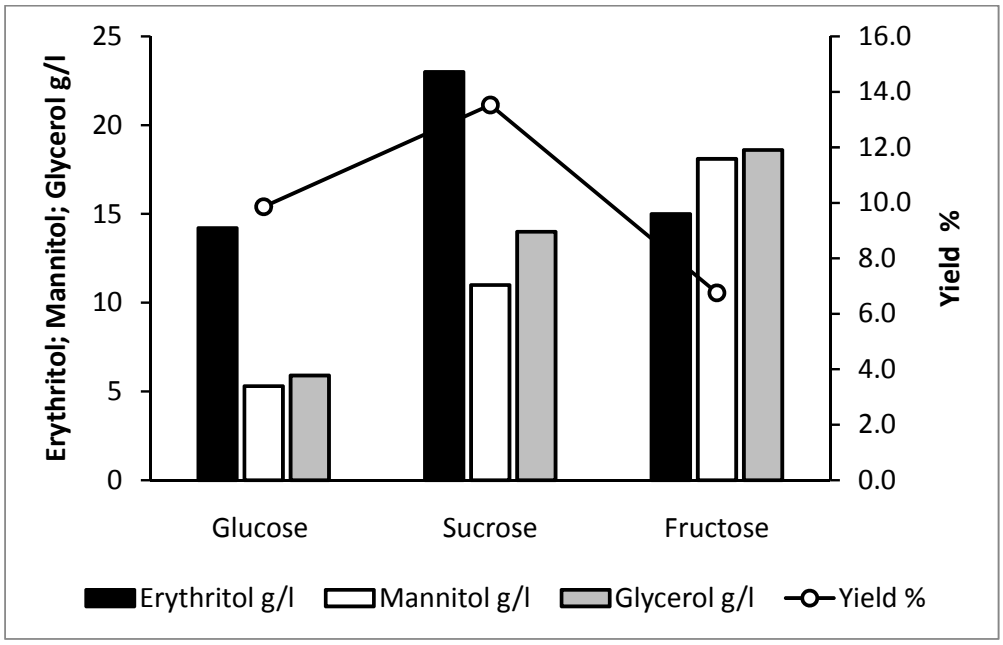

Figure 2.2 Choice of carbon source for *C. magnoliae* 3470

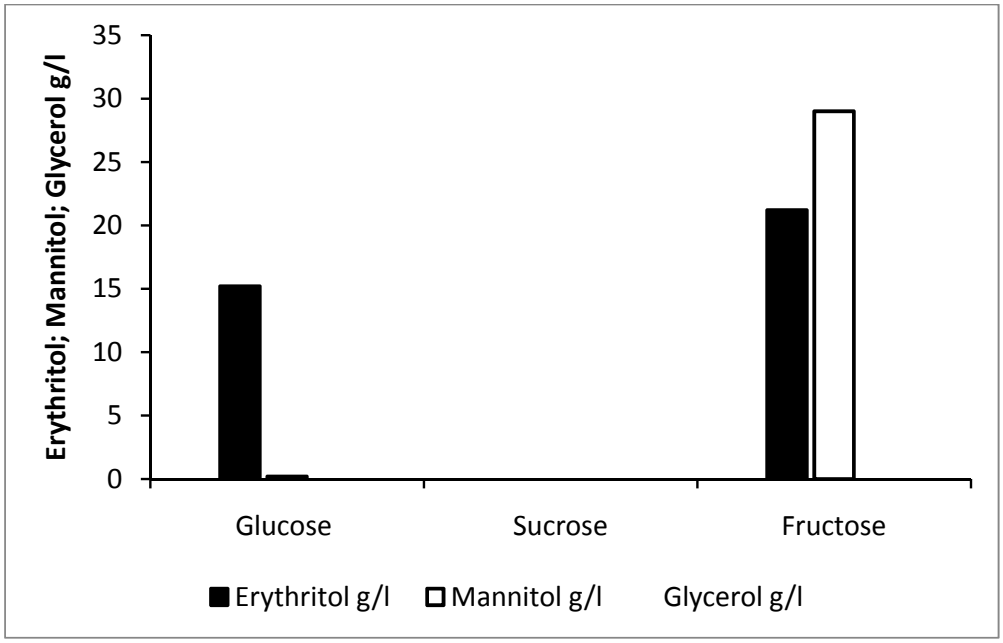

Figure 2.3 Choice of carbon sources for *Y. lipolytica* 3472

There was no erythritol formation when sucrose was used as the carbon source. *Y. lipolytica* was unable to utilize sucrose presumably due to lack of invertase enzyme (Förster et al., 2007). Although fructose seems to be the better carbon source for erythritol production, it produces fairly high amount of mannitol (27 g/l) as co-metabolite. When glucose was used as carbon source, mannitol and glycerol were not produced. Therefore glucose was used as carbon source for *Y. lipolytica* for successive experiments.

2.3.4 Evaluation of combinations of yeast extract and inorganic nitrogen sources for erythritol production by *Y. lipolytica* and *C. magnoliae*

Although yeast extract is a favorite nitrogen source in fermentation research, it is an expensive nitrogen source and probably enhances production of undesirable products. From the preliminary experiments it was found that both *C. magnoliae* and *Y. lipolytica* showed growth and erythritol production on majority of inorganic nitrogen sources when 80% of the nitrogen content was substituted with inorganic nitrogen source.

The influence of replacement of yeast extract with various concentrations of di-ammonium phosphate on the erythritol production by *Y. lipolytica* is presented in Fig. 2.4. For *Y. lipolytica,* 80 % of nitrogen from yeast extract could be replaced with di-ammonium phosphate without effecting yield and erythritol production. But when the yeast extract was completely eliminated from the medium, there was considerable decrease in erythritol concentration and yield although the biomass growth was comparatively equal to the other combinations with yeast extract.

The influence of replacement of yeast extract with di-ammonium phosphate on erythritol production by *C. magnolia* is shown in Fig. 2.5a, b. For *C. magnolia*, up to 60 % of nitrogen content from yeast extract can be replaced with di-ammonium phosphate without affecting much of erythritol production and yield. Whereas when sodium nitrate in the range of 20-40 % nitrogen content was used in combination with yeast extract more erythritol production was observed compared to control with 100% yeast extract (Fig. 2.5c, d). There was no erythritol formation when all the nitrogen was replaced in the form of inorganic salts.

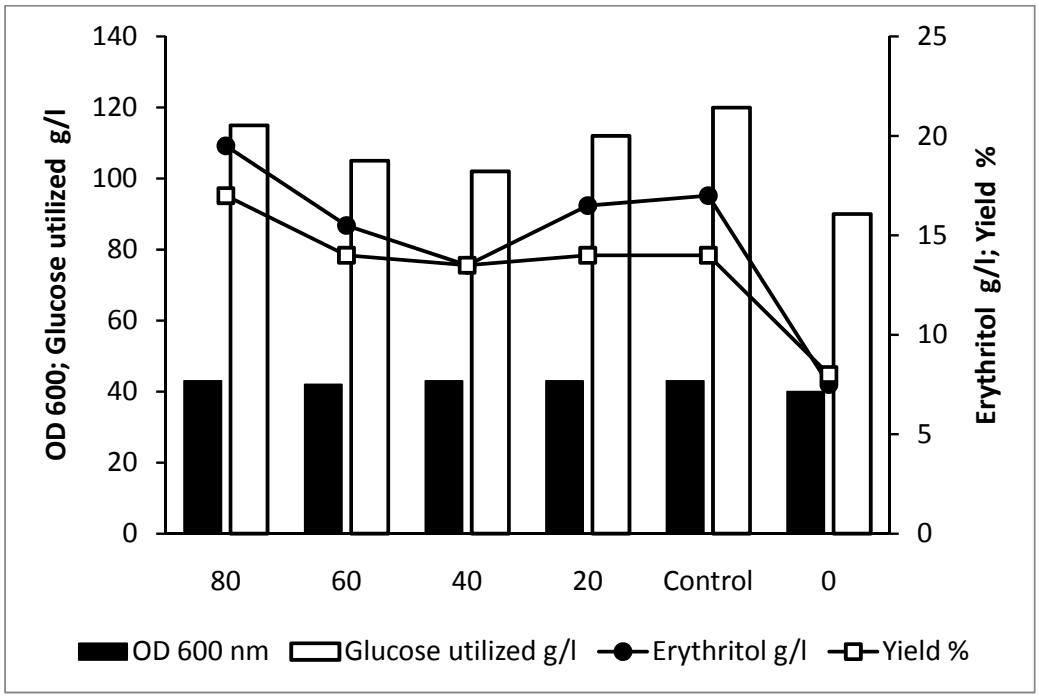

Figure 2.4 Effect of yeast extract and di-ammonium phosphate on erythritol production by *Y. lipolytica* with glucose as carbon source

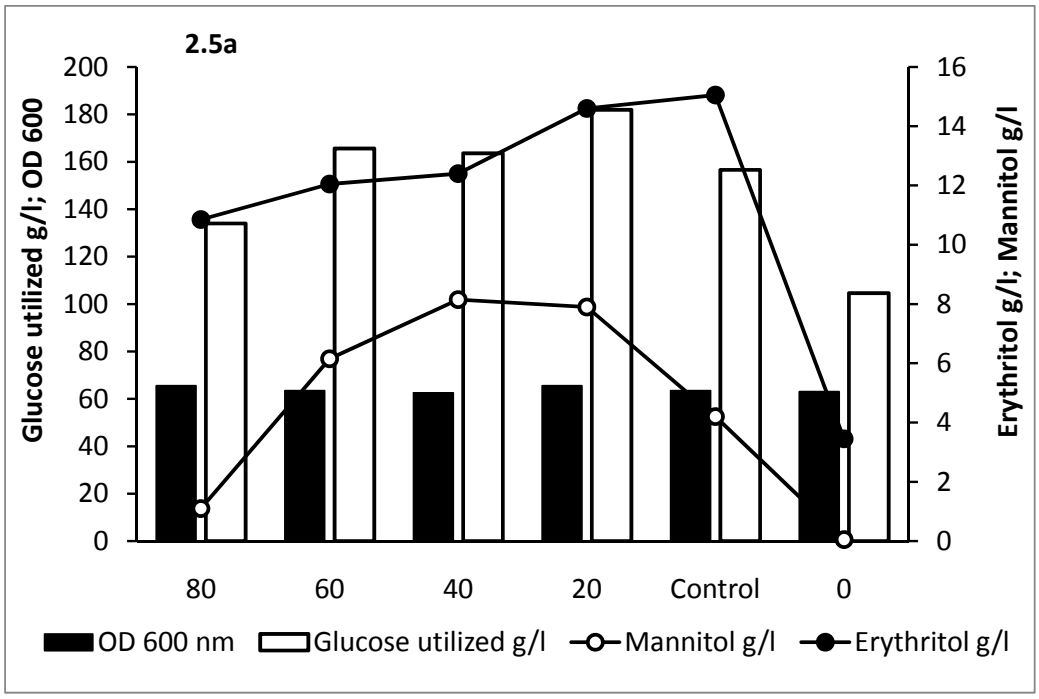

Figure 2.5a Effect of yeast extract and di-ammonium phosphate on erythritol production by *C. magnoliae* with glucose as carbon source

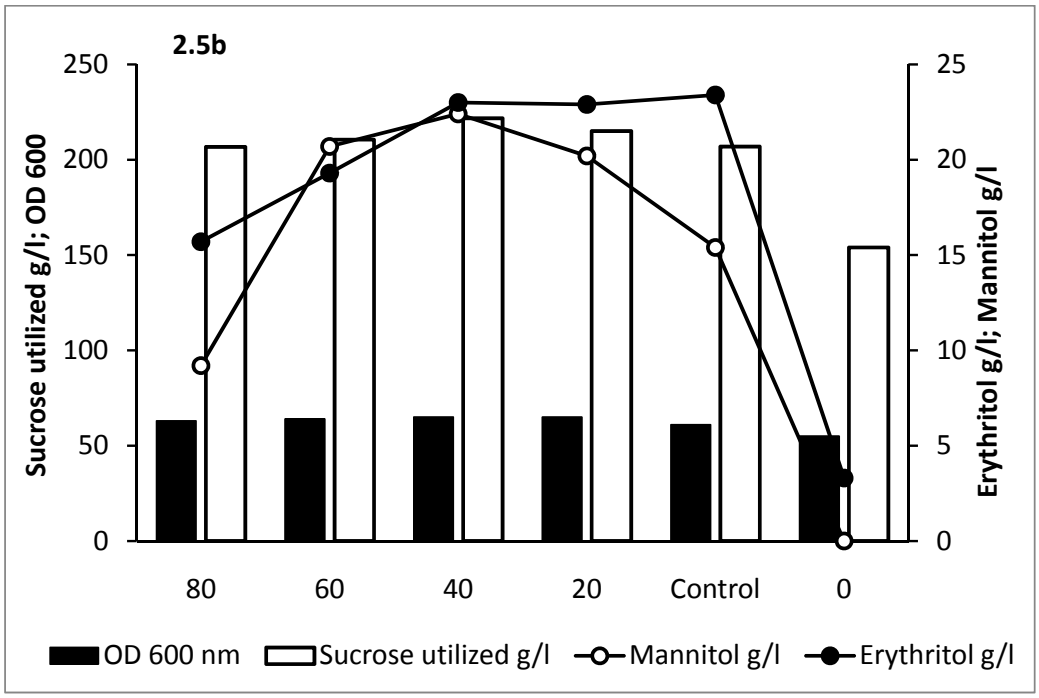

Figure 2.5b Effect of yeast extract and di-ammonium phosphate on erythritol production by *C. magnoliae* with sucrose as carbon source

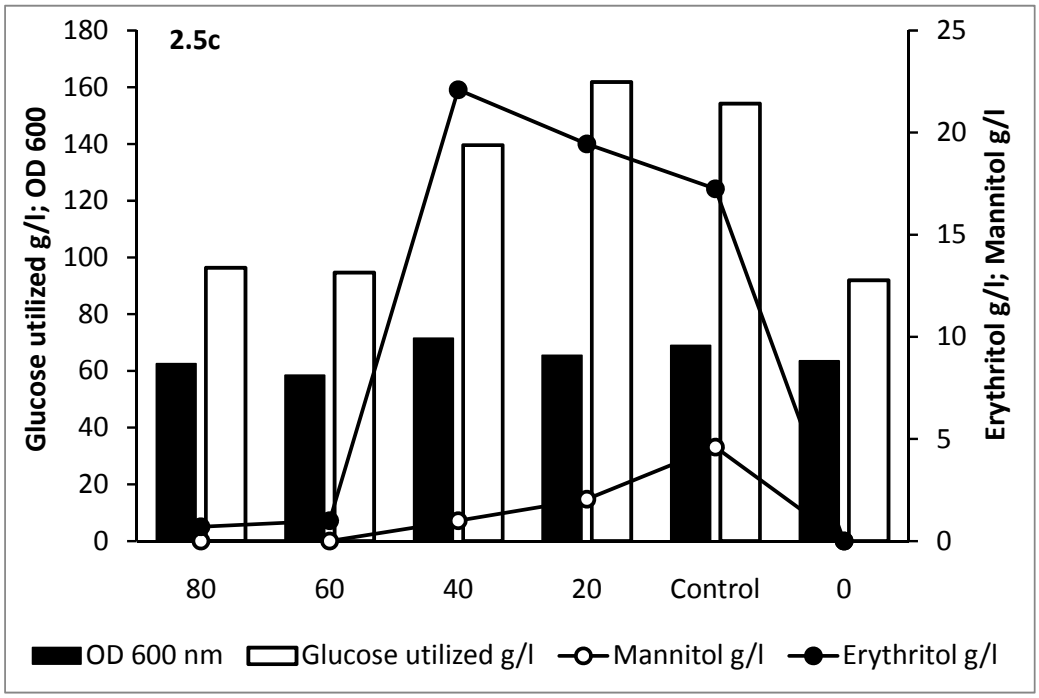

Figure 2.5c Effect of yeast extract and sodium nitrate on erythritol production by *C. magnoliae* with glucose as carbon source

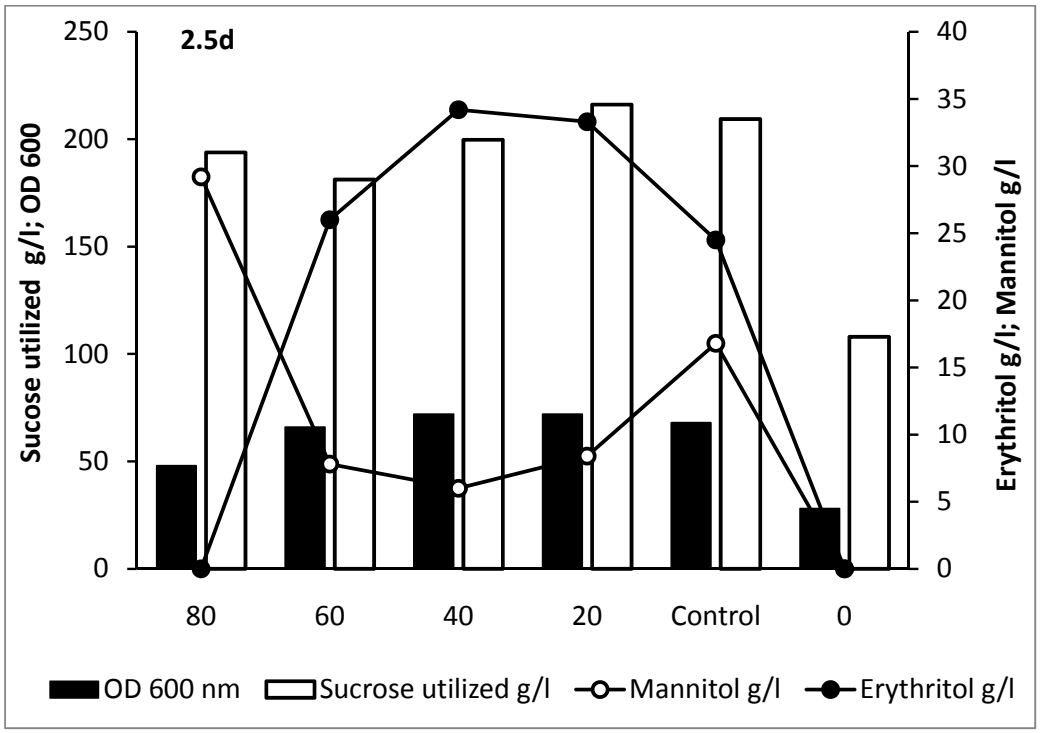

Figure 2.5d Effect of yeast extract and sodium nitrate on erythritol production by *C. magnoliae* with sucrose as carbon source

2.3.5 Effect of medium volume in shake flask for erythritol production

It was observed that the erythritol production is strongly influenced by the medium volume in the 250 ml Erlenmeyer flask which in turn affected availability of oxygen to the growing culture. Increase in medium volume in Erlenmeyer flasks results in decreasing oxygen transfer to the liquid cultures. In the present investigations, the amounts of erythritol, biomass concentration and yield decreased with the increase in medium volume in the flask where as the sugar utilization increased with the decrease in medium volume in the flask for both *Y. lipolytica* and *C. magnoliae* (Fig. 2.6 and 2.7).

The flask with 25 ml production medium resulted in highest yield of erythritol per gram sugar utilized. Interestingly, the ratio of glycerol: erythritol changed in favor of glycerol presumably because of higher availability of oxygen. The amount of sugar utilized increased with the increase in medium volume, but biomass concentration and polyols production decreased. The exact effect of oxygen on polyol production pattern needs to be investigated further for *C. magnoliae*.

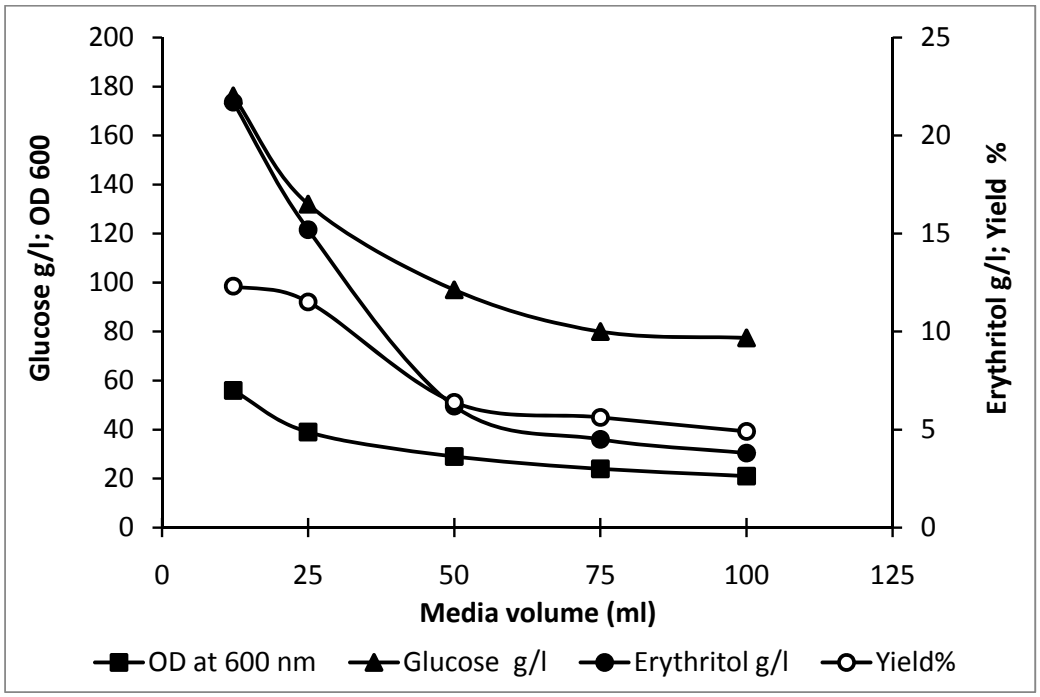

Figure 2.6 Effect of medium volume on erythritol production by *Y. lipolytica*

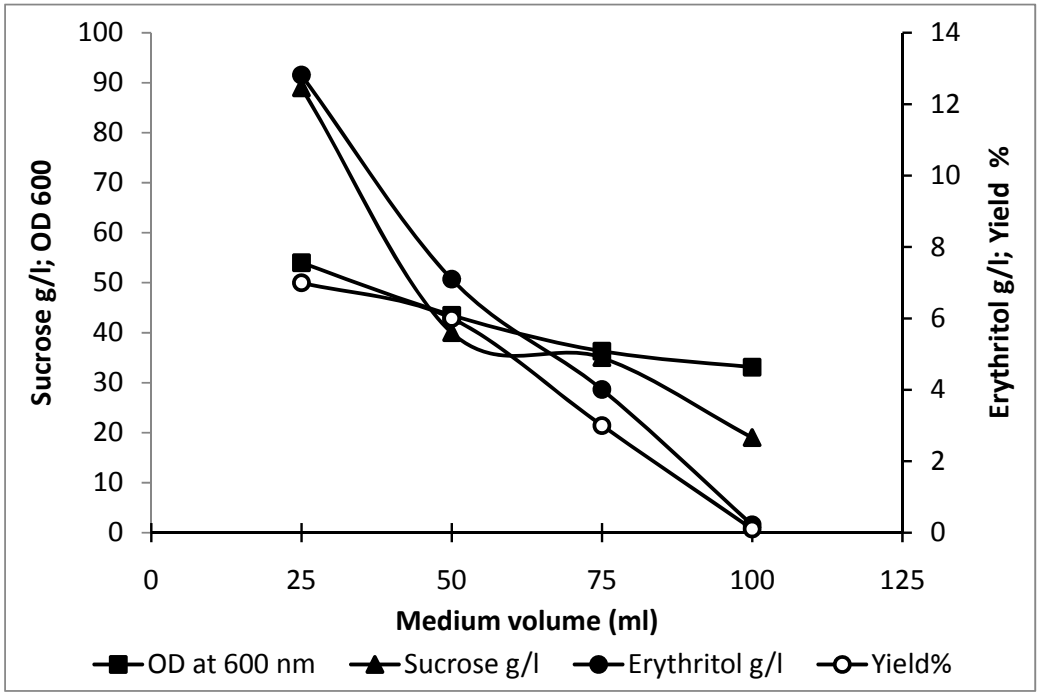

Figure 2.7 Effect of medium volume on erythritol production by *C. magnoliae*

2.3.6 Effect of pH on erythritol production by *Y. lipolytica and C. magnoliae*

Fig. 2.8a, b shows that erythritol production by *C. magnoliae* and *Y. lipolytica* was adversely affected in the presence of $CaCO_3$ as the neutralizing agent. It was observed that an attempt to control pH with calcium carbonate led to increase in undesirable polyol production by *C. magnoliae*.

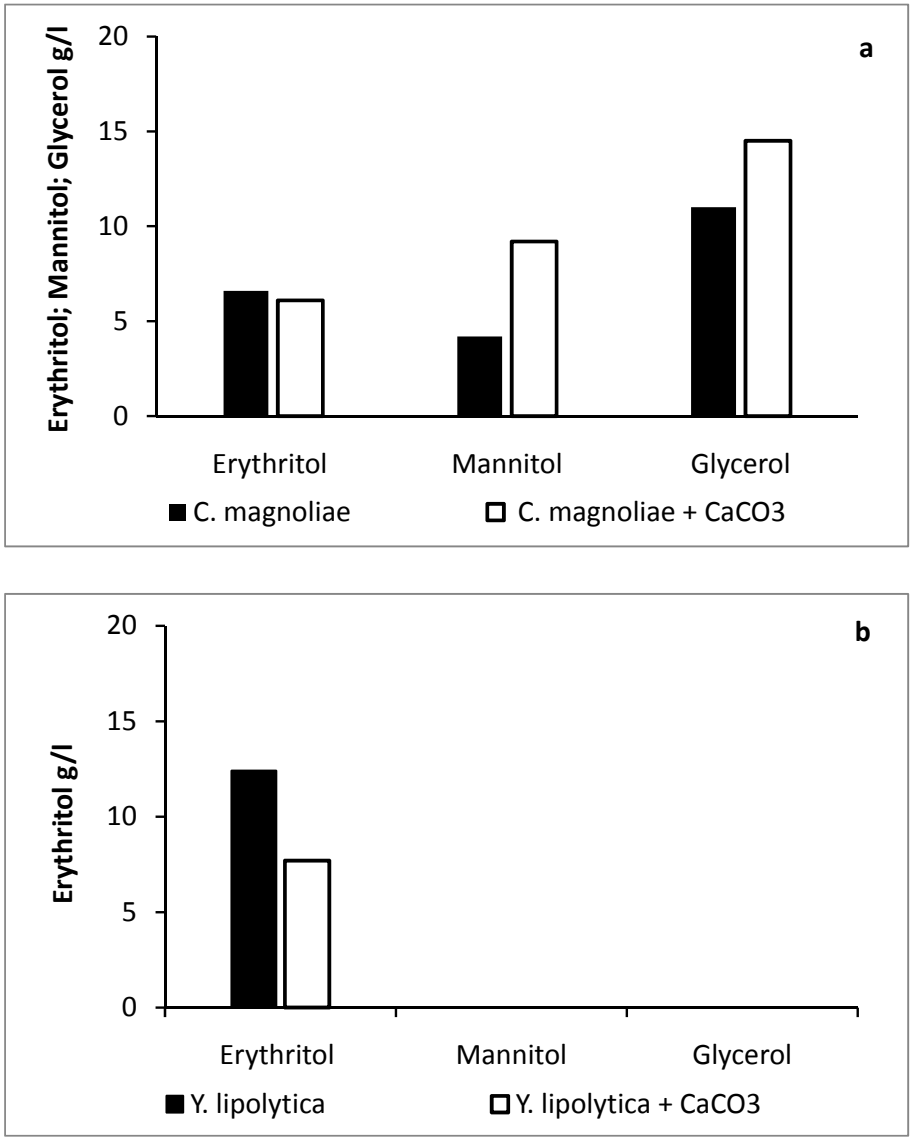

Figure 2.8a, b Effect of pH on erythritol production by *C. magnoliae* (a) and *Y. lipolytica* (b)

2.3.7 Evaluation of growth and erythritol production by *C. magnoliae* in a medium with different yeast extract concentrations

Increasing yeast extract concentration in medium, although increased sugar utilization, there was no significant difference in biomass generated as analyzed by measurement of OD. Increase in yeast extract concentration above 10 g/l decreased the concentration and yield of erythritol drastically. The increase in nitrogen source concentration in the medium allowed complete sugar utilization but did not result in erythritol production (Fig. 2.9). The experiments implied the need for carefully controlling the nitrogen concentration in the medium for erythritol production.

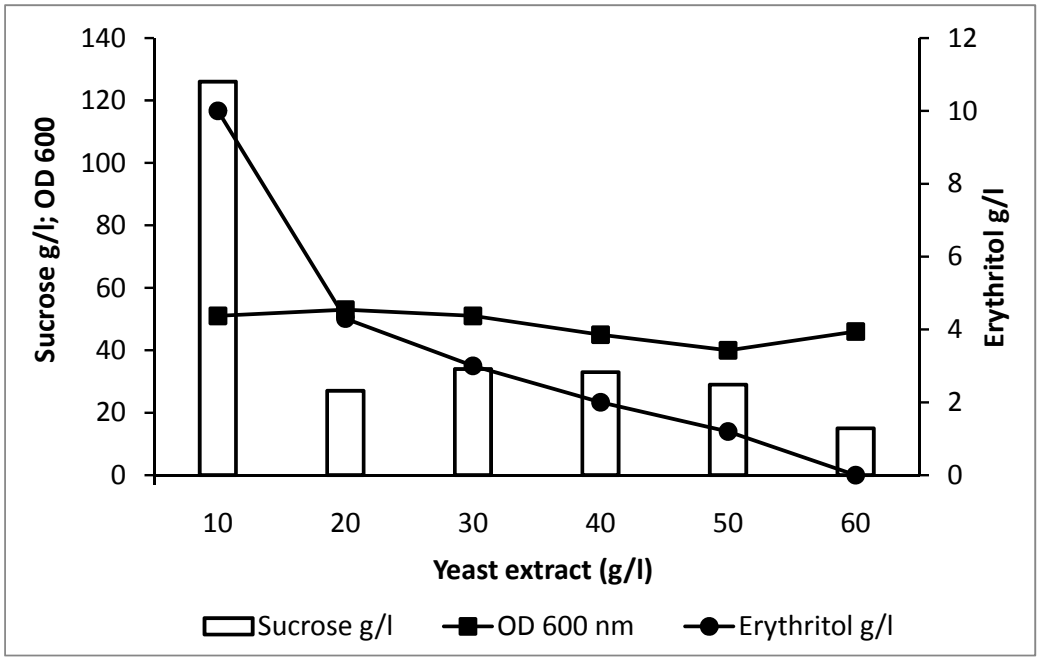

Figure 2.9 Effect of yeast extract on sugar utilization and erythritol production

2.3.8 Effect of initial sucrose concentration on erythritol production by *C. magnoliae*

The results show that the growth and erythritol production by *C. magnoliae* was not affected to a major extent by the differences in initial sucrose concentration, although 250 g/l sucrose concentration was slightly better in terms of the yield of erythritol per gram of sugar fermented. Initial sucrose content in excess of 250 g/l resulted in lower growth, yield, as well as specific erythritol production (Fig. 2.10). A sugar

concentration of 250 g/l was therefore used in subsequent experiments for erythritol production by *C. magnoliae.*

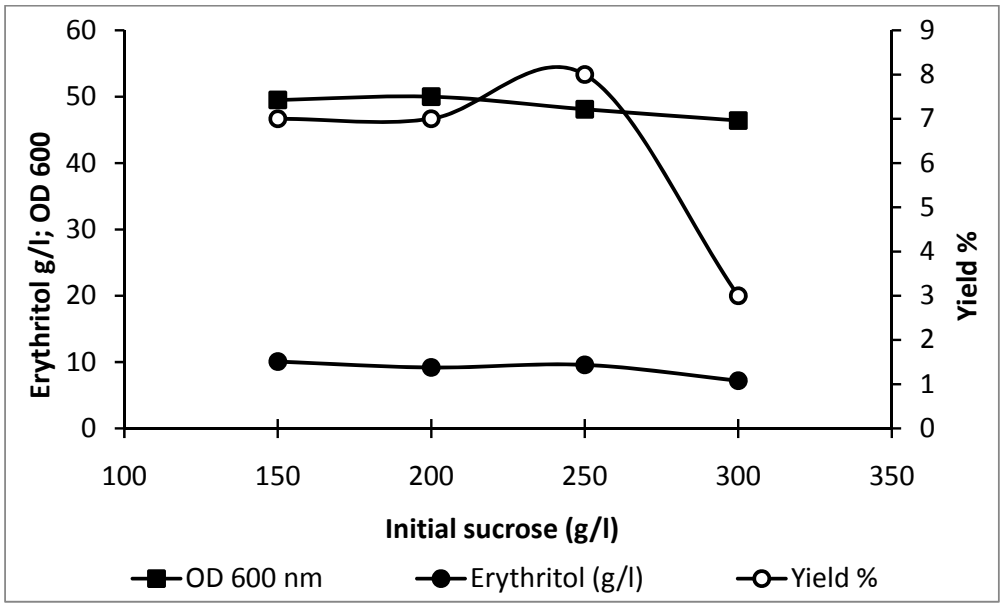

Figure 2.10 Effect of initial sucrose concentration on erythritol production

2.3.9 Requirement of phosphate for erythritol production by *C. magnoliae*

Erythritol concentration and yield were relatively better when 4 g/l potassium di-hydrogen phosphate was used. More importantly, there was reduction in unwanted polyols at this phosphate concentration for *C. magnoliae* as shown in Fig. 2.11.

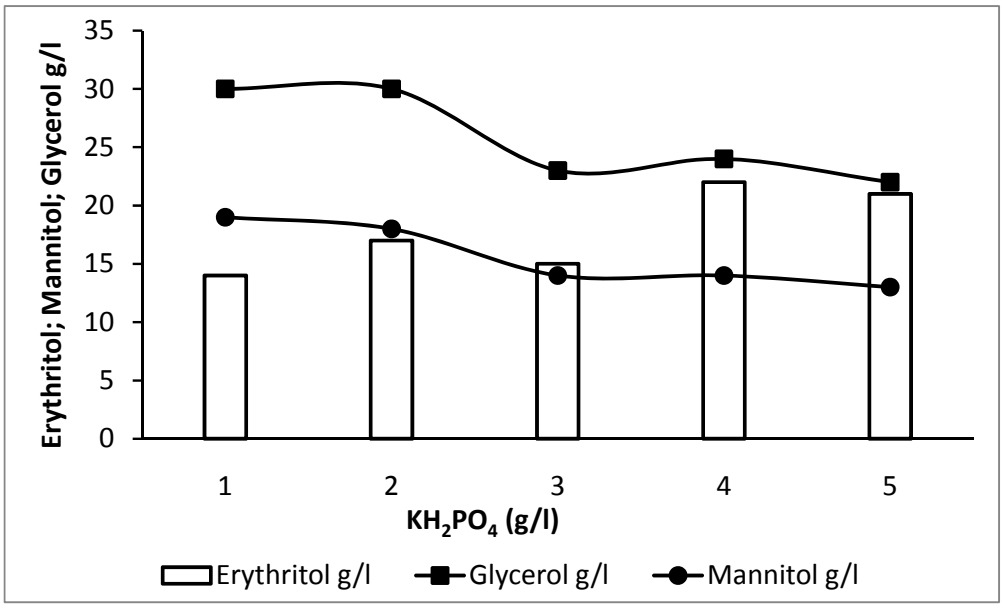

Figure 2.11 Requirement of phosphate for erythritol production by *C. magnoliae*

2.3.10 Time course analysis of erythritol production by *C. magnoliae* using sucrose as carbon source

Glucose-fructose uptake pattern and biomass growth of *C. magnoliae* on sucrose is illustrated in Fig. 2.12. *C. magnoliae* preferred glucose over fructose during the growth phase. However, beyond 96 h, the uptake pattern changed and the strain preferred fructose to glucose. During first 48 h, DCW increased rapidly and reached to 30 g/l towards the end of fermentation**.** It is seen that the glycerol that was produced initially was consumed by the culture during late hours. Maximum 42 g/l erythritol concentration was attained after consumption of 218 g/l of sugar (mixture of glucose and fructose) in 240 h. Erythritol yield of 19% was thus obtained at the end of 10 days (Fig 2.12).

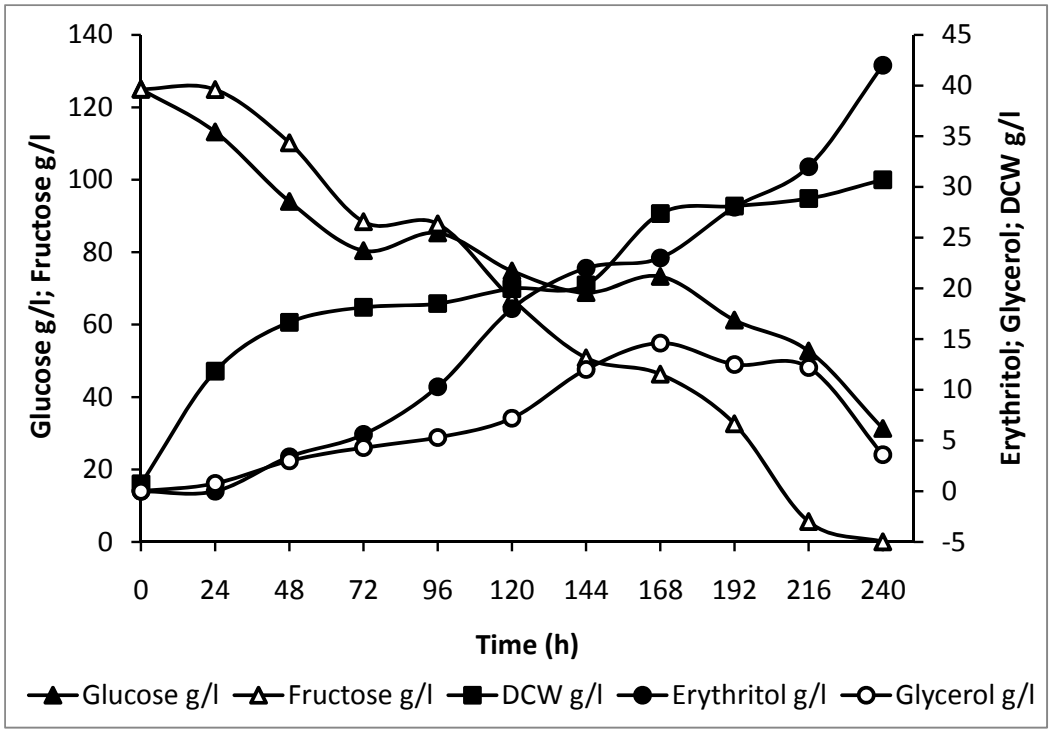

Figure 2.12 Time course of erythritol production by *C. magnoliae* using sucrose as carbon source

2.3.11 Effect of sucrose and glucose feeding on erythritol production by *C. magnoliae*

Based on results of time course fermentation of sugar uptake rate and erythritol production rate, a suitable nutrient feeding pattern of glucose and sucrose for fed-batch cultivation was evaluated to examine effect of substrate concentration and osmotic pressure. The sugar was fed in a particular pattern so that there was no excess unutilized sugar left in the broth. It was found that when sucrose was used as feed, fairly high amounts of erythritol and mannitol were produced, 46 and 54 g/l respectively (Fig. 2.13). On the contrary, when glucose was used, comparatively low amounts of erythritol and mannitol, 24 and 28 g/l, respectively were produced but the overall pattern was same (Fig. 2.14). A small amount of glycerol was produced when glucose was fed but was consumed later, which was not observed in case of sucrose feeding. Growth and sugar utilization patterns were similar when either of the sugar was used.

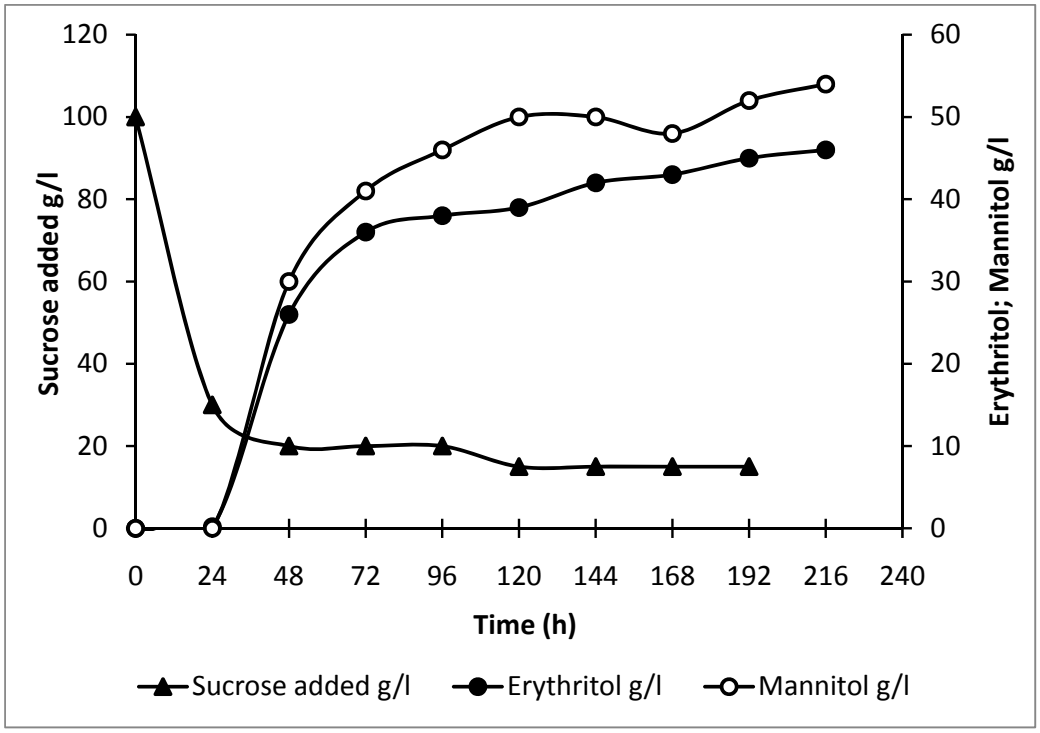

Figure 2.13 Effect of sucrose feeding on erythritol production for *C. magnolia*

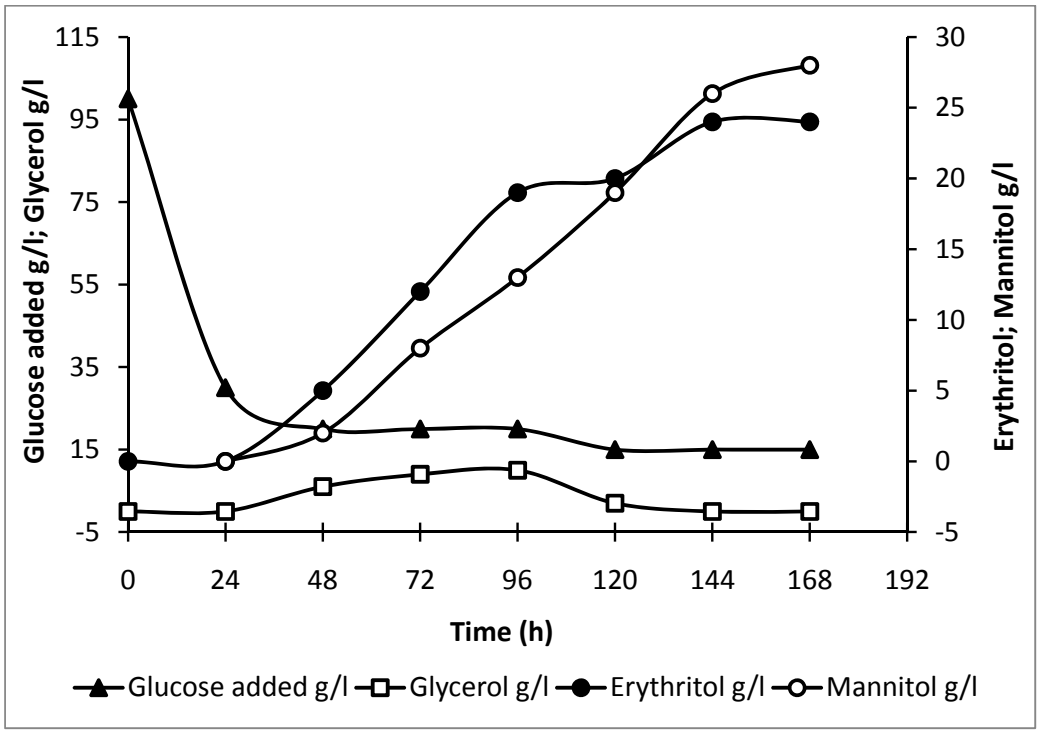

Figure 2.14 Effect of glucose feeding on erythritol production for *C. magnoliae*

2.3.12 Production of polyols by resting cells of *C. magnolia*

In the initial experimentation, *C. magnoliae* grew well in LFM at 250 g/l glucose or sucrose concentration and produced a mixture of erythritol, glycerol and mannitol. In the process in which *C. magnoliae* was grown in sucrose based medium and the resting cells were suspended in various carbon sources, the pattern of polyol produced was distinctly different (Fig. 2.15). The resting cells could not consume glucose effectively and only 36 g/l glucose was utilized in 96 h which resulted in 10 g/l erythritol and 3 g/l mannitol. On the other hand, fructose was completely consumed by the resting cells of *C. magnoliae* within 96 h and resulted in 44 g/l mannitol production. When sucrose was used, 35 g/l mannitol and 12 g/l of erythritol were produced. Interestingly, was completely consumed within 96 h and resulted in highest mannitol production (48 g/l).

During experiment with addition of 1 g/l yeast extract to keep the cells metabolically active during production phase, almost same pattern of polyols were observed with glucose, fructose and sucrose but with glycerol there was reduction in mannitol concentration to 37 g/l. *C. magnoliae* resting cells produced mannitol from glycerol in

aqueous solution under aerobic condition without any other nutrient. Most of the chemical and biological processes investigated earlier use fructose or glucose-fructose mixture as carbon source. Mannitol was the only metabolite produced from glycerol by resting cells of *C. magnoliae* with yield of mannitol as high as 48%.

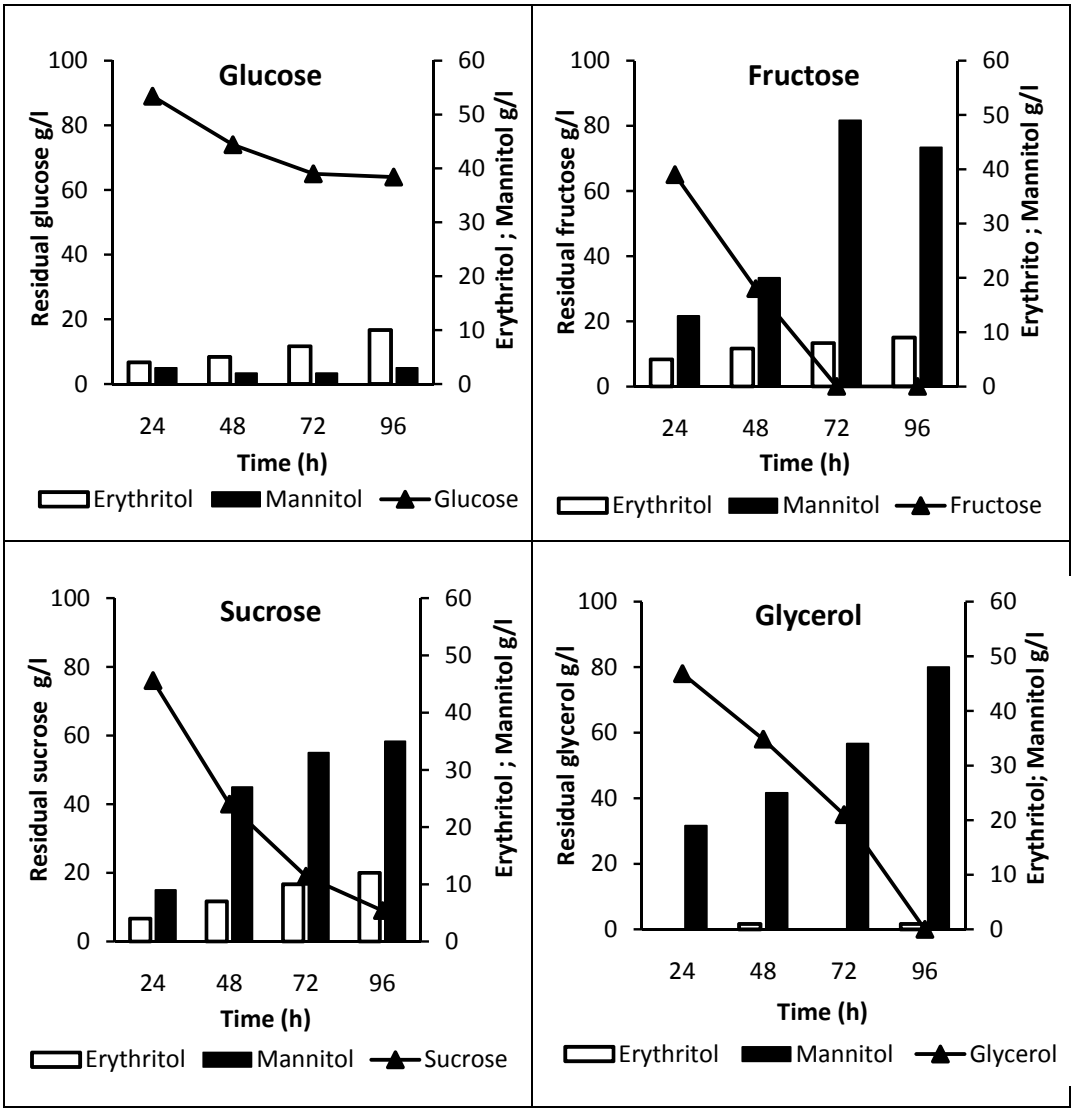

Figure 2.15 Production of polyols by resting cells of *C. magnoliae*

2.4 CONCLUSIONS

On screening osmo-tolerant yeast strains from NCIM and MTCC, *Y. lipolytica* NCIM 3472 and *C. magnoliae* NCIM 3470 that produced highest 17.1 g/l and 13.9 g/l erythritol with a respective yield of 12.4 and 9.5% were selected for further investigations. Studies on initial culture conditions illustrated that, glucose was the

best carbon source for *Y. lipolytica*, while *C. magnoliae* could produce erythritol from glucose and sucrose. Both the cultures could utilize most of organic and inorganic nitrogen sources studied and produce erythritol. These cultures required a small amount of yeast extract as vitamin and amino acid source for erythritol production. Erythritol production was found to be mainly dependent on oxygen availability and nitrogen content in the medium. Addition of yeast extract above 10 g/l drastically decreased the erythritol production. Time course fermentation profile illustrated that the erythritol production started towards the end of growth phase after the exhaustion of nitrogen source from the medium.

In a two-stage fermentation, the resting cells of *C. magnoliae* produced mannitol from fructose, sucrose and glycerol but not from glucose. Resting cells produced only mannitol irrespective of the carbon source used, in contrast to the earlier experiment where growing cultures produced mixtures of erythritol and mannitol. When resting cells with small amount of yeast extract in medium were used, mannitol production was less as compared to pure aqueous glycerol solution. Mannitol was the only metabolite produced from glycerol by resting cells of *C. magnoliae* with yield of mannitol as high as 48%.

Y. lipolytica produced erythritol as the only metabolite irrespective of the media constitutes and culture conditions, where as *C. magnoliae* produced small amount of mannitol and glycerol as co-metabolite.

C. magnoliae NCIM 3450 and *Y. lipolytica* NCIM 3472 were selected for mutagenesis programme for further enhancement in erythritol production.

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Chapter 3

Strain Improvement and Statistical Medium Optimization for Enhanced Erythritol Production

Abstract:

Mutants of C. magnoliae and Y. lipolytica were generated by UV irradiation, EMS and NTG treatment. Through screening of 1368 mutants for enhanced erythritol production, two of mutants of C. magnoliae named M572 and R23, producing higher erythritol were selected. Unexpectedly, two mutants of C. magnoliae named R1 and R9, producing mannitol from glucose were also isolated.

Optimization of medium components and culture conditions for the mutant M572 was carried out in shake flask by "one factor at a time". Statistical RSM approach of media optimization was employed for the mutant R23 to maximize erythritol production and minimize the formation of mannitol and glycerol. Maximum 60 g/l erythritol was produced by mutant R23 in shake flask in statistically-optimized medium.

To ascertain the reason for enhanced erythritol production by mutant R23, a comparison study between parent and its mutant R23 for two key enzymes involved in erythritol biosynthesis was conducted. Under identical growth conditions, at 96 h, erythrose reductase activity of mutant R23 was about two-fold higher than that of the parent C. magnoliae.

3.1 INTRODUCTION

Microorganisms are extremely good in producing array of valuable metabolites, although they tend to produce such compounds in smaller amount. Extensive strain improvement programs are usually necessary for obtaining a strain with a high product yield. Historically, strain improvement programme in industry had lead to increase in production of microbial metabolites by several hundred folds. The capability to modify selected microbial cultures for higher productivity has been a very important aspect in fermentation industry. Remarkable decreases in costs of fermentation products have come about mainly by strain improvement through mutagenesis or by application of recombinant DNA technology (Adrio & Demain, 2006; Demain & Adrio, 2008; Parekh et al., 2000).

The most common method used to obtain high yielding mutants is to treat cells of a desired culture with a mutagenic agent until a desired kill is obtained, plate out the survivors on suitable, preferably selective media and test each resulting colony or a randomly selected group of colonies for product formation in shake flasks. The most functional mutagens comprise *N*-methyl *N*-nitro *N*-nitroso guanidine (NTG), methyl methane sulfonate (MMS), ethyl methyl sulphonate (EMS), hydroxylamine (HA) and ultraviolet light (UV) (Adrio & Demain, 2006; Demain & Adrio, 2008).

Different classes of genetic controls are known for metabolite production viz. (i) structural genes coding for product synthesis, (ii) regulatory genes determining the onset and expression of structural genes (iii) resistance genes determining the resistance of the producer to its own product (iv) permeability genes regulating entry, exclusion and excretion of the substrate as well as product and (v) regulatory genes controlling pathways providing precursors and cofactors (Malik, 1979). Overproduction of microbial metabolites is effected by (i) increasing precursor pools, (ii) adding, modifying or deleting regulatory genes, (iii) altering promoter, terminator and/or regulatory sequences, (iv) increasing copy number of genes encoding enzymes catalyzing bottleneck reactions, and (v) blocking/deleting competing unnecessary pathways (Strohl, 2001).

Production of erythritol is a multi-gene activity; therefore the conventional mutagenesis is a preferred method for strain improvement in order to obtain enhanced

erythritol producing mutants. In addition to improvement in erythritol producing capabilities, undesirable properties, especially insufficient osmo-tolerance in media, undesirable metabolite formation and vigorous foaming under aerobic culture conditions have been eliminated by classical mutagenesis. Ishizuka et al., (1989) obtained a high erythritol-producing mutant of *Aureobasidium sp*. SN124A with 47.6% yield by UV irradiation and NTG treatment. *Penicillium sp*. KJUV29, a mutant derived from *Penicillium sp.* KJ81, exhibited a significantly improved erythritol production and reduced glycerol and foam formation compared with the wild-type strain (Lee & Lim, 2003).

A high erythritol-producing yeast strain was isolated from honeycombs and identified as *C. magnoliae* (Yang et al., 1999). In order to further improve the erythritol-producing ability, this strain was mutagenized by UV irradiation and NTG treatment and an osmotolerant mutant named M2 was generated which showed 25% increase in erythritol level (25 g/l) and 30% increase in productivity (0.54 g l^{-1} h^{-1}) than the wild strain (Yang et al., 1999). Further, optimization of fed-batch fermentation resulted in 200 g/l erythritol production with 1.2 g l^{-1} h^{-1} erythritol productivity and 43 % yield (Koh et al., 2003; Ryu et al., 2000). Although, this process resulted in a fairly high amount of erythritol production, it also led to the formation of substantially high amount of organic acids like gluconic, citric and butyric acid (Koh et al., 2003; Ryu et al., 2000). Apart from erythritol, *C. magnoliae* has also been reported to produce glycerol, mannitol xylitol, citric acid, gluconic acid, butyric acid and ethanol depending upon medium and environmental conditions (Sahoo & Agarwal, 2002; Song et al., 2002; Tada et al., 2004). Interestingly, the compositions of fermentation products of C. magnolia are dependent on the nature, composition and concentration of media constituents.

Erythritol yield of *Torula sp*. isolated by Kim et al. (2000) was improved to 48.9% by optimization of media constituents and process parameters. Fed-batch fermentation, supplemented with inositol and phytic acid, produced 196 g/l erythritol with 2.26 g $l^{-1}h^{-1}$ productivity. This strain did not produce glycerol and ribitol and therefore was considered on industrial scale for erythritol production (Kim et al., 2000; Lee et al., 2000; Lee et al., 2001; Oh et al., 2001).

To date, only a few microorganisms are known for erythritol production at industrially useful level. Erythritol is commercially produced by Bolak Corporation (Whasung, Kyungki-do, Korea), Cargill Food & Pharma Specialties (Blair, Nebraska, USA) and Mitsubishi Chemical Corporation (Tokyo, Japan). Glucose derived from wheat and corn starch is used as a major carbon source to produce erythritol by the fermentation using yeast-like fungi such as *Aureobasidium sp*. SN-G42, *Torula sp*. and *Moniliella pollinis* (Moon et al., 2010).

In previous chapter, seven erythritol producing yeast cultures were identified and out of them, *Y. lipolytica* NCIM 3472 and *C. magnoliae* NCIM 3470 were selected for strain improvement programme through mutagenesis. The objective of the present section of work was to enhance the erythritol production and minimize unwanted by-products of *C. magnoliae* and *Y. lipolytica* by classical mutagenesis and to optimize culture conditions for the selected mutants.

3.2 MATERIALS AND METHODS

3.2.1 Cultures

Candida magnolia NCIM 3470, *Yarrowia lipolytica* NCIM 3472 and the mutants generated were maintained on LFM agar slants. For long-term storage of the selected mutants, glycerol stocks were prepared and maintained at -20 ºC.

N-methyl *N*-nitro *N*-nitroso guanidine (NTG), Ethyl methyl sulphonate (EMS), Erythrose, Glucose-6-phosphate, NADPH, NADH, NADP, NAD, standard sugars and polyols were purchased from Sigma-Aldrich, USA. All media ingredients were purchased from HiMedia, Mumbai, India.

3.2.2 Mutagenesis

Four different approaches were employed for mutagenesis. A survival curves were drawn from the number of surviving cells with respect to time for each of the mutagen used and optimum time and concentrations were determined for the respective mutagens in order to get 90-95 % kill rate. Mutagenesis programme was initiated with UV, in which mutants were screened on morphological variations on high osmotic pressure agar medium. Subsequently, UV mutagenesis was coupled to chemical

mutagenesis and changes were made in screening medium including 1) increase in the sugar or salt concentration for higher osmo-tolerant mutants 2) incorporation of pH dyes for acid- negative mutants and 3) use of 2, 4, 5-triphenyl tetrazolium chloride to screen colonies with higher reductase activity.

(1) UV irradiation: A small mechanical device prepared from aluminum sheets with a UV lamp inside was used. The parent strains were grown in LFM for 48 h. The culture broth was centrifuged at 10,000 *g* and the cells were washed twice with sterile physiological saline by suspension followed by centrifugation. For *Y. lipolytica,* the pseudomycelium was removed by filtration over a glass funnel with sterile absorbent cotton to get well separated cells for mutagenesis. The cell count was adjusted to 1×10^5 cells/ml by counting cells under a microscope using Neubauer improved cell counting chamber (Marienfeld Germany). Two ml of the above cell suspension was UV irradiated using germicidal lamp (Sankyo Denki Co. Ltd., Japan) for 0 to 3 min at a distance of 20 cm.

(2) EMS treatment: Five ml cell suspension with 1×10^5 cells/ml was treated with 20 µl of EMS for 0 to 60 min. At fixed time interval, 0.5 ml filter sterilized, 5% sodium thiosulfate was mixed with 0.5 ml of treated cell suspension to inactivate EMS.

(3) NTG treatment: Five ml cell suspension with 1×10^5 cells/ml was treated with 50 µl (2 mg/ml) NTG, in 50 mMol acetate buffer pH 5.5, for 0 to 60 min. Sterile 5% sodium thiosulfate was used to inactivate NTG after treatment, at each time point.

(4) A combination UV and EMS/NTG treatment was carried out by irradiation of the cell suspension with UV for 30 sec followed by the EMS/NTG treatment for 20 min.

The sequence of mutagenesis procedures used in this study was as follows UV → EMS → UV + EMS → NTG → UV+NTG. After mutagenesis treatment, 20 µl mutagenized cell suspension containing approximately 2000 treated cells was spread plated on LFM agar plates with glucose/sucrose concentration between 400 and 600 g/l. To exert constant high selection pressure for selecting mutants in some of mutagenesis series, plates were incorporated with 200 g/l KCl with 20 g/l sugar. Bromocresol green was included in the media, at 0.1 g/l concentration to select non acid producing colonies. In some of the mutagenesis series, plates were incorporated

with (0.1 g/l) 2, 3, 5-Triphenyl tetrazolium chloride (TTC) to select the mutants with high reductase activity.

3.2.3 Mutant selection

After incubation of plates with treated cells at 28 °C for six to seven days, colonies were selected from those plates, which showed 5-10 % of survival. The colonies which were bigger, smooth, non-pigmented, non-sticky and acid-negative were selected from high osmotic pressure agar plates. The colonies which were larger in size and developed red colour presumably because of high reductase activity were selected from the plates in which TTC was added. The selected colonies were inoculated in tubes with 5 ml LFM or flasks with 25 ml LFM. The tubes and flasks were incubated on shaker at 210 rpm, 28 °C for 5 days. Preliminarily screening of the mutants was done in flasks and superior mutants were selected based on HPLC analysis. Later, an indirect mode of selection was used in which 5 ml liquid medium in 150 × 25 mm test tubes was inoculated with selected colonies and incubated at 210 rpm, at 28 °C for 3 days and residual glucose was analyzed. Mutants which showed higher glucose utilization compared to parent were selected and further evaluated in Erlenmeyer flasks with 25 ml medium. The mutants with enhanced erythritol production and minimal by-products were selected and used as intermediate parent strains for mutagenesis for further desired improvement. The selected mutants were maintained on LFM agar slants. For long-term storage, glycerol stocks were prepared and maintained at -20 °C. Intermediate mutants were also screened for enhanced erythritol production by optimizing key medium components (carbon and nitrogen sources) and the process parameters in shake flask.

3.2.4 Optimization of erythritol production by *C. magnoliae* mutant M572

3.2.4.1 Choice of carbon sources for erythritol production

Glucose, sucrose, fructose, glycerol and glucose-fructose mixture were investigated as carbon sources for polyol production by *C. magnoliae* mutant M572. Each of the carbon source was used at 250 g/l. Media were inoculated with 48 h seed culture and incubated at 28 °C, 210 rpm on rotator shaker. Samples were analyzed at 168 h as described earlier.

3.2.4.2 Effect of initial glucose concentration on erythritol production

Effect of initial glucose concentration on growth and erythritol production by *C. magnoliae* NCIM 3470 and mutant M572 was investigated by inoculating 2.5 ml 48 h old seed culture to flask containing different initial glucose concentrations between 150 and 400 g/l.

3.2.4.3 Effect of yeast extract concentration on erythritol production

Effect of yeast extract concentration on growth and polyol production by mutant M572 was investigated by varying yeast extract concentrations in LFM from 2 to 16 g/l. Later, in order to decrease the yeast extract requirement, 50% of yeast extract was replaced by utilizable inorganic nitrogen sources. The nitrogen content of the medium from all sources was adjusted to the level of 10 g/l yeast extract. A set of flasks with 10 g/l yeast extract was run simultaneously as control.

3.2.4.4 Effect of medium volume on erythritol production

Medium volume in Erlenmeyer flasks exerts effect on metabolism because of differences in oxygen transfer. To investigate this, *C. magnoliae* mutant M572 was grown in 25 ml LFM, for 48 h. Contents of several identical flasks were mixed at 48 h and the broth was redistributed aseptically in 9 flasks as 10, 20, 25, 30, 40, 50, 60, 80, 100 ml, respectively. The flasks were incubated under shaking till 168 h. Samples were withdrawn at an interval of 24 h and analyzed as described earlier. To determine solubility of oxygen in experimental conditions, several 250 ml Erlenmeyer flasks, each containing 25 ml sterile glucose solution were placed on shaker at 210 rpm, 28 °C for 24 h. The solutions used had glucose concentrations between 50 and 400 g/l at a difference of 50 g/l. After 24 h agitation, the dissolved oxygen concentration was estimated by Azide-Winkler method (Greenberg *et al.*, 1992).

3.2.4.5 Effect of metal ions on polyol production

Effect of Ca^{+2}, Co^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2}, Mo^{+2}, Zn^{+2} and B^{+2} on polyol production were studied in shake flask with 25 ml LFM. Three different concentrations of trace metals, 10, 50 and 100 mg/l, were added to individual flasks containing LFM. The flasks were incubated for 168 h and analyzed for growth and polyols production.

3.2.4.6 Nutritional requirements of mutant M572

In order to identify nutritional requirements of *C. magnoliae* mutant M572 for growth and erythritol production, the mutant was inoculated in 10 ml Yeast Nitrogen Base (YNB) medium in 150 × 25 mm test tubes. YNB with vitamins and amino acids (HiMedia M 139), YNB with vitamins but without amino acids (HiMedia M 878) and YNB without vitamins with amino acids (HiMedia M 208) were used. The specific vitamin requirement was investigated in YNB medium without vitamins and amino acids by omitting one vitamin at a time. Each of the water soluble vitamin solution biotin, folic acid, inositol, niacin, pyridoxine HCl, Ca-pantothenate, riboflavin and thiamine HCl, filter sterilized through 0.22 μm membrane filters was added separately to the medium to have 10 mg/l final concentration. Mutant M572 from freshly prepared slants was washed twice with saline by centrifugation and re-suspension and a loop-full was inoculated in 10 ml medium. The OD was measured after incubation for 72 h at 28 °C at 210 rpm. For confirmation of the essential vitamins, the above experiment was repeated with all vitamins, three of the essential vitamins biotin, pyridoxine and thiamine and by replacing one at a time. After the study of nutritional requirement, production of erythritol was investigated by inoculating mutant M572 in a minimal medium composed of (g/l) glucose 250, KH_2PO_4 5 and $MgSO_4$ 0.25, along with $NaNO_3$ or $(NH_4)_2SO_4$ as sole nitrogen source and 10 mg/l each of biotin, pyridoxine and thiamine. The flasks were incubated for 168 h and analyzed for growth and erythritol production.

In a separate experiment, effect of individual vitamins on erythritol production was studied. Biotin, Folic acid, Inositol, Niacin, Pyridoxine HCl, Ca-Pantothenate, Riboflavin and Thiamine HCl were added at 10 mg/l concentration to individual flasks containing LFM after 72 h of growth phase. Samples were analyzed at 168 h.

3.2.4.7 Effect of glucose feeding on erythritol production

Effect of glucose feeding on erythritol production for mutant M572 was investigated by inoculating 48 h old seed culture in 25 ml LFM with initial glucose concentration of 30 g/l. After 24 h incubation, sterile glucose solution was fed in the range from 10 to 50 g $l^{-1}d^{-1}$ at 24, 48, 72, 96 and 120 hours and samples were analyzed as described above. In another experiment to evaluate effect of relatively higher osmotic pressure

on erythritol production, glucose solution was fed to the flasks at a rate of 50 g $l^{-1}d^{-1}$ from 96 to 144 h to the flask in which initially 250 g/l glucose was used.

3.2.4.8 Comparison of *C. magnoliae* parent strain and its mutant M572

To compare the selected mutant M572 with the parent strain, respective seed cultures were grown in 25 ml LFM in 250 ml Erlenmeyer flasks as described above and samples were analyzed at 24 h interval using HPLC. Glucose uptake rate, erythritol productivity, specific erythritol productivity and erythritol yield for parent as well as mutant strain were compared at shake flask level.

3.2.4.9 Comparison of erythritol producing mutants M572 and R23 of *C. magnoliae*

Parallel mutagenesis was carried out using M572 mutant as parent for further improvement in erythritol production as described earlier. Mutants generated from M572 were screened for higher reductase activity on the plate medium containing TTC. All the morphologically different colonies with varying colour intensities were selected and analyzed by inoculating them in test tubes containing 5 ml LFM by incubating for 72 h. The colonies which showed higher sugar utilization than the parent strain were further analyzed in shale flask with 25 ml medium. Erythritol producing mutant R23 which showed higher erythritol production that M572 was compared in Erlenmeyer flasks in 25 ml LFM. Growth, sugar utilization and polyols produced were analyzed as described earlier.

3.2.4.10 Comparison of mannitol producing mutants R1 and R9 of *C. magnoliae*

During the mutagenesis, screening and selection programme, some mutants which produced high amounts of glycerol and mannitol instead of erythritol were also observed. Two mannitol producing mutants R1 and R9 were selected for further investigation. Mutant R1 and R9 were compared at flask level for their ability to produce mannitol production from glucose. These mutants were preserved at - 20 °C by preparing glycerol stocks.

3.2.5 Media optimization for maximum erythritol and minimum mannitol and glycerol formation from *C. magnoliae* mutant R23 using Response Surface Methodology (RSM)

Effect of individual medium components on erythritol production by mutant M572, by changing one factor at a time was studied initially. Evaluation of this results illustrated that formation of erythritol and other polyols (mannitol and glycerol) was mainly dependent on concentration of carbon, nitrogen and C:N ratio. Therefore combined effect of four media components was studied using central composite rotatable design (CCRD) of RSM. This model takes into account interactive effect of the media components as well as C:N ratio and predicts optimum solution. Moreover, medium optimization by "one factor at a time" is incapable of determining interactive effects of operating variables during the fermentation process and therefore, unable to determine the 'true' optimum. The limitations of the one factor at a time optimization can be eliminated by employing statistical optimization methods. RSM is a collection of statistical techniques which uses Design of Experiments (DoE) for building models, evaluating the interactive effects of factors and searching for the optimum conditions. In RSM, the operational variables (also called as input variables) are simultaneously varied and the corresponding experimental responses from the DoE are fitted to a quadratic equation. Using the quadratic equation, it is possible to optimize the medium which can selectively enhance the production of desired product by keeping the production of by-products at minimal possible levels. Since the separation of erythritol from the mixture of polyols is expensive and tedious, RSM appears to be an attractive method of media optimization especially to suppress production of other contaminating polyols.

Four media components viz. glucose, yeast extract, KH_2PO_4 and $MgSO_4$ were chosen for this study. A central composite factorial design of 2^4 = 16 plus 6 *centre points* plus 8 (i.e. 2 × 4) *star points* leading to a total of 30 experiments were performed in duplicate. Each flask was inoculated with 2.5 mL inoculum and incubated for seven days and analysis of polyols was done using HPLC as described earlier. The variable levels X_i were coded as x_i according to the *Eq. 3.1.*

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (\text{Where} i = 1,2,3..k) \text{ .....} \qquad \textit{- Eq. 3.1}$$

Where, x_i is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_0 is the real value of an independent variable at the center point and ΔX_i is the step change. The coded values of independent variables are given in Table 3.1.

The values of the three dependent responses (i.e. concentration of erythritol, mannitol and glycerol) were used to build RSM models. The second order polynomial coefficients were calculated and analyzed using the trial version of 'Design Expert' software (Version 8.0.2.0, Stat-Ease Inc., USA). Statistical analysis of the model was performed to evaluate the ANalysis Of VAriance (ANOVA). The overall predictive capability of the model is commonly explained by the coefficient of determination (R^2). The statistical significance of the fit of the polynomial model equation was checked by the F-test. The significance of the regression coefficient was tested by a t-test. The level of significance was given as values of Prob > F less than 0.05.

Coded values	***Glucose g/l***	***Yeast extract g/l***	***KH_2PO_4 g/l***	***$MgSO_4$ g/l***
-2	150	5	1	0.05
-1	200	7.5	3	0.15
0	250	10	5	0.25
1	300	12.5	7	0.35
2	350	15	9	0.45

Table 3.1 Coded values of independent variables

3.2.6 Characterization of R23 mutant of *C. magnoliae*

In the previous section, I have statistically optimized media components using RSM, to suppress the formation of undesirable polyols. Subsequently, R23 mutant was selected for optimization of fermentation parameters using statistically-optimized medium. Before optimization of process parameters, an attempt was made to study morphological and biochemical variations of the mutant. Morphological variations of the mutant R23, compared to its parent were examined by scanning electron microscopy (SEM). Respective cultures were grown in LFM for 48 h and washed twice with sterile physiological saline. Appropriately diluted cell suspension was applied on the SEM stubs and dried under vacuum for 30 min and subjected to SEM. Scanning electron microscopy (Quanta 200 3D, FEI) was used to observe the cells on

PDMS (Polydimethylsiloxane) stubs. Optical images were taken with Nikon Eclipse, E600-POL (Japan).

Carbohydrate utilization pattern of *C. magnoliae* 3470 and its mutant R23 was studied using various sugars such as arabinose, cellobiose, fructose, galactose, glucose, glycerol, maltose, mannose, melibiose, melezitose, raffinose, rhamnose, ribose, sucrose, trehalose and xylose. The media containing 25 g/l of each of the above mentioned sugars were prepared and dispensed in aliquots of 5 ml in 25 X 150 mm tubes, which were inoculated with the respective cultures and grown for 48 h at 28 °C, 210 rpm on rotary shaker. The growth in different sugars was compared by measuring optical density at 600 nm.

3.2.7 Studies on intracellular enzymes of mutant R23

To investigate the reason behind over production of erythritol by mutant R23, studies were undertaken to compare two of the intercellular enzymes namely erythrose reductase and Glucose 6-phosphate dehydrogenase (G6P-DH) which are key enzymes involved in biosynthesis of erythritol. ER catalyzes the final step of erythritol production, which reduces erythrose to erythritol with concomitant NADPH oxidation. NADPH provides the major reducing energy in microorganisms and is mostly generated in the pentose phosphate pathway by action of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (6PG-DH). Glucose-6-phospate (substrate of G6P-DH) is the branch point of glycolysis and PP pathway and catalyzed by G6P-DH. This enzyme is also responsible for the synthesis of NADPH which is required for the reduction of erythrose to erythritol. A high activity of PP pathway enzymes is required to produce abundant intermediates, required for higher erythritol productivity. Therefore it was decided to investigate these two intracellular enzymes.

3.2.7.1 Preparation of cell extracts

C. magnoliae and its mutant R23 were grown in LFM in several flasks for 144 h. The flasks of respective cultures were harvested at successive interval of 48 h by centrifugation at 10,000 *g* for 10 min. After washing the cells twice with 50 mM phosphate buffer (pH 6), two gram wet cells were suspended in disruption buffer for

30 min (50 mM phosphate buffer pH 6, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonylfluoride (PMSF)). This cell suspension was homogenized by grinding with 50 ml of 0.5 mm glass beads (Sigma) in a bead beater (Biospec Products Co., Bartlesville, USA.) for 5 cycles of one min with intermediate cooling of two min. Enzyme extracts was centrifuged at 10,000 *g* for 30 min at 4 °C and the supernatant was analyzed for ER and G6P-DH activities.

3.2.7.2 Erythrose reductase assay

ER assays were performed with some modifications of a previously described procedure by Lee et al., (2003c). The activity of ER was determined from the oxidation or reduction of NADPH, by measuring the increase or decrease in the absorbance at 340 nm at 50 °C. ER assay mixture (1.2 ml) for reduction consisted of 0.25 mM NADPH, 10 mM erythrose and 0.1 ml enzyme preparation in 50 mM phosphate buffer (pH 6). This reaction mixture was allowed to stand for one min to eliminate the endogenous oxidation of NADPH. The ER assay mixture for oxidation consisted of 0.5 mM NADP, 10 mM erythritol and 0.1 ml enzyme preparation in 50 mM phosphate buffer (pH 8.0). The reaction was started by the addition of substrate. One unit of enzyme activity is defined as 1 µmol of NADPH consumed or produced per min. ER activity of parent and mutant were expressed as U/g WCW

3.2.7.3 Glucose-6-P dehydrogenase assay

The activity of G6P-DH was determined by reduction of NADP, by measuring the increase in the absorbance at 340 nm at 50 °C. G6P-DH assay mixture (1.2 ml) consisted of 0.5 mM NADP, 10 mM glucose-6-phosphate and 0.1 ml enzyme preparation in 50 mM phosphate buffer (pH 8) with 1 mM $MgSO_4$. One unit of G6P-DH activity was defined as the amount of enzyme that produced one µmol of NADPH per min. G6P-DH activity of parent and mutant were expressed as U/g WCW.

3.2.7.4 Partial characterization of ER from mutant R23

Mutant R23 was grown for 96 h in LFM and ER was extracted as described earlier. The same enzyme preparation was used for characterization of the enzyme. For assays at different pH values, the reactions were performed with the following buffers (50

mM) of pH values (in parentheses): sodium citrate (4.0 and 5.0), potassium phosphate (6.0 to 8.0), carbonate:bicarbonate (9.0), and bicarbonate:NaOH (10.0). For thermal stability study of ER, aliquots of enzyme preparation were stored at 30, 40, 50, 60, and 70 °C and assayed for residual activity at successive interval of 30 min. The effects of metal ions, reducing agents and substrate and co-substrate specificity for oxidation and reduction reaction of ER were studied under above mentioned standard assay conditions. The obtained cell extracts showed specificity only for NADPH when erythrose was used as a substrate. Other substrate and co-substrate in the enzyme preparation negligibly interfered with the enzyme activity. Therefore the crude enzyme preparation was used to determine kinetic parameters and compare them to those reported in literature. The kinetic parameters were determined by Lineweaver-Burk plots for the enzyme activity vs. substrate concentrations. The *Km* and *Vmax* values were determined from the plots.

3.2.7.5 Effect of product and by-products on ER activity of mutant R23

Along with erythritol, a small amount of ethanol, glycerol and mannitol were formed during growth and fermentation, depending upon medium composition and environmental conditions. To determine, whether these by-products have inhibitory effect on ER reductase, the enzyme activity was estimated under assay conditions described above in the presence of erythritol, mannitol, glycerol and ethanol in concentration range between 50-600 mM.

3.3 RESULT AND DISCUSSION

3.3.1 Strain improvement for erythritol production by classical mutagenesis

Survival curve of *Y. lipolytica* and *C. magnoliae* after the mutagen treatments are presented in Fig. 3.1a, b. The figure illustrate that 90-95% kill was obtained on treating the cells with UV for 60-80 seconds for both the cultures, whereas in order to get 90-95% kill from the chemical mutagens (EMS and NTG) optimum time was found to be between 30 to 40 min. General protocol followed during the strain improvement programme for *C. magnoliae* and *Y. lipolytica* is presented in flow diagram and the same protocol was also followed for the intermediate mutants until a desired mutant was obtained (Fig. 3.2).

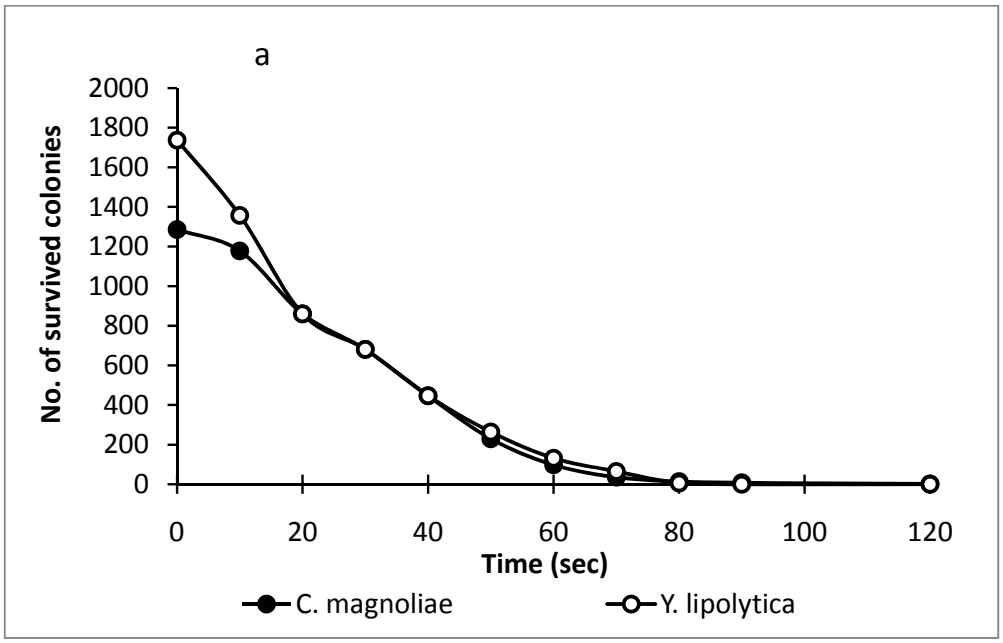


Figure 3.1a UV survival curve for *C. magnoliae and Y. lipolytica*

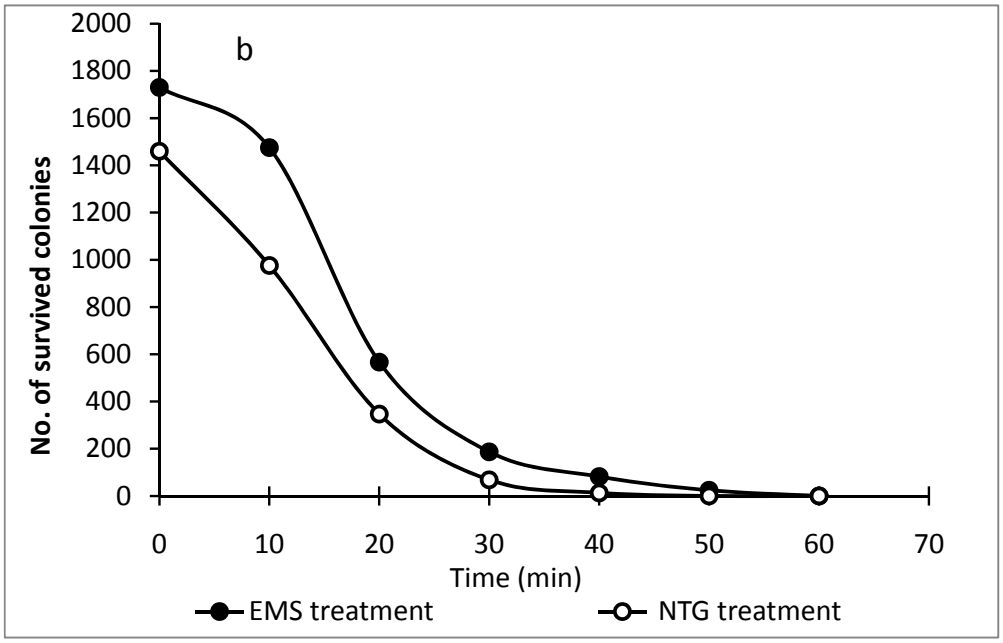


Figure 3.1b EMS and NTG survival curve for *C. magnoliae*

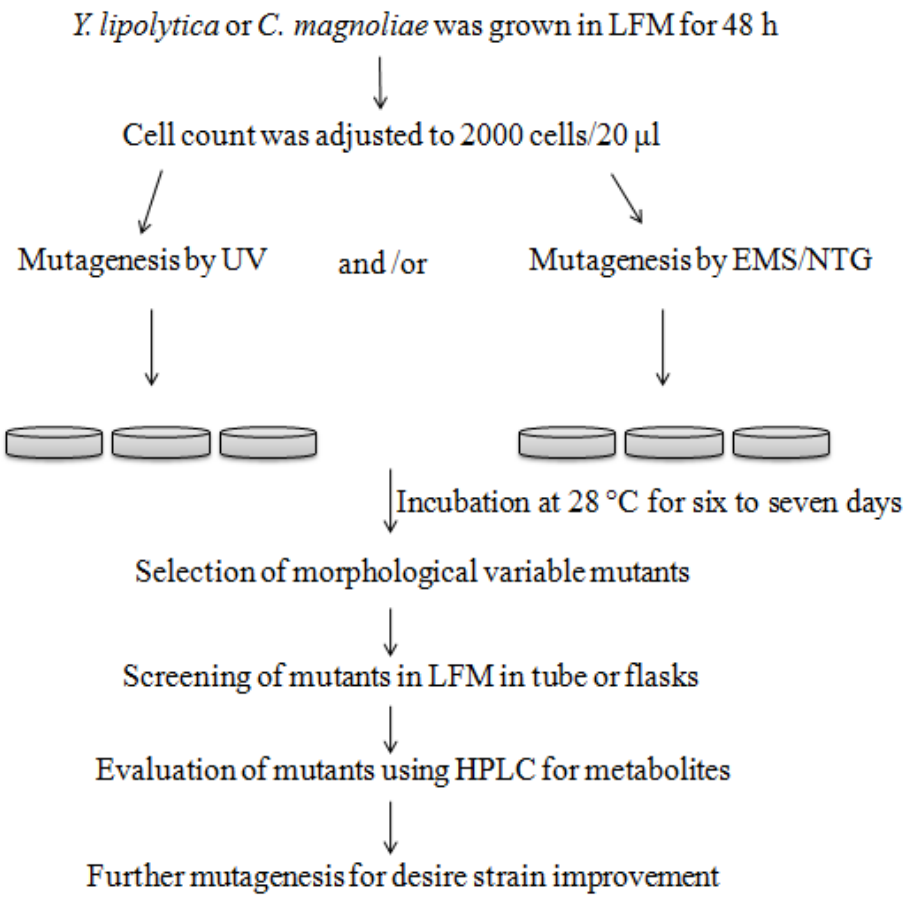


Figure 3.2 Methodology used for strain improvement by random mutagenesis

The detailed summery of each mutagenesis series, selection medium used for screening of the mutants, quantities of polyols produced and the selected mutant used as intermediate parents for next series of mutagenesis is given in Table 3.2. All together 52 mutation series were performed from which 1368 mutants were selected and screened for erythritol production.

Mutants generated from *C. magnoliae* strain showed gradual increase in erythritol production and sugar uptake. When the generated mutants and parent *C. magnoliae* were grown in 25 ml LFM, there were distinct differences in the extent of sugar utilization and product formation. The ratio of products formed was found to change as well as their concentrations. The comparison of parent and some of the selected mutants generated is shown in Fig. 3.3.

Series	***Parent used/ Intermediate parent***	***Mutagen***	***Selection medium g/l***	***No. of survivors selected***	***Screening environment***	***Mutant selected***	***OD 600 , Sugar utilized g/l***	***Erythritol Mannitol Glycerol g/l***
1-4	*C. magnoliae* NCIM 3470	UV	Sucrose 400	133	Flask-250 Sucrose	M16	60-145	E-13 M-5 G-6
5-6	M 16	UV	Sucrose 400	36	Flask-250 Sucrose	M 16-13	50-137	E-21 M-19 G-15
7	M 16-13	EMS	Sucrose 400	21	Flask-250 Sucrose	M 16-13-7	67-173	E-36 M-13 G-2
8	M 16-13-7	UV	Sucrose 500	30	5 ml Tube-250 Sucrose		48-202	M 20-30
9	M 16-13-2	EMS	Sucrose 500	29	5 ml Tube-250 Sucrose		43-250	M 40-42
10	M 16-13-7	UV, EMS and UV+EMS	Sucrose 600	23	Flask-250 Sucrose	M 16-13-7-3	72-183	E-42 M-15 G-13
					Flask-250 Sucrose	M 16-13-7-29	67-102	E-28 M-0
11-20	M 16-13-7-3	UV, EMS and UV+EMS	Glucose 450-600 / KCl 200	193	Tube GOD-POD Flask-250 Glucose	M572	76-230	E-53 M-27 G-9

Series	*Parent used/ Intermediate parent*	*Mutagen*	*Selection medium g/l*	*No. of survivors selected*	*Screening environment*	*Mutant selected*	*OD 600 , Sugar utilized g/l*	*Erythritol Mannitol Glycerol g/l*
21-23	M 16-13-7-3	UV, EMS and UV+EMS	Glucose 450-600 / KCl 200	68	Tube GOD-POD	ER8	78-250	M-58 E-13 G-14
24-33	M572	UV, EMS and UV+EMS	Glucose 450-600 / KCl 200	257	Tube GOD-POD			
34-39	M572	UV and NTG	Glucose 400 + TTC	198	Tube GOD-POD + HPLC 5 days	M1-UV R23 M1-UV R1 M4-UV R9	80-230 59-250 48-250	E-59 M-58 M-48
1-11	*Y. lipolytica* NCIM 3472	UV	Glucose 350-450	305	Tube GOD-POD + HPLC 5 days			
11-13	*Y. lipolytica* NCIM 3472	EMS	Glucose 350-450	75	Tube GOD-POD + HPLC 5 days			
52				**1368**		**M572, R23 R1,R9**		**E= 54,59 M= 48,58**

Table 3.2 Detail summary of mutagenesis series, selection medium, screening medium used mutant selection and mutants generated a* TTC (100 mg/l): 2, 3, 5-Triphenyl tetrazolium chloride (HiMedia RM470) , E= erythritol, M= mannitol and G= glycerol

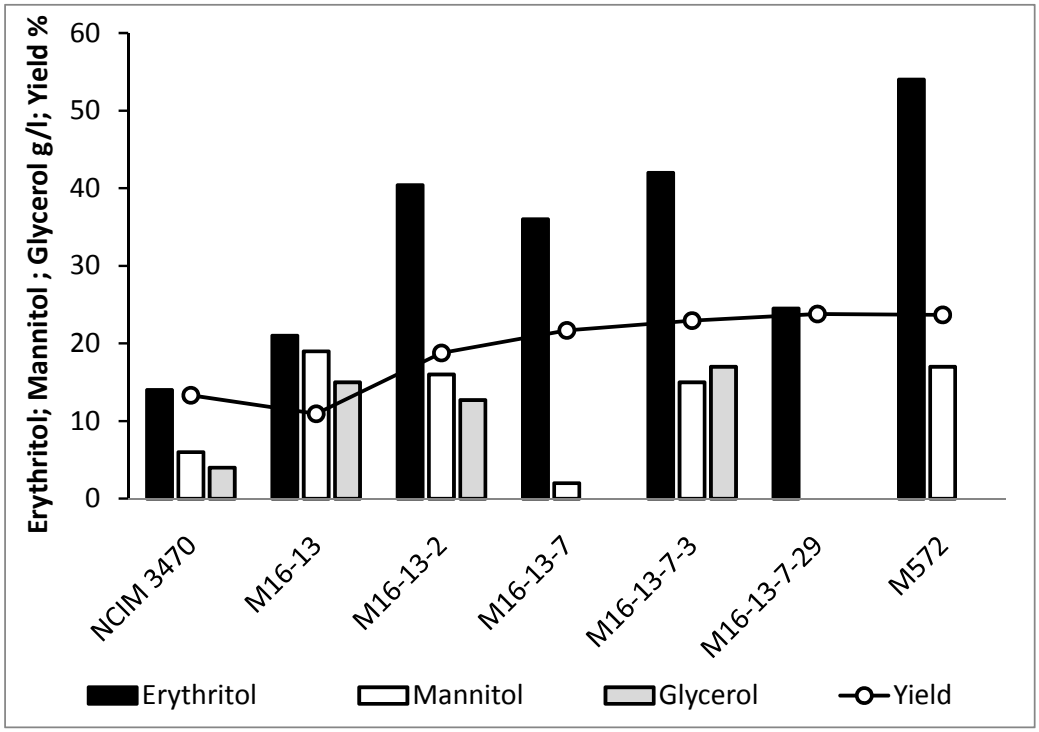


Figure 3.3 Comparison of respective parent and some of the selected mutants

Though, mutant M16-13-7-29 produced comparatively less erythritol than its parent M16-13-7-3 but still was selected and used for mutagenesis on the basis of its inability to produce the unwanted metabolites mannitol and glycerol. The subsequent mutant M572 generated from this mutant produced 54 g/l erythritol and was investigated in detail for further enhancement.

Resistance to higher sugar concentration for both the parent strains was evaluated by inoculating the respective cultures in tubes with varying concentration of glucose. *C. magnoliae* was more resistant to higher glucose concentration than *Y. lipolytica* and it could grow in a medium containing 600 g/l glucose (Table 3.3). Moreover, *C. magnoliae* and its mutants could tolerate and grow well in medium containing 200 g/l KCl (Table 3.4). At 250 g/l KCl, only mutant M16-13 and M16-13-7 grew comparatively better than the parent and other mutants. In general, when the sugar or salt concentration in the plates was increased, the colonies grew slowly and appeared smaller in size on plates, in such case TTC was used in the selecting medium and colonies were selected on the basis of change in the colour. The generated mutants were also tested for their ability to utilize mannitol or glycerol by inoculating the

mutants in the medium with mannitol or glycerol. All the tested mutants could utilize glycerol and mannitol for their growth (Table 3.5).

Glucose g/l	***200***	***250***	***300***	***350***	***400***	***450***	***500***	***600***
Y. lipolytica	20	17	12.5	7.7	3.5	-	-	-
C. magnoliae	30.3	28	23.9	20	18.3	16.0	15	8

Table 3.3 Growth of *C. magnoliae* and *Y. lipolytica* in media with varying glucose

Mutant	***OD 600 nm 100 g/l KCl***	***OD 600 nm 150 g/l KCl***	***OD 600 nm 200 g/l KCl***	***OD 600 nm 250 g/l KCl***
M16	15	14.2	2.1	0.1
M16-13	15.2	14.2	1.3	1
M16-13-2	15.9	13.2	1	0.1
M16-13-7	15.5	13.9	2.1	1
M16-13-7-29	15.4	5.3	0.1	0.1
M16-13-7-28	15.8	13.4	1.1	0.1
M16-13-7-7	16	12.3	1.4	0.1
M16-13-7-3	16	7.6	1.2	0.1
NCIM 3470	14.9	14.2	2.4	0.1

Table 3.4 Growth of *C. magnoliae* and its mutants in medium with varying KCl

Mutant	***Glucose***	***Mannitol***	***Glycerol***
M-16	30	29	35
M-16-13	32	30	32
M-16-13-2	30	30	33
M-16-13-7	35	33	32
M-16-13-7-3	34	31	30
M-16-13-7-29	31	30	28
M-572	32	35	29
NCIM 3470	31	33	36

Table 3.5 Screening of *C. magnoliae* and mutants for ability to use mannitol and glycerol

Mutants generated from *C. magnoliae* strain showed gradual increase in sugar uptake and erythritol production. Only those mutants, which showed higher glucose utilization than parent, were studied in flasks for polyol production. Mutant tree for parent *C. magnoliae* and its mutants with enhanced erythritol production is depicted in Fig. 3.4.

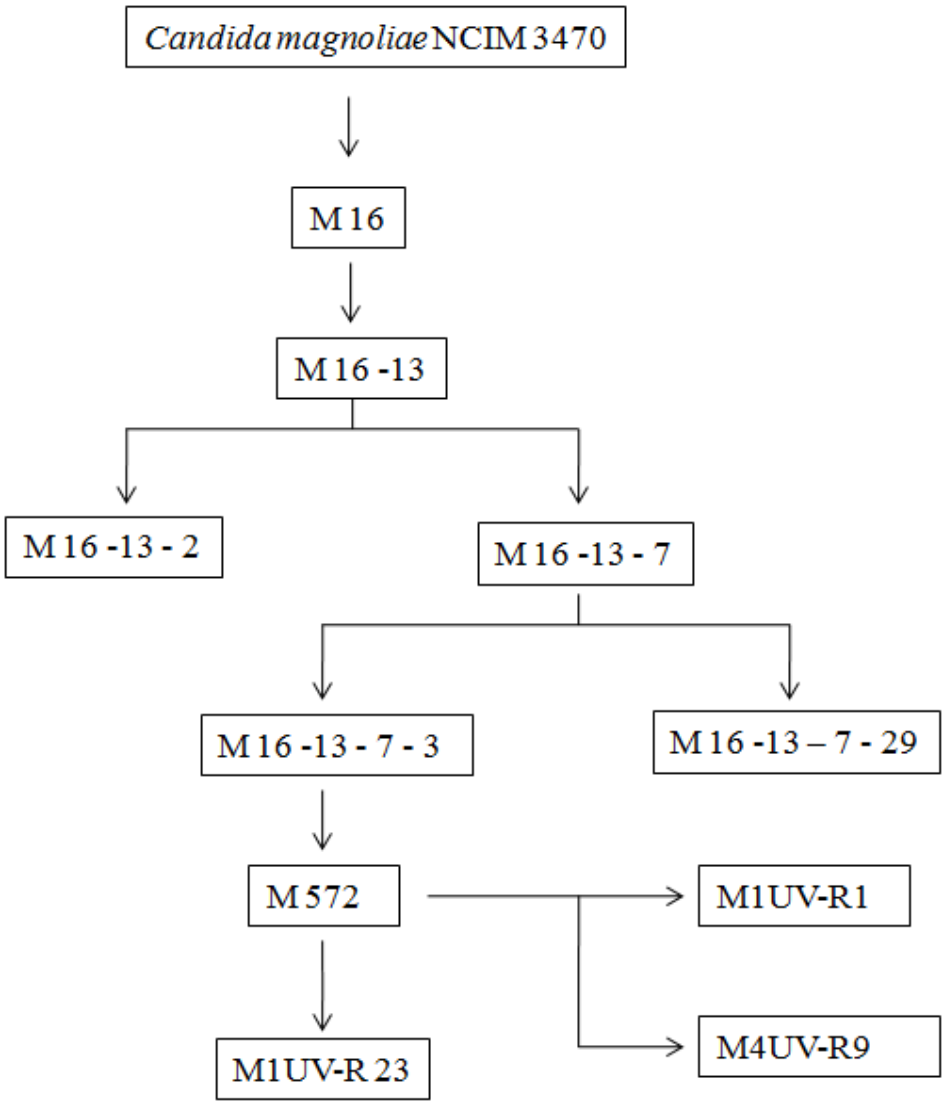


Figure 3.4 Mutant tree for parent *C. magnoliae* and its mutants with enhanced erythritol production

During screening of mutants, a mutant named M572 was selected because it produced considerably high amount of erythritol. The mutant M572 did not produce any organic acid as analysed using the plate assay and confirmed using HPLC. A small

amount of glycerol produced during growth phase was consumed by the cells later after the exhaustion of glucose from the medium. The selected mutant had marginal morphological difference from the parent *C. magnoliae* and the cells were slightly larger in diameter. Around 375 mutants of *Y. lipolytica* were also screened for increased glucose uptake and erythritol production but the effort was not successful.

Researchers have reported increased erythritol production by mutagenesis of different yeasts and fungi. Yang *et al.*, (1999) isolated an osmophilic strain of *C. magnoliae* and mutagenized it by the treatment with EMS. The isolated mutant M2 produced 25 g/l erythritol with 25% yield in a medium containing 100 g/l glucose. An acetate-negative mutant of *Y. lipolytica*, which did not produce citric acid, was isolated by Rymowicz *et al.*, (2009). When the mutant was grown in 300 g/l raw glycerol in fed-batch mode at pH 3, it produced 170 g/l erythritol with 56% yield. Similarly, a mutant of *Aureobasidium* sp that did not produce foam and by-products such as glycerol and ethanol was obtained by UV irradiation and NTG treatment. The generated mutant, SN-G42, converted 400 g/l glucose to 175 g/l erythritol with 43.7 % yield which was used for industrial production of erythritol in Japan Ishizuka *et al.*, (1989).

3.3.2 Optimization of erythritol production by the selected *C. magnoliae* mutant M572

3.3.2.1 Effect of carbon sources on erythritol production

Effect of various carbon sources on erythritol production by mutant M572 revealed that polyol production pattern (i.e. relative concentration of different polyols) by mutant M572 was greatly dependent on the type of carbon source used. Fructose resulted in higher production of mannitol (33 g/l) while glucose resulted in higher production of erythritol (45 g/l). Erythritol production from glycerol (20 g/l) was much lower compared to glucose. Mutant M572 produced maximum erythritol with glucose, in contrast to the initial shake flask study in which maximum erythritol was produced when sucrose was used as carbon source, More importantly, it produced very low amount of mannitol and glycerol (Fig. 3.5). Thus, independent of the other media constituents, glucose was found to be the most suitable carbon source and therefore was used as carbon source for subsequent experiments.

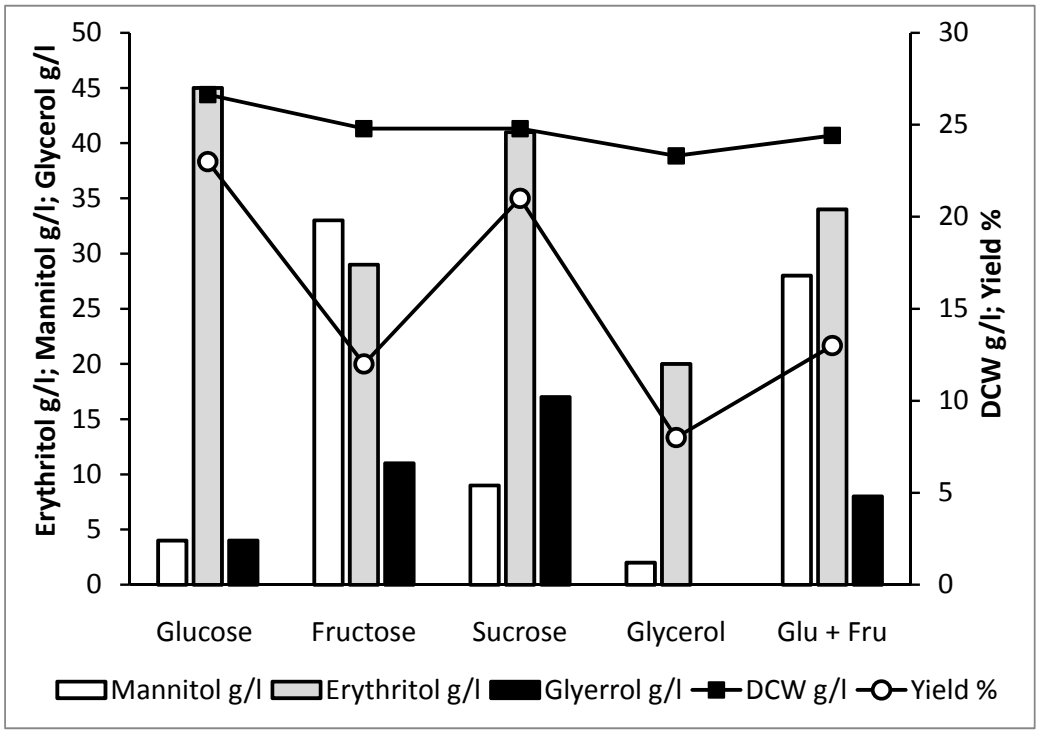


Figure 3.5 Effect of carbon sources on erythritol production by *C. magnoliae* mutant M572

3.3.2.2 Effect of initial glucose concentration on erythritol production by *C. magnoliae* and mutant M572

Osmophilic yeasts can produce erythritol, mannitol, xylitol, arabitol and glycerol that are used in a number of industrial applications. In addition, due of their tolerance to high osmotic pressure, osmophilic yeasts can easily ferment highly concentrated sugar solutions. Use of osmotolerant yeasts in a medium with high sugar concentration led to more concentrated product in the fermentation broth which results in easier product recovery, thus leading to reduced capital and operation costs.

In the present study as seen in Fig 3.6, the highest erythritol production and yield for both parent and mutant M572 was observed at 250 g/l initial glucose concentration. Further increase in glucose concentration caused decrease in biomass and erythritol production. Moreover, with the increase in initial glucose concentration above 300 g/l, glycerol started to appear in the broth as a by-product, which was similar to the results

obtained by Lee & Lim, (2003). Thus, the optimal concentration of glucose for erythritol production in shake flasks with the mutant M572 was found to be 250 g/l.

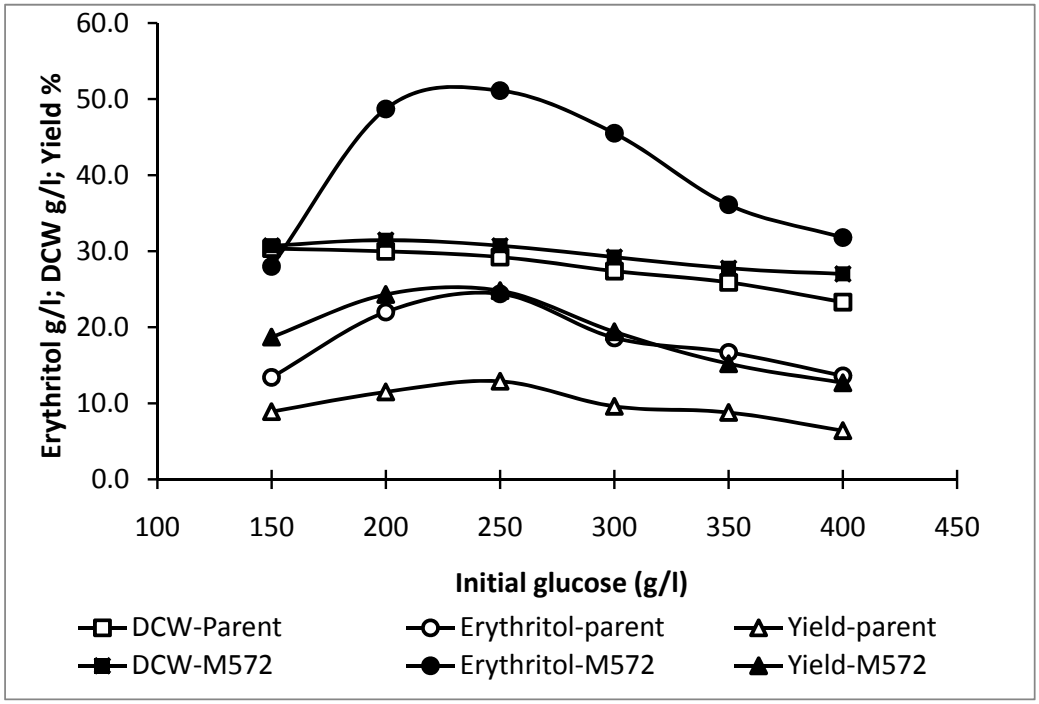


Figure 3.6 Production of erythritol by *C. magnoliae* and its mutant M572 in LFM with different initial glucose concentrations

3.3.2.3 Effect of yeast extract concentration on erythritol production

Yeast extract is a rich source of amino acids, polypeptides and vitamins and has been used by several investigators as an excellent nitrogen source for erythritol production (Kim *et al.*, 1999; Ryu *et al.*, 2000). In the present study, with the increase in yeast extract concentration from 2 to 10 g/l, the erythritol concentration gradually increased up to 57.3 g/l. Yeast extract concentration above 10 g/l resulted in decrease in erythritol production distinctly, presumably because of unsuitable C:N ratio in the fermentation medium (Fig. 3.7a). Biomass concentration (29.8 g/l) and erythritol yield (30%) were maximal when 10 g/l yeast extract used in the medium (Fig. 3.7b). When yeast extract concentration above 10 g/l was used, all the sugar was consumed but erythritol concentration, yield and productivity were very low (Fig. 3.7c); whereas mannitol production increased to 28 g/l which otherwise was not observed (Fig. 3.7 d). The experiment thus ascertains that yeast growth and dissolved oxygen

concentration must be carefully controlled by proper adjustment of nitrogen content in the medium to obtain maximal erythritol production.

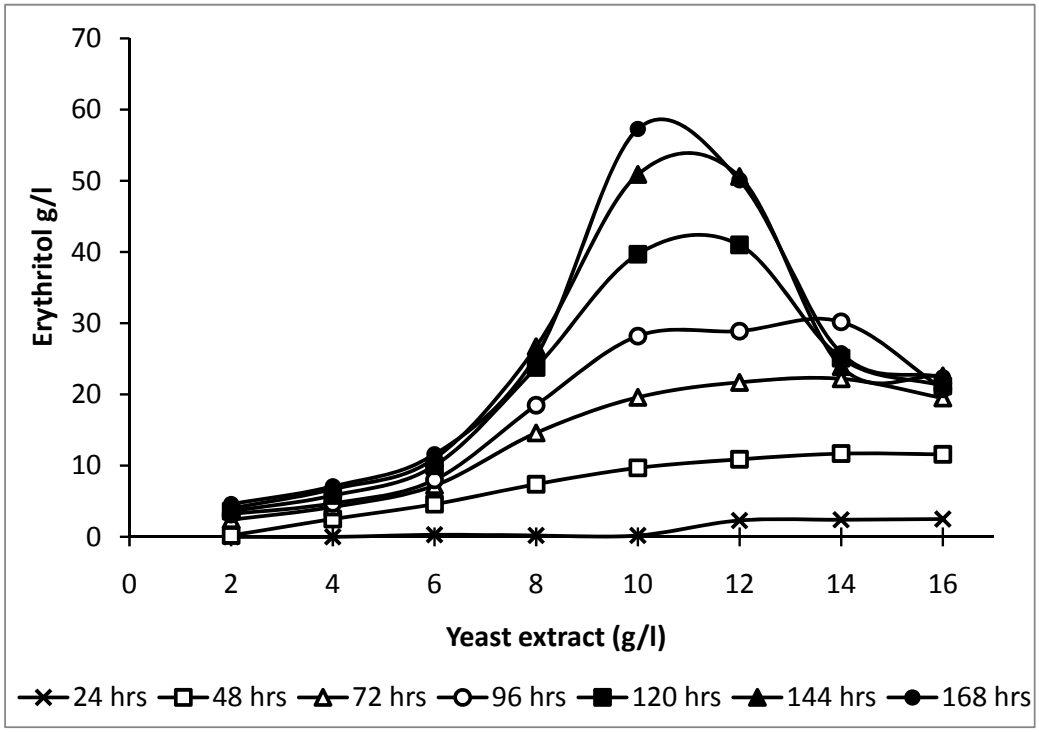


Figure 3.7a Effect of yeast extract on erythritol production by mutant M572

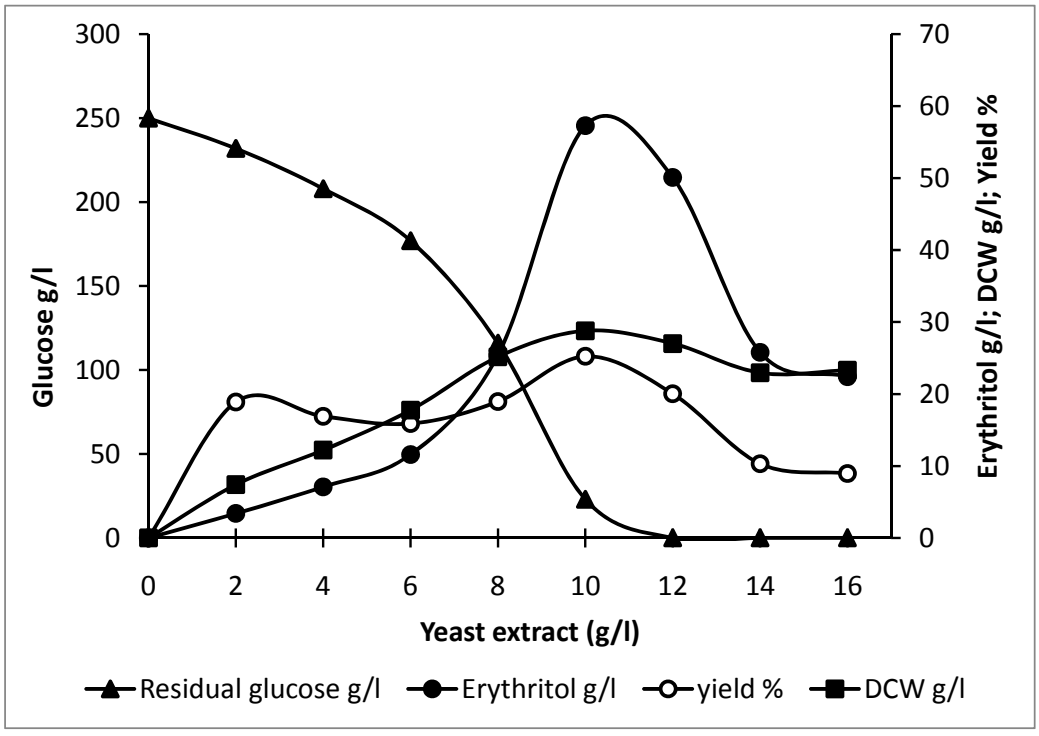


Figure 3.7b Effect of yeast extract on sugar uptake, biomass, erythritol concentration and yield by mutant M572

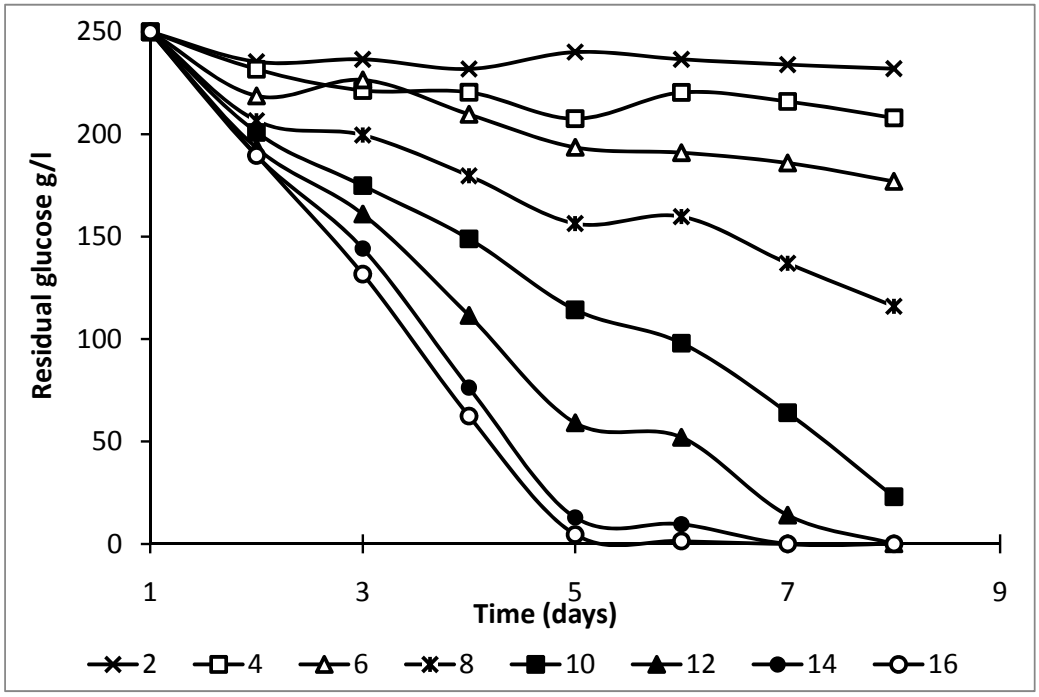


Figure 3.7c Effect of yeast extract on sugar uptake by mutant M572

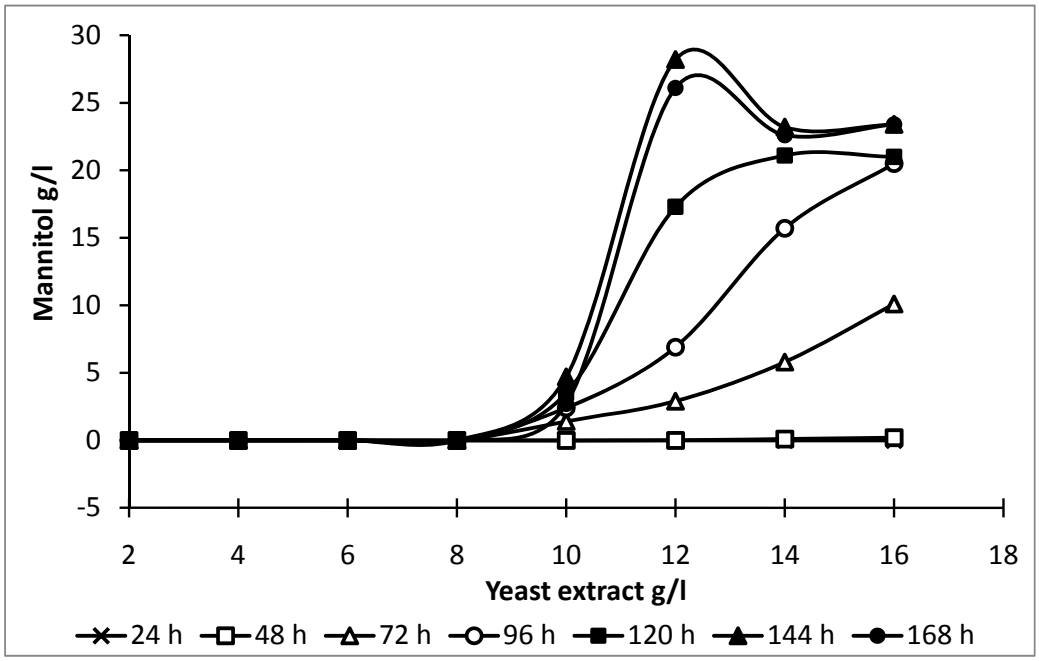


Figure 3.7d Effect of yeast extract concentration on co-metabolite mannitol formation by mutant M572

Although yeast extract is a favorite nitrogen source in fermentation research, it is rather expensive. In the present investigation an attempt was made to minimize the yeast extract requirement in the fermentation medium by supplementation with cheap inorganic nitrogen sources. The mutant M572 could produce almost equal amount of erythritol with similar yield when 50% of the nitrogen source was replaced by ammonium nitrate, urea, ammonium acetate or sodium nitrate as can be seen from Fig. 3.8. The lower concentration of erythritol produced in the medium with ammonium chloride, sulphate and phosphate may have been because of the lowering pH of the fermentation medium in shake flasks. The use of nitrate as nitrogen source requires reduction of nitrate to ammonium, which consumes two moles of NADPH per mole of nitrate. NADPH is mainly produced in the PP pathway. Therefore, the increased production of erythritol could be a consequence of overflow metabolism in the PP pathway due to the increased requirement for NADPH during growth on nitrate.

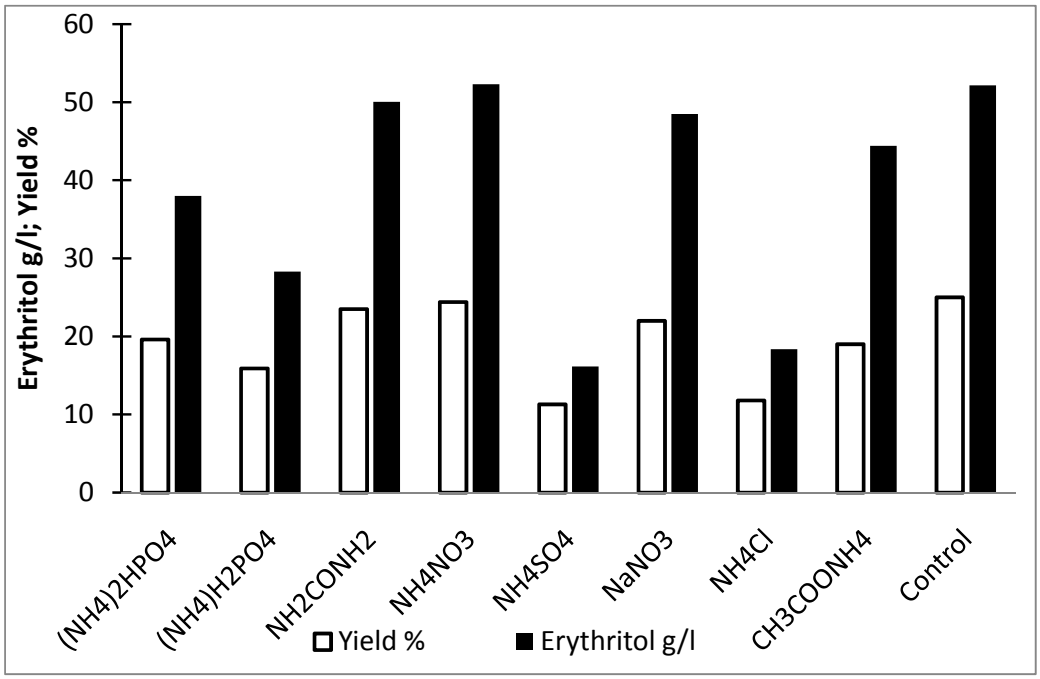


Figure 3.8 Production of erythritol by mutant M572 when 50% of the yeast extract was replaced with inorganic nitrogen sources

3.3.2.4 Effect of medium volume on erythritol production by M572

In the present study, it was observed that M572 resulted in maximal erythritol production when 25 ml medium was used in 250 ml Erlenmeyer flask. There was no major difference in DCW accumulated with the change in medium volume and the mutant did not produce organic acids or other polyols. Interestingly, at 10 and 20 ml volume, the erythritol production was very low. With increase in medium volume from 20 to 25 ml, erythritol concentration increased sharply, but it again declined when broth volumes were more than 30 ml. This was observed in the samples withdrawn at all the sample intervals during incubation and the trend remained uniform with age of the culture. As depicted in Fig. 3.9 with the change in medium volume on either side of 25 ml, there was more than 50% decrease in erythritol production.

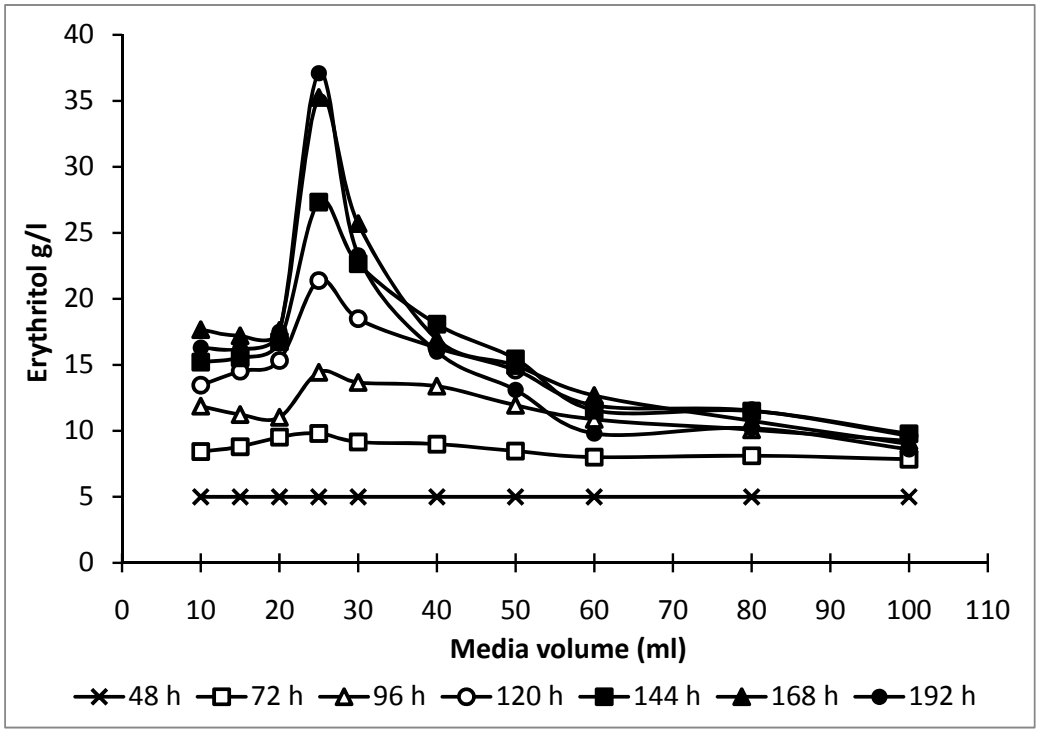


Figure 3.9 Effect of medium volume on erythritol production by mutant M572

It was found that with the increase in glucose concentration in the solution, there was a sharp decrease in solubility of oxygen (Fig. 3.10). At 250 g/l glucose concentration, the oxygen concentration in the sterile medium was only 1.6 mg/l even though it was shaken for 24 h at 210 RPM at 28 °C to reach saturation.

It is often reported that the yeasts require high osmotic pressure for erythritol production and this is commonly achieved by use of high sugar concentration in the fermentation medium. The observed need of high osmotic pressure for successful erythritol production may be in fact an indirect result of lower dissolved oxygen concentration, caused by low solubility of oxygen in high glucose containing medium. Careful control of dissolved oxygen is a must for further enhancement in erythritol productivity, and it is necessary to understand the role of high sugar containing medium in creation of high osmotic pressure against the lowering of dissolved oxygen concentration. It was evidenced that dissolved oxygen concentration is a key factor in erythritol production by *C. magnoliae* mutant M572 and either high or low DO_2 tension inhibited erythritol production. This observed effect was not a consequence of difference in the yeast growth because the culture broth was redistributed after 48 h and had equal and young biomass in all the flasks.

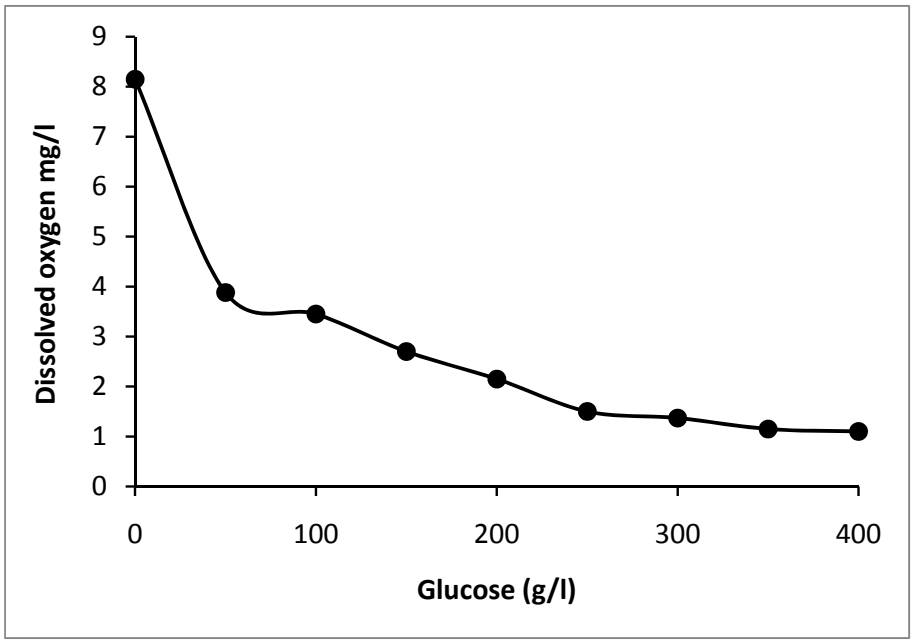


Figure 3.10 Effect of glucose concentration on solubility of oxygen

It has been reported earlier that oxygen availability caused dramatic alterations in the central carbon metabolism in *Aspergillus niger* (Diano *et al.*, 2006; Meijer *et al.*, 2007). Among the culture conditions tested for erythritol production by *Pseudozyma tsukubaenis*, dissolved oxygen was the key factor affecting not only cell growth but also erythritol production (Jeya et al., 2009). Kim et al. (2000) had suggested that for

maximum erythritol production by *Torula sp*, dissolved oxygen should be controlled in the range of 5-10% air saturation. Similarly, if the dissolved oxygen concentration for erythritol producing yeast *Moniliella tomentosa (pollinis),* decreased below 19% air saturation ethanol was formed as major product (Burschäpers *et al.*, 2002). The oxygen concentrations are normally expressed in terms of air saturation, but because of the differences in solubility of oxygen in medium with different sugar concentrations, it is difficult to compare these results. Earlier, Ryu et al., (2000) have investigated that with high glucose concentration during production phase of erythritol, *C. magnoliae* produced citric and butyric acid but with the increase in dissolved oxygen level there was formation of gluconic acid instead of citric acid. Polyol production studies from *Aspergillus niger* reported that the polyol concentration and the polyol pattern is strongly dependent on the source of carbon and nitrogen used as well as on dissolved oxygen concentration (Diano *et al.*, 2006). They have also reported that during oxygen limitation, there was an increase in CO_2 yield in C_{mol} per C_{mol} glucose, decreased biomass yield and increased production of several polyols. They concluded that deviation of carbon flux towards the PP pathway caused increase in erythritol production during limited oxygenation in *A. niger*. Mutant M572 of *C. magnoliae* also behaved in similar manner and responded to differences in oxygen availability. Although, organic acids were not produced by the mutant M572 in the present investigation either low or high oxygen condition but still erythritol production was strongly affected.

3.3.2.5 Effect of metal ion on polyol production by M572

In the present study, distinct enhancement in polyols production was observed upon addition of trace metals in the fermentation medium (Fig. 3.11a). With some of the metal ions like Zn^{+2}, Mn^{+2} and Ca^{+2}, the total amount of polyols produced was doubled. However, none of the trace metals selectively increased erythritol production. Ca^{+2}, Fe^{+2} and Zn^{+2} at higher concentrations (100 mg/l) enhanced erythritol production marginally. In a control medium without any additional metal salt, erythritol production accounted for more than 80% of the total polyols produced. The combined use of Ca^{+2}, Fe^{+2} and Zn^{+2} did not show any synergistic improvement in erythritol production. On the contrary, the combination of these three metal ions enhanced unwanted polyols.

Addition of Cu^{+2} in the fermentation medium resulted in slightly higher biomass production but there was 50% decrease in concentration of total polyols. The relative composition of polyol percentage changed to a greater extent with the use of diverse trace metals (Fig. 3.11b).

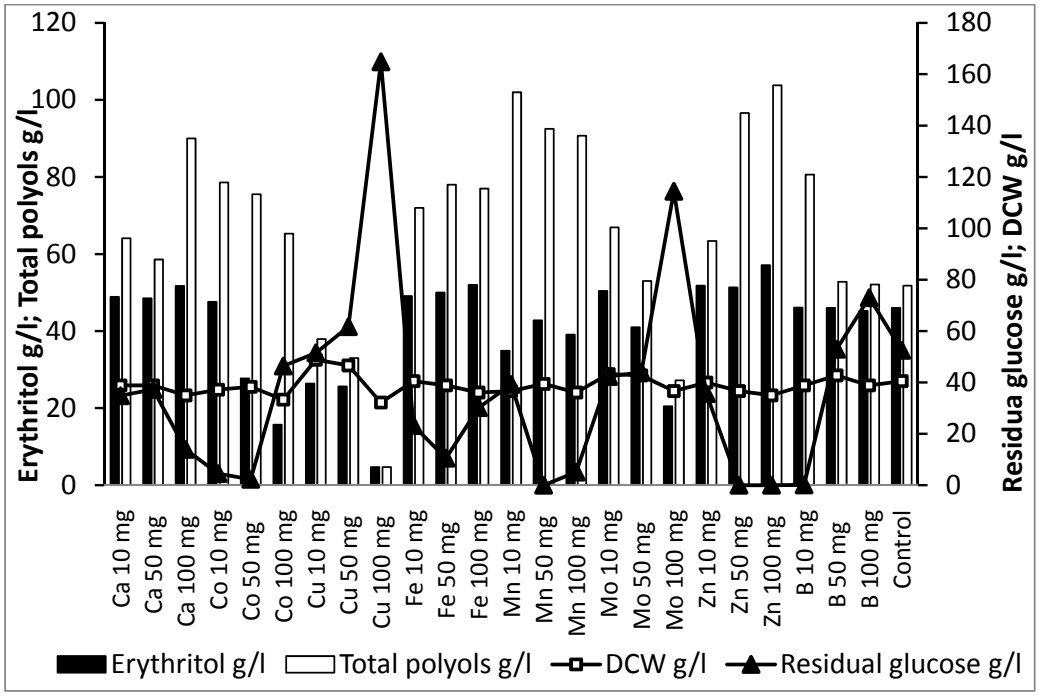


Figure 3.11a Effect of metals ions on polyol production by mutant M572

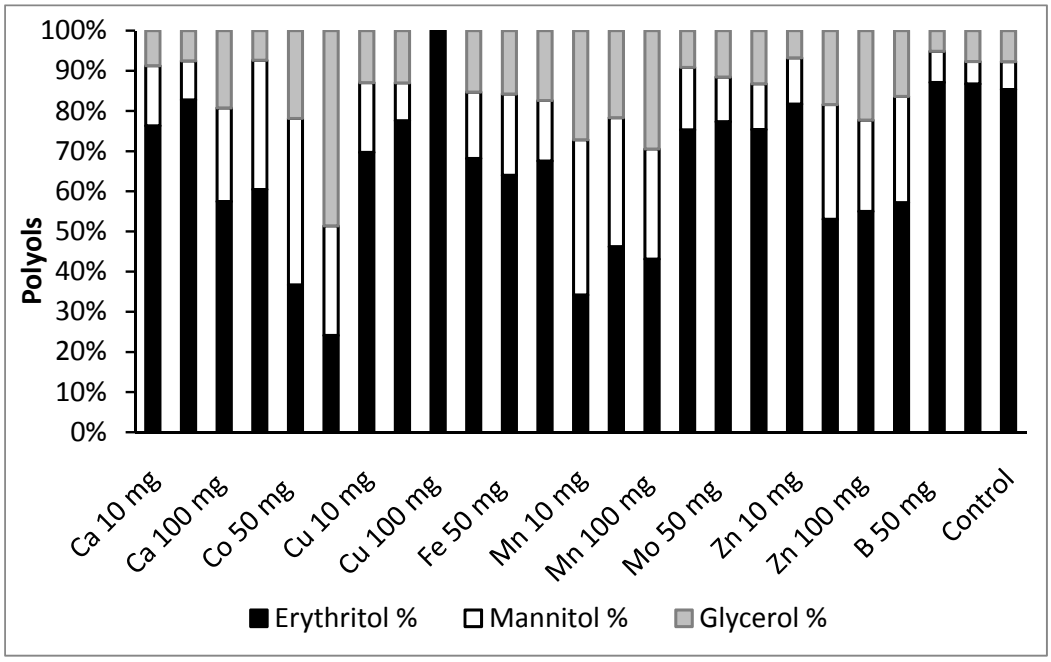


Figure 3.11b Effect of metal ions on proportion of polyols produced by mutant M572. Metal ion concentrations are in mg/l

Minerals are known to influence activity of enzymes involved in polyol biosynthesis and therefore affect the production of several polyols (Kim & Oh, 2003; Lee et al., 2000; Lee et al., 2002; Lee et al., 2007). Lee et al., (2007) investigated that there was substantial improvement in erythritol production by *Torula sp.* by supplementation of Mn^{+2} and Cu^{+2}. Their results stated that Mn^{2+} altered the permeability of cells and Cu^{+2} increased the activity of erythrose reductase, the key enzyme in erythritol biosynthesis. The effect of metal ions on erythritol production by *C. magnoliae* has not been studied yet.

3.3.2.6 Nutritional requirements of mutant M572

The study with varying concentrations of yeast extract in the medium indicated that the amount of nitrogen source in the medium was the controlling factor for erythritol production and yield. A synthetic medium would be economic and will minimize impurities and unwanted by-products and this may have an advantage in downstream processing for product recovery. The key ingredients in the medium, which would have the greatest effect on yeast growth and erythritol yield, were assumed to be the source and concentration of the nitrogen and one or more of the vitamins and amino acids. The inorganic nitrogen sources surveyed for *C. magnoliae* on which it can grow and produce erythritol were ammonium acetate, ammonium nitrate, ammonium phosphate, ammonium chloride, ammonium sulphate, sodium nitrate and urea (Fig. 3.8). YNB combinations of amino acids and vitamins revealed that mutant M572 requires one or more vitamins but can grow in a medium devoid of amino acids (Table 3.6)

Experiments were performed using media in which the YNB contained all the vitamins or in which the vitamins were omitted one by one (Table 3.7) and finally devoid of all the vitamins. When all the vitamins were omitted from the YNB medium, the mutant M572 did not grow in the liquid medium. The results illustrate that thiamine was an essential vitamin for growth of the mutant while biotin and pyridoxine caused improvement in growth of the mutant. For further verification of essential vitamins, when a synthetic medium with ammonium sulphate or sodium nitrate as nitrogen source was used by omitting one vitamin at a time, the results

confirmed that thiamine was an essential vitamin for growth of the mutant while biotin and pyridoxine caused improvement in growth of the mutant (Table 3.8).

YNB	***DCW (g/l)***
YNB with vitamins and amino acids	9.3
YNB with vitamins and without amino acids	9.3
YNB without any vitamins or amino acids	4.8

Table 3.6 Screening of vitamins and amino acids requirement for mutant M 572

Vitamins	***Tube Number***									
Tube No	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
Biotin	-	+	+	+	+	+	+	+	-	+
Inositol	+	-	+	+	+	+	+	+	-	+
Niacin	+	+	-	+	+	+	+	+	-	+
Ca- pantothenate	+	+	+	-	+	+	+	+	-	+
Riboflavin	+	+	+	+	-	+	+	+	-	+
Pyridoxine HCl	+	+	+	+	+	-	+	+	-	+
Thiamine HCl	+	+	+	+	+	+	-	+	-	+
Folic acid	+	+	+	+	+	+	+	-	-	+
DCW g/l	**6.7**	**7.8**	**7.5**	**8.1**	**8.3**	**7.2**	**1**	**8.8**	**0.7**	**8.1**

Table 3.7 Specific vitamin requirement of *C. magnoliae* mutant M572

LFM plus Nitrogen source 1g/l	***All vitamins v* 10 mg/l***	***Biotin Pyridoxine Thiamine 10 mg/l***	***Biotin Negative 10 mg/l***	***Pyridoxine Negative 10 mg/l***	***Thiamine Negative 10 mg/l***
$(NH_4)_2SO_4$ with a*	16	14.5	6.2	11.4	2.1
$(NH_4)_2SO_4$ without a*	17.3	16	6.6	12.5	1.6
$NaNO_3$ without a*	12.5	13	5	11.8	1.4

Table 3.8 Effect of essential vitamins on growth of *C. magnoliae* mutant M572 Values described OD_{600} nm [v*Biotin, Inositol, Niacin, Ca- pantothenate, Riboflavin, Pyridoxine HCl, Thiamine HCl and Folic acid 10 mg/l, a* Histidine, methionine and tryptophan 10 mg/l]

Lee et al. (2001) investigated that addition of inositol increased erythritol production by *Torula sp*. I had also studied the effect of individual vitamins on erythritol production in LFM medium with 25 mg/l of each vitamin enlisted in (Fig. 3.7) but the addition of any vitamin to the yeast extract containing medium did not further improve erythritol production (Fig. 3.12).

To check erythritol production in defined medium, experiments were performed in LFM in which yeast extract was replaced by ammonium sulphate or sodium nitrate as nitrogen source and supplemented with thiamine, biotin and pyridoxine. Use of such minimal medium resulted in 14 g/l DCW with $(NH4)_2SO_4$, and 19 g/l with $NaNO_3$ as sole nitrogen source respectively, but erythritol production was much less as compared to LFM with yeast extract. In sodium nitrate containing minimal medium, 2.4 g/l erythritol was produced while, in ammonium sulphate based medium, 16.7 g/l ethanol was produced without production of erythritol. More experiments are needed to investigate the exact cause of lower erythritol in minimal medium at fermenter level.

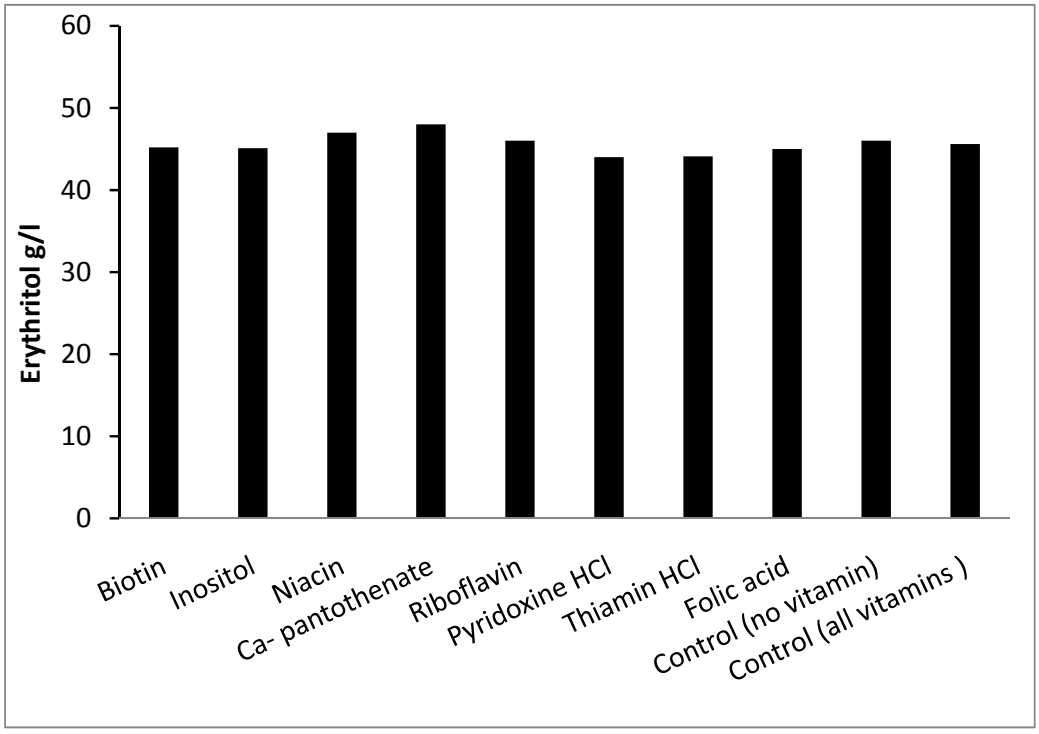


Figure 3.12 Effect of individual vitamin on erythritol production by mutant M572

3.3.2.7 Effect of periodic glucose feeding on erythritol production

When the effect of periodic addition of glucose to the shake flask cultures of M572 was studied, it was observed that there was an increase in glucose uptake and erythritol production with increase in glucose feeding from 10 to 40 g $l^{-1}d^{-1}$ as seen from (Fig. 3.13a). However, at 50 g $l^{-1}d^{-1}$ glucose feeding rate, there was a sharp decrease in erythritol production presumably because of the excess concentration of glucose in the medium. A maximum of 17 g/l erythritol was observed when 40 g $l^{-1}d^{-1}$ glucose was fed, which however was rather low as compared to the control experiments in which all the glucose was added initially.

Use of high initial glucose concentration in the medium and feeding additional glucose when glucose concentration in the medium decreased below 100 g/l, did not contribute to erythritol production. On the contrary, it led to an enhancement of other co-metabolites, mainly glycerol and mannitol (Fig. 3.13b).

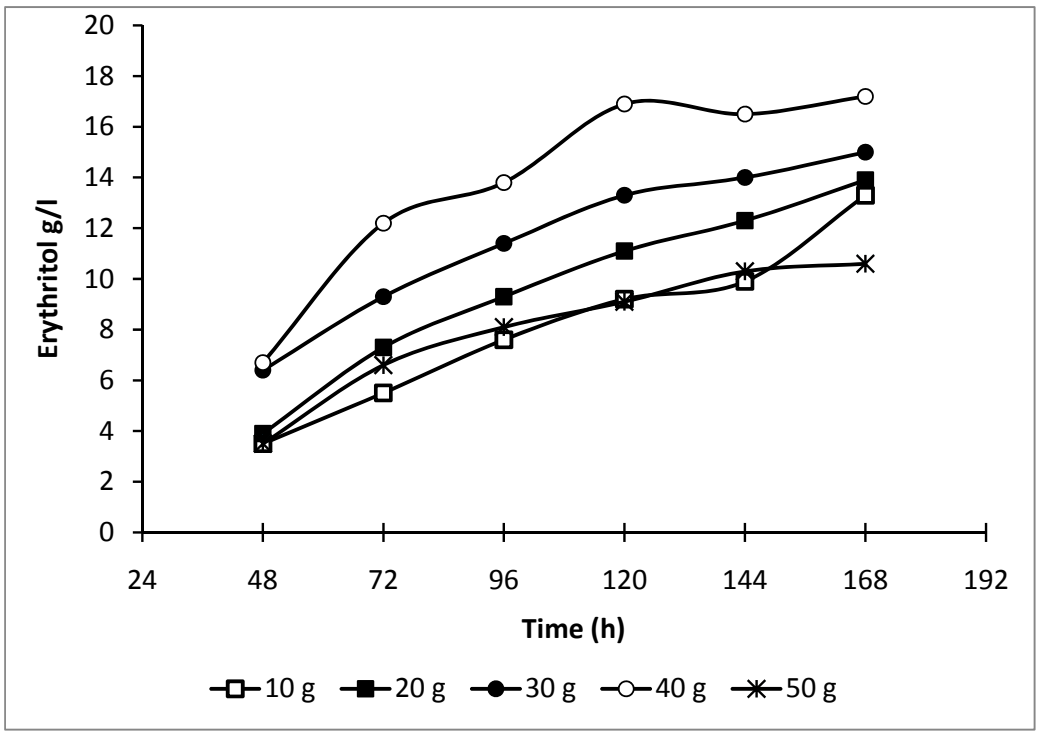


Figure 3.13a Effect of glucose feeding on erythritol production by mutant M572

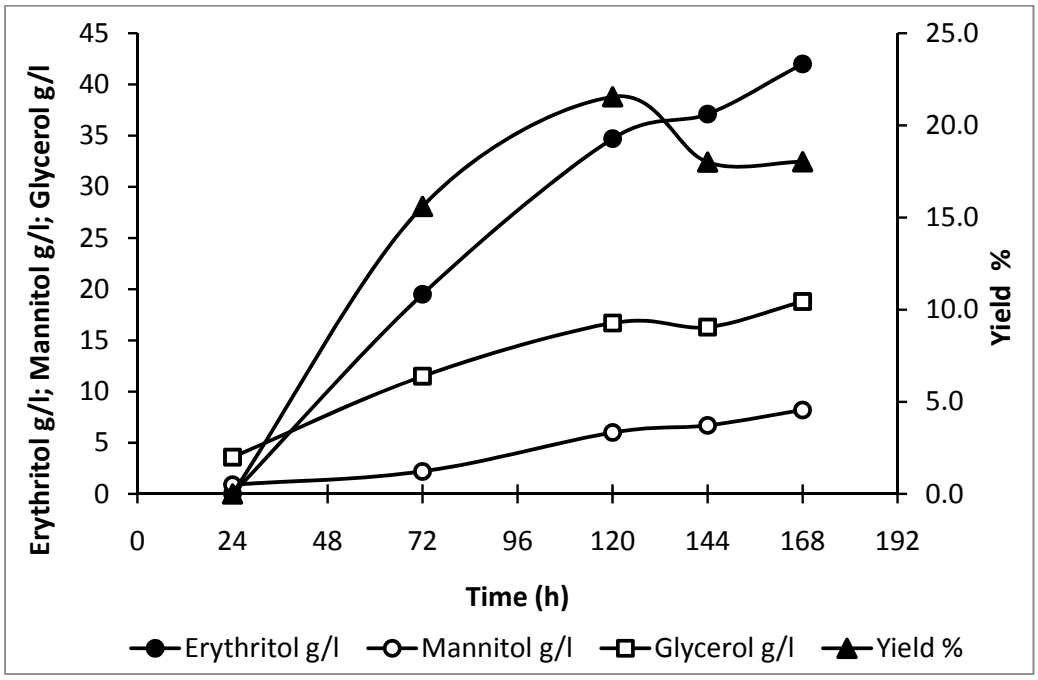


Figure 3.13b Effect of glucose feeding on erythritol production by mutant M572

3.3.2.8 Comparison of *C. magnoliae* parent strain and its mutant M572

The growth and erythritol production by parent and mutant M572 in LFM with 250 g/l glucose is depicted in Fig. 3.14a. It can be seen that there was a substantial increase in the erythritol production by mutant M572 as compared to its parent. The mutant produced 53 g/l erythritol with 23 % yield as compared to only 14 g/l by the parent with just 9 % yield. The overall erythritol productivity of the mutant was 7.5 g $l^{-1}d^{-1}$ as compared to 2 g $l^{-1}d^{-1}$ of parent (Fig. 3.14b). The growth rate for both the strains observed was similar and the final biomass of the mutant was almost equal to the parent strain. The specific erythritol production of mutant M572 was 1.7 g/g DCW as compared to 0.5 g/g DCW of the parent strain, which is 3.5 times higher than the parent. This illustrates that the mutant had substantially improved specific erythritol production and this was not an effect of only higher cell concentration. Erythritol production for both strains started at late exponential phase. The glucose utilization rates of these two strains were also different (Fig. 3.14c). During the initial growth phase, both parent and mutant consumed glucose at equal rate but during the production phase, glucose uptake rate of M572 was visibly higher. In 168 h fermentation, the glucose utilized by the mutant was 226 g/l as against only 145 g/l by the parent strain. In the present investigation, the yield of erythritol using the selected

mutant M572 was 2.42 fold higher and erythritol productivity was 3.79 fold more as compared to the parent grown under the identical conditions.

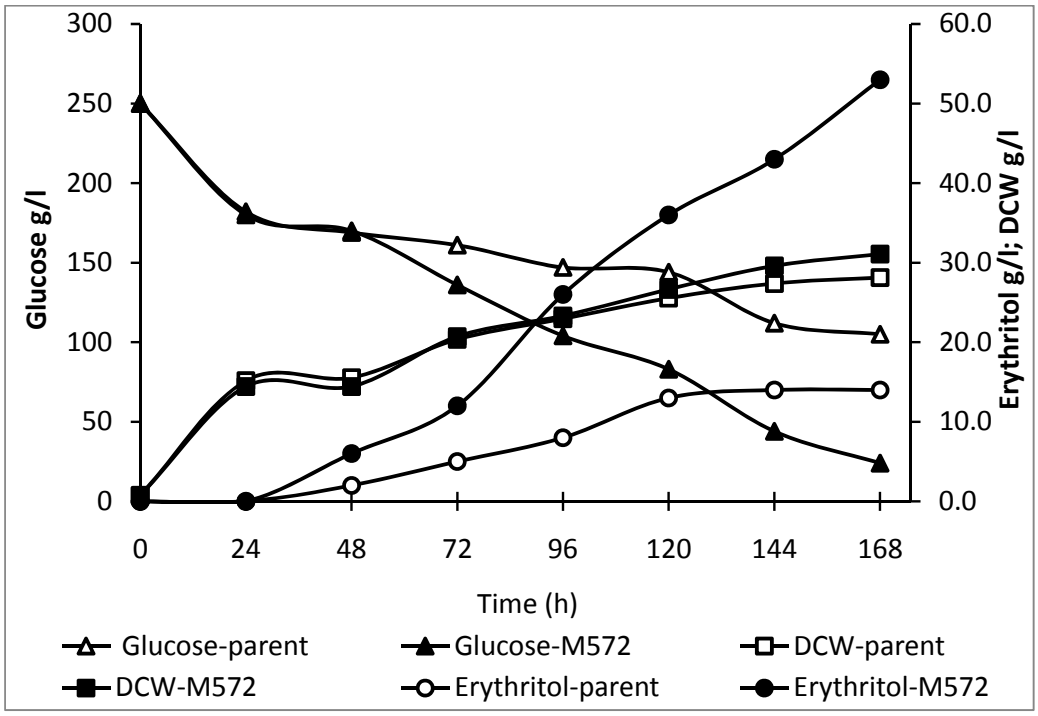


Figure 3.14a Comparison of growth and erythritol production of *C. magnoliae* and its mutant M572

Proposed pathway of erythritol biosynthesis in *C. magnoliae* suggests that glucose is converted to erythrose-4-phosphate via the PP pathway and then erythrose-4-phosphate is dephosphorylated into erythrose, which is further reduced to erythritol by erythrose reductase (Park *et al.*, 2005). Some of the key enzymes responsible for higher erythritol biosynthesis have been studied by many researchers. (Sawada *et al.*, 2009) investigated that the activities of PP pathway enzymes were higher than those of tricarboxylic acid cycle (TCA) cycle in those mutants in which more erythritol was produced during stationary phase. Erythrose reductase from mutants of *Aureobasidium* sp. and *C. magnoliae* showed the maximum activity when D-erythrose was used as substrate (Lee et al., 2003b; Tokuoka et al., 1992). Similarly, Lee et al. (2003) reported that *Torula corallina* yielded more erythritol without formation of other polyols due to high substrate specificity and V_{max} of erythrose reductase (Lee et al., 2003b). In the present study, there were distinct differences in the extent of glucose utilization and products formed between the mutant and the parent. The exact

mechanism behind improved erythritol production by the mutant is supported by investigating erythrose reductase and Glucose 6-phosphate dehydrogenase subsequently in this chapter.

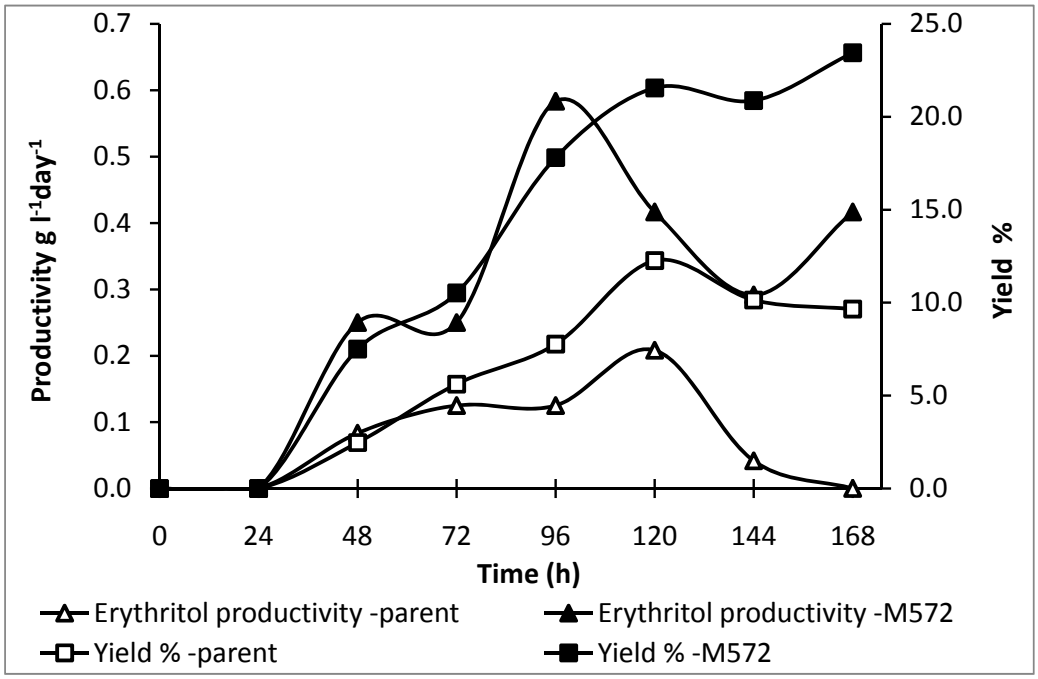


Figure 3.14b Comparison of erythritol productivity and yield of *C. magnoliae* and its mutant M572

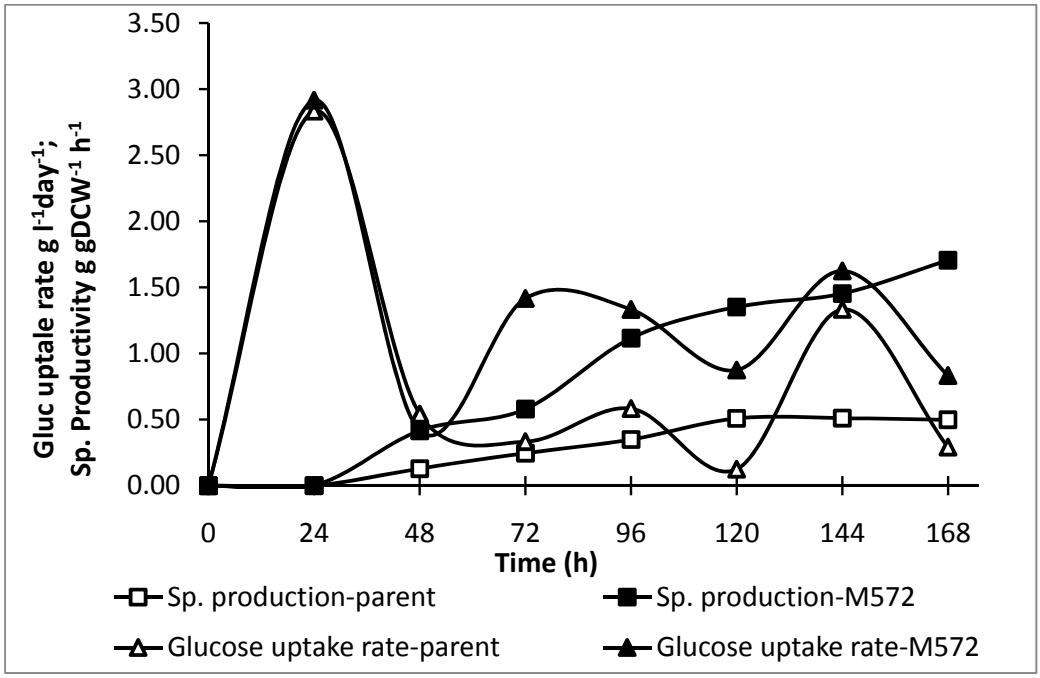


Figure 3.14c Comparison of glucose uptake rate and specific erythritol productivity of C. *magnoliae* and its mutant M572

During optimization of medium and culture conditions as described above, parallel mutagenesis programme was carried out using mutant M572 as parent strain for further improvement in erythritol production. The mutants generated from M752 were selected for higher reductase activity in plates in which TTC was incorporated. The mutants which produced dark red colonies were selected. On evaluating these mutants in tubes containing LFM, they showed fairly high glucose consumption than its parent. Mutant M572 consumed 149 g/l of glucose whereas mutant R1 consumed 191 and mutant R9 consumed 225 g/l of glucose in three days. On evaluating R1 and R9 mutants in shake flask it was found that it produced a fairly high amount of mannitol instead of erythritol. These mutants were maintained on LFM agar slants and used for further studies for mannitol production.

Another mutant named R23 generated from M572 showed better results as compared to M572 in shake flask and produced 60 g/l erythritol with 26.7 % yield although the biomass accumulation and glucose utilization pattern were similar to its parent, M572 Fig. 3.15.

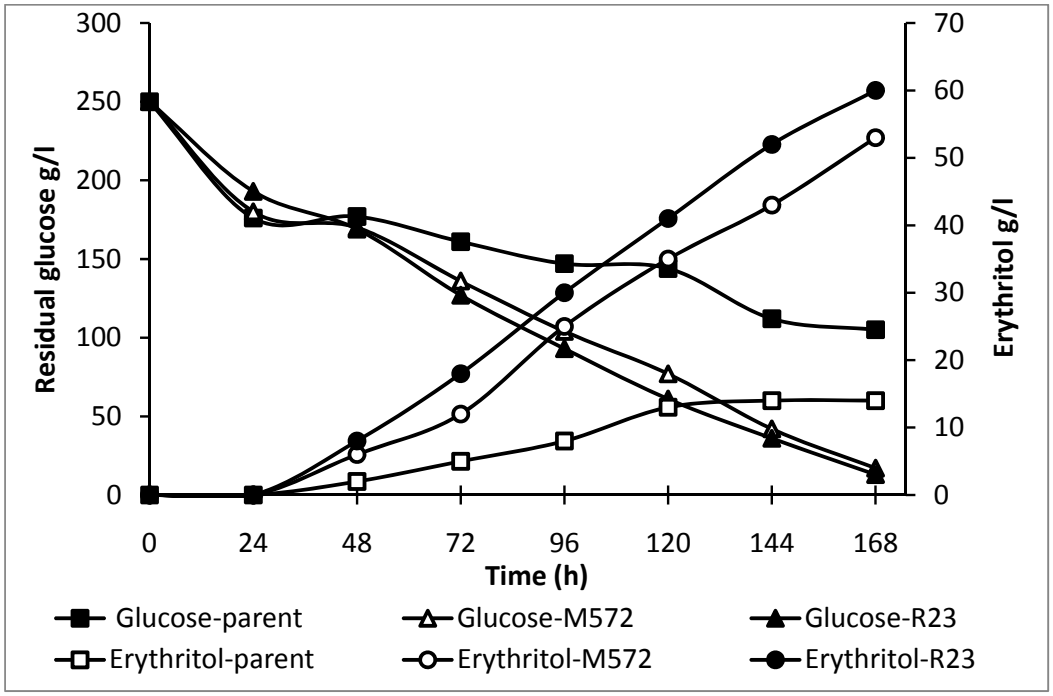


Figure 3.15 Comparison of C. *magnoliae* and its mutants M572 and R23

Comparison of R1 and R9 mutants in shake flask with LFM medium revealed that both of these mutants consumed all the glucose and produced mannitol instead of erythritol. The mutant R9 produced 46 g/l of mannitol within in 96 h with a yield of 18 % whereas R1 could produce 57 g/l mannitol in 168 h with 23 % yield (Fig. 3.16). The biomass of mannitol producing mutants were low (15 g/l DCW) compared (31 g/l DCW) to its parent, but still it could consume all 250 g/l glucose. Although mutant R1 could produce higher amount of mannitol than R9, overall glucose utilization rate and productivity was 35.7 and 8.1 g $l^{-1}d^{-1}$ which was low as compared to 62.5 and 11.5 of R9. These mutants were preserved in 30% glycerol solution at -20 °C.

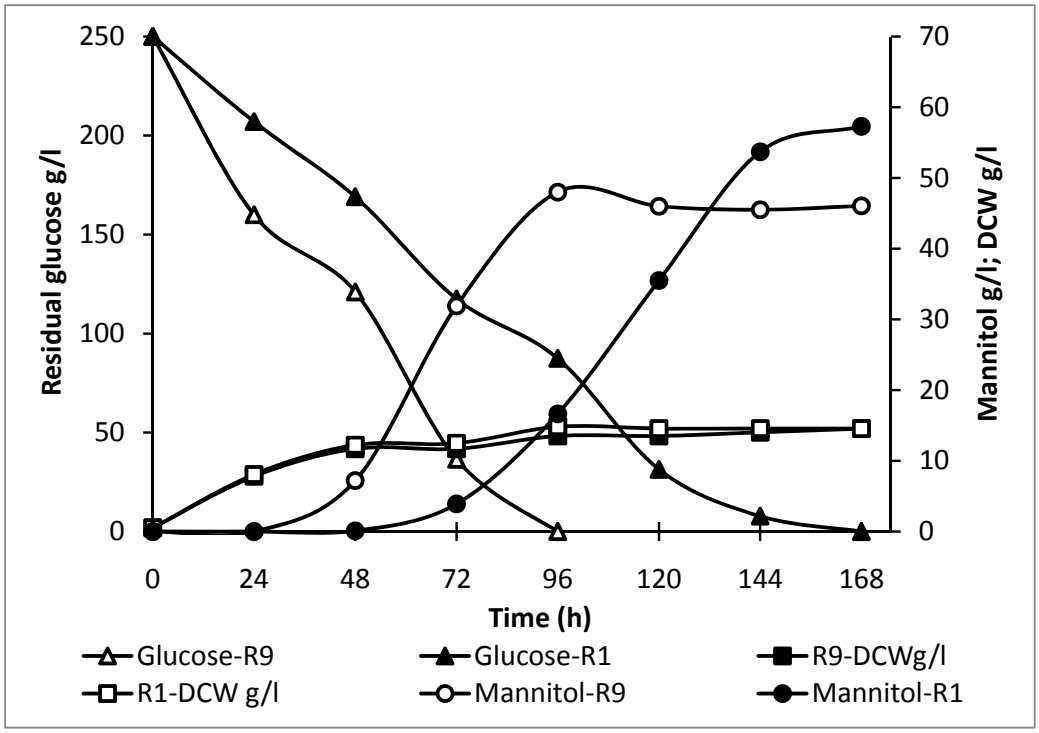


Figure 3.16 Comparison of mannitol producing mutants R1 and R9 of *C. magnoliae*

3.3.3 Media optimization for maximum erythritol and minimum mannitol and glycerol formation from *C. magnoliae* mutant R23 using RSM

The conventional method of media optimization by changing one parameter at a time while keeping the others at fixed levels is laborious and time consuming. Moreover, being linear, this method does not account for the synergistic effect between operational variables during fermentation process (Choudhari & Singhal, 2008; Lotfy et al., 2007). Hence in most of the cases, the conventional method is unable to predict

the 'true' optimum. These limitations of the conventional method can be overcome by using statistical approach for medium optimization (Pal et al., 2009; Tanyildizi et al., 2005).

Previous experimental data on mutant M572 illustrated that concentration and ratio of polyols produced was strongly influenced by medium composition. Since the production of polyols depends on environmental conditions created because of different media components, use of statistical method like RSM was thought to be a method of choice for R23 mutant. In the recent years, RSM has emerged as the most popular statistical optimization technique. In biotechnology, the technique has been used for broad range of primary as well as secondary metabolites like enzymes (Vohra & Satyanarayana, 2002); acids (Bustos et al., 2004) and terpenoids (Choudhari & Singhal, 2008). However, to the best of my knowledge, RSM has not yet been used for fermentative production of erythritol.

It was observed that mutant R23 produced 16 g/l mannitol and 4 g/l glycerol as side products. The preliminary screening experiments (one-factor at a time), suggested that glucose as carbon source and yeast extract as nitrogen source were the critical medium components for erythritol as well as by-products formation. Either increase or decrease in these medium components resulted in a drastic change in polyols pattern. To examine the combined effect of four media components (i.e. glucose, yeast extract, potassium di-hydrogen phosphate and magnesium sulphate) a four component-five level-three response CCRD of RSM model was used. The value of the dependent response (i.e. production of erythritol, mannitol and glycerol) are the mean of two independent duplicate experiments. The design of experiments and respective experimental and model predicted values of polyols are given in Table 3.9.

The second order polynomial equation was used to correlate the independent process variables with erythritol production. The second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert. The regression analysis gave second-order response model *(Eq. 3.2).*

$$\text{Erythritol (g/l)} = 59.10 + (3.60 \times A) + (2.28 \times B) + (0.57 \times C) + (0.42 \times D) - (5.66 \times A^2) - (8.76 \times B^2) + (0.079 \times C^2) - (0.17 \times D^2) + (6.49 \times AB) + (0.29 \times AC) - (0.25 \times AD) + (0.50 \times BC) + (0.89 \times BD) - (0.29 \times CD) \quad \text{- Eq. 3.2}$$

Where A: glucose, B: yeast extract, C: KH_2PO_4, D: $MgSO_4$, and A, B, A^2, B^2, AB were identified as significant model terms. Thus, the interactive effects between 'glucose and yeast extract' (AB) was predominant in the given system.

The model significance was evaluated using ANOVA (Analysis of Variance). The results of ANOVA are given in Table 3.10. Model F-value was calculated as a ratio of the mean square regression and mean square residual. The model F-value of 47.97 implied that the model was significant and there was only a 0.01% chance that a large 'Model F-value' could occur due to noise. The "P" value was used as a tool to check the significance of each of the coefficients which are necessary to understand the pattern of the mutual interactions between the operational variables. Smaller the magnitude of P, more significant is the corresponding coefficient. Values of P less than 0.05 indicate model terms are significant.

The model fitting values highlight model adequacy (Table 3.11). A low value of coefficient of variation (5.10 %) indicates very high degree of precision and a good reliability of the experimental values. The fit of the model can also be expressed by coefficient of regression R^2, which was found to be 0.978, indicating that 97.8% of the variability in the response could be explained by the model. Closer the R^2 value to 1, the better is the model fit to experimental data and less is the distance between the predicted and the experimental values. 'Adeq Precision' measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, a ratio of 23.936 indicates an adequate signal. The fit of the model is graphically represented by 'parity plot' which compares the predicted and experimental values of the response (Fig. 3.17). The diagonal indicates 0% error. Most of the points lie on or near diagonal indicating the high level of statistical significance of the model.

Std. run No.	***Glucose g/l***	***YE g/l***	***KH_2PO_4 g/l***	***$MgSO_4$ g/l***	***Mannitol g/l***	***Erythritol g/l***	***Glycerol g/l***	***Erythritol Predicted g/l***
1	-1	-1	-1	-1	3.3	45	0	45.4
2	1	-1	-1	-1	2.5	39.8	5.2	39.5
3	-1	1	-1	-1	8.9	35.4	0	34.2
4	1	1	-1	-1	21.8	50.1	4.7	54.3

5	-1	-1	1	-1	2.2	45.6	0	45.5
6	1	-1	1	-1	0	36.9	2	40.8
7	-1	1	1	-1	7	35	0	36.3
8	1	1	1	-1	19.6	58.8	6.3	57.5
9	-1	-1	-1	1	0.9	44.7	0	45.5
10	1	-1	-1	1	0	37	3.2	38.7
11	-1	1	-1	1	7.4	38.8	1.2	37.9
12	1	1	-1	1	31.6	57.3	3.8	57.0
13	-1	-1	1	1	2.6	45.7	0	44.5
14	1	-1	1	1	0.1	38	3.8	38.8
15	-1	1	1	1	10.2	39	0	38.8
16	1	1	1	1	25.8	56.5	5	59.1
17	-2	0	0	0	0	27.4	0	29.3
18	2	0	0	0	8.5	48	9.1	43.7
19	0	-2	0	0	0	21.2	0	19.5
20	0	2	0	0	27	29.4	0	28.6
21	0	0	-2	0	12.2	59.1	0.2	58.3
22	0	0	2	0	10.3	62.2	3.9	60.6
23	0	0	0	-2	13.4	59.7	0.2	57.6
24	0	0	0	2	14	59.6	0.2	59.3
25	0	0	0	0	4.5	58.3	0.2	59.1
26	0	0	0	0	4.9	58.8	0.4	59.1
27	0	0	0	0	6	60.2	0.9	59.1
28	0	0	0	0	4.5	58.3	0.2	59.1
29	0	0	0	0	4.9	58.8	0.4	59.1
30	0	0	0	0	6	60.2	0.9	59.1

Table 3.9 Central composite rotatable design matrix of independent variables and their corresponding experimental and predicted values of response

The perturbation plot for erythritol production (Fig. 3.18a) indicated that glucose (A) and yeast extract (B) were the influential media components whereas KH_2PO_4 (C) and $MgSO_4$ (D) had least influence on erythritol production. Likewise, the perturbation plot for mannitol production (Fig. 3.18b) suggested that glucose (A) and yeast extract (B) were the influential media components whereas KH_2PO_4 (C) and $MgSO_4$ (D) had least influence on mannitol production. The perturbation plot for glycerol production (Fig. 3.18c) indicates that the glycerol production was mainly dependent on glucose (A) while other media components i.e. yeast extract (B), KH_2PO_4 (C) and $MgSO_4$ (D) had least influence on glycerol production. Thus, from the perturbation plots, it was confirmed that concentration of glucose and yeast extract should be adjusted critically in order to maintain the unwanted mannitol and glycerol at minimum level.

The three-dimensional response and counter plots of the statistically significant interaction (i.e. interaction between glucose (A) and yeast extract (B) are shown in Fig. 3.19. The three-dimensional plot was obtained from the pair-wise combination of two independent variables (i.e. glucose and yeast extract), while keeping the other two variables (i.e. KH_2PO_4 and $MgSO_4$) at their center point levels. From the bump of three-dimensional plot or the central point of its respective contour plot, the optimal composition of medium components can be identified. The contour response plot gives the individual and interactive effects of these process variables on the response.

The three optimal media compositions were obtained by substituting levels of the factors into the regression equation using the Design Expert software. The criterion for obtaining optimal media compositions was chooses as: erythritol production at the maximum level and mannitol and glycerol at their minimum levels. The media composition for erythritol production and the corresponding predicted response (for erythritol, mannitol and glycerol) and experimental validation of model predicted values is summarized in Table 3.12.

The experimental values in all three cases never exceeded beyond ±4% of predicted values. The close conformity between predicted values and experimental values of polyols confirms the significance of the model. Among three optimal media compositions, the maximum erythritol production was observed in Case No. 3. Moreover, RSM-predicted medium effectively suppressed the production of unwanted

by-products as shown in Table 3.12. The optimal media composition for enhanced production of erythritol and minimal production of mannitol and glycerol was (g/l) glucose 238, yeast extract 9.2, KH_2PO_4, 5.16 and $MgSO_4$ 0.229.

Source	*Degree of Freedom*	*F - Value*	*Prob > F (P value)*
Model significant	14	47.97	< 0.0001
A	1	52.92	< 0.0001
B	1	21.13	0.0003
C	1	1.31	0.2701
D	1	0.74	0.404
A^2	1	149.41	< 0.0001
B^2	1	357.96	< 0.0001
C^2	1	0.03	0.8665
D^2	1	0.14	0.7173
AB	1	114.57	< 0.0001
AC	1	0.22	0.6421
AD	1	0.17	0.6858
BC	1	0.68	0.4223
BD	1	2.14	0.1638
CD	1	0.22	0.6421

Table 3.10 ANOVA analysis of the model

No.	*Model Terms*	*Values*
1	Coefficient of the variation	5.1
2	R^2	0.9
3	Adj R^2	0.9
4	Adeq Precision	23.9
5	Standard Deviation	2.4

Table 3.11 Model fitting values for RSM

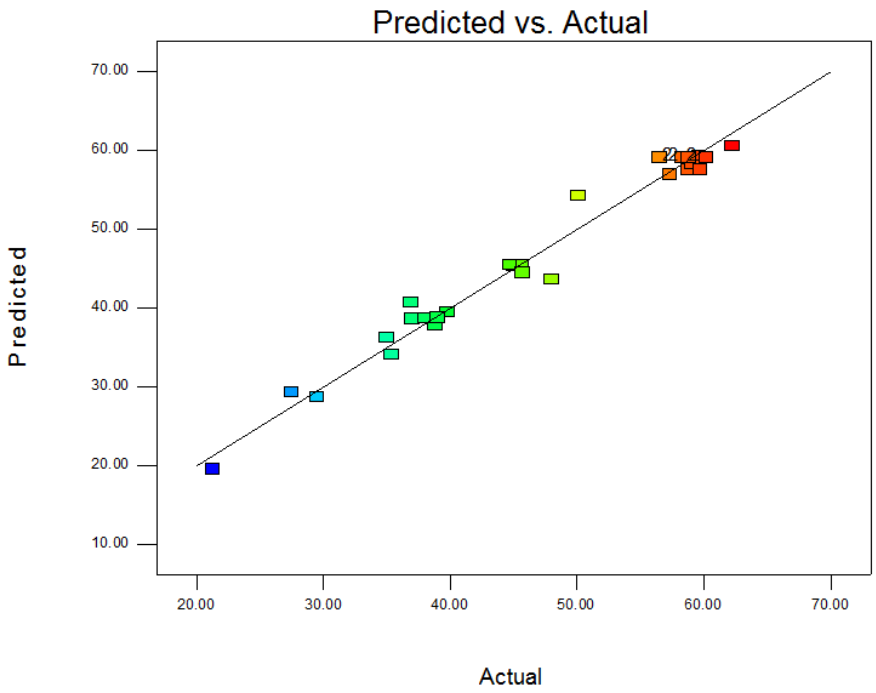


Figure 3.17 Parity plot (Actual values Vs Predicted values for erythritol production)

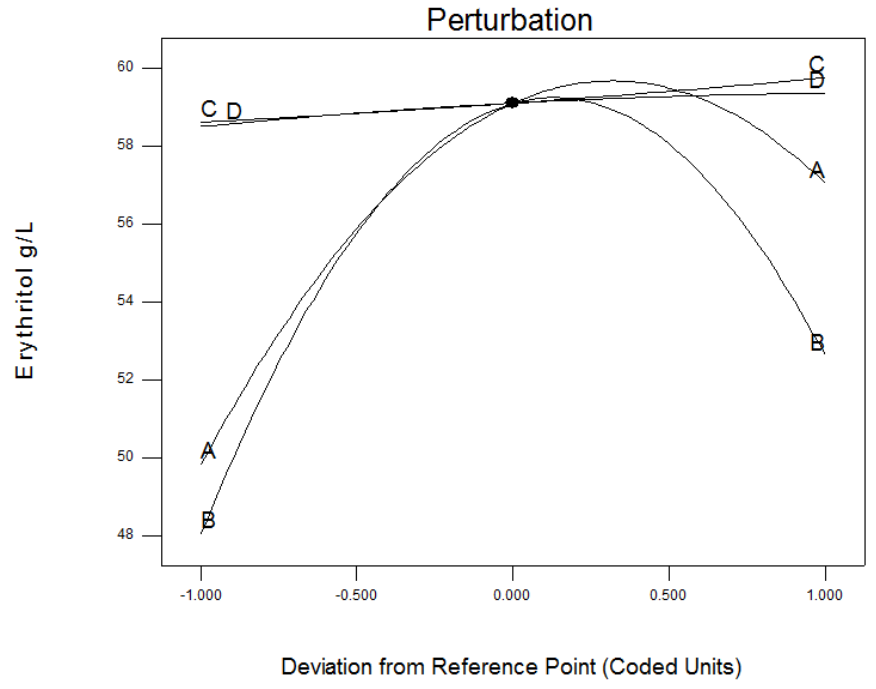


Figure 3.18a Perturbation plot for erythritol production

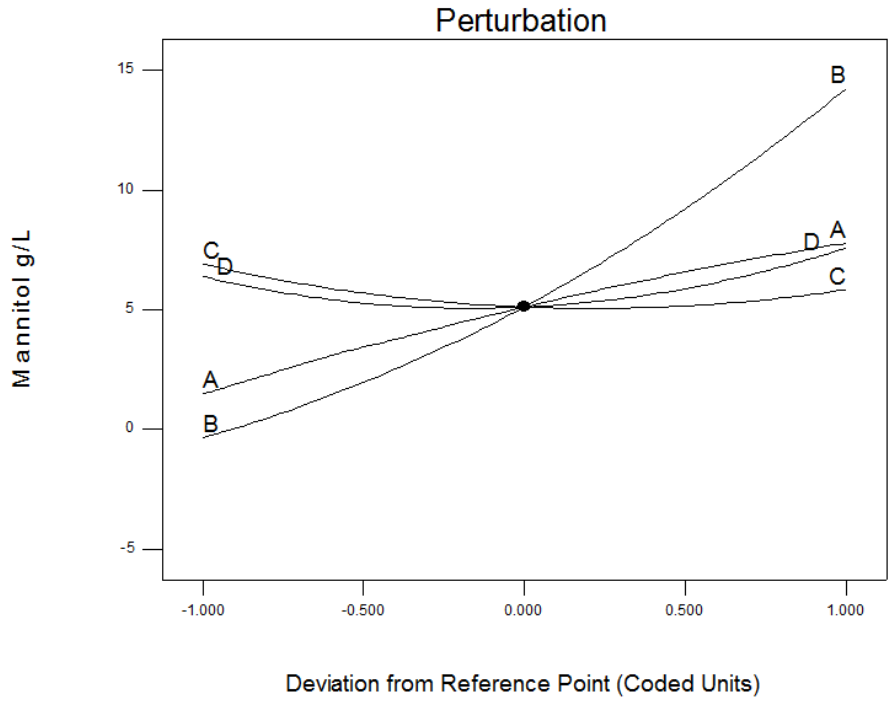


Figure 3.18b Perturbation plot for mannitol production

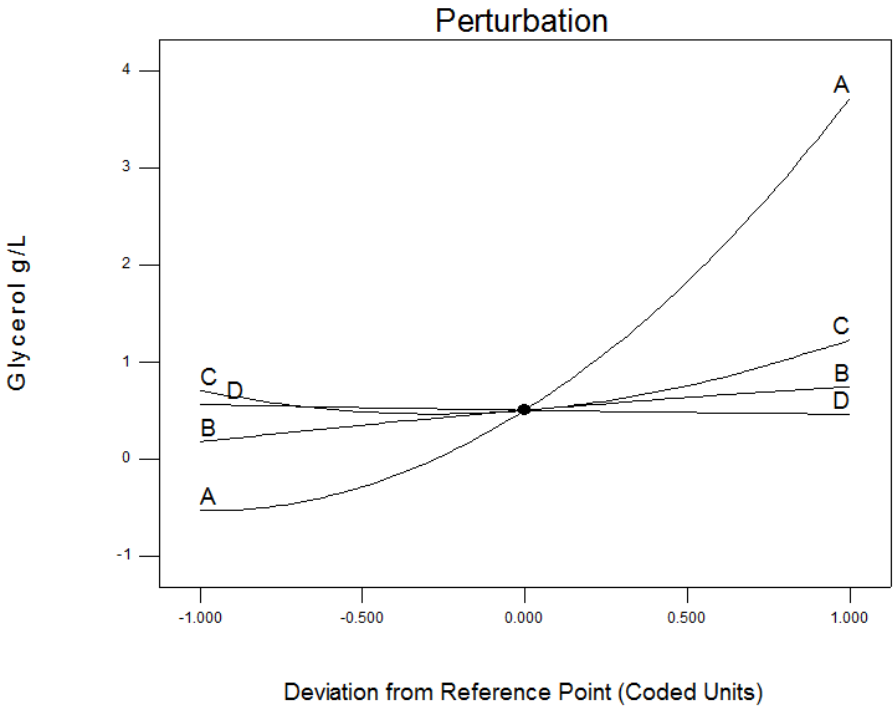


Figure 3.18c Perturbation plot for glycerol production

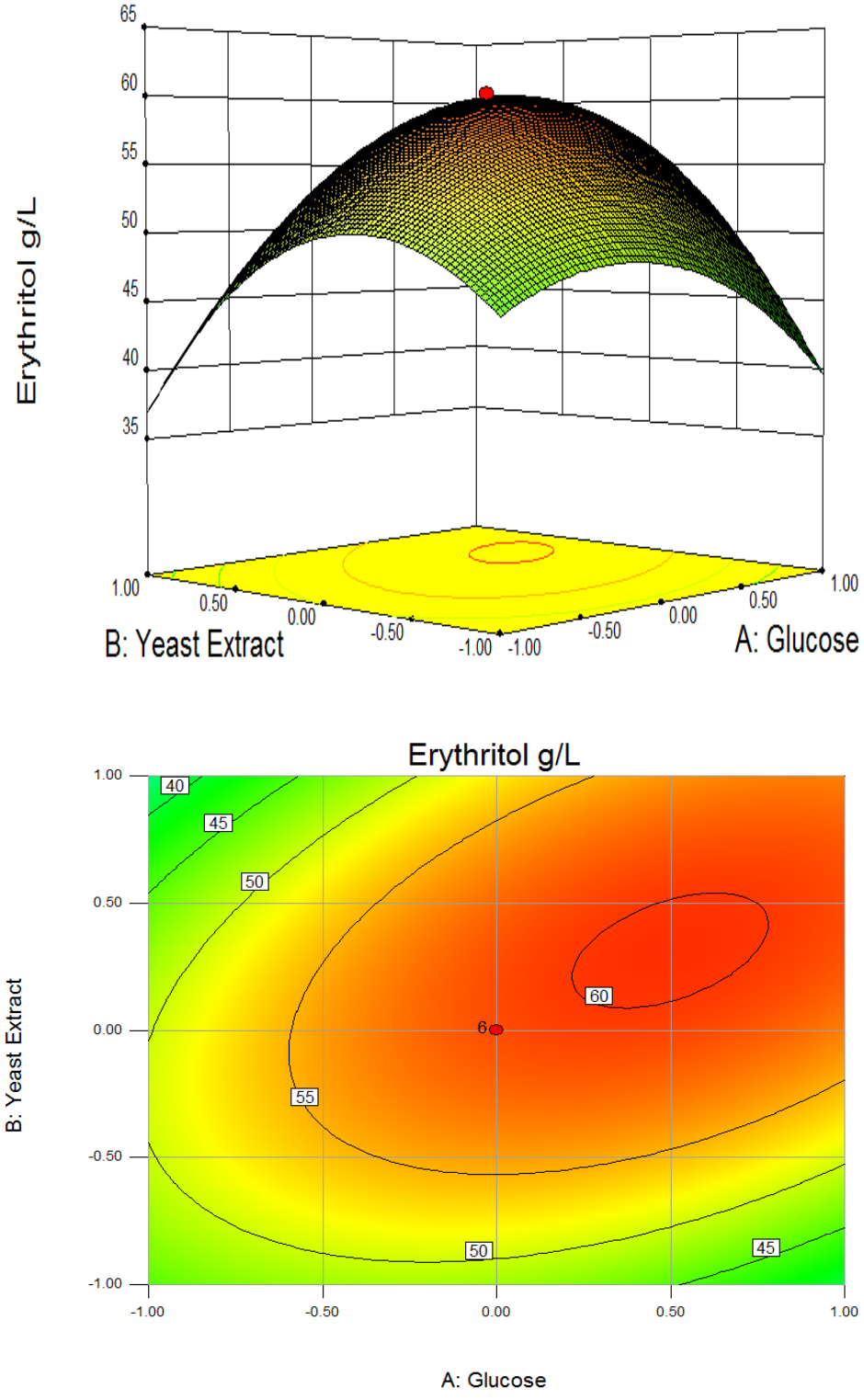


Figure 3.19 3-D response surface and contour plots of statistically significant interaction for erythritol production by *C. magnoliae* mutant R23

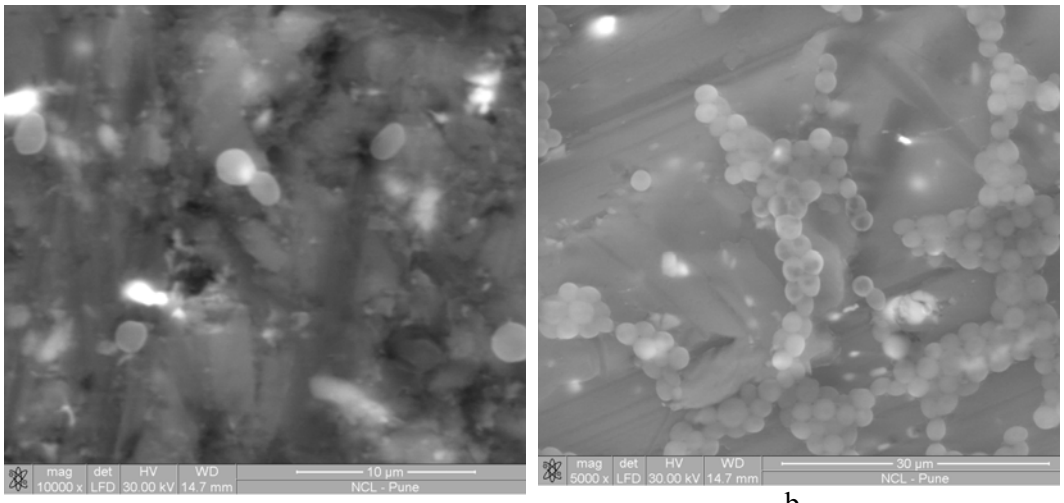
Case No.	Glucose (g/l)	Yeast extract (g/l)	KH_2PO_4 (g/l)	$MgSO_4$ (g/l)	Erythritol (g/l)		Mannitol (g/l)		Glycerol (g/l)	
					Actual	Predicted	Actual	Predicted	Actual	Predicted
1	238	9.2	5.2	0.228	57.3	56.793	3.2	2.51172	0.1	0.000028
2	238	9.17	5.2	0.227	58.8	56.7799	2.2	2.50124	00	0.000026
3	238	9.2	5.16	0.229	60.3	56.8052	3.3	2.52259	0.3	0.000011

Table 3.12 Experimental validation of model predicted values of polyol production

3.3.4 Characterization of R23 mutant of *C. magnoliae*

3.3.4.1 Morphology

Scanning electron micrographs (Fig. 3.20) illustrate that mutant R23 had morphological difference compared to its parent *C. magnoliae* NCIM 3470. The cells of mutant were larger in diameter as compared to parent and generally grew in chain or clumps as visualized under 5000-10000 X magnification.



a b

Figure 3.20 Scanning electron micrographs a) *C. magnoliae* NCIM 3470 b) Mutant R23

3.3.4.2 Carbohydrate utilization

C. magnoliae used in the current investigation showed carbohydrate utilization pattern similar to the type strain NCYC 2620 (National collection of yeast cultures). It could utilize fructose, galactose, glucose, glycerol, mannose, raffinose, ribose and sucrose for growth. It did not utilize arabinose, cellobiose, maltose, melezitose, melibiose, rhamnose, trehalose and xylose. There was no distinct difference in growth with the selected carbohydrate between parent and mutant strain as shown in Table 3.13 except in case of galactose.

Sugar	***C. magnoliae 3470***	***Mutant R23***
	OD at 600 nm	***OD at 600 nm***

Arabinose	-	-
Cellobiose	-	-
Fructose	25	27
Galactose	33	12
Glucose	28	26
Glycerol	29	28
Maltose	-	-
Mannose	27	24
Melibiose	-	-
Melezitose	-	-
Raffinose	10	6
Rhamnose,	-	-
Ribose	40	40
Sucrose	29	32
Trehalose	-	-
Xylose	-	-

Table 3.13 Growth of parent and mutant R23 on various carbohydrates

3.3.5 Studies in intracellular pathway enzymes of mutant R23

3.3.5.1 Assay of erythrose reductase and glucose-6-phosphate dehydrogenase in parent and mutant R23

Since ER is the key enzyme in the biosynthesis of erythritol, studies were undertaken to determine whether ER from mutant R23 reflects its role in increased production of erythritol compared to the parent strain. ER activity in the cell lysate of mutant R23 was reasonably higher at all the time points analyzed as compared to ER activity from parent strain, grown lysed and prepared under identical conditions. Maximum activity of 8078 units ER per g WCW was observed in the mutant R23, as compared to 6258 U per g WCW in the parent strain at 96 h. Towards the end of the growth phase, both parent and mutant strain showed elevated level of ER activity. During the growth phase, cells are metabolically active, continuously undergoing cell division where there is a continuous requirement of ribose for biosynthesis of nucleotides. Ribose is

an intermediate of PP pathway. This could be the reason for high level of ER activity during initial 48 h of growth phase.

Erythritol production started after the growth phase as the requirement for the nucleotides decreased and maximum production rate was observed between 72-96 h. In the present study, at 96 h there was distinct difference in ER activity and the mutant showed 7122 U per g WCW as compared to 3614 U per g WCW in the parent. These values represent 1.9 fold higher ER activity compared to parents strain grown under identical condition (Fig. 3.21). Beyond 96 h, the ER activity of parent and mutant strain decreased concomitant with erythritol production rate.

In contrast to ER activity, there was no distinct difference in G6P-DH activity, although the mutant R23 showed slightly higher G6P-DH activity at all the time analyzed as compared to parent strain (Fig. 3.22).

ER is present in a number of organisms and it reversibly catalyzes reduction of erythrose to erythritol and oxidation of erythritol to erythrose. It has been well documented that ER is a key enzyme in the biosynthesis of erythritol and most of the efficient erythritol producing strains possess higher ER activity (Lee et al., 2000; Lee et al., 2003b; Lee et al., 2003c; Tokuoka et al., 1992). Recently, a study on enzyme activities of PP pathway of *Trichosporonoides megachiliensis* SN-G42 which is used for industrial erythritol production was undertaken under various culture conditions to examine the production mechanism and the key-enzymes involved in higher erythritol biosynthesis. The proposed outcome of the study stated that, in the cultures in which erythritol was produced after completion of cell growth, the enzyme activities of the PP pathway were higher than those of the TCA cycle. In particular, transketolase appeared to be a key-enzyme for enhanced erythritol production in the *Trichosporonoides megachiliensis* SN-G42. A high activity of transketolase is required to produce abundant intermediates for high erythritol productivity (Sawada et al., 2009).

Studies on proteome analysis of *C. magnoliae* KFCC 11023 and its mutant having enhanced growth and erythritol production capability showed altered expression levels of some of the genes encoding TCA and glycolytic proteins. The levels of the TCA enzymes (citrate synthase, succinyl-CoA ligase, and fumarase) and the

glycolytic pyruvate decarboxylase were found to be increased in the mutant strain, whereas the level of enolase was found to be decreased compared to the wild-type strain (Lee et al., 2003a; Park et al., 2005).

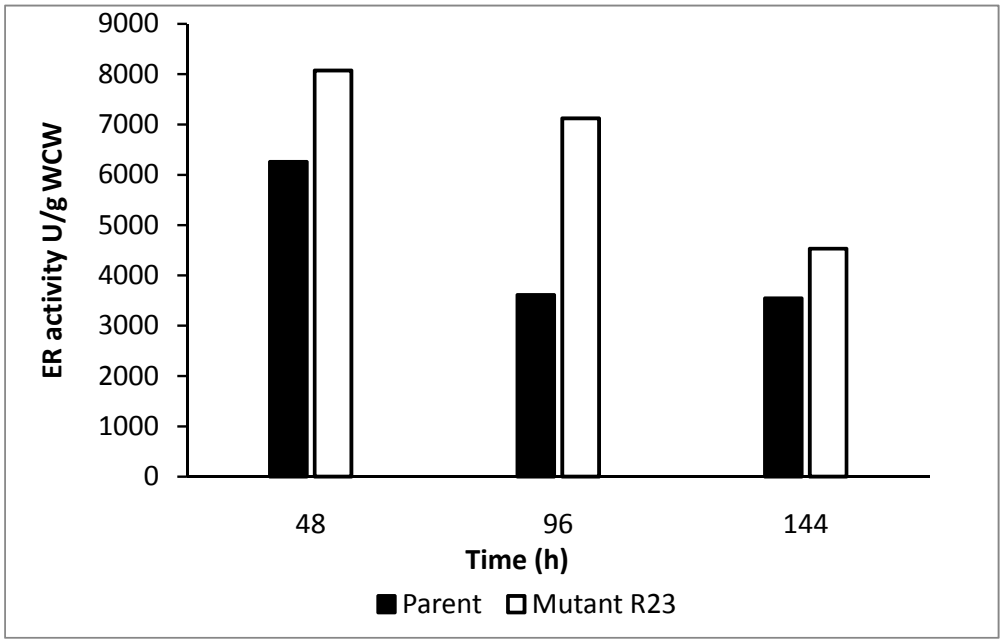


Figure 3.21 Comparison of ER from parent and mutant R23

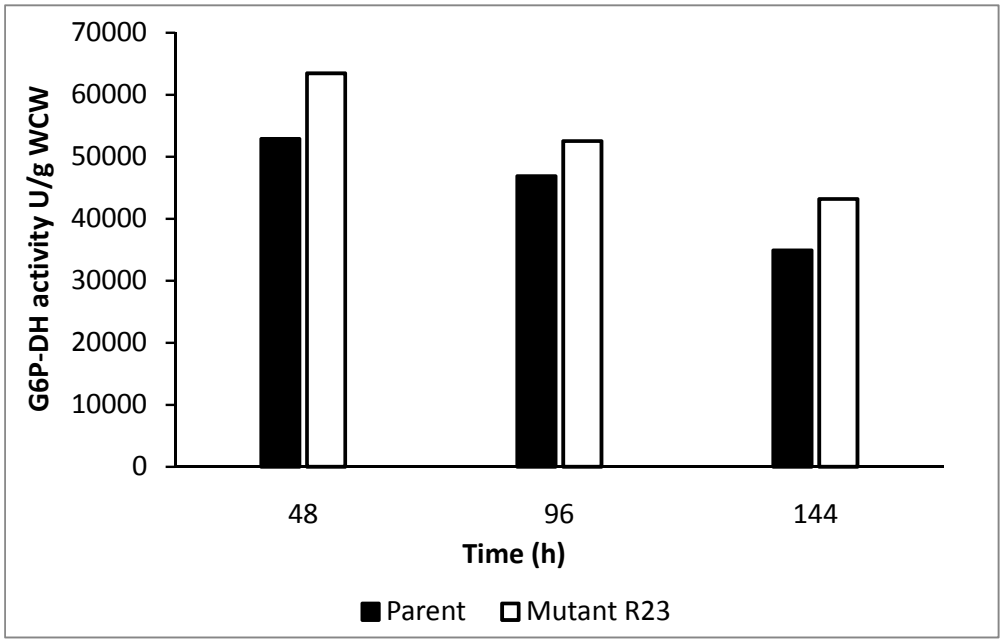


Figure 3.22 Comparison of G6P-DH from parent and mutant R23

3.3.5.2 Optimum pH and temperature of ER

The optimum pH for erythrose reduction by ER was 6, with 93 and 64% of the maximum activity at pH 5.0 and 7.0, respectively. The optimum pH for erythritol oxidation was 8.0, with 60% of the maximum activity at pH 7.0, whereas no activity was observed at pH 9.0 (Fig. 3.23). Maximal erythrose reduction activity at pH 6.0 and optimum erythritol oxidation activity at alkaline pH are common features of ER isolated from other erythritol producing yeasts (Lee et al., 2003b; Lee et al., 2003c). In the present investigation, the optimum temperatures for the reductive and oxidative reactions were 50 and 40°C, respectively.

As seen from the Fig. 3.23 erythritol oxidation was not observed at pH 5. Although it is known and also observed in our experiments that the erythritol oxidation activity increases in alkaline pH, it has no practical meaning in living cells because the pH inside the cells is slightly acidic. Therefore, ER is considered to catalyze reduction of erythrose to erythritol exclusively. Thermal stability of ER at various temperatures exemplified that at 30 °C almost 95% of its initial activities was retained till 30 min. With increase in temperature beyond 30 °C, negligible amount of activity was retained.

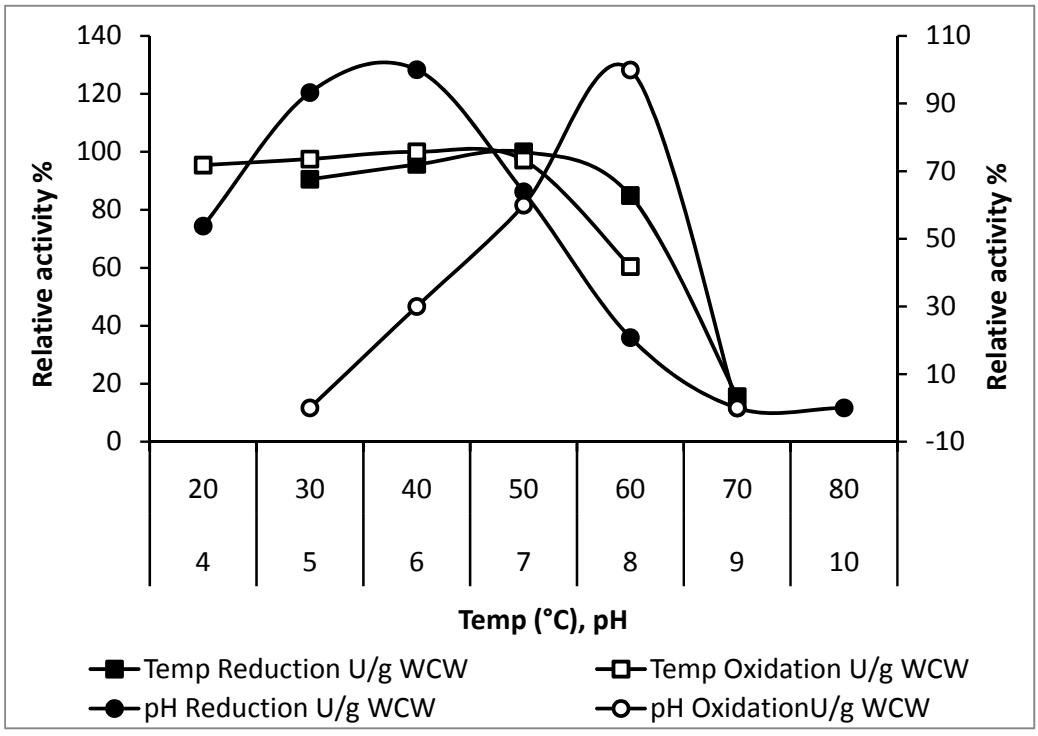


Figure 3.23 Effects of pH and temperature on the oxidation and reduction activity of ER from mutant R23

3.3.5.3 Substrate and co-substrate specificity

Most of the erythrose reductase are pyridine nucleotide linked and require either NADH or NADPH as a co-substrate. ER from *C. magnoliae* mutant R23 showed affinity only for NADPH and no activity was observed when NADH was used as co-substrate in the presence of 10 mM erythrose as substrate. When reduction of arabinose, fructose glucose, galactose, mannose, ribose, sucrose, trehalose and xylose, was examined at 10 mM concentration each with NADPH as a co-substrate, it was found that ER had a highest preference only for erythrose. None of the other substrates examined, except erythrose were reduced as seen in Fig. 3.24.

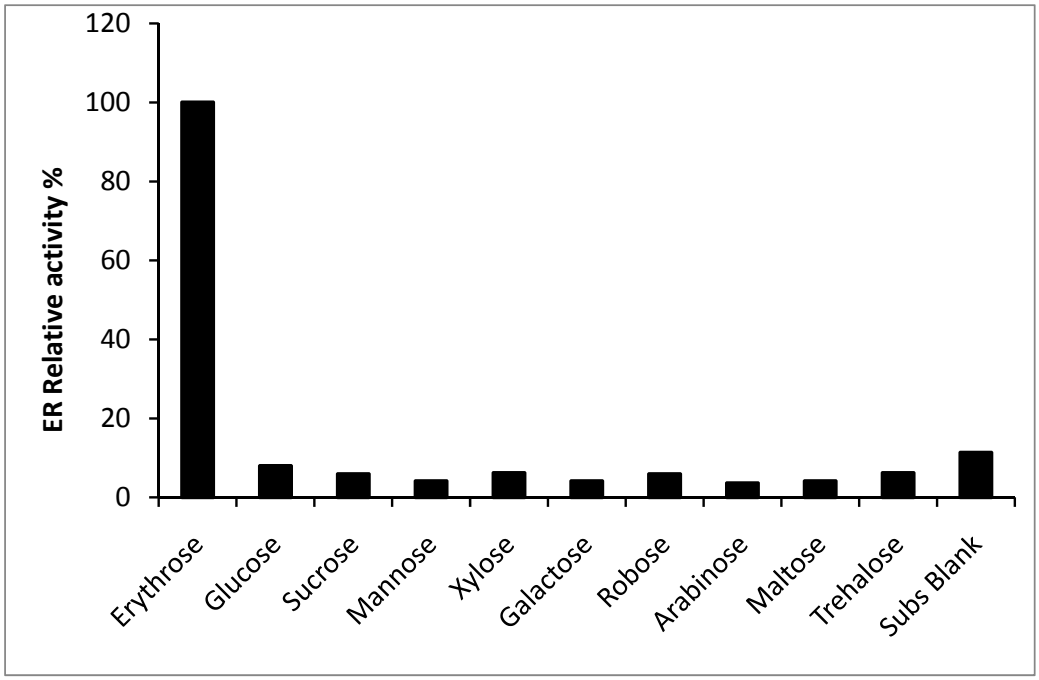


Figure 3.24 Co-substrate specificity of ER activity

3.3.5.4 Effects of metal ions and reducing agents on ER

ER activity was not affected by Ca^{+2}, Co^{+2}, Mn^{+2}, Mo^{+2}, Ni^{+2} and B^{+2} while, Cu^{+2} and Zn^{+2} ion showed inhibition of ER activity to the extent of 20 and 57%, respectively (Fig. 3.25). Addition reducing agents like 2-mercaptoethanol, glutathione, cysteine, or DTT at 1 mM concentration increased the enzyme activity by 15%, 24%, 32% and

43%, respectively. Dependence of the enzyme activity on sulfhydryl compounds has been reported for ER from *Torula coralline.* DTT was found to be the best reductant and served to keep the active enzyme in a reduced state (Lee et al., 2003b). In the present study, ER was neither inhibited nor activated by EDTA, mercaptoethanol, cysteine, or dithiothreitol at 10 mM. Lee et al., (2002) studied the mechanism behind the increased erythritol production by *T. coralline* on supplementation with Cu^{+2}. Their study revealed that the presence of Cu^{+2} in cultures decreased the formation of fumarate which is a strong inhibitor of ER. The decrease in fumarate content within the cells in turn enhanced ER activity and resulted in a high yield of erythritol.

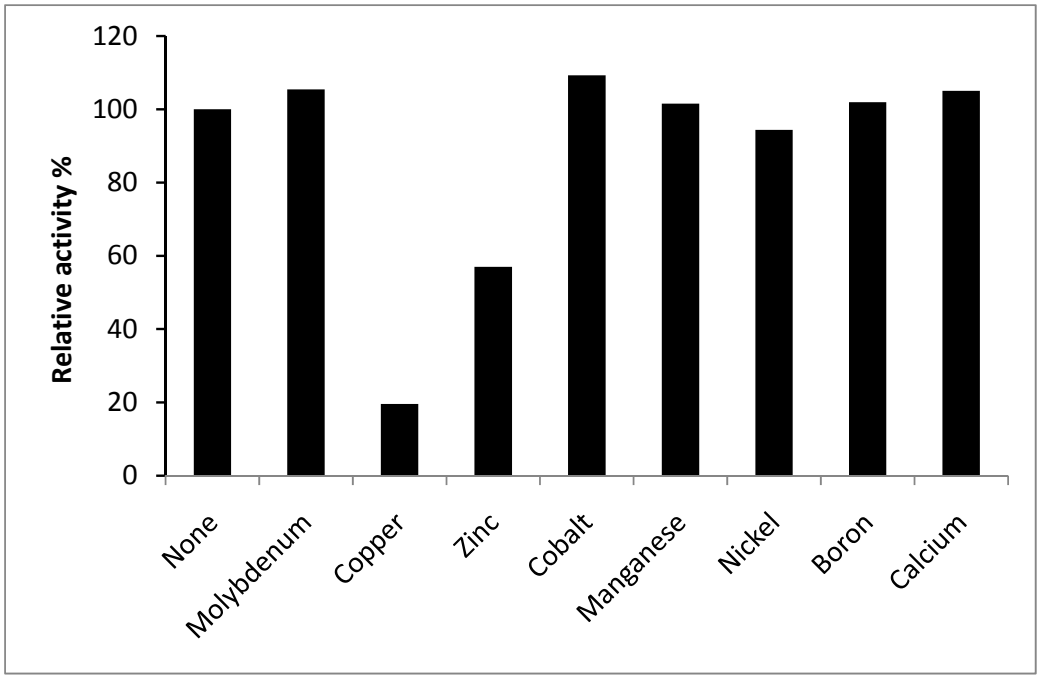


Figure 3.25 Effects of metal ions on ER activity

3.3.5.5 Kinetics

Initial-velocity studies performed with variable concentrations of erythrose as substrate in the presence of fixed concentrations of NADPH as co-substrate. The substrate, erythrose, showed hyperbolic saturation curve with increasing erythrose concentration representing typical Michaelis-Menten type kinetics. Maximum enzyme activity was obtained with an erythrose concentration of about 10 mM under the experimental conditions (Fig. 3.26). Plot of the reciprocal of the initial velocity against the reciprocal of the erythrose concentration gave a straight line that

intersected X axis in the left quadrant (Fig. 3.27). The *Km* and *Vmax* calculated from the Lineweaver-Burk plot for ER were found to be 1.55 mM and 0.26 mM ml^{-1} min^{-1}, respectively. This *Km* for D-erythrose is substantially lower than the values of ER from *Aureobasidium* sp, *T. coralline* and *C. magnoliae* which had the *Km* of 8.0, 7.1 and 7.9 mM respectively (Lee et al., 2003b; Lee et al., 2003c; Tokuoka et al., 1992). When NADPH was used as a variable substrate, similar straight lines intersecting each other were obtained (Fig. 3.28). These results indicate that the reaction proceeds via the formation of a ternary complex of the enzyme with NADPH and erythrose.

3.3.5.6 Effect of product and by-products on ER activity of mutant R23

The study on effect of erythritol and possible by-products of erythritol fermentation is illustrated in (Fig. 3.29). It was found that the increase in erythritol, mannitol and glycerol concentration from 50-600 mM did not affect ER activity. Almost 90% of its initial activity was observed even at 600 mM erythritol concentration. However, with the increase in ethanol concentration there was a distinct decrease in ER activity and at 600 mM ethanol ER activity was lowered to 70%.

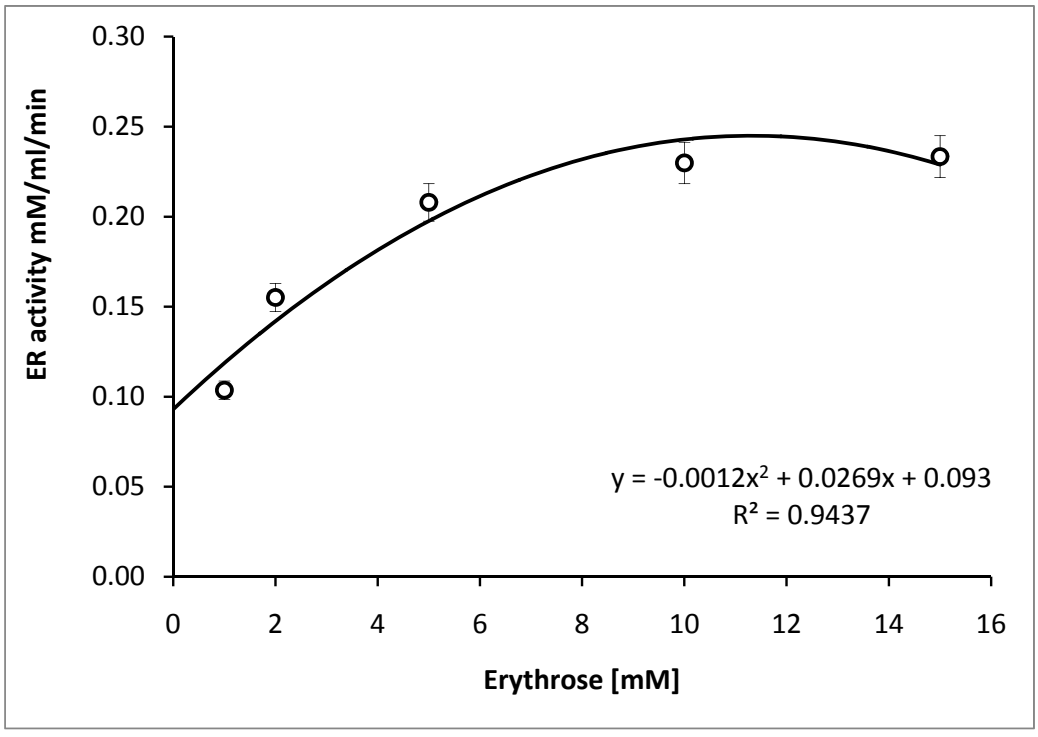


Figure 3.26 Effect of erythrose concentration on ER activity of mutant R23

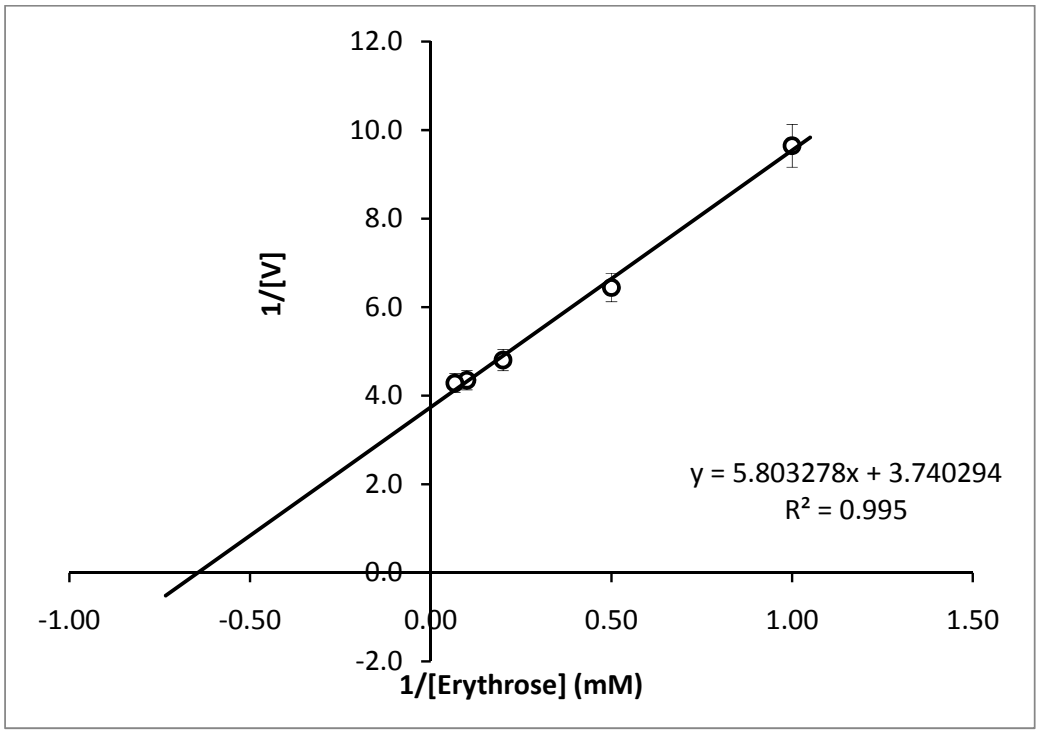


Figure 3.27 Lineweaver-Burk plot of initial velocity versus erythrose concentrations

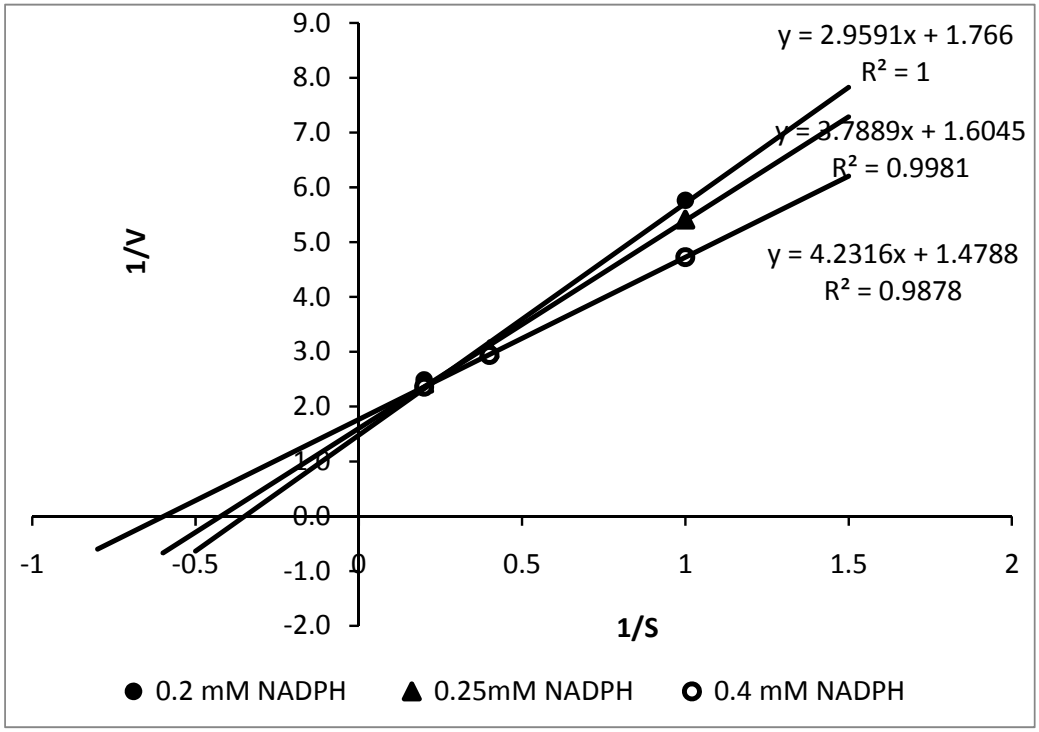


Figure 3.28 Double reciprocal plots for ER at varying NADPH concentrations

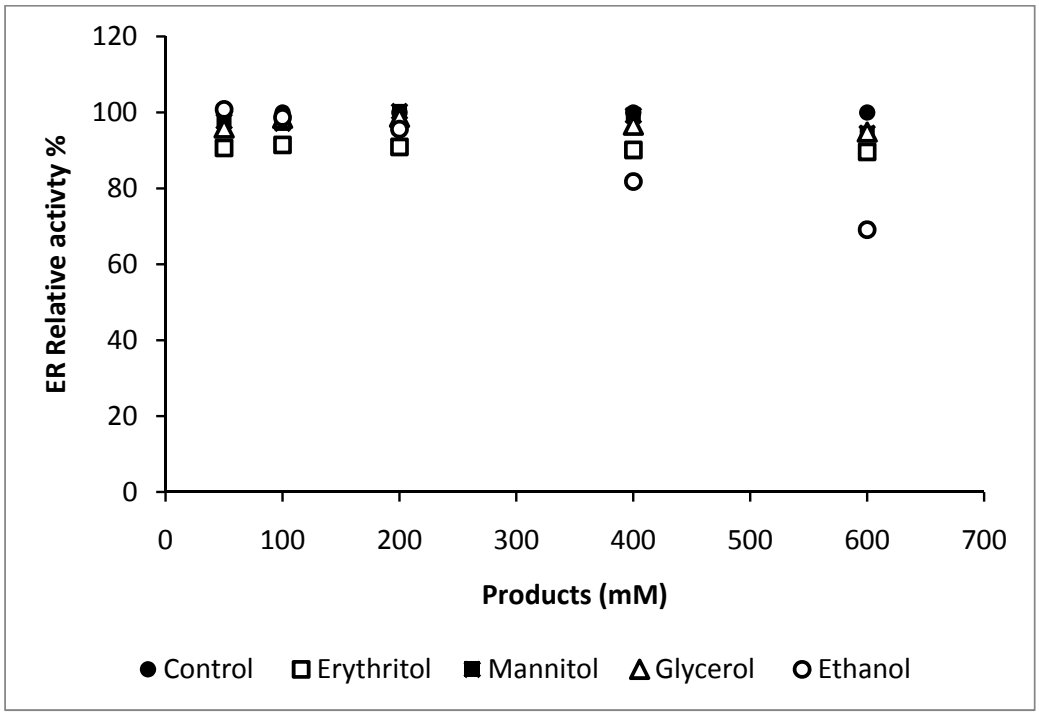


Figure 3.29 Effect of product and by-products concentration on ER activity

3.4 CONCLUSIONS

Mutants of *C. magnoliae* and *Y. lipolytica* were generated by UV irradiation, EMS and NTG treatment for desired improvement. Through screening of 1368 mutants for enhanced erythritol production, two mutants of *C. magnoliae* named M572 and R23 were selected because they produced considerably high amount of erythritol without formation of any organic acid. Efforts for improvement of *Y. lipolytica* were not successful. On screening of 380 mutants there was hardly any improvement in erythritol production.

Production of erythritol from glucose by *C. magnoliae* mutant M572 was found to be dependent on yeast extract concentration in the fermentation medium. In the optimized medium, the mutant produced erythritol 53 g/l in a flask culture with a yield of 0.23 g of erythritol per g of glucose consumed, which corresponded to 2.4-fold increase in the yield and 3.8-fold increase in erythritol productivity as compared to that of the parent. Half of the yeast extract could be substituted by inorganic nitrogen sources like ammonium nitrate, sodium nitrate or urea and still similar yield of erythritol and productivity could be achieved. This can save substantial raw

material cost because yeast extract is an expensive nutrient source. Detailed nutritional requirement for mutant M572 was investigated and based on this, a minimal medium was designed for erythritol production. The minimal medium although supported growth, but *C. magnoliae* was unable to produce erythritol in this medium.

Statistical RSM approach of media optimization for maximum erythritol and minimum mannitol and glycerol was employed for the mutant R23. Concentration of four media components, glucose, yeast extract, potassium di-hydrogen phosphate and magnesium-sulphate were optimized using central composite rotatable design (CCRD) of RSM. Coefficient of regression R^2, which was found to be 0.978, indicating that 97.8% of the variability in the response could be explained by the model. From the perturbation plot for erythritol production it was found that glucose and yeast extract were the most influential media components whereas $KH_2PO_4$and $MgSO_4$ were having least influence on erythritol production. Optimum medium composition obtained from RSM model in (g/l) was glucose 238, yeast extract 9.2, KH_2PO_4, 5.06 and $MgSO_4$ 0.253. This model was useful for operating the fermentation towards reduction in accumulation of unwanted metabolites.

Two mutants, R1 and R9, which produced mannitol as major product, were also isolated during the mutagenesis experiments. Mutant R1 produced 57 g/l mannitol in 168 h with 23 % yield while mutant R9 could produce 46 g/l of mannitol with a yield of 18 % in 96 h. Most of the chemical and biological processes investigated earlier for mannitol production use fructose as carbon source which has a rather high price as compared to glucose.

To ascertain the possible reason for erythritol overproduction by mutant R23, activity of the ER and G6P-DH, the enzymes involved in erythritol biosynthesis were compared in parent and mutant strain. It was observed that under identical growth conditions at 96 h, ER activity of mutant R23 was about 2 fold higher than that of the parent strain. During shake flask experiment, maximum erythritol production rate was observed at 96 h. Thus, higher ER activity in R23 is likely to be responsible for higher erythritol production than that of parent strain. The Lineweaver-Burk plot for ER

obtained from mutant R23 showed *Km* of 1.55 mM, which is about 3 to 4 fold lower than *Km* values of ER reported in literature.

My results indicate the potential of *C. magnoliae* mutant R23 for erythritol production with good yield and productivity and low amount of interfering by-products in shake flask. Optimization of fermentation for improving erythritol production in bench scale fermenter is discussed in the succeeding chapter.

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Chapter 4

Evaluation of Erythritol Production in Laboratory Fermenter

Abstract:

The present chapter describes a) evaluation of statistically-optimized medium in laboratory fermenter b) development of effective erythritol production strategy by optimizing various modes of fermentation c) purification and characterization of erythritol from the fermentation broth.

Among the culture conditions tested in batch fermentation, dissolved oxygen was the key factor affecting erythritol production. It was found that a low oxygen level is apparently required for effective erythritol production. By controlling DO concentration around 0-2% of air saturation, in fed-batch mode, the use of RSM-optimized medium resulted in 87.8 g/l erythritol and 31.1% yield, without formation of any by-products. Erythritol crystals of about 99% purity were obtained by using a simple purification protocol comprising activated carbon treatment, concentration and crystallization.

4.1 INTRODUCTION

From shake flask studies it was found that erythritol production using *C. magnoliae* and its mutants R23 were strongly influenced by medium composition and oxygen availability. Hence it was necessary for process to be studied by varying the parameters in a laboratory fermenter where accurate control of process variables is possible. Since larger samples can be taken, growth and production can be better analyzed.

In the previous chapter, strain improvement of *C. magnoliae* followed by statistical medium optimization for the selected mutant R23 was presented. Glucose and yeast extract were identified as critical medium components which indirectly govern the dissolved oxygen availability in shake flasks. The ratio of polyols produced by mutant R23 was mainly dependent on dissolved oxygen concentration. Strain improvement and statistical medium optimization resulted in a 4.3-fold enhancement in erythritol production by mutant R23. The reasons behind higher erythritol production by mutant than parent strain were investigated. Higher erythritol production by the mutant R23 was attributed to higher activity of erythrose reductase.

Apart from strain isolation and its improvement by chemical and physical mutagenesis, research efforts in erythritol production have been mainly focused on process optimization in batch and fed-batch fermentation (Table 1.3). Two to three fold increment in yield and productivity of erythritol has been reported in some of the strains by optimizing process parameters at the fermenter level. Higher values in erythritol production were mainly obtained through a high-cell density culture with control of process parameters, mainly dissolved oxygen and substrate concentration, which is not possible in shake flasks. Glucose obtained by chemical or enzymatic hydrolysis of wheat and corn starch is commonly used as a major carbon source for erythritol production. In erythritol fermentation, a yield of 0.15 to 0.6 g/g glucose utilized has been reported (Jeya et al., 2009; Kim et al., 1997).

At industrial scale, erythritol yield and production rate were reported to be 47% and 2.0 g $l^{-1}h^{-1}$ respectively, in a 100,000 L fermenter using *Aureobasidium sp.* SN-G42. Using optimized medium composition and by controlling oxygen transfer rate, formation of byproducts could be minimized (Moon et al., 2010). Recently, erythritol

production was scaled up to 200,000 L for commercial production by Sawada et al., (2009). Isolation of an erythritol-producing yeast *Pseudozyma tsukubaensis*, optimization of culture conditions at flask and fermenter level, scale-up studies from a laboratory scale (7L fermenter) to production plant scale (50,000 L) using dissolved oxygen as a scale-up parameter was published recently by Jeya et al., (2009). This study reports the highest erythritol concentration obtained at the plant scale (245 g/l) with a yield as high as 61%.

Batch and fed-batch fermentations in 30-500 L of bubble column and airlift tower loop reactors were studied in detail by Burschäpers et al., (2002a, b). In the preliminary investigation it was found that nitrogen source limitation caused foam formation whereas oxygen limitation enhanced ethanol formation. With a suitable aeration and a fed-batch regime by feeding a mixture of glucose and nitrogen source, ethanol and foam formation was minimized. An industrial process was developed by the combination of a well balanced medium composition and feeding strategy by Cerestar R&D Centre, Belgium with a cooperation partner (Cargill Corn Milling USA) in which up to 175 g/l erythritol concentration was achieved in 500 m^3 reactor (Schügerl, 2005).

It appears that, there is only one process which uses *C. magnoliae* KFCC 11023 for erythritol production. This strain was isolated from honeycombs and mutagenized by EMS treatment. The mutant M2 produced 25 g/l erythritol with 25% yield in a medium containing 100 g/l glucose. A fed-batch process was optimized by simultaneous feeding of glucose and yeast extract to achieve 75 g/l DCW which in turn resulted in 200 g/l erythritol with 1.2 g $l^{-1}h^{-1}$ productivity and 43 % yield. However, this process produced high amounts of organic acids namely gluconic acid, citric acid and butyric acid (Koh et al., 2003; Ryu et al., 2000; Yang et al., 1999).

The objectives of the work described in the present chapter were a) to evaluate the statistically-optimized medium, in laboratory fermenter b) to develop fermentation strategy for effective erythritol production and c) to purify erythritol from the fermentation broth and d) to chemically characterize erythritol produced in the fermentation broth.

4.2 MATERIAL AND METHODS

All medium ingredients, strain and analytical methods used were the same as described in chapter 3 unless otherwise mentioned. Standard mixture of fatty acid methyl ester (FAME 37) was purchased from Sigma-Aldrich Corporation, USA.

4.2.1 Culture conditions

Test tubes containing 5 ml statistically-optimized fermentation medium (g/l) glucose 238, yeast extract 9.2, KH_2PO_4, 5.16 and $MgSO_4$ 0.229 were inoculated with the cells of the mutant and incubated at 28 °C, 210 rpm for 48 h. Two and half ml of this seed culture was transferred into a 250 ml Erlenmeyer flask containing 25 ml fermentation medium and incubated at 28°C for 24 h. Five percent (v/v) of the above seed culture, grown in multiple flasks was then transferred into a fermenter. All the fermentation experiments were performed in 14 L laboratory fermenter (New Brunswick Scientific, Bio-flow 110 USA) with a working volume of 10 L, equipped with devices for control and measurement of pH, temperature, dissolved oxygen and agitation speed. Temperature was maintained at 28 °C by automatic heating or circulating chilled water. The pH of the medium was initially adjusted to 6 with 5N NaOH. The DO was measured with a dissolved oxygen probe (Mettler Toledo). The agitation speed was adjusted between 300-600 rpm and aeration rate was set at 0.5 vvm (volume per volume per minute). Online fermentation data of process parameters such as DO, pH, agitation and feeding strategy was acquired using NBS BioCommand Plus Software. Online biomass OD was recorded by measuring the turbidity at 1100 nm by using OD probe (Wedgewood Analytical Inc, Anaheim CA. USA with cell growth sensor BT 65 S).

4.2.2 Evaluation of M572 mutant for erythritol production in 10 L fermenter

A series of fermentation batches were carried out with the intermediate mutant M572 to evaluate fermentation conditions for erythritol production in LFM medium. For all the batches, glucose solution was autoclaved in the fermenter and yeast extract with salts was autoclaved separately in flask and later transferred to the fermenter aseptically. The DO probe was polarized for four hours after autoclaving. It was calibrated between 0 and 100% air saturation. Zero was adjusted electronically by disconnecting the cable for less than one minute, followed by saturating the medium

with oxygen by sparging air at 0.5 vvm and 600 rpm, this point was set as 100% air saturation. The fermentation batches were started by addition of 500 ml inoculum to 9.5 L LFM medium, which was initially grown for 24 h as shown in Fig. 4.1. The foam formation was controlled by periodic addition of silicon antifoam. Samples were analyzed at successive intervals for biomass OD, pH, DO, residual glucose and polyol production.

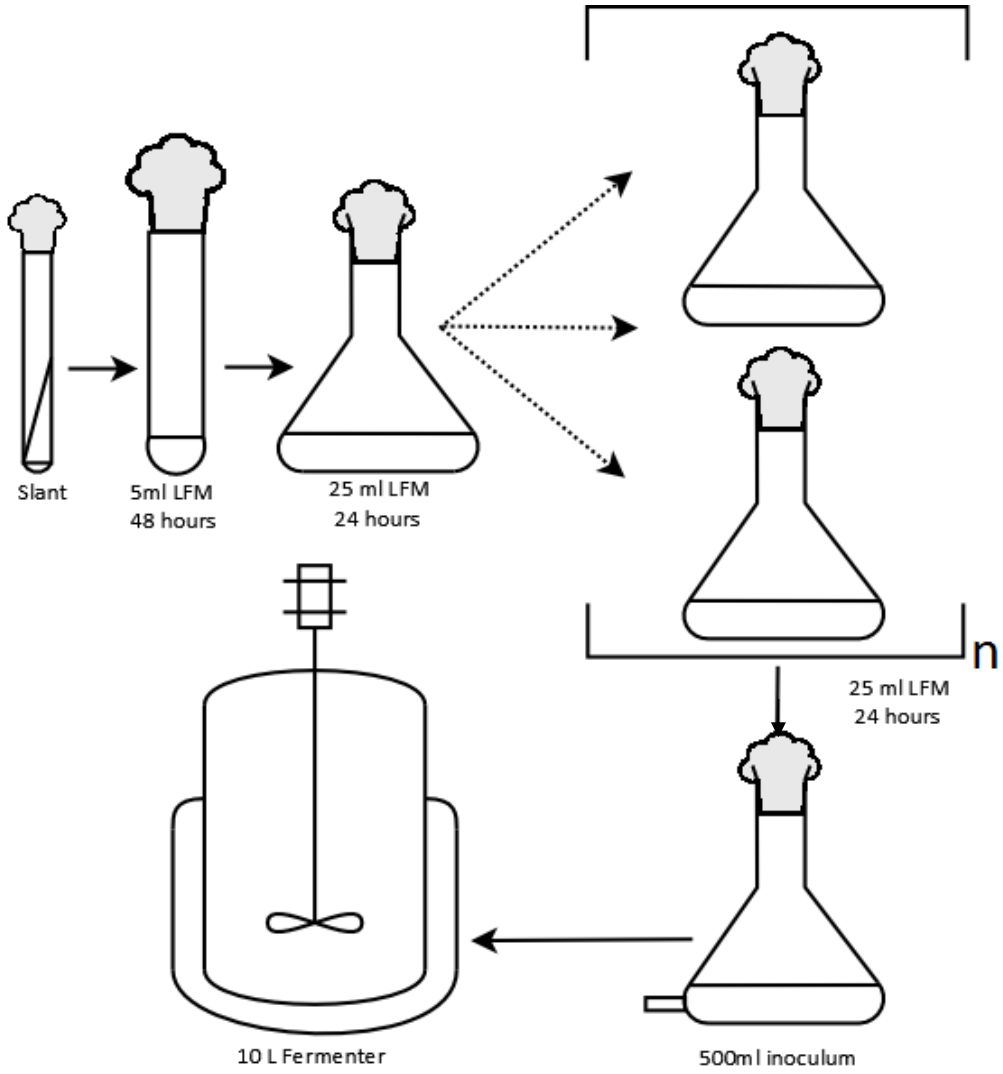


Figure 4.1 Schematic representation of inoculum preparation for 10 L fermenter

4.2.3 Effect of dissolved oxygen on erythritol production by R23 in 10 L batch fermenter

From the preliminary batch fermentation data on mutant M572, it was found that DO is the significant parameter in erythritol formation. To determine critical dissolved

oxygen concentration required for effective erythritol production for the newly generated mutant R23, three successive fermentation batches at different fixed rpm were performed in statistically-optimized medium. The agitator speed for the three successive batches was set at 300, 450 and 600 rpm, respectively. The aeration rate and temperature were kept constant at 0.5 vvm and 28 °C, respectively. DO concentration was recorded online by using NBS BioCommand plus software. Samples were analyzed at successive intervals as described above.

4.2.4 Evaluation of minimal medium for growth and erythritol production

Shake flask experiments in a defined medium, in which yeast extract from LFM was replaced with ammonium sulphate or sodium nitrate as nitrogen source resulted in good growth, but erythritol production was very less as compared to LFM with yeast extract. To evaluate erythritol production in minimal medium, more experiments were carried out at fermenter level using R23 mutant under controlled process parameters. Two separate batches were performed with $NaNO_3$ and $(NH_4)_2SO_4$ as sole nitrogen sources. All the media components and process parameters were same for both the batches, except nitrogen source. The nitrogen sources used were on equal nitrogen basis compared to yeast extract. Biotin, pyridoxine and thiamine, each at 25 mg/l, were filter sterilized and added to the fermented after autoclaving. The pH of the fermentation broth was maintained with 5 N NaOH during the growth phase and allowed to fall during the production phase. The agitation speed and aeration was fixed at 450 rpm and 0.5 vvm, respectively. Samples were analyzed at regular interval of time as described earlier.

4.2.5 Optimization of fed-batch fermentation for erythritol production

Development of a suitable feeding strategy is an important activity in fed-batch cultivations because nutrient feeding strategy determines cell growth and product formation. In order to enhance yield and productivity, six fed-batch fermentation experiments (R1, R2…R6) were performed using statistically-optimized medium with R23 mutant. First three experiments were aimed towards optimization of nutrient feeding strategy specifically glucose and yeast extract and subsequent runs were attempted to optimize DO concentration. For all fed-batch experiments, 50 g/l glucose along with the KH_2PO_4, $MgSO_4$ and yeast extract was used during the initial growth

phase. Feed solution (4 L) consisted of glucose solution (500 g/l) with or without yeast extract. Feeding strategy was modified according to the residual glucose and the product formed during the runs. The initial volume of the culture broth for all the experiments was 6 L which increased to 10 L on feeding glucose solution towards the end of fermentation. Agitation rates were adjusted during the course of fermentation depending upon the DO profile.

For the first fed-batch experiments named "R1", only 50 g/l glucose was added during the growth phase along with yeast extract and salts in order to reduce time for lag phase by reducing the osmotic pressure imparted by initial high glucose concentration. Remaining 200 g/l glucose was fed after the growth phase. In order to maintain cells in metabolically active phase, yeast extract was added in the feed glucose syrup at 5 g/l concentration. The feeding solution (4 L) consisted of 2000 g glucose with 50 g yeast extract. The addition of feed solution was started on the onset of stationary phase, based on on-line monitoring of biomass concentration, analyzed using OD probe. High agitation rate 500-600 rpm was maintained during growth phase to avoid DO limitation and during the production it was adjusted to 450-500 rpm depending upon the DO profile.

In the second fed-batch named "R2", the concentration of yeast extract in fermentation medium during growth phase was increased to 1.5 x (13.8 g/l) along with additional 5 g/l in feeding solution in order to increase initial biomass and in turn, erythritol productivity. During this run, agitation speed was set at 600 rpm during the growth phase, to avoid DO limitation as a high yeast extract concentration was used.

Data analysis of initial two fed-batch experiments hypothesized that erythritol production is mainly dependent on DO concentration and not on the residual glucose or nitrogen content during the production phase. To confirm this hypothesis in fed-batch run R3, feeding solution devoid of yeast extract was used and feeding rate of glucose solution was manually adjusted so as to maintain the residual glucose concentration below 25 g/l. After the growth phase, agitation rate was reduced from 600 rpm to 400 rpm gradually, after first 24 h.

In fed-batch experiments R4, R5 and R6, in order to increase erythritol productivity by increasing initial biomass concentration, the content of yeast extract in the fermentation medium was increased two-fold (18.4 g/l). The feeding rate of glucose syrup was controlled so as to maintain the glucose concentration in culture broth below 50 g/l. The only difference in these three experiments was that in run "R4" the agitation rate for the duration of growth phase was 600 rpm, where as for run "R5" and run "R6" was 700 and 450 rpm, respectively. Fed-batch runs illustrating changes made in the protocol of respective fermentation runs is presented in Table 4.1

No	***Growth medium***	***Changes made in protocol***
R1	Growth medium with 50 g/l glucose, 9.2 g/l yeast extract with salts	Feeding solution 200 g/l glucose with 5 g/l yeast extract
R2	Growth medium with 50 g/l glucose, 13.8 g/l yeast extract with salts	Feeding solution 200 g/l glucose with 5 g/l yeast extract
R3	Growth medium with 50 g/l glucose, 13.8 g/l yeast extract with salts	Feeding solution without yeast extract and residual glucose was maintained below 25 g/l all the time
R4	Growth medium with 50 g/l glucose, 18.4 g/l yeast extract with salts	Feeding solution without yeast extract and residual glucose was maintained below 50 g/l all the time. Agitation rate during growth phase was 600 rpm
R5	Growth medium with 50 g/l glucose, 18.4 g/l yeast extract with salts	Feeding solution without yeast extract and residual glucose was maintained below 50 g/l all the time. Agitation rate during growth phase was 700 rpm
R6	Growth medium with 50 g/l glucose, 18.4 g/l yeast extract with salts	Feeding solution without yeast extract and residual glucose was maintained below 50 g/l all the time. Agitation rate during growth phase was 450 rpm

Table 4.1 Fed-batch runs illustrating changes made in the protocol during fermentation

4.2.6 Volumetric oxygen transfer coefficient (K_La)

In an aerobic fermentation process oxygen is an important nutrient used by the microorganism for growth and production of metabolites. Therefore it is very important to ensure that there is enough supply of oxygen in the culture broth. Oxygen transfer rate from gaseous to liquid phase is given by the equation *(Eq. 4.1)* (Garcia-Ochoa et al., 2010).

$$OTR = K_L a(C_G - C_L) = K_L a.\Delta C \quad \ldots\ldots Eq.4.1$$

Where $K_L a$ is the volumetric transfer rate and (C_G-C_L) is driving force of oxygen transfer and is the measure of DO in the fermenter when there is no oxygen utilization. Therefore, determination of $K_L a$ is very useful tool in evaluation of effectiveness of the fermenter to deliver enough oxygen. Oxygen uptake rate (OUR) can be independently determined by the following equation *(Eq. 4.2)*

$$\left(\frac{dC}{dt}\right) = -qO_2.C_X \quad Eq\ 4.2$$

Where qO_2 is specific oxygen uptake rate of the biomass and Cx is the biomass concentration and (dC/dt) is the rate of change of dissolved oxygen. At steady-state, the rate of oxygen from the bubbles is equal to the rate of oxygen consumption by the cells therefore, $K_L a.\Delta C = OUR = OTR \quad \ldots\ldots Eq.4.3$

If air supply for the fermenter is stopped the dissolved oxygen will drop at the rate depending upon the biomass concentration and from the slope of the dissolved oxygen rate $K_L a$ can be calculated.

OTR in a sterile medium without culture at 28 °C was measured at three different agitation rates mainly 300, 450 and 600 rpm. On autoclaving the fermentation medium, DO probe was calibrated at 600 rpm agitation speed. Initially, the medium was deoxygenated by purging nitrogen gas. The rate of oxygen transfer was calculated by following the rate of re-saturation of the medium by sparging air for each of agitation speed. $K_L a$ during the growth phase was calculated by dynamic method, when the cells were in early exponential phase. The airflow to the fermentation broth was interrupted and the decrease in DO concentration was recorded by dissolved oxygen probe fallowed by, reintroduction of air under the same operational conditions. Under these conditions, the *Eq. (4.1)* was simplified to obtain $K_L a$ from the slope of the plot of DO concentration versus time after stopping air flow.

4.2.7 Quantification and characterization of intracellular lipid from mutant R23

4.2.7.1 Extraction of lipids

It is a common observation that microorganisms store intracellular lipids when nitrogen is limited. In the present investigation it was observed that on the basis of glucose utilized the yield of erythritol was low. Therefore to check whether the

mutant R23 diverted some amount of glucose to synthesize intracellular lipid under nitrogen limitation, the cellular lipids were extracted and estimated from mutant R23. Lipids from one gram of freeze-dried cells of R23 mutant were extracted with 25 ml of chloroform: methanol (1:2) for 2 h. The biomass was separated by centrifugation and re-extracted twice with the same amount of chloroform: methanol. The contents of all the three extract was mixed together and 10 ml of 0.9% saline was added to it for phase separation followed by centrifugation. The upper water and methanol phase was discarded. The bottom phase was washed twice in a separating funnel with distilled water and stored overnight with anhydrous Na_2SO_4. On filtering out Na_2SO_4, chloroform was removed by evaporating at 50 °C under vacuum. The lipid extracted was weighed and expressed as gram of crude lipids per gram dry cell mass.

4.2.7.2 Preparation of fatty acid methyl esters (FAME)

The extracted crude lipid was dissolved in hexane and a known amount of sample was used for preparation of FAMEs. FAMEs were prepared by reaction of 400 µl (50 mg/ml lipid) with two ml of 3N methanolic HCl, one ml of hexane and 0.5 ml of 2-methoxy propane at 60°C overnight in an agitated reaction vial. FAMEs were extracted with 3 volumes of hexane, the solution was treated overnight with anhydrous Na_2SO_4, used Na_2SO_4 was removed by filtration FAMEs were analyzed using gas chromatograph.

4.2.7.3 FAME analysis by gas chromatography

The extracted FAMEs in hexane were analyzed by capillary gas chromatography (Chemitto-1000 gas chromatography) with flame ionization detector (FID) on a 30-meter PAG column (0.25 mm ID, 0.25 µm film thicknesses) (Supelco, USA) using nitrogen as carrier gas. Injection port, column and detector temperature were at 220 °C. One µl of the sample was injected in a split mode. The split ratio was 1:20 and flow rate of carrier gas was 0.8 ml/min. Quantification was done using peak area normalization and external standard methods. FAME 37, the standard mixture was used for identification and calibration in gas chromatography

4.2.8 Purification and characterization of erythritol from the fermentation broth

Fed-batch experiment as described earlier was performed till all the residual glucose was consumed. Five hundred ml of the fermentation broth was centrifuged at 10000 *g*

for 10 min and supernatant was treated with 1% activated charcoal at 90 °C for twenty min under gentle agitation. The activated carbon was removed using glass microfiber filters discs (Whatman). The clear solution obtained was evaporated in a rotary evaporator at 50 °C under vacuum to concentrate erythritol to around 400 g/l. The concentrated solution was then allowed to cool to 20 °C under gentle agitation and seeded with a trace amount of erythritol to initiate crystallization. The solution was then incubated at 4 °C, overnight. Brittle white erythritol crystals formed were collected by filtration and washed twice with cold distilled water and dried at 50° for 2 h, under vacuum. The quality of erythritol was analyzed using HPLC. The purified product was further confirmed by melting point analysis, LC-MS and NMR spectroscopy.

Melting point analysis was performed using BUCHI melting point apparatus B-540 (Flawil, Switzerland) between 110-130 °C with gradient increase of 5°C. LC-MS analysis was performed using (Waters Alliance 2695 separation module) under the following conditions: source temperature 150°C, desolvation temperature 350°C, collision energy 10 V, capillary voltage 3.51 kV, and polarity ES positive. RP C18 column with mobile phase 80% methanol-water at a flow rate of 0.5 ml/min was used. Standard erythritol obtained from Sigma was injected as a reference at a concentration of 1 mg/ml for comparison. ^{13}C-NMR spectrum was measured using a NMR spectrometer (Bruker AC-200, Germany). ^{13}C-NMR spectra were obtained at 50 MHz with an acquisition time of 3.7 s. All samples were made up in 100% D_2O and locked internally.

4.3 RESULTS AND DISCUSSION

4.3.1 Evaluation of M572 mutant for erythritol production in 10 L fermenter

On inoculating the fermenter with 5% v/v M572 inoculum, it was found that within 12 h growth phase, DO declined to 10% of air saturation. In order to avoid DO limitation, the agitation speed was increased from 500 to 600 rpm at 24 h, which was later reduced to 550 rpm till 48 h. On attaining the required biomass, the rpm was further reduced to 400 rpm at 72 h. It can be seen that 90% of the biomass growth was achieved in initial 24 h (19.6 g/l DCW) which later on increased to 23 g/l DCW towards the end of batch. Out of 250 g/l initial glucose, around 50 g/l glucose was consumed for the biomass generation at the end of 36h. Beyond 36 h of growth phase,

there was a sharp decrease in sugar uptake rate. Batch was terminated at 120 h and at the end of the batch, 176 g/l glucose was left over and only 2-3 g/l of erythritol was produced throughout the entire batch (Fig. 4.2). The lower erythritol production in the fermenter could be attributed to unfavorable culture conditions. It was observed that there was DO limitation in early growth phase and at 24 h, DO concentration observed was only 5% of air saturation.

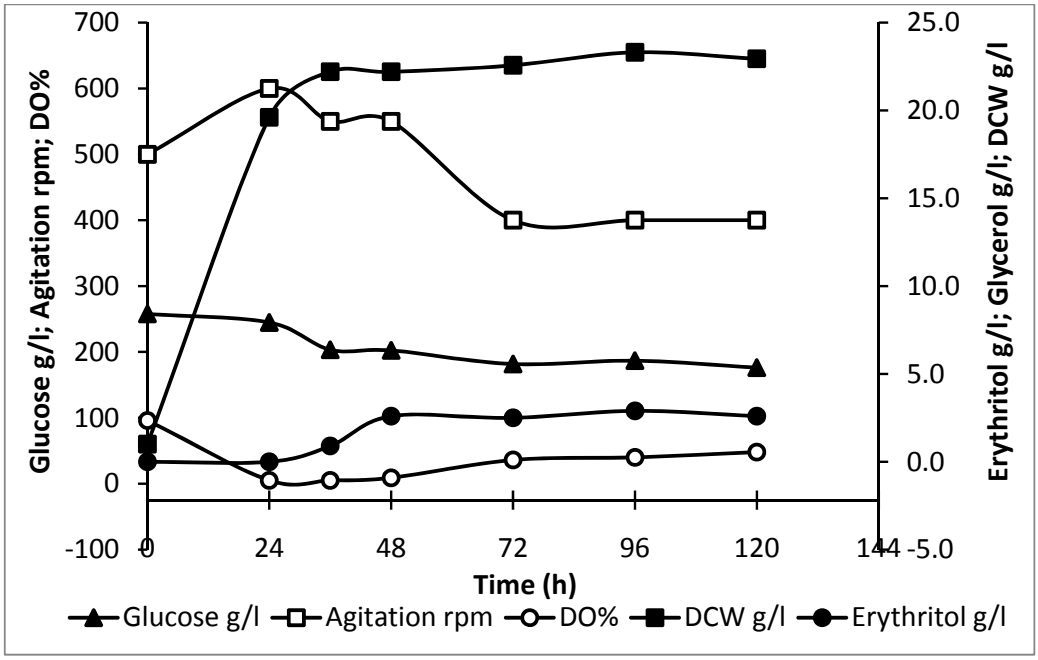


Figure 4.2 Fermentation profile of *C. magnoliae* mutant M574 in LFM medium

In the second batch, in order to maintain the DO concentration above 20% of air saturation, mixture of oxygen and air at the rate of 2 and 3 LPM respectively, was sparged into the fermenter. Surprisingly, there was no erythritol production in this batch although the cells grew well and the biomass concentration reached 24 g/l DCW within 24 h of growth phase.

In the third batch, which was carried out with constant agitation and aeration at 400 rpm and 0.5 vvm, respectively, 44 g/l of erythritol was produced at the end of 168 h. It can be observed from the Fig. 4.3 that DO concentration was observed to be below the detectable limit of the DO probe (0-1%), all throughout the entire batch. On continuing the batch till 240 h, final erythritol concentration 62 g/l was achieved with conversion yield of 30%. A small amount of glycerol was produced in the initial stage

but was consumed toward the end of the batch, whereas 4 g/l mannitol was formed as a by-product at the end of the batch. The overall erythritol productivity was found to be rather less, 0.25 g $l^{-1}h^{-1}$.

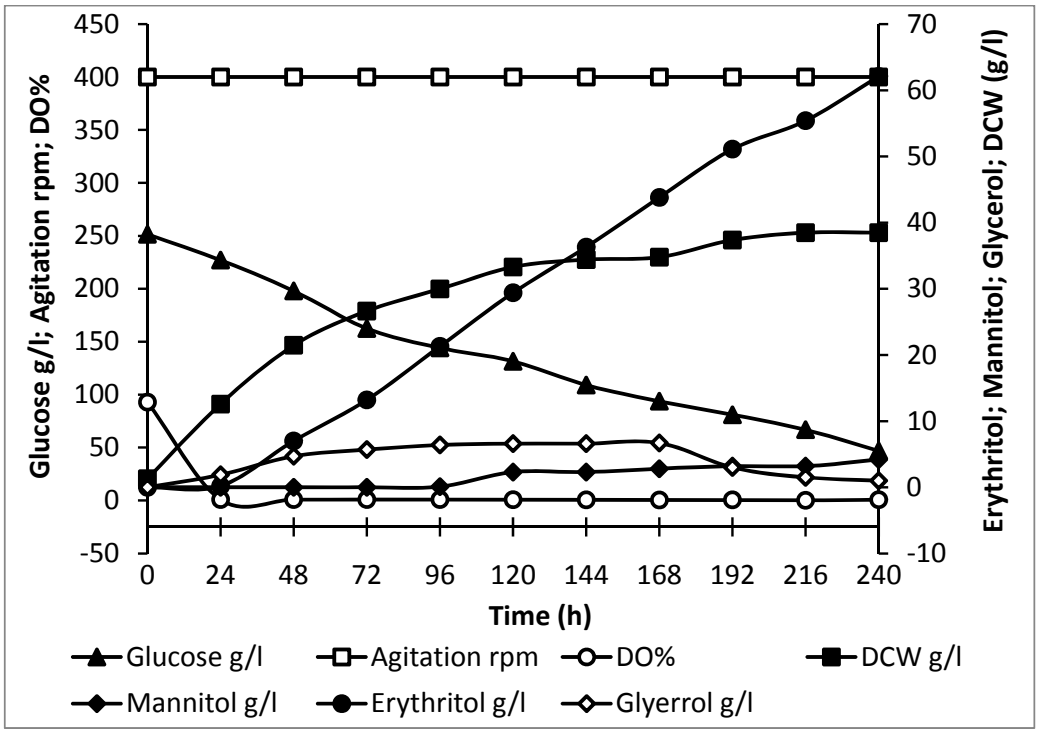


Figure 4.3 Fermentation profile of *C. magnoliae* mutant M574 in LFM medium at fixed agitation speed of 400 rpm

4.3.2 Effect of dissolved oxygen on erythritol production by R23 in 10 L batch fermenter

On careful evaluation of shake flask and fermentation data for mutant M574, it was concluded that DO in the medium is the key parameter for erythritol production and needs to be optimized critically for enhanced erythritol production. Optimization of DO concentration was carried out on mutant R23. The agitation speed caused change in the dissolved oxygen concentrations, which in turn, affected cell growth as well as erythritol and byproduct (ethanol and mannitol) formation. Respective DO of fermentation batches during growth and production phases at different agitation speed is illustrated in Fig. 4.4. DO during the production phase was 0% at 300 rpm, 0-2% at 450 rpm and 70-80% at 600 rpm. In the batch at 300 rpm, all the glucose was consumed whereas 29.4 g/l erythritol was produced with 12.3 % yield along with 21.6 g/l mannitol and 38 g/l ethanol as by-products. In a batch with 600 rpm, 21.6 g/l

erythritol was produced, but the glucose consumption diminished beyond 96 h and therefore 137 g/l glucose was leftover unutilized, even after 168 h. Highest 56 g/l of erythritol, with 31.6% yield of was observed in fermentation batch at 450 rpm without any by-products. Residual glucose concentration and erythritol production for all the three batches is illustrated in Fig. 4.5.

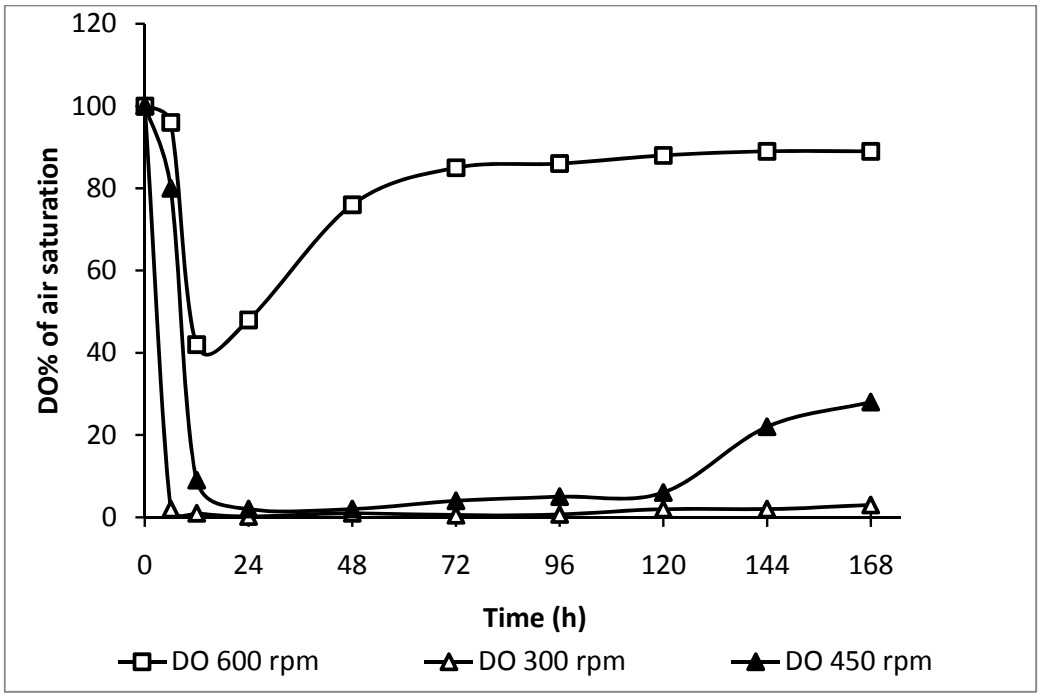


Figure 4.4 Effect of agitation on DO concentration in R23 mutant in batch fermentations

Although the biomass concentration (27 g/l DCW) was less in batch at 300 rpm, at 450 and 600 rpm batches it was similar 36 and 33 g/l DCW, respectively. Under oxygen limiting conditions (at agitation speed at 300 rpm), excess ethanol and mannitol were produced instead of erythritol. On the other hand in the case, cells were cultivated under aerobic conditions (600 rpm) there was hardly any ethanol or mannitol produced. Under slightly aerobic conditions (450 rpm), ethanol was not formed whereas mannitol production remained relatively low compared to that under anaerobic conditions. At this rpm the cell growth was not suppressed and resulted in relatively higher biomass, 36 g/l DCW. Thus, the limited oxygen supply could secure cell growth and higher glucose conversion into erythritol, instead of channeling it to CO_2 formation. Fermentation batches were also performed at controlled DO concentration between 5-10% and 15-25% of air saturation by cascading rpm to

maintain desired DO concentration, but the erythritol concentration was not more than 35 g/l in any of the case. This implicated that DO has to be maintained very critically over a specific range of 0-2 % of air saturation.

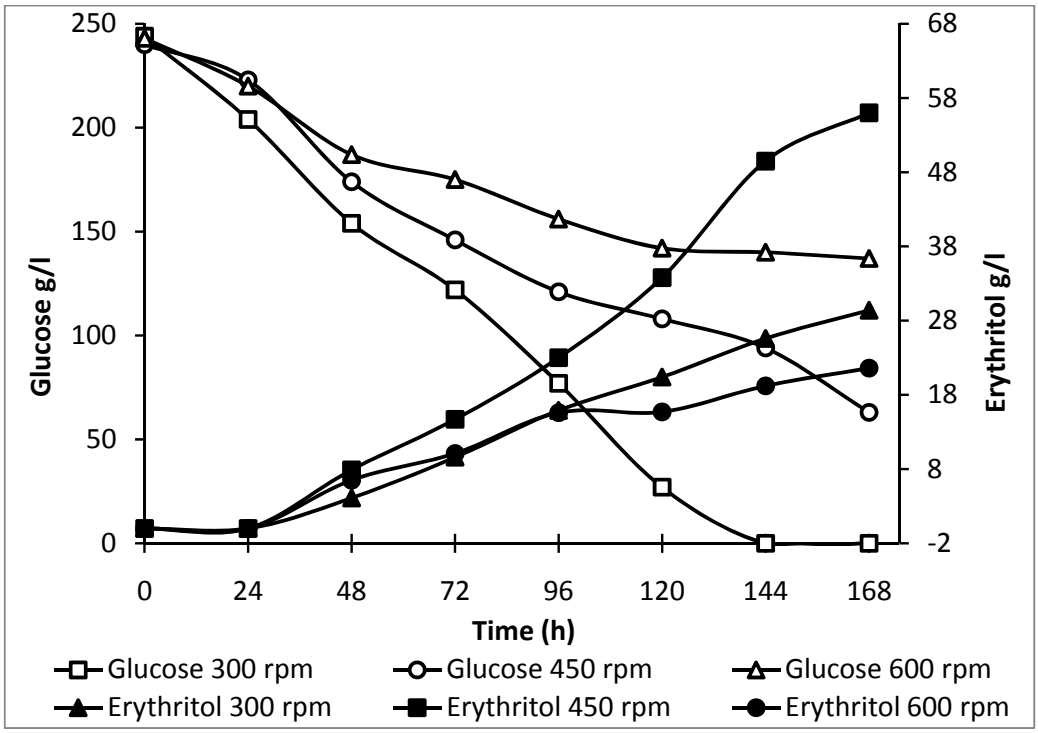


Figure 4.5 Effect of agitation on glucose uptake and erythritol production by mutant R23

It is well documented that DO is the key parameter for erythritol production. Jeya et al., (2009) studied effect of DO on growth and erythritol production in a 7 L fermenter by limiting the DO to 0–10%, 10–20%, 20–30%, and 30–40%, by adjusting the agitation speed after the growth phase. Maximum erythritol production was observed when DO was maintained between 20% and 30%. Using DO as a scale-up parameter, erythritol production was scaled up from laboratory scale to 300 L pilot and 50,000 L plant scales.

Similarly, Kim et al., (2000) suggested that for maximal erythritol production by *Torula sp*, dissolved oxygen should be controlled in the range of 5-10% of air saturation (Burschäpers et al., 2002a) investigated that, excess of ethanol was produced by an erythritol producing *Moniliella tomentosa (pollinis)* if the dissolved oxygen concentration decreased below 19% air saturation.

In *C. magnoliae* high glucose concentration during production phase produced citric and butyric acid, whereas with the increase in dissolved oxygen level there was formation of gluconic acid instead of citric acid (Ryu et al., 2000). The oxygen concentrations are normally expressed in terms of % air saturation. Because of the differences in solubility of oxygen in medium with different sugar concentrations it is difficult to compare these results.

4.3.3 Evaluation of minimal medium for growth and erythritol production

Although yeast extract is a favorite nitrogen source in fermentation and has the advantage of high production rate of erythritol, it is an expensive nitrogen source and probably enhances production of undesirable products. An attempt was made to eliminate or to minimize yeast extract from the fermentation medium by supplementation with cheap inorganic nitrogen sources. Previously, a chemically defined medium for mutant M572 was designed and the medium was evaluated in shake flask for erythritol production. Use of such minimal medium resulted in 14 g/l DCW with $(NH4)_2SO_4$, and 19 g/l with $NaNO_3$ as sole nitrogen sources respectively, but erythritol production was much less as compared to LFM with yeast extract. In minimal medium containing sodium nitrate, 2.4 g/l erythritol was produced while, in ammonium sulphate based medium, 16.7 g/l of ethanol was produced without production of erythritol.

The mutant R23 was evaluated for growth and erythritol production with $NaNO_3$ and $(NH_4)_2SO_4$ as sole nitrogen sources in 10 L laboratory fermenter. In the fermentation batch employing minimal medium containing $NaNO_3$, the mutant grew very slowly and it took 168 h to reach 17 g/l biomass, whereas in the batch with $(NH_4)_2SO_4$ as nitrogen source, the mutant R23 grew exponentially under aerobic condition and stationery phase was attended around 72 h (24 g/l DCW). There was no erythritol production when $NaNO_3$ was used as nitrogen source. This can be ascribed to higher DO concentration and lower biomass concentration observed throughout the entire batch. Moreover, use of nitrate as nitrogen source necessitates reduction of nitrate to ammonium, which requires two moles of NADPH per mole of nitrate consumed. Biosynthetic pathway for erythritol synthesis suggests that reduction of erythrose to erythritol requires NADPH as cofactor. Thus, consumption of NADPH for assimilation of nitrate may be the possible reason for lack of erythritol in $NaNO_3$

containing medium. As compared to $NaNO_3$ containing medium, higher biomass was obtained with use of $(NH_4)_2SO_4$ as nitrogen (28 g/l DCW) but it was less as compared to yeast extract containing medium Table 4.2. Although 142 g/l glucose was consumed in $(NH_4)_2SO_4$ containing medium, only 4 g/l erythritol was produced.

Time h	***DCW g/l***	***Glucose g/l***	***DO %***	***Erythritol g/l***	***DCW g/l***	***Glucose g/l***	***DO %***	***Erythritol g/l***
		$NaNO_3$				***$(NH_4)_2SO_4$***		
0	1	258	98	0	1	257	98	0
24	1	248	86	0	7	252	9	0
48	4	234	50	0	18	202	3	0
72	7	238	46	0	24	196	1.5	2
96	10	231	39	0	27	165	1	4.7
120	11	214	35	0	27	143	20	4
144	16	194	40	0	29	132	41	3.8
168	17	181	43	0	28	115	47	3.8

Table 4.2 Fermentation profile of mutant R23 in minimal medium containing $NaNO_3$ and $(NH_4)_2SO_4$

Recently, Edlauer & Trimmel, (2009) patented a process for erythritol production by *Moniliella sp.* which uses at least one inorganic nitrogen source, potassium nitrate or sodium nitrate, in amount of 45 to 65% as a pH regulator in the culture medium without formation of undesirable by-products. Results in the present investigation suggest that the mutant R23 can grow satisfactorily in medium with $(NH_4)_2SO_4$ as sole nitrogen source and can be used in supplementation with yeast extract to minimize the medium cost. Production of erythritol in minimal medium with $(NH_4)_2SO_4$ needs further experimentation for increasing biomass concentration with control over DO concentration.

4.3.4 Optimization of fed-batch fermentation

A high initial glucose concentration favors erythritol production by osmophilic microorganisms. Generally, an increase in the initial glucose concentration increases the production rate and yield in a batch process if the microorganism can tolerate a higher concentration of sugar and a higher osmotic pressure. In previous chapter using

RSM, it was observed that increase in initial glucose concentration above 250 g/l favors undesirable by-products formation, mainly glycerol, by R23 mutant.

To increase the volumetric productivity of erythritol and conversion ratio, six fed-batch fermentation batches were performed. In fed-batch fermentation, initial 50 g/l glucose was used during the growth phase to increase the growth rate of cells by lowering the osmotic pressure and then the osmotic pressure was increased by adding 200 g/l glucose during production phase to increase the erythritol production rate. Five g/l yeast extract was added to the glucose feed solution to avoid nitrogen source limitation so as to maintain the cells metabolically active during production phase.

Fermentation profile of fed-batch run R1 is shown in Fig. 4.6a. It illustrates that DO dropped to 6% in 12 h of growth phase. To compensate DO limitation, the agitation speed was increased to 600 rpm till 24 h and later decreased to 500 rpm on reaching stationary phase. Erythritol production started at the end of growth phase and reached 26 g/l at 72 h. Maximum erythritol productivity of 0.8 g $l^{-1}h^{-1}$ was observed at 54 h which suddenly dropped down to 0.1 g $l^{-1}h^{-1}$ at 96 h. Final erythritol concentration at the end of 168 h was only 34 g/l.

In the fed-batch run R2, since 1.5 fold initial yeast extract concentration was used to increase biomass concentration and the agitation rate was adjusted to 600 rpm till 96 h to avoid DO limitation. Although the DO concentration was maintained in the range of 0-2% of air saturation only 17 g/l of erythritol was produced Fig. 4.6b. After 72 h, DO started increasing and therefore the agitation rate was decreased to 500 rpm at 96 h. As the DO concentration increased, there was a decrease in sugar uptake rate and erythritol productivity (0.1 $gl^{-1}h^{-}1$), as a consequence of this, residual glucose concentration increased to 145 g/l, so the experiment was terminated at 144 h. From run R1 and R2 it was hypothesized that erythritol production by R23 mutant is not dependent on the osmotic pressure or the nitrogen content during the production phase. In run R3, yeast extract was eliminated from the feed solution to check whether there is requirement of nitrogen source for erythritol production during production phase. All the conditions were same as run R2, except that the feeding solution used was without yeast extract. After the growth phase, agitation rate was gradually decreased from 600 to 400 from 24-54 h Fig. 4.6c.

In fed-batch run R4, higher yeast extract concentration during the growth phase resulted in higher biomass concentration of 41 g/l DCW within 36 h of growth phase. During this period, higher agitation rate of 600 rpm was maintained to circumvent DO limitation. In run R4, highest erythritol production of 87.8 g/l was obtained from 250 g/l of glucose with a yield of 35%. These values represent 56.7% increase in erythritol and 11.1% in yield as compared to simple batch experiment. Towards the end of the run, biomass concentration dropped to 38.4 g/l presumably due to the dilution effect. Highest erythritol productivity of 0.8 g $l^{-1}h^{-1}$ was achieved between 36-54 h which decreased to 0.4 with the decrease in agitation speed from 600 to 400 rpm. This was thought to be because of DO decrease below critical value which could not be sensed by conventional oxygen probe, but increase in rpm to 550 rpm by stepwise increase of 50 rpm at a time resumed the productivity of 0.8 g $l^{-1}h^{-1}$ (Fig. 4.6d). This indicated that, a low oxygen level practically below DO probe sensitivity is required to maintain prolonged erythritol productivity.

In case of run R5, initial high agitation rate of 700 rpm was maintained to increase growth rate of mutant R23. After the growth phase, agitation rate was gradually decreased to 400 rpm. Unexpectedly, the erythritol production was very less (12 g/l). Highest productivity of only 0.3 g $l^{-1}h^{-1}$ was achieved between 48 to 60 h of production phase. Similar results were obtained in run R6 when initial agitation rate of 450 rpm which was later adjusted between 450-550 rpm depending upon the DO profile. The final erythritol concentration during this run was only 16 g/l.

In conclusion, either low or high DO during the growth phase adversely affected the erythritol production. For effective erythritol production, apart from maintaining DO at critical concentration, it is also important that how the culture is brought to the production phase. If high aerobic conditions are maintained beyond the growth phase and even though lower optimal DO concentration is maintained during the production phase the erythritol production is hampered by two to three fold. On the contrary, if low DO concentration is maintained during the growth phase it also results in lower erythritol production.

A representative screen view of online biomass OD monitored Wedgewood Analytical Inc probe by measuring the turbidity at 1100 nm and online fermentation

data of process parameters such as DO, pH, agitation and feeding strategy acquired by NBS BioCommand Plus Software of fed-batch run R4 is presented in Fig. 4.6e

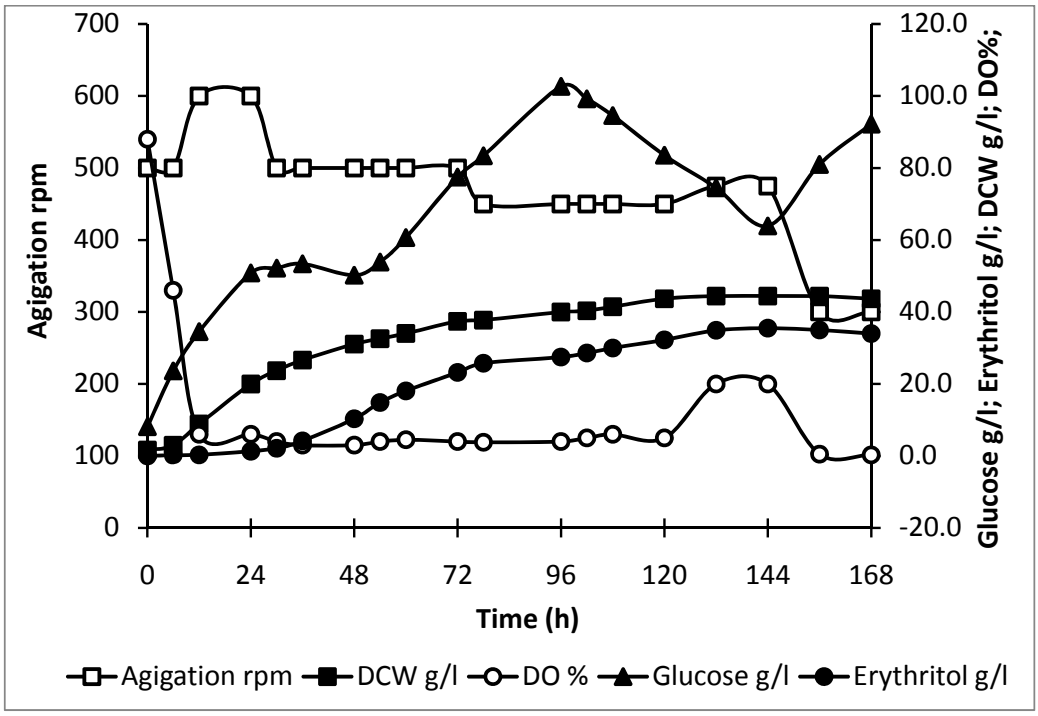


Figure 4.6a Fed-batch fermentation run R1 with mutant R23

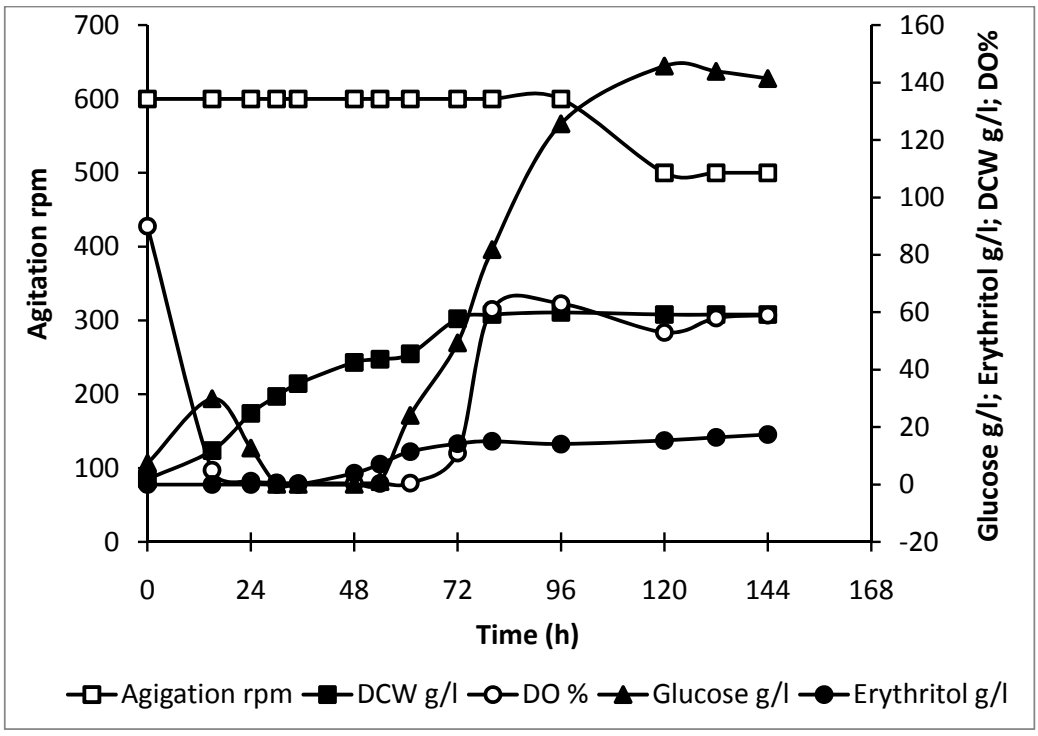


Figure 4.6b Fed-batch fermentation run R2 with mutant R23

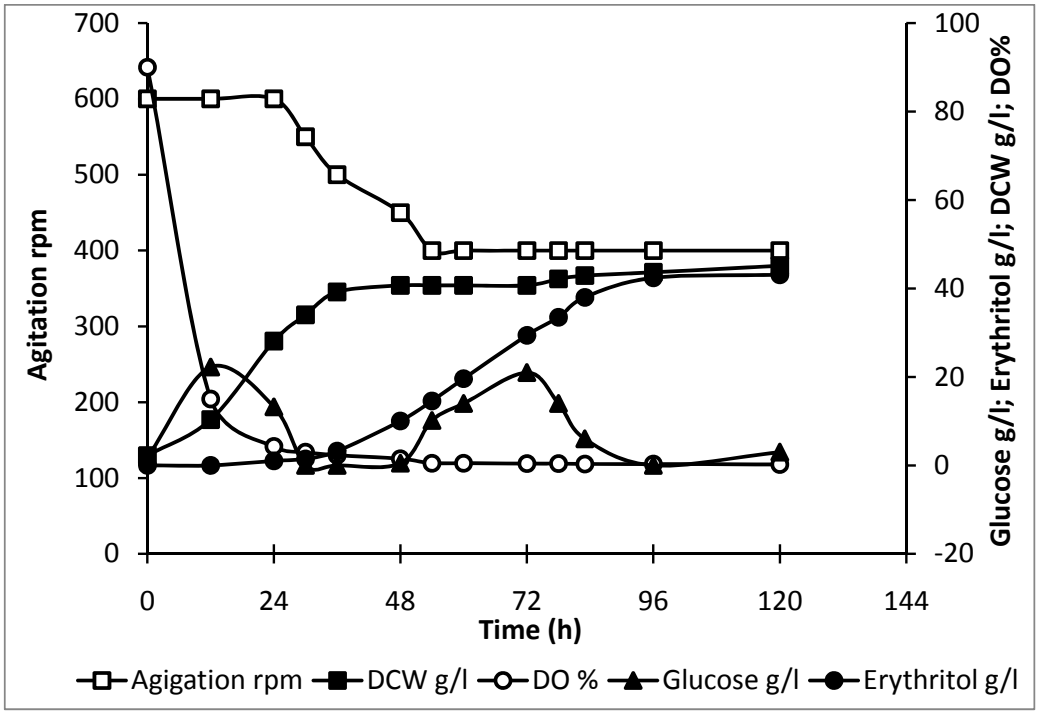


Figure 4.6c Fed-batch fermentation run R3 with mutant R23

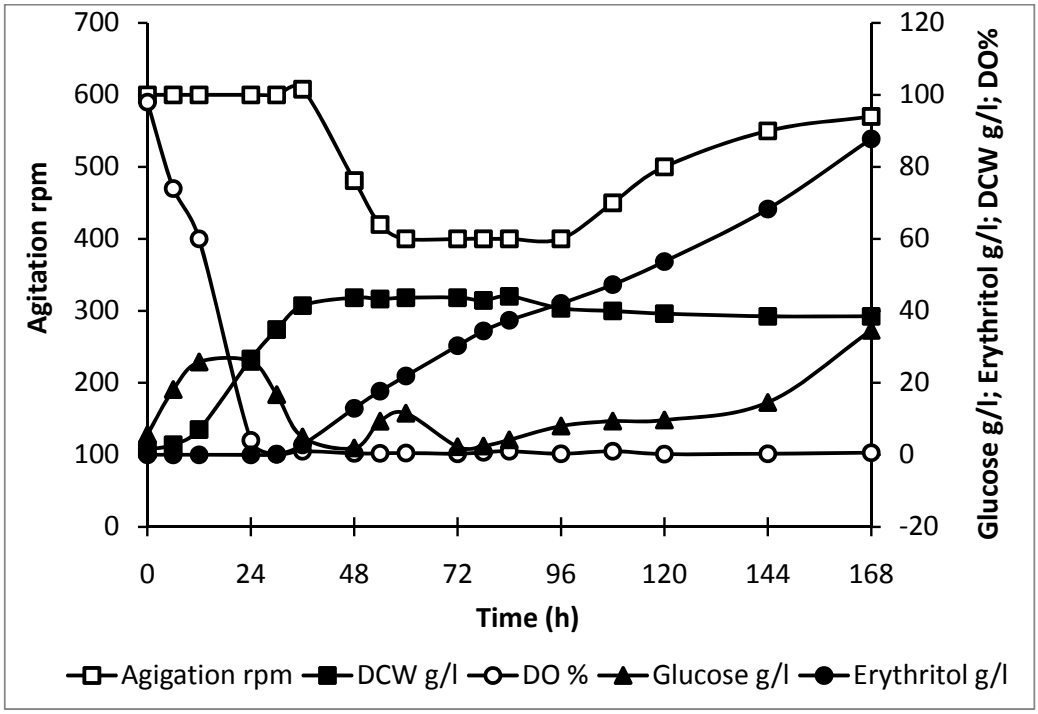
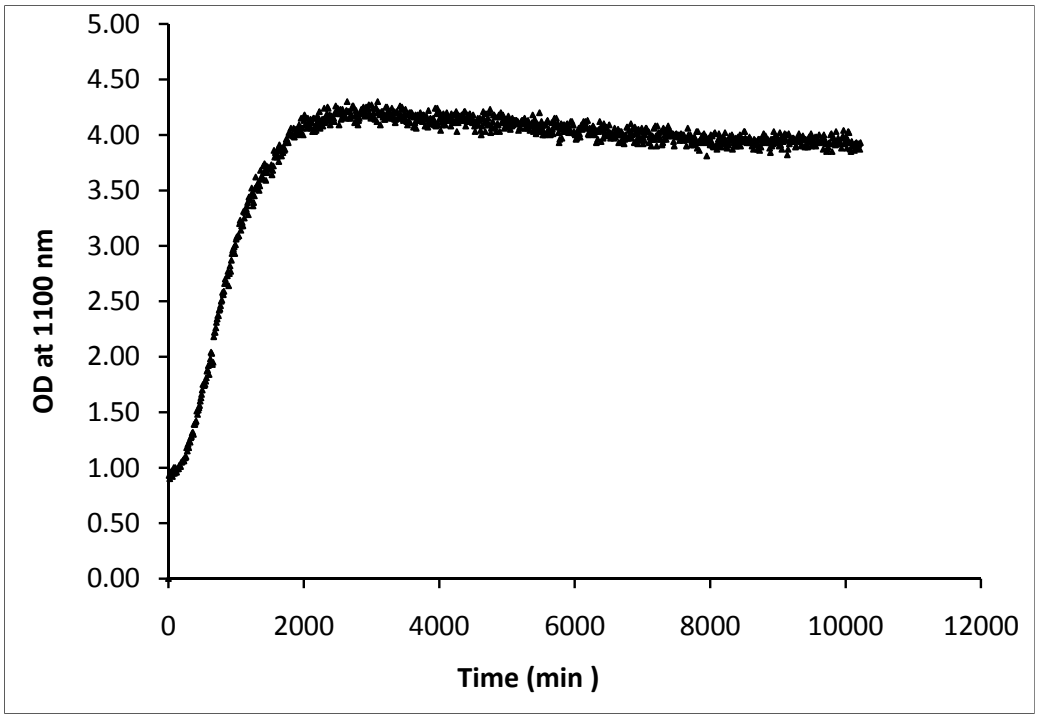


Figure 4.6d Fed-batch fermentation run R4 with mutant R23



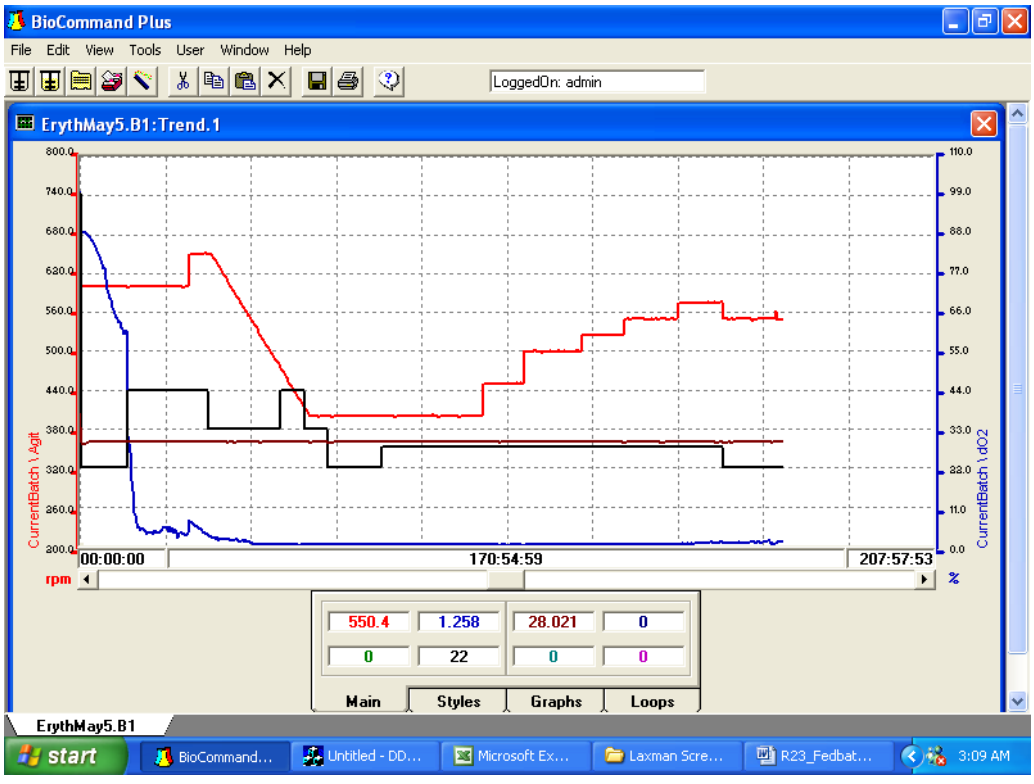


Figure 4.6e A screen view of online biomass OD and fermentation data of process parameters acquired by NBS BioCommand Plus Software of fed-batch run R4

The observations of other investigators also show similar findings. Kim et al., (1997) observed that specific erythritol production rate of *Trigonopsis variabilis* was increased from 0.09 to 0.19 g $g^{-1}d^{-1}$ by increasing the osmotic pressure from 1.3 to 3.9 kPa in glucose containing medium. But due to increase in osmotic pressure specific growth rate decreased. In order to maintain the productivity without affecting the growth rate, they optimized two-stage fermentation strategy which increased erythritol concentration by two-fold. During the growth phase, low osmotic pressure of 1.6 kPa was adjusted in order to increase the growth rate by using low initial glucose concentration of 100 g/l and then the osmotic pressure was increased to a higher level of 3.7 kPa by adding 200 g/l glucose during production phase.

Similarly, erythritol production was improved by controlling glucose concentration in a fed-batch culture of *Torula* sp. The volumetric productivity was maximal at a controlled glucose concentration of 225 g/l, because of reduction in the lag time of the erythritol production. A fed-batch culture with an initial glucose concentration of 300 g/l and later controlled around 225 g/l a final 192 g/l erythritol of was obtained from 400 g/l glucose in 88 h corresponding to 2.26 g $l^{-1}h^{-1}$ volumetric productivity and a 48% yield (Oh et al., 2001).

A biological process for erythritol production by a mutant of *C. magnoliae* was developed by optimizing the conditions of fed-batch fermentation (Koh et al., 2003; Ryu et al., 2000). Erythritol productivity of 1.2 $gl^{-1}h^{-1}$, erythritol concentration of 200 g/l and a yield of 43% were obtained by maintaining the specific glucose consumption rate in the fed-batch phase. Their study also suggested that supplementation of yeast extract as a nitrogen source was important for sustaining the constant specific glucose consumption rate during the erythritol production period, which was also demonstrated in pilot-scale (50 L) fermentation.

In the present investigation an attempt to optimize two-stage fed-batch fermentation by separating growth phase from production phase was made by maintaining critical DO concentration during production phase. Fed-batch experiments using R23 mutant suggested that DO concentration during the production phase is a critical parameter for erythritol production. Compositions of polyols produced are mainly dependent on DO concentration that needs to be critically maintained at 0-2% of air saturation during production phase for enhanced erythritol production. Using statistically-

optimized medium in fed-batch fermentation, erythritol production could be increased by 46 % with a 35% increase in the yield compared to batch fermentation.

4.3.5 Volumetric oxygen transfer coefficient

In aerobic process, oxygen has to be continuously supplied in order to achieve acceptable productivities. The OTR in a bioreactor depends on the liquid side mass transfer coefficient, K_L, the total specific surface area available for mass transfer a, and the driving force in terms of concentrations. Since the two parameters, K_L and a, cannot be measured individually, they are usually lumped together as one single parameter KLa. Dynamic method is most commonly used method for determination of K_La, due to its simplicity and reproducibility. Dynamic method is based on the respiratory activity of organisms actively growing inside the bioreactor. A profile of dissolved oxygen concentration from Eq. (4.1) during a cycle of turning aeration off and on is shown in Fig.4.7 When the aeration was turned on again, the DO concentration increased until it reached a steady-state concentration. In this condition both the oxygen transfer and oxygen uptake rate terms apply. The slope of the response curve at a given point was measured to get dC/dt, and *Eq. (4.1)* was solved for K_La which was found to be 0.008 sec^{-1} and OTR at 300, 450 and 600 rpm agitation rate found to be 0.009, 0.02 and 0.023 mol l^{-1} $sec^{-}1$, respectively.

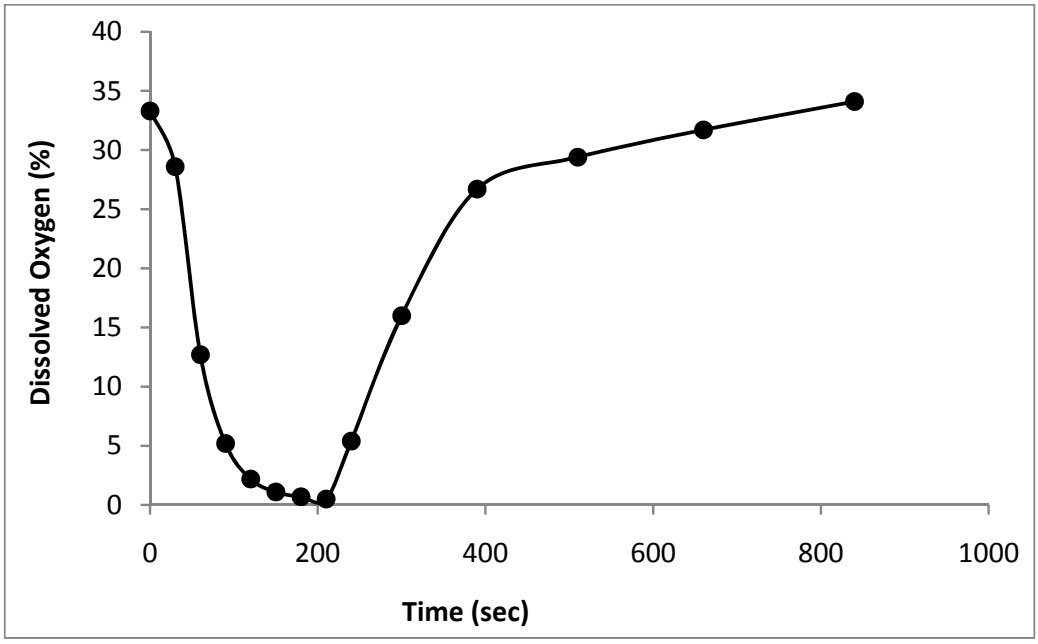


Figure 4.7 A profile of dissolved oxygen concentration during estimation of K_La

4.3.6 Estimation of intracellular lipid from mutant R23

The lipid extracted in chloroform was measured gravimetrically after evaporating chloroform. In both experimental sets, 160-165 mg lipid/g DCW was obtained which accounts for 16% of the total biomass. These results suggest that the total lipid concentration from the mutant R23 was below the average lipid concentration present in yeast thus indicating that glucose was not substantially diverted to lipid formation in nitrogen limitation condition by mutant R23. FAME analysis by GC-FID of parent and mutant strain is shown in Fig. 4.8. Out of the total lipid extracted, oleic acid accounted for 77% followed by 7% each of palmitoleic and stearic acid.

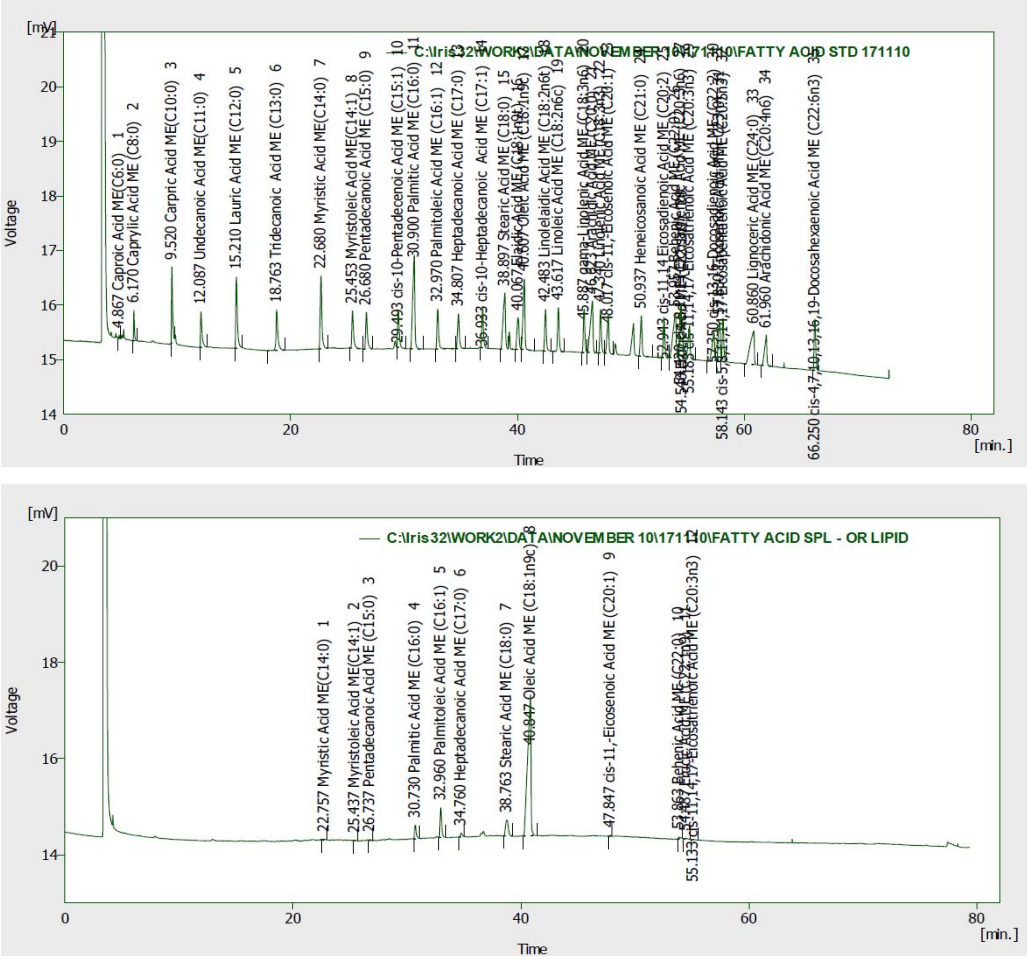


Figure 4.8 FAME analysis of standard mixture and R23 mutant

4.3.7 Purification and characterization of erythritol from the fermentation broth

Majority of techniques on downstream processing for purification of erythritol include separation of cells from fermentation broth by centrifugation, removal of coloured

impurities by adsorption on activated carbon followed by use of ion-exchange resins, concentration, crystallization, re-crystallization and drying. In the present investigation, on treating the fermentation broth twice with activated carbon, a transparent, colourless aqueous solution was obtained which was directly concentration and crystallized. White crystals of erythritol were obtained after cooling the mother liquor to 4°C overnight. The crystals were filtered using Whatman filter paper and washed twice with cold distilled water. The recovered crystals were 99% pure as analyzed by HPLC. HPLC chromatograms of the standard mixture of sugars along with polyols and purified erythritol are presented in Fig. 4.9. Melting point of the purified erythritol was found to be in the range of 118.9-119.8 which is comparable to the melting point 119 °C reported in literature (Kakiuchi et al., 1998).

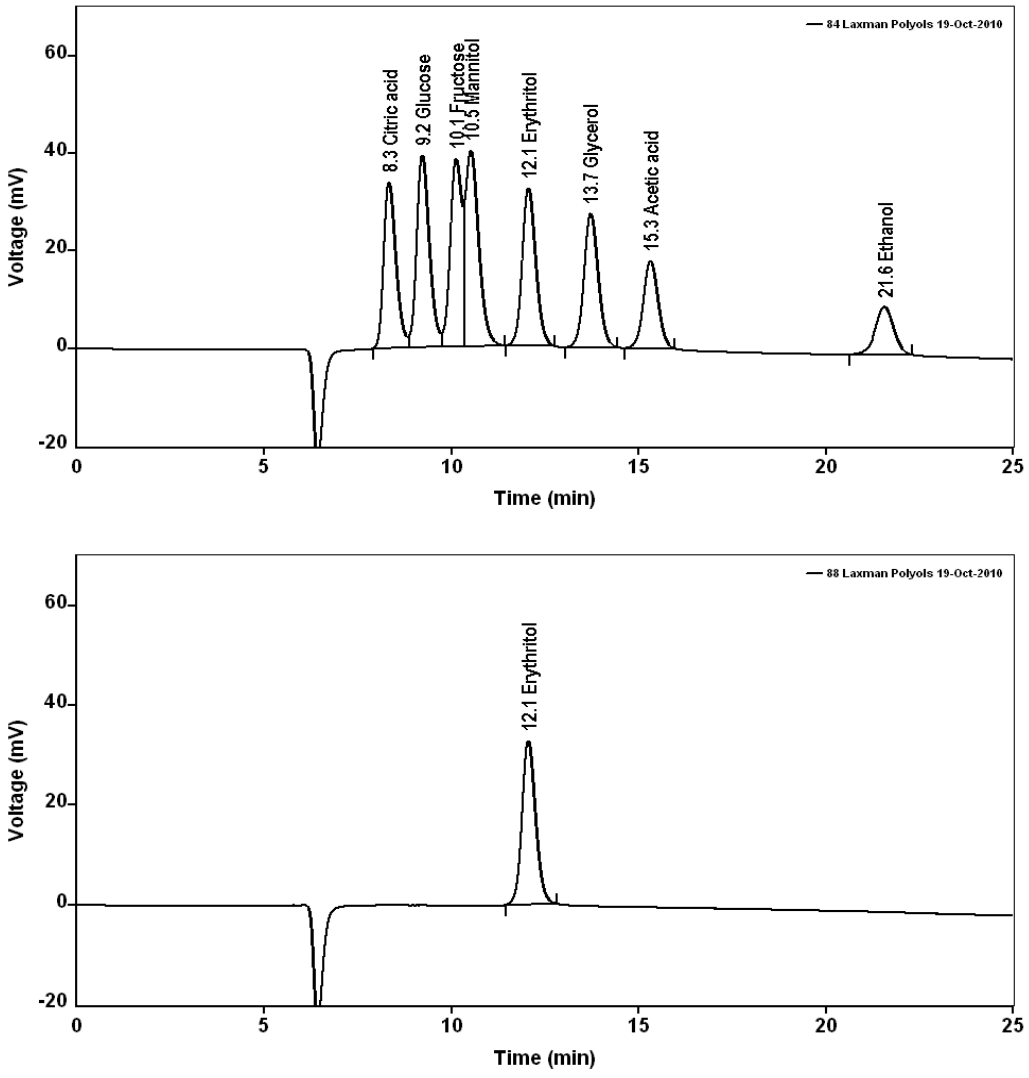
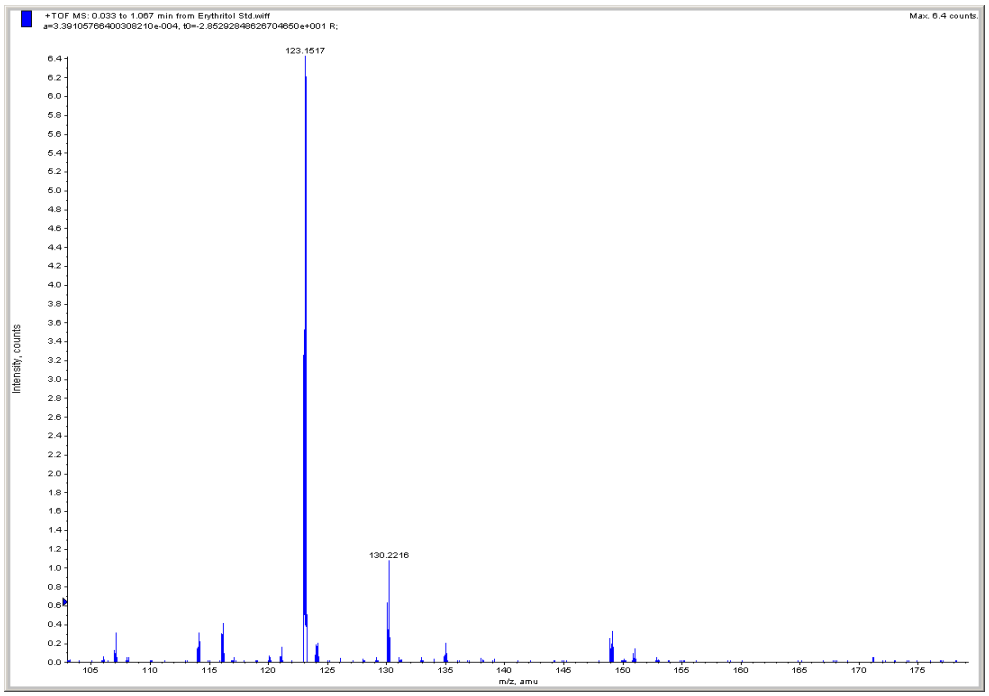


Figure 4.9 HPLC chromatograms of standard mixture sugars with polyols and purified erythritol

Purified erythritol crystals were characterized by LC-MS by comparison with mass spectra of authentic erythritol standard from Sigma. Mass spectra of authentic erythritol from sigma and purified erythritol are show in Fig. 4.10. Along with protonated peak with (*m/z*) 123.15, purified erythritol sample showed an additional adducted sodium ion spiked peak. Sodium added ions were detected as base peaks, and the molecular masses of the erythritol were determined by comparing $[M + Na]^+$ ions. Analysis in ES positive mode gave (m/z) 145.12 *ie.* [M + 23].

Number of carbon atoms from the purified erythritol was analyzed using ^{13}C-NMR. The ^{13}C-NMR spectrum Fig. 4.11 illustrated that the resultant molecule contains two sets of carbon atom represented as ^{13}C-NMR ($CDCl_3$, 50MHz): δ 62.6 (C1 and C4), δ 71.9 (C2 and C3).



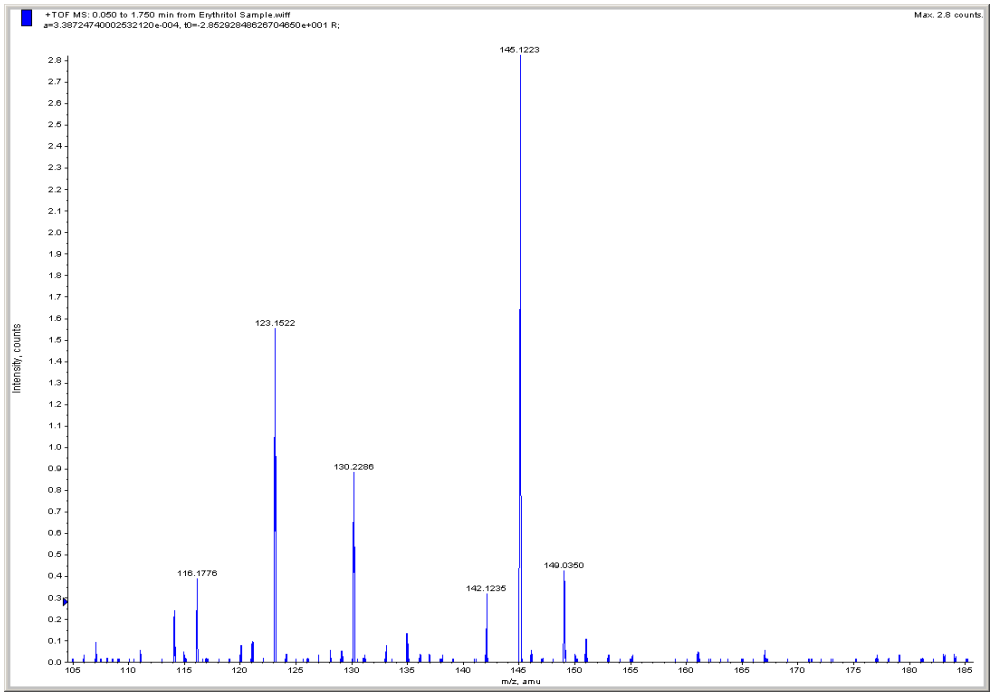


Figure 4.10 Mass spectra of authentic erythritol and purified erythritol from the fermentation broth.

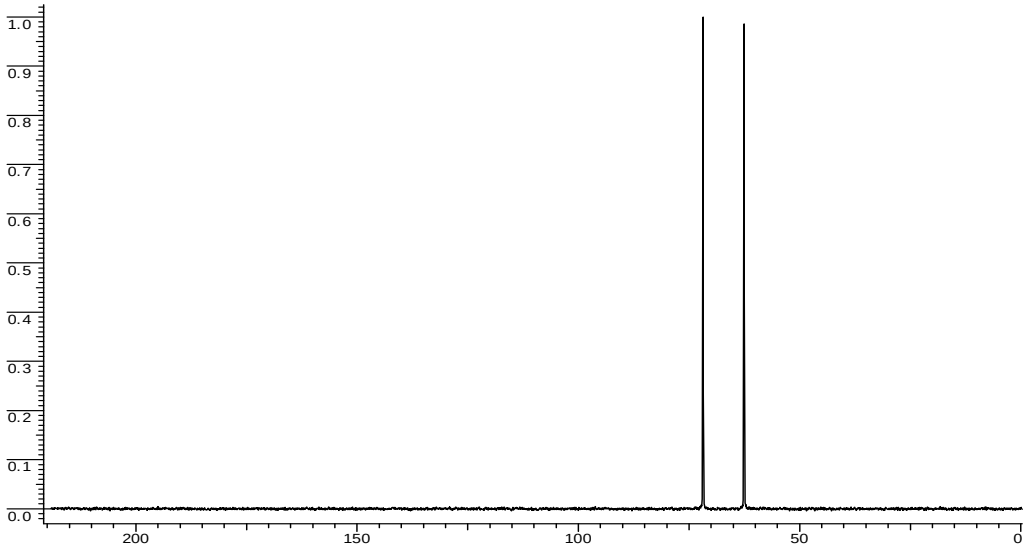


Figure 4.11 ^{13}C-NMR spectra of the purified erythritol from fermentation broth

4.4 CONCLUSIONS

Maximum erythritol production obtained in shake flask by mutant R23 using statistically-optimized medium was 60.3 g/l. This mutant was evaluated in a 10 L fermentation batch and fed-batch mode using statistically-optimized medium. Among

the culture conditions examined in batch process, DO was the key factor affecting erythritol production. Optimization of DO concentration in 10 L batch fermenter using polarographic DO probe was attempted. It was found that a low oxygen level (below DO probe sensitivity) is apparently required for effective erythritol production.

Controlling DO concentration at 0-2% of air saturation, in fed-batch mode, the optimized media resulted in 87.8 g/l erythritol and 31.1% yield, without formation of any by-products. Thus the present study involving strain improvement followed by media and process optimization resulted in 6.2 fold increase in erythritol production and 3.4 fold increase in the yield over the parent strain. Moreover, by using a simple purification protocol comprising activated carbon treatment, concentration and crystallization; erythritol crystals of 99% purity were obtained. The purified crystals of erythritol were characterized by LC-MS and ^{13}C NMR. In the present investigation a potential mutant R23, of *C. magnoliae* was isolated and key process parameters for erythritol production were optimized in laboratory fermenter.

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Chapter 5

Optimization of Fermentation Parameters for Mannitol Production from Glucose by *Candida magnoliae* Mutant R9

Abstract:

This chapter summarize mannitol production from glucose by mutant R9, using various bioprocess alternatives. RSM method of medium optimization was optimized for mutant the R9. In statistically-optimized medium, it produced 60 g/l mannitol with 20% yield which could not be improved further with optimization of fermentation parameters. The mutant R9, could produce 114 g/l mannitol from glucose-fructose mixture obtain from enzymatic isomerization of glucose. Acid-hydrolyzed sucrose was used in membrane cell recycle bioreactor (MCRB) for mannitol production. The biomass of mutant R9 could be used successively for three bioconversion reactions in MCRB, but mannitol concentration and yield were low.

To ascertain the reason behind overproduction of mannitol by mutant R9, activity of the mannitol dehydrogenase (MDH) the enzyme involved in mannitol biosynthesis, was compared in both parent and mutant strain. Under identical growth conditions, at 96 h, MDH activity of mutant R9 was about 2.4 fold higher than that of the parent strain. MDH from mutant R9 showed specificity only for fructose and NADPH with a Km value of 27.7 mM for fructose, which is comparable to 28.0 mM of Candida magnoliae HH1 which is used industrially for mannitol production from fructose.

5.1 INTRODUCTION

D-Mannitol is frequently found in pumpkins, celery, onions, grasses, olives, mistletoe, algae and lichens (Saha & Racine, 2011; Song & Vieille, 2009). It is also present in high levels in brown seaweeds (Ikawa et al., 1972). Although biotechnological mannitol production has been investigated in the past, major improvements in the yield and productivity have been achieved recently.

Heterofermentative LABs have been extensively studied for mannitol production from fructose (Wisselink et al., 2002). *Lactobacillus* sp, viz *L. brevis, L. buchneri, L. fermentum, L. intermedius, L. sanfranciscensis, L. mesenteroides, L. pseudomesenteroides* and *L. oeni* have been reported for mannitol production using batch, fed-batch and MCBR methods (Fontes et al., 2009; Saha, 2006; Saha & Racine, 2011; von Weymarn et al., 2002). Research on mannitol production is mainly focused on isolation of LABs, their improvement by mutagenesis for high mannitol yield with low unwanted metabolites and optimization of fermentation strategies such as batch, fed-batch and continuous mode with or without cell recycle mode (Helanto et al., 2005; Kim et al., 2002; Lee et al., 2003b; Racine & Saha, 2007).

Mannitol production using LABs results in formation of side products mainly lactic and acetic acid. Moreover, LABs are fastidious organisms and require rich complex medium containing amino acids, peptides and vitamins that are normally supplied in the form of yeast extract, peptones or corn steep liquor. LABs produce mannitol quantitatively from fructose, but production cost of mannitol from fructose is very high therefore cultures that can utilize cheaper raw material like glucose are advantageous.

Song et al., (2002) isolated an osmophilic *Candida magnoliae* which produced 209 g/l mannitol with 1.01 g l^{-1} h^{-1} productivity. Later, productivity of this strain was improved to 1.94 g $l^{-1}h^{-1}$ in fed-batch fermentation using (1:20) glucose:fructose mixture (Lee et al., 2003b). Recently, production of mannitol by resting cells of *C. magnoliae* under aerobic conditions from glycerol (waste product of biodiesel synthesis) was investigated in our laboratory (Khan et al., 2009).

As described in chapter 3, during mutagenesis programme of *C. magnoliae* for improved erythritol production, two of the mutants M1UV-R1 (R1) and M4UV-R9 (R9), produced mannitol as major polyol from glucose. The mutants R1 and R9 produced 57 and 46 g/l mannitol with respective yield of 22.9 and 18.4 % from 250 g/l glucose. In an endeavor to enhance mannitol production from glucose as sole carbon source using mutant R9, various bioprocess alternatives were investigated. Thus with the availability of mutant R9, it was decided to optimize fermentation medium using low-cost nutrients with a view to commercially exploit it for mannitol production.

5.2 MATERIALS AND METHODS

Candida magnoliae mutants, R1 and R9 which were generated during strain improvement programme for erythritol production produced more mannitol from glucose, were used in the present investigation. These mutants were maintained on LFM agar slants during the course of study. All media ingredients were purchased from HiMedia, Mumbai India. Sweetzyme, an immobilized isomerase, was a kind gift from Novozymes, Bangalore, India.

5.2.1 Analytical Methods

Concentrations of glucose, fructose, mannitol, erythritol, glycerol and other co-metabolites were determined using high performance liquid chromatography (HPLC) as described earlier in chapter 2. Cell mass was measured in terms of optical density (OD_{600}) using a spectrophotometer. DCW for R9 mutant was estimated from a calibration curve derived from the relationship between OD_{600} and DCW at 103 °C, wherein 1.0 OD_{600} was found to be equivalent to 0.26 g/l DCW.

5.2.2 Culture conditions

Respective cultures from freshly prepared slants were inoculated into 5 ml LFM in 150 × 25 mm test tubes and incubated at 28 °C, 210 rpm, for 48 h. Two and a half ml of these seed cultures were aseptically transferred to 250 ml capacity Erlenmeyer flasks containing 22.5 ml liquid medium. This base medium composition would vary with the type and nature of the experiment being carried out. Initial pH of the medium was adjusted to 6.0. Glucose was autoclaved separately for all the experiments. All

experiments were performed in duplicate. Flasks were incubated at 28 °C and agitated at 210 rpm for 96 h unless otherwise mentioned. Samples were withdrawn at fixed interval and analyzed for optical density at 600 nm, pH, residual glucose and polyol concentration. At the end of the incubation period, final volume of the culture broth was adjusted to 25 ml by addition of sterile distilled water for final analysis.

5.2.3 Mannitol production by mutants of *C. magnoliae* from glucose

To evaluate mannitol producing capability, mutants R1, R9 and *C. magnoliae* NCIM 3470 were grown in 25 ml LFM medium in 250 ml Erlenmeyer flasks as described above for seven days. Samples were analyzed at regular intervals of 24 h. Glucose uptake rate, productivity and mannitol yield for mutants were compared at flask level.

5.2.4 Choice of carbon source

Carbon sources, mainly glucose, fructose, glucose-fructose mixture, sucrose and glycerol were investigated for polyol production by mutant R9. Each of the carbon sources was used at a concentration of 250 g/l. The effect of initial glucose concentration in the LFM (containing 150 g/l, 200 g/l, 250 g/l, 300 g/l, 350 g/l and 400 g/l glucose) on mannitol production by *C. magnoliae* mutant R9 was studied. The media with respective glucose concentration were inoculated with 48 h seed culture and incubated at 28 °C, 210 rpm on rotary shaker. Periodic sample analysis was performed until 96 h of incubation.

5.2.5 Effect of varying yeast extract as nitrogen source

Effect of yeast extract concentration on growth and polyol production by *C. magnoliae* mutant R9 was investigated by inoculating the 48 h seed culture in LFM containing glucose at 250 g/l and with varying yeast extract concentrations from 2, 4, 6, 8, 10, 12, 14 and 16 g/l. The flasks were incubated at 28°C, 210 rpm on rotary shaker. Samples were analyzed periodically until 96 h of incubation.

5.2.6 Effect of medium volume in shake flask

Medium volume in Erlenmeyer flasks exerts effect on metabolism because of differences in the dissolved oxygen levels and hence the oxygen transfer rates. To investigate this, 48 h seed culture of *C. magnoliae* mutant R9 was grown in multiple

flasks containing 25 ml LFM, for 48 h at 28 °C, 210 rpm on rotary shaker. Contents of these flasks were mixed at 48 h and the broth was redistributed into flasks containing varying volumes of broth as 10, 20, 25, 30, 40, 50, 60, 80 and 100 ml, respectively. The flasks were re-incubated at 28 °C, 210 rpm on rotary shaker. Periodic sample analysis was performed until 96 h of incubation.

5.2.7 Effect of trace metal addition on mannitol production by mutant R9

Effect of Ca^{+2}, Co^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2}, Mo^{+2}, Zn^{+2}, B^{+2} and Cr^{+2} on polyol production was studied in shake flask with 25 ml LFM. Four different concentrations of trace metals namely 10 mg/l, 50 mg/l, 100 mg/l, 200 mg/l were added separately to individual flasks and the culture broths were analyzed for growth and polyol production.

5.2.8 Fed-batch fermentation for mannitol production from glucose in shake flask by mutant R9

A 48 h seed culture of mutant R9 was inoculated in multiple flasks containing 25 ml LFM, and incubated for 24 h at 28 °C, 210 rpm on rotary shaker. After 24 h, all the contents of flasks were mixed and then redistributed into six 250 ml flasks containing 25 ml of the culture broth and incubated at 28 °C at 210 rpm for 24 h. Flasks were named as 25, 50 and 75 g l^{-1} d^{-1} of glucose feeding in duplicates. The flasks cultures were fed with 3 ml sterile concentrated glucose solution, at a periodic interval of 24 h, to achieve final glucose concentrations of 25, 50 and 75 g $l^{-1}d^{-1}$. Samples were withdrawn at a periodic interval of 24 h and analyzed for biomass, sugar utilized and polyol concentrations using HPLC.

5.2.9 Optimization of medium components for enhanced mannitol production by *C. magnoliae* mutant R9 using RSM: Experimental design and statistical analysis

Previous experimental results demonstrated that production of polyols by mutant R9 mainly depends on source, concentration and ratio of media components, mainly glucose and yeast extract. Therefore use of statistical methods like Response Surface Methodology (RSM) was thought to be a method of choice to optimize mannitol production and minimize other undesirable by-products. Four media components viz. glucose, yeast extract, KH_2PO_4 and $MgSO_4$ were chosen for RSM optimization study.

An experiment with central composite factorial design of 22 (16 plus 6 centre points) plus 8 star points (i.e. 2 × 4) leading to a total of 30 experiments was performed in duplicate. Each flask was inoculated with 2.5 ml inoculum and incubated for four days and analysis of polyols was done using HPLC, as described earlier. The coded values of independent variables are given in Table 5.1. The values of the three dependent responses (i.e. concentration of erythritol, mannitol and glycerol) were used to build RSM models. The second-order polynomial coefficients were calculated and analyzed using 'Design Expert' software (Version 8.0.2.0, Stat-Ease Inc., USA). Statistical analysis of the model was performed to evaluate the ANOVA. The overall predictive capability of the model is commonly explained by the coefficient of determination (R^2). The statistical significance of the fit of the polynomial model equation was checked by the F-test. The significance of the regression coefficient was tested by a t-test.

Coded values	Glucose g/l	Yeast Extract g/l	KH_2PO_4 g/l	$MgSO_4$ g/l
-2	200	5	1	0.05
-1	250	7.5	3	0.15
0	300	10	5	0.25
1	350	12.5	7	0.35
2	400	15	9	0.45

Table 5.1 Coded values of independent variables

5.2.10 Time course study of mannitol and other by-products formation by mutant R9 in statistically-optimized medium

To evaluate the statistically-optimized medium in shake flask, 48 h seed culture of mutant R9 was inoculated in 8 flasks containing 25 ml statistically-optimized medium and incubated at 28 °C at 210 rpm on rotary shaker. Two flasks were harvested after successive intervals of 24 h and analyzed using HPLC.

5.2.11 Evaluation of mannitol production in 10-L fermenter by mutant R9

Statistically-optimized medium was evaluated in a 10-L laboratory fermenter (New Brunswick Scientific, Bio-flow 110 USA) equipped with devices for control and measurement of pH, temperature, dissolved oxygen and stirring speed. Glucose

solution was autoclaved in fermenter whereas yeast extract was autoclaved separately in flask and later added to the fermenter. In this fermenter pH of the culture broth was initially adjusted to 6 with 5N NaOH and temperature was maintained at 28 °C. Dissolved oxygen was measured with an oxygen probe (Mettler Toledo). The agitation speed was adjusted between 400-500 rpm and aeration rate was 0.5 vvm unless otherwise mentioned. Online fermentation data of process parameters such as DO, pH, agitation and feeding strategy was acquired using NBS BioCommand Plus Software Version 3.30. Online biomass OD was monitored by OD probe by measuring the turbidity at 1100 nm (Wedgewood Analytical Inc, Anaheim CA. USA with cell growth sensor BT 65 S).

In subsequent experiments, in order to obtain higher biomass for higher productivity, yeast extract concentration used was twice than that of RSM medium. The fermentation batches were initiated by addition of (5% v/v) inoculum to 9.5 L fermentation medium, grown for 24 h in multiple flasks as described in chapter 4 (during erythritol fermentation). The foam was controlled by periodic addition of food grade silicon oil as antifoam agent. Samples were analyzed at successive intervals for biomass, pH, DO, sugar utilized and polyol production.

Based on previous experience on erythritol production using R23 mutant of *C. magnoliae* and the results obtained from the batch fermentation with R9 mutant, two-stage fermentation was employed. In this experiment initial 50 g/l glucose, along other media components from statistically-optimized medium, was added during the growth phase and remaining 250 g/l glucose was fed after the growth phase (feeding solution consisted of 2500 g glucose in 4 L). The batch was initiated by the addition of 500 ml inoculum into 5.5 L growth medium. The agitation speed 400 rpm and aeration of 0.5 vvm were maintained constant through the fermentation. The DO and the pH during fermentation were not controlled. After 30 h, when the initial glucose was almost exhausted, feeding of the glucose solution was started at a rate of 1.5 l/d. The feed rate was adjusted further according to the glucose uptake rate of the mutant in the fermenter. Samples were analyzed at periodic intervals as describe earlier.

To check the ability of mutant R9 (grown and adjusted to a glucose containing environment) to convert fructose into mannitol, 100 ml culture broth was withdrawn

from the fermenter at 96 h and distributed 25 ml each into four 250 ml Erlenmeyer flasks. Sterile fructose powder was added initially to each of these flasks at concentrations of 25, 50, 75 and 100 g/l, respectively, and incubated at 28 °C, 210 rpm on rotary shaker for 48 h. Samples were withdrawn after periodic interval of 6 h and analyzed for utilization of fructose and production of polyols using HPLC.

5.2.12 Production of mannitol from isomerized glucose and acid hydrolyzed sucrose

In the preliminary experiments, with various carbon sources, it was observed that fructose was a preferred substrate for mannitol production in the presence of glucose-fructose mixture. In addition, when the fermentation broth of 96 h age, grown in glucose containing medium, was transferred to fresh fructose solution, concentration of mannitol produced increased from initial 40 g/l to 90 g/l, which corresponds to 50% conversion yield of the added fructose powder. However, fructose is an expensive substrate as compared to glucose and sucrose. Further, experiments with glucose as carbon source were not encouraging, even on optimizing culture conditions and fermentation parameters as it was not possible to increase the mannitol yield from glucose beyond 20%.

5.2.13 Acid hydrolysis of sucrose

Sucrose is an economical substrate as compared to fructose and could be used after inversion, instead of pure fructose. Hydrolysis of sucrose was carried out in a jacketed vessel. Sucrose solution was acidified by 50% H_2SO_4 to a pH of 2 and maintained under agitation at 90-95 °C for 3 h by passing steam through the jacket.

5.2.14 Isomerization of glucose to fructose by using commercial glucose isomerase

In another approach, it was decided to convert glucose to fructose by a commercially available immobilized glucose isomerase enzyme Sweetzyme®, (Novozymes). Each gram of the immobilized enzyme contained 400 I.U of the enzyme activity (one I.U. of glucose isomerase = 1 µM glucose converted to fructose, per minute). According to the specifications, the optimal pH of the enzyme was 7.5 and the optimal temperature was 55 °C. Isomerization of glucose to fructose was carried out with 50 ml of 25 %

glucose solution in 250 ml flasks. After calculation of the units of activity, 1.45 g immobilized enzyme was added to the glucose solution and incubated for 3 h at 55 °C under shaking. Samples were withdrawn after periodic interval of 30 min and enzyme reaction was terminated by adding equal volume of 0.1M HCl to the sample withdrawn and analyzed for glucose and fructose concentrations in the solution using HPLC.

5.2.15 Production of mannitol by mutant R9 from isomerized glucose-fructose mixture

Two gram freshly grown saline-washed cells were aseptically weighed and re-suspended in 25 ml of the glucose-fructose mixture produced by enzymatic isomerization of 300 g/l glucose. The flasks were incubated at 28 °C at 210 rpm on rotary shaker and samples were periodically analyzed for the consumption of glucose/fructose and mannitol. When all of the fructose in the medium was consumed, the broth was aseptically centrifuged and cells were recovered. The cell-free reaction mixture was then subjected to second round of enzymatic isomerization to obtain fructose from the residual glucose. After this process, the recovered cells were re-suspended in the fructose enriched solution and incubated at 28 °C at 210 rpm on rotary shaker and samples were analyzed as above.

5.2.16 Membrane cell-recycle bioreactor for mannitol production using acid hydrolyzed sucrose (MCRB)

Based on results obtained by using glucose-fructose mixture obtained from enzymatic isomerization of glucose, a MCRB system was designed and evaluated for mannitol production using hydrolyzed sucrose. In this process, initially *C. magnoliae* mutant R9 biomass was generated aerobically in 10-L batch fermentation as described earlier and was later used repeatedly in MCRB. The batch fermentation for growth was carried out in statistically-optimized medium containing 50 g/l glucose. On achieving the cell concentration of about 15 g/l DCW, the cells were concentrated to about 20% (v/v) using hollow fiber microfiltration membrane module with membrane area of 0.35 m^2 (AgTech USA). The cells were washed with equal volume of sterile physiological saline and 8-L acid-hydrolyzed sucrose solution was added to the fermenter to achieve the final sugar concentration of 300 g/l (glucose-fructose mixture

in 50:50 proportion). When the first bioconversion batch of fructose to mannitol was finished, the cells were retained using the same hallow fiber membrane module and 8-L fresh inverted sucrose solution was added to the bioreactor and the process was repeated for one more time using the same initial cell biomass. The agitation was adjusted between 400-500 rpm and the aeration was set to 0.5 vvm. Samples were withdrawn at periodic intervals and analyzed for biomass, sugar utilized and polyol concentration using HPLC.

Permeate of the first bioconversion batch which contained unutilized glucose and mannitol was collected aseptically in a separate bioreactor equipped with pH and temperature controllers. The pH of the second bioreactor was maintained at 7.5 using 5N NaOH and the temperature was controlled at 60 °C. In view to convert the residual glucose to fructose which can be used again for mannitol production, cell free fermentation broth from the second reactor was circulated through a glass column containing immobilized glucose isomerase. The schematic representation of the above process is shown in Fig. 5.1

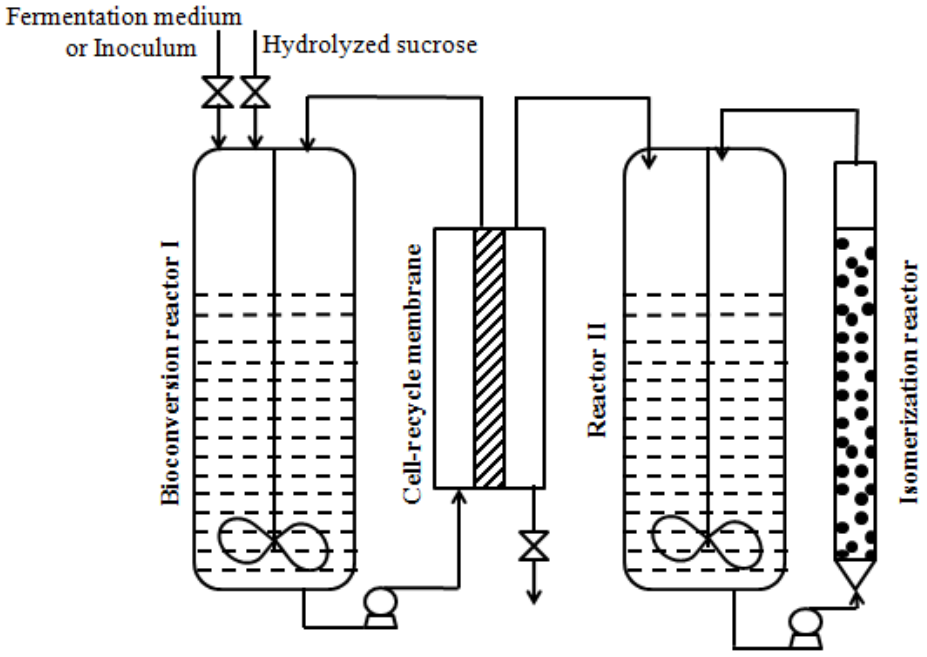


Figure 5.1 MCRB process diagram for mannitol production using hydrolyzed sucrose

5.2.17 Microbiological characterization of *C. magnoliae* mutant R9

Morphological variations of the mutant R9, compared to its parent were examined by phase contrast and SEM. Respective cultures were grown in LFM for 48 h and

washed twice with sterile physiological saline. Appropriately diluted cell suspension was directly visualized under phase contrast microscope or applied on the SEM stubs and dried under vacuum for 30 min and visualized under SEM. Scanning electron microscopy (Quanta 200 3D, FEI) was used to observe the cells on PDMS stubs. Optical images of polymer films were taken with Nikon Eclipse, E600-POL (Japan).

Carbohydrate utilization pattern of *C. magnoliae* 3470 and its mutant R9 was investigated for various sugars such as arabinose, cellobiose, fructose, galactose, glucose, glycerol, maltose, mannose, melibiose, melezitose, raffinose, rhamnose, ribose, sucrose, trehalose and xylose. The media containing 25 g/l each of the above mentioned sugars were prepared and dispensed in aliquots of 5 ml in 25 ×150 mm tubes, inoculated with the respective cultures and grown for 48 h at 28 °C, 210 rpm on rotary shaker. Their growth was compared by measuring optical density at 600 nm.

5.2.18 Studies on mannitol dehydrogenase (MDH, EC 1.1.1.67)

5.2.18.1 Preparation of cell extracts

MDH is the key enzyme in the mannitol biosynthesis. Studies were therefore undertaken to determine whether MDH from mutant R9 reflects its role in overproduction of mannitol as compared to the parent strain. *C. magnoliae* and its mutant R9 were grown in LFM medium in multiple flasks, for 96 h. A set of flasks of the respective cultures were harvested at successive interval of 24 h by centrifugation at 10,000 *g* for 10 min. After washing the cells twice with 50 mM phosphate buffer (pH 6), two gram wet cells were resuspended in disruption buffer (50 mM phosphate 6 pH buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF) for 30 min. This cell suspension was homogenized by grinding with 50 ml of 0.5 mm glass beads (Sigma) in a bead beater (Biospec Products Co., Bartlesville, Okla.) for 5 cycles of one min each with intermediate cooling for two min. Enzyme extracts were centrifuged at 10,000 *g* for 30 min and MDH activity was estimated.

5.2.18.2 Mannitol dehydrogenase assay

MDH assays were performed with some modifications of a previously published procedure (Lee et al., 2007a). The activity of MDH was determined from the oxidation or reduction of NADP(H), by measuring the increase or decrease in the absorbance at 340 nm at 60° C unless mentioned. MDH assay mixture (1.2 ml) for

reduction consisted of 0.25 mM NADPH, 10 mM fructose and 0.1 ml enzyme preparation in 50 mM phosphate buffer (pH 6). This reaction mixture was allowed to stand for 1 min to eliminate the endogenous oxidation of NADPH. The MDH assay mixture for oxidation consisted of 0.5 mM NADP, 10 mM mannitol and 0.1 ml enzyme lysate in 50 mM phosphate buffer (pH 8.0). The reaction was started by the addition of substrate. One unit of enzyme activity is defined as 1 µmol of NADP(H) consumed or produced per min.

5.2.18.3 Partial characterization of MDH from mutant R9

Mutant R9 was grown for 96 h in LFM and cells were subjected to lysis, as described above. The crude enzyme preparation was used to study the characteristics of MDH.

For assays at different pH values, the reactions were performed with the following buffers (50 mM) of pH values (in parentheses): sodium citrate (4.0 and 5.0), potassium phosphate (6.0 to 8.0), carbonate:bicarbonate (9.0), and bicarbonate:NaOH (10.0). For thermal stability study of MDH, aliquots of enzyme lysate were held at 30, 40, 50, 60, and 70 °C and assayed for residual activity at successive interval of 30 min. The effects of metal ions, various reducing agents, substrate and co-substrate specificity for oxidation and reduction reaction of MDH were studied under above mentioned assay conditions.

The obtained cell extract showed specificity only for fructose and NADPH. Other substrate and co-substrate in the enzyme preparation showed negligibly interference with the enzyme activity. Therefore the crude enzyme extract was further used to determine kinetic parameters and compared them to those reported in literature. Kinetic parameters were determined by Lineweaver-Burk plots for the enzyme activity vs. substrate concentrations. The *Km* and *Vmax* values were determined from the plots.

5.3 RESULTS AND DISCUSSION

5.3.1 Evaluation of mannitol production by mutants of *C. magnoliae*

The mannitol producing mutants generated during the mutagenesis programme of erythritol production as described in Chapter 3 were re-evaluated for their mannitol production ability from glucose. When the mutants and the parent strain were grown

in 25 ml production medium with 250 g/l glucose as carbon source, there were distinct differences in the extent of glucose utilization and products formed. It can be seen from the Fig. 5.2 that there was a substantial increase in the mannitol production by mutants, from glucose, as compared to the parent strain. The mutants R1 and R9 produced 57 and 46 g/l mannitol with respective yield of 22.9 and 18.4 % as compared to 6 g/l mannitol by the parent with mere yield of 4.1 %. The parent strain grew better as compared to its mutants (19.7 g/l DCW) but sugar uptake rate and mannitol production rate of the parent strain were substantially low. During 168 h of fermentation, the mutants could utilize all 250 g/l glucose as compared to only 145 g/l by the parent strain. Mutant R9 utilized all the glucose at the rate of 2.6 g $l^{-1}h^{-1}$ with mannitol production rate of 0.5 g $l^{-1}h^{-1}$ in 96 h of fermentation whereas mutant R1 required 168 h for complete utilization of glucose and produced mannitol at rate of 0.34 g $l^{-1}h^{-1}$. Based on mannitol productivity, mutant R9 was selected for further experimentation.

The polyol production pattern (i.e. relative concentration of different polyols) by *C. magnoliae* mutant R9 was greatly dependent on the carbon source used (Fig. 5.3). Within 96 h, glucose resulted in the highest mannitol production (42 g/l) while fructose resulted in a higher glycerol production (50 g/l). Mannitol production from sucrose and glycerol (9 g/l and 1.5 g/l) was much lower as compared to glucose. But highest biomass production was observed in the medium containing glycerol as the carbon source. Thus, independent of the other media constituents, glucose was found to be the most suitable carbon source for mannitol production. Therefore it was used in further experiments.

Fructose is the commonly used carbon source for mannitol production by heterofermentative LABs. Fructose is reduced to mannitol by NAD(P)H dependent MDH. Although a few homofermentative LABs produce mannitol from glucose, its production level is very low (Wisselink et al., 2002). Kaup et al., (2005) engineered *E. coli* for mannitol production from glucose in a whole cell biotransformation by supplementation with extracellular glucose isomerase which resulted in the formation of 145.6 g/l mannitol from 180 g/l glucose.

Song et al., (2002) isolated a strain of *C. magnoliae* which produced 67 g/l mannitol in fructose containing medium in 168 h shake flask culture. Recently, Khan et al., (2009) investigated that the resting cells of *C. magnoliae* produced mannitol from fructose, sucrose and glycerol but not from glucose. With glycerol as carbon source they got highest yield of 45 %. In contrast, the growing cells of mutant R9 generated from the *C. magnoliae* produced high amount of mannitol from glucose. Comparatively glucose is cheaper substrate than fructose. Therefore it was decided to optimize mannitol production from glucose with R9 mutant.

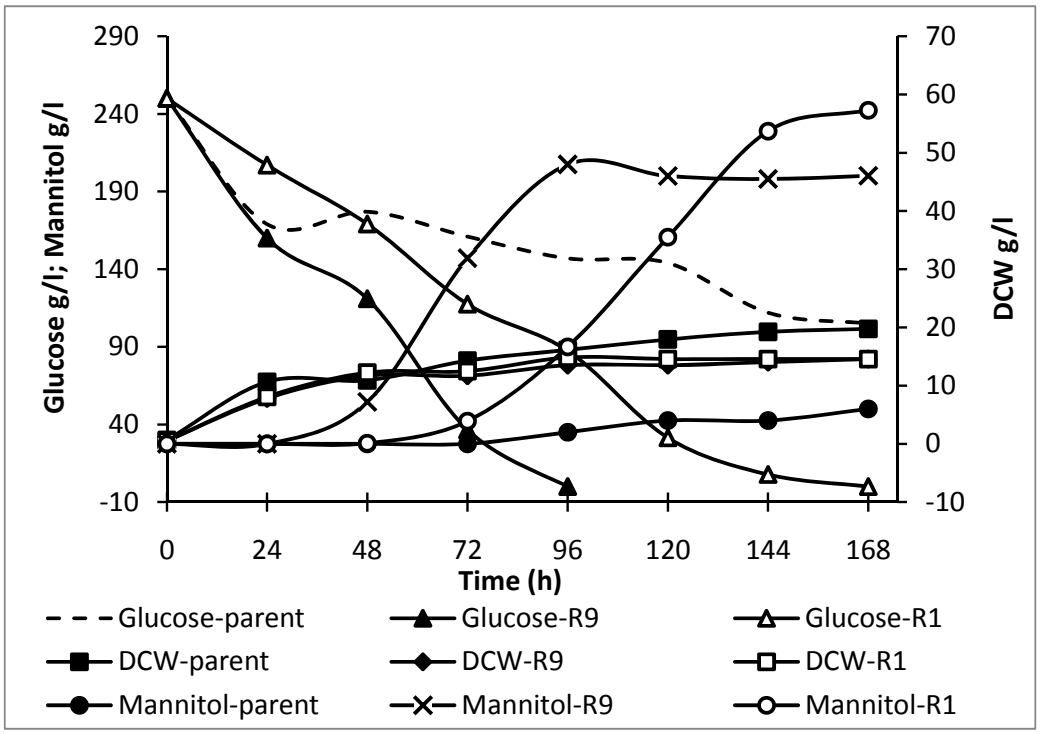


Figure 5.2 Comparison of *C. magnoliae* and its mannitol producing mutants on LFM

The effect of initial glucose concentration in the fermentation medium on mannitol production by mutant R9 is illustrated in Fig. 5.4. The maximum mannitol production using *C. magnoliae* mutant R9 was obtained at 300 g/l initial glucose concentration. Further increase in glucose concentration (beyond 300 g/l) caused decrease in biomass and mannitol production. When the initial glucose concentration was increased above 250 g/l, an unwanted by-product, glycerol, was produced at fairly high concentration.

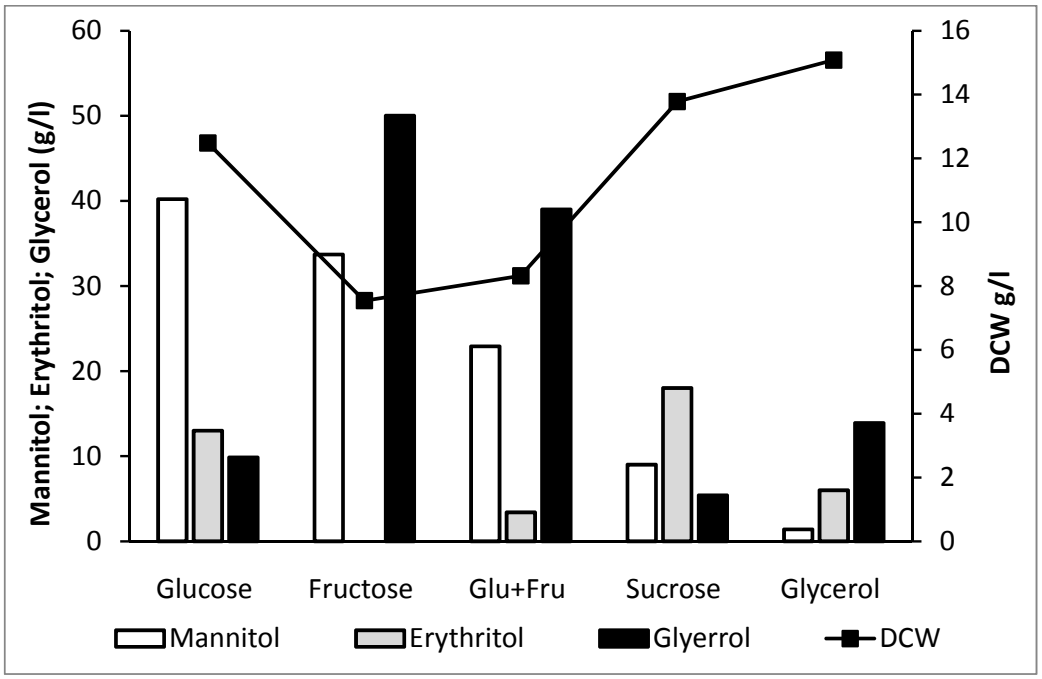


Figure 5.3 Effect of various carbon sources on mannitol production by mutant R9

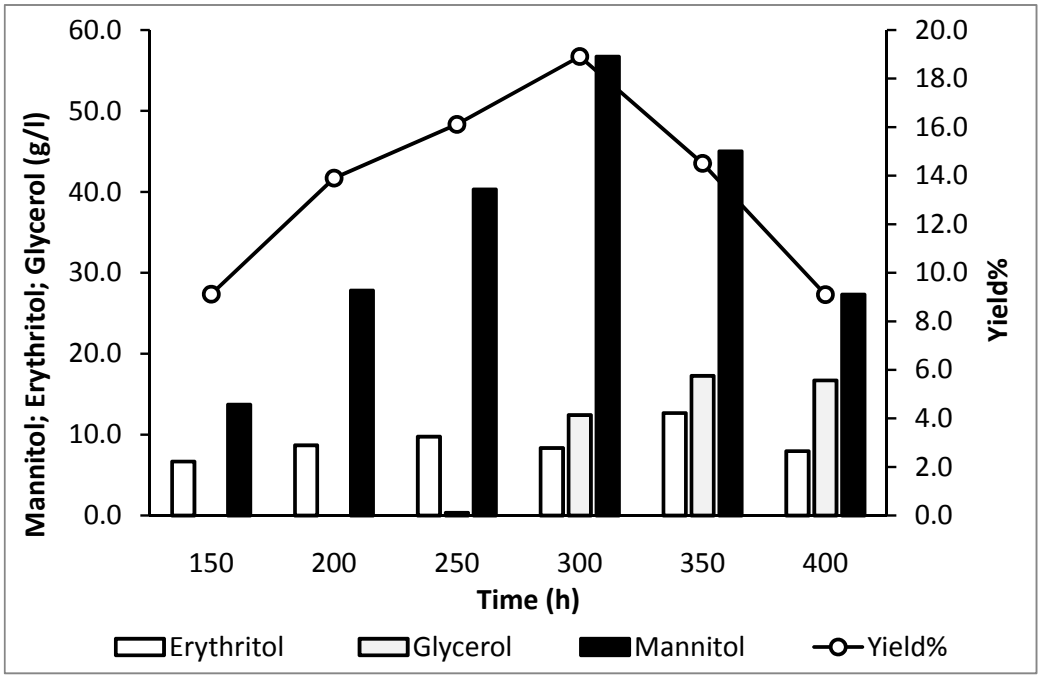


Figure 5.4 Effect of initial glucose concentration on mannitol production by mutant R9

5.3.2 Effect of nitrogen source

Yeast extract is a commonly used nitrogen source for mannitol production (Song et al., 2002; von Weymarn et al., 2003). In the preliminary experiment, different organic nitrogen sources were studied for erythritol production by *C. magnoliae* parent NCIM 3470. During these experiments it was observed that of all the organic nitrogen sources used, yeast extract resulted in maximum mannitol production i.e. 4.7 g/l (Table 2.2). Mutant R9 was selected based on its high mannitol production and increase in yeast extract for the growth of R9 in shake flask at concentrations from 2 to 12 g/l resulted in higher mannitol production. The maximum mannitol (56 g/l) was observed above 10 g/l yeast extract concentration (Fig. 5.5).

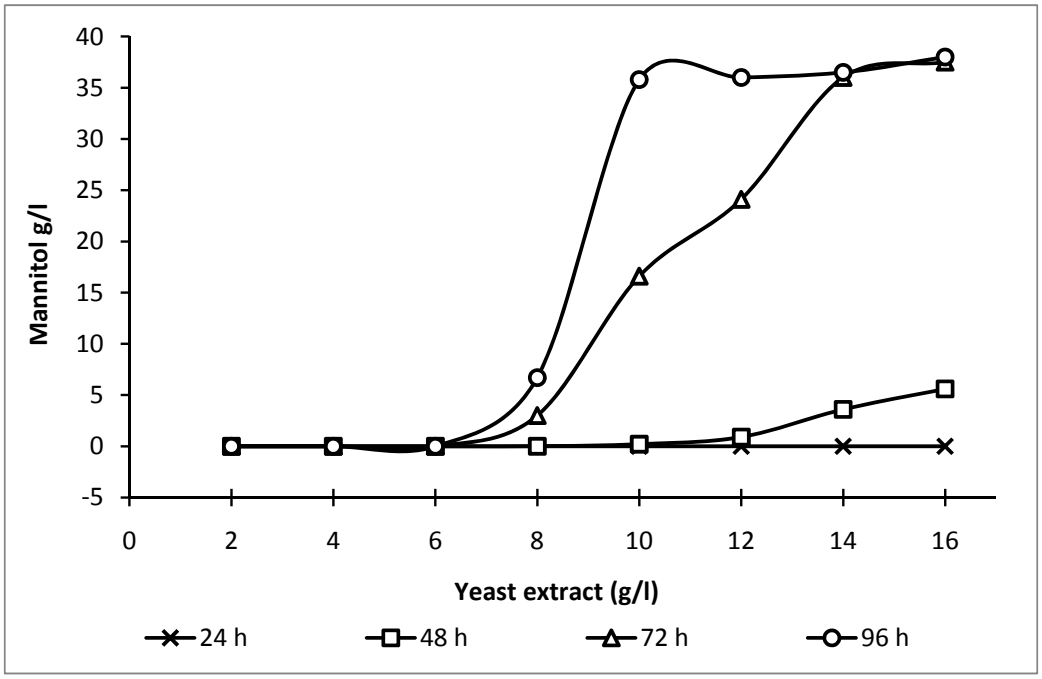


Figure 5.5 Effect of yeast extract concentration on mannitol production by mutant R9

5.3.3 Effect of media volume in shake flask

The dissolved oxygen level in the LFM was found to have profound effect on the production of mannitol. With increase in the volume of fermentation medium, the amount of mannitol production increased and maximum mannitol production was observed at the 25 ml volume. Further increase in the volume beyond 25 ml led to decreased amount of mannitol production (Fig. 5.6). It was also observed that with

increasing volume of LFM in the shake flask there was consistent decrease in growth as well as glycerol and erythritol levels while ethanol production increased. As the LFM volume in the shake flask increased, the dissolved oxygen levels decreased, resulting in increased anaerobic conditions and the increased production of ethanol. The effect of aeration and medium volume has been highlighted in the work done by Onishi & Suzuki, (1968), wherein the increase in the medium volume in the shake flask led to decreased mannitol production. Also it was observed that under oxygen limiting conditions, mannitol was the major polyol produced by *A. niger* strain BO-1, in contrast to glycerol whose production was highest during the growth phase (Diano et al., 2006).

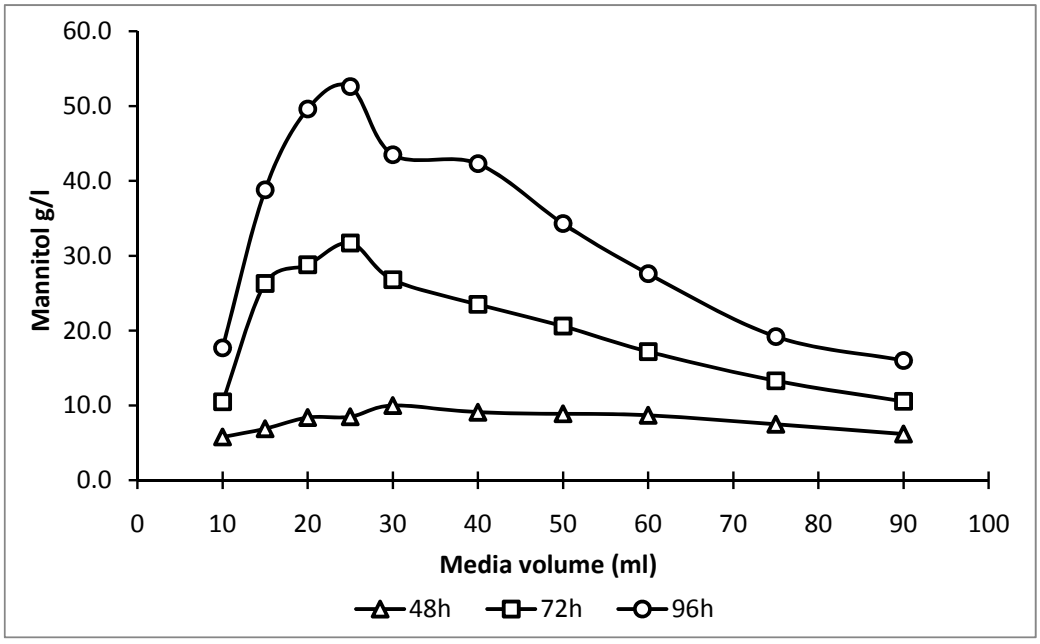


Figure 5.6 Effect of media volume on mannitol production by mutant R9

5.3.4 Effect of trace metals on mannitol production by mutant R9

In the present study, with various metal ions, none of the trace metals specifically increased mannitol production to a large extent. Maximum mannitol production up to 65 g/l was observed in case of Ca^{+2}, Fe^{+2}, Zn^{+2} and B^{+2}. On the contrary, addition of Co^{+2} increased formation of unwanted by-product glycerol up to 60 g/l. The concentration of total polyols decreased drastically when Cu^{+2} was added to the medium even though there was higher biomass. Lowest growth levels were observed in case of addition of Co^{+2} Fig. 5.7a, b.

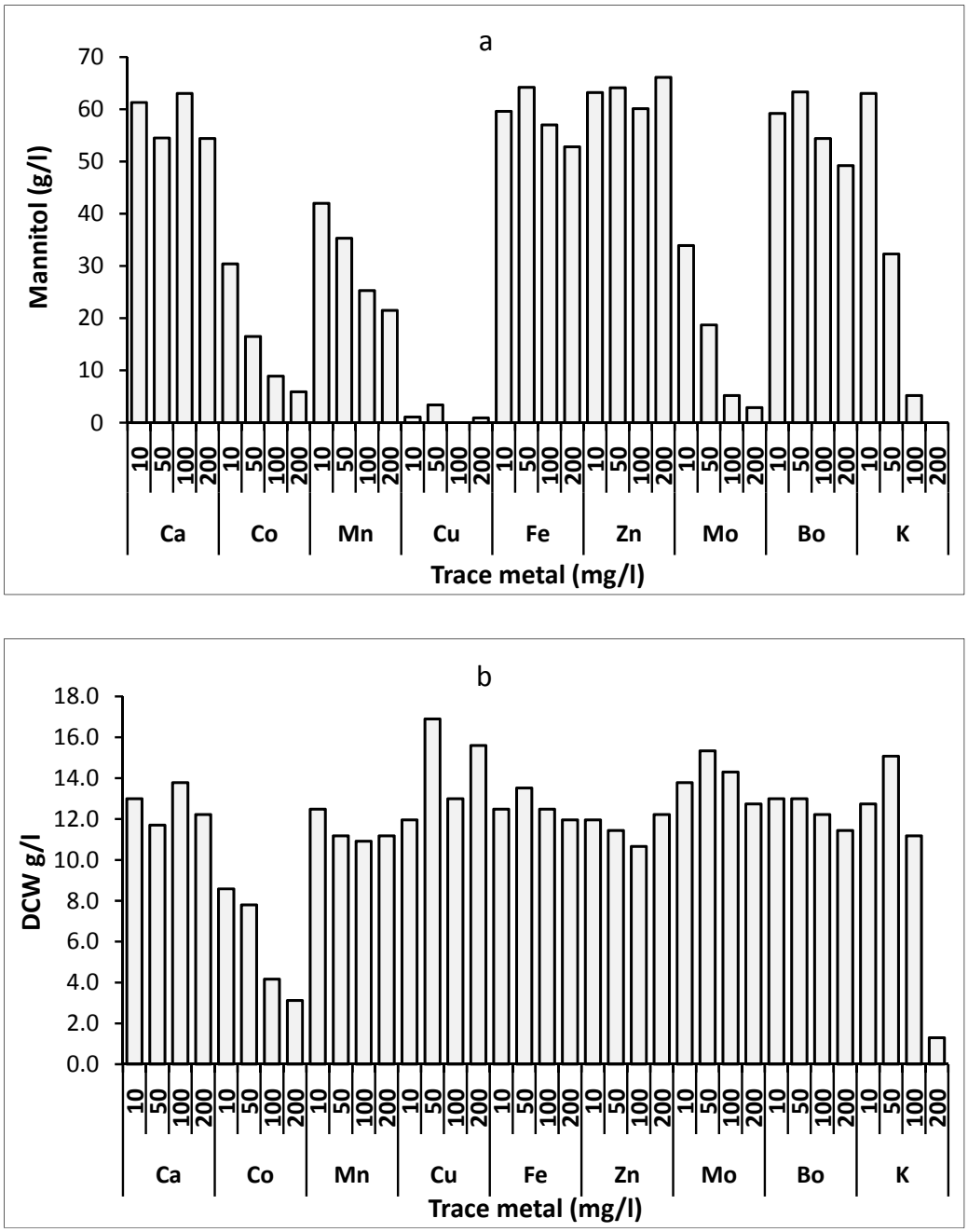


Figure 5.7a, b Effect of trace metal on growth and mannitol production by mutant R9

Minerals have been reported to influence the production of some sugar alcohols and the activity of enzymes involved in sugar alcohol synthesis (Lee et al., 2007a; Lee et al., 2002; Lee et al., 2007b). Lee et al., (2007a) investigated that Ca^{+2} works by altering the permeability of cells to mannitol whereas Cu^{+2} increases the activity of an enzyme responsible for mannitol biosynthesis.

5.3.5 Fed-batch fermentation in shake flask for mannitol production from glucose by mutant R9

When the effect of periodic addition of glucose to the shake flask was studied, it was observed that there was an increase in mannitol production with increase in glucose feeding from 25 to 50 g $l^{-1}d^{-1}$ as seen from (Fig. 5.8). However, at 75 g $l^{-1}d^{-1}$ glucose feeding rate, there was a sharp decrease in mannitol production presumably because of the excess concentration of glucose in the medium.

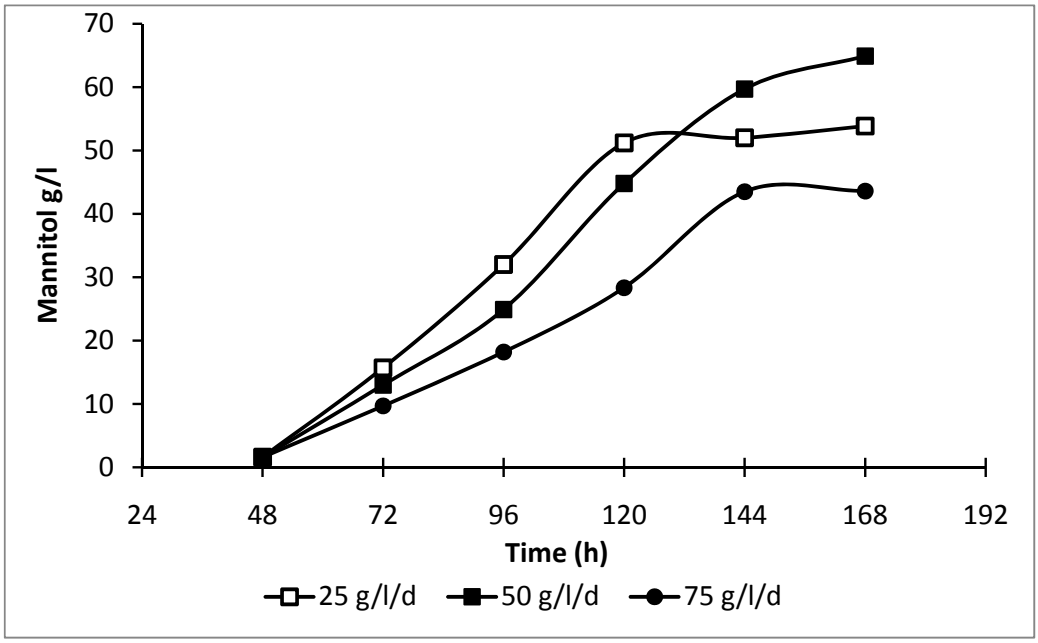


Figure 5.8 Fed-batch fermentation in shake flasks for mannitol production from glucose by mutant R9

5.3.6 Optimization of medium components for enhanced mannitol production by *C. magnoliae* mutant R9 using RSM

To study combined effect of the effective media components, RSM method of media optimization was applied for mutant R9. In recent years, RSM has emerged as the most popular statistical optimization technique and has been used for production of a broad range of microbial metabolites and enzymes (Choudhari & Singhal, 2008; Mahajan et al., 2010; Mullai et al., 2010; Padma & Singhal, 2010). von Weymarn, (2002) studied combined effect of pH and temperature on mannitol production by resting cells of *L. mesenteroides* ATCC-9135 using RSM. However, to the best of my

knowledge, RSM has not yet been used for fermentative production of mannitol by yeasts. It was observed that mutant R9 produced 16 g/l erythritol and 20 g/l glycerol as side products. The preliminary screening experiments (one-factor at a time), suggested that glucose as carbon source and yeast extract as nitrogen source were the critical medium components for mannitol as well as by-products formation. Either increase or decrease in these medium components resulted in a drastic change in polyols pattern. The design of experiments and respective experimental and model-predicted values of mannitol are given in Table 5.2. The second order polynomial equation was used to correlate the independent process variables with mannitol production. The second order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert software. The regression analysis gave second-order response model (*Eq. 5.1).*

$$\text{Mannitol (g/l)} = 54.50 - (1.95 \times A) + (4.23 \times B) - (1.22 \times C) - (0.52 \times D) - (6.76 \times A^2) - (5.63 \times B^2) - (0.24 \times C^2) - (0.39 \times D^2) + (0.075 \times AB) + (0.21 \times AC) - (0.14 \times AD) + (1.33 \times BC) + (0.18 \times BD) - (0.69 \times CD) \quad \text{- } Eq.\ 5.1$$

Where B: yeast extract, A^2 Glucose and B^2 were identified as significant model terms. The model significance was evaluated by using ANOVA (Analysis of Variance). The results of ANOVA are given in Table 5.3. Model F-value was calculated as a ratio of mean square regression and mean square residual. The model F-value of 8.15 implied that the model was significant and there was only a 0.01% chance that a large 'Model F-value' could occur due to noise. P values of less than 0.05 indicate model terms are significant. The model fitting values highlight model adequacy (Table 5.4). A low value of coefficient of variation (10.70 %) indicates very high degree of precision and a good reliability of the experimental values. The fit of the model can also be expressed by coefficient of regression R^2, which was found to be 0.884, indicating that 88.4% of the variability in the response could be explained by the model. 'Adeq Precision' measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, a ratio of 9.7 indicates an adequate signal. The fit of the model is graphically represented by 'parity plot' which compares the predicted and experimental values of the response (Fig. 5.9). The diagonal indicates 0% error. Most of the points lie on or near diagonal indicating the high level of statistical significance of the model. The perturbation plot for mannitol production (Fig. 5.10a) indicates that glucose (A) and

yeast extract (B) were the influential media components whereas KH_2PO_4 (C) and $MgSO_4$ (D) had least influence on mannitol production. Likewise, the perturbation plot for erythritol production (Fig. 5.10b) suggests that yeast extract (B) is the influential media component as compared to glucose (A) whereas KH_2PO_4 (C) and $MgSO_4$ (D) had least influence on erythritol production. The perturbation plot for glycerol production (Fig. 5.10c) indicates that the glycerol production was mainly dependent on glucose (A) as compared to yeast extract (B), while the other media components i.e. KH_2PO_4 (C) and $MgSO_4$ (D) had least influence on glycerol production. Thus, from the perturbation plots, it was confirmed that concentration of glucose and yeast extract should be adjusted critically in order to maintain the unwanted metabolites erythritol and glycerol at minimum level.

The three-dimensional response surface and contour plots of the statistically significant interaction (i.e. interaction between glucose (A) and yeast extract (B) in mannitol production) is shown in Fig. 5.11. The three-dimensional plot was obtained from the pair-wise combination of two independent variables (i.e. glucose and yeast extract), while keeping the other two variables (i.e. KH_2PO_4 and MgSO4) at their center point levels. From the bump of three-dimensional plot or the central point of its respective contour plot, the optimal composition of medium components can be identified. The contour response plot gives the individual and interactive effects of these process variables on the response. Three-dimensional response surface plots of statistically significant interaction responsible for the by-products erythritol and glycerol formation in shown in Fig. 5.12

The optimal media compositions were obtained by substituting levels of the factors into the regression equation using the Design Expert software. The criterion for obtaining optimal media compositions was chosen as: mannitol at the maximum level and erythritol and glycerol at their minimum levels. The media composition for the mannitol production and the corresponding predicted response and experimental validation of model predicted values is summarized in Table 5.5. The close conformity between predicted values and experimental values of polyols confirmed the significance of the model. The optimal media composition for enhanced production of mannitol and minimal production of erythritol and glycerol was (g/l) glucose 300.5, yeast extract 10.8, KH_2PO_4, 1.24 and $MgSO_4$ 0.34.

Std-Run order No.	*Glucose*	*Yeast extract*	*KH_2PO_4*	*$MgSO_4$*	*Predicted Mannitol (g/l)*	*Actual Mannitol (g/l)*
1	-1	-1	-1	-1	41.90	44.90
2	1	-1	-1	-1	37.70	41.60
3	-1	1	-1	-1	47.22	46.90
4	1	1	-1	-1	43.32	37.30
5	-1	-1	1	-1	37.77	40.60
6	1	-1	1	-1	34.42	33.40
7	-1	1	1	-1	48.38	43.30
8	1	1	1	-1	45.33	47.30
9	-1	-1	-1	1	42.17	42.40
10	1	-1	-1	1	37.42	40.00
11	-1	1	-1	1	48.18	46.70
12	1	1	-1	1	43.73	43.10
13	-1	-1	1	1	35.28	38.80
14	1	-1	1	1	31.38	33.90
15	-1	1	1	1	46.60	44.90
16	1	1	1	1	43.00	37.50
17	-2	0	0	0	31.35	30.70
18	2	0	0	0	23.55	24.50
19	0	-2	0	0	23.53	14.60
20	0	2	0	0	40.47	49.70
21	0	0	-2	0	55.98	55.20
22	0	0	2	0	51.12	52.20
23	0	0	0	-2	53.98	54.20
24	0	0	0	2	51.92	52.00
25	0	0	0	0	54.50	56.20
26	0	0	0	0	54.50	53.60
27	0	0	0	0	54.50	54.90
28	0	0	0	0	54.50	53.70
29	0	0	0	0	54.50	54.90
30	0	0	0	0	54.50	53.70

Table 5.2 Central composite rotatable design matrix of independent variables and their corresponding experimental and predicted values of mannitol

Source	*Sum of Squares*	*Degree of Freedom*	*Mean Square*	*F Value*	*p-value (Prob > F)*
Model Significant	2541.23	14	181.52	8.15	0.0001
A-Glucose	91.26	1	91.26	4.1	0.0611
B-Yeast Extract	430.11	1	430.11	19.32	0.0005
C-KH2PO4	35.53	1	35.53	1.6	0.2258
D-MgSO4	6.41	1	6.41	0.29	0.5995
AB	0.09	1	0.09	$4.04E^{-03}$	0.9501
AC	0.72	1	0.72	0.032	0.8595
AD	0.3	1	0.3	0.014	0.9088
BC	28.09	1	28.09	1.26	0.279
BD	0.49	1	0.49	0.022	0.884
CD	7.56	1	7.56	0.34	0.5687
A^2	1254.35	1	1254.35	56.34	< 0.0001
B^2	867.86	1	867.86	38.98	< 0.0001
C^2	1.55	1	1.55	0.069	0.7957
D^2	4.12	1	4.12	0.18	0.6732

Table 5.3 ANOVA analysis of the model

No.	*Model Terms*	*Values*
1	Coefficient of the variation	10.7 %
2	R^2	0.88
3	Adeq Precision	9.76
4	Standard Deviation	4.72

Table 5.4 Model fitting values for RSM

A	***Glucose (g/l)***	***Yeast extract (g/l)***	***KH_2PO_4 (g/l)***	***$MgSO_4$ (g/l)***	***Predicted values (g/l)***		
					Mannitol	***Erythritol***	***Glycerol***
	300.5	10.8	1.24	0.34	56.30	10.51	16.81

B	***Glucose (g/l)***	***Yeast extract (g/l)***	***KH_2PO_4 (g/l)***	***$MgSO_4$ (g/l)***	***Observed values (g/l)***		
					Mannitol	***Erythritol***	***Glycerol***
	300.5	10.8	1.24	0.34	54.48	10.69	16.03

Table 5.5a, b Experimental validation of model predicted values of polyol production

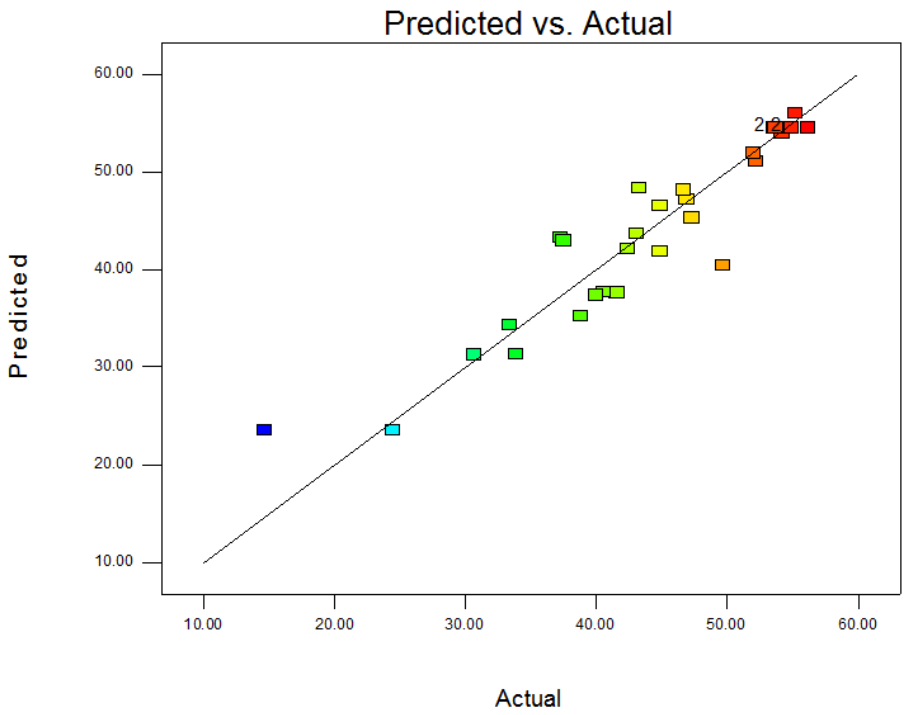


Figure 5.9 Parity plot (Actual values Vs Predicted values for mannitol production)

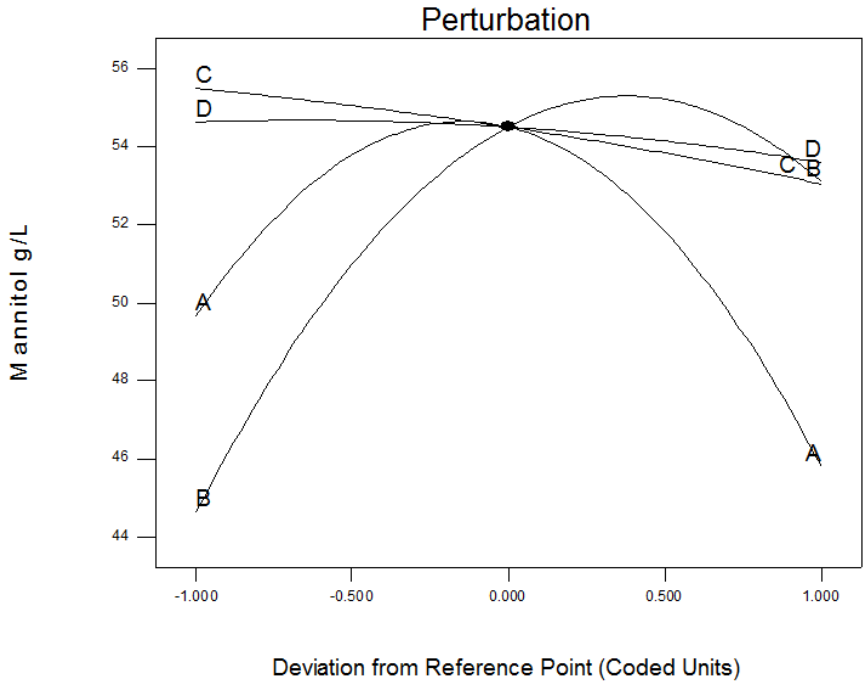


Figure 5.10a Perturbation plot for mannitol production

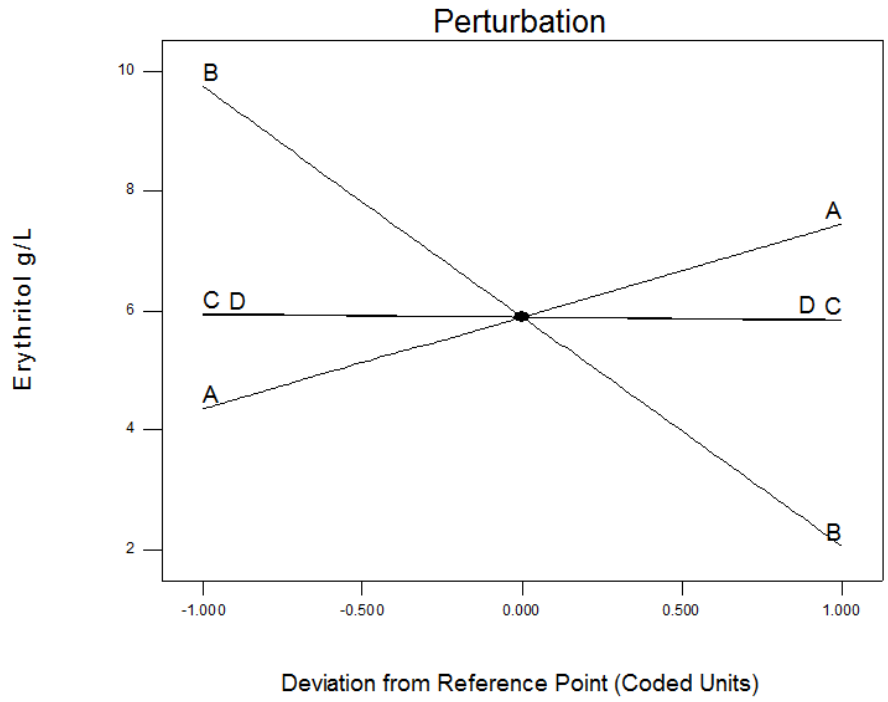


Figure 5.10b Perturbation plot for erythritol production

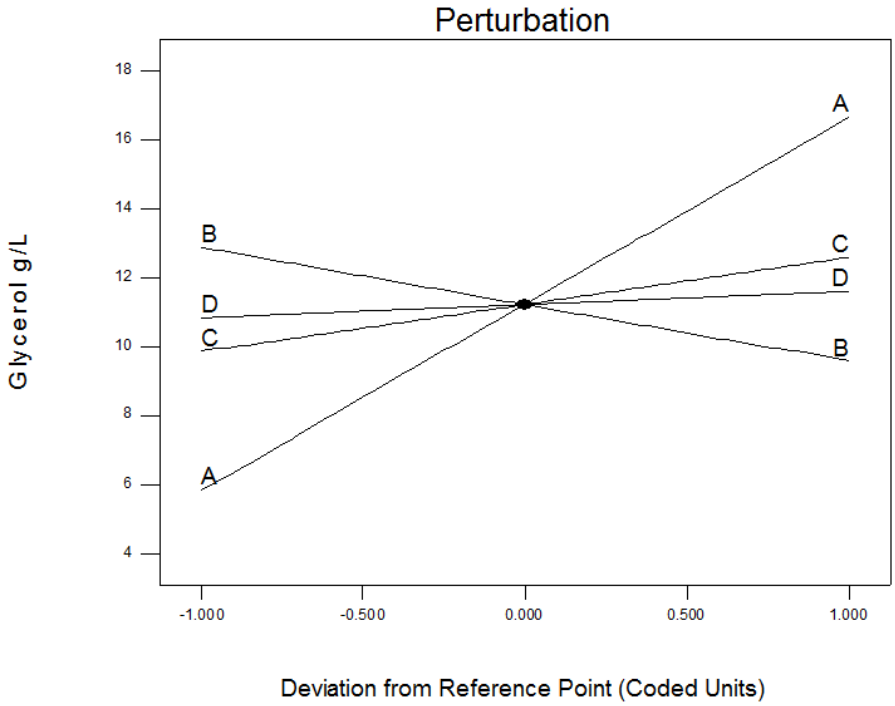


Figure 5.10c Perturbation plot for glycerol production

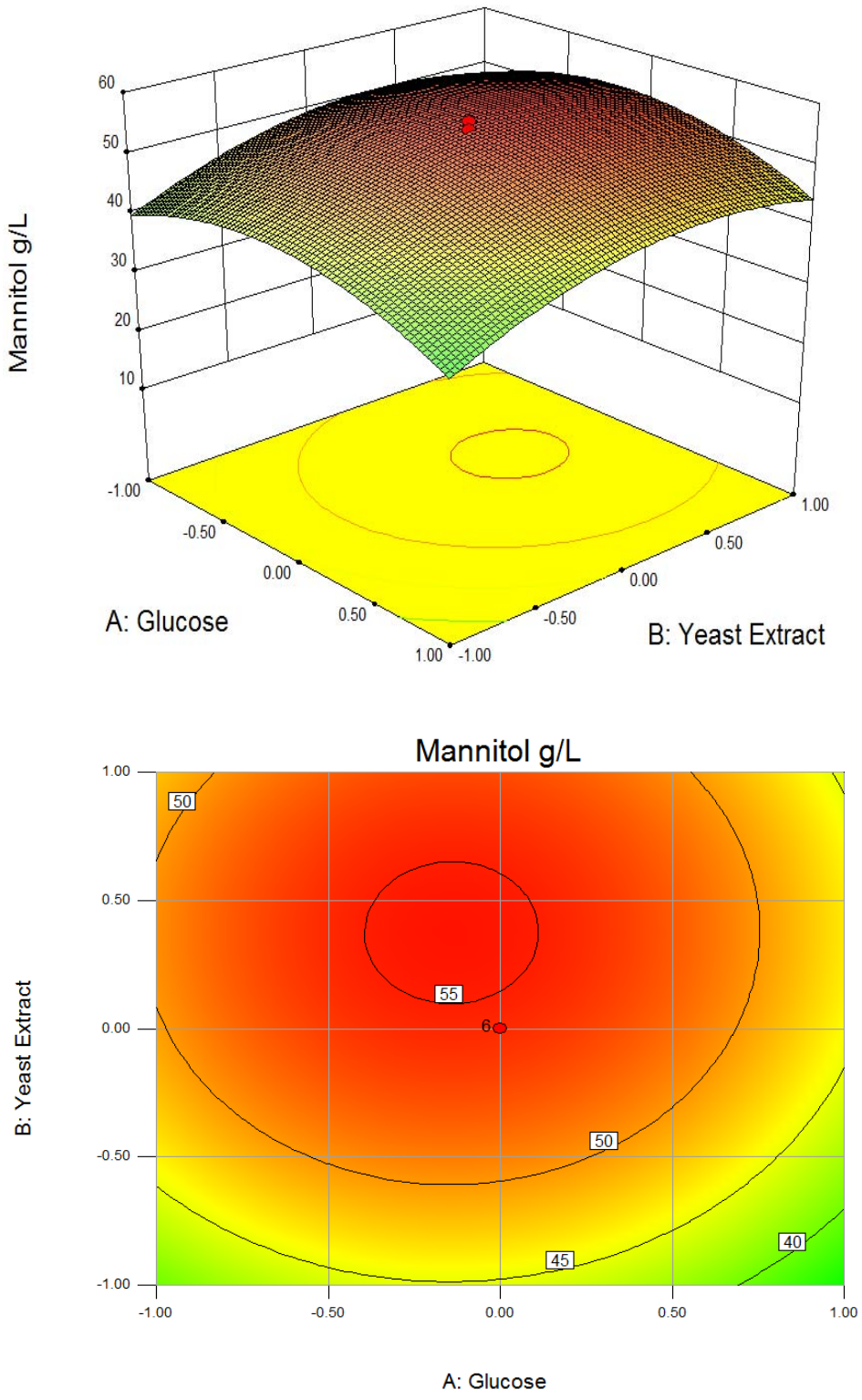


Figure 5.11 3-D response surface and contour plots of statistically significant interaction for the mannitol production

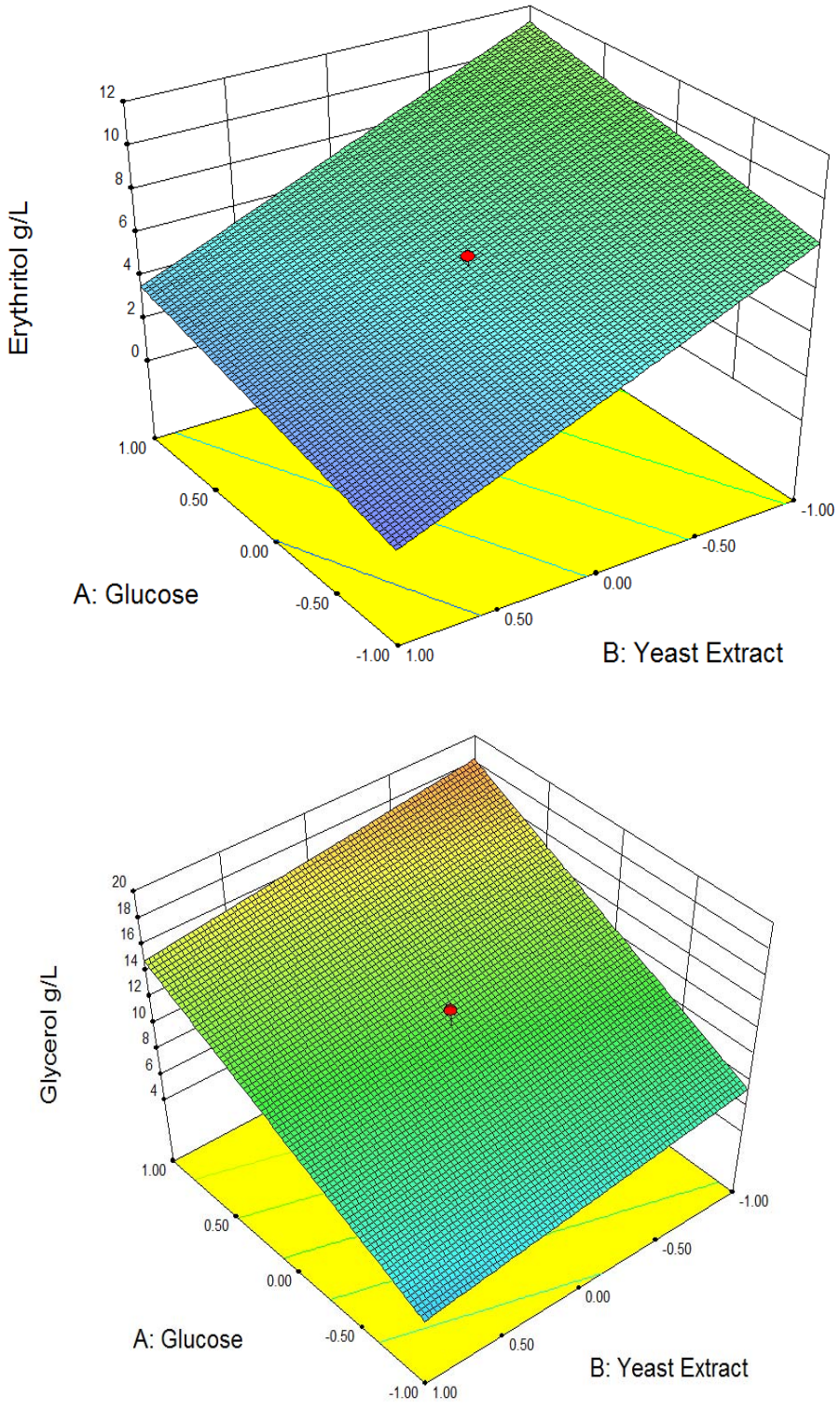


Figure 5.12 3-D response surface plots of statistically significant interaction for the by-products erythritol and glycerol production

5.3.7 Time course study of mannitol and other by-products formation by mutant R9 in statistically-optimized medium

It can be observed from time course profile of growth and mannitol production that the growth phase lasted till 48 h after which mannitol formation occurred and reached up 60 g/l at 96 h. The formation of by-products glycerol and erythritol took place during the initial stages and their levels remained constant after 48 h. Maximum mannitol 60 g/l was produced at 96 h and there is no residual glucose Fig. 5.13

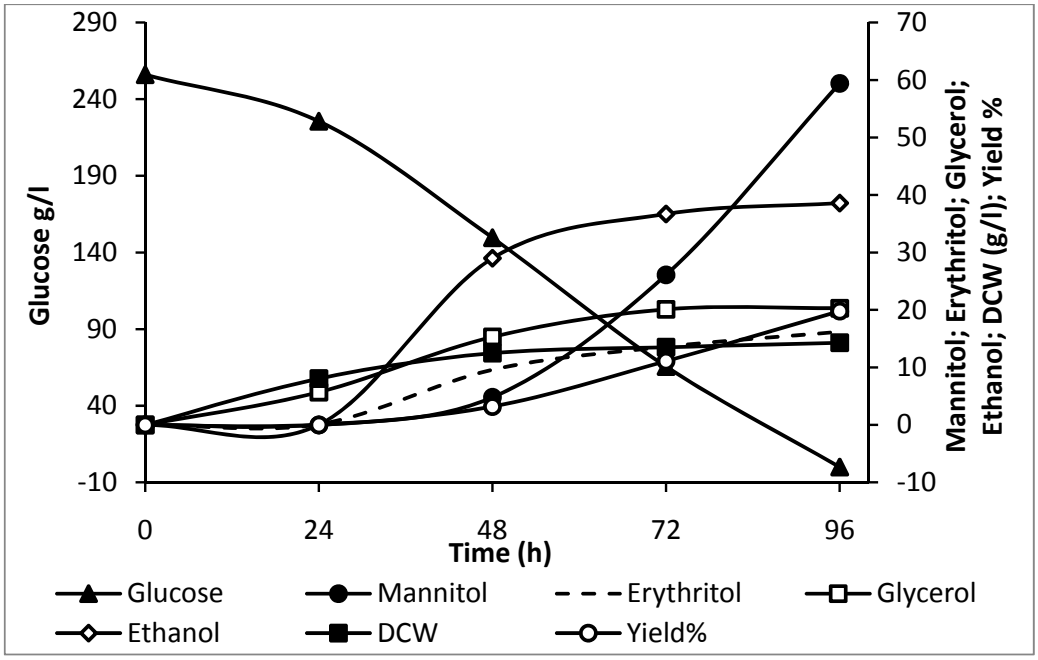


Figure 5.13 Time course profile of mannitol and other by-products produced in statistically-optimized medium

5.3.8 Evaluation of mannitol production in 10L fermenter from mutant R9

In fermenter, during the initial 48 h of growth phase, the biomass concentration increased to 16 g/l DCW and reached 18 g/l at the end of the batch. Initially, there was continuous increase in concentration of erythritol and glycerol which can be attributed to the aerobic conditions prevalent in the fermenter. After 60 h, the agitation was reduced to 500 and then to 425 rpm. From this point onwards, the ethanol concentration in the broth increased rapidly. This rapid increase in the ethanol was attributed to excessive glucose levels in the fermenter and due to the partial anaerobic conditions. A final mannitol concentration of 11 g/l was obtained in the batch fermenter (Fig. 5.14). By observing the results of the batch fermenter, it was

hypothesized that feeding glucose under partial aerobic conditions would increase the production of mannitol.

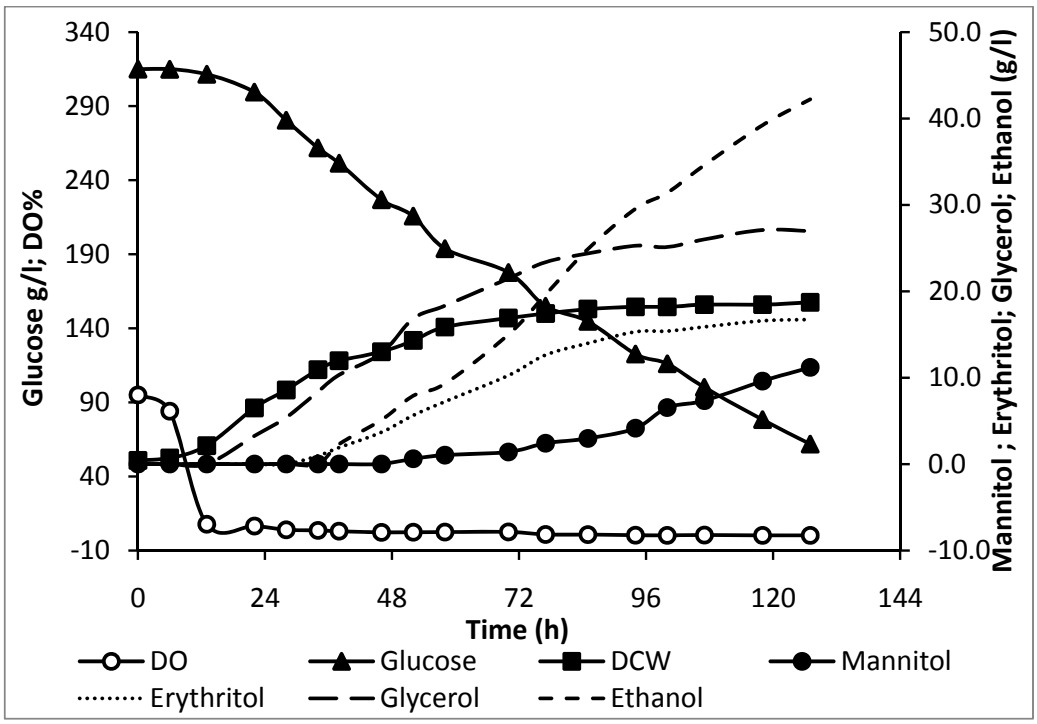


Figure 5.14 Batch fermentation profile using glucose by mutant R9

In the second two-stage fermentation batch, agitation was set at 400 rpm during the growth phase. At 28 h, glucose level in the fermenter decreased below 10 g/l and glucose feeding was started at this point. There was an increase in the level of ethanol and glycerol which could be attributed to low DO concentration. With a view of limiting the glucose level in the fermenter below 35 g/l, the feeding was paused for some period of time and resumed at 65 h. There was a consistent increase in the mannitol concentration whereas, ethanol and glycerol became stable at 72 h and did not increase further. The fermentation was continued till 135 h. The final mannitol concentration in the fermenter was 50 g/l and around 55 g/l glucose remained unutilized (Fig. 5.15)

On transferring 25 ml of fermentation broth to four flasks containing sterile fructose at concentration of 25, 50, 75 and 100 g/l, it was observed that within 48 h it could consume all the fructose and produce mannitol. It can be seen from the Fig. 5.16 that with the increase in fructose concentration there was an increase in mannitol production. Highest mannitol (90 g/l) was produced in the flask containing 100 g/l

fructose concentration. Thus, 50% of the added fructose was converted to mannitol even on using mutant R9 cells grown on glucose for 96 h. Thus, the cells could convert fructose into mannitol even after growing initially in a glucose containing medium. This led us to the use of glucose-fructose mixture for mannitol production, generated by inversion of sucrose or by isomerization of glucose.

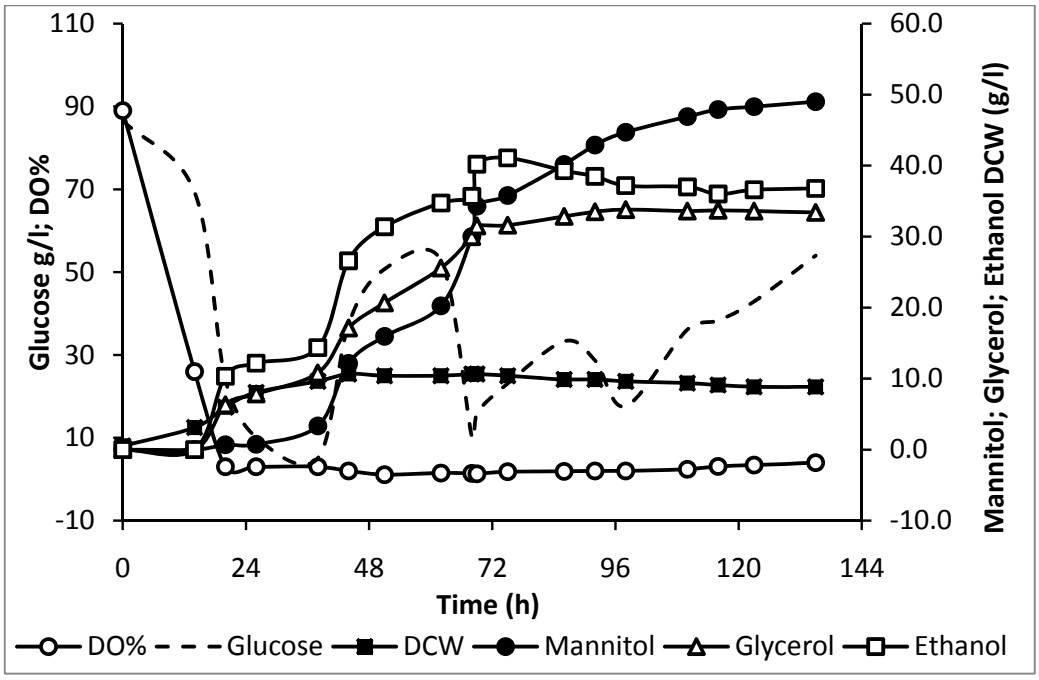


Figure 5.15 Two-stage fermentation processes for glucose to mannitol production by mutant R9

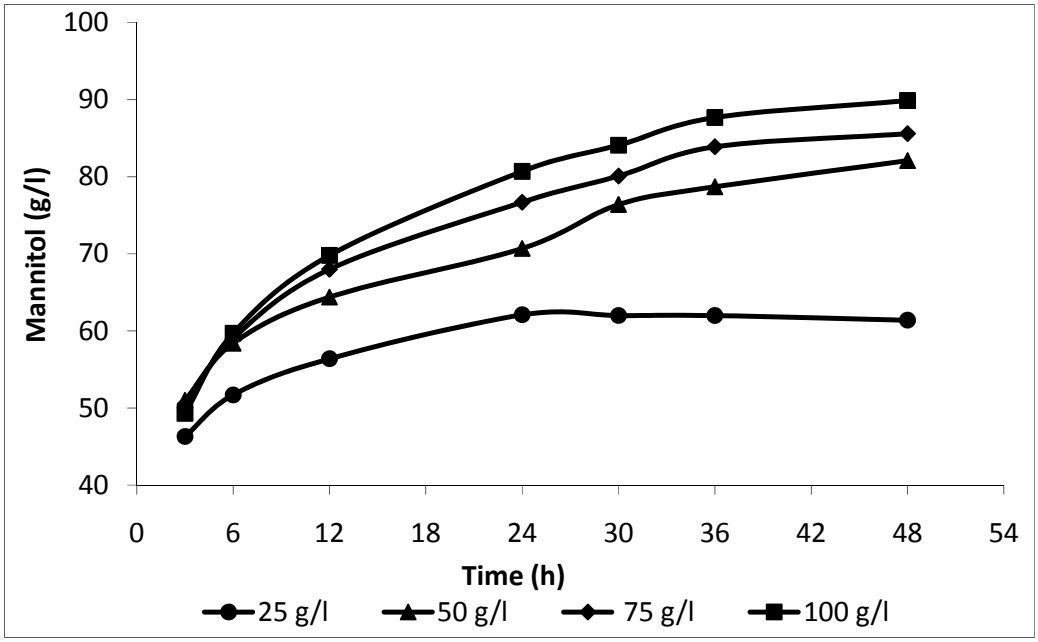


Figure 5.16 Conversion of fructose to mannitol by mutant R9 grown initially in a glucose containing medium.

5.3.9 Production of mannitol from isomerized glucose

The time required for complete isomerization of 250 g/l glucose solution using 1.45 g of glucose isomerase enzyme, at 55 °C, pH 7.5 to a 50:50 mixture of glucose-fructose was 3 h under the experimental conditions (Fig. 5.17). At 65 °C, 250 g/l glucose was isomerized to a 50:50 mixture of glucose-fructose in 2 h using 1.45 g of immobilized glucose isomerase enzyme (Sweetzyme). Although the enzyme showed increased activity at higher temperatures, the stability of the enzyme reduces over time when incubated at higher temperatures as reported by Converti et al., (1997). Hence the isomerization of glucose was done subsequently at 60 °C.

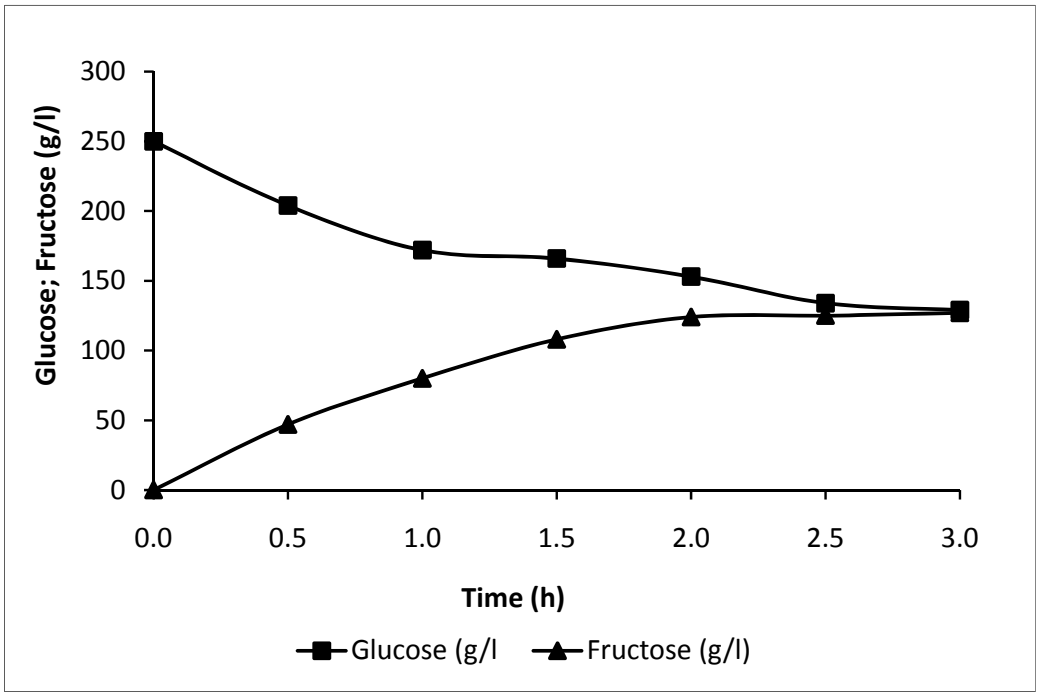


Figure 5.17 Isomerization of glucose to fructose over time by glucose isomerase at 55 °C, pH 7.5

5.3.10 Production of mannitol by mutant R9 from glucose-fructose mixture produced by enzymatic isomerization of glucose

From the glucose-fructose mixture produced by the enzymatic isomerization of glucose, fructose was consumed preferentially over glucose by the resting cells of mutant R9. In initial 9 h of incubation, 72 g/l mannitol was produced from 128 g/l of fructose with 56% yield. At this point, there was 122 g/l glucose left over unutilized. It was observed that fructose is converted more efficiently into mannitol. Therefore residual glucose which was left over was isomerized to produce additional 38 g/l fructose by immobilized isomerase, which was converted to mannitol using the same cells. A total of 114 g/l mannitol was produced after 30 h. Apart from mannitol 42 g/l of ethanol was produced at the end along with small amounts of erythritol and glycerol. Production of mannitol and other byproducts from glucose-fructose mixture prepared by enzymatic isomerization of glucose is shown in Fig. 5.18. It can be see that on consumption of fructose, the cells rapidly utilized glucose and converted it to ethanol.

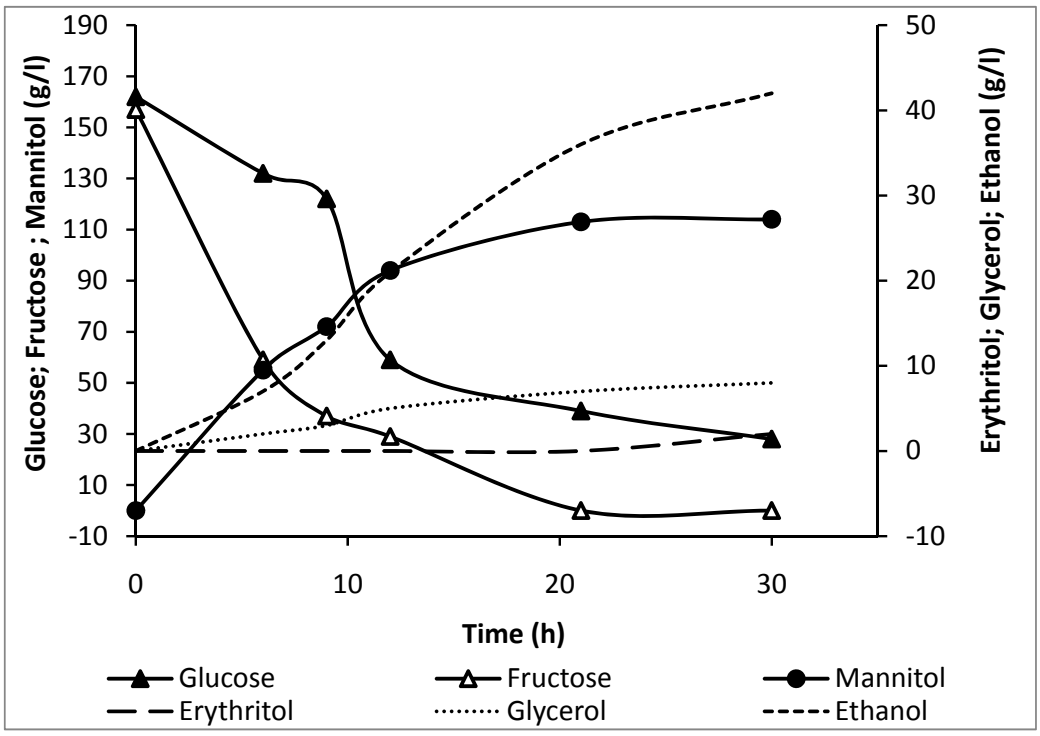


Figure 5.18 Production of mannitol and other byproducts from glucose-fructose mixture produced by enzymatic isomerization of glucose

5.3.11 Membrane cell-recycle bioreactor for mannitol production using acid hydrolyzed sucrose

During the growth phase, aerobic conditions were maintained using higher rpm and aeration rate in the fermenter. Within 30 h of growth phase, the biomass concentration reached about 16 g/l DCW and all the glucose added was consumed. On reaching the desired biomass, the fermentation broth was filtered through hallow fiber cross flow membrane filtration module and the recovered cells were washed with equal volume of saline.

The first bioconversion batch was started with addition of hydrolyzed sucrose and agitation was set at 500 rpm. The fructose got rapidly consumed with rapid increase in the mannitol level after 10 h of lag phase. During this period, ethanol concentration increased up to 20 g/l. Therefore to minimize the ethanol formation at 15 h, agitation was increased to 550 rpm. The ethanol formation rate dropped but, on the contrary, production of another by-product, glycerol, increased. At 35 h, the agitation was increased to 575 rpm. It was observed that fructose in the fermenter was completely consumed in 42 h incubation. The final mannitol concentration in the fermenter was 51 g/l, while 138 g/l glucose remained unutilized.

Permeate of microfiltration containing residual glucose, mannitol and other by-products was aseptically transferred to bioconversion reactor II and circulated through a column containing glucose isomerase. When the experiment for isomerization of unutilized glucose to fructose using immobilized isomerase in the fermented broth was performed, it was seen that only 36 g/l glucose could be isomerazed even after circulation for 12 h. This presumably may be because of one of the products formed during bioconversion inhibited the activity of the isomerase enzyme. The suspected isomerase-inhibitory products were mannitol, glycerol and ethanol since they were at a high concentration in the supernatant. On evaluating the effect of the above by-products on isomerization of glucose to fructose, it was found that in the presence of 50 g/l glycerol in the medium the isomerase activity was substantially inhibited.

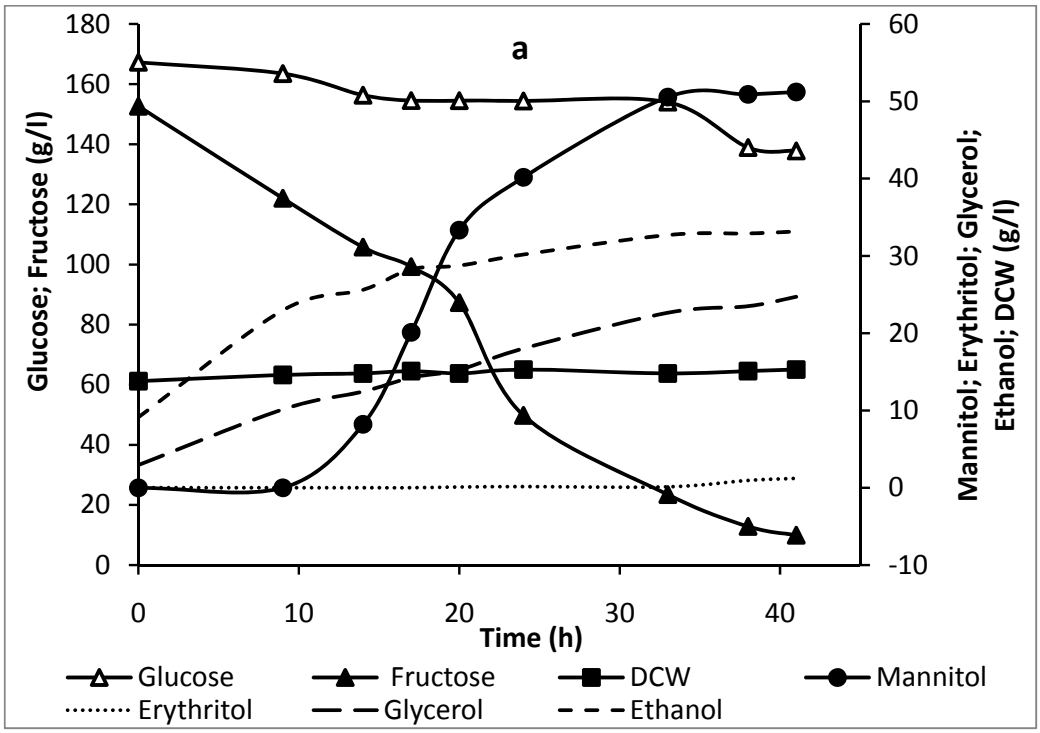
In the second cycle of bioconversion of hydrolyzed sucrose solution to mannitol, on feeding of hydrolyzed sucrose, the agitation was increased to 600 rpm. Fructose in the broth was consumed rapidly to produce mannitol with comparatively high levels of

glycerol as by-product. Ethanol remained at a very minimal level in the fermenter. After all the fructose was consumed by 60 h, the rate of mannitol production slowed down. The final mannitol concentration in the fermenter was 60 g/l while 115 g/l glucose remained unutilized. Finally, 82 g/l of glycerol was produced and the aerobic conditions led to glycerol formation.

In the third bioconversion cycle, the agitation rate was adjusted to 400 rpm to minimize glycerol formation, due to which only 23 g/l mannitol was produced by consuming 101 g/l fructose. Although the glycerol formation decreased to 18 g/l, ethanol formation increased to 41 g/l therefore the batch was terminated at 26 h.

In conclusion, highly aerobic conditions during bioconversion lead to formation of erythritol, as by-product while in oxygen limiting conditions, ethanol was formed. These results indicate DO concentration and residual glucose governs direction of carbon flow towards mannitol production or undesirable by-products. A photograph of MCRB assembly used is presented as Fig. 5.19. Production of mannitol and other byproducts from hydrolyzed sucrose in MCRB-batch I and II is shown in Fig. 5.20a, b.

Figure 5.19 Photograph of MCRB setup



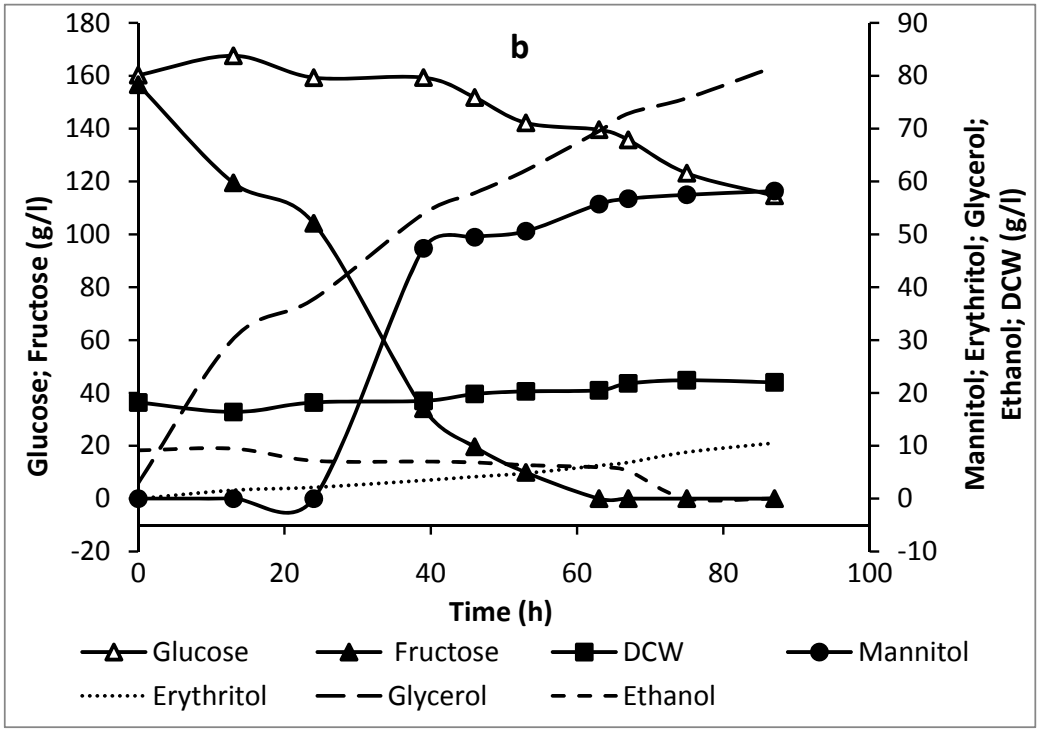


Figure 5.20a, b Production of mannitol and other byproducts from sucrose hydrolysis in MCRB-batch I and II.

5.3.12 Characterization of *C. magnoliae* mutant R9

Phase contrast and SEM of parent and mutant are shown in Fig. 5.21. The cells of mutant were slightly larger in diameter as compared to parent and generally grew in clumps. Carbohydrate utilization pattern of *C. magnoliae* and mutant R9 presented in Table 5.6 revealed that both parent and mutant strains have ability to utilize fructose, glucose, glycerol, mannose, mannitol, ribose and sucrose for growth and they do not have ability to utilize arabinose, cellobiose, galactose, maltose, melezitose, melibiose, raffinose, rhamnose, trehalose and xylose. The above observations suggest that the carbohydrate utilization pattern of *C. magnoliae* and mutant R9 was similar to that of *C. magnoliae* NCYC 2620.

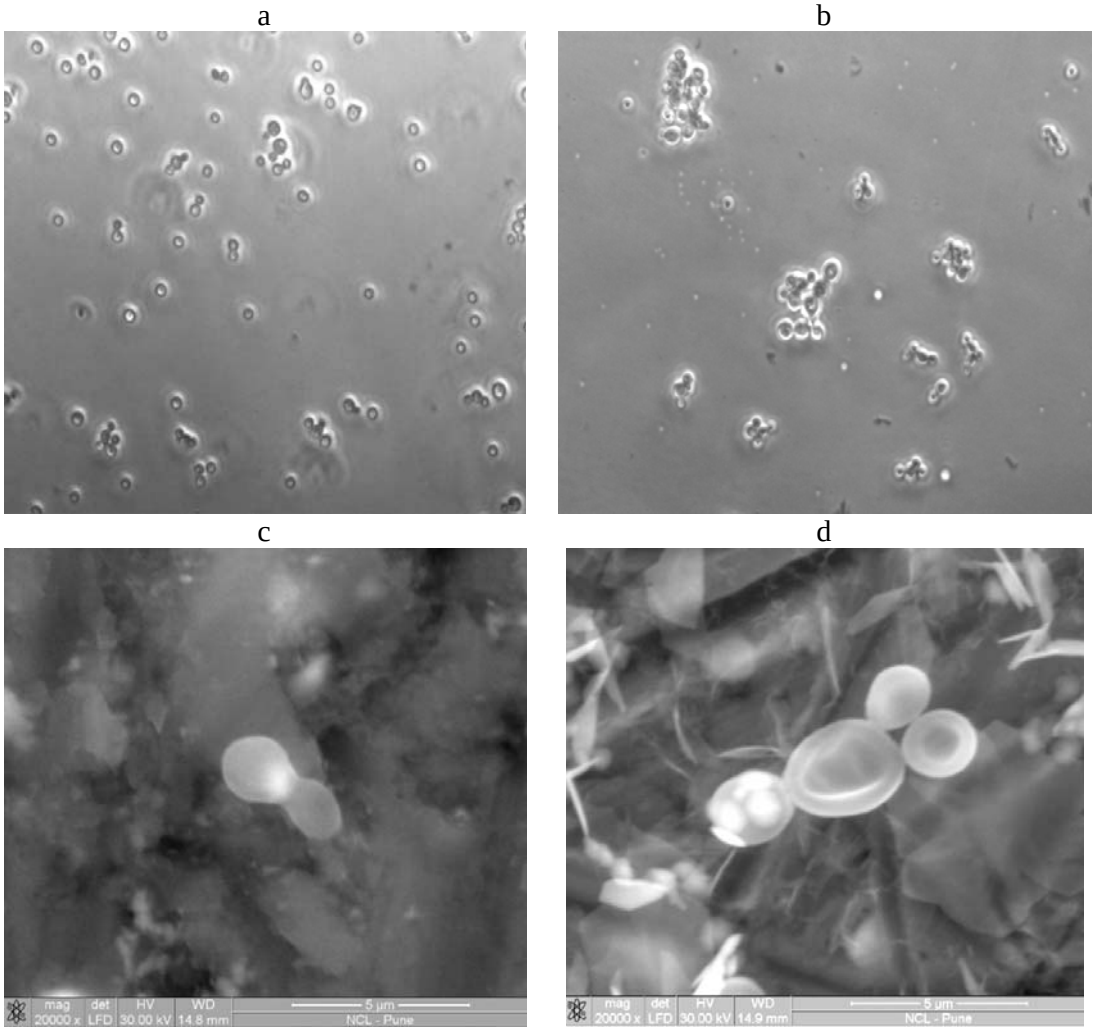


Figure 5.21 Phase contrast and scanning electron microscopy of A, C parent and B, D mutant respectively.

Sugar	*C. magnoliae 3470 (O.D. 600)*	*Mutant R9 (O.D. 600)*
Arabinose	-	-
Cellobiose	-	-
Fructose	25	24
Galactose	-	-
Glucose	30	20
Glycerol	38	40
Maltose	-	-
Mannose	28	20
Melibiose	-	-
Melezitose	-	-
Raffinose	-	-
Rhamnose,	-	-
Ribose	40	28
Sucrose	29	25
Trehalose	-	-
Xylose	-	-

Table 5.6 The carbohydrate utilization pattern of *C. magnoliae* and mutant R9

5.3.13 Study on mannitol dehydrogenase

MDH is the key enzyme in the biosynthesis of mannitol, therefore studies were undertaken to determine whether MDH from mutant R9 plays a role in increased production of mannitol compared to its parent strain. MDH activity in the enzyme preparation of mutant R9 was reasonably higher at all the time points as compared to MDH extracted from parent strain grown under identical conditions. Maximum activity of 7933 U/g WCW was observed at 96 h. On the contrary, MDH activity in parent strain reached 3279 U/g WCW at 96 h (Fig. 5.22).

These values represent, 2.4 fold increase in MDH activity compared to parent strain under identical conditions which may be due to increase in activity or expression level of MDH enzyme.MDH is present in a number of plants and microorganisms. It catalyzes the oxidation and reduction of D-mannitol and D-fructose (Niehaus Jr &

Dilts Jr, 1982; Saha, 2004). In *C. magnoliae*, mannitol is synthesized from fructose by a reaction catalyzed by NAD(P)H dependent mannitol dehydrogenase (Lee et al., 2003a).

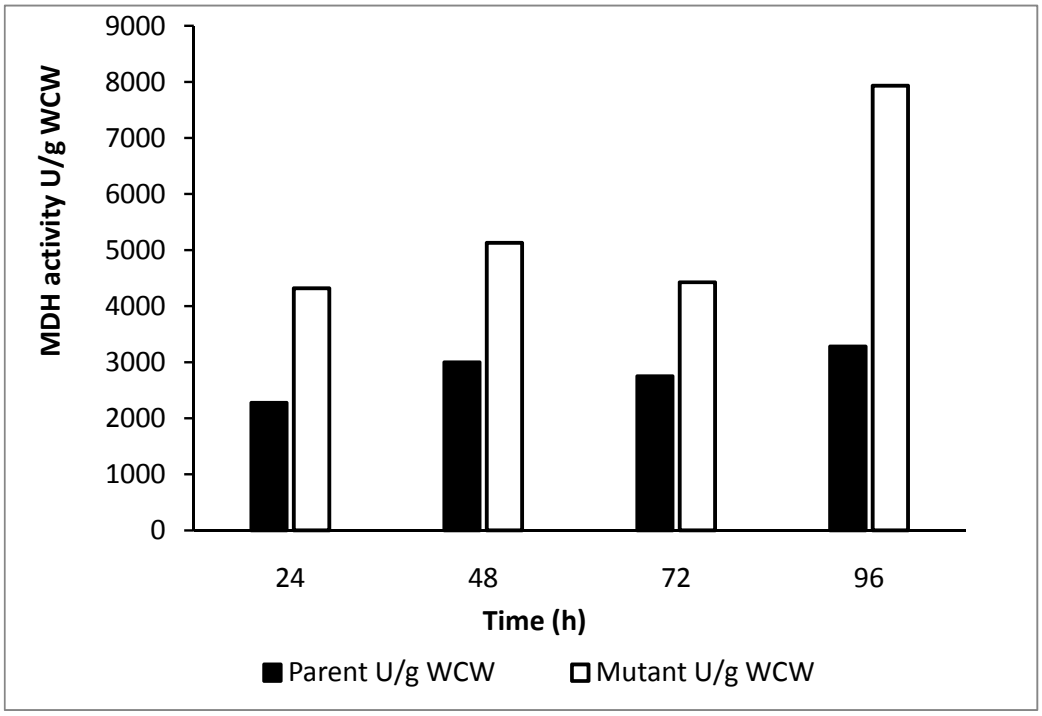


Figure 5.22 Comparison of MDH from parent and mutant R9

5.3.13.1 Optimum pH and temperature

The optimum pH for fructose reduction by MDH was 6, with 94.1 and 74.5% of the maximum activity at pH 5.0 and 7.0, respectively. The optimum pH for mannitol oxidation was 8.0, with 61.8 and 5.5% of the maximum activity at pH 7.0 and 9.0, respectively (Fig. 5.23). Maximal fructose reduction activity at pH 6.0 and an alkaline pH optimum for mannitol oxidation are common features of MDH isolated from diverse microbial cultures (Lee et al., 2003a; Stoop et al., 1998; Trail & Xu, 2002). In the present investigation, the optimum temperatures for the reductive and oxidative reactions were 70 and 60°C, respectively. This is a striking feature of MDH obtained from this mutant compared to MDH from *C. magnoliae* HH1 with an optimum temperature of 37 and 40 °C (Lee et al., 2003a). In thermal stability study, interestingly it was observed that at temperature 30, 40 and 50°C almost 100 % of its initial activities were retained even after 3.5 h (Fig. 5.24).

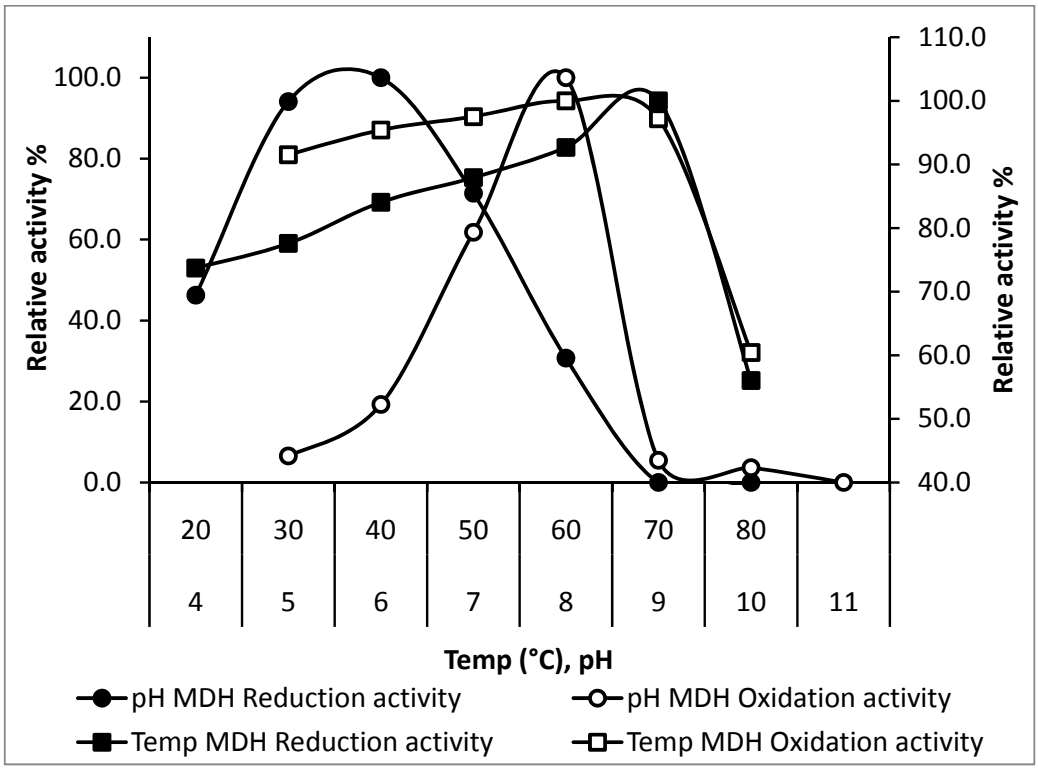


Figure 5.23 Effects of pH and temperature on the oxidation and reduction activity of MDH from mutant R9

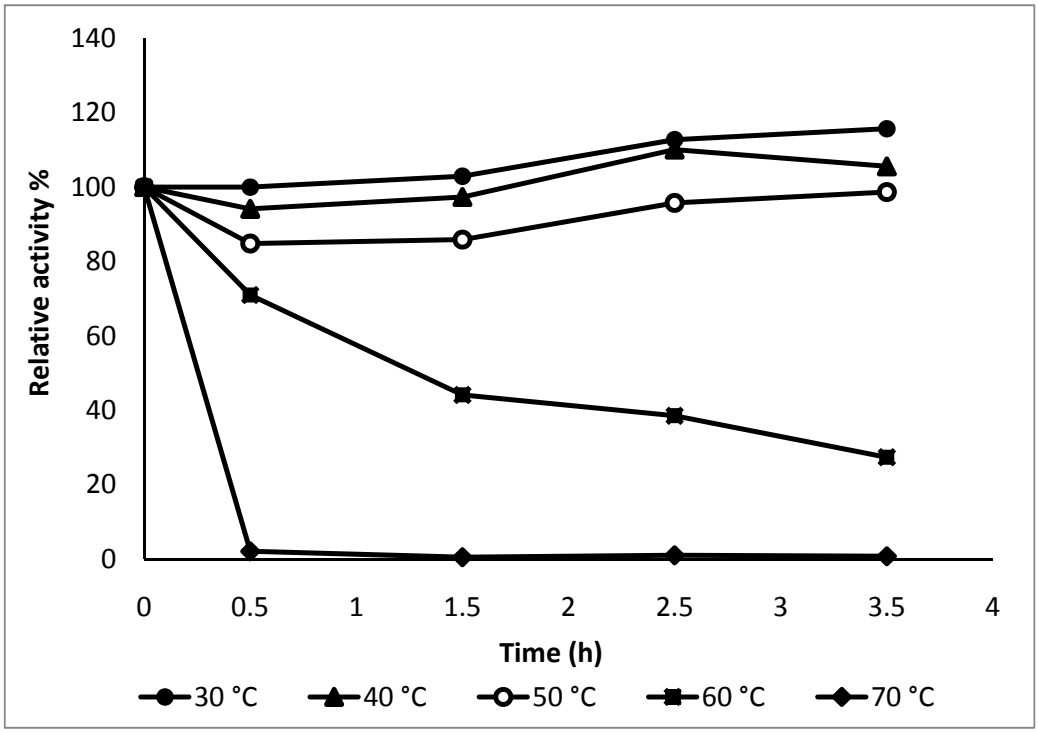


Figure 5.24 Thermal stability of MDH from mutant R9 at various temperatures

5.3.13.2 Substrate and co-substrate specificity

The rate of mannitol oxidation by present MDH was 3.5% of fructose reduction activity at pH 5. Although it is known and also observed in our experiments that the mannitol oxidation activity increases in alkaline pH, it has no practical meaning in living cells because the pH inside cells is slightly acidic. Therefore, MDH is considered to catalyze reduction of fructose to mannitol exclusively. Most of the mannitol oxidizing and reducing enzymes described till date, are pyridine nucleotide linked, requiring either NADH or NADPH as a co-substrate. MDH from *C. magnoliae* mutant R9 showed affinity only for NADPH and no activity was observed when NADH was used as co-substrate in the presence of 10 mM fructose. When reduction of arabinose, glucose, galactic, sucrose, mannose, ribose, trehalose and xylose, (all at 10 mM), with NADPH as a co-substrate, were examined it was found that MDH had a high preference only for fructose (Fig. 5.25). When oxidation of mannitol, erythritol, xylitol, glycerol sorbitol and ribitol was studied it could only oxidize mannitol (Fig. 5.26).

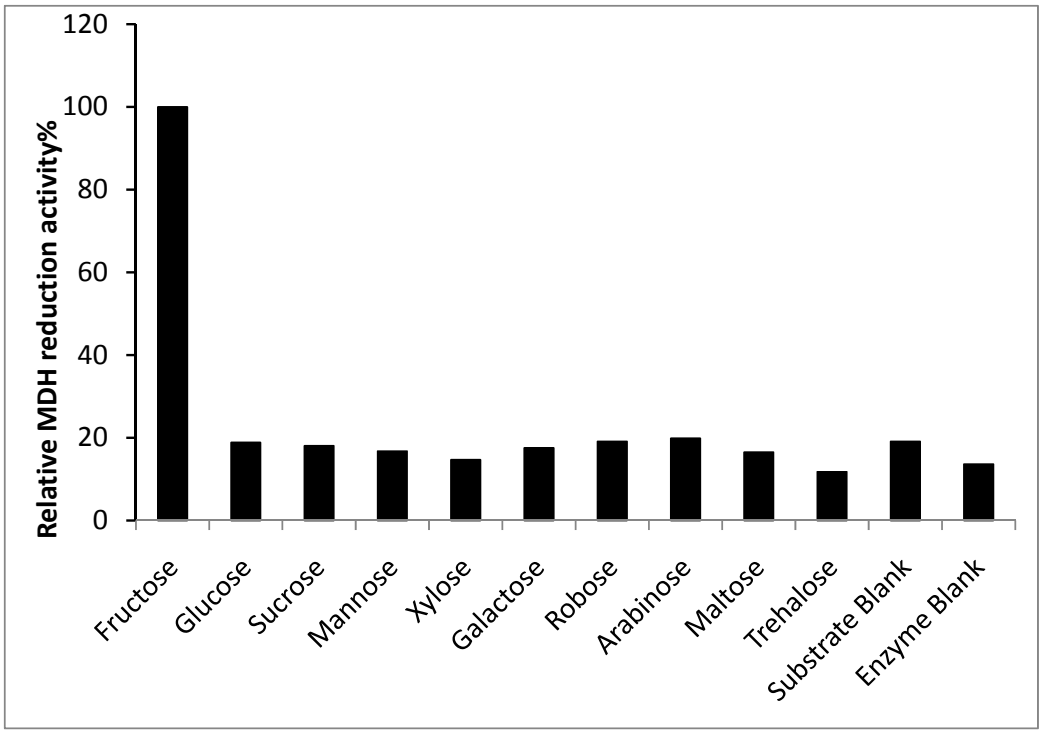


Figure 5.25 Substrate specificity for MDH reduction activity from mutant R9

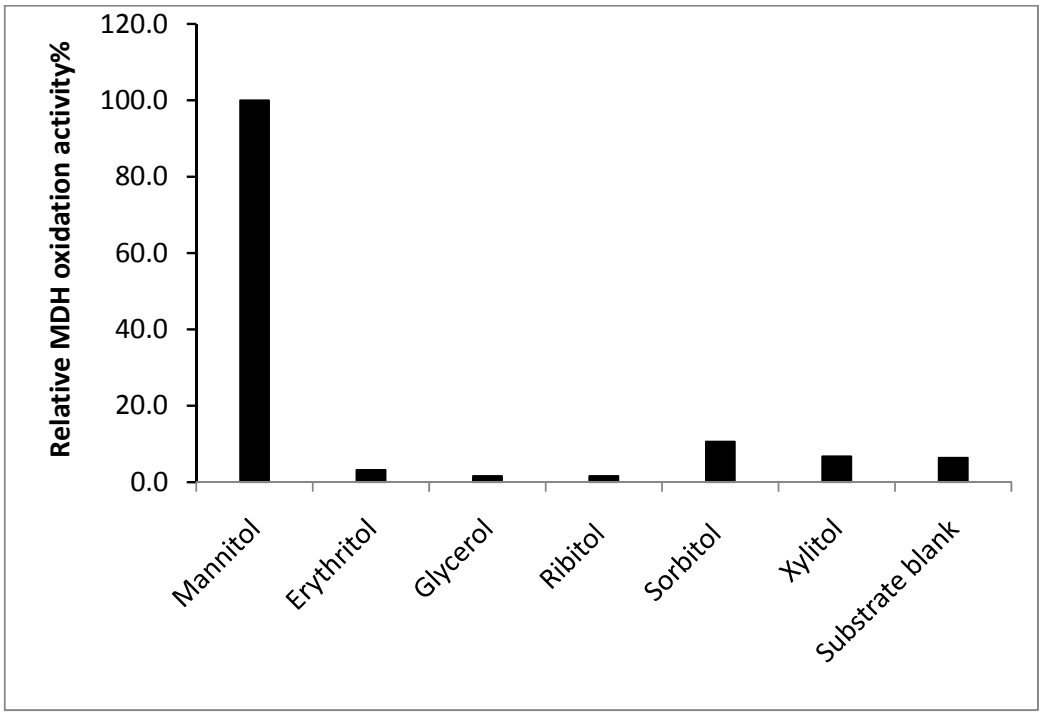


Figure 5.26 Substrate specificity for MDH oxidation activity from mutant R9

5.3.13.3 Effects of metal ions and various compounds

MDH activity in the present investigation was not affected by Ca^{+2}, Mn^{+2}, Mo^{+2}, Ni^{+2}, B^{+2} or Fe^{++} wherein, Co^{+2} $Cu+^{2}$ and Zn^{+2} ion relatively showed significant inhibition of MDH activity, 59.2, 53.9 and 49.5%, respectively. MDH was neither inhibited nor activated by EDTA, mercaptoethanol, cysteine, or dithiothreitol at 10 mM Table 5.7.

Chemical	***Relative MDH activity%***
None	100
Copper (1mM)	59.2
Zinc (1mM)	53.9
Cobalt (1mM)	49.5
Calcium (1mM)	99.7
Manganese (1mM)	96.5
Molybdenum (1mM)	98.6
Nickel (1mM)	95.8
Boron (1mM)	102.2

Ferric chloride	99.2
BME (10mM)	91.6
EDTA (10mM)	95.3
DTT (10mM)	90.6
Cysteine (10mM)	97.5

Table 5.7 Effects of metal ions and reducing agents on MDH reduction activity

5.3.13.4 Kinetics

MDH had hyperbolic saturation curve with increasing fructose concentrations representing typical Michaelis-Menten-type kinetics Fig. 5.27. Lineweaver-Burk plot for the conversion of D-fructose to mannitol showed the *Km* 27.7 mM (Fig. 5.28). When NADPH was used as a variable substrate, similar straight lines intersecting in the left quadrant were obtained (Fig. 5.29) indicating that the reaction proceeds via the formation of a ternary complex of the enzyme with NADPH and D-fructose. MDH obtained from our mutant R9 has a high affinity for the substrate. Its *Km* value of 27.7 mM for fructose is comparable to previously reported 28.0 mM of *C. magnoliae* HH1, used industrially for mannitol production (Lee et al., 2003a).

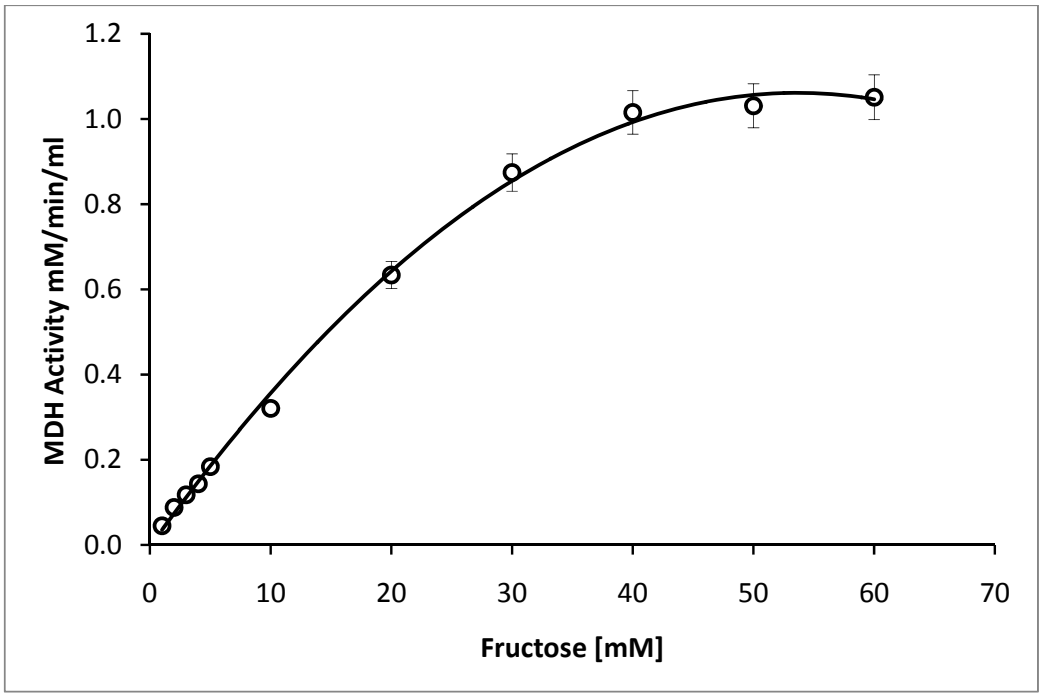


Figure 5.27 Effect of fructose concentration on MDH reduction activity of mutant R9

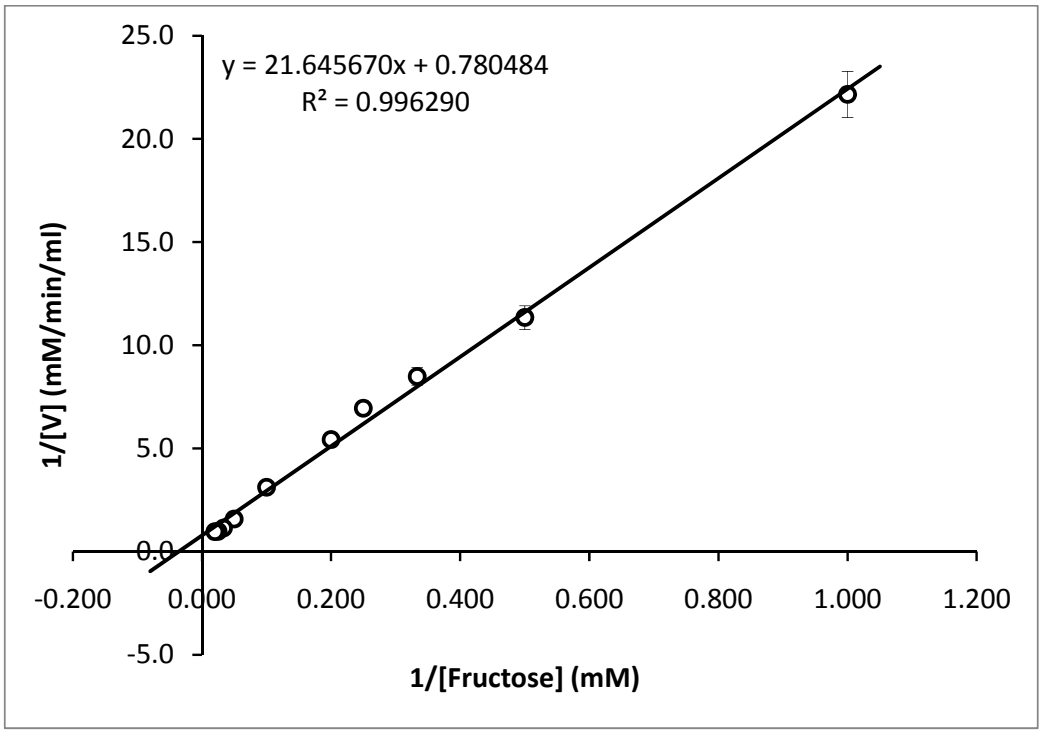


Figure 5.28 Lineweaver-Burk plot of initial velocity versus fructose concentrations

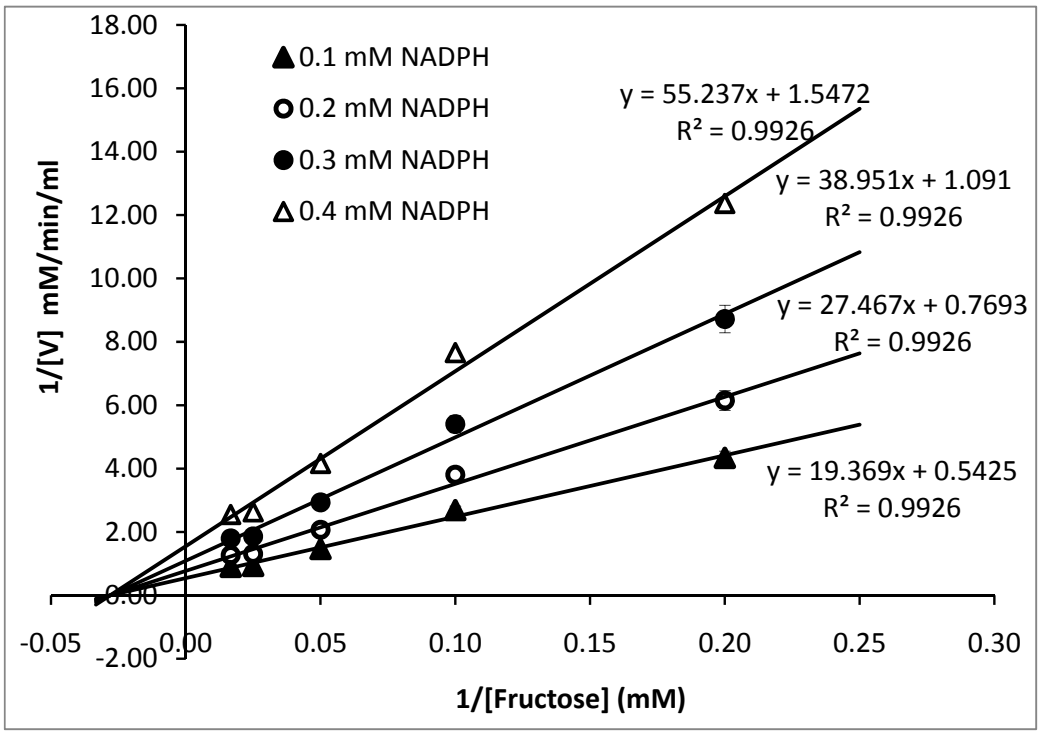


Figure 5.29 Double reciprocal plots for MDH at varying NADPH concentrations

5.4 CONCLUSIONS

There are no reports on mannitol production from glucose which is relatively cheap substrate for mannitol production. The mutant had a potential for further improvement in mannitol production by optimization media and fermentation parameters. RSM method of medium optimization was applied for mutant R9 to study combined effect of the effective media components. The optimal media composition for enhanced mannitol production was (g/l) glucose 300.5, yeast extract 10.8, KH_2PO_4, 1.24 and $MgSO_4$ 0.34. In statistically-optimized medium, the mutant produced 60 g/l mannitol with 20% yield. Mannitol production could not be improved further even on optimizing fermentation parameters. However, the cells could convert fructose into mannitol even after growing initially in a glucose containing environment. This led to the use of glucose-fructose mixture for mannitol production, generated by inversion of sucrose or by isomerization of glucose. From glucose-fructose mixture produced by the enzymatic isomerization of glucose, 114 g/l mannitol was prepared after 30 h. Acid hydrolyzed sucrose a cheap substrate available locally was evaluated for mannitol production in MCRB. The same cells were successively used for three bioconversion reaction in MCRB, but mannitol concentration yield could not be improved. Isomerization of glucose to fructose in MCRB setup posed difficulty because of the presence of by-products ethanol and glycerol and a better control over fermentation is necessary to minimize the by-products formation.

To ascertain the exact mechanism of mannitol overproduction by mutant R9, activity of the MDH involved in mannitol biosynthesis was compared in parent and mutant strain. Under identical growth conditions at 96 h, MDH activity of mutant R9 was about 2.4 fold higher than that of the parent strain. The MDH in cell extract showed specificity only for fructose and NADPH. Other substrate and co-substrate in the enzyme preparation negligibly interfered with the activity. MDH obtained from mutant R9 had a *Km* value of 27.7 mM for fructose which was comparable to previously reported 28.0 mM of *C. magnoliae* HH1 which is used industrially for mannitol production from fructose. This led to hypothesis that the same mutant could be potential candidate for mannitol production using fructose.

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Chapter 6

Production of Mannitol by *Candida magnoliae* Mutant R9 from Fructose

Abstract:

This chapter deals with optimization of culture conditions for mannitol production from fructose by growing and resting cells of the mutant R9 in shale flask and laboratory fermenter. Optimization of two-stage fermentation process resulted in production 240 g/l mannitol, highest ever reported, from 300 g/l fructose with a fairly high volumetric productivity of 4 g $l^{-1}h^{-1}$ and 81.2% yield.

Resting cells of mutant R9 were successfully employed to produce 200 g/l mannitol for five successive cycles in an aqueous 300 g/l fructose solution with 7.3 g l^{-1} h^{-1} productivity without formation of any by-products. Moreover, the resting cells could also convert 600 g/l inverted sucrose solution to 204 g/l mannitol. Mannitol production by resting cells was mainly dependent on biomass concentration and temperature.

In two-stage fermentation, using chemically defined medium, 172 g/l mannitol was produced with 84.4% yield and 1 g $l^{-1}h^{-1}$ productivity. The purified mannitol was characterized using HPLC, LC-MS and ^{13}C NMR. About 99% pure mannitol crystals were obtained by using a simple purification protocol comprising activated carbon treatment, concentration and crystallization.

6.1 INTRODUCTION

Several filamentous fungi produce mannitol from various carbon sources. Smiley et al., (1967) reported mannitol production from *Aspergillus candidus* using glucose. Similarly, Hendriksen et al., (1988) found *Penicillium scabrosum* producing mannitol and glycerol from sucrose. The volumetric mannitol productivity of 0.14 g l^{-1} h^{-1} was similar to that reported for *A. candidus*. However, yield 56.7 mol% was better. Both Smiley et al., (1967) and Hendriksen et al., (1988) noted that when the initial sugar was consumed, the cells started to utilize the sugar alcohols produced.

Onishi & Suzuki, (1968) isolated yeast species belonging to genus *Torulopsis* and they found *Torulopsis versatilis* to be good mannitol producer. A few years later, they reported another species, *T. mannitofaciens*, exclusively producing mannitol from both glucose and glycerol (Onishi & Suzuki, 1970). The yield of mannitol from glycerol with *T. mannitofaciens* was about 31 mol%. Using glucose as the carbon source, a volumetric mannitol productivity of 0.23 g l^{-1} h^{-1} was obtained with this yeast. In summary, although yeast and fungi have the ability to produce mannitol from glucose, the volumetric productivities achieved are simply too low for industrial production.

Till date, only one research group from Korea has got promising results on mannitol production using *Candida magnoliae* HH-01, which was isolated from fermentation sludge by screening of over 1,000 microorganisms. In flask culture, in a medium containing fructose, it yielded 67 g/l mannitol after 168 h. In fed-batch culture with fructose, production of mannitol reached a maximum of 209 g/l in 200 h (Song et al., 2002). Improvements of the fed-batch conversion process have since included co-feeding glucose with fructose and supplementing the culture with Ca^{+2} and Cu^{+2} (Baek et al., 2003; Lee et al., 2007; Lee et al., 2003). In *C. magnoliae* mannitol is produced from fructose by an NADP-dependent mannitol dehydrogenase. The co-feed of glucose, rather than fructose was used for cell maintenance and NADPH regeneration (Lee et al., 2003). Although, final mannitol concentration 226 g/l was among the highest reported, volumetric productivity 1.94 g $l^{-1}h^{-1}$ was relatively low.

The use of resting cells has became a common approach in order to obtain pure compounds with fewer production steps, higher yields and cleaner processes as

compared to traditional strategies of chemical synthesis or production by growing cells (Marques et al., 2010). A system which uses growing cells is more complex, since they require fulfillment of both growth and bioconversion requirements along with aseptic conditions. Besides, a large number of compounds other than the intended product are usually released into the media. Bioconversion processes that rely on resting cells, recovered from a suitable fermentation broth, require no sterile environment and are usually performed in a simple aqueous buffered solution containing the substrate without any media ingredients.

Mutant R9, developed from mutagenesis of *C. magnoliae*, which produced mannitol from glucose, was described in chapter 5. In an optimized fermentation process the mutant R9 produced 60 g/l mannitol, with 0.62 g l^{-1} h^{-1} productivity and 20% yield from 300 g/l glucose. Although, the mutant R9 produced mannitol from glucose, the volumetric productivity is too low for industrial production.

In previous chapter, it was presented that, resting cells of mutant R9, grown in glucose containing medium, could effectively convert fructose to mannitol. Literature review on mannitol production, from fructose, led to the hypothesis that the same mutant strain may be useful for mannitol production from fructose. The present chapter deals with optimization of culture conditions for mannitol production from fructose by growing and resting cells of the mutant R9 in shale flask and laboratory fermenter. Purification and chemical characterization of mannitol from the fermentation broth is also presented.

6.2 MATERIALS AND METHODS

All media ingredients, strain and analytical methods were same as described in chapter 5 unless otherwise mentioned. A loop-full of R9 mutant from freshly prepared slant was inoculated into 25 ml growth medium in 250 ml Erlenmeyer flask and incubated at 28 °C, 210 rpm, for 48 h. The above seed culture 5% (v/v) was transferred either to a 250 ml Erlenmeyer flask containing 25 ml fermentation medium or 2.5 L fermenter with 2 L working volume (Brunswick Scientific Bio-Flo 115). The growth medium was composed of (g/l) glucose 50, yeast extract 10, KH_2PO_4 5 and $MgSO_4 \cdot 7H_2O$ 0.25. The production medium contained 250 or 300 g/l glucose or fructose in the above medium.

6.2.1 Production of mannitol from fructose by growing cells

6.2.1.1 Comparison of mannitol production from fructose by *C. magnoliae* and mutant R9

To compare the mannitol producing mutant R9 with parent *C. magnoliae* NCIM 3470, respective seed cultures were grown for 24 h in growth medium in 250 ml Erlenmeyer flasks. These inoculums (5% v/v) were transferred to four Erlenmeyer flasks containing 25 ml growth medium and incubated for 48 h. The broths were pooled together and 25 ml of respective cultures were redistributed in flask containing sterile fructose powder to achieve 300 g/l fructose concentration and incubated on shaker at 28°C at 210 rpm. Samples were withdrawn at 24, 48, and 72 h intervals and analyzed for mannitol using HPLC.

6.2.1.2 Two-stage fermentative production of mannitol by mutant R9

Seed culture 5% (v/v) grown for 24 h in growth medium in 250 ml Erlenmeyer flasks was transferred to a 2.5 L fermenter with 1.7 L of growth medium containing 20 g/l yeast extract. Temperature was maintained to 28 °C. The pH of the culture broth was maintained at 5 with 5N NaOH. The dissolved oxygen tension (DO) was measured with an oxygen probe (Mettler Toledo). The agitation speed was adjusted between 400-600 rpm and aeration rate was 0.5 vvm, to keep the level of dissolved oxygen concentration above 10% of air saturation till the biomass reached stationary phase, as monitored by online OD probe. The production phase was initiated by disconnecting air, reducing the agitation to 300 rpm and addition of fructose powder to reach 300 g/l concentration.

To further increase mannitol productivity by increasing biomass concentration, the content of glucose and yeast extract in growth medium was increased to 100 and 30 g/l, respectively. During the growth phase, the fermenter was run in fed-batch mode by feeding nitrogen source till 20 h. Agitation and aeration rates were adjusted between 500-700 rpm and one vvm, respectively, in order to avoid the DO limitation. During the growth phase, temperature was maintained at 28 °C. The temperature was raised to 35°C during production phase in order to overcome the solubility limit of mannitol. Samples were withdrawn at fixed time interval and analyzed for optical density at 600 nm, pH, residual substrate and the end products using HPLC.

6.2.2 Production of mannitol by resting cells of *Candida magnoliae* mutant R9

6.2.2.1 Preparation of resting cells

During optimization of mannitol production using growing cells, simultaneous experiments were carried out to evaluate production of mannitol by using resting cells physically separated from the fermentation broth. A loop-full of mutant R9 culture from freshly prepared slant was inoculated into 25 ml growth medium in 250 ml Erlenmeyer flask and incubated at 28 °C, 210 rpm, for 36 h. The broth was aseptically centrifuged at 10000 *g* for 10 min. The cell pellet was washed twice with sterile physiological saline by centrifugation and re-suspension. Fresh cells were prepared for each experiment. Carbon sources were autoclaved separately for all the experiments. All experiments were performed in triplicate and the values were presented as a mean of triplicate experiment. Viability of cells was checked intermittently by methylene blue staining procedure. One hundred micro-liter cell suspension was mixed with with 0.1 ml methylene blue solution (10 mg/ml) and incubated for 10 min at room temperature. The cells were observed microscopically and classified as blue and colourless, representing dead and alive cells, respectively.

6.2.2.2 Choice of carbon source for mannitol production by resting cells

Resting cells (3 g wet weight) were suspended in 25 ml sterile aqueous solutions of glucose, fructose, sucrose, glucose-fructose mixture and glycerol (300 g/l) individually, in 250 ml Erlenmeyer flasks and incubated on a rotary shaker. Effect of glucose-fructose ratio on the yield of mannitol was investigated by suspending 3 g resting cells to various combinations of fructose-glucose mixture maintaining total sugar concentration at 300 g/l.

6.2.2.3 Effect of resting cell mass concentration and temperature on mannitol production

To investigate effect of cell mass concentration on mannitol production, 0.5-3 g of freshly grown wet resting cells of mutant R9 were weighed aseptically and suspended in 25 ml sterile fructose solution (300 g/l) in 250 ml Erlenmeyer flasks and incubated at 220 rpm, 28 °C for 96 h. Effect of temperature on the conversion of fructose to mannitol was investigated by aseptically suspending equal quantities (3 g) of freshly

grown resting cell to 250 ml Erlenmeyer flasks containing 25 ml sterile fructose solution (300 g/l) and by incubating each set of flasks at different temperatures *viz* 28, 33, 37, 42 and 48 °C.

6.2.2.4 Repeated batch bioconversion

Repeated batch bioconversion was investigated at 28, 37 and 48 °C by known amount of resting cells suspended in 25 ml aqueous fructose solution (300 g/l). Periodic samples were withdrawn and analyze for fructose and mannitol concentration. When all the fructose was consumed, cells were separated by centrifugation at 10000 *g* for five min and re-suspended in fresh fructose solution for the next cycle of batch fermentation. Three g wet cell mass was used at 37 and 48 °C where as five gram of cell mass was used for the bioconversion at 28 °C in order to maintain higher volumetric productivity.

6.2.3 Production of mannitol from hydrolyzed sucrose by mutant R9

The price of fructose is significantly higher in the world market. Therefore, from a mannitol production perspective, it would be desirable to find alternative sources of fructose. Sucrose, a disaccharide of glucose and fructose, is considerably less expensive than fructose and could be used in the bioconversion, instead of pure fructose. In preliminary experiments with equal ratio of glucose:fructose mixture, 111 g/l mannitol was produced by the mutant. Mannitol production using sucrose was investigated by suspending 3 g resting cells in 25 ml acid hydrolyzed sucrose solution (600 g/l) and incubation on rotary shaker at 37 °C. Samples were withdrawn periodically and analyzed as described above.

6.2.4 Production of mannitol by mutant R9 in chemically defined medium

Experiments on resting cells of mutant R9 confirmed that mannitol can be produced from fructose in aqueous solution without any media ingredients. In order to produce mannitol economically at industrial level, replacing expensive nitrogen source by cheap inorganic nitrogen source would be preferred. Previously, we have developed chemically defined medium for mutant M572 and the medium was evaluated in shake flask for growth and erythritol production (chapter 3). Use of such minimal medium resulted in 14 g/l DCW with $(NH4)_2SO_4$ as sole nitrogen source. To evaluate whether

the biomass produced using chemically defined medium could produce mannitol, mutant R9 was grown in the defined medium and the biomass using this medium was evaluated for mannitol production. Production of mannitol was investigated in 2.5 L fermenter using minimal medium with $(NH_4)_2SO_4$ as sole nitrogen source and 10 mg/l each of the vitamin; biotin, pyridoxine HCl, and thiamine HCl. The production phase was initiated by addition of fructose powder (300 g/l) as described earlier. Fructose utilization rate and mannitol production rates were compared with the batch containing complex medium with yeast extract.

6.2.5 Purification and characterization of mannitol

Cell-free broth of successive repeated batches was pooled together and treated with 1% (w/v) activated charcoal at 90 °C for 10 min under gentle agitation. The activated carbon was filtered out through glass microfiber filter discs (Whatman). A clear solution obtained was evaporated in a rotary evaporator, at 50°C, under vacuum to a mannitol concentration of around 300 g/l. The concentrated solution was then allowed to cool down to 20°C under gentle agitation and seeded with a pinch of mannitol to initiate the crystallization. The solution was then placed in cold room at 4°C, overnight. Brittle white needles of mannitol crystals obtained were filtered out and washed twice with distilled water and dried at 50° for 2 h. The purity of the mannitol was be checked using HPLC and further confirmed by LC-MS and ^{13}C NMR.

6.3 RESULTS AND DISCUSSION

6.3.1 Production of mannitol from fructose by growing cells

The mutant R9, generated from *C. magnoliae* NCIM 3470, exclusively produced mannitol (60 g/l) from glucose with a 20% yield. Production of mannitol could not be increased further by optimization of culture condition in shake flask or at fermenter level. Undoubtedly, the most efficient substrate for mannitol production published in literature is fructose, although glucose and sucrose can also be converted into mannitol but with lower yield. Several microorganisms have been reported to produce mannitol from fructose and small amount of glucose with a very high yield and productivity (Table 6.1).

On evaluating mannitol production from fructose in shake flask by the parent *C. magnoliae* and its mutant R9, there was a distinct difference in fructose utilization and mannitol production by these two cultures (Fig. 6.1). After the growth phase on glucose, mutant R9 could consume 272 g/l of fructose in 73 h production phase whereas parent could consume only 128 g/l fructose. Moreover, the mutant was able to produce 108 g/l mannitol with 39.7% yield and 0.9 g $l^{-1}h^{-1}$ overall productivity which correspond to 8.2 fold increase in mannitol yield and 130 fold increase in productivity compared to parent culture under the identical conditions. As the mutant showed clearly a better mannitol production capability in the flask, it was used for studies in a 2.5 L laboratory fermenter.

Song et al., (2002) have isolated *Candida magnoliae* HH-01 (KCCM-10252), that was used for the industrial production of mannitol. This strain produced up to 223 g/l mannitol from fructose with a yield of 88% upon optimization of media and fermentation conditions (Baek et al., 2003; Lee et al., 2007; Lee et al., 2003). Final mannitol concentrations obtained by this yeast was among the highest reported. However volumetric productivity is low, 1.94 g $l^{-1}h^{-1}$ (Table 6.1).

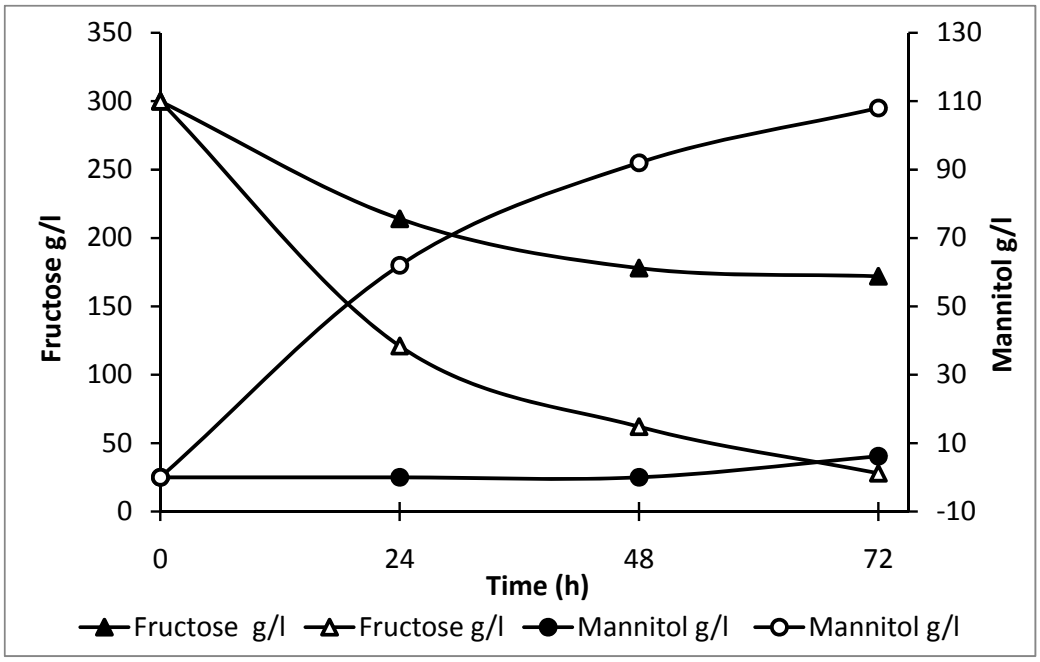


Figure 6.1 Fermentation profile of mannitol production from fructose by *C. magnoliae* and mutant R9 in shake flask. Open symbols for mutant R9 and closed for parent *C. magnoliae*

Culture	***Mode of operation***	***Substrate (g/l)***	***Yield% (g/g fru)***	***Productivity ($g\ l^{-1}h^{-1}$)***	***Mannitol (g/l)***	***Reference***
Lactobacillus sp KY107	Batch	Fructose 100	70	0.87	70	(Yun et al., 1996)
Lactobacillus mesenteroides	Fed-batch	Fru 200	89.9	1.79	179	(Kim et al., 2002)
Lactobacillus fermentum	Batch	Glu 50+Fru 100	89.6	7.6	83	(von Weymarn et al., 2002a)
Leuconostoc mesenteroides	Resting cells MCRB	Glu 50+Fru 100	97	26.2	98	(Von Weymarn et al., 2002b)
Lactobacillus intermedius	Batch Fed-batch	Fructose 300 Glu/fru ratio 1:2	66.1 67.5	1.45 2.2	198.3 202.5	(Saha & Nakamura, 2003)
Leuconostoc mesenteroides	Resting cells MCRB	Glu 50+Fru 100	87	18.8	87	(von Weymarn et al., 2003)
Candida magnoliae HH-01	Fed-batch	Glu 50 fru 250	84	1.94	213	(Lee et al., 2003)
Lactobacillus mesenteroides	Batch	Fru/sucrose 1:1 (150)	87.5	4.7	105	(Saha, 2006a)
Lactobacillus mesenteroides	SSF	Inulin 300 Fru 150+Inulin 250	69 57	2.88 2.1	207 227	(Saha, 2006b)
Lactobacillus intermedius	Fed-batch MCRB	Glu 33.5+Fru 67 Glu 50+Fru 100	94.6 93	5.9 28.4	176 95	(Racine & Saha, 2007)
Candida magnoliae HH-01	Fed-batch	Glu 30 fru 250	88	1.72	223	(Lee et al., 2007)
Lactobacillus mesenteroides	Batch	Cashew apple juice Glu/fru ratio 1:2	66.2	1.8	18	(Fontes et al., 2009)

Table 6.1 Mannitol production by fermentation by different microorganisms

6.3.1.1 Two-stage fermentative production of mannitol by mutant R9

Two stage fermentation process with initial 1.7 l growth medium in a 2.5 L fermenter was employed for mannitol production by mutant R9. During the growth phase, the cells grew exponentially in aerobic condition in glucose based medium and biomass reached 19.8 g/l DCW in 24 h. The growth phase was continued up to 30 h till all the nitrogen was exhausted from broth. Mannitol was not observed during the growth phase. The production phase was initiated by creating anaerobic condition by discontinuing the air flow, reducing the agitation speed and adding fructose powder to reach fructose concentration around 300 g/l. On addition of fructose powder, the biomass concentration decreased to 16.3 g/l DCW due to dilution effect and remained constant till the end of production phase. As shown in Fig. 6.2, fructose was depleted in 60 h with a concomitant production of 202.5 g/l mannitol with 70% yield and 2.2 g $l^{-1}h^{-1}$ overall productivity. These values represent 87 % increase in mannitol concentration, 76% increase in mannitol yield and 150% increase in productivity compared to the experiments performed using mutant R9 in shake flasks.

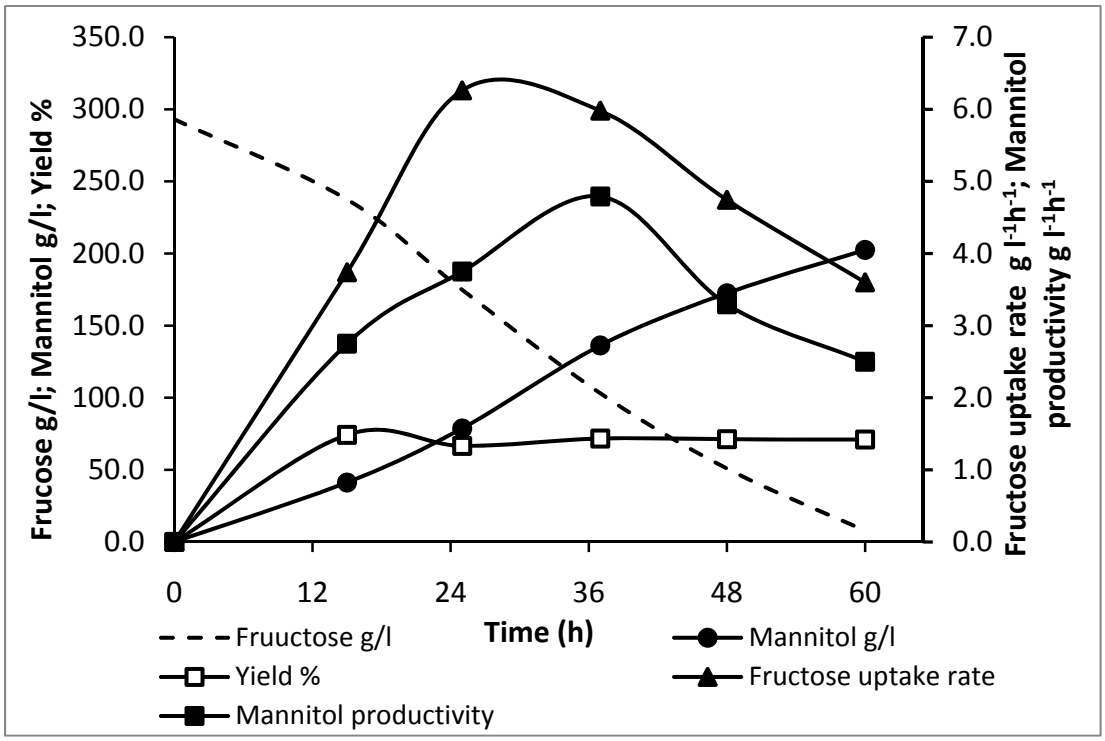


Figure 6.2 Two stage batch fermentation profile of mutant R9 during production phase at 28 °C in 2.5 L fermenter

In order to further increase mannitol yield and productivity, a fed-batch process was employed with initial glucose concentration of 100 g/l and feeding of 30 g/l yeast extract solution till the biomass reached 25 g/l DCW. After 36 h of growth phase, production phase was initiated as described earlier. Production was carried out at 35°C at pH 5.0, to overcome mannitol solubility limit. The biomass concentration during the production phase was 20.3 g/l DCW which was almost constant throughout the production phase.

Final mannitol concentration 240 g/l obtained was (highest among all previously reported organisms) within 24 h of production phase with an excellent yield and productivity of 80% and 10 g $l^{-1}h^{-1}$, respectively. Fermentation profile of mannitol production, substrate uptake rate, productivity and yield during production phase is depicted in Fig. 6.3. Fructose uptake rate and mannitol productivity were at its maximum level 17.4 and 16 g $l^{-1}h^{-1}$ respectively, between t=0 h and t=12 h of production phase. The production rate gradually decreased towards the end of the batch. The overall productivity of 4 g $l^{-1}h^{-1}$ is reasonably higher representing 77% increase in productivity and 14% increase in yield than previous batch.

Although the initial fructose concentration of 300 g/l which was substantially higher than all previously published investigations, the mutant converted fructose to mannitol rapidly during the early production phase. Moreover, it did not consume mannitol even when all supplied fructose had been utilized. Most importantly this mutant did not produce any other polyols or organic acid by-products.

A small amount of ethanol, 15.2 g/l, was observed which can be easily removed. A fermentation process has several advantages compared to the chemical synthesis, such as a complete conversion of fructose to mannitol, absence of side products (like sorbitol) that are difficult to remove, moderate production conditions and no requirement of highly purified substrates (Wisselink et al., 2002).

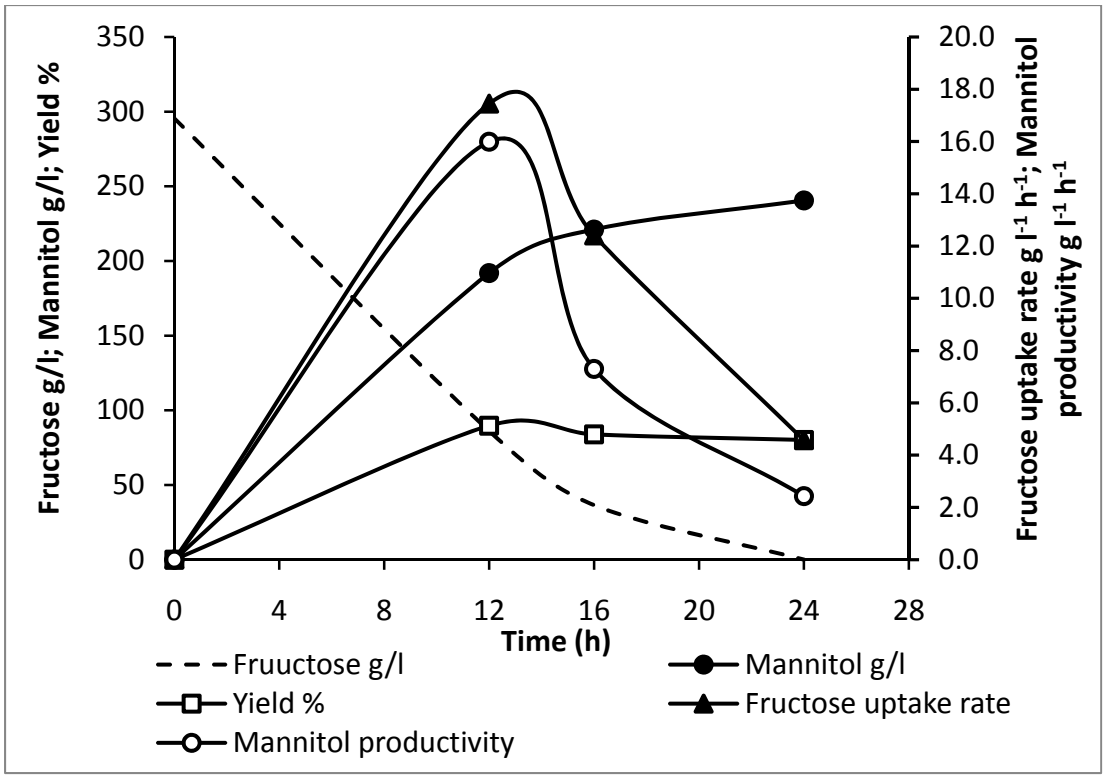


Figure 6.3 Two stage batch fermentation profile of mutant R9 during production phase at 37 °C in 2.5 L fermenter

Mannitol production by fermentation with mutant R9 may be an interesting alternative to the LABs as the present mutant does not produce any organic acid as by-products unlike those by LABs. Apart from this, *C. magnoliae* is not fastidious organism. Thus a simple inexpensive medium supplemented with cheap inorganic nitrogen sources could be used for growth and mannitol production. Moreover, the selected mutant R9 produced 240 g/l mannitol, highest ever reported by a mannitol-producing microorganism with reasonably higher productivity (4 g $l^{-1}h^{-1}$) and yield (81.2%).

At the end of the production phase, with the decrease in temperature of fermentation broth to 28 °C, mannitol crystals were formed within the reactor as seen in Fig. 6.4. The samples withdrawn for analysis in test tube from the fermenter also formed crystals with the reduction in temperature. To my knowledge this is the highest mannitol production by any microorganism reported till date.

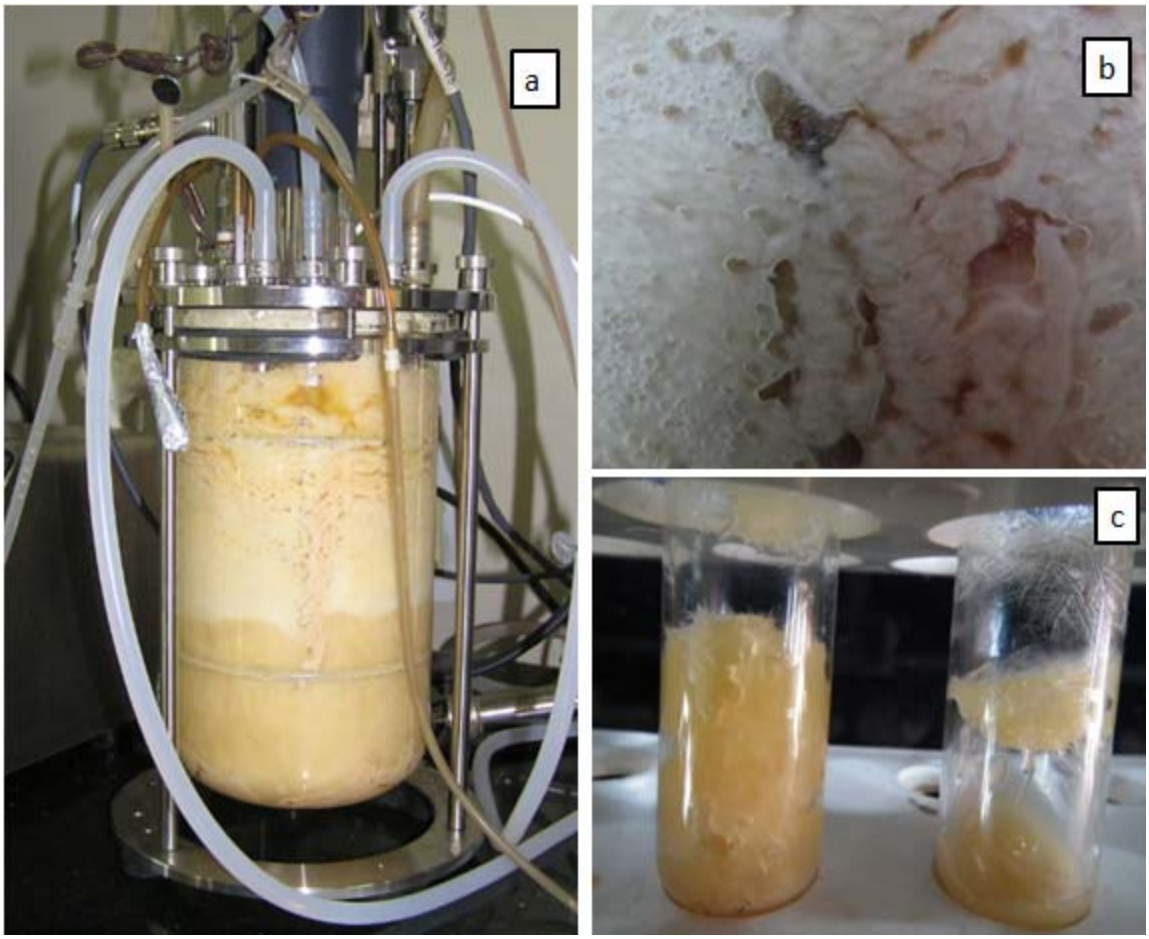


Figure 6.4 Photographs of fermentation broth with mannitol crystals

6.3.2 Production of mannitol by resting cells of *C. magnoliae* mutant R9

Use of whole cells for mannitol production has several advantages over other methods of mannitol production. Compared to the chemical reduction process, only negligible amounts of by-products are formed. Regeneration of cofactors by the intact cells and the general stability of intracellular enzymes make this production mode more favorable. Furthermore, most importantly these conversions are generally carried out in aqueous, buffered solutions so the final product can be easily recovered with low processing cost.

6.3.2.1 Choice of carbon source

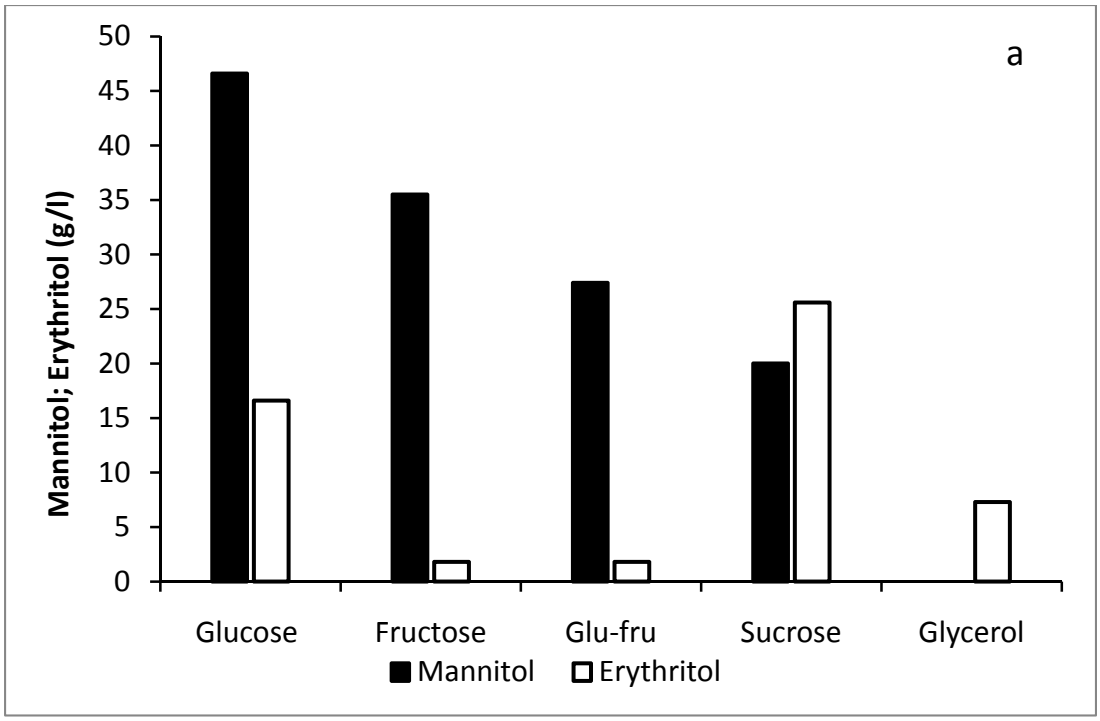
In our laboratory, Khan et al., (2009) investigated that the parent *C. magnoliae* grew well in growth medium at 250 g/l initial sugar concentration and produced a mixture of

erythritol and mannitol in the medium containing glucose and sucrose but produced only mannitol from fructose and glycerol. Resting cells could not consume glucose effectively, whereas fructose and glycerol were completely consumed within 96 h and resulted in 44.5 and 51 g/l mannitol, respectively. In contrast to the earlier results, with 250 g/l initial sugar, growing cells of mutant R9 produced 46.6 g/l mannitol from glucose, 35.5 from fructose, 27.4 from glucose-fructose mixture and 20 g/l from sucrose whereas erythritol was the only metabolite produced from glycerol (Fig. 6.5a).

Surprisingly, resting cells of mutant R9 produced 180 g/l mannitol from fructose, 111 g from glucose-fructose mixture and only 10 g mannitol from glucose. Mannitol was not formed when sucrose or glycerol was used as carbon source. Since fructose was completely consumed within 72 h and resulted in highest mannitol production, subsequently fructose was used as a substrate for mannitol production. In contrast to the earlier experiment where growing cultures produced mixtures of erythritol and mannitol, the resting cells produced only mannitol irrespective of the carbon source (Fig. 6.5b).

A microbial process for efficient mannitol production from fructose requires continuous regeneration of NADPH, the cofactor for mannitol dehydrogenase. The best way to increase mannitol yield is simultaneous transport of a co-substrate along with fructose into the cell. Role of co-substrate (glucose) in maximizing the conversion yield of fructose into mannitol by *C. magnoliae* HH-01 was investigated (Baek et al., 2003). The co-substrate was primarily used for NADPH regeneration and energy supply while fructose was converted to mannitol without being metabolized further.

When effect of glucose-fructose ratio on mannitol production by mutant R9 was studied, conversion of fructose to mannitol was higher when glucose was used as co-substrate than with fructose alone, suggesting that fructose is spared by using glucose to generate NADPH. But 10-20 g/l ethanol was formed when glucose was used as co-substrate. Maximum mannitol yield of 70.4% was achieved when 10 % of fructose was substituted with glucose, as compared to 65.3% yield in control without any glucose (Fig. 6.6). The only metabolite mannitol (185 g/l) was produced when fructose was used. Therefore, fructose was used for subsequent experiments.



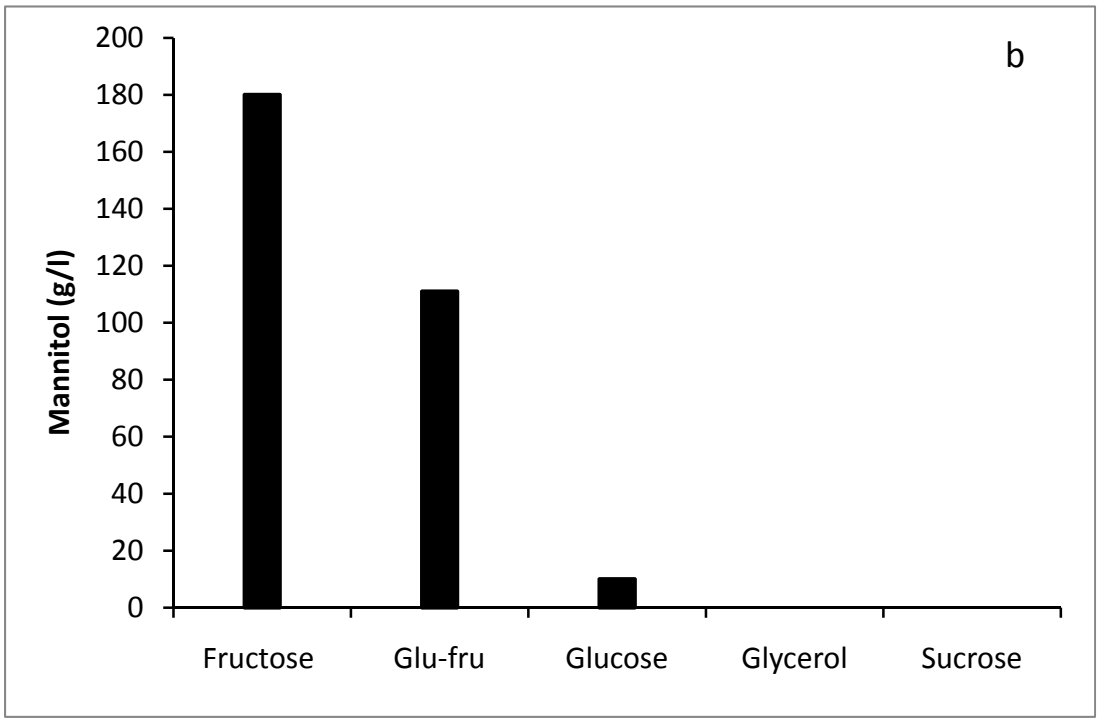


Figure 6.5a, b Polyol production by *C. magnoliae* mutant R9 in different carbon sources a) Growing cells b) Resting cells

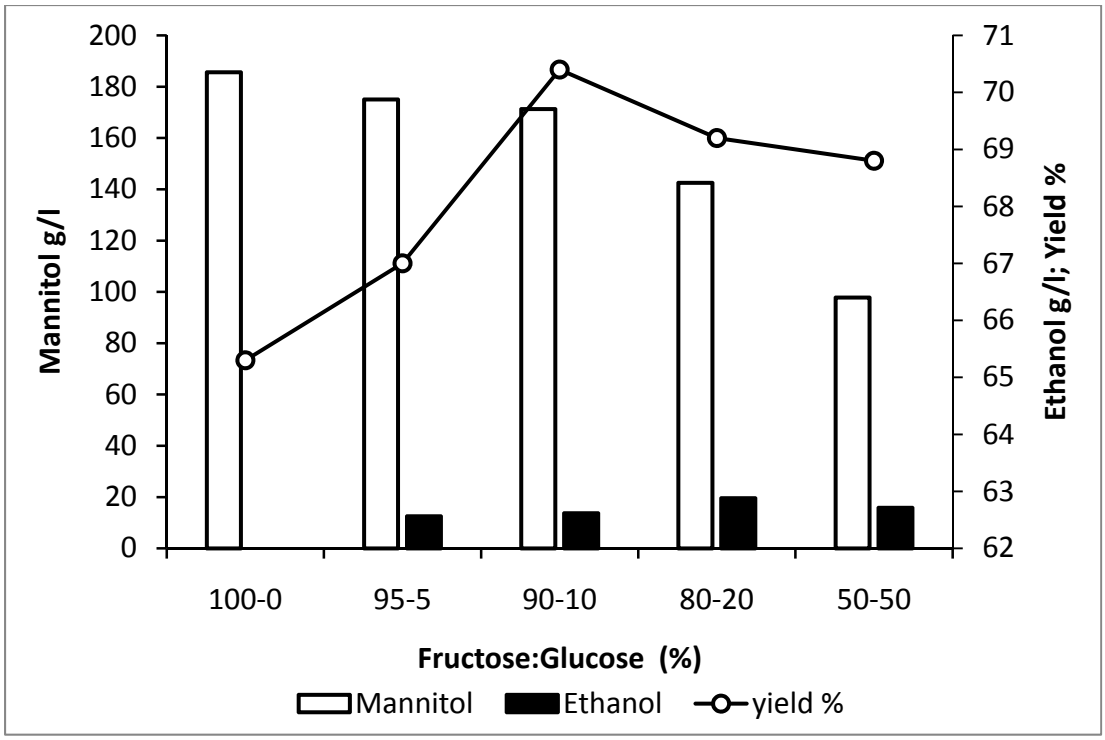


Figure 6.6 Effect of fructose-glucose ratio on mannitol production with 300 g/l initial substrate concentration

6.3.2.2 Effect of biomass concentration and temperature on mannitol production

Higher volumetric productivity is required for industrial biotechnological production of mannitol. Volumetric productivity can be improved by increasing both specific productivity and biomass concentration. Increase in specific productivity can be achieved by screening higher producing strain or their mutants and optimization of culture conditions for the same. The increase in biomass concentration can be rapidly achieved by high cell density culture using various modes of fermentation. In the present study effect of biomass concentration on mannitol production illustrated that the production of polyols from resting cells was mainly dependent on biomass concentration per unit volume which in turn, controlled dissolved oxygen tension in the shake flasks. At lower biomass concentration (higher dissolved oxygen tension) unwanted metabolites like glycerol and ethanol were formed. At about 1.5 g biomass concentration in 25 ml medium, highest amount of glycerol (54 g/l) was observed which decreased further with increase in biomass concentration similar to the ethanol (Fig. 6.7). Increase in the resting

cell mass concentration specifically increased mannitol production. At biomass concentration of about 3 g wet cell mass, exclusively mannitol was formed from fructose with negligible amount of ethanol (4 g/l). This indicated that dissolved oxygen concentration is a key factor in mannitol production by resting cells of *C. magnoliae* mutant R9.

In *Aspergillus niger* it has been reported that oxygen availability had significant impact on the physiology of cells which caused dramatic alterations in the central carbon metabolism and caused huge changes in polyol production pattern (Diano et al., 2006; Meijer et al., 2007). Similarly, Baek et al., (2003) investigated mannitol production from *C. magnoliae* HH-01, in which aerobic conditions were maintained to promote cell growth during growth phase and then switched to anaerobic conditions by reducing the agitation speed and stopping aeration. In a study with resting cells, it was observed that mannitol productivity was strongly influenced by temperature (Fig. 6.8).

In the present investigation during initial 12 h, the volumetric mannitol productivity sharply increased from 3 g l^{-1} h^{-1} at 28 °C to 17.4 g l^{-1} h^{-1}. These values represent 5.8 fold increase in productivity without any reduction in yield and concentration of mannitol. To the best of my knowledge, this is the highest mannitol productivity ever reported by any microorganism in flask or fermenter level. At 48 °C, resting cells converted all fructose to mannitol within 12 h with 69.6% yield. On the other hand only 53% yield of fructose to mannitol was observed at 28 °C in 48 h. At higher temperature (33 to 48 °C) conversion yields of fructose to mannitol was almost constant around 70%. Similarly, von Weymarn et al., (2002a) investigated the influence of growth temperature on the volumetric mannitol productivity by *L. fermentum*. A change in growth temperature from 25 to 35 °C brought about an approximately twofold increase in the volumetric mannitol productivity. In their investigations the specific mannitol productivities (volumetric productivity divided by the optical density) were also clearly higher at 35 °C than at 25 °C.

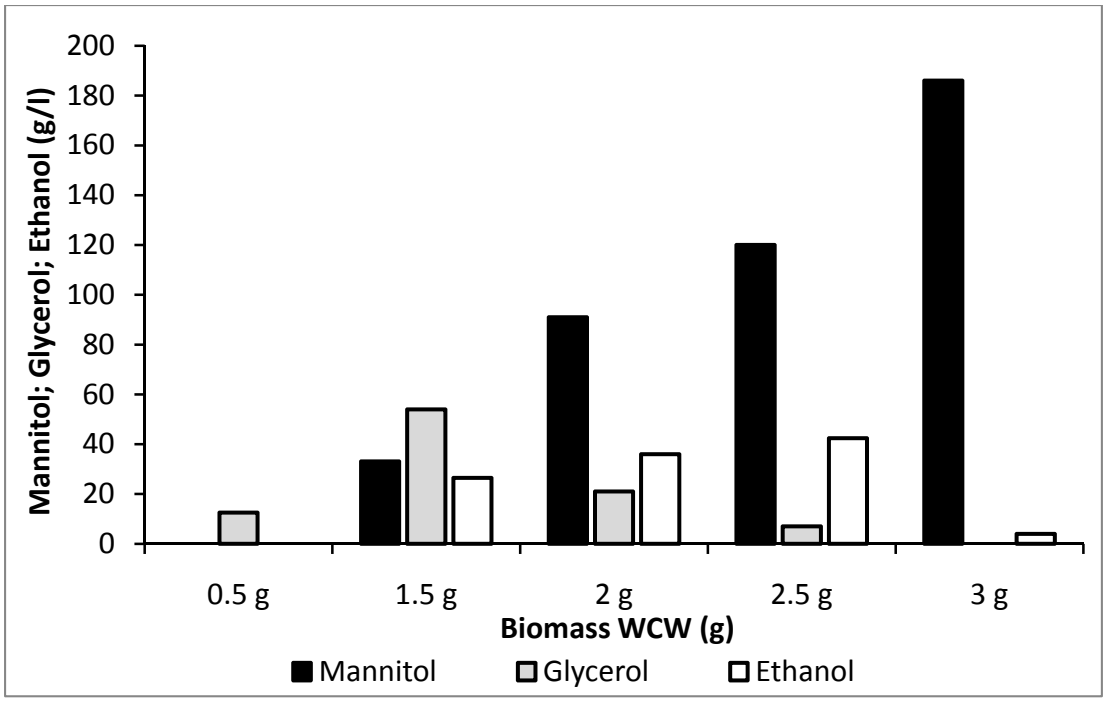


Figure 6.7 Effect of resting cells concentration on polyol production

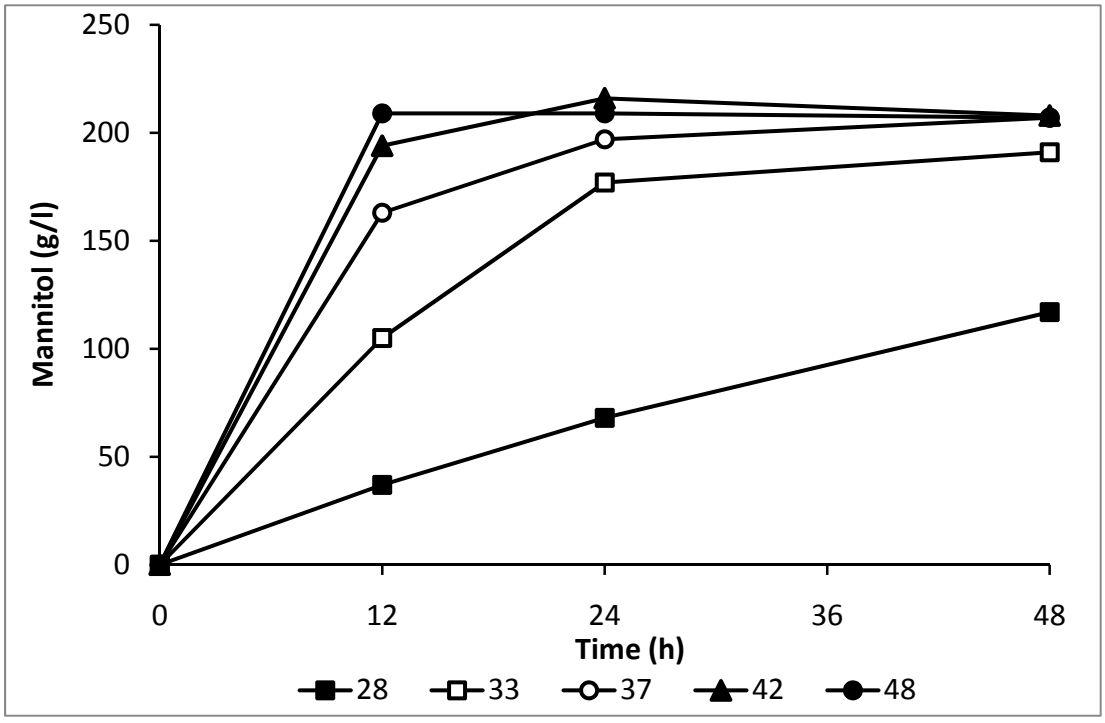


Figure 6.8 Effect of temperature on mannitol production by resting cells of *C. magnoliae* mutant R9

6.3.2.3 Repeated batch bioconversion

Mannitol productivity was further enhanced in repeated batch fermentation by reusing the same biomass for successive batches. The same biomass was successfully used for five times at 28 °C in aqueous fructose solution which produced a total of 1021 g mannitol from 1436 g fructose in 138 h, which corresponds to an average mannitol concentration of 204 g/l for each recycle (Fig. 6.9a). The average time of each batch was 27.6 h. The volumetric productivity and mannitol yield from fructose were 7.39 g l^{-1} h^{-1} and 71.1%, respectively. On extending the bioconversion for sixth cycle, it could only produce 110 g/l mannitol with 52% mannitol yield in 36 h. At 37 °C this mutant produced 734 g of mannitol from 1042 g of fructose in 96 h with an average volumetric productivity 7.64 g l^{-1} h^{-1} and mannitol yield of 70.4 % (Fig. 6.9b).

Although highest volumetric productivity 17.4 g l^{-1} h^{-1} was observed at 48 °C it could convert fructose to mannitol only in first cycle, By reusing the biomass for second cycle there was no mannitol formation. It was observed that with increase in time and temperature, the viability of cells dropped substantially as observed under microscope by visualizing viability of the cells by methylene blue staining. Thus, substantially higher volumetric productivity of approximately 7.5 g l^{-1} h^{-1} was achieved using resting cells at higher biomass concentration at (5 g wet cell mass) with lower temperature 28 °C or at lower biomass concentration (3 g wet cell mass) at higher temperature 37° C. One of the important finding was that specific mannitol productivity increased from 1.5 to 2.54 g g^{-1}WCW h^{-1} representing 59% increase in specific mannitol productivity. The final average mannitol concentration of 204 g/l and yield of 70 % was comparatively equal to all previously reported mannitol producing microorganisms (Table 6.1). Use of resting cells in repeated batch bioconversion resulted in 87.5 % increase in volumetric productivity, compared to 4 g $l^{-1}h^{-1}$ in previously optimized two-stage fermentation process with growing cells. In this process, clean resting cells, physically separated from the fermentation broth were used, thus avoiding all the impurities released by the cell and medium components during growth phase.

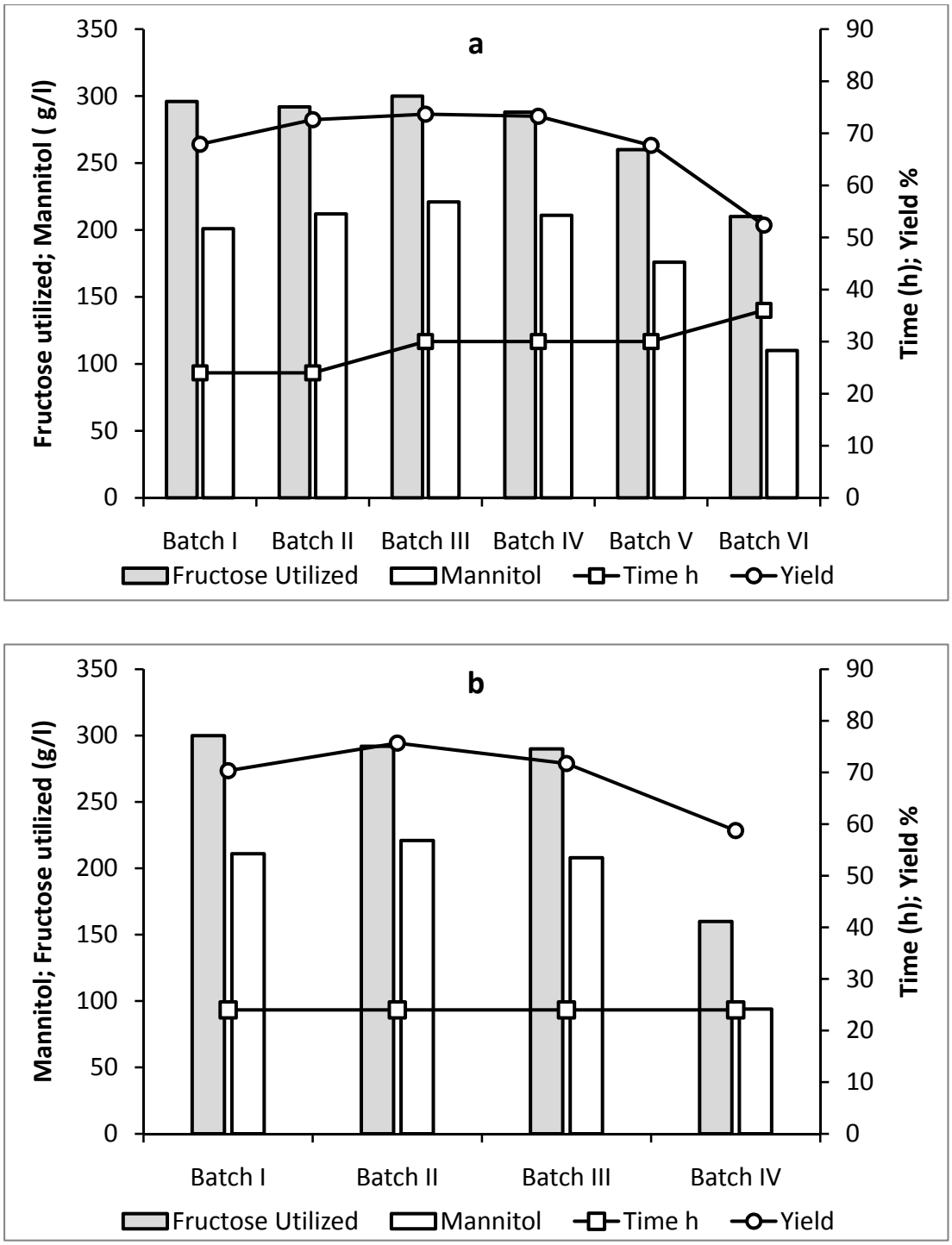


Figure 6.9a, b Repeated batch bioconversion cycles by resting cells of *C. magnoliae* mutant; a) at 28° with 5 g WCW b) at 37° with 3 g WCW

Another commonly used approach in LABs based mannitol production processes is bacterial biomass is first grown, and then the resting cells are used repeatedly in MCBR (von Weymarn et al., 2003; Von Weymarn et al., 2002b). The scalability of this MCBR mannitol production process was tested at a 100-l pilot plant scale (von Weymarn et al. 2003). Even volumetric productivity up to 28.4 g l^{-1} h^{-1} was obtained with *L. intermedius* in a continuous cell-recycle fermentation process, by continuous sugar syrup feeding to overcome limitations caused by high substrate concentrations (Racine & Saha, 2007). The highest volumetric productivities using MCBR have been reported for mannitol production by LAB, but they are reported to produce a fairly high amount of organic acids as co-metabolites that adds up to the additional downstream processing cost. On the other hand, enzymatic conversion process requires cofactor regeneration. Strong product inhibition of mannitol dehydrogenase, the high *Km* value for fructose and increased costs of a two-enzyme system makes the enzymatic process unfeasible (Song & Vieille, 2009). Whole-cell transformation process investigated in the present study has the potential to produce mannitol essentially without by-products. The new bioprocess is simple and requires only a bioreactor with pH and temperature control and slow mixing with mild conditions. The contamination risk of the new bioprocess is low and the downstream processing protocol is simple and cost-effective. Further, continuous production with increased productivity may be easily achieved using more sophisticated bioprocess alternative such as MCBR technique and optimizing the critical process parameters.

6.3.3 Production of mannitol from hydrolyzed sucrose by mutant R9

To produce mannitol commercially on industrial scale, more economical carbon and nitrogen sources are required to replace costly fructose, yeast extract and peptone. Earlier, in this regard, mannitol production by *L. intermedius* NNRL B-3693 was evaluated by replacing 50% of fructose with molasses as an inexpensive carbon source. This bacterium produced 104 g/l mannitol from molasses and fructose syrup with total sugars 150 g/l (Saha, 2006a).

Our initial experiments with 1:1 ratio of glucose-fructose mixture using this strain could produce 111 g mannitol from 150 g fructose with 1.54 g l^{-1} h^{-1} productivity and 74%

conversion yield at 28°C. On evaluating the mutant at 37°C in high concentration inverted sucrose solution (600 g/l), 204 g/l mannitol was produced with 68.1% conversion yield in 48 h with a volumetric productivity of 4.25 g l^{-1} h^{-1}. These values represent highest mannitol concentration, conversion yield and mannitol productivity from easily available cheap substrate, sucrose, as compared to fructose by any microorganism, ever reported.

6.3.4 Mannitol production in chemically defined medium

Although yeast extract is a favorite nitrogen source in fermentation, it is very expensive and probably enhances production of undesirable products. In order to produce mannitol cost-effectively on an industrial scale by fermentation, cheaper nitrogen source is required to replace peptone and yeast extract. Corn steep liquor is commonly used as nitrogen, vitamin and amino acid source. The changes in the chemical constituents of corn steep liquor can cause difficulty in fermentation and also product purification. A synthetic medium is economic, helps in minimizing impurities and consequently has an advantage in downstream processing.

In the chemically defined medium, during the growth phase, the mutant R9 grew exponentially under aerobic condition and biomass reached stationery phase at 36 h (14.3 g/l DCW). By the addition of fructose the biomass concentration during the production phase decreased to 13 g/l DCW which remained unaltered throughout the production phase. During 136 h production phase, 172.4 g mannitol was produced with overall mannitol conversion yield of 84.4% and productivity of 1 g l^{-1} h^{-1}. In the present investigation, maximum fructose uptake rate and productivity observed was 2.7 and 2.3 g l^{-1} h^{-1} respectively, between t=30 h and t=66 h of production phase which gradually decreased towards the end of production phase (Fig. 6.10).

This study demonstrated that mannitol can be produced effectively by mutant R9, grown in a chemically defined medium instead of a complex medium. Although mannitol productivity was less as compared to the batch in which the cells grown with yeast extract were used, still has an advantage in terms of cost of fermentation medium and ease in downstream processing. Moreover, in the chemically defined medium the mutant

did not produce any other by-products. These results are comparable to previously reported mannitol production using *C. magnoliae* HH-1 (KCCM-10252) in a complex medium with yeast extract which produced 209 g mannitol l^{-1} with 1.03 g l^{-1} h^{-1} productivity and 83% yield (Song et al., 2002). This is probably the first report where defined medium was used for mannitol production.

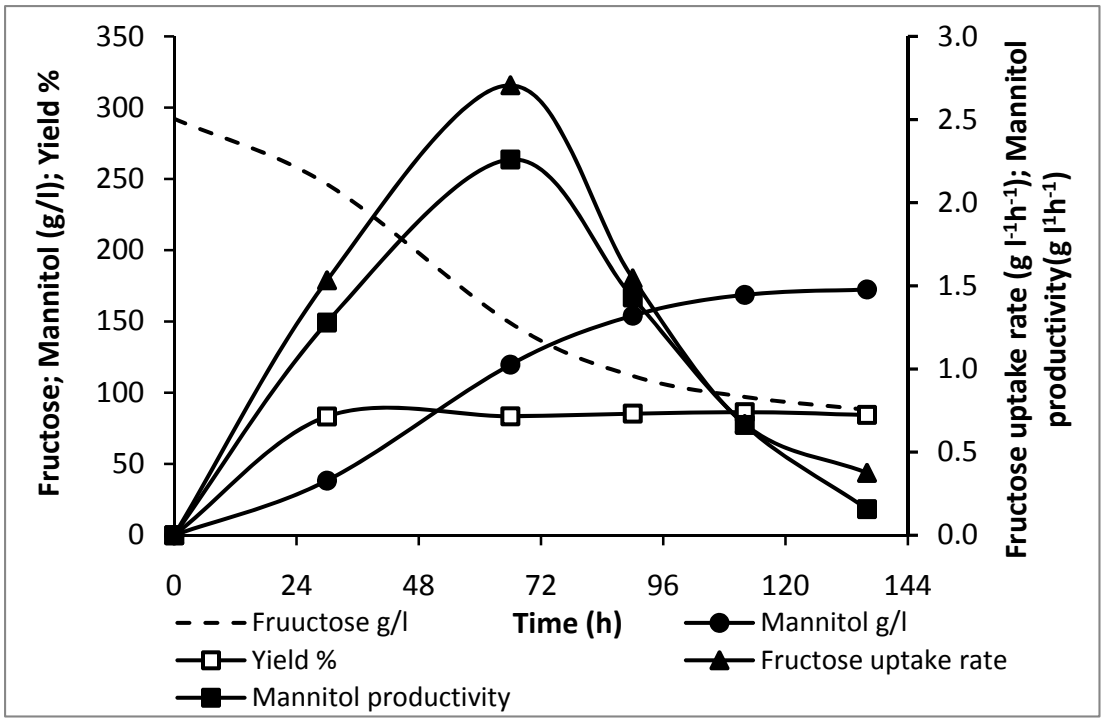


Figure 6.10 Fermentation profile of mannitol production by cell mass grown in a chemically defined medium

6.3.5 Purification and characterization of mannitol

Majority of reports on downstream processing of mannitol are based on separation of cells from broth by centrifugation, removal of coloured impurities by activated carbon treatment, electro-dialysis, crystallization-recrystallization and drying. Chromatographic methods are also employed to separate acetate and lactate fractions from mannitol especially when LABs are used for fermentative production.

In the present investigation, pure fructose with resting cells in aqueous solution was used and there was hardly any by-product observed as analyzed using HPLC. After complete

conversion of fructose into mannitol, cells were separated by centrifugation. On treatment of the cell-free broth with activated carbon, a transparent colourless solution was obtained which was directly used for crystallization by concentration and seeding. The recovered crystals were over 99% pure as analyzed using HPLC. The peak area of standard and purified mannitol solution at 1 g/l concentration was almost identical. HPLC chromatogram of purified mannitol is shown in Fig. 6.11. Melting point of the purified mannitol was found to be 165.2 ℃ which is equivalent to the melting point quoted in literature (Saha & Racine, 2011).

Purified mannitol crystals were analyzed by LC-MS by comparison with mass spectra of authentic mannitol standard from Sigma Fig. 6.12. Both standard and purified sample were spiked with sodium ion to produce adducts rather than a protonated species. Sodium added ions, were detected as base peaks, and the molecular masses of the mannitol were determined by comparing $[M + Na]^+$ ions. Analysis in ES positive mode gave (m/z) 205.12 [M + 23]. Number of carbon atoms from the purified erythritol was analyzed using ^{13}C-NMR and the spectrum (Fig. 6.13) showed that the resultant molecule contained two sets of carbon atom represented as ^{13}C-NMR ($CDCl_3$, 50MHz): δ 63.2 (C1 and C6), δ 69.2 (C2 and C5), δ 70.7 (C3 and C4).

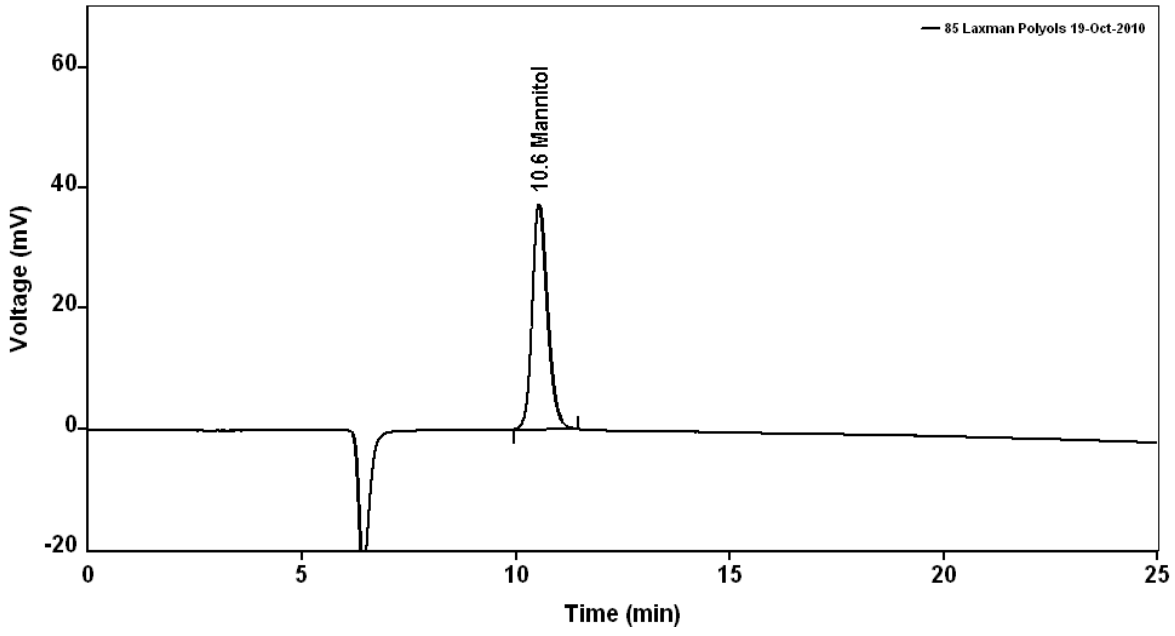


Figure 6.11 HPLC chromatogram purified mannitol

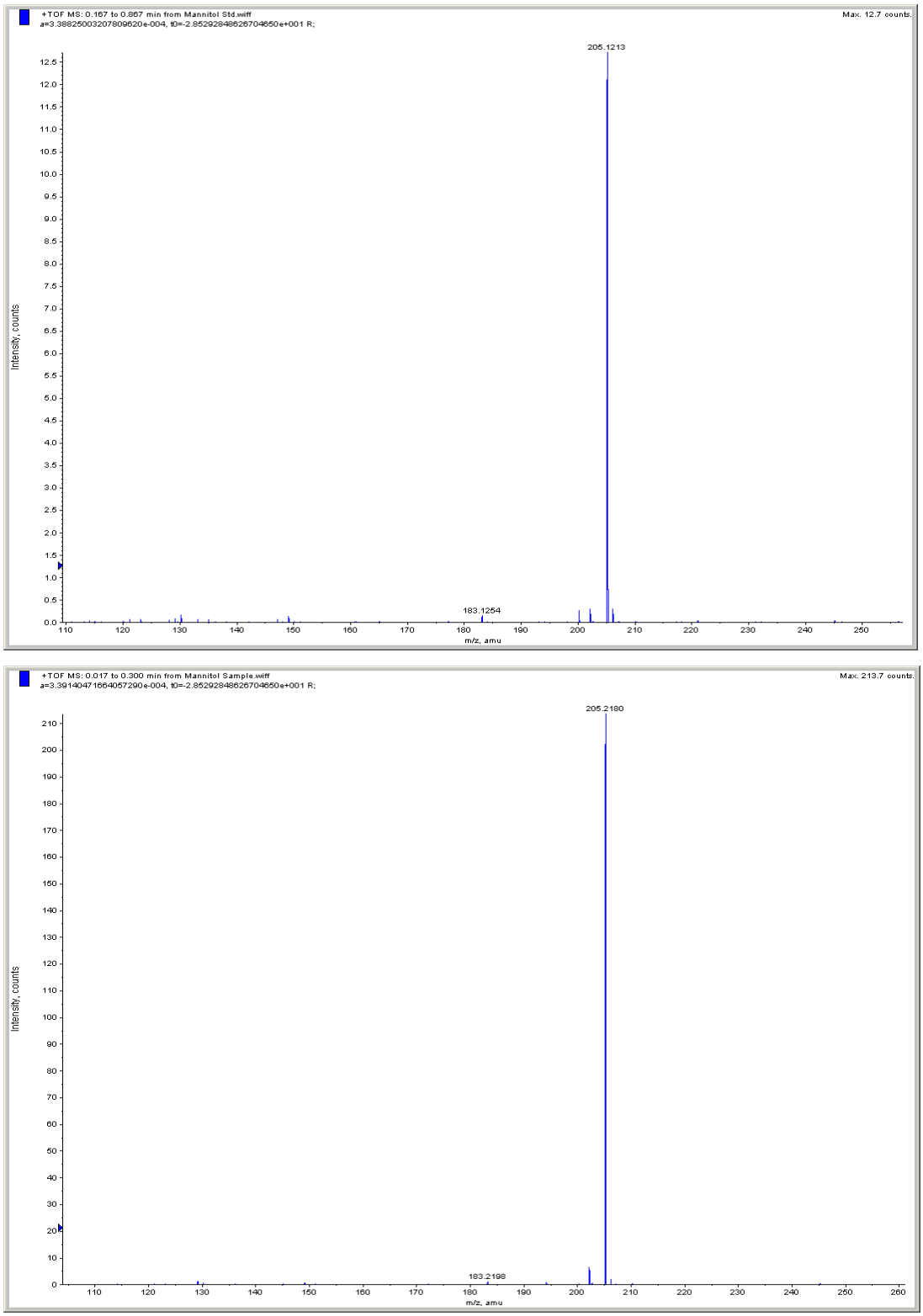


Figure 6.12 Mass spectra of authentic mannitol and purified mannitol

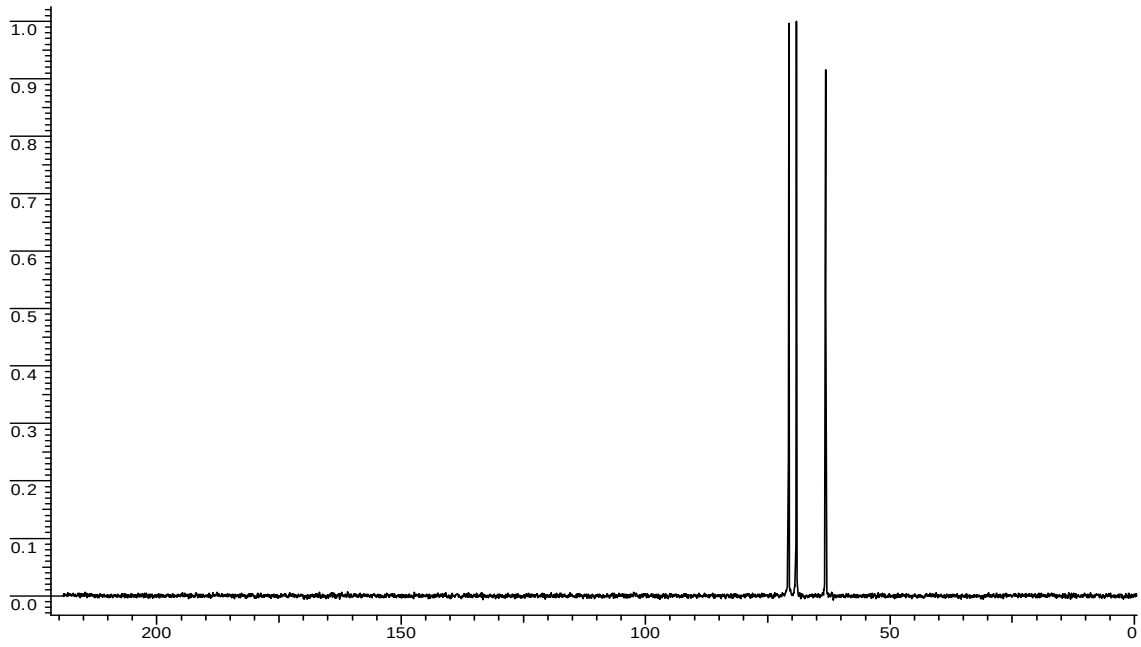


Figure 6.13 ^{13}C-NMR spectra of the purified mannitol from fermentation broth

6.4 CONCLUSIONS

Production of mannitol from fructose was investigated using the mutant R9 of *C. magnoliae*. Optimization of two-stage fermentation process resulted in production of 240 g/l mannitol, highest ever reported, from 300 g/l fructose with a fairly high volumetric productivity of 4 g $l^{-1}h^{-1}$and yield of 81.2%. This mutant is superior in several aspects compared to previously published reports. First, it produced fairly high mannitol concentration with a very good productivity among mannitol-producing microorganisms. Second, it did not produce any by-products. Third, its specific productivity is 28.6 fold higher as compared to parent strain at flask level fermentations. Consequently, the loss of substrate which is incorporated to built the yeast cell mass is low.

Studies on resting cells of *C. magnoliae* mutant R9 confirmed that mannitol production by resting cells of mutant R9 is mainly dependent on biomass concentration and temperature. In the present investigation, resting cells of *C. magnoliae* mutant R9 were successfully employed to produce more than 200 g/l mannitol with highest productivity of 7.3 g l^{-1} h^{-1} for successive five cycles in an aqueous 300 g/l fructose solution without

producing any by-products (lactic or acetic acid). Moreover, resting cells of mutant R9 could also convert 600 g/l inverted sucrose solution to 204 g/l mannitol. This is highest mannitol production ever reported from sucrose. The mannitol produced was purified and characterized. The purified mannitol was more than 99% pure and was confirmed using HPLC, LC-MS and ^{13}C NMR.

A chemically defined medium with ammonium sulphate as sole nitrogen source supplemented with vitamins (Biotin, Pyridoxine HCl and Thiamine HCl) was substituted for the complex medium containing yeast extract for growth of *C. magnoliae* mutant R9. In a two-stage batch fermentation using chemically defined medium for initial growth, 172 g/l mannitol was produced with overall mannitol yield of 84.4% and productivity of 1 g l^{-1} h^{-1}. This is probably the first report on mannitol production by microbial cells grown in a chemically defined medium. Although the overall mannitol productivity was less as compared to the batch in which cell mass was produced in a medium with yeast extract, it still has an advantage in terms of cost of fermentation medium and ease in downstream processing. Moreover, in the chemically defined medium, the mutant did not produce any other by-products.

The prospect of mannitol production by using resting cells of *C. magnoliae* mutant R9 using a chemically defined medium with hydrolyzed sucrose as carbon source looks very promising in near future.

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1. **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.
2. **Savergave, L.S.**, Jogdand, V.V., Gadre, R.V. High-level mannitol production by a mutant of *Candida magnoliae* (Manuscript under preparation).
3. **Savergave, L.S.**, Jogdand, V.V., Gadre, R.V. Enhanced mannitol production rate by resting cells of *Candida magnoliae* mutant NCIM 3649 (Manuscript under preparation).
4. **Savergave, L.S.,** Dhule, S.S., Jogdand, V.V., Nene, S.N., Gadre, R.V. 2008. Production and single step purification of cyclodextrin glycosyltransferase from alkalophilic *Bacillus firmus* by ion exchange chromatography. *Biochemical Engineering Journal*, **39**(3), 510-515.
5. Jaitak, V., Kaul, V.K., Kumar, N., Singh, B., **Savergave, L.,** Jogdand, V., Nene, S. 2009. Simple and efficient enzymatic transglycosylation of stevioside by cyclodextrin glucanotransferase from *Bacillus firmus*. *Biotechnology Letters*, **31**(9), 1415-1420.

Awards and Fellowships

1. Recipient of **Senior Research Fellowship** (2008) sponsored by Council Scientific & Industrial Research (CSIR), Government of India.
2. Awarded **Certificate of Merit** (University Second Rank) by Swami Ramanand Teerth Marathwada University Nanded, 2003-2004.