KINETICS, SIMULATION AND SCALE-UP OF PROCESS FOR FERMENTATIVE PRODUCTION OF GLYCEROL AND ARABITOL USING *HANSENULA ANOMALA*

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Dedicated to

My beloved father

Late Dr. Vasantrao M. Patil

M. S., Ph. D., D. Sc.

Kinetics, simulation and scale-up of process for fermentative production of glycerol and arabitol using *Hansenula anomala*

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This is to certify that the work incorporated in the Ph. D. thesis entitled "**Kinetics, simulation and scale-up of process for fermentative production of glycerol and arabitol using *Hansenula anomala*"** submitted by Mr. Sanjay V. Patil was carried out under my supervision. Material obtained from other resources has been duly acknowledged in the thesis.

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I hereby declare that the thesis entitled "**Kinetics, simulation and scale-up of process for fermentative production of glycerol and arabitol using *Hansenula anomala***" submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of Dr. B. D. Kulkarni and has not formed the basis for the award of any degree in this or any other university. I further declare that the material obtained from other resources has been duly acknowledged in this thesis.

Place: PUNE

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GC	Gas Chromatography	DCW	Dry Cell Weight
pO_2	Percentage Saturation Oxygen	TRS	Total Reducing Sugars
OV	One-Variable-at-a-time	GA	Genetic Algorithm
rpm	Revolution per Minute	ppm	Parts Per Million
ts	Total Sugars	su	Sugar Utilized
µl	Micro liter	ml	Milliliter
mg	Milligrams	g	Grams
l	Liter	hr	Hour
M	Meter	^oC	Degree Celsius
Cal	Calorie	Kcal	Kilo Calorie
cP	Centipoise	µs/cm	Micro Siemens/centimeter
W/m.K	Watt per meter Kelvin	D^{25}	Density at 25^oC
d^{25}	Density at 25^oC	d^{20}	Density at 20^oC

$bp_{3.5}$	Boiling point of glycerol at 3.5 mm Hg
bp_4	Boiling point of glycerol at 4 mm Hg
bp_{100}	Boiling point of glycerol at 100 mm Hg
bp_{760}	Boiling point of glycerol at 760 mm Hg
vvm	Gas Volume flow per liquid volume per minute

ABSTRACT

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Increasing prices of petroleum feed-stock have made it necessary to look for alternate energy sources as well as alternate routes to produce downstream petro-chemicals such as glycerol. At the same time, gradual increase in oil-seed prices and replacement of soaps by detergents have caused decline in production and availability of glycerol in the country. India is still importing this vital industrial chemical.

On the other side, the increasing stocks of sugar in the world as well as in the country have resulted in decline of sugar (sucrose) prices. Sugar cane and sugar beet processing products such as molasses, sugar cane/beet juice or even the crystalline sucrose are considered as renewable raw materials and are available easily in a country like India. In the past decades, the significant advances in Biotechnology have led to a renewed interest in the production of chemicals from renewable sources of carbohydrates, so-called green chemistry.

Production of glycerol and related polyols e. g. mannitol, xylitol, erythritol, D-arabitol etc by fermentation of sugary raw materials by osmophilic yeasts is an interesting research area of Yeast Biotechnology. With recent developments of separation techniques, such as ultrafiltration, reverse osmosis, ion-exchange, ion-exclusion etc microbial production of glycerol and other polyols, especially by osmophilic yeast, deserves a fresh consideration.

Glycerol has wide industrial applications. Other polyols are usually used as additives in food industry. Erythritol is also now reported to be produced commercially by fermentation. Work is also in progress on development of process for fermentative production of D-arabitol. At present, arabitol and erythritol have limited commercial applications. However, the market potential of these polyols will further develop when they will be produced cheaply on a large scale. Sucrose being cheaper in a developing country like India, it would be desirable to study the production of these polyols from sucrose-based media.

This work involves the study of kinetics of glycerol and arabitol formation in the fermentation of sucrose-based media by an osmophilic yeast *Hansenula anomala.*

The data generated in our previous study was used to undertake simulation studies using lazy learning technique of robost regression. The version of lazy

learning method employed in this work is a memory based local learning requiring the storage of training data in the memory.

The polyols yield prediction problem is solved with lazy learning. In this case, local quadratic model with 2.212 % root mean square error (RMSE) is found to be performing better than kernel principal component regression (KPCR) and kernel partial least squares (KPLS) with 4.009 % and 4.417 % RMSE, respectively. Local combination of models achieved the same RMSE as local quadratic model. The technique is especially useful in instances where fast training and online updation of process models is required.

With studies in shake flask experiments it was found that with sucrose as substrate, the selected organism gave substantially less yield. Therefore, it was decided to optimize the nutrients concentration with sucrose substrate on shake flask scale using genetic algorithm (GA)-based approach. The composition of optimized media obtained by the genetic algorithm approach was (g/l), Sucrose: 300.5, yeast extract: 1.25, Urea: 1.10, and $MgSO_4$: 0.256 and was slightly different from that obtained with conventional approach. The final concentration as well as the yield of polyols, particularly during early period of fermentation, was higher with medium optimized by the GA approach. Maximum polyols yield on the basis sucrose utilized was found to be 76.43 % and final polyols yield on the basis of total initial sugars was 30.23 %. It was also found that addition of invertase enzyme during fermentation in shake flask improves the final concentration of polyols with sucrose as substrate.

Media optimized by genetic algorithm approach was used for scale-up and further process optimization experiments in an automated fermenter at 2.5 liter capacity. The effect of pH, temperature, inoculum size, oxygen % saturation (pO_2), agitation rate and external invertase addition on polyols production was studied.

Based on the investigations carried out, the optimum conditions for production of polyols using sucrose as substrate in batch fermentation mode are, Inoculum siz: 15 %, pO_2 controlled at 50 % saturation, Agitation rate: 600 rpm, Temperature: 32.5°C, pH: 8.0, Invertase dose: 0.5 g/l. Under these conditions it was found that sugar utilization is completed in a period of 148 hours with polyols concentration and polyols yield reaching to the maximum value of 143.5 g/l and 47.84 %, respectively. The polyols productivity and sugar utilization rates also reached the maximum value of 0.983 g/l/hr and 2.055 g/l/hr, respectively.

The Gas chromatographic analysis used for qualitative estimation indicated 4: 1 ratio of glycerol to arabitol in the final fermentation broth sample.

Fed-batch fermentation aspect was investigated in two sets of experiments. In the first set of experiments, 10 % inoculum was used at 30°C fermentation temperature without pO_2 control. In the second set 15 % inoculum was used at 32.5°C fermentation temperature and pO_2 controlled at 50 %.

In the first set of fed-batch experiments, optimum results were achieved where the dose of nutrients was reduced to 50 % of the initial dose with maximum overall polyols yield of 32.2 %. In the second set of fed-batch experiments the maximum polyols concentration achieved was 142.9 g/l as compared to 230.0 g/l achieved in the first set. The maximum overall polyols yield of second set (27.55 %) is less than that obtained in the first set (32.20 %). However, overall yields of both the sets of fed-batch experiments are considerably less than that obtained in batch fermentation (47.84 %). Similarly, the polyols productivity and sugar utilization rates in fed-batch fermentations are lower than those obtained under optimum conditions in batch fermentations. These observations suggest that the optimum conditions for fed-batch fermentation with respect to inoculum size, temperature of fermentation, optimum pO_2 level can be different from the optimum conditions of batch fermentation process. Therefore, it would be necessary to adjust these parameters as well as sucrose and nutrients feed strategy carefully to achieve maximum yield and productivity of polyols in fed-batch experiments.

In the best experiment of fed-batch fermentation it was possible to achieve 230.0 g/l polyols concentration at an overall yield of 32.20 %, polyols productivity of 0.552 g/l and sugar utilization rate of 1.776 g/l/hr.

Finally, a kinetic model for batch fermentation based on the data generated with sucrose as the substrate has been developed, which takes into account the effect of dissolved oxygen concentration. This model will be useful for design purposes and for further scale-up studies.

CHAPTER-1

INTRODUCTION AND REVIEW OF LITERATURE

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1.1 INTRODUCTION

Low carbohydrate diets contributing to declining or stable sugar consumption in some developed countries, free trade agreements, an oversupply of sugar in the world market and in many individual countries and other factors are providing new challenges to the research community. Large stocks of sugar in the world in the last ten years have resulted in decline of sugar (sucrose) prices. The sugar and sugar-ethanol industries are facing serious sustainability issues. However, sugar in a few years, is also expected to be "new oil" as sugar is being looked upon as a superior feed-stock for the production of platform chemicals for the manufacture of range of end-products, e.g. bio-plastics, industrial solvents and chemicals (1).

At the same time, the soaring costs of petroleum products have shown that supplies of fossil fuels are indeed finite. With petroleum crude prices crossing $ 100 a barrel and some pundits predicting that it could go all the way to $ 150, the need for alternate uses of sugar is now apparent and appears more feasible (2). In addition, sugarcane and sugar (sucrose) are renewable raw materials and offer an advantage over exhausting fossil fuels for development of value added products. World production, consumption and stocks of sugar are given in Table 1.1 (3).

Table 1.1: World production, consumption and stocks of centrifugal sugar (Oct/Sept, 1000 tonnes, raw value)

Year	World production	World consumption	Ending stocks	Stocks as % of consumption
2001/02	138,288	134,845	59,655	44.24
2002/03	150,488	139,829	69,086	49.41
2003/04	143,841	141,665	68,191	48.14
2004/05	141,027	144,541	61,731	42.71
2005/06	151,098	146,840	64,170	43.70
2006/07	166,459	152,871	72,610	47.50
2007/08	166,616	159,474	73,771	46.26
2008/09	151,873	159,343	62,252	39.07
2009/10	158,622	161,076	56,462	35.05
2010/11	166,880	164,097	57,764	35.20

The term fermentation has been used in strict biochemical sense to mean an energy generating process in which organic compounds act as both electron donors and terminal electron acceptors. The biotechnologists have extended the term fermentation to describe any process for the production of product by manipulation and mass culture of microorganisms.

The biotechnology or classical fermentation industry has evolved in the last century (4). Prior to 1990, the product of fermentation industry was confirmed to potable alcohol and vinegar. This phase was marked by the development of a bioreactor known as Packed Bed Generator for vinegar production and it was the first aerobic fermenter to be developed. Between the years 1900 to 1940, the new products developed were citric acid, lactic acid, glycerol, yeast biomass and acetone-butanol. Fed-batch fermentation process for the production of Baker's yeast was introduced in this phase. Aseptic aerobic fermentation and use of steam sterilizable sparger for aeration were also established in this period.

The next phase in the development of biotechnology industry started with the need to produce penicillin in submerged culture under aseptic conditions and the subsequent strain improvement programmes, which became a dominant feature of antibiotic industry. Many new processes were established in this phase including other antibiotics, vitamins, gibberellins, amino acids, enzymes and steroid transformations. This phase saw the development of the large mechanically stirred bioreactors.

Starting from early 1960s, in the fourth phase, investigations were carried out towards production of biomass as a source of feed proteins utilizing hydrocarbons as a substrate under highly aerobic conditions. These requirements led to the development of pressure jet and pressure cycle fermenters, which eliminated the need for mechanical stirring. To overcome the economical constraints, continuous fermentation systems were developed. Computer control systems for continuous sterilization of feed streams and operation cycles were also developed during this phase.

The fifth phase, starting from 1970s, has been initiated by the developments in genetic engineering and molecular biology, which not only allow the transfer of genes between unrelated organisms but also enable the extremely precise alteration of the genome of the organism. Products such as insulin and interferon, normally produced by higher cells, can now be synthesized by microbial cells. It is believed that the

techniques of genetic manipulation will revolutionize the biotech industry in the coming years and give rise to a large number of new products and processes. In 2001, the sequence of human genome was published in Science and Nature, making it possible for researchers all over the world to begin developing genetically based treatments for diseases.

Various micro-organisms like bacteria, moulds and yeasts are being used commercially to produce value added products. Yeast a group of unicellular fungi, has been described as man's domesticated microorganism. They exhibit an ability of rapid multiplication, can utilize variety of sugars and tolerate higher concentration of sugars and salts. Species of yeast have been commercially used for the manufacture of various alcoholic beverages, production of industrial chemicals and fuel-ethanol and as a source of human and animal feed in the form of single cell protein. Use of yeast as a Bio-control agent in spoilage of food is also being investigated (5).

Polyols or polyhydroxy alcohols are important class of products produced by several species of yeast. The glycerol formation accompanying ethanol production in fermentation of sugars has been described since the days of Pasteur (6). During normal alcoholic fermentation to produce ethanol or in brewing and wine making, 2.5 – 3.0 % of sugar fermented is converted to glycerol.

Beside yeasts, many micro-organisms including bacteria, molds and algae are known to produce glycerol (7). It has been shown that osmophilic yeasts can produce other polyols along with glycerol. Among the other polyols produced by yeasts are arabitol, erythritol, xylitol, mannitol etc. Interesting research has been carried out for more than 60 years on production of polyols by osmophilic yeasts. Higher yields have been obtained from species that show a relatively strong tolerance for high concentration of sugars and salts (8, 9, 10, 11).

1.2 GLYCEROL

Glycerol is an important industrial chemical and is used in large variety of applications. Reported world production of glycerol in the year 2002 was 600,000 tonnes per annum (12). Though recent data on glycerol production in India is not available, the import statistics indicate that India continues to import this vital polyol.

Glycerol was discovered in 1779 by Scheele. In 1811, Chevrenl showed that fats are glycerine esters of fatty acids. He also gave glycerol its name, after the Greek

word meaning sweet. The first industrial use of glycerol was reported in 1886 when Nobel produced dynamite.

Table 1.2: Physical Properties of Glycerol (13, 14)

No.	Property	Value
1.	Melting point	18.17°C
2.	Boiling point - b_4 - b_{100} - b_{760}	147.9°C 222.4°C 290.0°C
3.	Freezing point	18.0°C
4.	Density	D^{25} 1.2617 (100% Glycerol) -d^{25} 1.2488 (95% Glycerol)
5.	Refractive Index	η_D^{20} 1.47399
6.	Vapour pressure at 50°C 100°C 150°C 200°C	 0.0025 mm 0.195 mm 4.30 mm 46.0 mm
7.	Surface tension	63.4 dynes/cm at 20°C (100 % glycerol)
8.	Specific heat	0.5795 cal/gm at 26°C
9.	Viscosity	1499 cP at 20°C (100 % Glycerol)
10.	Heat of vapourization	21,060 Cal/mole at 55°C
11.	Heat of solution to infinite dilution (Exothermic)	1381 Cal/mole
12.	Heat of formation	159.60 Kcal/mole
13.	Thermal conductivity	0.28 W/(m.K) at 0°C
14.	Flash Point	Open cap – 177°C Closed cap – 199°C
15.	Fire Point	204°C
16.	Auto-ignition temperature on glass	429°C
17.	Specific electric conductivity	at 20°C 0.1 μS/cm
18.	Relative dielectric constant	at 25°C 42.48
19.	Miscibility	Glycerol is miscible with water and ethyl alcohol in all proportions. It is immiscible with *ether, benzene, chloroform* etc.

Glycerol (1, 2, 3-propanetriol or glycerin) is the simplest alcohol with two terminal primary hydroxyl groups and one internal secondary hydroxyl group. The primary hydroxyl groups are more reactive than secondary hydroxyl group. It is a clear, water white, viscous, hygroscopic liquid with a characteristic sweet taste. The physical properties of glycerol are listed in Table 1.2.

1.3 APPLICATIONS OF GLYCEROL

Glycerol is an industrially important chemical used widely in many commercial applications. Its largest application is in the manufacture of drugs and oral care products including toothpaste, mouthwash and oral rinses. In addition, glycerol is used in foods and cosmetics, tobacco, wrapping and packaging materials, lubricants, urethane polymers, gaskets, cork products, cement compounds, soldering compounds, compasses, cleaning materials, detergents, wetting agents, emulsifiers, skin protective, asphalt, ceramics, photographic products, leather, wood treatment and adhesives (14).

Techno-commercial aspects and global market scenario of glycerol production have been revised in the past by various authors (15, 16, 17). Specifications for crude and refined grade glycerin [IS:1796 (1961)] are given by Bureau of Indian Standards.

Because of its particular combination of physical and chemical properties, glycerol is an extremely important product with a large variety of applications. Lack of colour and odour and non-toxicity are very important properties of glycerol. It also possesses good solvent properties for many organic as well as inorganic compounds. Due to its hygroscopicity, it keeps preparations moist and hinders unwanted crystallization.

The poly functional reactivity of glycerol is used in the production of alkyd resins and in cross-linked polyesters from mono and di-carboxylic acids and polyols. Glycerol can be used to prepare polymeric ester plasticizers. Glycerol terepthalic acid polyester coatings are widely used in wire coating formulations.

The trinitrate derivative of glycerol, popularly known as nitroglycerine, is a powerful explosive. Commercial manufacture of nitroglycerin was first commenced by Alfred Nobel in Sweden in 1960s.

Along with other flavouring agents, glycerol is applied at a rate of 3 % of the weight of the tobacco to prevent the leaves from becoming friable and thus crumbling during processing. Glycerol finds application in food processing and packaging

because of its unique properties such as water solubility, hygroscopicity, viscosity, taste etc. Glycerol is non toxic and easily digestible. It is used in flavouring and colouring food products. Glycerol acts as a moistening agent and is also a vehicle ingredient of syrups. In candies and icing, it is added to prevent crystallization of sugar. Glycerol in the form of its derivative glycerol monolaurate on monooleate is used as an emulsifying and dispersing agent in the manufacture of food products. As a sweetening agent, it is added to food products to provide the necessary bulking and bodying action.

In medicines and drugs, glycerol is an ingredient of many elixirs, tinctures, jellies and ointments. Derivatives of glycerol are used as tranquilizers (e.g. glyceryl guaiaolate) and to reduce elevated blood pressure and as a heart stimulant in asthama, Round's syndrome and to reduce pain in angina pectoris.

In cosmetics, glycerin is used in many creams, lotions and industrial skin protectives to make the skin soft and replace skin moisture. It is the basic medium in which toothpaste is formed and maintained. It is also used in soaps and detergents.

Consumption pattern in 2002 in USA and in other major glycerol consuming countries is given in Table 1.3

Table 1.3: Consumption pattern in USA, Europe, Japan & China (17)

	Uses, (%)			
	USA (160,000 tones/year)	**Europe (190,000 tones/year)**	**Japan (50,000 tones/year)**	**China (80,000 tones/year)**
Drugs	39.5	23.1	34.0	5.2
Tobacco	15.8	2.5	5.3	7.3
Glycerine triacetate	ND	14.4	ND	ND
Food	14.5	5.6	ND	ND
Polyether alcohol	10.5	13.1	11.6	5.2
Polyether alcohol	9.2	13.1	19.5	49.0
Paints	2.0	4.4	3.8	1.5
Cellophone	0.6	3.1	1.9	3.1
Dynamite	ND	ND	ND	16.0
Cosmetics	ND	ND	ND	6.3
Miscellaneous	7.9	20.6	23.9	7.2

ND = No data

It should be also noted that newly proposed bio-diesel plants world over are expected to add to the glycerol supply significantly. These plants are expected to produce substantial quantity of glycerol as a by-product. However, there is still some uncertainty about future developments in this sector in India.

1.4 MANUFACTURING PROCESSES FOR GLYCEROL

Glycerol can be obtained by several different routes, which can be classified as those producing natural or synthetic glycerol. Natural glycerol is produced from naturally occurring raw materials through processes such as conversion of fats and oils to fatty acids or fatty acid methyl esters, fermentation of sugars and catalytic hydrogenation of carbohydrates. Synthetic glycerol is produced from propylene. Glycerol is now also produced as a by-product of the emerging bio-diesel industry.

Most of the glycerol produced in India comes as a by-product of soap industry (Saponification). In this process, oils and fats are boiled with caustic soda solution and salt. Triglycerides react with caustic soda to form soap and glycerol.

The presence of salt causes a separation into two layers. The upper layer is soap and the lower layer, called as spent lye, contains glycerol, water, salt and excess caustic. Spent lye contains 8 to 15 % glycerol. The lye is made alkaline by adding sodium hydroxide and then evaporated in a double effect evaporator system (18, 19). The separated salt is recycled and the glycerol is removed from the remaining mass by steam distillation.

Continuous high pressure fat splitting process for fatty acid manufacture is commonly used world-over for fatty acid manufacture. The fatty acids are withdrawn from the top of the column, and the glycerol containing aqueous phase is withdrawn from the bottom. Concentration of the sweet water by evaporation results in a product called hydrolysis crude or 88 % saponification crude glycerol. This glycerol is low in ash and has less inorganic salts (18, 20).

The third source of natural glycerol is the transesterification of oils and fats with alcohol to produce fatty acid esters. Oils and fats usually react with methanol or ethanol in the presence of alkali catalyst such as sodium methoxide to produce methyl or ethyl esters and glycerol. The latter may be separated by water washing and acidulation with HCl, followed by removal of the residual methanol to produce a crude grade glycerol (direct concentration 90-92%) with a few percent of salt content. The methyl esters are reduced to the corresponding fatty alcohols, marketed as fatty esters, or used as Bio-diesel.

Petrochemical based propylene can be converted to glycerol by various routes (18, 21, 22). In the first route propylene can be chlorinated in high yields to allyl chloride. The allyl chloride produced is oxidized with hypochlorite to dichlorohydrin i.e. a mixture of 1, 2 – 1, 3 dichlorohydrins. These are converted to epichlorohydrins

by treating with sodium or calcium hydroxide, which is then hydrolysed with sodium hydroxide or sodium carbonate to glycerol. This is the most widely used method for glycerol manufacture from propylene.

In the second method, propylene is selectively oxidized in high yield to acrolein followed by the reduction of acrolein to allyl alcohol. The allyl alcohol is epoxidized to form glycidol, which is then hydrolyzed to glycerol.

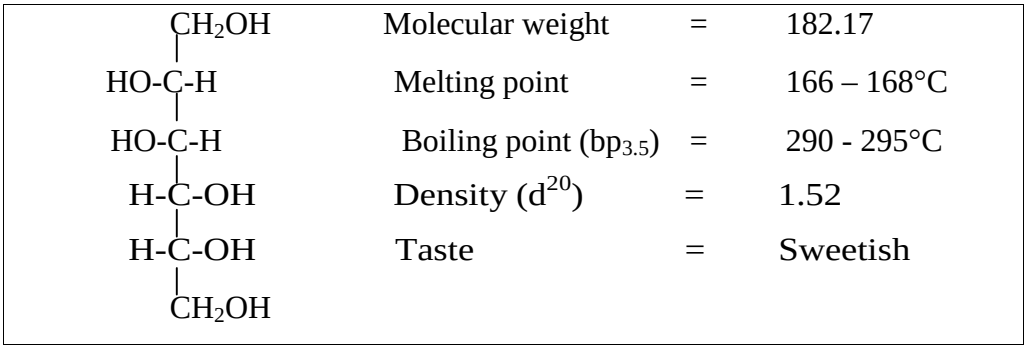
In the third method, first epoxidation of propylene to propylene oxide takes place. The isomerization of propylene oxide to allyl alcohol is the second step. A second epoxidation is carried out to produce glycidol, which is then hydrolysed to glycerol. Glycerol is also produced on commercial scale to a small extent by simultaneous catalytic hydrogenation and hydrogenolysis of refined sugar.

1.5 PROPERTIES AND COMMERCIAL APPLICATIONS OF OTHER POLYOLS

These polyols have characteristics similar to sugar and are used to improve the nutritional profile of food products owing to health-promoting properties such as lower calorific content, non-carcinogenicity and low glycemic index and insulin response. Other auspicious qualities as food additives include high enthalpies of solution and lack of reactive carbonyls. These polyols additionally find many applications in pharmaceuticals, chemicals production, oral and personal care and animal nutrition.

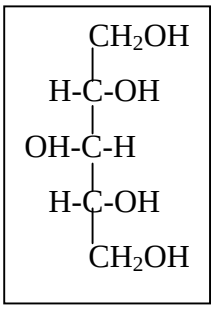
1.5.1 Mannitol

Mannitol is a six-carbon sugar alcohol with a variety of clinical applications, in addition to its use as sweetener. Physical properties of mannitol (also known as D-mannitol, manna sugar, cardyceptic acid, manical, Mannidex, Disonol, Osmitrol, Osmosal) are given below,



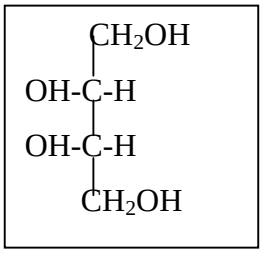
Mannitol is derived by the catalytic hydrogenation of its parent reducing sugar. Of the other polyols, mannitol ranks next to glycerol in their applications. Mannitol is used with boric acid in the manufacture of dry electrolytic condensers for radio applications, in making artificial resins and plasticizers, in pharmacy as excipient and diluent for solids and liquids, in analytical chemistry for Boron determination and in manufacture of mannitol hexanitrate. It is also used in food industry as anticaking and free flow agent, flavouring agent, lubricant and release agent, stabilizer and thickner and nutritive sweetner.

1.5.2 Xylitol



Xylitol is a five-carbon sugar alcohol obtained from xylose reduction. The annual xylitol market is estimated to be $ 340 million and is priced at $ 4.0-5.0 per kg (23). Xylitol is commercially produced by hydrogenation of xylose originating from the hemicelluloses occurring in birch bark, almond shells and corn cobs. It is now also produced from sugar cane bagasse in China.

1.5.3 Erythritol

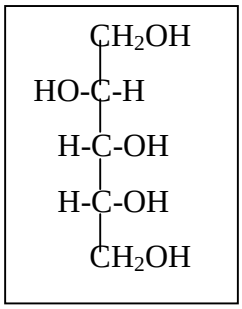


It is also known as 1, 2, 3, 4-Butanetetrol, meso-erythritol, tetrahydroxybutane, erythrol or erythrite. The molecular weight, melting and boiling points are 122.0, 121.5°C and 329-331°C, respectively. Erythritol is the only sugar alcohol produced commercially by fermentation (24) using the yeast *Aurobaridium sp*. Erythritol has recently drawn considerable attention as a sweetener that is safe for diabetics. Erythritol is 70-80 % as sweet as sucrose and although it is rapidly absorbed in the intestines, over 90% is excreted from the body without being metabolized. It is also shown to be non-carcinogenic and non-caloric (0.3 Kcal/g) sweetener. Erythritol has very high negative heat when dissolved in solution, providing a strong cooling effect. Because, it is a small molecule, erythritol has strong colligative properties, including freezing point depression, boiling point elevation and high osmotic pressure. With its low hygroscopicity and viscosity in solution, it is very useful for reducing and controlling

water activity in foodstuffs (25, 26, 27, 28). Erythritol is also used as plasticizer and in manufacture of certain explosives such as penta-erythritol tetranitrates. In some cases para-erythritol is also used to substitute glycerol in alkyd resins.

1.5.4 Arabitol

D- arabitol is a five-carbon sugar alcohol. Arabitol is not known to be produced commercially. It is possible to oxidize D-arabitol to D-xylulose by a membrane bound D-arabitol dehydrogenase and then reduce to xylitol by a NAD-dependent xylitol dehydrogenase. Therefore, an efficient conversion of sugar to D- arabitol is essential to xylitol production process.



1.6 PRODUCTION OF POLYOLS VIA BIOCHEMICAL PATHWAYS

1.6.1 Glycerol production with algae

Many reports have appeared on glycerol production with green algae *Dunaliella* from renewable sources (29, 30). These salt tolerant algae accumulate glycerol as an osomo regulator strategy to counteract cell dehydration (31). These algae produce intracellular glycerol from CO_2 and light by photosynthesis in response of high concentration of NaCl. They lack a rigid polysaccharide wall and respond to the changes in osmolarity of the surrounding medium instantaneously as osmometers; they shrink under hypertonic or swell under hypotonic conditions. Following this volume change, synthesis or elimination of glycerol continues until the cell volume returns to its original value (29).

The special features of this bioconversion route are –

a) Utilization of CO_2 as a cheap and renewable resource
b) Utilization of solar energy as the major energy source
c) Possible recovery of valuable by-products such as protein and β-carotene

Since *Dunaliella* has the potential of converting solar energy to glycerol, experiments for large-scale outdoor cultivation have been conducted (29, 32). A productivity of 8.0 g glycerol per M^2 per day was achieved in short-term experiments in pilot plants, whereas long-term experiments resulted in about half this value.

1.6.2 Microbial production of glycerol and other polyols

Glycerol fermentation accompanying ethanol production in fermentation of sugars has been described since the days of Pasteur (6). About 2.5 – 3.5 g per 100 g of sugar fermented during normal alcoholic fermentation is converted to glycerol. Besides glycerol, most osmotolerant yeasts produce a variety of polyhydroxy alcohols (8, 33) and may convert as much as 60 % of utilized sugar to a mixture of polyols (7) depending on the yeast strain used and the cultural conditions prevailing over the course of fermentation.

No major attempts were made to produce glycerol by fermentation until the First World War. At that time, Neuberg (34, 35) reported the increase in the amount of glycerol produced during alcoholic fermentation in the presence of sulfites or alkaline conditions. Eoff et al. (36, 37) established that glycerol production can be increased if fermentation is carried out under alkaline conditions by addition of alkali salts. These processes were based on interruption of the normal alcoholic fermentation, using strains of S*accharomyces cerevisiae*, by addition of "steering agents" such as sulfites or alkalis so that the normal amount of ethanol formed was considerably decreased and that of glycerol was increased.

Nickerson and Caroll (38) first reported that an osmophilic yeast strain, then classified as *Zygosaccharomyces acidifaciens*, produced glycerol in yields up to 22 % of the sugar fermented along with ethyl alcohol, acetic acid, lactic acid and CO_2. During 1960's Spencer (39) and Onishi (40) demonstrated that glycerol and variety of polyols can be produced in the absence of steering agents by osmophilic yeasts which include species of *Saccharomyces, Zygosaccharomyces, Torulopsis, Candida, Hansenula, Debaromyces* etc. They can synthesize a great variety of polyhydroxy alcohols of C_3, C_4, C_5, C_6 and C_7 from pentoses and hexoses: glycerol, erythritol, D-arabitol and mannitol from glucose; glycerol, D-arabitol and dulcitol from galactose; xylitol, meso-glycero-ido-heptitol and D-glycerol-d-ido-heptitol from xylose; L-arabitol from L-arabinose and ribitol from D-ribose.

1.6.2.1 Metabolic basis of glycerol production in yeast

A simplified scheme of the metabolism in *S. cerevisiae* is shown in Fig.1.1 Glycerol serves at least two functions in yeast a) As a sink for the excess NADH which is produced by anabolic reactions during anaerobic conditions (41, 42, 43, 44),

and b) As an osmolyte balancing a high external osmotic pressure during salt or sugar stress (45).

Glycerol is formed via the Embden–Mayerhof pathway in *S. cerevisiae*, *S. rouxii* and other osmophilic yeasts. *S. cerevisiae* possesses a very efficient means of redox regulation, since the cells grow rapidly under aerobic as well as anaerobic conditions (12). Under normal conditions of growth, most of the glucose is converted to ethanol. During the normal alcoholic fermentation NAD^+ is first reduced to NADH by glycolytic oxidation of glyceraldehydes-3-phospate to 1, 3-diphosphoglycerate then re-oxidized during reduction of acetaldehyde in the final stage of ethanol formation. Thus, the fermentation of glucose to ethanol by itself is redox neutral. A little of the NADH is diverted and used up in the reduction of dihydroxy acetone phosphate to glycerol-3-phosphate, which is then dephosphorylated yielding glycerol.

1.6.2.2 The role of glycerol as an osmolyte

Most cells possess mechanism that maintains the intracellular osmolarity higher than that of the extracellular medium. Many organisms use osmolytes, or organic solutes, to adjust their intracellular osmolarity (46). Intracellular accumulation of osmolytes that are compatible with protein and membrane structure, allows for osmotic adjustment without becoming detriment to cellular functions. Since the selected organic solutes used for this purpose tend to stabilize cell structures during adverse conditions, increased synthesis can be triggered not only by dehydration stress, but also by other stresses such as heat stress. In yeast and filamentous fungi, glycerol is repeatedly found as the major osmolyte (45). In yeasts exposed to strong osmotic stress, the intracellular glycerol concentration can reach to molar levels (47, 48). The increased intracellular accumulation of glycerol during osmotic stress is due to both increased production of glycerol and enhanced retention within the cells. In *S. cerevisiae*, the glycerol efflux is partly controlled by a glycerol facilitator channel that closes during hyperosmotic stress and opens during hypoosmotic stress (12).

1.6.3 Processes for Glycerol production by non-osmophilic yeast.

Non-osmophilic yeasts are known to produce glycerol as the only polyol. In these yeast, the overall “cost” of 1 mole of glycerol produced is 0.5 mole of glucose, 1 mole of NADH and 1 mole of ATP.

1.6.3.1 Steering chemicals – The sulfite process

During the First and Second World Wars large scale glycerol was produced by the well-known "Protol" process based on addition of sulfite. It was developed in Germany by Connstein and Ludecke (49). This process was also used in England (50) and good description of the process has been given by Harris (51). In this process, "steering agents" such as sulfite or a mixture of sulfite and bisulfite is added to the fermentation medium. The mechanism is the formation of a complex between bisulfite and acetaldehyde, preventing the reduction of acetaldehyde to ethanol. Glycerol formation results from the cellular need to reduce the NADH formed together with the acetaldehyde (52). Consequently, accumulated NADH, then reacts with dihydroxyacetone phosphate yielding glycerol (Fig 1.1). The maximum theoretical yield of glycerol from glucose in the absence of products other than acetaldehyde and CO_2 is 1 mol/mol (corresponding to 0.51 g/g). However, in practice some ethanol formation is needed to provide extra ATP for sustaining glycolysis (53). Therefore, any product formation besides acetaldehyde will give a decreased glycerol yield. Since, sulfite is toxic to the cells and can inhibit cell division, it should be added gradually to maintain a sufficient production rate.

In one of the processes operated during World War I, 2000 tonnes of crude glycerol and 1000 tonnes of dynamite grade glycerol were obtained from 6000 tonnes of beet sugar (54). The process was carried out as follows.

10 % beet sugar solution having 3 % Na_2SO_3 and other usual salts were sterilized and inoculated with baker's yeast in a fermenter of 1000 M^3 capacity. The anaerobic fermentation was carried out at 30-35°C for 2-3 days. The maximum yield of glycerol in this process was about 20 % based on sugar metabolized. The fermented broth contains 3 % glycerol, 2 % ethanol and about 1 % acetaldehyde.

In England, Cooking and Lilly (55) used a mixture of Na_2SO_3 and Na_2HSO_3 at a slightly acidic pH values. Na_2HSO_3 was added at different intervals of time. This mixture is not as toxic as Na_2SO_3 alone. Other sulfites at pH values where they are more soluble have been recommended e.g. $CaSO_4$ at 5.0 pH, $MgSO_4$ at pH 4.7 and $(NH_4)_2SO_4$ at pH 6.5.

In a continuous modification of bisulfite process, where the bisulfite concentration itself was controlled continuously a yield of 25 % glycerol, 17 % ethanol and 11 % acetaldehyde based on sugar utilized has been reported (51).

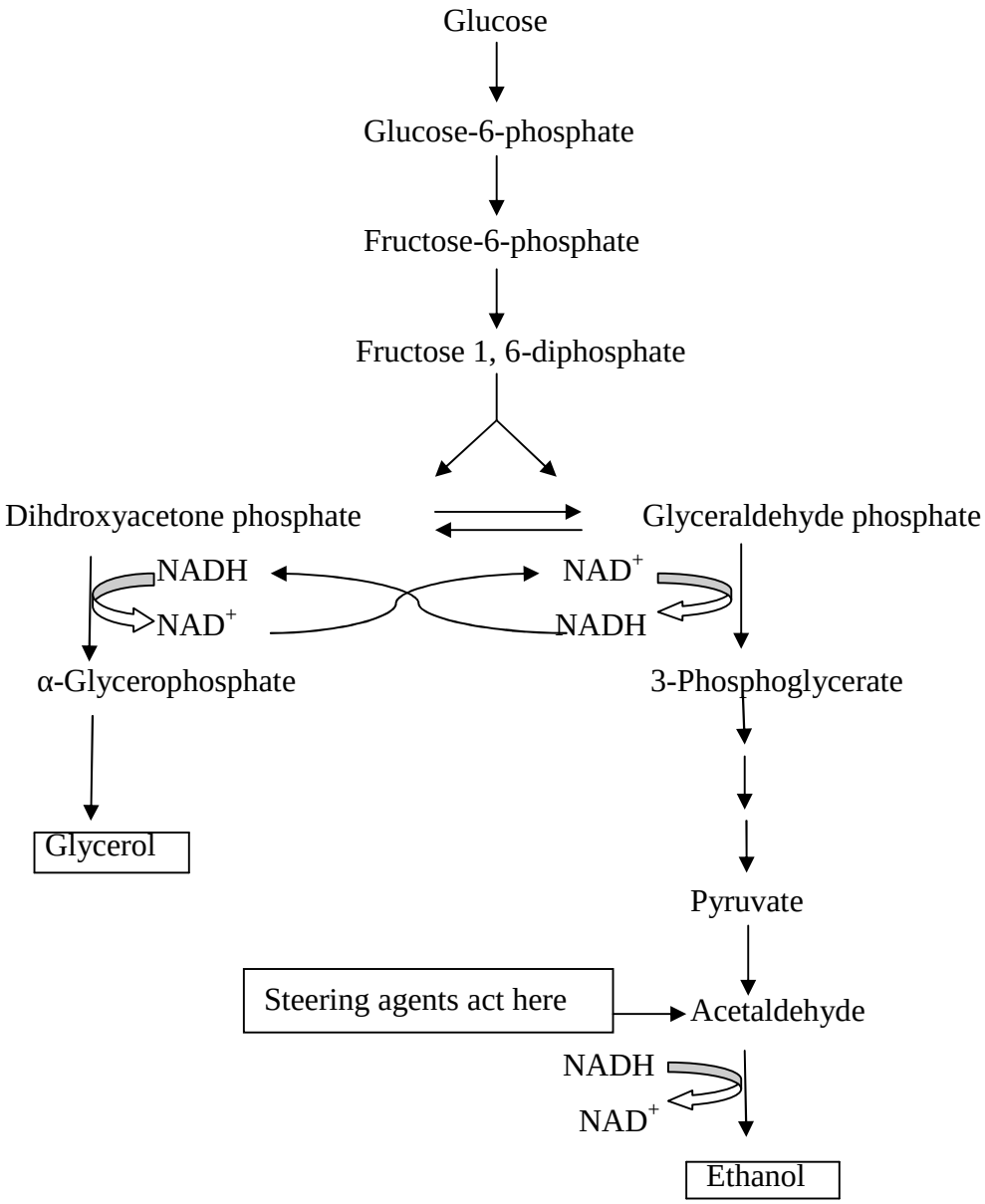


Fig. 1.1 Formation of Glycerol and ethanol via the Embden-Meyerhof pathway

Another way of minimizing the required amount of sulfite is to operate the fermentation under vacuum or with continuous sparging of CO_2 (56, 57). In this way acetaldehyde can be stripped off, during fermentation. In another report, a continuous fed batch vacuum fermentation system in presence of sulfite has been described. A glycerol concentration of 80 g/l was achieved with a productivity of 30 g/l.day at a dilution rate of 0.4/day which is twice that from a vacuum batch process (15 g/l.day) or four times that obtained without vacuum (58).

Fixed-bed studies on glycerol production in presence of sulfite using κ-carrageenan (59), polyacrylamide hydrazide (60) and sintered glass rasching rings (61) have been described. A continuous fermentation system, using immobilized

cells, application of vacuum distillation technique and the addition of CO_2 gas has led to a yield of 67 % as a percentage of metabolized sugar and glycerol concentration of 85 g/l in the fermented broth (62).

Actual glycerol yields in the sulfite process seldom exceed about 0.39 mol/mol (0.2 g/g) of the utilized sugar (53), although yields up to 0.68 mol/mol (0.35 g/g) have been reported (63).

1.6.3.2 The Alkaline Process

Glycerol production by yeast is also influenced by alkalis and alkaline salts. The alkaline process, also known as Neuberg's third form of fermentation, is probably as old as the sulfite process (34, 64). The basis of this fermentation is that, in the Embden Mayerhof pathway, two molecules of acetaldehyde undergo the Cannizaro's reaction, and are thereby converted to acetic acid and ethanol in the molecular proportion of 1:1. Acetic acid formation is accompanied by formation of NADH, which must be balanced by glycerol formation during anaerobic conditions.

In the process of Eoff et al. (37) blackstrap molasses and other sources of sugars were fermented in the presence of sodium carbonate. The blackstrap molasses was fermented by *Saccharomyces ellipsoideus* and sodium carbonate was added stepwise (up to 5 % of the wash), so that the medium always had an alkaline pH. The molasses mash contained about 20 % sugar. Glycerol yields in the same range as for the sulfite process (0.2 g/g) have been reported (36). Apart from sodium carbonate, other salts such as Na, K or NH_4 carbonate, bicarbonate, acetate, phosphate and hydroxide have been used as buffers to increase the pH value for alkaline glycerol production (65, 66, 67).

The main processes for glycerol production with non-osmophilic yeast, operated during World War I and II are compared in the Table 1.4. These processes were short lived and could not compete with post-war methods for synthetic glycerol production. The drawback of these processes has primarily been the low yield of glycerol from carbohydrates and difficulties in the recovery of glycerol from the fermented broth.

Table 1.4: Fermentation Processes for manufacture of glycerol

Process	Operating conditions	Products
Connstein and Ludecke process (or Protol Process)	Temperature= 30-32°C Sugar in broth = 10 % Na_2SO_3 added = 75-85 % of sugar weight Ferm^n Period = 2-3 days	Sugar conversion = 95 % Glycerol formed = 27.5 – 32.5 % of sugar consumed By products = Acetaldehyde + CO_2 + Ethanol
Eoff process (or alkaline or American carbonate process)	Temperature= 30-32°C Sugar in broth = 17-20 % Na_2CO_3 added = 5 % of mash weight Ferm^n Period = 5 days	Sugar conversion=94 % Glycerol formed=20-25 % of sugar consumed By products = Acetic acid + CO_2 + Ethanol
Cocking and Lilly Process	Temperature= 36°C Sugar in broth = 12-16 % Na_2SO_3 : $NaHSO_3$ mixture added (in 1:1 weight ratio) = 75-85 % of sugar weight Ferm^n Period = 6-9 days	Sugar conversion=95 % Glycerol formed = 42.5 – 47.5 % of sugar consumed By products = Acetaldehyde + CO_2 + Ethanol

1.6.4 Polyols production by osmophilic yeasts

Yeasts, like many other microorganisms, are able to produce polyhydroxy alcohols as an integral part of normal growth processes. The osmophilic yeast tolerates high concentration of salts and sugars and produce variety of polyols (glycerol, arabitol, erythritol, sorbitol, mannitol etc.) essentially under aerobic conditions and in absence of sulfites for alkaline conditions. Glycerol, sorbitol and mannitol are bulk produce with a low price and are available by alternate routes. Erythritol, arabitol, ribitol and DL-threitol are specialities with high added values (68). Osmophilic yeasts show interesting industrial potential. Because of their high tolerance for osmotic pressure, they can easily ferment highly concentrated sugar solutions (20 %, w/v and above). Excellent basic research on polyols production by osmophilic yeasts was performed many years ago by Onishi (69, 70), Spencer (7) and

Peterson's group (71, 72). In a growth associated process, these yeasts can convert as much as 60 % of sugars utilized to polyols (73). Interesting developments in polyols biosynthesis by osmophilic yeasts can be found in recent studies (74, 75, 76, 10, 77) and reviews (78, 12, 62, 79).

Formation of polyhydroxy alcohols is an integral part of the normal growth processes of the various yeasts, which produce them and the requirement of growth determines the requirement for polyols production. Yields of the different polyhydroxy alcohols, however, can be influenced by the condition of growth and production. Of these, the composition of the medium, especially the choice of the carbon and nitrogen sources, the level of aeration and the temperature have the greatest effect on the yields and rate of fermentation. The essential difference in the process from the "steered" fermentation were that, they employed aerobic rather than micro-aerophilic or anaerobic conditions of growth, a relatively high content of nitrogenous compounds and considerably higher sugar content in the medium, an improved rate of conversion of sugar to product, and a much improved yield of polyols.

In addition to the factors mentioned above, an adequate, but not excessive, supply of oxygen is essential for maximum yields of products in osmophilic yeasts. The growth of the yeast under conditions of sub optimum aeration results in increased quantities of ethanol being formed and a considerable decrease in the yield of glycerol. Thus, the metabolic pathway of glycerol formation in osmophilic yeasts is essentially same as in *S. cerevisiae* (8).

Several species of osmophilic yeasts have been investigated for polyols production. These include species such as *Saccharomyces, Zygosacharomyces, Torulopsis, Hansenula, Candida, Debaryomyces, Kluyveromyces,* etc. The nature of the yeast strain used has a profound effect on the yield of polyhydroxy alcohols produced.

Besides glycerol, most osmophilic yeasts produce a variety of polyols (8, 33) and may convert as much as 60 % of the utilized sugar to a mixture of polyols (11). The physiological role of higher polyols may serve as substitute compatible solutes for glycerol in cells, exposed to hyperosmotic stress, to allow for more efficient retention of the protective solute under such conditions (45).

At least two mechanisms are proposed in the production of D-arabitol and erythritol (8). The main pathway involves the decarboxylation of 6-phosphogluconate

via the hexose monophosphate pathway. The second mechanism involves the participation of transketolase enzymes, which transfer two-carbon units from ketoses or ketose phosphates to a variety of acceptors. In case of arabitol, the acceptor is one of the triose phosphate fragments formed by action of aldolase on fructose 1, 6-diphosphate and the result is the formation of 5-carbon chain. Similarly, erythritol is probably formed principally by the action of transketolase on fructose-6-phosphate, which leaves 4-carbon fragment that is probably reduced and deposphorylated. Since these yeasts have an aerobic metabolism, commercial production of polyols from osmophilic yeasts would require aerobic conditions. Very little is known regarding the metabolic control in these polyols formation. Nothing is known of the mechanism, that determine which of the polyols will be produced by any particular yeast.

Factors influencing polyols production

Besides the strain used, other factors, which can influence the polyols yield and ratio of polyols, are given below. The concentration of sugar, nitrogen and other components of the medium can influence the yield of polyols.

Carbon sources

Osmophilic yeasts utilize relatively few sugars. Kregar-van Rij has briefly summarized utilization of different sugars by different species of yeast (80). Most of the studies for polyols production with osmophilic yeast have been carried out using synthetic glucose media. Working with *Candida magnoliae* and glucose based media, Sahoo and Agarwal have detected small amount of arabitol and erythritol along with glycerol as major fraction of total polyols produced (76). Lucca et al. have reported production of glycerol and arabitol in glucose based media using an intergeneric osmotolerant yeast, PB2 (75). Park et al. have reported production of glycerol and erythritol in fed-batch fermentation in synthetic glucose media using *Trichosporon sp.* (26). In pilot plant production of glycerol, using *Torulopsis magnoliae* I_2B strain, Button et al. have used glucose based synthetic media. *Torulopsis magnoliae* assimilates glucose, galactose, raffinose and sucrose, but not maltose and trehalose. *Saccharomyces rouxii* uses glucose, maltose and sometimes galactose, trehalose and sucrose and produces glycerol and arabitol during growth on these substrates. *Pichia farinosa* uses a wider range of common sugars, including as well sarbose, cellobiose, xylose, ribose and some times Lactose and L-arabinose. However, according to the standard description of *P. farinosa* (80), the type strain does not metabolise sucrose.

It has been shown that *P. farinosa* (81) exhibits good growth on glucose, fructose, glycerol, mannitol and bagasse, rice straw and starch hydrolysates, but lacks the ability to produce glycerol from sucrose. In practice, only a few of the possible sugars have been tested as carbon sources for production of polyols.

The concentration of sugars and salts affects the yield and the ratios of the polyols formed (7, 83). The fermentation with osmophilic yeasts have been carried out in the sugar concentration range of 10-40 %. However, one of the intergeneric hybrid, PB_2, has been shown to grow even at 70 % glucose concentration (75). Working with *Saccharomyces rouxii*, Spencer et al. (39) found that increasing initial sugar concentration from 23 to 29 %, increased the yield of glycerol from 23 to 103 mg/ml, while the yield of arabitol fell from 55 to 51 mg/ml. However, the yield of polyols on the basis of glucose utilized, remained almost constant at approximately 50 %. Increasing the sugar concentration above 30 %, increased the rate of sugar utilization but lowered the percentage conversion to polyols. Peterson et al. (72) and Hajny et al. (71) observed similar increased total yields of polyols with increasing initial glucose concentrations in *Zygosaccharomyces* and *Torulopsis magnoliae* fermentations. However, the percentage yield of glycerol per unit of glucose consumed was reduced as the glucose concentration was increased. Sahoo et al. (76) have reported that increase in initial glucose concentration from 10.3 to 25.4 % resulted in two–fold increase in glucose uptake rate and a three-fold increase in glycerol productivity. A further increase in glucose concentration from 25.4 to 30.0 %, had little effect on the final glycerol concentration, as about 10 % of initial glucose remained unutilized. Onishi (82) working with *Pichia miso* found an optimum concentration of sugar for production of glycerol and arabitol approximately at 29 %. With increasing sugar concentration, the yield of polyols increased up to 43 %. If the sugar concentration is raised above 48 %, the yield fell considerably to 33 % and only about 30 % of the sugar was metabolized.

Ramachandran and Sulebele (83) investigated the effect of sugar concentration on *Torulopsis datilla*. Increasing the glucose content in the medium from 10 % to 40 %, increased the yield of glycerol from 16 g/l to 60 g/l, while the yield of erythritol rose from 37 g/l to 59.5 g/l. The highest efficiency of conversion of glucose to glycerol was realized at 30 % glucose concentration in the medium, while the efficiency of conversion to erythritol was maximum at a sugar concentration of 10 % in the medium.

Nitrogen Sources

The type and concentration of nitrogen supplied, greatly influences the yield of polyols. Polyols production using osmophilic yeasts investigated so far, have used yeast extract and urea as the major nitrogen sources. Use of supplementary nitrogen sources such as malt, malt sprouts, distillers dried solubels (84), beef extract, polypeptone and corn-steep liquor (82) and salts such as ammonium lactate, ammonium nitrate, ammonium phosphate etc has been reported.

Most of the osmophilic yeasts studied for polyhydroxy alcohol can use urea and ammonium salts as a source of nitrogen, except *Torulopsis magnolia* (85) and *Trichosporon oedocephalis* (86), which can utilize nitrate as a source of nitrogen. Most yeast, however, can use a variety of amino acids and other nitrogen compounds and complex nitrogen sources are usually used in media for polyols production.

It has been in general observed that reduction in the nitrogen levels increases glycerol yields. Increased nitrogen level favours cell growth with however, a decrease in sugar utilization. In some cases, the effects of availability of oxygen and increased cell growth were not separated from those of increased nitrogen concentration, so that lower yields of polyols may have been brought about by conversion of an appreciable fraction of the sugar to ethanol under conditions of diminished oxygen supply, i.e. the increased cell population, resulting from an increased concentration of nitrogen, depleted the amount of dissolved oxygen in the medium, actually available to the cells. Thus, the level of nitrogen and aeration as well as carbon/nitrogen ratio requires careful adjustment to maximise the polyols production.

With *Pichia miso*, Onishi et al. (82) studied the effect of yeast extract concentration in the range of 0.1 to 4.0 %. With increasing level of yeast extract, the conversion of glucose to ethanol increased from 0.6 to 64 % and conversion of polyols decreased from 49.0 to 6.0. This may be due to diminished oxygen supply per unit cell under conditions of presence of higher concentration of nitrogen.

Spencer et al. (87) investigated the effect of yeast extract concentration in the range of 0.5 to 1.0 % with *Saccharomyces rouxii* and found an increase in polyols yield from 3.7 to 7.0 % and reduction in fermentation time from 17 to 7 days. The pH of the mash was considerably reduced, but adjustment of the pH with calcium carbonate did not improve the yield. Substituting with corn steep liquor was not a satisfactory nitrogen source. However, when the concentration of corn steep liquor

was reduced to 0.75 % and that of urea was increased from 0.1 to 0.35 %, yields of polyols comparable to those obtained with yeast extract as a nitrogen source were obtained. Most workers used small concentration of urea as a supplement to the yeast extract, obtaining slightly improved yields thereby. The effect of urea in the fermentation medium is the reduction in the fermentation time. However, it may not affect the polyols yield directly.

Hajny et al. (84) found excessive cell growth and reduced erythritol yield at higher concentration of yeast extract and urea. Satisfactory yields were obtained in media containing 10 % glucose, 0.5 % yeast extract and 0.1 % urea. They also found that corn-steep, blackstrap molasses, malt sprouts, malt and distiller's dried solubles could be used as nitrogen sources. The addition of urea allowed the concentration of these products to be reduced while maintaining the yield of erythritol. Changes in the concentrations of corn steep liquor, urea and glucose showed very clearly the importance of control of the C:N ratio in erythritol production by this organism. This data is an excellent demonstration of the importance of the use of the optimum C:N ratio in media designed for optimum yields of polyols.

Similar observations have been reported by Sahoo and Agarwal (76). In their study with *Candida magnolia* higher C/N ratios resulted in poor cell growth, slow glucose uptake and reduced glycerol productivity, but the yield of glycerol based on glucose utilized increased and only a small amount of ethanol was produced. With decrease in C/N ratio, a greater cell mass was produced, and the glucose uptake rate increased. This did not lead to enhanced glycerol accumulation by the yeast. The organism produced a maximum amount of glycerol (78.5 g/l) at a C/N of 49.5, and a decrease or increase in C/N ratio from this resulted in a lower glycerol production. They also showed that accumulation (of a small amount) of glycerol at the initial growth phase was independent of C/N ratio, and a large variation in glycerol yield pattern occurred later in fermentation. Although the yield of glycerol per unit of glucose utilized was high at higher C/N ratios (88.0 and 71.0), a C/N ratio of 49.5 was optional in terms of total glycerol production and productivity.

Effect of Salts:

Sodium and potassium salts are important for increasing the osmotic tension of the medium, rather than for any direct involvement in the metabolism of the organisms.

Onishi (40) extensively studied the osmophilic yeasts isolated from salty environments, which displayed good salt-tolerance or perhaps even a requirement for NaCl. Working with *Saccharomyces rouxii* (88), he reported increased glycerol yields, in a medium containing 9.23 % initial glucose concentration and 18 % NaCl, of 43.0 % as compared with 27.4 % yield in a medium containing 30 % initial glucose concentration and no salts. However, in the salt free medium, glucose utilization was more rapid.

Hajny et al. (84) found a considerable decrease in glucose utilization as the concentration of KCl was increased from 0 to 3.0 M, and at the same time, erythritol yield decreased and glycerol, which was not formed in the potassium chloride free medium appeared in increasing yield. Onishi (88) found a similar type of phenomenon with *Pichia farinosa* in the presence of 18 % NaCl. In the absence of NaCl, the yeast produced both polyols but, as the salt concentration was increased, the concentration of arabitol decreased until at 6 % NaCl, it had virtually ceased to be produced and the sole product formed, was glycerol.

The mechanism of increased yield of polyols and the shift in the product formation in presence of salts is not yet understood.

Effect of Phosphate:

Phosphate was found to exert profound influence on polyols yield by osmophilic yeasts. Spencer and Shu (89) investigated the effect of oxygen tension and inorganic phosphate concentration on the production of polyols by *Saccharomyces rouxii*. They observed that increasing the concentration of inorganic phosphate, decreased the yield of glycerol and arabitol and increased the yield of ethanol. The change in yields of polyols with increasing phosphate concentration was most pronounced at a partial pressure of oxygen of 280 mm of Mercury. Further studies indicated that the effect was due to increased cell growth and consequent exhaustion of oxygen from under aerated medium. This results in decrease in oxidative metabolism and an increase in fermentation, with a consequent decrease in glycerol and increase in ethanol. However, it was also shown that changes in phosphate concentration did not significantly affect the yield of polyols other than glycerol (8).

However, with *Pichia miso*, Onishi (82) observed that increasing the phosphate concentration up to 2 %, the glycerol yield remained fairly constant and the glucose utilization was rapid and complete.

In view of these conflicting reports, the precise role of phosphate in the regulation of polyols production is not clearly understood. However, much of the effect can be explained in terms of cell growth and the consequent effects on the available oxygen.

Effect of vitamins and amino acids

Various growth factors (84) and amino acids (90) are required by osmophilic yeasts. Yeast like fungus used for erythritol production (84), required the presence of thiamin for optimum product formation and growth. However, Onishi (82) found no effect on polyols synthesizing ability of *Pichia miso* of external addition of vitamins and trace elements, but addition of casamino acid decreased polyols formation and increased ethanol yield. Most of the yeasts can utilize many amino acids as sole nitrogen sources for growth and would likely use them for polyols production.

Effect of temperature

Increase in rate of product formation and sugar utilization, was observed with increasing temperature (87). With *Saccharomyces rouxii*, increasing temperature, at an initial sugar concentration of 20 %, has been shown to increase the final concentration from 39 mg/ml to 64 mg/ml and that of arabitol from 61 mg/ml to 70 mg/ml. However, with elevated sugar concentration of 30 % and 35 %, no major increase in polyols production was observed. Similar results were obtained by Peterson et al. (72) with various strains of *Zygosaccharomyces*. Hajny et al. (71) found that increasing temperature from 30°C to 35°C increased the rate of glucose utilization by *Torulopsis magnoliae* with a slight increase in glycerol yield. For *Endomycopsis chodatti*, the optimum temperature for arabitol production was between 30°C to 35°C, yields were somewhat lower at 25°C and both yields and cell growth greatly reduced at 40°C.

For *Candida Magnoliae* (91), the optimum temperature for glycerol production was found to be 35°C.

Effect of pH

It has been well established with non-osmophilic yeasts, that alkaline pH increases glycerol production. However, yeast growth is optimum between pH 4.0 to 4.5.

With osmophilic yeast, *Pichia farinosa*, it has been reported (77) that polyols yield increased under alkaline conditions. Na_2CO_3 used to produce alkaline conditions, also helps to improve the rate of sugar utilization.

Patil and Sastri (92) have shown that with *Hansenula anomala* in 30 % glucose media, increasing the pH improves the polyols yield. It was observed that the controlled pH at 8.0 gave the same sugar utilization rate as obtained without pH control. However, improved polyols yields were obtained under alkaline conditions.

With *Candida magnoliae* (76) no improvement of glycerol production could be obtained by maintaining pH constant at 3.5, 4.0, 4.5, 5.0 and 6.0 as compared to uncontrolled pH experiments. Therefore, it was concluded that pH control was not necessary in this fermentation.

There are no other reports available on effect of controlled pH on fermentation by osmophilic yeasts.

Effect of Oxygen Transfer

Production of polyols by osmophilic yeasts, is an aerobic and growth associated process. The effects of oxygen transfer on the osmophilic yeast fermentation are clear and unequivocal. Campbell and Hogbom (93) have already shown the effect of aeration and related factors in influencing the growth and product formation in yeast fermentation. They found that under conditions of rapid growth and high cell yield, the ethanol yield also increased.

Saccharomyces rouxii like *Saccharomyces cerevisiae*, exhibits the Pasteur Effect as the level of aeration is varied, so that, at low levels of aeration, conversion of fermentable sugar to ethanol is relatively higher and decreases as the level of aeration is increased.

With most of osmophilic yeasts, presence of ethanol in fermented broth, is an indication of inadequate aeration and low yields of polyols could often be due to sub optimal oxygen supply. In all cases, increased aeration was necessary for maximum yields in media, containing higher concentration of sugar, but aeration rates which were too great were detrimental for polyols production (7).

Working with *Pi farinosa*, Vijaikishore and Karanth (94) have shown that aeration and agitation should be maintained above a critical value to give a dissolved oxygen of 40 % saturation for enhanced glycerol and negligible ethanol yields.

Liu et al. (95) have applied oxygen limitation strategy to enhance glycerol production by *C. krusei* in a bioreactor. Glycerol dissimilation could be effectively inhibited due to oxygen limitation and at the same time the residual sugar concentration was also decreased. The final glycerol concentration reached 51.8 g/l at 96 hr and 54.9 g/l at 116 hr, which was 18 and 60 % higher than the control (without oxygen limitation), respectively.

Djelal et al. (96) have investigated the effect of dissolved oxygen on the bio-production of glycerol and ethanol by *Hansenula anomala* growing under salt stress conditions. The experimental ratio of ethanol on glycerol produced, was 5.1 when the available oxygen was lowered (low stirring rate, 500 rpm) and increased to 10.2 in absence of aeration. Extracellular glycerol production was optimal for a moderate stirring (1000 rpm) and aeration (1.4 vvm) rates. These optimal conditions resulted in an experimental ratio ethanol on glycerol produced of 4.1.

Parekh (97) working with *H. anomala* has made detailed study of oxygen transfer rate in conventional and draft tube fermenters, using sulfite oxidation method. The values of oxygen transfer rate and volumetric mass transfer coefficient are summarized in Table 1.5.

Maximum yield of 72 g/l of glycerol in draft tube fermenter at 800 rpm stirrer speed and 1.2 vvm air flow rate has been reported. At these conditions, he observed an increase in 5 % in biomass value and 14 % in glycerol production in draft tube fermenter as compared to the conventional fermenter. For maximum glycerol production, the optimum OTR is estimated to be 67.8 mMole/l/hr.

In a glucose based fermentation media and *H. anomala*, Patil and Sastri (98) have obtained optimum polyols yield at an air flow rate of 0.5 vvm and agitation rate of 600 rpm. The volumetric mass transfer coefficient (K_La) and the maximum oxygen transfer rate (OTR) has been estimated to be 132/h and 6.6 mMol/l/h, respectively.

Hajny et al. (84) observed similar results during the production of glycerol by *Torulopsis magnolia*. They obtained an optimum yield of glycerol, somewhere between aeration rate of 42 and 200 mMole O_2/l/h. There was a considerable decrease in glycerol yield when the aeration rate was raised from 200 to 360 mMole O_2/l/h, but yields were still better than those obtained at 42 mMole/O_2//l/h.

Table 1.5: Effect of aeration and agitation on oxygen transfer rate and volumetric mass transfer coefficient (97)

Fermenter Type	Agitation speed Rpm	Aeration Vvm	OTR mMole/l.h	K_La (h^{-1})
2 litre	450	0..50	19.6	98.0
conventional fermenter		1.20	34.6	173.0
		1.50	41.2	206.0
		1.75	49.6	248.0
	800	0..50	33.0	165.0
		1.20	55.2	276.0
		1.50	59.2	295.0
		1.75	64.9	324.5
	1020	0..50	38.2	191.0
		1.20	64.0	322.0
		1.50	76.0	380.0
		1.75	80.2	401.0
Draft tube fermenter	800	0.85	50.0	250.0
		1.20	67.8	339.0
		1.50	78.0	390.0
	1020	0.85	56.0	280.0
		1.20	85.0	425.0
		1.50	98.0	490.0
Shake flask	Volume of the medium			
500 ml, 200 rpm	40		47.2	236.0
	50		40.2	201.0
	75		36.0	180.0
	100		25.0	125.0

Effect of Inoculum size

If the cell density is increased, the oxygen supply must be also increased, or the cells will be starved for oxygen, and ethanol will be produced instead of polyols. If the density of nitrogen and phosphate are kept low, the density of yeast population will remain relatively low and a low level of oxygen may suffice for maximum yields of polyols. If the concentration of these nutrients is increased, the cell population density will increase and thus the oxygen requirement.

The rate of oxygen supply per unit of yeast cell mass, which is the critical factor determining the conversion of glucose to polyhydroxy alcohols is determined

by the density of yeast population in relation to the rate of solution of oxygen in the medium. This in turn, is a function of other nutritional factors in the medium, especially the nature and concentration of the nitrogen source and the carbon/nitrogen ratio in the medium (8).

Sahoo, D. K. and Agarwal G. P. (99) working with *Candida magnolia* have reported that cell growth as well as glycerol production is strongly affected by oxygen supply. Improvement in OTRs resulted in increased cell growth and glycerol yield. However, at high OTRs, there was a reduction in glucose uptake rate, indicating Pasteur Effect, and glycerol accumulation was also reduced at K_La of 253 h^{-1}. The availability of oxygen per unit of cell mass was found to be the most important factor that controlled the cell growth, glucose uptake, and glycerol yield.

Thus, the solubility of oxygen in the prevailing environment and nutritional conditions and the amount of oxygen available to the yeast cells is of paramount importance in the production of polyols by osmophilic yeasts.

Bioreactor Design

Being an aerobic fermentation, the choice and design of a suitable bioreactor system, able to achieve optimum oxygen transfer rate under prevailing nutrients and environmental conditions, is most important in the development of an economical process for bioglycerol production. Use of bioreactor also allows the possibility of accurately controlling the pH and dissolved oxygen, which has indeed been found to be an important parameter in a number of genetic and physiological approaches of glycerol enhancement (12).

Polyols production with osmophilic yeast studied so far, have used a batch reactor. Vijaikishore and Karanth (78) have reviewed the possible implications of various types of bioreactor designs on glycerol production with osmophilic yeasts.

Fed-batch operation is experimentally somewhat more demanding, but gives certain advantages. The most important advantage of fed-batch compared to batch cultivation is that, effects of inhibitors, such as steering chemicals, can be minimized, since their concentration in the medium can be kept low (100, 101, 102, 103). In addition, by using fed-batch technique, it is possible to maintain limitation of a certain substrate component, such as phosphate in the cultivation of the osmophilic yeast, *Torulopsis magnolia* (104). In such pilot plant system, the cell growth phase was separated from the glucose to glycerol conversion phase. The glycerol production

phase was extended several-fold by periodic glucose addition. The yeast cell count was kept constant by limiting the phosphate concentration, a deficiency required for efficient glycerol production. Fed-batch culture approach has been also used for improving the productivity of erythritol (26). Burschäapers et al. (74) have used fed-batch operations to achieve high erythritol concentrations (up to 170 g/l) and high polyols concentrations (up to 188 g/l) as compared to erythritol concentration of 90 g/l and polyols concentrations of 150 g/l in batch fermentation mode.

Continuous fermentation shares several advantages with fed-batch fermentation, although the risk of contamination increases. A continuous process based on sulfite method was found to work satisfactorily (51). A multistage Cascade bioreactor has been also used to improve the glycerol productivity (105). In a six stage Cascade bioreactor, when the dilution rate was increased from 0.023 to 0.085 h^{-1}, the glycerol yield, based on biomass, remained constant, but specific productivity increased by 94.7 %. Productivity increased by only 46.6 % due to low biomass concentration in the bioreactor at the higher dilution rate.

Cell immobilization or recycling has been applied in order to provide a high cell density in the bioreactor, centrifuging and recycling of cells resulted in increased production rate of glycerol (106), and cell immobilization was reported to significantly increase the glycerol production rate in batch (107), fed-batch (99) and continuous cultivation (108, 109, 110). Various supports such as agarose (110), Ca-alginate and polyacrylamide hydrazide (60), sintered glass (111, 61) and κ-carrageenan (59) have been used. Obviously, the benefit of increased volumetric productivity must be weighed against the added cost for cell recycling or immobilization.

Polyols production employing 80 L bubble column and 80 L airlift tower loop reactor, has been also reported (112). At high initial concentration of glucose (500 g/l), high erythritol concentration and yield of 175 g/l and 43 %, respectively as well as high total polyols concentration and yield of 250 g/l and 60 % has been achieved.

1.6.5 Recovery and Purification of Polyols

In steered fermentations employing non-osmophilic yeasts, glycerol is the only polyol that is present in the broth. Some reports with osmophilic yeasts (76) also indicate that glycerol is the only polyol produced during fermentation. To recover

glycerol cost-economically, such processes should lead to fermentation solutions with (62),

- High concentration of glycerol in broth
- Absence of by-products e.g. ethanol and other polyols
- Low amounts of yeast cells,
- Absence of residual substances, e.g. sulfite, salts, molasses etc.

A process will have a good chance for industrial use, if most of these conditions are met. Many recovery methods from fermentation broths have been described. Most important are those reported by Bernhaler (113) and Weixl–Hofmann (114).

In unsteered aerobic fermentations employing osmophilic yeast mixture of polyols is obtained. Recovery of individual polyols from the mixture and their purification is expected to increase the cost of downstream recovery process.

For separation of arabitol and glycerol from fermentation broth Onishi (115) added Zinc Sulfate to the broth, filtered and concentrated it under reduced pressure. Water was then removed by lyophilization, the dried material was extracted twice with 99 % ethanol to get crystals of arabitol and then the glycerol was vacuum distilled.

Roxburgh et al. (116) recovered glycerol from a mixture of polyols using a method which consisted of first removing the yeast cells by settling or centrifugation after acidification of the broth. Most of the water was then removed by evaporation, preferably under vacuum. Hot ethanol was then added, the gums were filtered off and the solution was decolourized with active charcoal. Erythritol and arabitol are only slightly soluble in cold mixtures of ethanol as compared to glycerol, and they crystallize from the solution on cooling. After the solids are removed by filtration, alcohol was recovered from the liquid by distillation at atmospheric pressure, and glycerol was vacuum distilled.

Some researchers (117, 67, 118) have used latest separation techniques such as dialysis, ion-exclusion, ion-exchange chromatography, ultrafilteration and reverse osmosis (119) for separation and purification of polyols. Chinese scientists working at Research Center for Industrial Microorganisms and Research and Design Center for Glycerol Fermentation, School of Biotechnology, Wuxi University of Light Industry, China, have reported (17) development of an advanced distillation technique called

carrier distillation for recovery of glycerol with a glycerol distillation efficiency of greater than 90 %. More work is needed for developing an effective recovery process for commercial application.

1.7 EVOLUTIONARY ALGORITHM, ARTIFICIAL NEURAL NETWORKS, LAZY LEARNING AND MATHEMATICAL MODELING IN FERMENTATION STUDIES

Evolutionary Algorithms

An algorithm is a sequence of unambiguous instructions for solving a problem, i.e. for obtaining the required output for any legitimate input in a finite amount of time. It is a sequence of instructions (or set of instructions) to make a programme more readable.

An algorithm is a step by step instruction to execute a program which must be unambiguous. Think of an algorithm as a mathematical recipe - it describes the process for completing some task via a sequence of steps.

The word “algorithm” comes from the name of a Persian author, Abu Ja’far Mohammed ibn Musa al Khowarizmi (c. 825 A.D.), who wrote a textbook on mathematics.

The conventional way for fermentation is to add yeast to the wort and wait for some time, letting the yeast consume substrates and produce product. Fermentation can be accelerated with an increase of temperature but some contamination risk (Lactobacillus, etc.) and undesirable by-products yields could appear. With the data obtained experimenting in the laboratory, it has been possible to develop a new model of the fermentation dynamic behaviour based on the activity of suspended biomass. Thus, some equations of the model are devoted to the biomass behaviour: part of it settles slowly and is inactive, while the active biomass awakes from latency to start growing and producing product, etc.

The classical approaches to optimize biotechnological processes are time consuming and allow only a single–dimensional search. Therefore, statistical-mathematical methods represent a great improvement in experimental design. Among these methods genetic algorithms (GA), which mimic the natural selection, seems to be promising. GA is a search/optimization technique based on natural selection. Successive generation evolve more fit individuals based on Darwinian survival of the

fittest. The genetic algorithm is a computer simulation of such evolution where the user provides the environment (function) in which the population must evolve.

Evolutionary algorithms (EAs) is an umbrella term used to describe computer based problem solving systems which use computational methods of some of the known mechanisms of evolution as key element in their design and implementation. A variety of Evolutionary algorithms (EAs) have been proposed. The major ones are: Genetic algorithm, evolutionary programming, evolution strategies, classifier systems and genetic programming. They all share a common conceptual base of simulating the evolution of individual structures via processes of selection, mutation and reproduction. The processes depend on the perceived performance of the individual structures as defined by the environment. More precisely, EAs maintain a population of structures, that evolve according to the rules of selection and other operators, that are referred to as “search operators” (or genetic operators), such as recombination and mutation. Each individual in population receives its measure of fitness in the environment. Recombination focuses attention on high fitness individuals, thus exploiting the available fitness information. Recombination and mutation perturb those individuals, providing general heuristics for exploration. Although simplistic from a biologist’s viewpoint, these algorithms are sufficiently complex to provide robust and powerful adaptive search mechanisms.

Evolutionary algorithms (EAs) use several variables of a problem to provide an optimum solution. They are preferred alternative method for monitoring state variables in biotechnological processes (120). EAs techniques include the method of Neuro-computing, evolutionary computing, probabilistic computing, belief networks, fuzzy logic (FL) and chaotic computing (121). The optimization techniques that have been applied to solving complex problems in biotechnology are dynamic programming (DP), stochastic dynamic programming (SPD) and heuristic programming such as genetic algorithms (GA), differential evolution (DE), shuffled complex evolution, fuzzy logic (FL) and artificial neural networks (ANNs) (122).

The rapid development of artificial intelligence, computer technology and software have been found to be advantageous over the conventional methods in dealing with system modelling and optimization problems especially those involving nonlinear and complex mathematical approaches (123). Three evolutionary algorithms commonly used for the optimization of fermentation processes are

artificial neural networks (ANNs), genetic algorithm (GA) and differential evolution (DE).

Genetic algorithm is a stochastic optimization technique that searches for an optimal value of a complex objective function and is used to solve complicated optimization problems by simulation or mimicking a natural evolution process (124). It involves repeated procedures with an initial population of potential solutions, a fitness evaluation via the application of genetic operators and the development of a new population (125). In addition, GA has been successfully used as a tool in computer programming, artificial intelligence, optimization, neural network training and information technology since its introduction by Holland (126). GA starts with an initial set of solutions called population and each solution in a population is called chromosome or individual which are evolved through successive iterations called generations by genetic operators such as selection, crossover and mutation that mimic the principle of natural evolution (127). “Selection” means that two individuals from the whole population of individuals are selected as “parents”, which depends on the value of the fitness function of each individual. “Crossover” exchanges the segments of selected parents between each other by probability. Crossover allows the exploration of the feature space to find a near to optimal solution. Mutation randomly alters the value of each element of the chromosome according to a probability called mutation probability.

Artificial neural networks

Artificial neural networks (ANNs) models are designed to mimic the human learning processes by creating linkages between process input and output data. They also ‘learn’ how to reproduce an output from the input parameters without any prior knowledge of the relationship between them (128). ANNs have incredible arbitrary decision boundary capabilities, capacity to adapt to different types and structure of data easily. They can predict, analyse, associate and emulate the connectivity of biological neurons to solve complex problems in the same manner as the human brain (124; 129). ANNs model architectures and algorithms of neuro-computing have been developed and applied successfully. In the study of theoretical aspect of ANNs, the potential and capabilities of interconnecting of several basic components based on the model of neuron was pioneered by McCulloch and Pitts (130).

In the 1980s, Hopfield (131) applied a particular nonlinear dynamic structure to solve optimization problem. Rosenblatt (134) coined the name perceptron and devised architecture, which are recognised as helpful tools for dynamic modelling (121). Neurons of the network are arranged into several groups called layers. ANNs can be a multi-layer neural network that has hidden and output layers. The most commonly used neural network for solving nonlinear regression problems is the multi-layer feed forward neural network called multi-layer perceptron (MLP). Algorithm used for the optimization problems during training of the ANNs is by means of back propagation (BP) algorithm. It involves the minimization of performance function commonly called mean-squared error (MSE) including linear programming (LP) and non-linear programming.

Lazy Learning

Lazy learning algorithms are machine learning algorithms (133) that are welcome members of procrastinators anonymous. Purely lazy learners typically display the following characteristics (134):

1. Defer: They delay the processing of their inputs until they receive requests for information; they simply store their inputs for future use.
2. Demand-Driven: They reply to information queries by combining information from their stored samples.
3. Discard: They delete the constructed query and any intermediate results. In contrast, eager algorithms greedily replace their inputs with an abstraction and use it to process queries.

Lazy learners have been designed to induce decision trees (135, 136), rule sets (137, 138), and improve speedup learning algorithms (139). However, the most frequently studied group of lazy learners are those that use similarity functions to answer queries. These include k-nearest neighbor classifiers and algorithms identified by names satisfying the following grammar:

(case, exemplar, instance, memory) -based (learning, reasoning).

Eager learning algorithms assume their learning bias is appropriate for the performance task. When this assumption is correct, this can yield performance benefits (e.g., increased query response speed). However, there is a risk that this assumption is wrong, and that information lost during eager abstraction is crucial for generating accurate responses to queries. Thus, a key advantage of lazy algorithms is

that they can respond to unanticipated queries in ways not available to all eager learners (140).

Lazy algorithms have three other computational advantages. First, they have small raining costs (e.g., store a sample and update some indices), although this is frequently balanced by higher costs for generating predictions unless a fast indexing scheme is implemented., where a data stream continually updates the set of input samples. Second, lazy problem solvers provide efficiency gains through solution reuse; they can store and adapt solutions for subsequent problems, which can greatly reduce problem solving effort. Finally, lazy algorithms can generate precedent explanations, which are preferable to abstract explanations for many tasks. Only purists and teachers remain interested in purely lazy, standalone learners. Most research and practice with lazy learners involves some form of caching, which can be used to one tune a lazy learner (e.g., by storing information on prediction quality) so as to improve its performance.

Mathematical Modelling

Mathematical models describing the behavior of microbiological systems are important as they provide a mathematical description of mechanism of the process and that they are required for optimization and control. The objective of mathematical modelling is to obtain expressions that quantitatively describe the behavior of the process under consideration. The generality of a model depends upon several factors, which include the complexity and information available concerning the process. Given a suitable model, optimization and control system design of the process can be performed. With this viewpoint, mathematical modelling of fermentation processes has been attempted in the past (141, 142, 143, 144).

Parameter estimation is often an essential step in the verification and subsequent use of a mathematical model in many fields of science and engineering. In biochemical engineering problems, it is often necessary to estimate the model parameters of nonlinear algebraic or differential equations. The mathematical estimation of model parameters is based on minimization of some quantity that can be calculated and is a function of parameters to be estimated. If the model under consideration is linear, the estimation will be generally an easy task. Linear regression or plot procedures are well known and do not pose any major problems. There exists,

however, no unique method for nonlinear models. Several approaches have been suggested to estimate the parameters of nonlinear models (145, 146, 147, 148).

The unstructured models of bioreaction are usually described by a set of nonlinear differential equations. Parameter estimation in unstructured growth models is often performed with the aid of continuous fermentation. These continuous experiments are time-consuming and complex in nature. In contrast, more simple experiments can be achieved with batch fermentation. However, the kinetic parameters of the Monod model are difficult to identify from such experiments. From the application of sensitivity analysis, Baltes et al. (149) demonstrated that the sensitivity functions were strongly correlated. Optimal experimental design of fed-batch experiments has been introduced to alleviate such a correlation (149, 150, 151). A kinetic model established from batch experimental observations is, in general, applied to evaluate the concentration profiles of cell mass, substrate and product for fed-batch fermentation processes. Such a kinetic model may not be perfectly applied to predict the concentration profiles for a fed-batch fermentation process. A dynamic model of fed-batch fermentation processes includes the dilute term. This dilute effect causes different morphogensis of microorganisms from the one in batch fermentation. On the other hand, the kinetic model established from fed-batch experimental observations may be unsuitable to predict the concentration profiles for batch fermentation. To overcome such drawbacks, both experimental data have to be simultaneously used to estimate the parameters of the kinetic model.

The modelling of fermentation processes is a basic part of any research in fermentation process control. Since all the optimisation work to be done, is based on the reliability of the model equations, they are important for the right design. The understanding and study of any process, requires a mathematical representation or model of the process. The process may have an input-output representation or a time series. The model is based on the prior physical or subjective knowledge about the process itself, the measured data on the inputs and the outputs, and the physical and engineering laws governing the working of the process. If the model is a complete and exact representation of the process, it is called a deterministic model, and the process is called a deterministic process. The parameters of such a model are precisely known, and the model can be used to produce exact prediction of the process response from the past data. Most real life processes cannot be represented by this kind of model, because of the dynamic nature of the process and the lack of information and other

uncertainties being associated with the available data. A model that incorporates noise or disturbance terms to account for such imprecision in the knowledge of the process is called a stochastic model.

1.8 SCOPE AND OBJECTIVES OF THE PROPOSED WORK

Increasing prices of petroleum feed-stocks has made it necessary to look for alternate energy sources as well as alternate routes to produce downstream petro-chemicals such as synthetic glycerol. At the same time, because of gradual increase in oil-seed prices and replacement of conventional soaps by detergents have caused decline in production and availability of natural glycerol in the country. India is still importing this vital industrial chemical. Interesting developments are also taking place in developing new products based on glycerol such as syngas by means of electric arc generated plasma (152).

On the other side, the increasing stocks of sugar in the world as well as in the country have resulted in decline in sugar (sucrose) prices. Sugar cane and sugar beet processing products such as molasses, sugar cane/beet juice or even the crystalline sucrose are renewable raw materials that can be used to produce new fermentation based products.

Production of glycerol and related polyols e. g. mannitol, xylitol, erythritol, arabitol etc. by fermentation of sugary raw materials by osmophilic yeasts is an interesting research area of yeast biotechnology. At present, polyols such as arabitol and erythritol have limited commercial applications. However, the market potential of these polyols will further develop when they will be produced cheaply at a large scale. Sucrose being cheaper in developing country like India, it would be desirable to study the production of these polyols from sucrose based media.

In our previous study a strain of osmophilic yeast *Hansenula anomala* was selected for polyols production using glucose as the basic carbon source. Using shake flask experiments, optimization of fermentation medium and environmental parameters was carried out. Investigations were also carried out on fermenter scale to study the effect of aeration and agitation on the oxygen transfer during polyols production. The kinetics of glucose utilization, product formation and effect of pH with reference to polyols yield and specific rate of product formation was also studied.

In the proposed investigation, the data generated in the previous study as mentioned above will be used to carry-out simulation studies to predict polyols production on higher scale of operation. Lazy learning, a recently introduced memory based local learning method will be used to conduct the simulation studies.

Further investigations and process development work will be carried-out with *Hansenula anomala* and using sucrose as the basic carbon source. Shake flask experiments will be conducted to optimize the nutrients concentration. We propose to use the latest tools such as evolutionary algorithm, artificial neural networks, colony optimization etc. to model the input-output relations and for accurate optimization of nutrients concentrations of the fermentation media. We also propose to optimize the nutrients concentration using conventional method of one-variable-at-a-time approach and compare the results obtained by both the methods.

Various analytical methods required to analyze samples of fermentation broths will be developed and standardized. Attempts will be made to develop method for simultaneous estimation of individual polyols and sugars during polyols production. Since in the case of *Hansenula anomala*, the viable and dead cells can be easily examined and counted in suspension under microscope, it was decided to base the studies and express the results in terms of viable count instead of a routinely used method of estimation of dry cell weight.

Using shake flaks optimized sucrose based media; investigations will be also carried-out on fermenter scale to optimize process parameters such as pH, temperature, inoculum size, rate of agitation etc. Effect of oxygen transfer will also be investigated by controlling the oxygen percent saturation (pO_2) during the course of batch fermentation. Attempts will be made to improve the overall yield and productivity of polyols.

Fed-batch experiments will be conducted to investigate the product inhibition aspect of polyols formation by osmophilic yeasts. Objectives of fed-batch experiments will be to maximize the product formation and reduce the overall cost of production of polyols.

Based on the data generated, a kinetic model will be developed for design purposes and prediction of final product yield on larger scale of operations.

Presentation of the work

The work carried out in this investigation is presented in six chapters. Chapter-1 reviews the work carried out by various researchers and groups on polyols production by osmophilic yeasts. This chapter also highlights the latest tools available for simulation and modelling for fermentation studies. The scope and objectives of the present investigations are also highlighted in this chapter. Chapter-2 describes the analytical methods used to study the fermentation experiments. Merits and limitations of various analytical methods studied are explained in this chapter. Chapter-3 briefly describes our previous work using glucose as carbon source. The method of lazy learning is also introduced in this chapter and used to process the data generated in our previous study. Chapter-4 mainly deals with shake flask studies employing genetic algorithm and one-variable-at-a-time approaches. The media optimized in shake flask studies is used in Chapter-5 to conduct fermenter scale experiments related to optimization of parameters of batch process. Fed-batch experiments conducted in an automated fermenter are also explained and discussed in this chapter. The results are expressed in terms of overall yield and productivity of polyols. Finally, Chapter-5 also explains the development of a kinetic model based on the data generated in batch and fed-batch experiments. Chapter-6 summarizes the conclusions of the investigations and recommends the future work required to be conducted in this area.

CHAPTER-2

ANALYTICAL METHODS AND CULTURE MAINTENANCE

CHAPTER-2

ANALYTICAL METHODS AND CULTURE MAINTENANCE

2.1 INTRODUCTION

Thin layer chromatography, liquid chromatography, gas chromatography and spectrophotometry based methods have been used in determination of polyols, reducing sugars (glucose and fructose), sucrose and ethanol from fermentation broths.

High performance liquid chromatography is one of the most versatile methods for simultaneous determination of polyols and sugars in broths of osmophilic yeast fermentations. Many workers (25, 26. 27, 61, 74, 75, 103, 153. 154. 155) have used HPLC for studies with polyols production for reliable, accurate and fast estimations. However, during our course of investigation such facility was not available for routine use.

Many groups working on osmophilic yeasts for glycerol and other polyols production have also used a spectrophotometric method based on periodate oxidation (72, 73, 76, 83, 97, 104, 106, 156). This method estimates quite accurately the total polyols in presence of sugars in fermentation broth.

Direct injection of fermentation broths by Gas chromatography for estimation of polyols is reported in the literature (157). We tested this method in our laboratory after pre-filtration of fermentation broth samples. However, we were not able to reproduce the results reported. We, therefore, tried to develop a method based on derivatisation of sugars and polyols in fermentation broths and separating the derivatives by gas chromatography. The details of the method which is developed and tested are reported below.

Spectrophotometric methods used for estimation of sugars and ethanol from fermented broths are also reported below. The quantity of yeast in fermentation broths was measured as viable cell count under microscope as well as by estimating dry cell weight.

2.2 ANALYTICAL METHODS

Estimation of polyols

Total polyhydroxy alcohols from the fermentation medium are estimated by the combination of the colorimetric method of Frizzel et al. (158) and Lambert and Neish (159) as outlined by Hanahan and Olley (160).

Reagents

1. Chromotropic acid reagent

   1 g of chromotropic acid was dissolved in 100 ml of distilled water to which was added with constant stirring, 400 ml of 24 N H_2SO_4 solution. The solution was kept in ice bath throughout the addition and allowed to cool down. The reagent was transferred to an amber coloured bottle and stored in the refrigerator. The reagent can be preserved for two weeks, after which it is discarded.

2. 0.1 M Sodium metaperiodate
3. 10 % sodium metabisulfite
4. 10 N H_2SO_4
5. 10 % Thiourea.

Principle

The method depends upon the quantitative oxidation of glycerol and other polyols to formaldehyde by periodate. The reaction can be represented as follows:

$$\begin{array}{l} CH_2OH \\ | \\ n\,(CHOH) + (n+1)\,HIO_4 \rightarrow 2HCHO + nHCOOH + (n+1)\,HIO_3 + H_2O \\ | \\ CH_2OH \end{array}$$

The liberated formaldehyde forms a coloured complex with chromotrophic acid reagent under acidic conditions. The intensity of this colour is proportional within the limits of the polyhydroxy alcohol concentration of 0-100 μg/ml.

Procedure

After suitable dilution, to 2 ml aliquot of samples containing 0-200 μg of polyols were added with subsequent shaking 0.1 ml of 10 N H_2SO_4 and 0.5 ml of 0.1 M sodium meta periodate. This mixture was allowed to stand for 5 minutes at room temperature and 0.5 ml of 10 % sodium metabisulfite solution was added at the end of this period. The solutions were diluted to 10 ml with distilled water and 1 ml

aliquots were treated with 5 ml of the chromotropic acid reagent and the tubes were kept in a boiling water bath for at least 30 minutes. The contents in the tube were cooled to room temperature and 1 ml of 10 % thiourea solution added to remove the red colour blank imparted by the reagents. The resulting violet colour was read at 570 nm on a Shimadzu UV-240 spectrophotometer.

The procedure is simple, quite fast and very sensitive in the range of 0 – 100 µg/ml. It is reported (159) that if the periodate oxidation is completed within 5 minutes then the interference from reducing sugars is negligible. Authors have mentioned that the error due to the presence of sugars is quite small, being in the range of 2.5 to 5.0 %.

A typical calibration curve for estimation of polyols is given below.

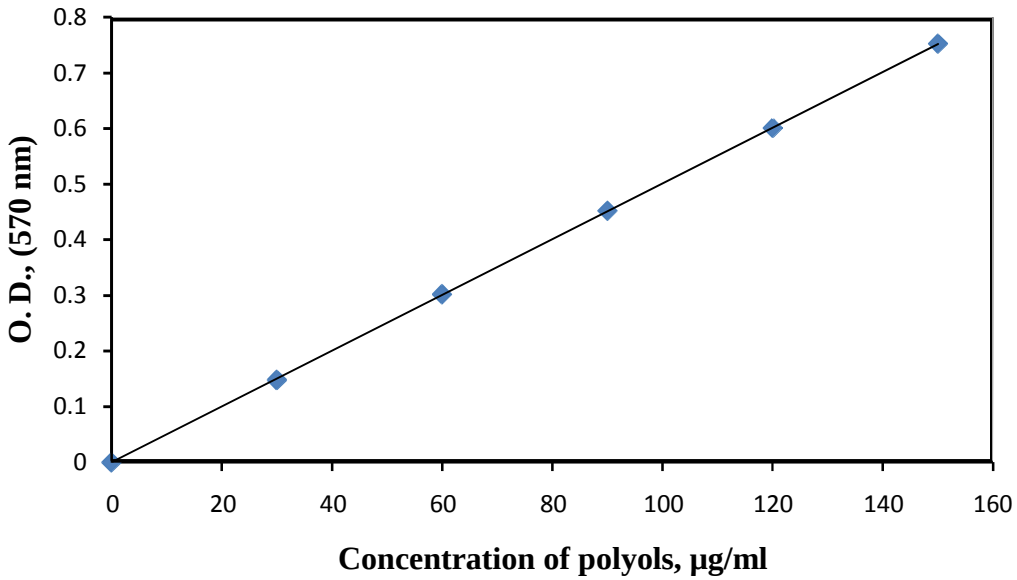


Fig.2.1: Calibration curve for estimation of polyols

Estimation of sugars

Sugars were determined spectrophotometrically with the alkaline copper reagent of Somogyi (161) and the arsenomolybdate reagent of Nelson (162). **Reagents**

A. Alkaline Copper Reagent

Dissolve the following ingredients sequentially in distilled water.

Na_2CO_3 (Anhydrous)	25 g
Rochelle salt (K-Na tartarate)	25 g
$NaHCO_3$	20 g

Na_2SO_4 (Anhydrous) 200 g

and dilute to 1 litre with distilled water. Store the solution above 20°C for few days and filter the sediments which form after standing.

B. Copper Sulfate Solution

Dissolve 15.0 g of $CuSO_4.5H_2O$ in distilled water and dilute to 100 ml. Add 1-2 drops of concentrated H_2SO_4.

C. Working Alkaline Copper Reagent

Mix 5 ml of A and 1 ml of B prior to estimation.

D. Arsenomolybdate Reagent

a. Dissolve 25.0 g of ammonium molybdate in distilled water and make the volume to 450 ml. Add 21 ml of concentrated sulfuric acid to the above solution.

b. Dissolve 3.0 g of $Na_2HAsO_4.7H_2O$ (sodium arsenate) in distilled water and make the volume to 25 ml.

Add (b) to (a) and incubate for 24 hours at 37°C. Store the arsenomolydbate reagent in a glass stoppered brown bottle.

Principle

Reducing sugars will reduce copper under alkaline conditions. Reduced copper forms a coloured complex with arsenomolybdate which can be quantitatively estimated spectrophotometrically.

Procedure

To 1 ml of sufficiently diluted sample, containing not more than 300 μg of sugars, in a 25 ml volumetric flask was added 0.1 ml of HCl and incubated in a boiling water bath for 20 minutes. To this solution was added 1 ml of working alkaline copper reagent. The contents after proper mixing were heated in a boiling water bath for 20 minutes. The contents were cooled to room temperature and 1 ml of arsenomolybdate reagent was added. After mixing, the contents were diluted to 25 ml with distilled water and the intensity of the resulting blue colour measured at 520 nm on Shimadzu UV-240 spectrophotometer. The method is very sensitive between 5 to 300 μg of sugars.

A calibration curve for estimation of sugars is given on next page.

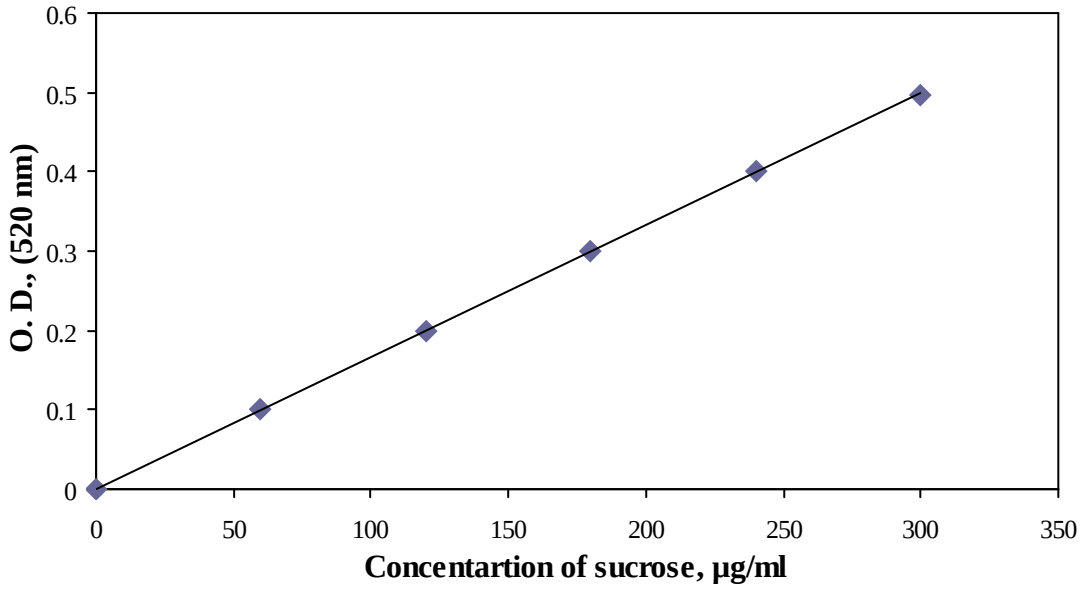


Fig.2.2: Calibration curve for estimation of sugars

Simultaneous estimation of sugars and polyols by gas chromatography

Simultaneous estimation of sugars and polyols in fermentation broth by gas chromatography has been reported for the first time. The method involves clarification of fermentation broth and conversion of sugars and polyols into their trimethylsilyl ethers followed by quantitation of polyols and sugars using two internal standards. The method is useful in investigating the formation of different polyols and utilization of sugars by osmophilic yeasts. Preliminary results agree with those obtained by the conventional methods; both for polyols and sugars.

For the analysis of fermentation broth we tried methods based on thin layer chromatography but found it cumbersome and of qualitative use only. Similarly, chemical methods though found to be effective for estimation of total polyols and total sugars they were found to be inadequate in resolving the complex mixtures. Enzymatic methods for the above type of analysis are known but their utility is restricted to specific components only. HPLC can resolve sugars and polyols in complex mixtures but such facility was not available for routine analytical purpose. Therefore, a need was felt to have a rapid and accurate method for simultaneous determination of substrates (sugars) and products (polyols) in the fermentation broth. Inherent advantages of GC viz. small sample size, high sensitivity and fast speed prompted us to develop this technique for the above analysis. Dooms et al. (7) have

estimated C3-C5 polyols in fermentation media by GC by directly injecting the samples in a packed column. Matarese (163) and Fenton (164) have also determined glycerol in serum and cerebral tissues by this method. The technique developed by us, on the other hand, can simultaneously determine polyols (viz. glycerol, arabitol) and sugars (viz. sucrose, glucose, fructose) in the fermentation media. Further, for estimation of sucrose, we have successfully employed lactose in place of trehalose as the internal standard. The method was developed to study kinetics of reactions involving these moieties where one is formed at the expense of the other. Results obtained by this method and those by the conventional method are in good agreement.

Materials and methods

All reagents including sugars and polyols were of AR grade. Calibration standards were prepared in such a way that their concentration matched the expected concentration of each component in the fermentation broth.

Polyols Determination – 10 N sulphuric acid, 0.1 M sodium periodate, 10 % sodium metabisulfide and chromotropic acid reagent were prepared by the known method. Sugar Determination – HCl, alkaline copper reagent and arsenomolybdate were prepared by the known method.

GC analysis was performed using an Agilent plus 6890 model gas chromatograph equipped with the split-splitless injector, flame ionization detector and Chemstation chromatography software. The capillary column (15 m x 250 μm) coated with 50 % phenyl 50 % methyl polysiloxane (Restek Corporation, U.S.A.) was used and conditioned overnight at 280°C before use.

Procedure

Samples of fermented broth were withdrawn at intervals aseptically, centrifuged at 5000 rpm to separate the cell mass, the supernatant passed through 0.2 μm Millipore membrane filter and the filtrate subjected to derivatisation and chemical analysis.

(i) Derivatisation

The above samples (5 μl) were converted to trimethylsily ethers by the known method (165, 166) which involved the following steps:

a) **Oximation reagent** – Fresh oximation reagent was prepared every day prior to use by dissolving hydroxylamine hydrochloride (2.5 g) in pyridine (100 ml)

followed by careful addition of dimethylaminoethanol (55 μl/ ml) to the above solution.

b) **Calibration standards** – Aqueous solutions of various polyols and sugars were prepared as given in Table 2.1. The indicated quantity of each component in the standard is comparable to that expected in the fermentation process.

(ii) Oximation and Silylation

To the calibration standard (5 μl) taken in a 5 ml screw capped hypovial the oximation reagent (500 μl) was gradually added, the vial sealed with screwcap and heated in a block heater at 80°C for 10 minutes. Subsequently the contents of the vial were cooled to room temperature and mixed under agitation with hexamethyl disilazane (450 μl). Addition of trifluoroacetic acid (50 μl) with stirring followed. The mixture was sealed in vial and reheated at 80°C for 10 minutes and then allowed to cool to room temperature when the resultant precipitate settled down. The supernatant (1 μl) was subjected to GC analysis. The fermented media were derivatised analogously.

(iii) Gas Chromatography

For optimum results, the oven temperature was raised @ 7.5°C/min from 80°C to 210°C, where it was held for 8 minutes, thus completing the GC analysis in about 25 minutes. The split injector was maintained at 260°C with a split ratio of 10:1 and split flow of 5 ml/min. The flame ionization detector was maintained at 280°C with the flow of nitrogen gas at 0.5 ml/min and constant column plus make up flow at 10 ml/min. For calibration standard and fermented sample 1 μl injections were used.

Glycerol, arabitol, glucose and fructose were quantified using mannitol where as sucrose was estimated using lactose as the internal standard. The multilevel calibration programme of Chemstation software employed in the analysis ensured minimal instrumental errors.

(iv) Estimation of Polyols

Total polyols (glycerol+arabitol) in the fermented broth were estimated spectrophotometrically (159). Precision of the method is ± 2.5 %.

(v) Estimation of Sugars

Total sugars (glucose + fructose + sucrose) in the fermented broth were determined spectrophotometrically, after inversion with HCl, with the alkaline copper reagent of Somogyi and arsenomolybdate method of Nelson (161).

RESULTS AND DISCUSSION

Fermented broth is a complex system comprising inorganic salts, suspended impurities, yeast cells, residual substrates and the products. It is, therefore, necessary to remove interferences, if any, due to these congeners. No simultaneous estimation of sugars and polyols in such media has been reported earlier by GC. The proposed method fulfilled this requirement and was found to be rapid.

For the estimation of sucrose, the reported methods (165, 166) employed as an internal standard, trehalose which is an expensive and rather uncommon trisaccharide. We used an inexpensive disaccharide, lactose, as an internal standard. This can lead to reduction in cost per injection.

The conditions for derivatistion of samples viz. reagent concentration, heating time, temperature and heating block design were arrived at after evaluating several combinations of these to ensure complete derivatization. Proper precaution must be taken during cleaning and washing of glassware used for weighing, derivatisation of standard compounds, otherwise the method gives erroneous results (166).

Fermentation Kinetics

During the preliminary investigations, concentration of reactants (sugars) and products (polyols) in the fermentation broth as arrived at by parallel estimations using GC and chemical methods were found to be in good agreement (Table 2.2). Advantage of the GC method lies in the fact that it estimates individual polyols and sugars which helps to investigate kinetics of formation/utilization of these compounds. The presence of small quantity of glucose and fructose indicates that the osmophilic yeast (or externally added invertase) is able to hydrolyse part of sucrose before being taken up in the cell. Accumulation of polyols in the fermentation medium was found to start after about 10 hrs. Glycerol was produced much faster than arabitol and the ratio of glycerol to arabitol was found to be 4:1.

The GC method proposed can be very useful to understand the profile of sucrose inversion as well as glucose and fructose uptake by the osmophilic yeast.

Calibration, Linearity and Accuracy

The GC instrument was calibrated everyday by analyzing standards with appropriate concentrations of components (Table 2.1). The calibration standards were prepared in triplicate and each sample was chromatographed twice. The detector response of each component to different concentrations was linear throughout (Fig. 2.3-2.7). Figure 2.8 illustrates the separation of individual components of the standard and fermented broth samples. Resolution of individual components including internal standard was quite good. Statistical evaluation of results (Table 2.3) confirmed the validity of the method for components under study. For standard mixtures, the results are in good agreement with those obtained by conventional methods. Advantages of the proposed method over other methods include simultaneous detection of individual polyols and sugars and ease of operation.

Reproducibility and consistency

Though the method developed initially gave encouraging result, it was found that it lacked consistency and reproducibility of results with fermentation broth samples. This may have occurred because of salts, other nutrients, or some unknown by-products of fermentation which could interfere during derivitisation process. Attempts were made to take maximum precaution during all preparation stages. However, we faced serious problem of reproducibility. Therefore, this method was used for routine qualitative analysis.

It is necessary to investigate in-depth the reasons for inconsistency and lack of reproducibility.

Table 2.1: Calibration mixtures of sugars and polyols

(Amounts expressed in g/100 ml)

Sr. No.	Glycerol	Arabitol	Mannitol	Fructose	Glucose	Sucrose	Lactose
1.	0.15	0.10	0.12	0.33	0.33	9.30	9.30
2.	0.58	0.25	0.40	0.25	0.25	7.10	7.10
3.	0.87	0.39	0.60	0.21	0.21	5.83	5.83
4.	1.17	0.50	0.90	0.18	0.18	4.67	4.67
5.	1.75	0.75	1.25	0.08	0.08	2.33	2.33
6	3.33	1.00	1.50	0.01	0.01	1.00	1.00

Each sample was chromatographed twice & every mixture was prepared in triplicate.

Table 2.2: Comparison of results by GC and Spectrophotometric methods

Time (hr)	GC			CHE-Total Polyols (% w/v)	GC-Total Sugars (% w/v)	CHE-Total Sugars (% w/v)
	Glycerol (% w/v)	Arabitol (% w/v)	Total Polyols (% w/v)			
0	0.000	0.000	0.000	0.000	30.16	29.40
29	0.555	0.175	0.730	0.750	24.39	25.00
77	2.716	0.654	3.370	3.410	15.03	15.40
185	3.052	0.738	3.790	3.815	13.72	13.50

GC – Gas Chromatography, CHE – Chemical methods.

Table 2.3: Statistical evaluation of GC analysis results

Analyte	Standard Deviation	Relative Error
Glycerol	0.019	0.014-0.043
Arabitol	0.012	0.020-0.067
Glucose	0.022	0.003-0.050
Fructose	0.028	0.013-0.083
Sucrose	0.410	0.0002-0.160

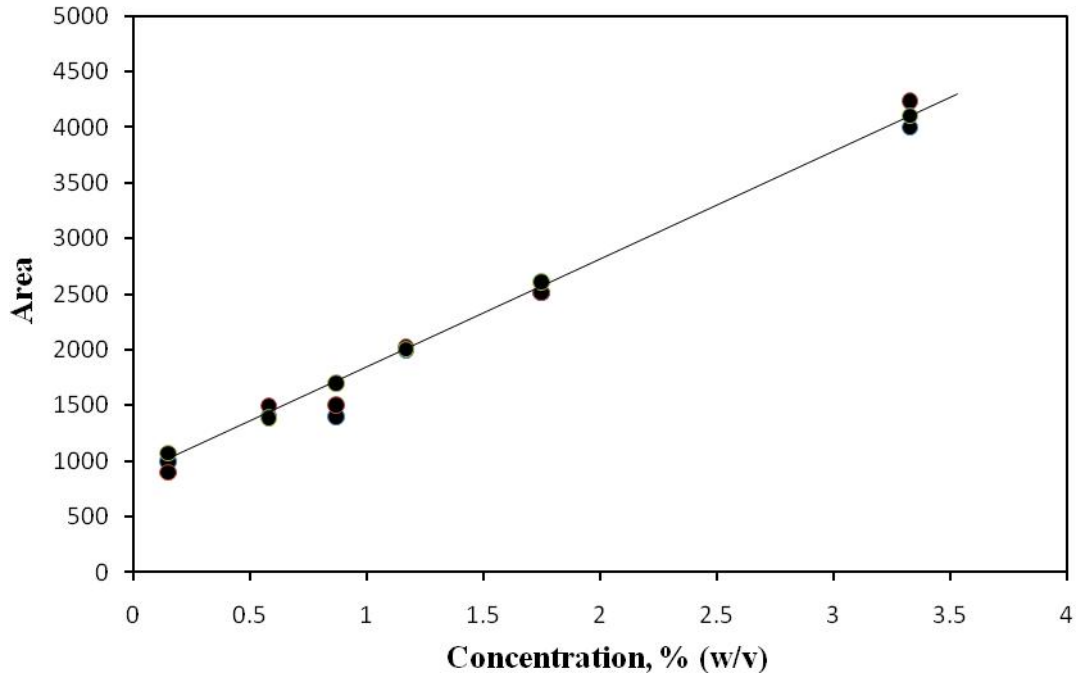


Fig. 2.3: Glycerol Linearity Curve

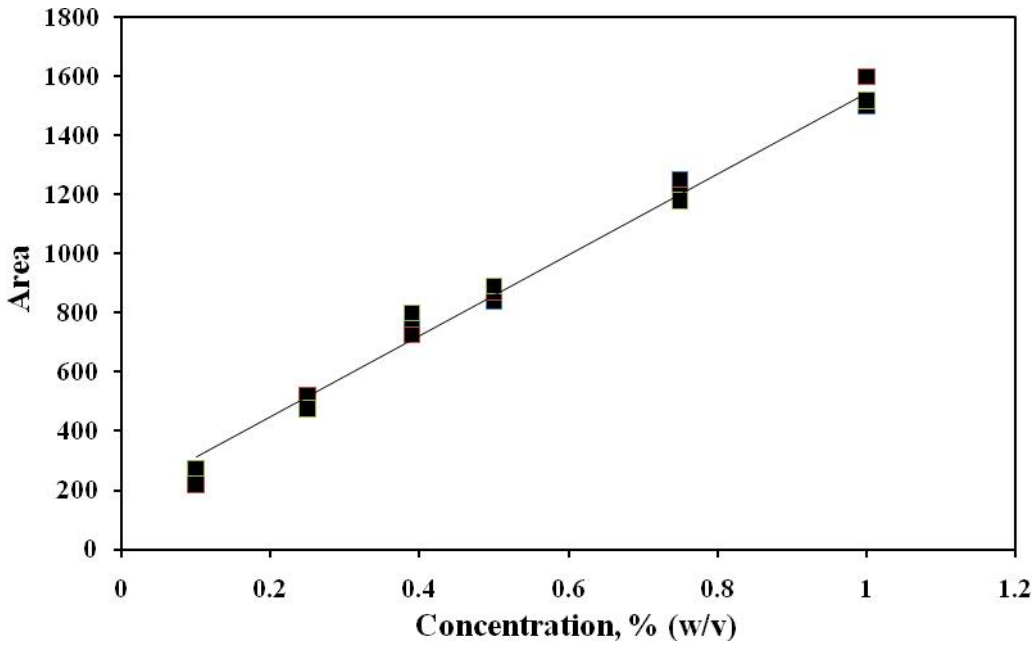


Fig. 2.4: Arabitol Linearity Curve

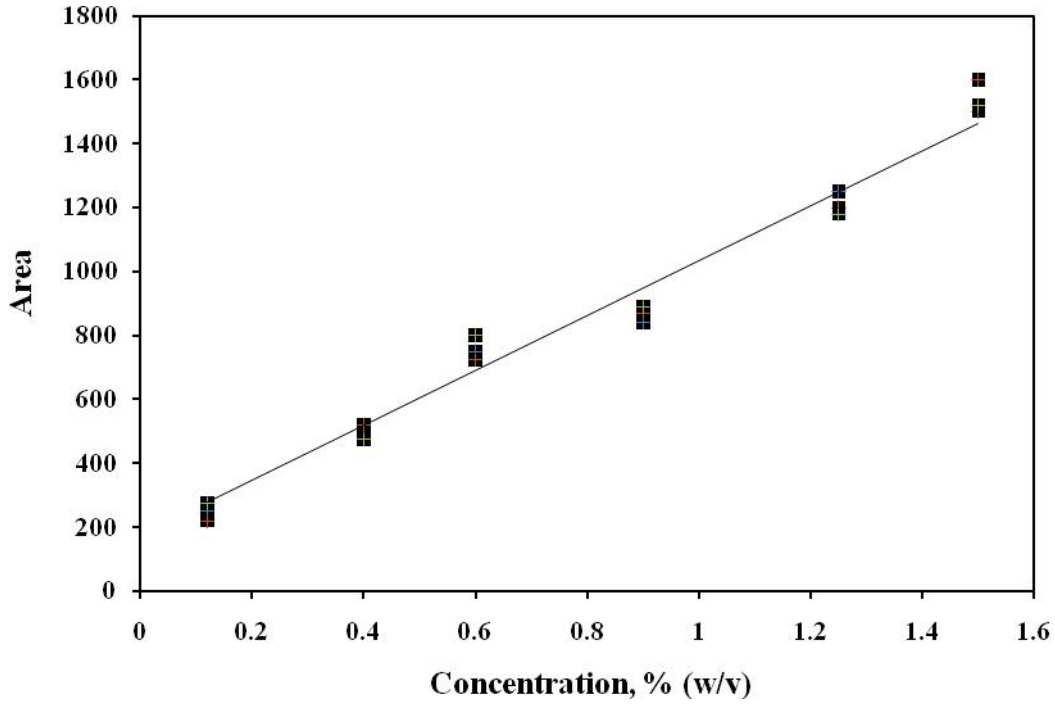


Fig. 2.5: Mannitol Linearity Curve.

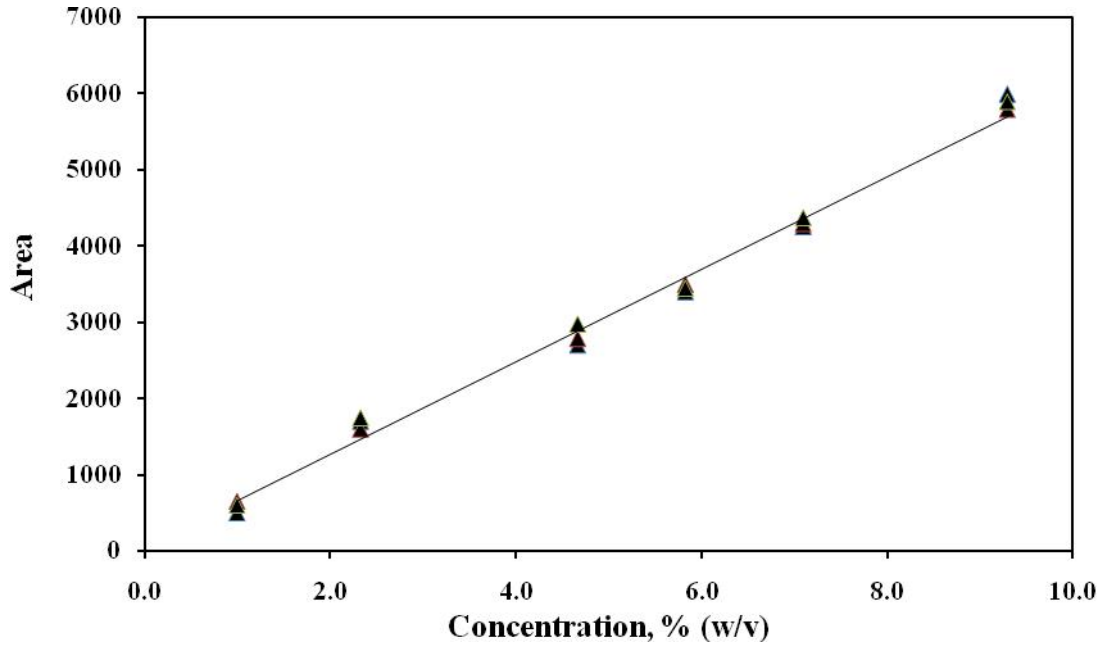


Fig. 2.6: Sucrose Linearity Curve

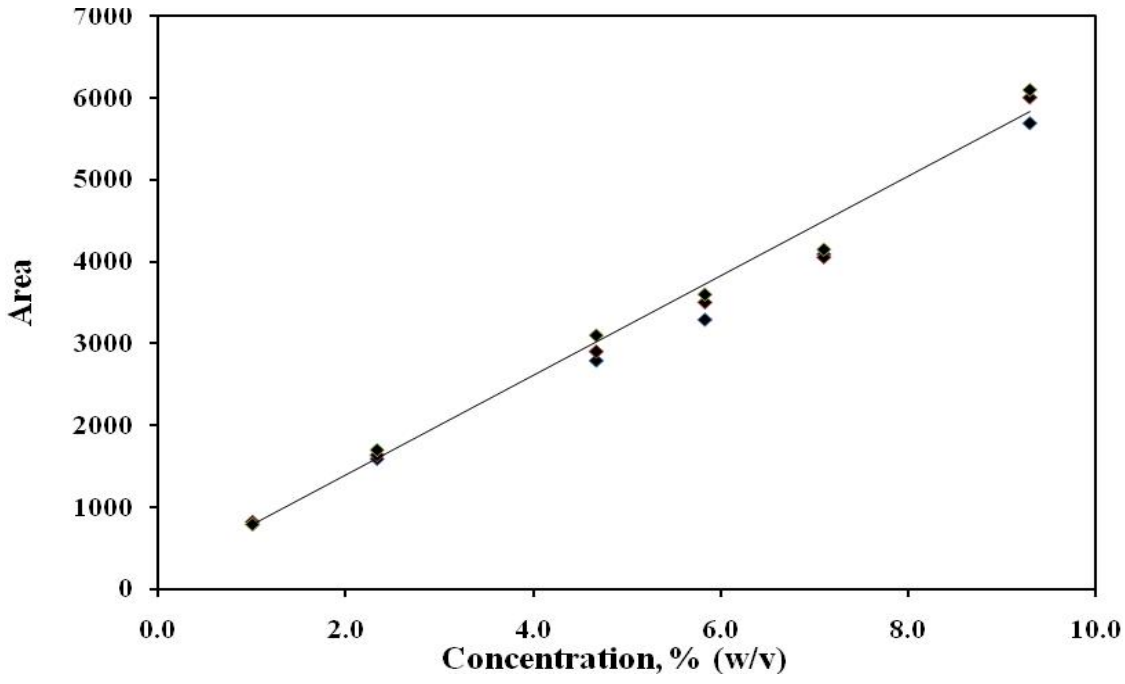
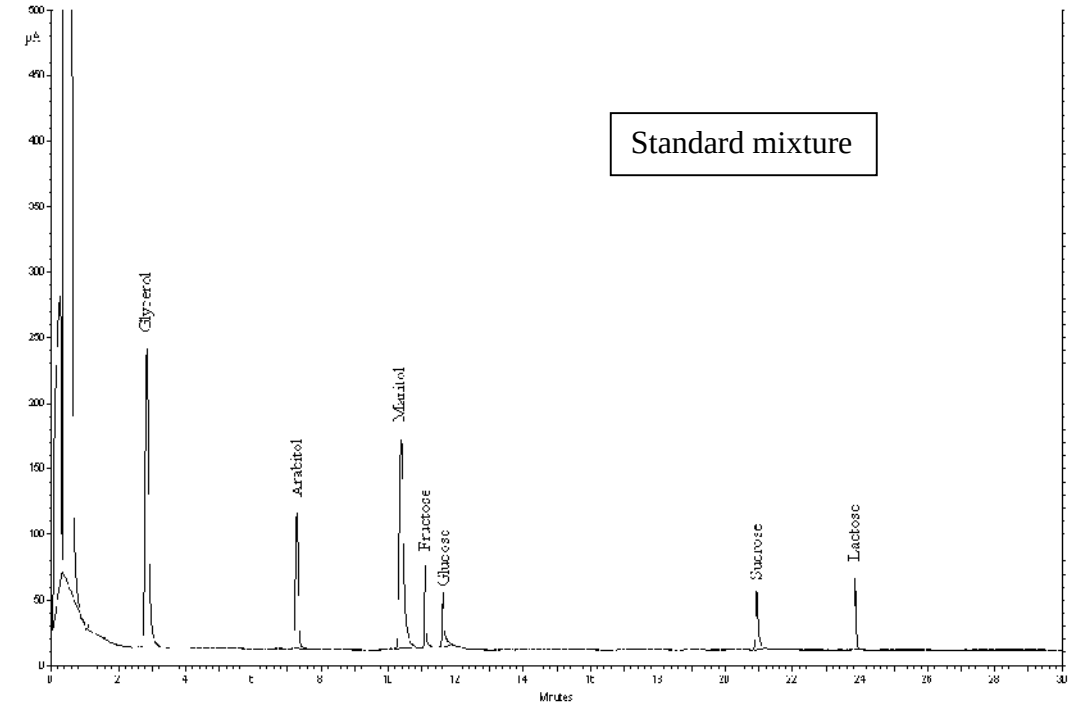


Fig. 2.7: Lactose Linearity curve



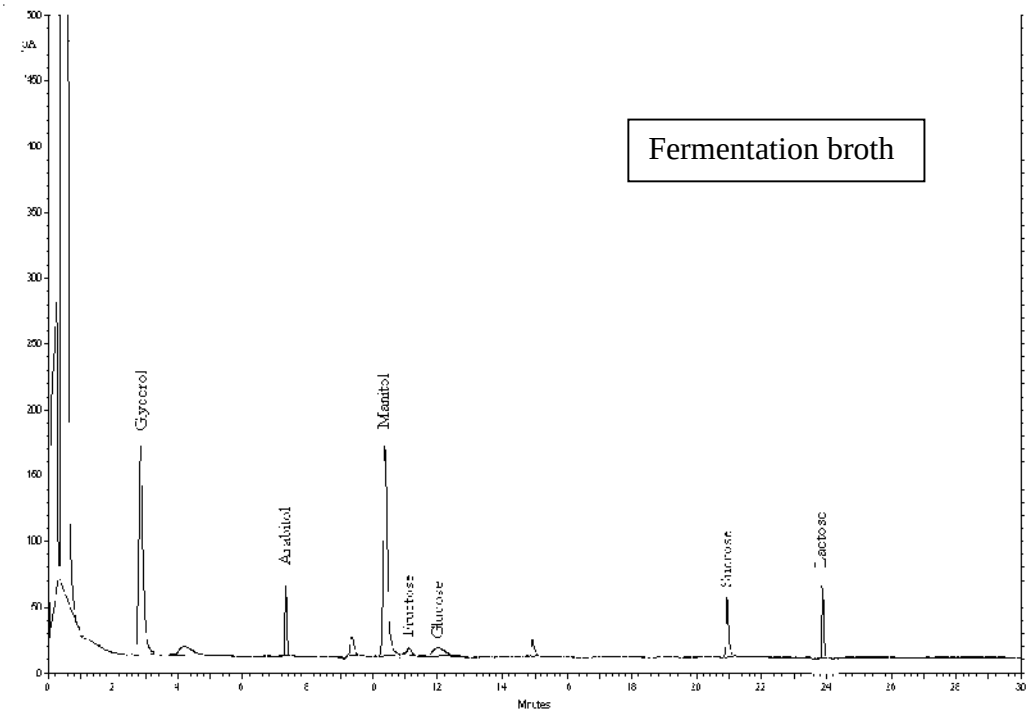


Fig. 2.8: Gas Chromatogram of standard mixture and fermentation broth.

Estimation of Ethanol

Ethanol in the fermented broth sample was determined by the dichromate reduction method as described by Caputi (167).

Reagent

Dichromate reagent: 34 g of potassium dichromate was dissolved in 500 ml of distilled water in a 1 litre volumetric flask. The volumetric flask was placed in a container of ice and 325 ml of concentrated H_2SO_4 was added carefully under constant stirring so that minimum heat was generated. The solution was mixed and volume was made up to 1 litre.

Principle

The method is based on the principle that dichromate is reduced by ethanol and a chromic complex is formed, the relative quantity of which is measured spectrophotometrically.

Procedure

1 ml aliquot of fermented sample was transferred to a 250 ml flask containing 50 – 70 ml distilled water. It was connected to a condenser when the delivery end of it was already submerged in the 25 ml dichromate reagent in a 50 ml volumetric flask. Distillation was carried out on a hot plate. After collecting about 20 ml distillate, the flask was lowered and the drop of dichromate remaining on the tip was rinsed in the flask with distilled water. The flask was then incubated in water bath at 60°C for 20 minutes, cooled and brought to the mark with water. After mixing, optical density of the mixture was measured at 660 nm against a blank with Shimadzu UV-240 spectrophotometer. Percent alcohol was determined from standard graph with a known ethanol (1-10 %) water mixture.

The method is capable of good accuracy and reproducibility. A calibration curve for estimation of ethanol is shown on next page.

Estimation of Cell Mass (Dry cell weight)

The popular method for estimation of cell mass is total yeast count by Petroff-Haussar Counter. Viable yeast count can be estimated by plating on Saborand’s agar plates. The latter method is time consuming and osmophilic yeast usually require more time for development of colonies. The particular osmophilic yeast strain chosen in this investigation was seen as independent cells under microscope in suspension.

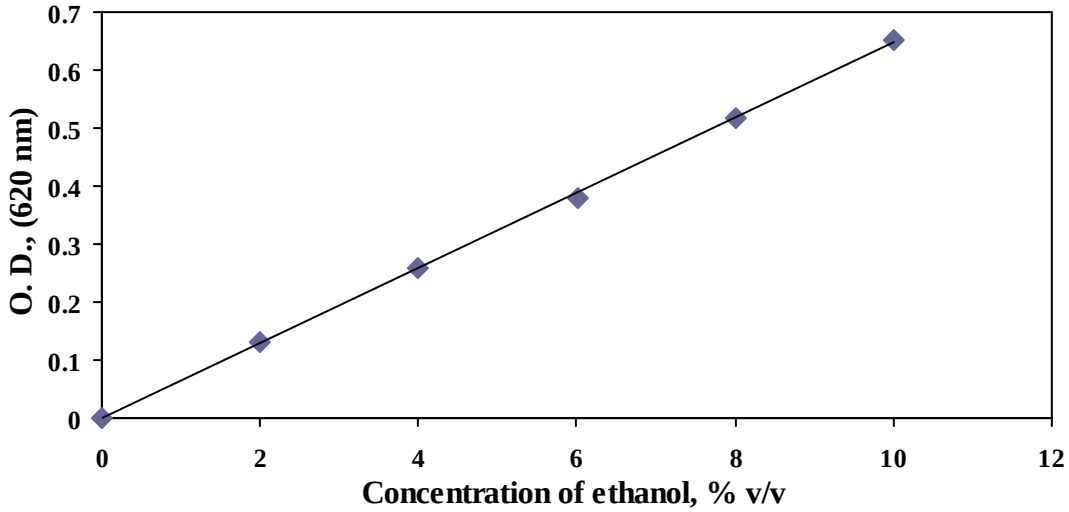


Fig. 2.9: Calibration curve for estimation of ethanol

Previous investigators have used volume of yeast cell after centrifugation as criterion for cell mass estimation, but this is also not very reliable method.

In present study, the cell mass was estimated as dry cell weight as well as viable cell count and as optical density. It was found that all three methods are in good agreement with each other and represent the same cell growth behavior.

Procedure

3 ml of fermented sample was centrifuged at 4000 rpm for 20 minutes. The residual cell mass was washed twice with distilled water and dried in a vacuum oven at 105°C to a constant weight. The results are expressed as gram of dry cell weight per litre of fermentation sample.

Microscopic estimation of yeast cell count

For microbiology, cell culture, and many applications that require use of suspensions of cells, it is necessary to determine cell concentration. One can often determine cell density of a suspension spectrophotometrically, however that form of determination does not allow an assessment of cell viability, nor can one distinguish cell types.

A device used for determining the number of cells per unit volume of a suspension is called a counting chamber. The most widely used type of chamber is called a hemocytometer, since it was originally designed for performing blood cell counts (168).

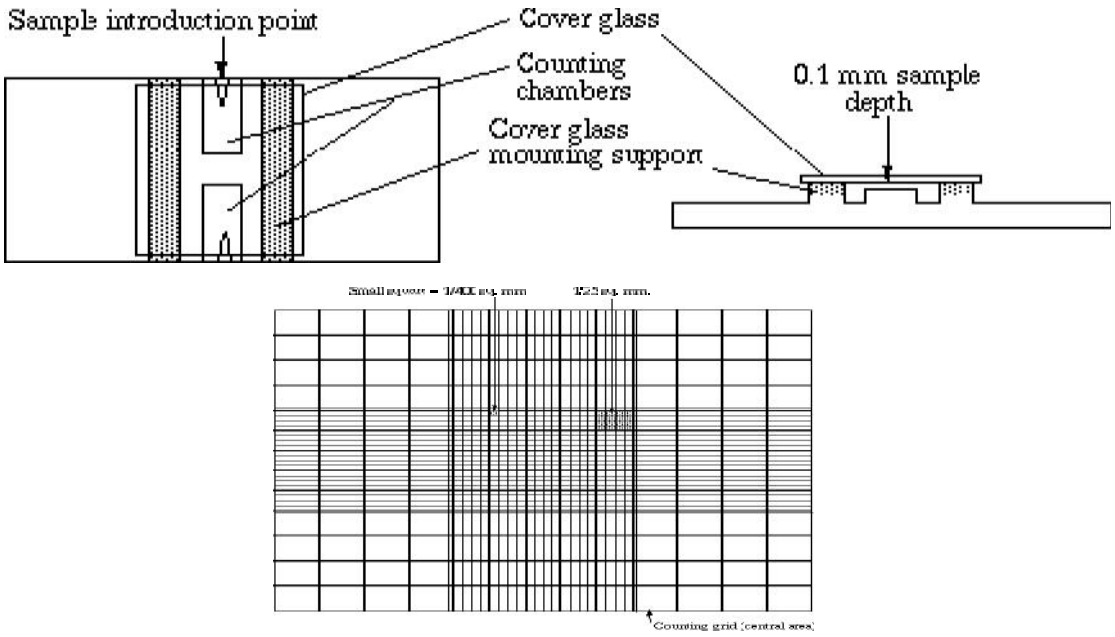


Fig. 2.10: Hemocytometer or counting chamber

To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting on the cell suspension. The suspension is introduced into one of the V-shaped wells with a Pasteur or other type of pipette. The area under the coverslip fills by capillary action. Enough culture suspension should be introduced so that the mirrored surface is just covered. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at high power objective.

In hemocytometer, the main divisions separate the grid into 9 large squares. Each square has a surface area of one square mm and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm. The ruled area used

for yeast cell counting is the central block of the nine ruled blocks, composed of 25 squares, each of which contains 16 smaller squares. This entire ruled central area measures 1 mm on each side, with a total area of 1 mm^2. Each of the 25 squares measures 0.2 mm, with an area of 0.04 mm^2. Depth of the counting area is determined by the cover glass supports, which are usually 0.1 mm above the counting area. The volume of liquid directly over the ruled area can be calculated using the formula:

Volume (v) =length (L) × width (W) × height (H)

Where L =1 mm, W = l mm and H = O.l mm. Then the volume becomes l mm × l mm × 0.1 mm or 0.1 mm^3 or converting to 0.0001 cm^3 = 1 x 10^{-4} mL.

Requirements:

1. Diluents- 0.5 % sulphuric acid
2. Microscope
3. Hemocytometer- open type with improved Neubauer ruling and with hemocytometer cover slip
4. Micropipette and tips

Procedure:

1. Cleaning the counting chamber:

The counting chamber must be clean and dry before use. Dirty counting chambers may influence the sample volume over the counting area and therefore lead to erroneous results. Clean the counting chambers and coverslip with tap water. Dry the chamber and cover slip using silk or other lintless material.

2. Positioning the cover slip:

The cover slip should be centered over the counting area so that both counting sections are equally covered.

3. Preparation of the yeast cell suspension:

The sample to be counted must be well mixed, degassed and diluted. If necessary, dilute the sample so that we can observe countable number of cells. After every dilution, mix it well using vortex machine.

4. Filling the hemocytometer:

Because the counting area and the correctly positioned cover glass determine the volume over the counting area, it is extremely important to fill the hemocytometer correctly. The sample is constantly stirred during the replicate counting period. Using micropipette, load the yeast cell suspension in counting chamber. The entire counting

area must be filled completely, but no part of the sample should extend into the moat. Let the prepared slide stand for a few minutes to settle yeast.

5. Counting:

Yeast cells within the $1mm^2$ ruled area showing 25 squares will be counted. To eliminate the possibility of counting some yeast cell twice, it is necessary to standardize the counting technique. To minimize the counting error, at least 3-5 times count should be taken for each sample. Cells touching or resting on the bottom or left boundary lines are counted. Yeast cells that are budded are counted as 1 cell if the bud is less than one half the size of the mother cell. If the bud is equal to or greater than one half the size of mother cells, both cells are counted. To obtain an accurate yeast cell count, it is advisable that the count should not be below 75 cells on the entire $1mm^2$ ruled areas and not more than about 48 cells in one of the 25 squares. Counts from both sides of the slide should agree within 10%, if a dilution is used. The dilution factor must be used in the calculation.

Calculation:

Number of cells/ mL = total cells in central 25 square ruled area × Dilution factor (if any) × 1×10^4

Preparation of standard methylene blue solution (MB)

The most desirable concentration being 0.025% (w/v) of methylene blue is used to differentiate the viable (colourless) and nonviable (blue coloured) yeast cells. It was also found that full strength Ringer solution provides the best environment to maintain the viability of yeast cells although one-fourth strength Ringer solution was suggested for bacteria. Some glucose (at least 1% w/v) was required in this stain to create a reduced environment for yeasts. The standard Methylene blue solution consists of the following components: Methylene blue, 0.025 g: NaCl, 0.9 g:, KCl, 0.042 g: $CaCl_2.6H_2O$, 0.048 g: $NaHCO_3$, 0.02 g: glucose, 1 g: distilled water to 100 ml.

Standard procedure of methylene blue staining for estimation of viable cell count.

Samples with dense cell concentrations taken from fermentation broths need to be diluted down to approximately (2-4) × 10^8 cells/ml with a suitable diluent, i. e., Ringer salt solution. Then 0.1 ml of the diluted cell suspension is mixed with 0.9 ml of Methylene blue solution (169). A hemocytometer is then loaded with this solution

and the standard counting procedure is performed as explained above. Colourless cells (viable) and blue-coloured cells (dead) are counted (each square should contain 80-160 cells). The viability index is calculated by

Viability index= No. of coloured cells/ no. of colourless cells+ No. of blue cells

A concentration of approximately 80-160 cells/square is suggested for statistical reasons. Although yeast cell viability remains stable in the methylene blue solution for a long period of time, the yeast cells were counted within 10 minutes.

Estimation of dissolved oxygen concentration

Principle

The manganese sulfate reacts with NaOH to form white precipitate which in the presence of oxygen is converted into brown colour complex. In the strong acid medium this brown colour precipitate reacts with NaI to form free iodine. The liberation of free iodine is proportional to the amount of O_2 present (170).

Reagents

1. Manganese sulfate solution: Dissolve 480 g $MnSO_4.4H_2O$ or 400 g $MnSO_4$ $.2H_2O$ or 364 g $MnSO_4.H_2O$ in distilled water, filter and dilute to 1 liter. The manganese sulfate solution should not give a colour with starch when added to an acidified solution of KI.
2. Alkaline-Iodide Sodium azide reagent: Dissolve 500 g NaOH and 135 g NaI in distilled water. Add 10 g sodium azide, NaN_3, dissolved in 40 ml. of distilled water and dilute to 1 liter. Potassium and sodium salt may be used interchangeably. This reagent should not give a colour with starch solution when diluted and acidified.
3. Sodium thiosulfate stock solution 0.1 N: Dissolve 24.82 g $Na_2S_2O_3$, $5H_2O$ in boiled and cooled distilled water and dilute to 1 liter. Preserve by adding 5 ml. chloroform or 1 g NaOH/l.
4. Standard sodium thiosulfate titrant 0.025 N: Prepare by diluting 250 ml. sodium thiosulfate stock solution to 1000 ml.
5. Standard potassium dichromate solution 0.025 N: Dissolve 1.226 g/l potassium dichromate.
6. Concentrated sulfuric acid. 7. Starch.

Procedure

1. Standardization of 0.025 N $Na_2S_2O_3$

   Dissolve 2 g KI in an Erlenmeyer flask with 50 ml distilled water, add 10 ml diluted (1+9) H_2SO_4, then 20 ml of standard potassium dichromate. Place it in dark for 5 min, then add 70 ml. of water and titrate against $Na_2S_2O_3$ using starch indicator.

2. To the sample as collected in a 250 to 300 ml. sterilized bottle, add 2 ml manganese sulfate solution, followed by 2 ml alkali-iodide azide reagent well below the surface of the liquid. Stopper carefully to exclude air bubbles and mix by inverting the bottle at least 10-15 times. When the precipitate settles, leaving a clear supernatant above the manganese hydroxide flock, and 2 ml concentrated sulfuric acid by allowing the acid to run down the neck of the bottle, restopper, and mix by gentle inversion until dissolution is complete. Distribute the iodine uniformly throughout the bottle before decantation of the amount needed for titration. Use 200 ml volume for the titration with 0.025 N sodium thiosulfate. Titrate till pale straw colour, then add about 1 ml starch solution and continue the titration to the first disappearance of blue colour.

   For 200 ml of original sample, 1 ml of 0.025 N $Na_2S_2O_3$ = 1 mg/l of DO.

2.3 CULTURE MAINTENANCE

Variety of yeast cultures either belonging to osmophilic or osmotolerant category as described by Kreyer-Van Rij (80) were obtained from National Collection of Industrial Microorganisms (NCIM, NCL, Pune).

All the osmophilic yeast cultures were maintained on Malt extract – Glucose – Yeast extract – Peptone (MGYP) agar slants and were transferred every 3 to 4 weeks. The composition of the MGYP agar slant was as given in chapter 4.

CHAPTER-3

REVIEW OF THE WORK DONE WITH GLUCOSE AS SUBSTRATE AND SIMULATION STUDIES

CHAPTER-3

REVIEW OF THE WORK DONE WITH GLUCOSE AS SUBSTRATE AND SIMULATION STUDIES

3.1 INTRODUCTION

This chapter deals with our previous studies on production of polyols using glucose as substrate. During these studies screening of various species of osmophilic yeasts was carried out for selection of suitable strain for polyols production. On the basis of screening experiments an osmophilic yeast strain of species *H. anomala* (NCIM-3341) was selected for further evaluation.

Optimization of nutrient and environmental parameters was carried out using shake flask experiments. In the fermenter scale experiments, the effect of aeration, agitation, initial glucose concentration and pH on the time course profiles of glucose utilization and polyols formation was investigated. Particular emphasis was given on study of the effect of oxygen transfer on the yields and kinetics of product formation. The rates of oxygen transfer in the polyols production by *H. anomala* were evaluated by the sulfite oxidation method and the dynamic method of gassing out (171).

In the following part of this chapter, a brief review of the work carried out using glucose as substrate is given. The results and conclusions of producing polyols using *H. anomala* starting with shake flask level to fermenter scale are also explained.

In the later part of this chapter, the data generated in our previous work was used to carry-out simulation studies. “Lazy learning”, a memory based local learning method was used to process and simulate the experimental data to predict polyols yield in real world applications (172).

3.2 SCREENING STUDIES

Screening studies are usually carried out on shake flask scale as it allows to screen number of cultures at the same time and under identical environmental conditions. However, shake flask experiments are limited in their oxygen transfer capacity.

Survey of literature has revealed that most of the workers have used higher sugar concentration in the medium since this is known to favour polyols production in osmophilic yeast.

Following factors are known to be crucial for polyols production by osmophilic yeasts.

1. Rate of oxygen supply per unit weight of biomass.
2. Concentration of the substrate.
3. Concentration of nitrogen in the medium and
4. Concentration of phosphate in the medium.

If the concentration of nitrogen and phosphate is kept low, then the percentage conversion of substrate to ethanol should remain low.

A variety of yeast cultures either belonging to osmophilic or osmotolerant categories as described by Kreyer-Van Rij (80) were obtained from National Collection of Industrial Microorganisms (NICM, NCL, Pune). In all, sixteen cultures of osmophilic yeast were screened for polyols production. The method of preservation of osmophilic yeast, propagation of yeast culture and shake flask fermentation experiments are reported in the reference (171).

The results of screening studies revealed that most of the yeast cultures were able to grow and utilize sugar at a very slow rate. Under the conditions of screening *S. rouxii* (NCIM-3385) gave the maximum yield of polyols (36 %) on the basis of sugar utilized. However, much of the sugar remained unutilized even after 240 hrs of fermentation. *P. fermentans* (NCIM-3408) gave good yield of ethanol even under aerobic conditions employed for screening. *H. anomala* (NCIM-3341) and *T. magnolia* (NCIM-3470) also gave good yield of polyols (above 20 g/100 g of sugar consumed). TLC analysis of fermented wash from *T. magnolia* showed that it produces erythritol, unidentified polyol of very low r_f value and glycerol. The proportion of glycerol to other polyols was approximately 1:1. *H. anomala* (NCIM-3341) produced glycerol as the major polyol along with small amount of arabitol. Glycerol accounted to almost 80 % of total polyols. The rate of sugar utilization was also quite fast as most of the glucose was consumed in 168 hrs.

H. anomala (NCIM-3341) was, therefore, selected for further studies on the basis of yield of polyols (20 % of sugar utilized), rate of fermentation and the percentage of glycerol in total polyols.

3.3 OPTIMIZATION OF NUTRIENT AND ENVIRONMENTAL PARAMETERS

Shake flask experiments were conducted to optimize the nutrient and environmental factors. At a shaker speed of 180 rpm, the optimum inoculum level of *H. anomala* was found to be 0.15 (% w/v) DCW corresponding to about 10 % liquid inoculum volume. Low concentration of yeast extract favoured polyols formation and 0. 25 % level was found to be optimum. Urea concentration of 0.1 % was found to be optimum. Sugar concentration of 30 % gave the maximum yield of polyols. Casein hydrolysate and magnesium sulfate at a level of 0.1 % and 0.025 %, respectively were found to improve the yield of polyols and sugar utilization rate. Phosphate at all concentrations reduced the yield of polyols and the highest yield of polyols was obtained without any added phosphate. Sodium sulfide, calcium chloride, calcium carbonate failed to improve yield of polyols and sugar utilization rate.

The shake flask experiments also indicated the existence of optimum dissolved oxygen concentration corresponding to the maximum yield of the product. Aeration had an important part and a ratio of 0.08 of medium volume to flask volume which corresponds to approximate oxygen transfer rate of 40-50 mMol of O_2/l/hr (97) was found to be optimum. It was also found that aeration efficiency affects both the cell mass and product yield. The maximum polyols yield obtained was 42.80 % after optimization of nutrient parameters and aeration rate on shake flask level. The addition of Na_2CO_3 in small lots to maintain the pH near alkaline conditions increased the product yield to about 46 % based on the sugar consumed.

These studies indicated that in polyols production by *H. anomala* oxygen supply for fermentation requires a careful adjustment. If oxygen levels are higher than the optimum, cell mass yield and sugar utilization rate increases with consequent decrease in polyols formation. Lower than the optimum level of oxygen results in anaerobic conditions, thus favouring the ethanol formation.

3.4 OXYGEN TRANSFER STUDIES

The production of polyols by osmophilic yeasts is essentially an aerobic process and therefore, requires the provision of oxygen.

In this study, attempt was made to probe in detail the oxygen transfer in polyols production by *H. anomala* and to quantify the factors which would influence

the rate of transfer of oxygen into solution. The effect of variation of aeration rate and agitation speed on polyols production was carried out on fermenter scale. To quantify the aeration efficiency of the fermenter and the effects of operating variables on the provision of oxygen, volumetric oxygen transfer rate (OTR) was estimated. Sulfite oxidation and dynamic method of gassing out were used to estimate OTR. Since solubility of oxygen in sugar solution is important from the view point of determining the maximum oxygen saturation level, experiments were carried out to assess the effect of increasing sugar concentration on dissolved oxygen.

Effect of air-flow rate

If the metabolic processes consuming oxygen are faster than the oxygen transfer rate, the rate of transfer of oxygen is given by the following equation

$$N_A = \frac{dC_L}{dt} = K_L a(C^* - C_L) \qquad \ldots\ldots\ldots\ldots\ldots\ldots \qquad [1]$$

where, N_A = Volumetric oxygen transfer rate, in (mMol/l/hr)

C_L = Concentration of dissolved oxygen in fermentation broth, in (mMol//l)

t = time, in (hr)

$\frac{dC_L}{dt}$ = is the rate of change of dissolved oxygen at any time, in (mMol/l/hr)

K_L = Mass transfer coefficient, in (cm/hr)

a = Gas liquid specific surface area, in (cm^{-1})

C* = Saturated or equilibrium dissolved oxygen concentration under the prevailing conditions, in (mMol/l).

K_La is the measure of the aeration capacity of a fermenter under the test conditions, the larger the K_La, the higher the aeration capacity of the system.

Air flow rate was studied at the air flow rate values of 0 vvm, 0.18 vvm, 0.36 vvm, 0.54 vvm and 0.72 vvm. The effect of agitation rate was studied at 500 rpm, 600 rpm and 700 rpm.

Since the product and substrate concentration showed a linear variation with time, they have been fitted to a straight line equation by least square fitting method.

The cell mass concentration variation with time has been fitted to a second degree polynomial. The oxygen % saturation pO_2 profile shows that starting with oxygen saturated medium, the % oxygen saturation falls steeply to a low value before showing a rise. The slope of this line was found to be constant and independent of air flow rates. The lowest pO_2 value reached increased with increasing aeration rates.

The typical results expressed as overall yield based upon the total sugar consumed (Y p/s) are shown in Fig 3.1. The overall yield of product increased steeply with increasing air flow rate up to 0.2 vvm. The rate of increase became smaller between 0.2 to 0.6 vvm and declined sharply beyond an air flow rate of 0.6 vvm. The overall yield of product was clearly related to the volumetric mass transfer (K_La) which increased with increasing flow rate. But, beyond an air flow rate of 0.6 vvm the volumetric mass transfer coefficient decreased due to the flooding effect as it was observed during the sulfite oxidation experiments.

The rate of agitation studied in the range of 500 to 700 rpm exhibited a maximum in overall yield in the early phase of fermentation but this effect was nullified in the later phase. Optimum yield was obtained at an agitation rate of 600 rpm.

The time course profile of cell mass increase clearly identified three phases in the process. The first phase involved the rapid growth of the cells under oxygen limiting conditions with little polyols formation. In fact, at lower aeration rates, near anaerobic conditions are created in the fermenter due to high rates of oxygen consumption by the fast growing organisms. It was found that the end of fast growth for the cells is reached at about18 hrs after the start of the fermentation process with an inoculum size of 1.6 g/l. The percentage oxygen consumption in this phase falls linearly with time.

The second phase of fermentation is characterized by an increase in dissolved oxygen concentration with time. In this phase the product formation also increased reaching a maximum value at the point of total utilization of sugar. In general, it was found that the polyols yield increased with increased % saturation of oxygen in the medium. This would mean higher product yields correspond to higher dissolved oxygen concentrations.

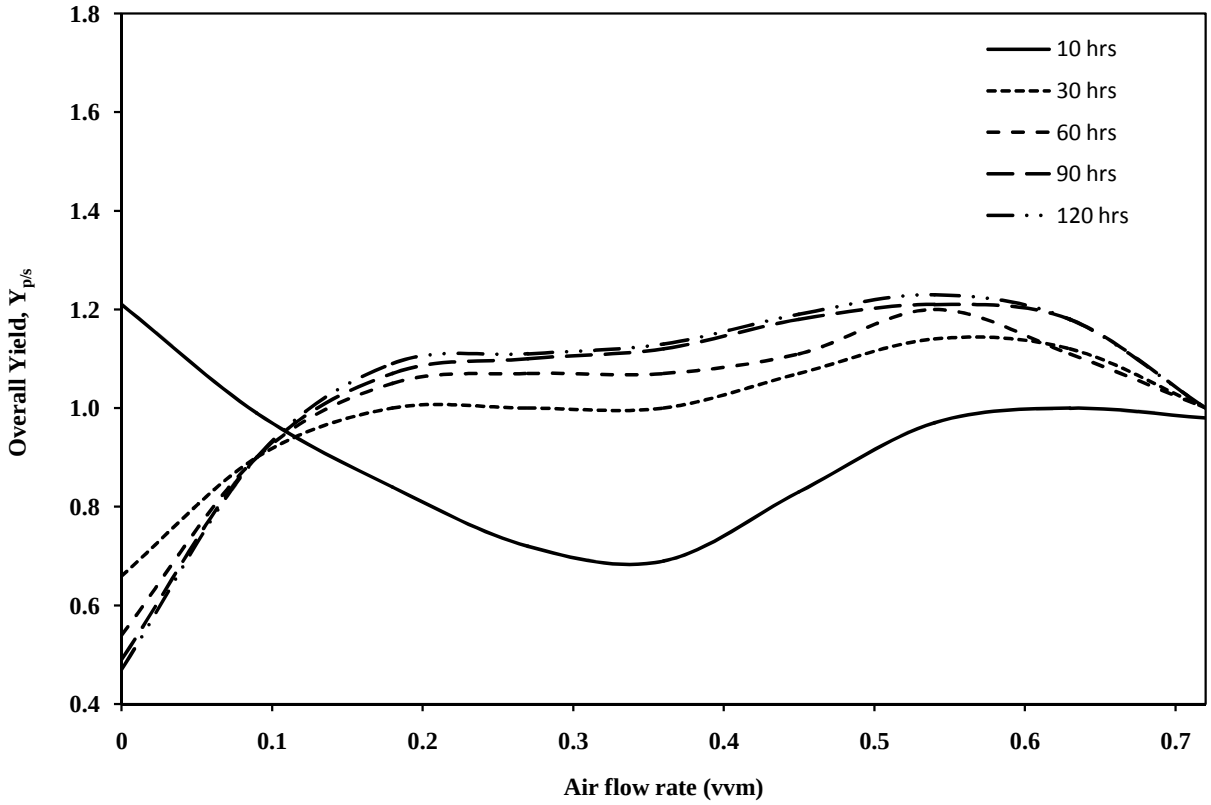


Fig.3.1 Effect of air flow rate on overall yield at different fermentation times.

The third stage of the process begins at the end of total sugar consumption by showing rapid growth of cell mass again, presumably, due to the switch over of metabolism of the organism from glucose to glycerol as substrate. From the process development point of view, fermentation should be terminated at the beginning of this phase.

It was also found that aeration improved the yield of the product up to 0.54 vvm aeration rate, after which the yield was adversely affected. This might be due to the "flooding" effect, which involves a drastic decrease in the gas liquid interfacial area available for oxygen transfer.

Two techniques were used to study the rate of mass transfer of oxygen (OTR). These were sulphite oxidation method (173, 174) and the dynamic method of gassing out (175, 176). The first method is purely chemical in nature and the second one takes into account the microbial activity of the actual fermentation process.

The sulphite oxidation method allowed only the calculation of oxygen transfer rate but not K_La. The OTR values obtained by the sulfite oxidation method ranged from 43.5 to 136.0 mMol/l/hr. The values found in this study agreed well with values reported in literature (177).

The volumetric mass transfer coefficient (K_La) was estimated by the dynamic method of gassing out, which is based on the following equation.

$$C_L = C^* - \frac{1}{K_La}\left[rX + \frac{dC_L}{dt} \right] \quad \ldots\ldots\ldots\ldots\ldots [2]$$

Where, r = Specific oxygen uptake rate per unit weight of cells, in (mMole/g/hr)

X = dry weight of cells per unit volume, in (g/l).

The quantity in the bracket on the right hand side of the above equation is the net change of dissolved oxygen concentration due to metabolic consumption and oxygen transfer.

The value of K_La obtained as per the equation 2 in this study was 132 hr^{-1}. In agitation systems with aeration the K_La values reported in literature are in the range of 180 to 240 hr^{-1} for *S. cerevisiae* by dynamic method. The reasonable agreement with

the reported value of K_La showed that the dynamic method employed in the study gave reliable results.

The specific oxygen uptake rate of 0.3 g/g.hr determined in the study also compared well with maximum specific growth rate of 0.256 g/g.hr reported in the literature (178). The volumetric oxygen demand rate of microorganism, rX was estimated to be 0.0084 mg/l/sec.

The K_La obtained by the dynamic method gives a maximum oxygen transfer rate of 6.2 mMol/l/hr, whereas the measured OTR by the sulfite oxidation method was 121 mMol/l/hr. This disagreement between the oxygen transfer rates found by the two methods chiefly arises due to the fact that the sulfite oxidation results strictly apply to systems having very high oxygen consumption rates. In the system studied the oxygen consumption rates were quite moderate as evidenced by the existence of finite concentrations of dissolved oxygen in liquid phase. These observations are further supported by the fact that all osmophilic yeasts are slow growing.

3.5 KINETICS OF SUBSTRATE UTILIZATION AND THE EFFECT OF pH

High concentration of sugar is necessary to obtain maximum yield of polyols (87, 179, 180). The function of high sugar is to create an environment of a high osmotic pressure and thus force the organism to produce polyols.

The substrate used in this investigation was glucose and its concentration was investigated at 10 %, 20 %, 30 % and 40 % levels.

The increase in cell mass concentration in all the experiments, showed an initial lag phase of around 6-8 hrs, an exponential growth phase, followed by a quasi-stationary phase and then a phase of rapid growth. The overall yield of the product based on total sugar consumed increased steeply from 20 % to 30 % glucose concentration and then remained almost constant up to 40 % sugar concentration. However, in the early phase of fermentation the overall yield of the product was higher in case of 30 % glucose as compared to 40 % glucose concentration.

The pH of fermentation decreased from initial value of 6.0 to 2.8- 3.0 and then became steady till all the sugar was consumed. It was observed in all the experiments, where pH was not controlled, that the pH again starts increasing sharply at the end of fermentation when most of the sugar has disappeared from the medium. The fall in

pH is mainly due to the net accumulation of CO_2 produced in the rapid cell growth in the aqueous phase. In the later phase a low constant level of pH is maintained mainly because of balancing by the rate of CO_2 formation and desorption by bubbling the air.

The effect of pH was studied at 6.0, 7.0, 8.0 and 9.0 pH. To control the pH, 2.5 N Na_2CO_3 solution was used. The effect of pH on the yield of the product based on sugar consumed showed an interesting trend. Between pH 6.0 to 7.0, there is sharp increase in the yield of product. This increase flattened to some extent between pH 7.0 and 8.0. From pH 8.0 to 9.0, there is once again a steep increase in the polyols yield. Although generally higher yields were obtained at 9.0 pH, it was found that the yield at 8.0 pH increased with increasing time whereas at 9.0 pH the yield decreased with increasing time. At pH 9.0, about 50 % of the sugar remained unutilized even after 125 hr of fermentation. At pH 8.0, the sugar utilization was completed within 125 hrs. These observations indicated that pH 8.0 is optimum for polyols production. The percentage yield at the end of fermentation, based on sugar utilized worked out to be 50 % at pH 8.0. This probably results from the fixing of acetaldehyde at pH 8.0 because of addition of Na_2CO_3, thus eliminating the ethanol formation and diverting the excess NADH towards glycerol formation.

The effective oxygen transfer efficiency of the fermenter improved the polyols productivity to 1.11 g/l/hr as compared to 0.6 g/l/hr on shake flask scale.

The overall optimum conditions for the maximum product yield were found to be pH 8.0, Aeration rate = 0.54 vvm, Agitation rate = 600 ppm, initial glucose concentration = 30 %. The maximum yield obtained under optimum conditions was 51 % based on glucose utilized and this mixture consisted of glycerol and arabitol in 4:1 ratio.

3.6 SIMULATION STUDIES: MEMORY BASED LOCAL LEARNING

3.6.1 INTRODUCTION

Linear and nonlinear regression techniques, developed over the years, are widely applied to obtain solutions to number of different problems in various fields of science and engineering. System identification, process monitoring, fault detection and diagnosis, nonlinear modeling, optimization and control etc. are typical problems

common in process industries. The common goal here is to provide an accurate input/output mapping that is adequate for the purpose at hand.

Various regression methods can be broadly classified as parametric and nonparametric. Parametric models, most of the time, are easy to understand and simple to compute. General linear regression, ordinary least squares regression, simple logistic regression, partial least squares, time-discrete models etc. are some of the more commonly used parametric models. These are a kind of global models since single model is used in fitting all the training data. The main disadvantage of these models is that if wrong functional form is chosen, the model remains highly biased in prediction task (181).

Nonparametric models do not make a prior assumptions about the functional form. They are flexible and found to be capable of capturing subtle nonlinear relationships (181). Neural networks, support vector regression, rules, kernel regression, smoothing splines etc. are some of the nonparametric methods. The statistical complexity and computational cost are the main disadvantages of some of these methods. Also, they require large amount of data for training. The whole procedure is divided in training, validation and assessment of the generalization of the model by applying it to unseen data. This procedure is time consuming and can badly affect the performance in the cases where time is a limiting factor (for e.g. control related problems). Hybrid models combining the models from parametric, nonparametric methods and/or mechanical models have also been proposed (182).

The input/output mapping [$y = f(\mathbf{x})$] of a highly nonlinear process may exhibit reasonably smooth behavior over a certain domain of variables and may possess very uneven or peaky surfaces for certain other domain of variables. The global models try to obtain a relationship that approximates the actual mapping with least overall error over the entire domain of variables. It is possible that the model is very accurate in certain regions and not so much in certain other regions. In other words, the single global model may have different extents of error in different regions of the variables span. It is more advantageous in such cases to build local models. The idea of using local techniques as an alternative to such methods originated in nonparametric statistics and then it was rediscovered and developed by the machine learning community (183).

There are three different steps involved in local learning: local representations, local selection, and locally weighted learning. In local representation, each new data point, also called as query point, affects a small subset and thus to answer a query involves only a small subset of the parameters. Local selection methods, on the other hand, store all (or most) of the training data and use a distance function to determine the points relevant to query point. Finally, locally weighted learning stores the training data explicitly and only fits parameters to the training data when a query point is known. The seemingly global models, for e.g. rules, decision trees and parametric models, can be converted to their local equivalents by using a locally weighted training criterion. Nearest neighbor, weighted average and locally weighted regression are some of the local models in common use (184).

Lazy learning is a recently introduced memory-based local learning method using local selection of parameters. It defers the computations till a request for prediction is received (i.e. query based). It answers the query point by interpolating locally relevant examples according to a distance measures. Therefore, each prediction of query point requires local modelling procedure. This local modelling procedure is composed of parametric and structural identification. Given a model structure, parametric identification involves optimization of the parameters of the local approximator. The structural identification, on the other hand, comprises the selection of a family of local approximators, a metric to evaluate relevant examples and *bandwidth* which indicates the size of the region in which the data are correctly modeled by the members of the local approximators chosen earlier. Since it is a memory-based technique, no separate training is required to answer the query points, which greatly improves the speed of implementation. Secondly, it predicts by locally interpolating the relevant points based on a distance measure. This is particularly useful when limited amount of input/output data is available and an accurate prediction is required. Lastly, it is less susceptible to the noise contamination (185). All these features greatly improve the overall performance of the learning from input/output data as compared to other parametric as well as nonparametric methods. The advantages are of considerable relevance and interest to number of problems in process industries and hence the present work illustrates in more detail these and such features by considering example of polyols production.

In this part, section 3.6.2 explains the details of the lazy learning. Sections 3.6.3 and 3.6.4 are on case study and results and discussion and addresses the example of prediction of polyols production in detail and bring out the salient features of the technique in comparison with the contemporary methods. Section 3.6.5 summarizes the results of the present work.

3.6.2 LAZY LEARNING

The version of the lazy learning method employed in this work is a memory based local learning technique requiring storage of training data in the memory. This method is termed as lazy learning method because it delays processing of data until a query is required to be answered. Being a memory based technique; the unknown function is estimated by giving the whole attention to the region surrounding the point (query point) where the estimation is required. The relevant data is measured using a distance function. Lazy learning attempts to fit the training data only in a region around the location of the query point (186). As it postpones the computations till the query is received for prediction, local modelling procedure is required for each query point. The local modelling procedure consists of parametric and structural identification. Given a model structure, parametric identification involves the optimization of the parameters of the local approximator. The structural identification, on the other hand, comprises the selection of the family of local approximators, a metric to evaluate relevant examples and *bandwidth* which indicates the size of the region in which the data are correctly modeled by the members of the local approximators chosen earlier. After generating candidate models, the generalizing ability of each of the models needs to be assessed by mean squared error (*mse*). It is customary to provide a reliable assessment of *mse* employing a cross-validated approach. Normally, such a cross validation approach requires heavy computational requirements. For linear models, however, the PRESS (Prediction Sum of Squares) statistic procedure can rapidly estimate the leave-one-out cross validation estimate and at a very nominal computation load. This work takes advantage of this methodology for identifying the optimal local structure around every query point. The entire procedure is explained below (185).

Consider two variables $\mathbf{x} \in \Re^m$ and $y \in \Re$ for unknown input-output mapping $f: \Re^m \rightarrow \Re$ known through a set of n examples $\{(\mathbf{x}_i, y_i)\}_{i=1}^{n}$. Let this mapping be represented as:

$$y_i = f(\mathbf{x}_i) + \varepsilon_i \qquad [1]$$

where $\forall i$, ε_i is a random variable such that $E[\varepsilon_i] = 0$ and $E[\varepsilon_i \varepsilon_j] = 0, \forall j \neq i$, and such that $E[\varepsilon_i^r] = \mu_r(\mathbf{x}_i), \forall r \geq 2$, where $\mu_r(\cdot)$ is the unknown r^{th} moment of distribution of ε_i and is defined as a function of $\mathbf{x}_i$.

Now given a query point $\mathbf{x}_q$, the parameter of a local linear approximation of $f(\cdot)$ in a neighborhood of $\mathbf{x}_q$ is obtained by solving the local polynomial regression:

$$\sum_{i=1}^{n} \left\{ (y_i - \mathbf{x}_i' \beta)^2 K\left(\frac{d(\mathbf{x}_i, \mathbf{x}_q)}{h}\right) \right\} \qquad [2]$$

where, given a metric on the space $\Re^m$, $d(\mathbf{x}_i, \mathbf{x}_q)$ is the distance from the query point to the i^{th} example, $K(\cdot)$ is weight function, and h is the bandwidth. To consider a constant term in the regression, a constant value of 1 is appended to each input vector $\mathbf{x}_i$.

The solution of the above weighted least squares problem to find is

$$\hat{\beta} = (X'W'W)^{-1}X'W'W'y \qquad [3]$$

Where X is a matrix whose i^{th} row is $\mathbf{x}_i'$, y is a vector whose i^{th} element is y_i, W is a diagonal matrix whose i^{th} diagonal element is

$$w_{ii}\sqrt{K(d(\mathbf{x}_i, \mathbf{x}_q), h)} \qquad [4]$$

Replacing, $Z = XW$ and $v = Wy$ in equation [3]

$$\hat{\beta} = (Z'Z)^{-1}Z'v = PZ'v \qquad [5]$$

It is assumed that matrix P is nonsingular so that its inverse is defined.

Once the local linear polynomial approximation is obtained, a prediction of y_q = f $(\mathbf{x}_q)$is given by

$$\hat{y}_q = \mathbf{x}_q' \hat{\beta} \quad [6]$$

The cross-validation procedure gives the assessment of the mean-squared-error as

$$mse = E\left[\left(y_q - \hat{y}_q\right)^2\right] \quad [7]$$

Cross-validation requires a large computational effort to be performed due to the series of training steps. Instead, the PRESS statistic, which returns the leave-one-out cross validation error at the reduced computational effort, is used extensively in case of linear models. The PRESS statistic (20) is given by:

$$e_j^{cv} = y_j - \mathbf{x}_j' \hat{\beta}_{-j} \quad [8]$$

where$e_j^{cv}$is the leave-one-out-error and $\hat{\beta}_{-j}$ is the estimated regression parameter with the j^{th} sample removed from the available set of examples.

The above equation can be simplified as:

$$\frac{y_j - \mathbf{x}_j' P Z' v}{1 - \mathbf{z}_j' P \mathbf{z}_j} = \frac{y_j - \mathbf{x}_j' \hat{\beta}}{1 - h_{jj}} \quad [9]$$

Where

$$\mathbf{z}_j = w_{jj} \mathbf{x}_j \quad [10]$$

which is nothing but the j^{th} row of *Z*.

h_{jj} is the j^{th} diagonal matrix of the *Hat matrix*, *H*, given as

$$H = ZPZ' \quad [11]$$

From equation (9), it is evident that it is possible to calculate leave-one-out error without having to explicitly identifying $\hat{\beta}_{-j}$. This simplification greatly reduces the computations making the lazy learning regression algorithm very robust and competitive. Having provided the general procedure we now proceed to explain how to recursively estimate the parameters, obtain the leave-one-out errors and generate the models.

Recursive Algorithm associated with leave-one-out estimation

To start with, consider the first-degree linear approximators. The algorithmic extension to generic polynomial approximators of any degree is simple. It is assumed that metric on the space $\Re^m$ is given. Naturally, the problem of bandwidth selection will be the rivet of attention. For the weight function *K* (.), the following indicator function is adopted (18).

$$K\left(d(\mathbf{x}_i,\mathbf{x}_q)/h\right) = \begin{cases} 1 & if\, d(\mathbf{x}_i,\mathbf{x}_q) \le h \\ 0 & otherwise \end{cases} \qquad [12]$$

By doing this, the optimization of the parameter *h*, is reduced to the optimization of the k neighbors to which unit weight is assigned in the local regression i.e. the problem of bandwidth selection is reduced to a search in the space of $h(k) = d(\mathbf{x}(k),\mathbf{x}_q)$,where $\mathbf{x}(k)$ is k^{th} nearest neighbor of the query point.

By adopting weight function as given in equation [12], it becomes easier and inexpensive to calculate $\hat{\beta}(k+1)$ by simply updating the parameter $\hat{\beta}(k)$ of the model identified with *k* nearest neighbors. By using standard steps of the recursive least squares algorithm it is shown that (21).

$$\left.\begin{aligned} P(k+1) &= P(k) - \frac{P(k)\mathbf{x}(k+1)\mathbf{x}'(k+1)P(k)}{1+\mathbf{x}'(k+1)P(k)\mathbf{x}(k+1)} \\ \gamma(k+1) &= P(k+1)\mathbf{x}(k+1) \\ e(k+1) &= y(k+1) - \mathbf{x}'(k+1)\hat{\beta}(k) \\ \hat{\beta}(k+1) &= \hat{\beta}(k) + \gamma(k+1)e(k+1) \end{aligned}\right\} \quad [13]$$

Where$P(k) = (Z'Z)^{-1}$ when $h = h(k)$ and $\mathbf{x}$ (k+1) is the (k+1)th nearest neighbor of the query point.

With these recursive equations, local model candidates are generated. Once the matrix *P* (k+1) is calculated from equation (13), the leave-one-out errors can be calculated directly without any further model identification as:

$$e_j^{cv}(k+1) = \frac{y_j - \mathbf{x}_j'\hat{\beta}(k+1)}{1-\mathbf{x}_j'P(k+1)\mathbf{x}_j}, \qquad [14]$$

$$\square j: d(\mathbf{x}_j\mathbf{x}_q) \le h(k+1)$$

This gives for each *k*, the $[k\mathbf{x}'1]$ vector $e^{cv}(k)$ that contains all the leave-one-out errors associated with the model $\hat{\beta}$ (*k*).

Model selection

Before starting the algorithm, the range (k_{min}, k_{max}) of nearest neighbors to be employed is selected a *priori*. With $k = k_{min}$, P (*k*) and $\hat{\beta}(k)$ are calculated using equations (3-5). The recursive algorithm returns for a given query point a set of parameters, $\hat{\beta}(k+1)$and a vector containing associated leave-one-out errors. $e_j^{cv}(k+1)$ respectively (equation (13)). After each estimation, a null hypothesis is formulated to check whether $e_j^{cv}(k)$and $e_j^{cv}(k+1)$belong to the same distribution. This hypothesis can be evaluated by a permutation test (For details, see 22). This test facilitates determination of the maximum number of nearest neighbors to be employed. The above procedure of estimating the parameters and the leave-one-out-errors can be done with a set of local models like e.g. local constant, local linear and local quadratic models. Now the question to be answered is how to make final prediction based on the local models generated? This is nothing but the model selection problem. There are mainly two approaches by which a final prediction is done: *winner-takes-all* (competitive) approach, local combination (cooperative) approach.

winner-takes-all selects the best approximator based on some given criterion, *mean-squared-error* (*mse*) being the most convenient and classical one. So, prediction obtained for each value of *k* is compared based on mean square error and the final prediction is done as:

$$\hat{y}_q = \mathbf{x}_q'\hat{\beta}(\hat{k}) \qquad [15]$$

with,

$$\hat{k} = \arg\min_{k \epsilon S} mse^{cv}(k) \qquad [16]$$

Where *S* is a range from which the optimal number of neighbors are selected.

In the other approach for model selection, a local combination of *b* best models is chosen based on the weighed average of *mse*. Here *b* is a user-defined parameter. If the predictions $\hat{y}_q(k)$ and the errors vectors $e^{cv}(k)$ are ordered creating a sequence of integers $\{k_i\}$ such that $mse(k_i) \leq mse(k_j), \forall i < j$ The final prediction is made as:

$$\hat{y}_q = \frac{\sum_{i=1}^{b} \zeta_i \hat{y}_q(k_i)}{\sum_{i=1}^{b} \zeta_i} \qquad [17]$$

where the weights are the inverse of the mean square errors: $\zeta_i = \frac{1}{mse^{cv}(k_i)}$

Using equation [17], lazy learning combines a number of best models for optimal performance. The complete lazy learning algorithm is explained in a step-by-step algorithmic form below.

1) Input the date $\{(\mathbf{x}_i, y_i)\}_{i=1}^{n}$ where $\mathbf{x}$ represents the attributes of the data and *y* the corresponding outputs.
2) Input the query points $\mathbf{x}_q$ to be identified.
3) Define the range of the *k*-nearest neighbors (k_{min}, k_{max}) to identify the local models like local constant, local linear, local quadratic or combination of these models.
4) For a query point $\mathbf{x}_q$, based on the nearest neighbor range and the indicator function given by equation [12], calculate the weights by using equation [4].
5) Use these weights to calculate P matrix in equation [5].
6) Use the recursive algorithm to calculate different $\hat{\beta}$ parameters for the given range of the nearest neighbors and associated errors using PRESS statistic.
7) To select the best number of nearest neighbors, form null hypothesis and evaluate it using a permutation test. If the hypothesis identifies $e_j^{cv}(k+1)$ different from the distribution $e_j^{cv}(k)$ stop including more neighbors and go to step 8 else go to step 6.

8) Use the model selection approach (*winner-takes-all* or local combination) based on mean-squared-error to select the best local approximator for the query point, $\mathbf{x}_q$.

9) Make the final prediction with equation [6] using the best approximator given by step (8).

10) Repeat steps 1 to 9 with different query points. Employ appropriate performance metric (mean-absolute-error, root-mean-squared-error etc.) to calculate the final prediction error.

In data based modelling methods, regularization ability of the modelling method plays an important role. In function approximation problems, the learning bias is typically expressed as a smoothness criterion to optimize. But in case of memory-based local learning, smoothness constraint is not explicit. Smoothing or bandwidth parameter can be manipulated to improve generalization and regularization capabilities. Apart from the form of equation used in this work, there exists many other ways to use the bandwidth parameter. These include fixed bandwidth selection, nearest neighbor bandwidth selection, global bandwidth selection, point-based bandwidth selection etc. A detailed discussion on bandwidth selection can be found in Atkeson et al. (184). Ridge regression and *PCA* dimensionality reduction have also been employed for the purpose of regularization.

With new incoming data the size of the database grows. There are several ways to find relevant data so that lazy learning does not slow down while processing queries. A few methods have been described in literature for accelerating the search without discarding data. These include techniques like binning, use of k-d trees binary data structure etc (184). Once the relevant data is identified, lazy learning method can be automatically used without retraining or incremental learning.

Based on this theoretical foundation, we now explain its application to problem of polyols production.

3.6.3 CASE STUDY

The regression capability of the lazy learning paradigm was tested with polyols production data generated in our previous study. The details of this example are provided before discussing the results that are obtained.

Example

This example presents the real world data in which the effect of composition of the medium on the polyols yield is studied. Production of glycerol and related polyols by the fermentation route is assuming increasing importance in view of escalating prices of petroleum crude and shortage of fats and oils. The option of using fermentative production of these chemicals from raw materials such as glucose, sucrose or sugarcane molasses using osmophilic yeast seems to be promising. The yield of polyols can be influenced by the conditions of growth and production. Of these, the composition of the medium, especially the choice of carbon and nitrogen sources, has the greatest effect on the yield and rate of fermentation. The variables considered in carrying out the experiment are cell mass, glucose concentration, yeast extract, urea concentration, casein hydrolysate concentration, magnesium sulphate concentration, calcium chloride concentration and potassium dihydrogen phosphate concentration (171, 190). By changing these variables on a wide range of operating conditions, fermentation runs were carried out and polyols yield was noted. Data from 39 experimental runs were taken. Randomly selected 8 query points were used to test the efficacy of the algorithms. The performance metric used in this case was root mean squared error (RMSE).

RESULTS AND DISCUSSION

We shall now use the proposed method to obtain solutions to the case example. To make the final prediction, both the approaches, viz. *winner-takes-all* and local combination, were used. In the problem considered, we have used Euclidean distance to measure the distances between observations. There are other options such as Manhattan distance, Mahalanobis distance etc. which can also be used. The results are shown in the Tables 3.1 and 3.2. The final prediction errors reported are based on average values of 100 simulation experiments in the example considered. The simulations were carried on Pentium-IV, 512 MB RAM machine. KPCR and KPLS both performed equally well but the computational time is found to be more in case of KPCR and KPLS as compared to local approaches.

Table 3.1: Prediction results based on winner-takes-all approach

Example	Approximator model	No. of query points	MAE (%)	SSE (%)	RMSE (%)
Polyols data	LCM	8	-	-	2.693
	LLM	8	-	-	2.693
	LQM	8	-	-	2.212
	KPCR	8	-	-	4.009
	KPLS	8	-	-	4.417

MAE: Mean absolute error, SSE: sum of squared errors, RMSE: root mean squared error, LCM: local constant model, LLM: local linear model, LQM: local quadratic model, KPCR: kernel principal component regression, KPLS: kernel partial least squares.

Table 3.2: Prediction results based on local combination of the models

Example	Approximator Model	No. of query points	MAE (%)	SSE (%)	RMSE (%)
Polyol data	Local combination	8	-	-	2.212

MAE: mean absolute error, SSE: sum of squared errors, RMSE: root mean squared error.

The polyols yield prediction problem is solved with lazy learning. In this case, local quadratic model with 2.212 % RMSE is found to be performing better than KPCR and KPLS with 4.009 % and 4.417 % RMSE respectively. Local combination of models achieved the same RMSE as local quadratic model. The local nature of the model, in our opinion, would have a greater influence in the number of neighbours to be incorporated, than the number of problem variables.

The case example as illustrated above involves the use of simulated or laboratory experimental data. For real world application, it is necessary to have sufficient amount of carefully monitored historical database. Lazy learning tool can then be directly incorporated into the monitoring system. The technique is especially useful in instances where fast training and online updation of process models are required. These situations arise as for example in incipient fault detection and diagnosis. Also many systems involve time varying parameters, such as in deactivating systems where faster tracking and updation of models would be a great advantage (191, 192). Future work will focus on implementation of the methodology to such real world examples.

3.6.5 CONCLUSIONS

This work presents a lazy learning approach for robust regression. The method employs a memory based local learning approach and processes the data only when a query is posed. The relevant neighborhood is estimated by simple distance function. The adoption of recursive algorithm along with the model selection criterion makes lazy learning distinct from the other local learning techniques. Employment of local weighted regression for parametric identification and PRESS statistics to assess a local model in cross-validation for structural identification, are the two unique features of the lazy learning. Local weighted regression is a fast parametric technique to determine in a single step, the best local parameters. The PRESS statistic is a well-founded and easiest way to validate the local model. It is this judicious use of linear techniques for parametric and structural identification that makes lazy learning an attractive alternative to the contemporary methods. Even with the simple metric used in the study, the algorithm performed better than some of the state-of art approaches.

NOTATION

d	distance function
e	leave-one-out error
h	bandwidth
k	number of nearest neighbors
K	weight function
x	input vector
y	output vector

Greek letters

β β β β	regression parameter for local linear approximation
$\hat{\beta}$	regression parameter of weighted least squares problem
Č	random variable in equation [1]
ζ	Weights in equation [17]

Subscripts

i	number of the input/output sample
ii	diagonal element of the corresponding matrix
j	sample number in equation [8]
jj	diagonal element of the corresponding matric
q	query point
min	minimum

Superscripts

m	dimension of the input vector
n	total number of input/output samples
cv	Cross validation

CHAPTER-4

MEDIA OPTIMIZATION BY GENETIC ALGORITHM: SHAKE FLASK EXPERIMENTS WITH SUCROSE AS SUBSTRATE

CHAPTER-4

MEDIA OPTIMIZATION BY GENETIC ALGORITHM: SHAKE FLASK STUDIES WITH SUCROSE AS SUBSTRATE

4.1 INTRODUCTION

Micro-organisms are specific in their nutritional requirements. There exists a diverse pattern of preference towards different nutrients by various micro-organisms. All commercial exploitations of micro-organisms and their activities arose from their primary need to increase their cell numbers and during such process; they elaborate enzymes, whose activities on the substrate result in the synthesis of wide range of chemical substances. It has been revealed that apart from the genetic make-up of the cells, environmental conditions markedly influence the activities of specific enzymes or enzyme system which ultimately determine the nature and the quantity of the product elaborated. Such external conditions include primarily the oxygen transfer, temperature of fermentation, the pH of the medium, the nature and the concentration of the substrates and growth parameters (nitrogen source) and the presence of salts in optimum amounts (97).

Shake flask experiments are primarily used to optimize the nutrient parameters in many fermentation studies. Shake flask experiments provides aerobic environment. Shaking of a flask on a rotary shaker improves the surface area of liquid in contact with air and hence facilitates oxygen transfer. The shape of the flask, the ratio of the volume of the medium to the volume of the flask, the shaker speed, presence of the baffles etc. determine the oxygen transfer efficiency during the shake flask experiments.

The temperature of fermentation is another factor which may influence the metabolic pattern of the organism and this fact is made use of in several instances to synthesize a specific substance (193). For example, in the commercial production of ethanol from sugarcane juice or molasses, the fermentation temperature is controlled at 32–33°C (194). In the synthesis of polyols by yeasts, a temperature between 30-35°C is reported to be optimum (87). Lowering the temperature causes a decrease in the yields in spite of the growth remaining unaffected.

Physical condition like pH also influences the yield of the product (195). The maximum production of the desirable substance is limited to a narrow range of pH values. In a number of cases, with the progress of fermentation, a fall in the pH is generally observed which is attributed to the production of CO_2 and organic acids. It thus becomes necessary to incorporate buffers into the medium (195) or use automatic pH control systems.

In formulation of fermentation medium, it is necessary to include 1) Carbon and/or energy source 2) Nitrogen source, 3) Source of Phosphate 4) Growth factors and vitamins and 5) Necessary trace elements.

In preliminary studies, pure carbon sources such as glucose, fructose or sucrose are used which usually also serve as a source of energy. From the viewpoint of commercial exploitation, cheaper carbon sources such as sugarcane or sugarbeet juice, molasses, hydrolysate of cereal grains (e.g. corn or sorghum), waste sulphite liquor etc. can also serve the purpose. However, they are complex in nature and hence may not give realistic effect on fermentation process. Sometimes, it is also necessary to subject these materials for pre-treatment before fermentation for removal of inhibitory substances.

Nitrogen requirement of micro-organisms is generally met by addition of complex substances such as yeast extract, malt extract, peptone, casein hydrolysate, corn steep liquor, soyabean meal etc. They not only serve as a source of nitrogen but also provide the necessary growth factors. Urea and ammonium salts are also useful as a source of nitrogen for yeast.

Phosphate is necessary for growth. Similarly, other trace elements such as K^+, Na^+, Mg^{++}, Ca^{++} etc. are also necessary for the growth of micro-organisms as they act as functional components of proteins or enzyme activators or stabilizers of proteins.

The inoculum size and the metabolic status of the cells of the inoculum can influence the product yields and fermentation times. Oxygen transfer efficiency in shake flask experiments, which is a function of medium volume to flask volume, can also influence the product yields.

Optimization of nutrient parameters of fermentation media on shake flask level can be carried out in several ways. The simplest and conventional approach is the One–Variable–at–a–time approach (OV approach). This approach has been used

by researchers for several years. This approach is however, inefficient in locating a true optimum when interaction effects are present.

Multi-variable methods for optimization of media are used to overcome problems with interaction effects. Two categories of methods, the sequential design approach and simultaneous design approach, are used for this purpose. In the sequential method experiments are successfully performed in the direction of improving the performance index until the optimum is reached. Although several methodologies exist for sequential design, the simplex approach has been the most widely used method. It can handle many variables and starts with a design consisting of a simplex with n+1 dimensional space. Subsequent improvements are obtained by reflection. The other popular method is the simultaneous statistical design methodology. These methods allow simultaneous and efficient variation of all media components (196-199). Both single and two-level factorial experiments have been designed for optimization of media.

The common goal in the fermentation industry is to reduce the overall development time and to locate a true optimum when interaction effects are present. A critical part of this development is to fix an equation for the manufacturing process. Because of the competitiveness in the market this has to be done in a constrained time frame. It is, therefore, imperative to optimize the process rapidly and maximize the information from fewer numbers of experiments. Recently, evolutionary methods such as genetic algorithms, simulated anneling, tabu search and ant colony methods are becoming increasingly popular. Artificial neural networks have also been widely employed in various bioprocess engineering applications including optimization of media (200-202). In the present study, we have used the genetic algorithm procedure for optimization of culture media with a view to maximize the polyols yield.

Genetic Algorithm: An overview

Genetic algorithms are stochastic search techniques that mimic the process of natural selection (203, 204). They have the inherent ability of simple representation to encode highly complicated structures and use simple transformations to improve such structures to reach optimal solution. In the natural evolution, each species searches for beneficial adaptation in a complicated and dynamically differing environment. The knowledge gained in the search is embodied in the chromosome of its members. Random mutation, numerical creeping, and crossover are the genetic operators, which

alter the chromosomal makeup. Conventional genetic algorithm as an optimization procedure basically involves five components: a chromosomal representation of solutions, an evaluation function mimicking the role of the environment, rating solutions in terms of their current fitness, genetic operators that alter the compositions of children during reproduction, and values of the parameters that the algorithm uses (such as population size, probabilities of applying genetic operators).

Recently, several improvements in genetic algorithms over the basic algorithm have been proposed. These include problem-specific crossover operators, elitist selection strategy, and diversity measures. Problem-specific crossover operators improve algorithm efficiency in the speed and quality of obtaining global solutions. Elitism ensures that the best solution from the previous generation is preserved in the current generation by replacing the weakest new individual. Incest prevention is used to maintain genetic diversity. This is usually implemented by allowing mating to occur only if the Hamming distance is above an acceptable threshold. Genetic algorithms have the following features, which make them different from the conventional (deterministic) algorithms:

1. They work on a population of points instead of a single point.
2. They work on the representation rather than on the variables.
3. They facilitate a global optimum.
4. They are inherently parallel.
5. They do not require derivatives for fitness function.

Genetic algorithms have been used in a wide variety of fields for optimization purposes. One of the earliest applications of genetic algorithms in bioprocess engineering was for obtaining optimal media composition (205). Genetic algorithms were found to be able to bring out highly optimized media experimentally even in noisy systems. Matsuura et al. (206) calculated the optimal trajectories for fermentation processes using genetic algorithms. A genetic algorithm was used to estimate parameters in fermentation dynamic models, and its effectiveness was tested with three typical models: an equation for biomass determination, an equation for substrate consumption, and an equation for products. The estimation results obtained were better than the Marquardt optimization procedure. Weuster-Botz et al. (207) developed a fed-batch process for the production of L-lysine with *Corynebacterium glutamicum* in a stirred-tank reactor. Optimizations of medium

composition in parallel shake flasks were done using genetic algorithms. The other relevant applications of genetic algorithms, which are useful in bioprocess optimization, can be found in references 208 to 211.

The conventional binary coded genetic algorithm having discrete search space has been very successful in obtaining solutions for the combinatorial optimization problems. However, in solving problems with continuous search space, coding of the real valued variables in finite length strings causes a number of difficulties: inability to obtain arbitrary precision in the obtained solution, fixed mapping of the problem variable, inherent hamming difficulty associated with binary coding, and processing of Holland's schemata in continuous space (212). Several real coded genetic algorithms were used in the recent past to overcome these difficulties (213). We have employed one such strategy for optimizing the media variables in the production of polyols. We have prepared our own software, and the details of our algorithm for optimizing the media components to maximize polyols yield are discussed under genetic algorithm approach.

As explained in the Chapter-2, we have carried out screening experiments and optimization of medium components using glucose as the carbon source. Most of the work carried-out so far is also based on glucose as the carbon source.

In a country like India, sucrose is available in the form of plantation white sugar and it is also present in readily available feed-stock such as sugarcane juice and molasses. Sucrose being cheaper in developing countries like India, it would be desirable to produce polyols from sucrose based media. Therefore, throughout this investigation, sucrose has been used as the carbon source. In fact, one of the objectives of this study is to develop process for fermentative production of polyols from sucrose-containing media. In the present investigation, we have attempted to optimize the fermentation media by both the approaches, i.e. by One-Variable-at-a-time approach as well as by using Genetic Algorithm.

4.2 MAINTENANCE OF YEAST CULTURE

As described in the previous Chapter the selected osmophilic yeast *H. anomala* NCIM-3341 was maintained on Malt extract-Glucose-Yeast extract-Peptone (MGYP) agar slants and was transferred every month. The composition of the MGYP agar slant was as given in the Table on next page.

Table 4.1: MGYP Agar slant composition

Sr. No.	Components	Concentration g/100 ml.
1.	Malt extract	0.30
2.	Glucose	30.00
3.	Yeast extract	0.30
4.	Peptone	0.50
5.	Agar-agar	2.50
6.	pH	5.50

The stock culture slants were layered with sterile liquid paraffin and preserved at 4°C. For acclimatizing the culture to high osmotic conditions it was deliberately maintained on MGYP agar slants containing 30 % glucose.

4.3 PREPARATION OF MEDIA

It is often best to sterilize sugars separately because they may react with ammonium ions and amino acids to form black nitrogen containing compounds which will partially inhibit the growth of many micro-organisms. This also avoids the caramalization of sugars (214). Yeast extract and urea were sterilized together at 1.1 bar for 15 minutes. Salts such as $MgSO_4$, KH_2PO_4, $CaCl_2$ etc. were sterilized together as and when they were required. Prior to incubation, the various sterilized solutions were cooled to room temperature and mixed together.

4.4 PREPARATION OF INOCULUM

The composition of the inoculum medium used was as given in the following Table.

Table 4.2: Inoculum medium composition

Sr. No.	Components	Concentration (g/100 ml)
1.	Sucrose	10.0
2.	Yeast extract	0.5
3.	Urea	0.1
4.	pH	5.5 – 6.0

A loop full of culture from the agar slant was transferred to the sterile 100 ml inoculum medium contained in a 500 ml conical flask under aseptic conditions. The inoculated flasks were incubated at 30 ± 2°C for 48 hrs on a rotary incubator shaker at 180 rpm with an eccentricity of one inch.

Usually, the volume of the inoculum required for seeding the fermentation flask was around 10 % of the main fermentation medium. To avoid the dilution of the fermentation medium it was necessary to centrifuge the grown inoculum medium. Centrifugation was carried out in sterile stoppered centrifuge tubes at 4°C and 4000 rpm (2225 g) for 20 minutes. The centrifuged wet cell mass corresponding to dry cell weight as indicated in the individual experiments was used to inoculate the experimental flask.

4.5 FERMENTATION

The fermentation medium was sterilized and prepared in similar manner as inoculum medium except that the sucrose concentration was raised to 30 %. The 500 ml Erlenmeyer flask containing 40 ml of fermentation medium was inoculated and incubated on a rotary shaker at 180 rpm The temperature of the incubator rotary shaker was maintained at 30 ± 2°C and experiments were conducted for 216 hrs. 3 ml samples were removed aseptically at 216 hrs or at required intervals and centrifuged. The clear supernatant was used for estimation of final polyols and residual sugar concentration. Detailed composition of fermentation media is indicated in the respective Tables.

4.6 ANALYTICAL METHODS

The detailed analytical methods used throughout the course of this investigation are given in Chapter-2. Total polyols were determined by the spectrophotometric method of Lambert and Neish (159) as outlined by Hanahn and Olley (160). Gas chromatography of fermentation broth samples as explained in Chapter 2 was performed only to get qualitative idea of individual polyols present in the fermentation medium.

Sucrose after inversion with HCl was estimated with the alkaline copper reagent of Somogyi (161) and arsenomolybdate reagent of Nelson (162). Ethanol in

the fermented broth sample was determined by the dichromate reduction method as described by Caputi et al. (167).

4.7 ONE-VARIABLE-AT-A-TIME APPROACH (OV APPROACH)

In the present study, following parameters were investigated in order to examine the final polyols concentration at the end of 216 hrs by the selected strains of *H. anomala,*

1. Effect of inoculums size.
2. Effect of yeast extract concentration.
3. Effect of urea concentration.
4. Effect of initial sucrose concentration.
5. Effect of casein hydrolysate concentration.
6. Effect of magnesium sulphate ($MgSO_4.7H_2O$) concentration.
7. Effect of calcium chloride ($CaCl_2$) concentration.
8. Effect of potassium dihydrogen phosphate (KH_2PO_4) concentration.
9. Effect of aeration.
10. Comparison of using sucrose, glucose and fructose as carbon source.
11. Effect of fermentation temperature.

Literature survey on production of polyols by osmophilic yeasts has revealed that it is necessary to keep the level of nitrogen and phosphate sources at minimum so as to maximize the polyols production and to reduce the conversion of sugar to ethanol. High sugar concentration and high efficiency of aeration help to increase the polyols yield. Keeping this in mind, following medium and environmental conditions were decided to start with.

Table 4.3: Starting conditions of fermentation

Sr. No.	Components/parameters	Concentration/Values
1.	Sucrose	30 g/100 ml
2.	Yeast extract	0.25 g/100 ml
3.	Urea	0.10 g/100 ml
4.	pH	5.8 to 6.0
5.	Ratio of medium volume to flask volume	0.08
6.	Shaker speed	180 rpm
7.	Temperature	30 ± 2°C

4.7.1 RESULTS AND DISCUSSION

Effect of inoculums size

The size of inoculum used is directly related to the rate of oxygen supply per unit biomass, which determines the percentage conversion of sugar to polyols. Higher inoculum would result in better sugar utilization but polyols yield would be hampered. The results of variation of inoculum size are given in Table 4.4. At the end of 216 hrs of fermentation, the final polyols concentration was maximum (60.20 g/l) at the lowest inoculum level of 0.78 g/l DCW. This was closely followed by inoculum size of 1.56 g/l DCW with final polyols concentration of 59.35 g/l and went on decreasing to 16.25 g/l at 6.24 g/l DCW probably because of competition amongst the cells of the population resulting in inadequate oxygen supply. However, sugar utilization rate was maximum at inoculum size of 1.56 g/l. Therefore, in all the subsequent experiments, 1.56 g/l DCW inoculum was used as optimum inoculum size.

It is to be noted that in all shake flask as well as scale-up experiments in an automated fermenter, we could not detect ethanol formation probably because of fully aerobic conditions used and absence of externally added phosphate.

Effect of yeast extract concentration

It has been reported that in *T. magnolia* (71) higher level of yeast extract led to a decrease in glycerol yield and increased ethanol production. Yeast extract not only serves as a nitrogen source but also as an excellent source of growth factors. Yeast extract has been used extensively in studies in production of various polyols. Concentrations of yeast extract that is used have ranged from 0.1 to 0.4 % depending upon the conditions of the experiment, and researchers have not always agreed upon the optimum concentration (215).

The results of varying yeast extract concentration in the range of 0.0 to 20 g/l are given in Table 4.5. It can be noted that without any yeast extract the final polyols concentration at the end of 216 hr was just 14.60 g/l which increased to 50.50 g/l with introduction of yeast extract at a concentration of 2.5 g/l. For higher values of yeast extract, the final polyols concentration went on decreasing.

Effect of urea concentration

External addition of urea as a cheap source of nitrogen in fermentation industry is a common practice. Urea is added as the third component in the standard

Table No 4.4: Effect of Inoculum size

Constant Parameters, (g/l) Yeast extract=2.50, Urea=1.00, Sucrose=300.0, Casein Hydrolysate=0.00,$MgSO_4.7H_2O$=0.00, $CaCl_2$=0.00, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) Inoculum (DCW)	0.78	1.56	3.12	4.68	6.24
Polyols Concentration, (g/l) at 216 hr	60.20	59.35	35.50	27.75	16.25

Table No 4.5: Effect of Yeast Extract concentration

Constant Parameters, (g/l) Inoculum=1.56, Urea=1.00, Sucrose=300.0, Casein Hydrolysate=0.00,$MgSO_4.7H_2O$=0.00, $CaCl_2$=0.00, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) Yeast Extract	0.00	2.50	5.00	10.00	20.00
Polyols Concentration, (g/l) at 216 hr	14.60	50.50	23.70	9.55	8.80

Table No 4.6: Effect of Urea concentration

Constant Parameters, (g/l) Inoculum=1.56, Yeast Extract=2.50, Sucrose=300.0, Casein Hydrolysate=0.00,$MgSO_4.7H_2O$=0.00, $CaCl_2$=0.00, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) Urea	0.00	0.50	1.00	2.00	4.00
Polyols Concentration, (g/l) at 216 hr	54.20	56.35	58.75	52.55	50.30

medium in addition to sucrose and yeast extract. Most researchers have used small concentrations of Urea as a supplement to the yeast extract, obtaining slightly improved yield thereby (215).

The results of the experiments of varying the urea concentration in the range of 0.0 g/l to 4.0 g/l are given in Table 4.6. It can be seen that maximum total polyols concentration of 58.75 g/l at the end of 216 hr was obtained at an optimum dose of 1.0 g/l urea. Above and below this concentration of urea, the final polyols concentration was slightly reduced.

Effect of sucrose concentration

Working with *S. rouxi*, Spencer et al. (87) have found that increasing the initial sugar concentration from 23 to 29 % increased the yield of glycerol from 23 to 103 mg/ml while the yield of arabitol fell from 55 to 51 mg/ml. Onishi and Suzuki (156) have reported that increasing the sugar concentration from 10 to 29 % increased the yield of polyols (and in particular the yield of glycerol) from 50 to 71 % on the basis of sugar utilized.

The sucrose concentration in the fermentation medium was varied from 150 g/l to 350 g/l and the results are given in Table 4.7. It can be noted that the total polyols concentration at the end of 216 hrs of fermentation was maximum for 300 g/l initial sugar concentration. Above and below this value, the final polyols concentration was reduced. Therefore, 300 g/l initial sucrose concentration seems to be optimum for polyols production by our strain of *H.anomala*.

Effect of casein hydrolysate

Onishi (216, 217) has used casein hydrolysate in his studies with *S. rouxi* and *P. miso*. Casein hydrolysate is a polypetone and serves as a good source of amino acids.

The effect of casein hydrolysate concentration on final polyols concentration is illustrated in Table 4.8. Maximum total polyols (glycerol + arabitol) concentration of 60.00 g/l was obtained at 1.0 g/l concentration of casein hydrolysate. Below 1.0 g/l concentration of casein hydrolysate, the final polyols concentration went on decreasing. Similarly, above 1.0 g/l concentration of casein hydrolysate, the final polyols concentration was also reduced. Therefore, 1.0 g/l of casein hydrolysate can be considered as optimum concentration for polyols production by *H. anomala* under the given set of conditions.

Table No 4.7: Effect of Initial Sucrose concentration

Constant Parameters, (g/l) Inoculum=1.56, Yeast Extract=2.50, Urea=1.00, Casein Hydrolysate=0.00,$MgSO_4.7H_2O$=0.00, $CaCl_2$=0.00, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) Sucrose	150.0	200.0	250.0	300.0	350.0
Polyols Concentration, (g/l) at 216 hr	15.85	29.65	52.25	55.85	52.70

Table No 4.8: Effect of Casein Hydrolysate concentration

Constant Parameters, (g/l) Inoculum=1.56, Yeast Extract=2.50, Urea=1.00, Sucrose =300.0,$MgSO_4.7H_2O$=0.00, $CaCl_2$=0.00, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) Casein Hydrolysate	0.0	0.25	0.50	1.00	2.00
Polyols Concentration, (g/l) at 216 hr	49.60	52.90	54.40	60.00	30.10

Table No 4.9: Effect of $MgSO_4.7H_2O$ concentration

Constant Parameters, (g/l) Inoculum=1.56, Yeast Extract=2.50, Urea=1.00, Sucrose =300.0, Casein Hydrolysate=1.00, $CaCl_2$=0.00, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) $MgSO_4.7H_2O$	0.0	0.13	0.25	0.50	1.00
Polyols Concentration, (g/l) at 216 hr	60.10	61.05	65.10	48.00	41.60

Effect of $MgSO_4.7H_2O$ concentration

Mg^{++} is known to influence the activity of various intracellular enzyme involved in metabolic pathways. The $MgSO_4.7H_2O$ concentration was varied between 0.00 g/l to 1.00 g/l as shown in Table No.4.9. It can be seen that 0.25 g/l concentration of $MgSO_4.7H_2O$ is optimum under the given set of conditions yielding 65.10 g/l concentration of total polyols at the end of fermentation.

Effect of $CaCl_2$ concentrations

As a source of Ca^{++}, the concentration of $CaCl_2$ was varied between 0.0 g/l to 2.0 g/l in the fermentation medium and the effect is shown in Table No.4.10. It can be seen that at all concentrations, $CaCl_2$ reduced the final concentration of polyols. This indicates that Ca^{++} is not required by *H. anomala* for polyols production.

Effect of KH_2PO_4 concentration

The KH_2PO_4 concentration was varied between 0.0 g/l to 0.20 g/l in fermentation studies on shake flask level. Surprisingly, KH_2PO_4 was also found not to improve the polyols formation (Table 4.11). Probably, the PO_4^{-2} available from yeast extract and as contaminant with other salts was sufficient enough for *H. anomala* metabolic activity during polyols formation.

Therefore, in all subsequent experiments, no external sources of Ca^{++} and PO_4^{-2} were used.

Effect of aeration

In shake flask experiments, it is possible to increase the aeration rate or oxygen transfer efficiency by either increasing the ratio of medium volume (Mv) to flask volume (Fv) or by increasing the shaker speed. The results of varying the volume of medium from 25 ml to 50 ml in a 500 Erlenmayer flask are reported in Table No.4.12. It can be observed that the final polyols concentration at the end of 216 hrs was maximum (65.10 g/l) at 0.08 ratio of medium volume to flask volume and it decreases on both sides.

Comparison of using sucrose, glucose and fructose as carbon source

As mentioned previously, we wanted to develop fermentation process based on sucrose as carbon source which is cheaply available in a country like India. It is known that many yeast produce extracellular invertase, which is responsible for hydrolysis of sucrose outside the cell. The hydrolysed products i.e. glucose and

Table No 4.10: Effect of $CaCl_2$ concentration

Constant Parameters, (g/l) Inoculum=1.56, Yeast Extract=2.50, Urea=1.00, Sucrose =300.0, Casein Hydrolysate=1.00, $MgSO_4.7H_2O$ =0.25, KH_2PO_4=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0	
Variable Parameter, (g/l) $CaCl_2$	0.00	0.05	0.10	0.20
Polyols Concentration, (g/l) at 216 hr	65.10	50.10	39.50	32.20

Table No 4.11: Effect of KH_2PO_4 concentration

Constant Parameters, (g/l) Inoculum=1.56, Yeast Extract=2.50, Urea=1.00, Sucrose =300.0, Casein Hydrolysate=1.00, $MgSO_4.7H_2O$ =0.25, $CaCl_2$=0.00			**Constant Parameters** Mv/Fv=0.08, Temp=30°C, Initial pH=6.0		
Variable Parameter, (g/l) KH_2PO_4	0.000	0.025	0.050	0.100	0.200
Polyols Concentration, (g/l) at 216 hr	60.75	26.50	23.05	23.95	22.70

Table No 4.12: Effect of Aeration

Constant Parameters (g/l) Inoculum=1.56, Yeast Extract=2.50, Urea=1.00, Sucrose =300.0, Casein Hydrolysate=1.00, $MgSO_4.7H_2O$ =0.25, $CaCl_2$=0.00, KH_2PO_4=0.00		**Constant Parameters** Temp=30°C, Initial pH=6.0	
Variable Parameter Mv/Fv	0.05	0.08	0.10
Polyols Concentration (g/l) at 216 hr	43.85	65.10	45.85

fructose, are then transported inside the cell for further utilization (218). The various sugars used in fermentation can also exert osmotic pressure of different degrees on the yeast cell. For example, fructose is known to exert more osmotic pressure on the yeast cell as compared to sucrose and glucose (219). We wanted to verify the effect of using different sugars for polyols production in *H. anomala* as given in Table 4.13. In these experiments, samples were drawn at 144 hr and 216 hr for estimation of sugars, polyols and dry cell weight. During this experiment, each flask was run in duplicate and sample 1 at 144 hrs was drawn from 1st flask and sample 2 at 216 hrs was drawn from the 2nd flask. This precaution was taken to avoid excessive reduction in the volume in the shake flask, which could change the oxygen transfer efficiency and thus the final polyols yield.

It can be seen from Table 4.13 that maximum polyols yield on the basis of total initial sugars (p/ts) and related final polyols concentration are all on the higher side for fructose (p/ts – 24.77 %) followed by 21.67 % for glucose and 19.98 % for sucrose. This also validates our hypothesis that fructose exerts more osmotic pressure on the yeast cells thereby increasing the polyols formation. The reduction in yield and final polyols concentration with sucrose as carbon source may be related to the extent of extracellular inversion of sucrose by *H. anomala*.

We are probably the first researcher to report this aspect in polyols production by osmophilic yeast.

Effect of temperature

All industrial fermentation processes are governed by optimum temperature of fermentation. For example, in industrial alcoholic fermentation process the ethanol producing yeast, *S. cervevisiae*, requires optimum temperature of 32–33°C (194). Production of lactic acid by fermentation with *Lactobacillus* requires an optimum temperature of 45°C (220).

It was, therefore essential to investigate the effect of temperature on polyols production by *H. anomala*. These experiments were conducted with different sugars at different temperatures as given in Table No. 4.14. Experiments were performed in duplicate flasks and samples were withdrawn at 144 hrs and 216 hrs as explained in previous part. The effects of temperature at 30.0°C, 32.5°C, 35.0°C and 37.5°C are reported in the Table No.4.14.

Table 4.13: Effect of different sugars on polyols formation in OV optimized media

Sugar	Time	Residual sugar	Polyols	Sugar utilization rate	Polyols yield on the basis of sugar utilized (p/su)	Polyols yield on the basis of total initial sugar (p/ts)
	(hr)	(g/l)	(g/l)	(g/l/hr)	(%)	(%)
Sucrose	0	300	0.00	0.00	0.00	0.00
	144	148	29.30	1.02	19.27	9.70
	216	120	59.95	0.83	33.30	19.98
Glucose	0	300	0.00	0.00	0.00	0.00
	144	139	36.30	1.11	22.54	12.10
	216	60	65.00	1.11	27.08	21.67
Fructose	0	300	0.00	0.00	0.00	0.00
	144	138	48.10	1.12	29.69	16.03
	216	44	74.30	1.19	29.02	24.77

Table 4.14: Effect of Temperature

Sugar	Temp	Time	Residual sugar	Polyols	Sugar utilization rate	Polyols yield based on sugar utilized (p/su)
	(^{o}C)	(hr)	(g/l)	(g/l)	(g/l/hr)	(%)
Sucrose	30.0	0	300	00.00	0.00	0.00
	30.0	144	148	29.30	1.02	19.27
	30.0	216	120	59.95	0.83	33.30
	32.5	0	300	00.00	0.00	0.00
	32.5	144	152	27.60	1.03	18.65

Continued……..

…..……Continued

	32.5	216	171	41.30	0.60	32.02
	35.0	0	301	00.00	0.00	0.00
	35.0	144	129	46.55	1.02	27.06
	35.0	216	123	43.85	0.64	24.70
	37.5	0	300	00.00	0.00	0.00
	37.5	144	236	7.70	0.53	11.93
	37.5	216	216	13.00	0.50	15.38
Glucose	30.0	0	300	00.00	0.00	0.00
	30.0	144	139	36.30	1.11	22.54
	30.0	216	60	65.00	1.11	27.08
	32.5	0	300	00.00	0.00	0.00
	32.5	144	138	47.60	1.15	10.53
	32.5	216	104	72.30	1.04	35.90
	35.0	0	301	00.00	0.00	0.00
	35.0	144	84	88.80	1.28	41.01
	35.0	216	27	89.65	1.42	32.77
	37.5	0	300	00.00	0.00	0.00
	37.5	144	242	7.50	0.48	12.83
	37.5	216	212	18.40	0.52	20.79
Fructose	30.0	0	300	00.00	0.00	0.00
	30,0	144	138	48.10	1.12	27.69
	30.0	216	44	74.30	1.23	29.02
	32.5	0	301	00.00	0.00	0.00
	32.5	144	138	48.10	1.15	28.80
	32.5	216	34	75.10	1.41	28.23
	35.0	0	301	00.00	0.00	0.00
	35.0	144	105	84.75	1.16	43.35
	35.0	216	96	78.20	1.06	38.23
	37.5	0	301	00.00	0.00	0.00
	37.5	144	195	36.90	0.90	34.00
	37.5	216	162	49.20	0.82	35.00

It can be seen that with maximum yield (38.23 %) at 216 hrs on the basis of sugar utilized has been obtained with fructose at fermentation temperature of 35.0°C. With glucose as substrate, the *H. anomala* could tolerate temperature up to 32.5°C and resulted in maximum yield of 35.90 % at 216 hrs. The final polyols concentration is high for experiments at 35^{o}C with glucose and fructose as substrates. With fructose as carbon source the polyols yield goes on increasing from 30.0°C to 35.0°C and then drops at 37.5°C. The final polyols concentration achieved with glucose increases from 65.0 g/l to 89.65 g/l from 30.0°C to 35.0°C, respectively and then drops at 37.5°C. With fructose, the polyols concentration increases from 74.30 g/l to 78.20 g/l from 30.0°C to 35.0°C. At 37.5°C, the yield is drastically reduced for all three sugars. Maximum polyols yield with sucrose is obtained at 30.0°C as compared to other sugars. Polyols yield at 32.5°C with sucrose as substrate is slightly reduced as compared to yield at 30.0°C. With sucrose as the substrate, the yeast is unable to tolerate higher temperatures of 35.0°C and 37.5°C. We suspect that this reduced yield may be because of the reduced extracellular invertase activity at higher temperatures.

Therefore, for glucose and fructose the optimum temperatures are 32.5 and 35.0°C, respectively for *H. anomala* and for sucrose based medium it can be around 30.0°C to 32.5°C. It would be necessary to investigate this aspect again during scale-up studies on fermenter scale.

4.8 GENETIC ALGORITHM APPROACH (GA APPROACH)

As explained earlier, we wanted to optimize the media composition using both the approaches. The purpose was to verify the difference in the composition of the optimized media and the final polyols production.

Following experiments were conducted.

1. Optimization of media using genetic algorithm.
2. Comparison between GA optimized media and OV optimized media.
3. Effect of addition of invertase.
4. Comparison between *H. anomala* and *P. farinosa* for polyols production

OPTIMIZATION OF MEDIA

The Algorithm

The algorithm's steps are initialization, selection, crossover, and mutation, which are discussed below.

Step 1: Initialization

The algorithm is initiated by creating a population having a certain size. This is done by randomly generating solutions having the values of the variables *x1* to *x5* (representing inoculum size, sugar concentration, yeast extract concentration, urea concentration, and magnesium sulfate concentration, respectively) within the allowable ranges specified. After generating the random populations, we have to assign a fitness for all the members of the population. The fitness for our problem is the yield of polyols.

Step 2: Selection

For the sake of convenience, the population members are sorted in the order of decreasing fitness (objective function value) with a view to identifying the strong and weak members of the population. The strong members are called parents and are selected to take part in crossover operations. In these operations, they are suitably combined to produce children. These children then replace the existing weak members. The population of the succeeding generation thus consists of the strong parents and the children produced by genetic combination of the strong parents. In our work, we have termed the top 50 % of the population as parents.

Step 3: Crossover

In the crossover operation, a certain number of the weaker populations are removed. These are replaced by new solutions, which are created by genetic like combinations of fitter solutions. Crossover operations are done as follows: A fitter solution, i.e., a parent from the top half of the population, is selected randomly (weaker solutions are not selected for crossover operation), and the value of the first variable (*x1*) of the selected parent is set as the value of the first variable of the newly created solution. The value of the second variable (*x2*) of the new solution is set to the value of the corresponding variable of the randomly chosen second parent with a probability equal to the crossover probability, *CP*. Subsequent values of the variables

of the new solution are chosen by the same procedure. Thus, for *CP = 1*, each variable of the new solution has a different parent, and for *CP = 0*, the new solution is identical to a randomly selected single parent. After crossover operation is performed, the new solution replaces the weakest solution in the population. Similarly, other weaker solutions are replaced by repeating the process.

Step 4: Mutation

The crossover operator is mainly responsible for the search aspect of genetic algorithms, but a mechanism is needed to keep and maintain the algorithm's diversity; otherwise, the algorithm very quickly converges to the local optimum and never reaches the global optimum. The mutation operator is used for this purpose. There are several ways in which mutation can be performed in the real coded algorithm. We used the following method for mutation: Select a variable randomly and add a perturbation randomly (positive or negative direction is chosen with equal probability) with the specified mutation probability (*MP*). If a population member at a given generation number **t** is $\bar{x}(x_1, x_2, \ldots, x_m)$ and if x_i is selected for mutation, it is replaced with

$$x_i^{new} = \begin{cases} x_i + \Delta(t, -X_i^u - x_i) \\ \quad or \\ x_i - \Delta(t, x_i - X_i^l) \end{cases} \quad Both\ with\ equal\ probablities$$

in which X_i^l and X_i^u are, respectively, the lower and upper bounds for the variable x_i. The function **Δ** (t, y) returns a value in the range **[0, *y*]** in which ***y*** is any real number. The function is designed in such a way that the probability of **Δ (*t*, *y*)** being close to zero increases as *t* increases and is defined as

$$\Delta(t, y) = y \cdot (1 - r^{[1-(t/T)]^B})$$

in which *r* is a random number from [0, 1], *T* is the maximum number of generations, and *B* is the nonlinearity parameter. Each variable x_i of each population member is mutated as shown with a probability equal to the mutation probability, *MP*.

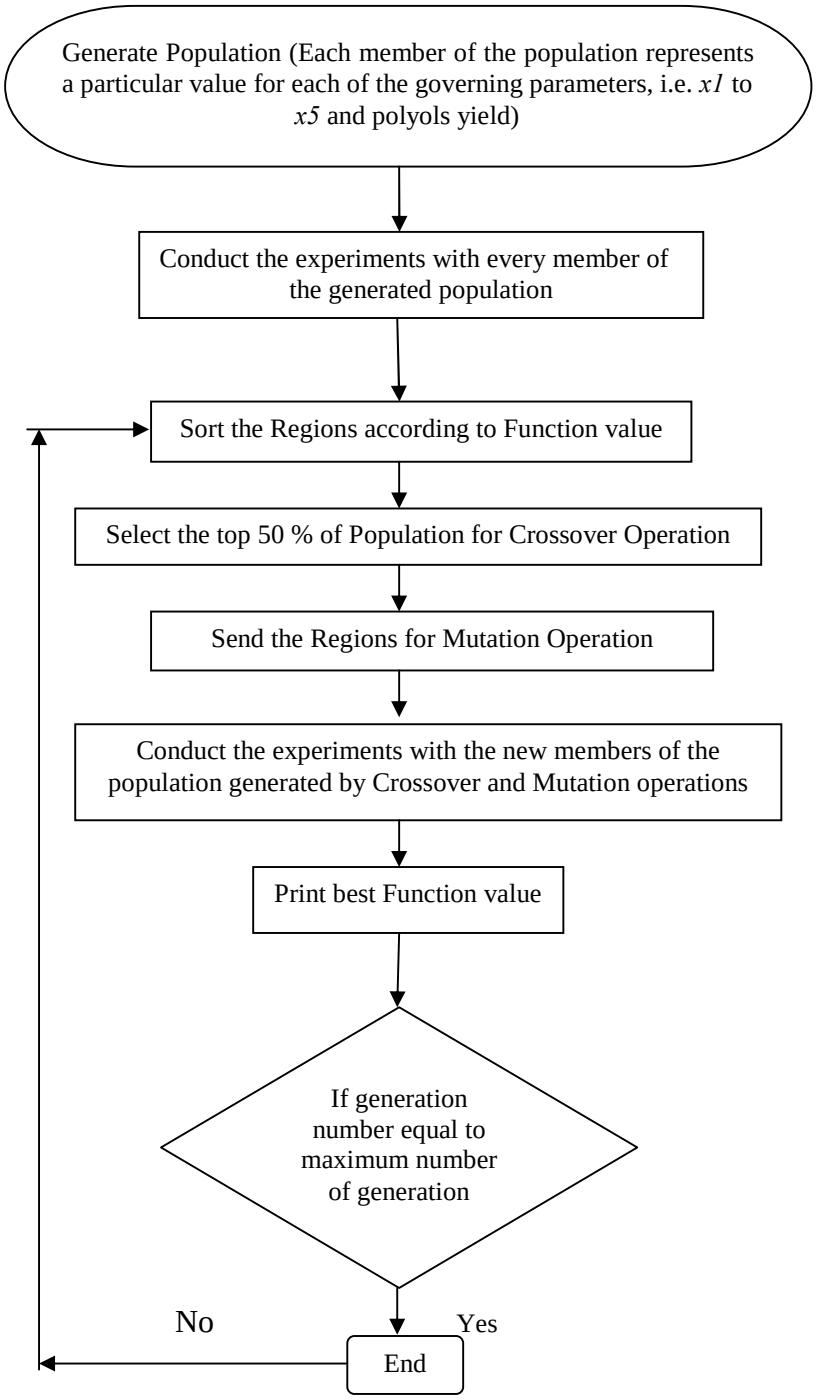


Fig. 4.1: Flow chart of real coded genetic algorithm.

The last three steps - selection, crossover, and mutation - are repeated for a fixed number of generations. These two operations are carried out repeatedly until the required number of generations is completed.

4.8.1 RESULTS AND DISCUSSION

As mentioned above, the optimization of media was carried out in parallel shake-flask experiments, using real coded genetic algorithm. To begin with, the genetic algorithm randomly generates 20 sets of governing parameters. With these 20 sets of values experiments were performed. Each experiment was performed in duplicate to check the reproducibility. It was found that the results in terms of polyols yield (based on sugars utilized) varied with a maximum of 7.5 %. The average yield values of the duplicate experiments are reported here. The 20 different experimental values of polyols yield were transferred to the algorithm. The algorithm used this information to combine the parameter values of the best 10 experiments and suggested six new sets of parameter values (with the help of cross over and mutation operations) for performing experiments in the next generation. This procedure was repeated for 15 generations. The progress of the algorithm in obtaining the maximum in polyols yield is shown in Fig. 4.2. It can be seen that algorithm moves steadily toward the maximum with progression of generations. After the twelfth generation, we observed that the maximum yield of polyols did not change. Maximum polyols yield on the basis of sucrose utilized was 76.43 % and final polyols yield on the basis of total initial sugars was 30.23 %. The best process variable concentrations to achieve this yield are given in Table No. 4.15. This optimum combination was obtained by doing a total of 104 experiments. The algorithm's parameters are given in Table No. 4.16.

Table 4.15: Optimized Medium Composition and Maximum Polyols Yield

Inoculum size (ml)	Sucrose (g/l)	Yeast extract (g/l)	Urea (g/l)	$MgSO_4.7H_2O$	Maximum polyols yield (based on sugar utilized) (%)
4.05	300.5	1.25	1.10	0.256	76.43

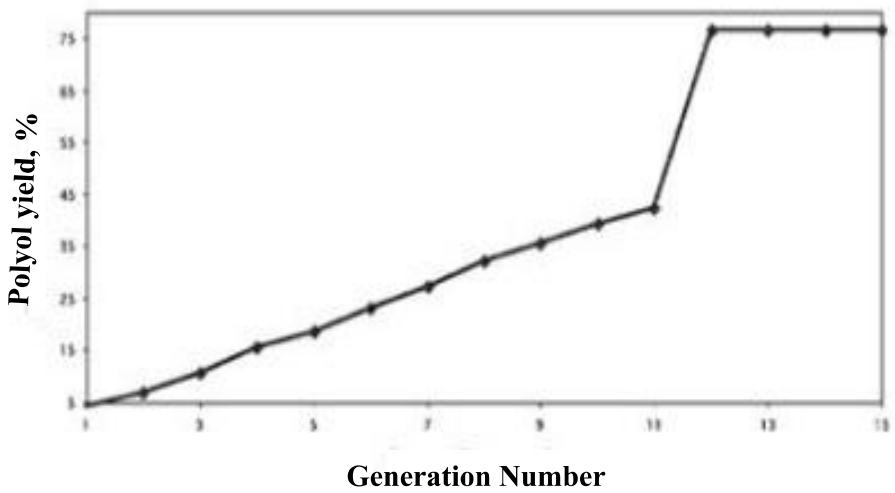


Fig. 4.2: Optimization of medium for polyols production with real coded genetic algorithm

Table 4.16: Genetic Algorithm Parameters

Population	Weaker regions removed per generation	Crossover possibility	Mutation possibility	Maximum no. of generations
20	6	0.8	0.1	15

Comparison of polyols production by One-variable-at-a-time approach vs Genetic algorithm approach optimized media.

To validate the results obtained as mentioned above, experiments were run simultaneously with one-variable-at-a-time optimized media and genetic algorithm optimized media. The results of the experiments are given in Table No. 4.17. It can be seen that the maximum polyols yield based on sugar utilized at 100 hrs was 70.02 % in case of genetic algorithm approach optimized media as compared to the yield of 39.73 % with one-variable-at-a-time approach optimized media. The final polyols yield at 216 hrs based on total initial sugars was also on higher side (30.16 %) in genetic algorithm approach optimized media as compared to one-variable-at-a-time approach (19.92 %) optimized media.

Therefore, in all subsequent experiments, genetic algorithm optimized media was used for process optimization and scale-up studies on 2.5 liter capacity in an automated fermenter.

Table 4.17: Comparison of polyols yields between GA optimized media and OV approach optimized media.

Particulars of medium	Time (hr)	Residual Sucrose (g/l)	Polyols (g/l)	Sucrose Utilized (g/l)	Polyols yield on the basis of sucrose utilized (p/su), %	Polyols yield on the basis of total initial sucrose (p/ts), %
GA	0	300.5	0.00	0.00	--	--
Optimized	96	235.0	45.86	65.50	70.02	15.26
Medium	216	85.0	65.00	215.50	30.16	21.67
Conventional	0	300.5	0.00	0.00	--	--
Approach	96	275.0	10.13	25.50	39.73	3.37
Optimized	216	15.0	59.85	285.50	20.96	19.92
Medium						

Effect of addition of invertase

As compared to our previous experience with glucose as carbon source, it was found that the final polyols concentration achieved with sucrose was always lower than that obtained with glucose and substantial amount of sucrose remained unutilized even after 216 hrs of fermentation.

We suspected that this reduced polyols formation with sucrose as substrate may be related to the invertase activity of *H .anomala*. It is reported in the literature that *H. anomala* has invertase activity and can metabolise sucrose (221). However, it seems that the invertase activity of this strain may not be strong enough to metabolize sucrose under the conditions of fermentation. Therefore, it was decided to use external invertase and evaluate its effect on polyols production by *H. anomala*.

Invertase enzyme was supplied by Biocon India Ltd. The invertase was added after inoculation at the rate of 0.5 g/l of fermentation medium. During this investigation, parallel shake flasks were also run using glucose and fructose as substrate, and the results in terms of final polyols concentration at 168 hrs are given in following Table No.4.18.

Table 4.18: Effect of addition of invertase and use of glucose and fructose in GA optimized medium.

Sr. No.	Particulars	Medium composition (g/l)				Polyols conc. at 168 hrs (g/l)
		Sucrose (S) Glucose (G) Fructose (F)	Yeast extract	Urea	$MgSO_4 \cdot 7H_2O$	
1.	GA optimized sucrose media	300 (S)	1.25	1.1	0.25	60.8
2.	GA optimized sucrose media plus invertase	300 (S)	1.25	1.1	0.25	73.0
3.	GA optimized fructose media	300 (F)	1.25	1.1	0.25	81.0
4.	GA optimized glucose media.	300 (G)	1.25	1.1	0.25	75.0

The results indicate that addition of external invertase has resulted in substantial improvement in final polyols production at 168 hr from 6.08 % to 7.30 %. This was probably because of inversion of sucrose outside the cell by the externally added invertase. The Table No.4.18 also indicates the effect of using glucose and fructose with GA optimized medium. With glucose, the final polyols concentration is almost equal to that with sucrose plus invertase. With fructose, the final polyols concentration was higher probably because of the high osmotic pressure exerted by fructose on yeast cell as compared to glucose and sucrose.

This experiment indicated that with sucrose as the substrate, there may be a need to add external invertase. This aspect need to be studied in the scale-up experiments using automated fermenter.

Comparison of polyols production by *H. anomala* and *P. farinosa*

Various species of osmophilic yeasts have been used by different workers. Salt tolerant strain of *Saccharomyces rouxii* has been reported to produce good yields of glycerol and related polyols (70). *Pichia farinosa* has been also used under alkaline conditions for glycerol production (77).

Under the conditions of fermentation optimized so far, we were interested to know the performance of our strain of *H. anomala* in comparison with other species reported in the literature. Shake flask experiments were conducted using *H. anomala* and *P. farinosa*. Both the cultures were propagated under similar conditions. Fermentations were also carried out under similar conditions and the results are given in following Table No.4.19.

It can be seen that *H. anomala* achieved higher polyols concentration at the end of 216 hrs of fermentation as compared to *P. farinosa* under the given conditions of propagation and fermentation. The gas chromatographic analysis of final product by *H. anomala* indicated that it contains glycerol and arabitol in an approximate proportion of 4:1.

Table 4.19: Comparisons of performance of *H. anomala* and *P. farinosa* in sucrose based media

Sr. No.	Particulars	Medium composition (g/l)				Polyols conc. at 216 hr. (g/l)
		Sucrose	Yeast extract	Urea	$MgSO_4 \cdot 7H_2O$	
1.	*H. anomala* with GA optimizes media and invertase	300	1.25	1.10	0.25	8.07
2.	*P. farinosa* with GA optimizes media and invertase	300	1.25	1.10	0.25	5.86

Based on the findings described above and the optimized conditions, *H. anomala* was used for further scale-up studies.

CHAPTER-5

SCALE-UP AND PROCESS OPTIMIZATION: BATCH AND FED-BATCH EXPERIMENTS AND MODELLING STUDIES

CHAPTER-5

SCALE-UP AND PROCESS OPTIMZATION: BATCH AND FED-BATCH EXPERIMENTS AND MODELLING STUDIES

5.1 INTRODUCTION

In aerobic production of polyols by osmophilic yeasts supply of oxygen is a critical factor and requires a careful adjustment. This aspect has been covered in our previous study (98) and briefly reported in Chapter-3.

Oxygen is sparingly soluble gas with maximum solubility of 9.2 mg/l in distilled water at 20°C. This solubility is strongly influenced by and inversely related to the temperature and solute concentration of the medium (222).

In laboratory shake flask experiments, aeration and agitation are accomplished by the rotary action of the shaker. In shake flasks, the oxygen absorption rate decreases as the shaker speed is decreased. Similarly, as one increases the liquid volume in the flask, the oxygen transfer interfacial area per unit volume of the solution as well as the degree of turbulence would decrease. As a consequence the volumetric adsorption rate would be affected adversely when the broth volume in the shake flask is increased (223). In shake flask experiments, optimum polyols concentration was achieved at a medium volume to flask volume ratio of 0.08, which probably supplied the limited but adequate oxygen for maximum polyols production. Above this critical ratio of medium volume to flask volume the oxygen supply in the medium becomes inadequate and thus adversely affects the polyols production.

Djelal, H. et al. (96) working on glycerol production by *H. anomala* have reported 40 % reduction of the initial dissolved oxygen concentration at 14 hrs after inoculation for the highest medium volume of 300 ml in an 500 ml Erlenmeyer shake flask studies. The reduction of the initial dissolved oxygen concentration was 50 % after 21 hrs of inoculation for lowest working volumes of 100 and 50 ml. Therefore, it is really difficult to understand the exact influence of oxygen on shake flask level.

Shake flask experiments also do not allow investigating the effect of controlled pH on cell growth and product formation. In many industrial fermentation processes it is necessary to monitor and control pH so as to maximize the product formation. It is also

difficult to run the shake flask experiments in fed-batch or continuous fermentation mode.

To overcome such limitations and as a part of scale-up studies, the shake flask experiments are always followed by fermenter scale studies. The laboratory scale fermenter can be automated to control or monitor various parameters such as pH, temperature, redox potential, dissolved oxygen, intermittent addition of nutrients and feed substrate etc. It is also possible to run an automated fermenter in batch, fed-batch or continuous fermentation mode. The automated fermenter also allows the study of rate of aeration and agitation which ultimately determines the oxygen transfer efficiency of the fermenter. Small laboratory-scale fermenters used in groups of two to three or more allow great flexibility in research for the development of fermentation processes. Thus, several experimental variables with adequate controls can be tested side by side in these small fermenters. In addition, the optimum fermentation conditions, as determined in these fermenters, often are applicable in further “scale-up” to fermentations in much larger fermentation tanks (224).

This chapter deals with scale-up studies of the results obtained in shape-flask experiments on a 2.5 liter CHEMAP make laboratory fermenter. Following important factors concerned with development of process for polyols production were investigated in fermenter scale experiments.

1. Optimization of pH and effect of invertase addition.
2. Effect of temperature.
3. Effect of use of fructose as carbon source.
4. Effect of inoculum size without pO_2 control.
5. Effect of controlling oxygen percent saturation (pO_2).
6. Solubility of oxygen in fermentation medium.
7. Effect of inoculum size with pO_2 control.
8. Effect of agitation with pO_2 control.
9. Fed-batch fermentation.
10. Development of kinetic model for optimum polyols production.

5.2 MAINTENANCE OF CULTURE AND PREPARATION OF INOCULUM

As described in Chapter-4, *H. anomala* culture was maintained on MGYP agar slants.

A 500 ml Erlenmeyer flask containing 100 ml of inoculum preparation medium, described in Chapter-4 was inoculated with loopful of culture from a fresh slant. After 24 hrs of growth on the incubator shaker at 30 ± 2°C and 180 rpm, the flask was transferred to ten freshly sterilized and cooled flasks having the same medium under aseptic conditions. These ten flasks in turn were incubated on a rotary shaker at 180 rpm at 30 ± 2°C for 24 hrs. The liquid culture volume corresponding to 10 % of fermenter medium volume or at higher percentage level as required was centrifuged at 2225 g at 4°C for 20 minutes under aseptic conditions. The residual cell mass was resuspended in the fermentation medium and used to inoculate the main fermenter. Aseptic conditions were maintained during all operations mentioned above so as to avoid contamination.

5.3 EXPERIMENTAL

The fermentations were carried out in a CHEMAP make 7.5 liter capacity glass fermenter at 2.5 liter of fermentation volume. The CHEMAP fermenter was equipped with sterilizable pH and pO_2 electrodes (Ingold make) and the temperature was maintained at 30°C by circulating chilled water through hollow baffles. The glass vessel was fitted with bottom entering stirrer with double mechanical seal and having two numbers of four bladed turbine impellers. The stirrer speed was controlled by a variable speed controller. The air was supplied by an oil free reciprocating compressor after passing through a sterilizable ceramic filter. The rotameter incorporated into the fermenter assembly measured the air flow rates, which was also controlled by an air control valve. The antifoam controller controlled the foam level but its use was limited since the volume of the broth in the vessel was small compared to the total volume of the fermenter.

Polymethyl siloxane based antifoam emulsion (Sigma, A5758) was used for controlling foam level.

The details of the fermenter vessel dimensions and operating parameters were as follows.

Total working capacity	=	7.5 liters
Actual working capacity used	=	2.5 liters
Length of the glass vessel	=	312 mm
Diameter of the glass vessel	=	200 mm
Diameter of the impeller	=	75 mm
Height of the first impeller from the bottom	=	37 mm
Height of the second impeller from the bottom	=	170 mm
Sparger	=	Single orifice type
Height of the fermentation medium (2.5 liter)	=	94 mm

The losses due to evaporation were kept at minimum by passing the exhaust gases through a reflux condenser.

Fermentation medium composition

The composition of nutrients in all the fermentations carried out on 2.5 liter was as optimized by the Genetic Algorithm (GA) approach and described in Chapter-4.

As explained previously our attempt was to use sucrose exclusively as the carbon source. The GA optimized medium composition is given in the following Table 5.3.1.

The sucrose used was of commercial nature (plantation white sugar) readily available in the local market. The sucrose solution was sterilized in situ in the glass fermenter along with the pH, pO_2 electrodes and antifoam sensor.

Table 5.3.1: Fermentation medium composition

Sr. No.	Parameters	Concentration g/l
1.	Sucrose	300.5
2.	Yeast extract	1.250
3.	Urea	1.100
4.	$MgSO_4 \cdot 7H_2O$	0.256

The yeast extract solution was sterilized independently and the solution of urea and magnesium sulfate was sterilized separately in an autoclave at 15 psi for 15 minutes. These sterilized solutions were then pumped aseptically in the fermenter to make up the

final fermenter volume to 2.5 liters. The pH of the medium after sterilization was adjusted to 6.0 with 0.25 N NaOH.

Just before inoculation, the fermentation medium was supplied with high aeration rates and stirring speed so as to bring the % saturation of oxygen to about 100 % level. Suitable quantity of freshly prepared fermentation medium was removed aseptically and used to resuspend the wet cell mass prepared by centrifugation. This solution was then again pumped back aseptically to the fermenter to initiate the fermentation.

pH control

The pH of fermentation was controlled by an automatic pH controller system of the CHEMAP fermenter. 2.5 N Na_2CO_3 was used to control the pH. Na_2CO_3 solution was sterilized by Seitz filtration.

It was observed in our previous studies that addition of Na_2CO_3 in the initial phase of fermentation does not help to improve the yield of polyols. On fermenter scale studies, a policy of controlling the pH, only after completion of 18 hrs of fermentation was employed. At the beginning of fermentation, as described above, the pH is adjusted to 6.0, which after 18 hrs falls to a value of around 2.9 to 3.0. At this point pH is brought to the desired level and then maintained throughout with automatic addition of Na_2CO_3.

Due to addition of Na_2CO_3, there is always an associated increase in the volume of the fermentation broth. It is not possible to measure the broth volume during the fermentation experiment because of entrapment of air bubbles and foam formation. The final broth volume is measured only after the end of fermentation. The results of these experiments are, therefore, calculated after correcting for the increase in the volume of the broth to 2.5 liters.

Analytical methods

After inoculation, at regular intervals, samples were withdrawn aseptically. Part of the sample (1 ml) was diluted suitably with distilled water and used for estimation of viable cell count by methylene blue staining as described in Chapter-2. The results are expressed as cells/ml × 10^8. Part of the sample was also used for estimation of dry cell weight (DCW) and optical density (OD) of fermentation broth. Most of our results are expressed on the basis of viable cell count. However, data on DCW and OD has been also used in few cases.

Other part of the sample was centrifuged at 2225 g for 20 minutes. The supernatant liquid was passed through a 0.22 μm Millipore membrane filter and used for

estimation of polyols and total reducing sugars (TRS) after inversion with HCl as described in detail in Chapter-2. Total reducing sugars is a sum of glucose, fructose and sucrose present in fermentation broth samples.

Final sample at the end of fermentation was centrifuged, filtered and used to prepare the silyl derivative as explained in Chapter-2. The derivatized sample was injected in GC to estimate qualitatively the ratio of glycerol to arabitol. As explained in the previous part, we did not use GC method for quantitative estimation because of reproducibility problems.

5.4 OPTIMIZATION OF pH AND EFFECT OF INVERTASE ADDITION

In the present investigation, the effect of pH was studied at uncontrolled pH, pH 4.0, pH 5.0, pH 6.0, pH 7.0 and pH 8.0.

Experiments were also conducted to study the effect of addition of an external invertase (β-D-fructofuransidase EC 3.2.1.26). A liquid invertase (Sucrose L 300) sample was supplied by Biocon India Ltd. with an enzyme activity of 15,000 summer units/ml (sp. gravity = 1.4). It was informed by Biocon India Ltd. that the liquid enzyme has good activity even in the near alkaline pH range.

The liquid invertase was used at a dose of 0.5 g/l on the basis of fermentation broth prepared or approximately 0.167 % of sucrose used. The effect of invertase addition was investigated at pH 8.0 and pH 8.5. The enzyme was added under aseptic conditions at the start of fermentation after inoculation.

The investigational data of individual experiments are presented in the form of the time course profiles of a) Total polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig. 5.4.1 to 5.4.8.

RESULTS AND DISCUSSION

As mentioned earlier, we have monitored the cell growth of *H. anomala* by taking viable cell count as well as by measuring dry cell weight. Dry cell weight is more commonly used method by researchers in this area. The reason for taking viable cell count was that *H. anomala* cells can be easily stained with methylene blue and examined under microscope in suspension form. The yeast cell count measurement procedure is more rapid than dry cell weight measurement. The dry cell weight measurement can

sometimes overestimate the cell mass particularly when sugar or product concentrations are on higher side. We have expressed the results in terms of % polyols yield based on sugar utilized (p/su) and % overall polyols yield based on initial total sugars (p/ts). We have also calculated the yield of number of cells of *H. anomala* on the basis of cell count measured. The % cell mass (count) yield is expressed as x × 10^8 cells/ml per 100 g of sugar utilized (x_{cc}/su) and % overall cell mass (count) yield is expressed as x × 10^8 cells/ml per 100 g of initial total sugars (x_{cc}/ts). Productivity of polyols formation is calculated as grams of polyols formed per liter per hour (g/l/hr). Productivity of cell mass (or cell count) is calculated and expressed as x × 10^8 cells/ml/hr. In all calculations and results related to cell count, x stands for number of cells or cell count. Utilization rate of sugar (sucrose) is also calculated and expressed in g/l/hr.

Uncontrolled pH: After rapid growth phase for about 50 hrs, the cell count of *H. anomala* remained almost constant. The plot of log of number of viable cells versus time gives a typical batch growth curve with initial exponential growth phase followed by stationary phase (Fig. 5.4.1). Sucrose utilization was also rapid and parallel to the growth phase of cells and continued up to about 90 hrs. Sucrose consumption was almost nil from 90 hrs onwards. It was observed that when pH was not controlled, it dropped to 2.86 in first 18 hrs and then gradually reduced to 2.60 in about 77 hrs and remained constant thereafter. Polyols concentration slowly increased up to about 90 hrs. A shift in metabolism occurred after 90 hrs when the organism stopped utilizing sucrose and started consuming the polyols produced earlier. This may have occurred because of the adverse pH conditions in the fermenter. Similar observation is also reported by Liu, Yongqiang et al. (225) working with *Candida krusei* for glycerol production. Therefore, the polyols concentration after 90 hrs went on decreasing from 29.5 g/l to almost 2.0 g/l at the end of 185 hrs. Maximum overall polyols yield (p/ts) and maximum cell mass yield (x_{cc}/ts) based on the initial total sugars were 9.97 % (at 89 hrs) and 3.42 x 10^8 cells/ml per 100 g of sucrose consumed (at 184 hr), respectively. This indicates that slow cell growth occurred during the later part of fermentation using the polyols as carbon source. A substantial amount of sucrose remained unutilized even after 185 hrs of fermentation. The oxygen % saturation of the medium dropped from initial value for 98.9 % to about 42 % in first 30 hrs because of rapid growth of yeast cells. After 30 hrs the oxygen % saturation value (pO_2) went on increasing gradually indicating decrease in metabolic activity of the organism. The polyols productivity increased up to

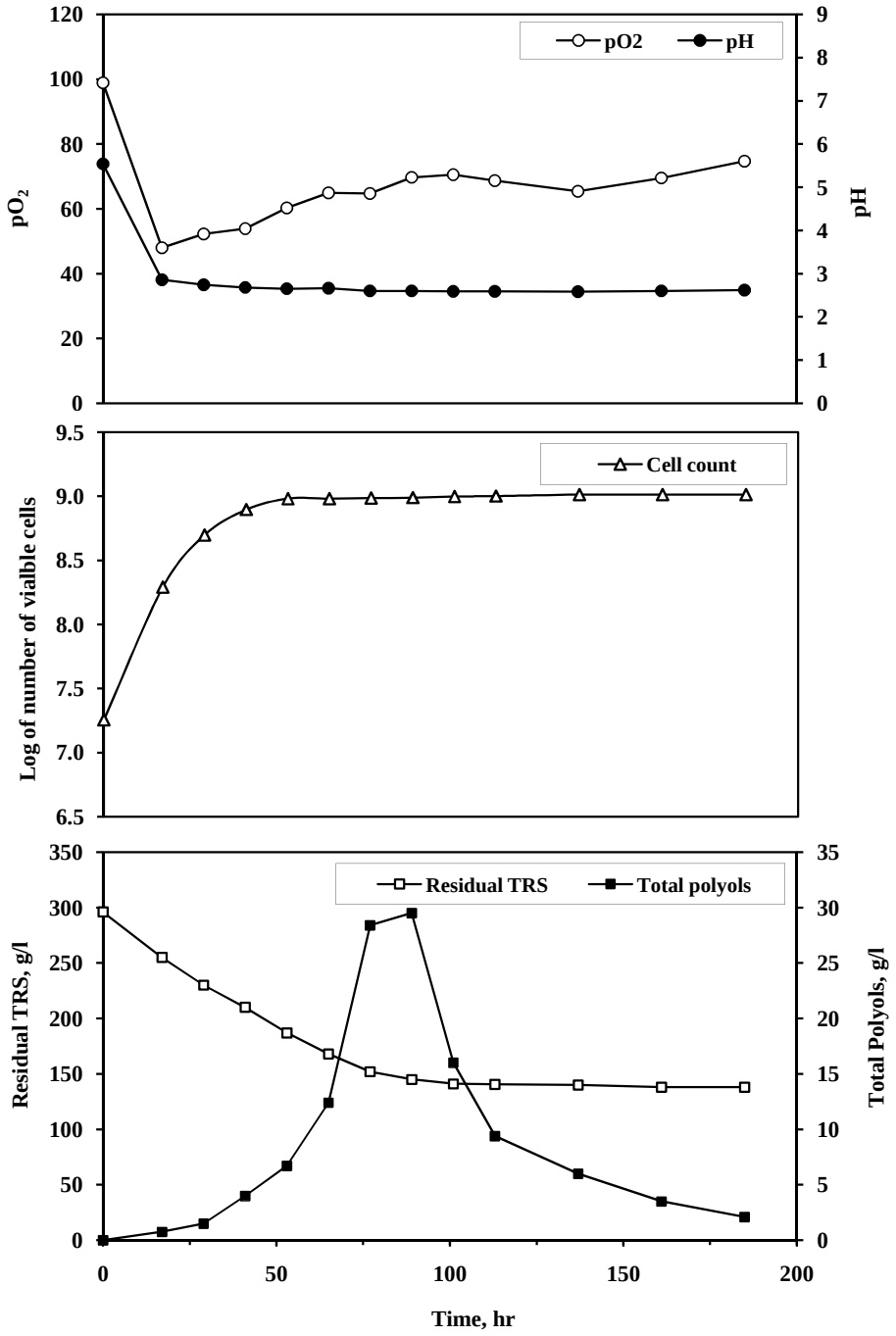


Fig. 5.4.1: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at uncontrolled pH

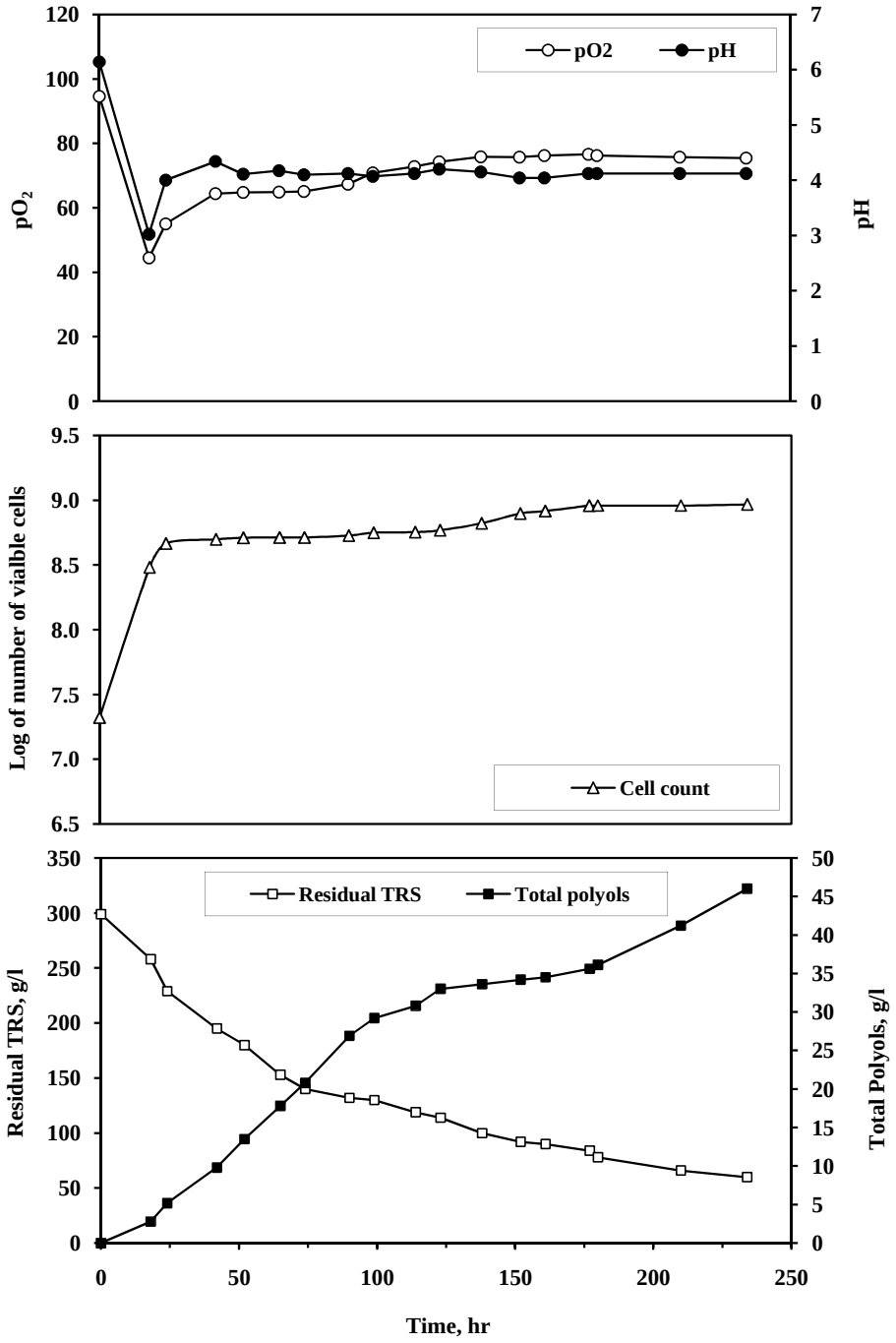


Fig. 5.4.2: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 4.0

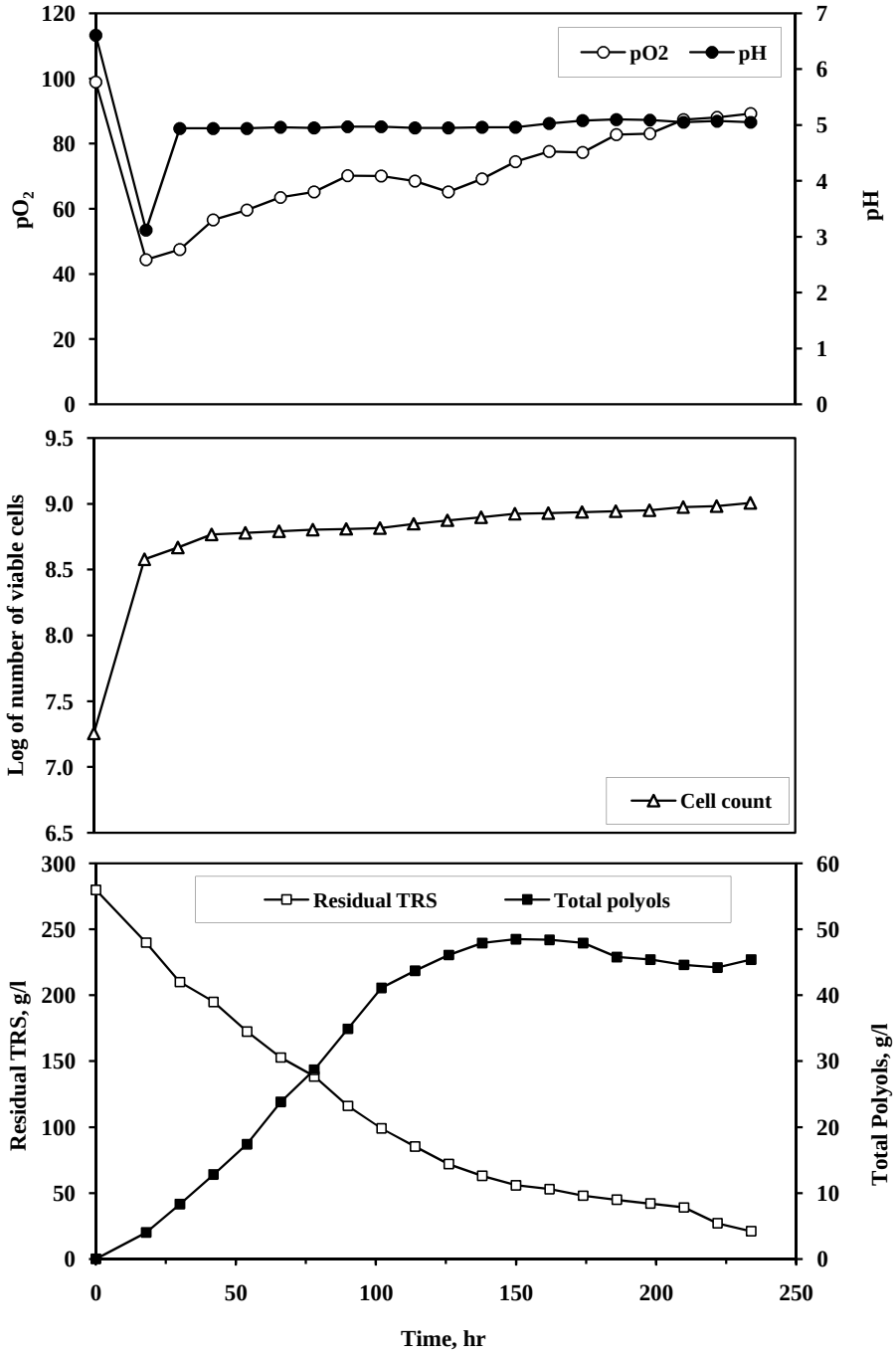


Fig. 5.4.3: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 5.0

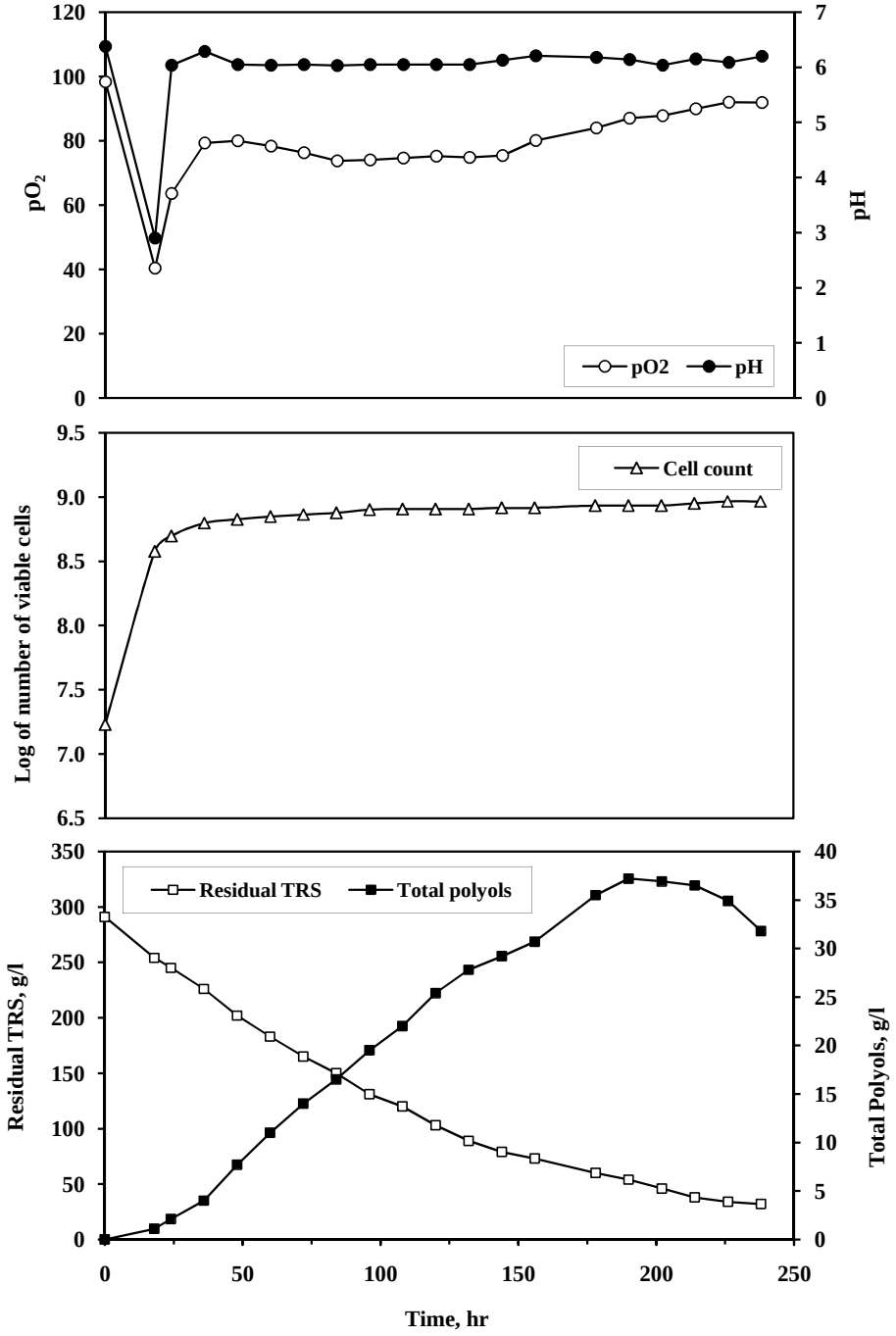


Fig. 5.4.4: Time course profile of a)Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 6.0

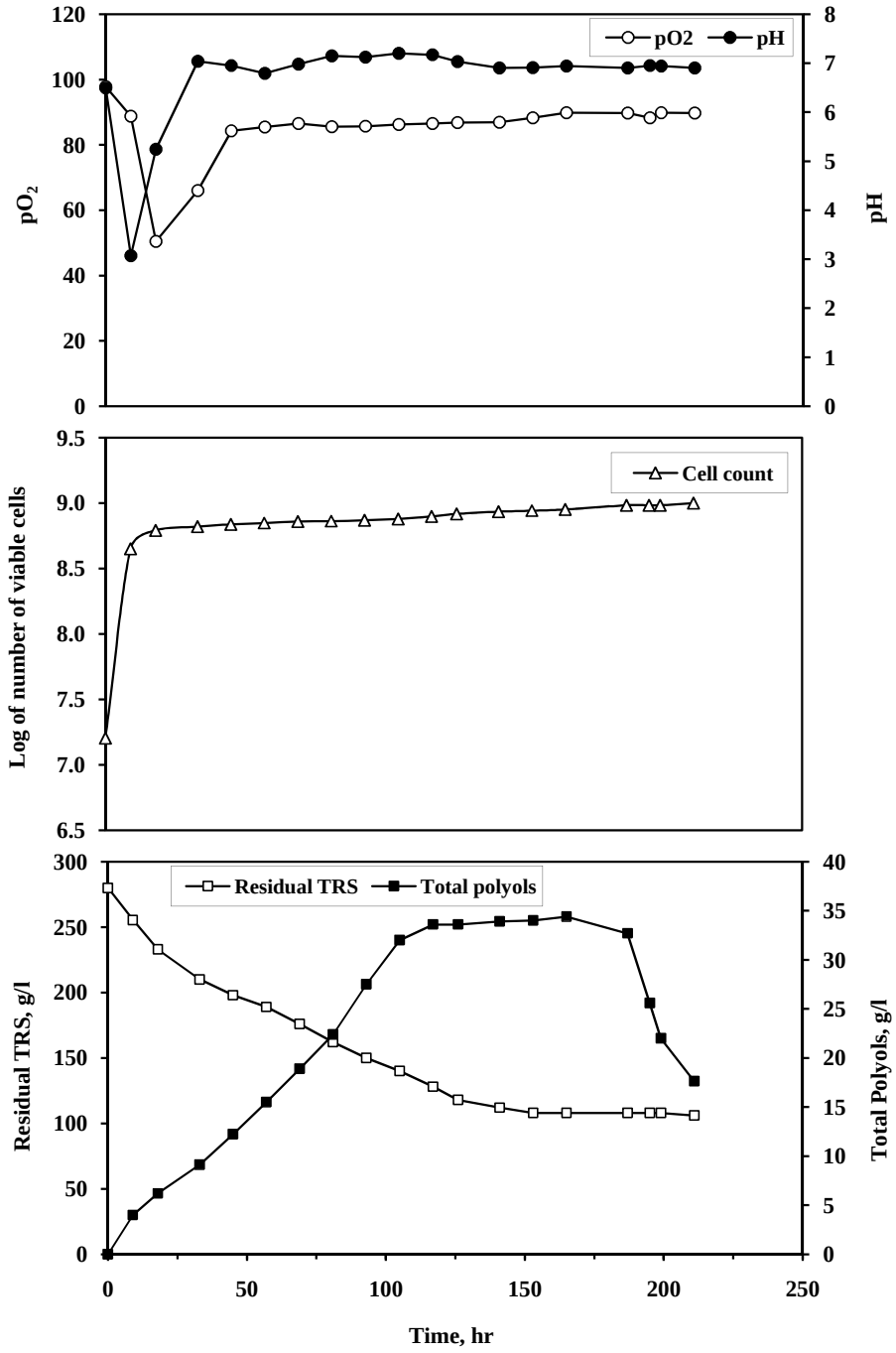


Fig. 5.4.5: Time course profile of a) Total Polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 7.0

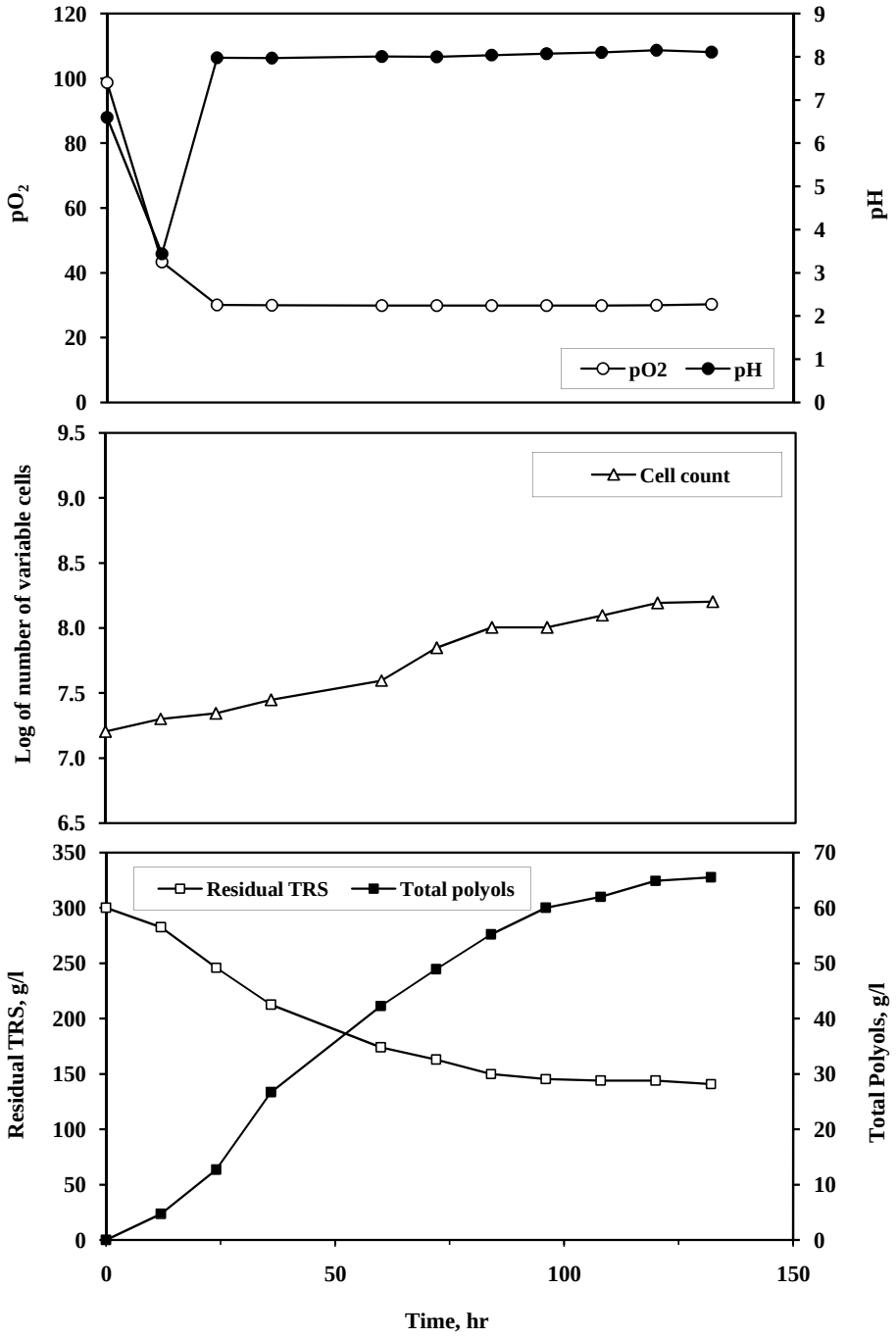


Fig. 5.4.6: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 8.0

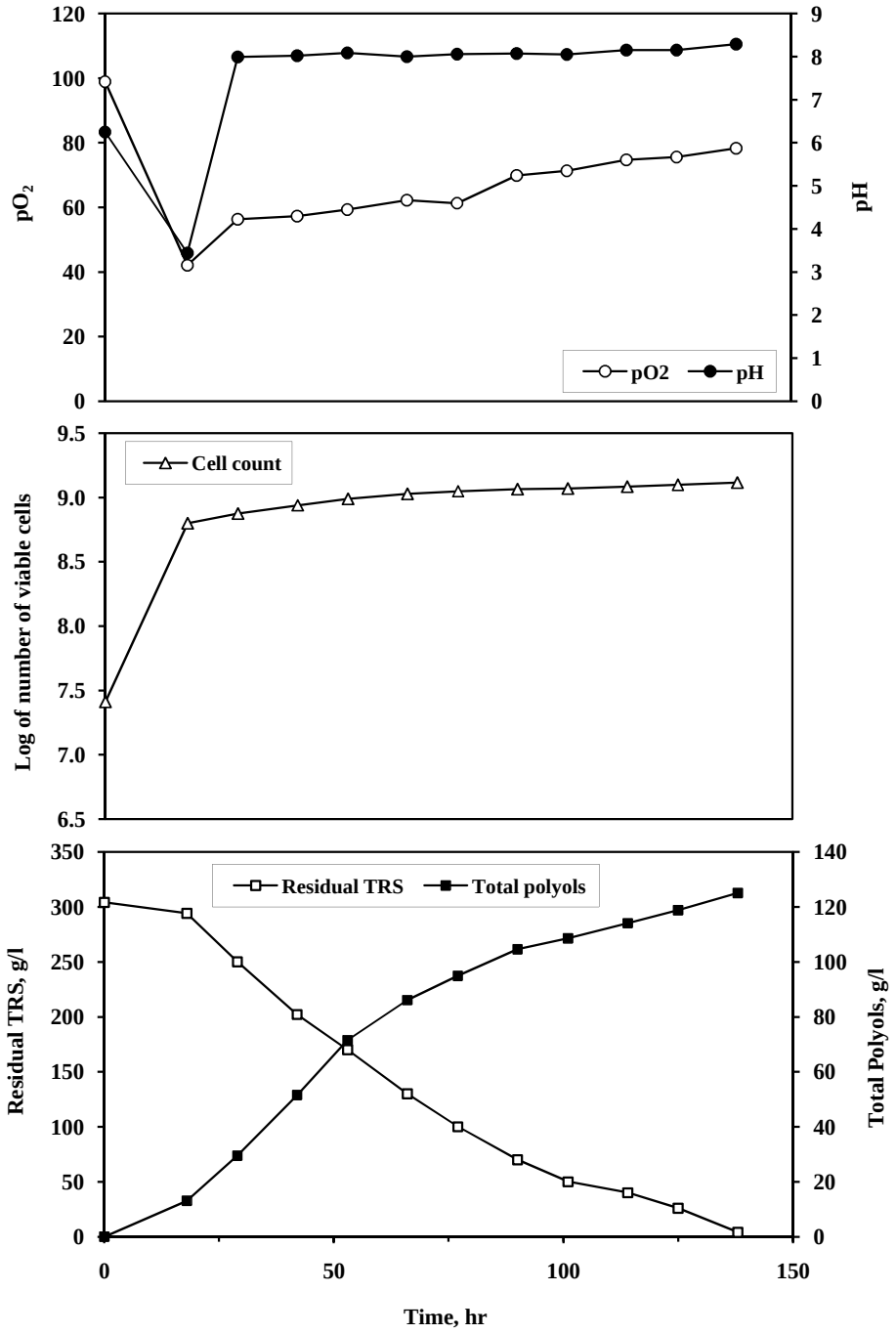


Fig. 5.4.7: Time course profile of a) Total polyols, b)Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 8.0+ Invertase

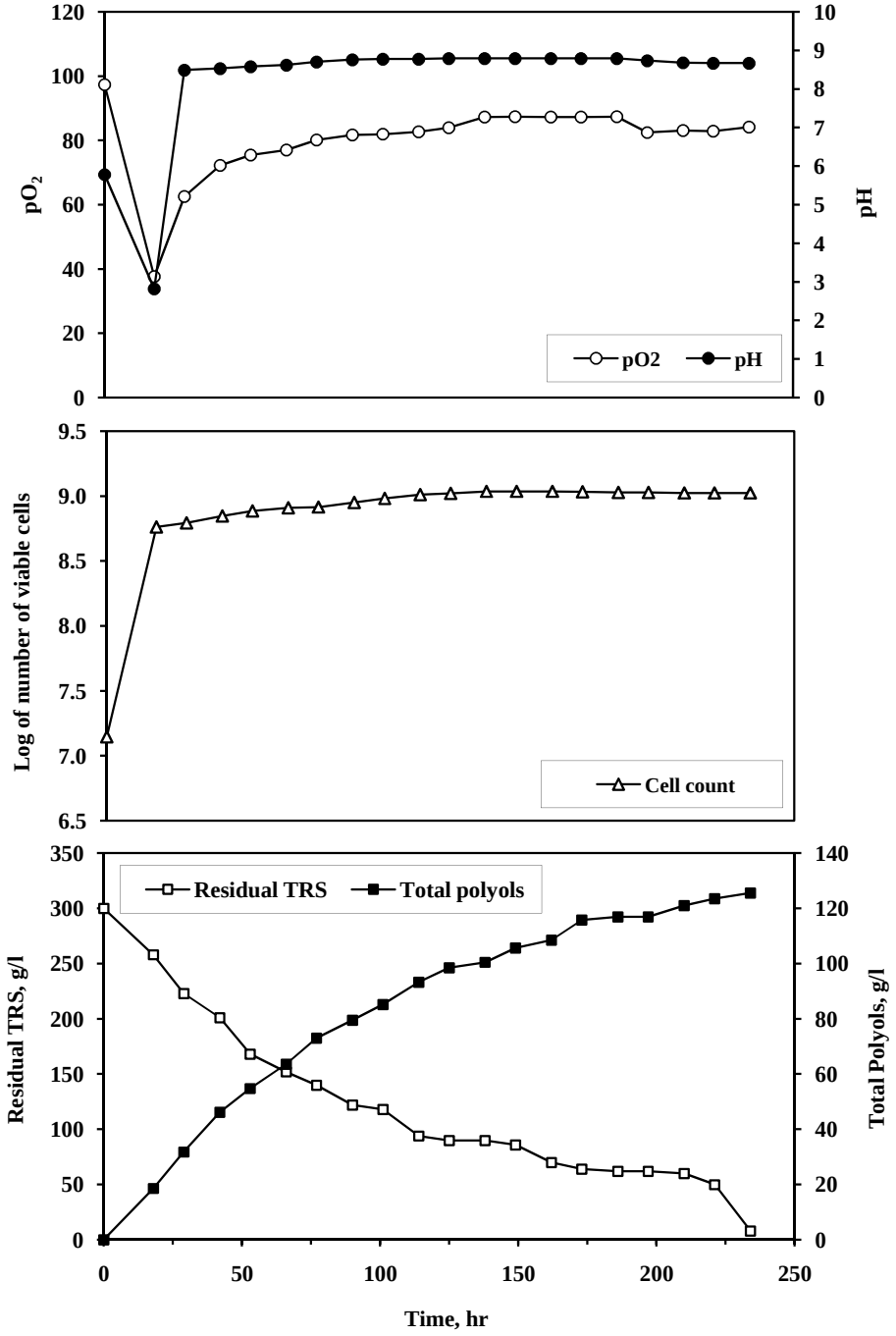


Fig. 5.4.8: Time course profile of a) Total Polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 8.5+ Invertase

0.369 g/l/hr (77 hrs) and then gradually dropped to 0.011 g/l/hr.

Controlled pH: With all pH controlled experiments, the initial pH before inoculation was adjusted to 6.0 with sterile 0.25 N NaOH. The pH normally dropped to 2.85 to 2.95 in the first 18 hrs, which covers the early period of rapid growth. The strategy of adjusting the pH to the required value only after 18 hrs of growth was adopted as explained earlier.

Some yeast species give increased polyols yield in alkaline conditions. Eoff (37) has suggested mode and amount of Na_2CO_3 to be added during glycerol production by non-osmophilic yeast. Vijaikishore and Karanth (77) have also used alkaline conditions for improving the polyols yield by osmophilic yeast *Pichia farinosa*. They have found significant increase in glycerol yield in the alkaline range giving the highest yield at pH 8.2.

pH 4.0 : It can be seen from Fig 5.4.2 that pH dropped from 6.14 to about 3.02 in first 18 hrs. During this first phase, the cell growth was exponential. However, after adjusting the pH to 4.0 the rate of cell growth declined by a considerable amount. Up to about 180 hrs, the cell growth remained slow but steady. Sucrose utilization rate was considerably fast till 74 hrs and declined thereafter. Even after 234 hrs of fermentation, about 6.6 % sugar remained unutilized in the broth. Similarly, polyols productivity increased up to about 99 hrs and then declined thereafter. Final polyols yield based on total sugars (p/ts) reached to 15.38 % and yield of cell mass (x_{cc}/ts) based on total sugars reached to a value of 3.03 x 10^8 cells/ml per 100 g of sugar utilized. Oxygen percentage saturation value (pO_2), which was not controlled, dropped from about 95 % to 44 % in the first 18 hrs because of rapid cell growth and then gradually went on increasing due to the slower rate of cell growth in the later phase.

pH 5.0 : Sahoo, D. K. and Agarwal, G. P. (76) working with an osmophilic yeast *Candida magnolia* have concluded that a pH in the range of 3.5 – 5.0 did not have a significant influence on glycerol production. Djelal, H. et al. (96) and Liu, Youggiang et al. (105) has also used starting pH of 4.5 to 5.5 for glycerol production with osmophilic yeasts.

Similar trends as explained for controlled pH 4.0 were obtained with pH 5.0 (Fig. 5.4.3). The cell growth was rapid in the first 18 hrs before the pH was brought and controlled at 5.0, it then slowed down up to about 60 hrs and further declined in the later phase. Sugar utilization rate was considerably fast in the initial phase but slowly declined

in the later phase. Even after 234 hrs of fermentation, about 2.1 % TRS remained un-utilized and final sugar utilization rate came down to 1.107 g/l/hr. Polyols productivity increased up to about 100 hrs and then started decreasing. The overall polyols yield based on total sugars (p/ts) at the end of 234 hrs was 16.22 % whereas the overall cell mass yield (x_{cc}/ts) was 3.55 x 10^8 cells/ml per 100 grams of sugar utilized.

pH 6.0: Similar trends of rapid initial growth followed by reduced growth rate were observed (Fig. 5.4.4). Sugar utilization rate declined slowly and even after 238 hrs of fermentation about 3.2 % sugar remained un-utilized. From about 200 hrs, the total polyols concentration also declined indicating the possibility of utilization of polyols as substrate by the yeast in this phase. Oxygen percentage saturation value (pO_2) gradually increased and remained in between 80 to 90 % in the later phase indicating very slow uptake of oxygen by the organism. Overall polyols yield (p/ts = 10.92 %) as well as cell mass yield (x_{cc}/ts = 3.13 %) also reduced indicating that pH at 6.0 is not supporting to polyols production.

pH 7.0: Similar initial trends (Fig. 5.4.5) but sugar utilization almost stopped after 153 hrs of fermentation. In fact, after 153 hrs, the organism shifted over to polyols as carbon source indicated by decrease in polyols concentration. Even after 211 hrs of fermentation, about 10.6 % TRS remained un-utilized and final sugar utilization rate came down to 0.825 g/l/hr. This resulted in reduction of overall polyols yield based on total initial sugars (p/ts) to 6.30 % again indicating that pH 7.0 is not at all supporting polyols formation.

pH 8.0: The cell growth is reduced drastically resulting in final cell mass yield of 0.48 x 10^8 cells/ml per 100 grams of sucrose consumed (Fig. 5.4.6). It is not clear why the initial rapid growth was not seen in this case. After about 108 hrs of fermentation, sugar utilization almost stopped. However, the polyols yield based on sugar utilized (p/su) increased to 41.19 % indicating that moderate alkaline conditions favour polyols production. This probably results from fixing of acetaldehyde by Na_2CO_3, thus eliminating the ethanol formation and diverting the excess NADH towards polyols formation. Though, substantial amount of sugar remained unutilized (14.1 %), the overall yield of polyols based on total sugars (p/ts) increased to 21.83 % with final polyols concentration reaching to a value of 6.55 %.

Between pH 6.0 to pH 8.0, sucrose was never utilized completely and in the later phase, the *H. anomala* shifted its metabolism towards consuming the polyols produced in

the earlier phase. This was contrary to our observations with glucose as substrate where the organism shifted over to polyols consumption only after all the sugar was utilized.

As explained in Chapter-4, we suspected that such reduced sucrose utilization rates between pH 6.0 to pH 8.0 as compared to utilization of glucose by the same organism may be due to weak invertase activity of *H. anomala* at these pH values. It is generally known that in yeast, invertase action takes place outside the cell resulting in to the formation of glucose and fructose, which are then transported inside the yeast cell for utilization in the Embden-Mayerhof pathway. Yeast cannot transport sucrose inside the cell.

pH 8.0 + invertase: To verify our hypothesis, we added external invertase (Sucrose L 300 from Biocon India Ltd.) at a dose of 0.5 g/l or 1.01 ml for 2.5 liter of fermentation broth. Our aim was to hydrolyse sucrose outside the cell and improve the sucrose utilization rate by increasing the hexose uptake rate by the yeast. It can be seen from Fig. 5.4.7 that sucrose utilization as well as the yeast growth rate improved substantially. Sugar was almost completely consumed in 138 hrs. Polyols productivity improved to 0.904 g/l/hr and overall polyols yield (p/ts) increased to 41.13 %. The yield of cell mass (x_{cc}/ts) also improved to 4.19 x 10^8 cells/ml per 100 grams of sugar utilized. The sugar utilization rate also improved and in 138 hrs almost all sugar was consumed at a rate of 2.174 g/l/hr. This indicates that externally added invertase was able to hydrolyse sucrose outside the cell and the transported hexoses were efficiently converted to polyols inside the cell under alkaline conditions.

pH 8.5 + invertase: Similar trends as at pH 8.0 with invertase were obtained at pH 8.5 with invertase (Fig. 5.4.8). However, complete utilization of sucrose required longer time (234 hrs) indicating that cell growth rate was reduced under these conditions. Though the overall polyols yield (p/ts) was almost the same (41.86 % at 234 hrs) as that of pH 8.0 (41.13 % at 138 hrs), the productivity of polyols and cell mass as well as sugar utilization rate were substantially reduced, which indicates the reduced metabolic activity of *H. anomala* at pH 8.5.

Above observations indicate that pH 8.0 with externally added invertase provides suitable conditions for the yeast to produce maximum polyols.

Fig. 5.4.9 and Fig. 5.4.10 illustrate the relationship between a) Overall polyols yield, b) Cell mass productivity, c) Polyols productivity, and d) Sugar utilization rate and time at different pH values and with addition of invertase. It indicates that overall polyols

yield (p/ts) is minimum for uncontrolled pH, improves slightly for pH 4.0 and pH 5.0, drops again at pH 6.0 and pH 7.0 and improves again at pH 8.0. With addition of invertase, the overall polyols yield improves substantially at pH 8.0 and pH 8.5. Similar trends are also observed for polyols productivity for different pH. Maximum overall yield and polyols productivity is achieved at pH 8.0 with addition of external invertase. Maximum sugar utilization rate is also achieved at pH 8.0 with invertase addition. Cell mass yield was very low at pH 8.0 as compared to other pH values. The reason for this low cell mass yield is not clearly understood.

From process development point of view, overall polyols yield based on total sugars is an important criterion. Overall yield and the duration of fermentation at different pH values and with addition of invertase are compared in Fig. 5.4.11. Overall polyols yield was lowest at uncontrolled pH, maximum at pH 5.0 in the acidic range, reduced at pH 6.0 and 7.0 and increased significantly at pH 8.0. Maximum polyols yield was obtained at pH 8.0 with externally added invertase. One possible explanation for this improvement is fixing of acetaldehyde in the glycolytic pathway under alkaline conditions, thus diverting the sugar and NADH towards glycerol formation (62, 7). Similar results have been reported with glucose (226, 98) with complete utilization of glucose within 110 hrs and maximum polyols yield of about 50 % (based on total sugars).

However, with sucrose as substrate, *H. anomala* is unable to utilize the sugar completely and substantial amount of sucrose remained unfermented. At pH 8.0 and without invertase addition, the overall polyols yield based on total sugars (p/ts) and yield based on sugar utilized (p/su) was 21.83 % and 41.19 %, respectively. This indicates that pH 8.0 is favourable for polyols synthesis but the endogenous invertase activity of *H. anomala* is weak at this pH, which resulted in incomplete utilization of sucrose. With addition of external invertase, sucrose was consumed much more rapidly i.e. within 138 hrs. At pH 8.5 and in the presence of invertase, the polyols yield based on sucrose utilized was almost the same, but the time required for complete sugar utilization was significantly longer (about 234 hrs). This indicated that pH 8.0 with external invertase is the optimum condition for polyols production by *H. anomala* with sucrose as substrate.

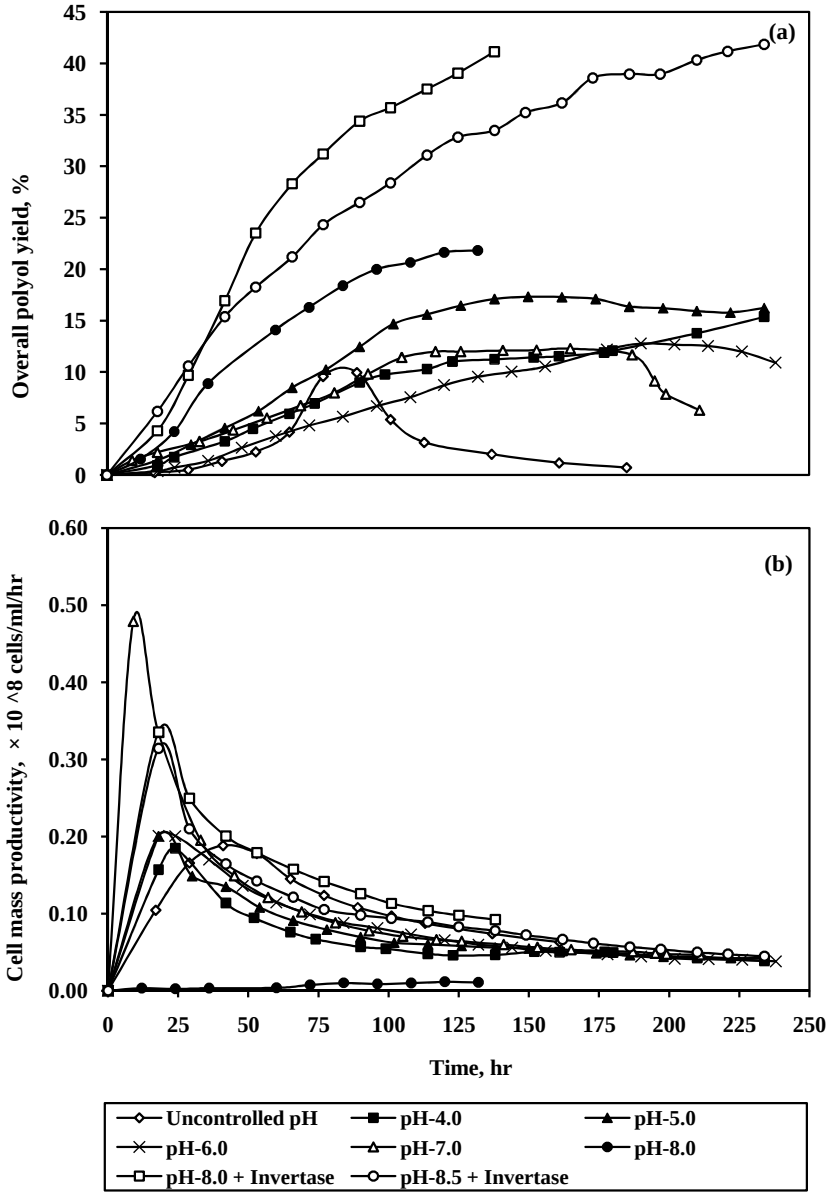


Fig. 5.4.9:Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for different pH with invertase addition

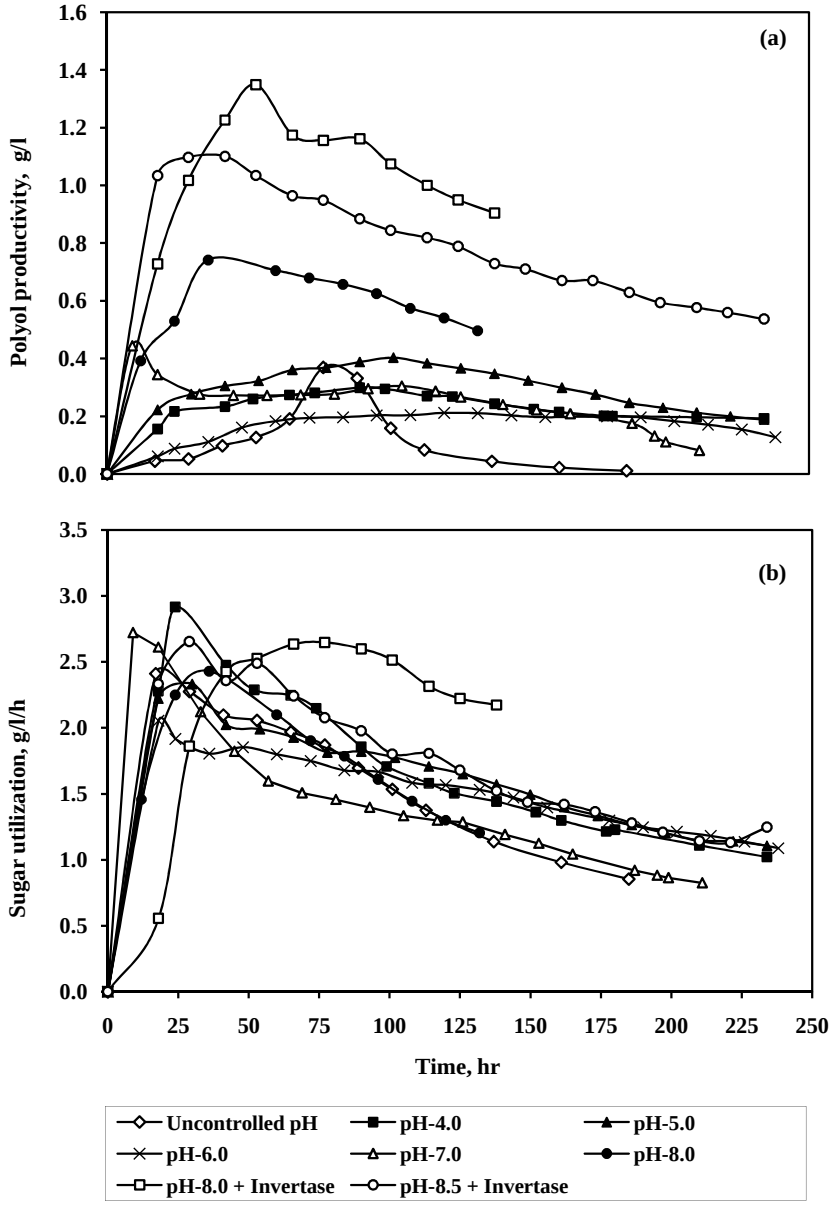


Fig. 5.4.10 : Relationship between a) Polyol productivity and Time & b) Sugar utilization rate and Time for different pH with invertase addition

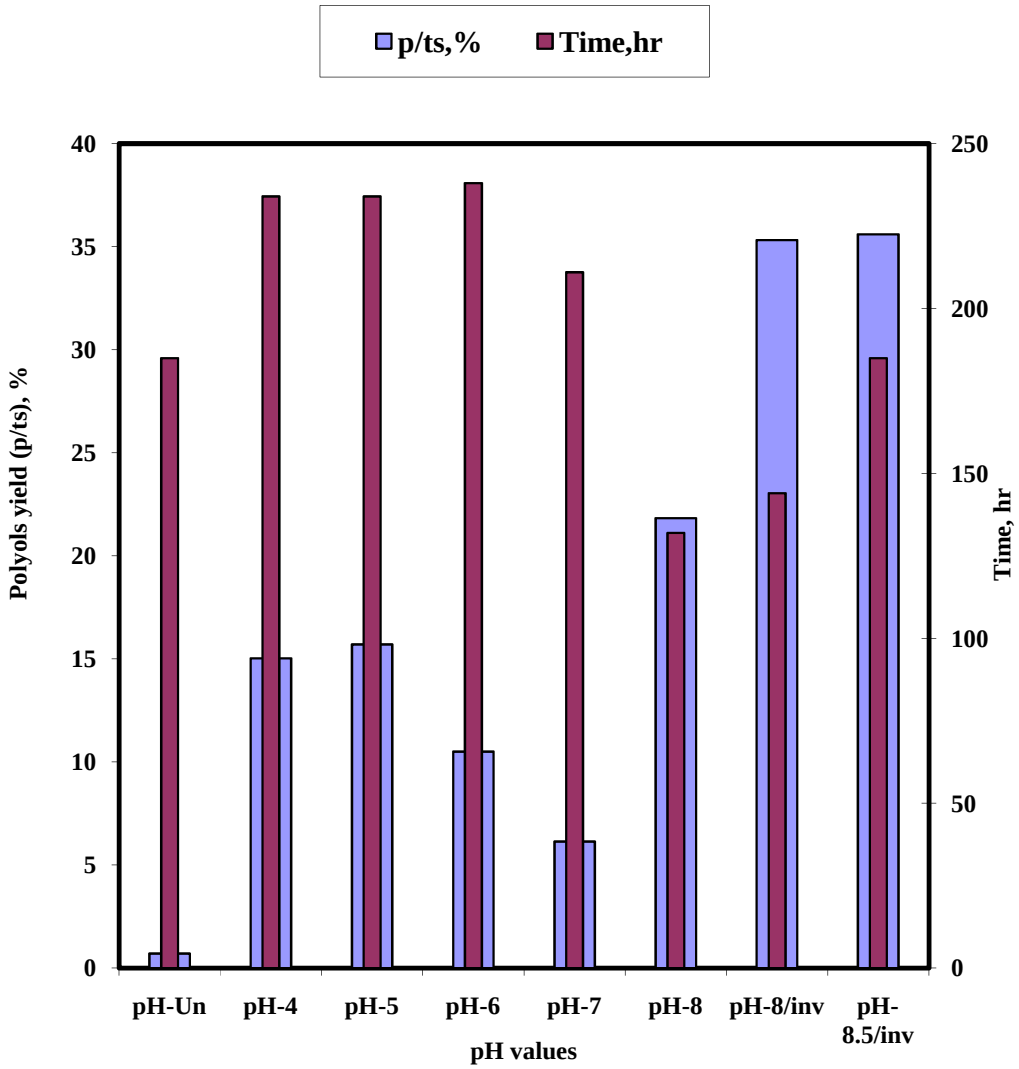


Fig.: 5.4.11 Effect of pH and invertase addition on final polyols yield based on total initial sugars

5.5 EFFECT OF TEMPERATURE

The optimum temperature for polyols production by osmophilic yeasts is reported to be in between 30°C to 35°C. With *Torulopsis magnolia* (71), increasing the temperature from 30°C to 35°C gave a better rate of glucose consumption with a slightly increased yield of glycerol. For *Endomycopsis chodatti*, the optimum temperature for glycerol production was in between 30°C to 35°C, yields were somewhat lower at 25°C and both yields and cell growth greatly reduced at 40°C.

Chinese researchers working at Tsinghua University, Beijing and Chinese Academy of Sciences, Beijing for glycerol production with an osmophilic yeast strain of *Candida krusei* have used 35°C as optimum temperature (95, 105, 227). Vajaikishore, P. (77) working with *Pichia farinosa* has used 30°C as optimum temperature. Sahoo, D. K. (76) has used *Candida magnolia* for glycerol production at 35°C. Djelal, et al. (96) working with *H. anomala* have used a temperature of 28°C for glycerol production from glucose containing media.

In Chapter-4, we have reported that the temperature tolerance of our strain of *H. anomala* was different with different carbon sources. With fructose and glucose, the organism was able to withstand a temperature of 35 and 32.5°C, respectively. However, with sucrose as substrate, the temperature tolerance was in between 30 to 32.5°C. These shake flask experiments were conducted without invertase addition. Therefore, it was necessary to investigate the effect of temperature at higher scale of operation and at pH 8.0 in presence of externally added invertase.

Experiments were conducted at 30, 32.5 and 35.0°C at pH 8.0 plus invertase. The investigational data of individual experiments are presented in the form of the time course profiles of a) Total polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig. 5.5.1 to 5.5.3.

RESULTS AND DISCUSSION

Temperature 30îC: Rapid growth was noticed in the first 18 hrs before adjustment of pH to 8.0. The growth rate was somewhat reduced from 18 hrs to about 66 hrs. After 66 hrs, the growth rate reduced further. The polyols formation rate and sugar utilization rate were quite fast and almost complimentary to each other. The final

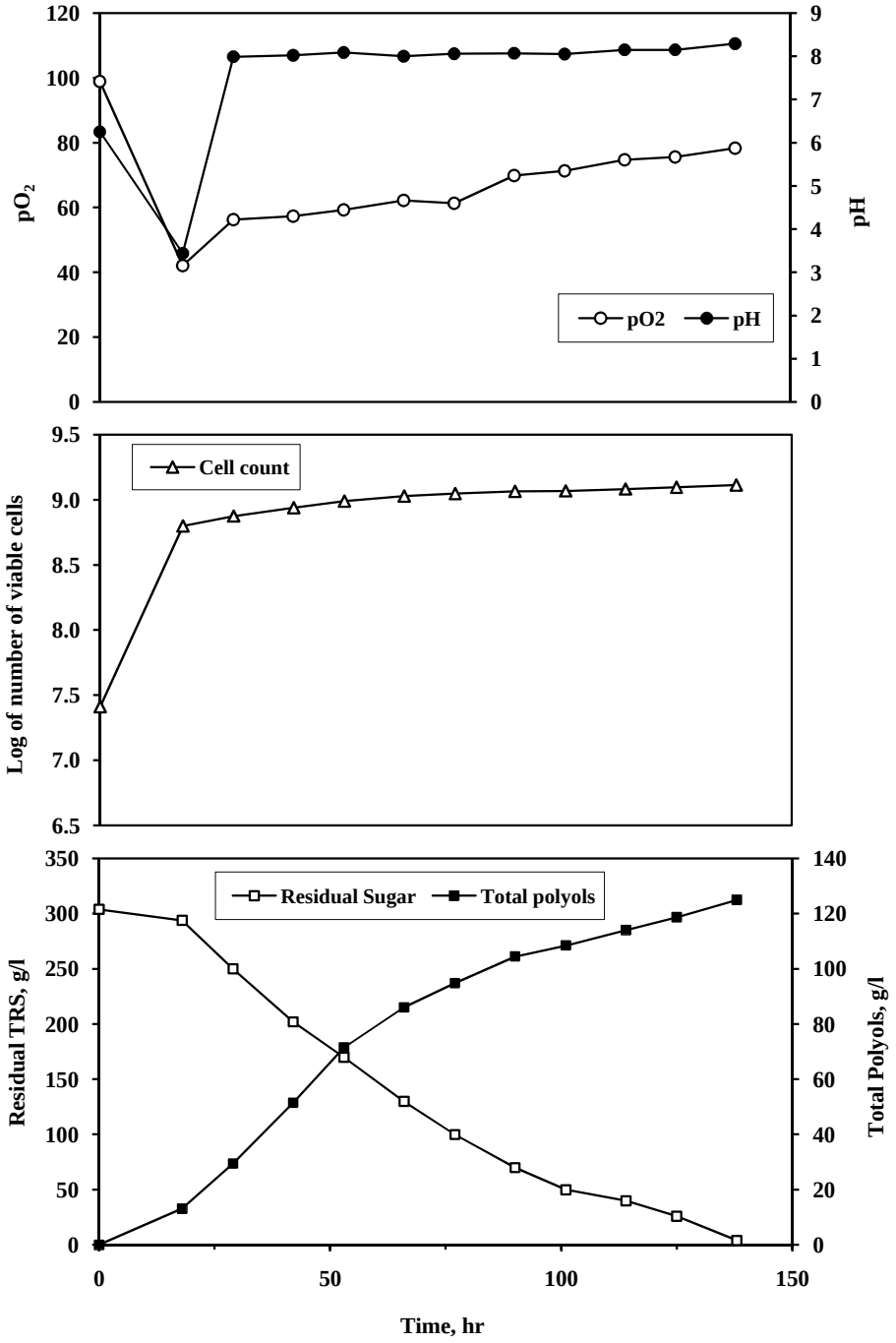


Fig. 5.5.1: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 8.0 + Invertase + 30°C

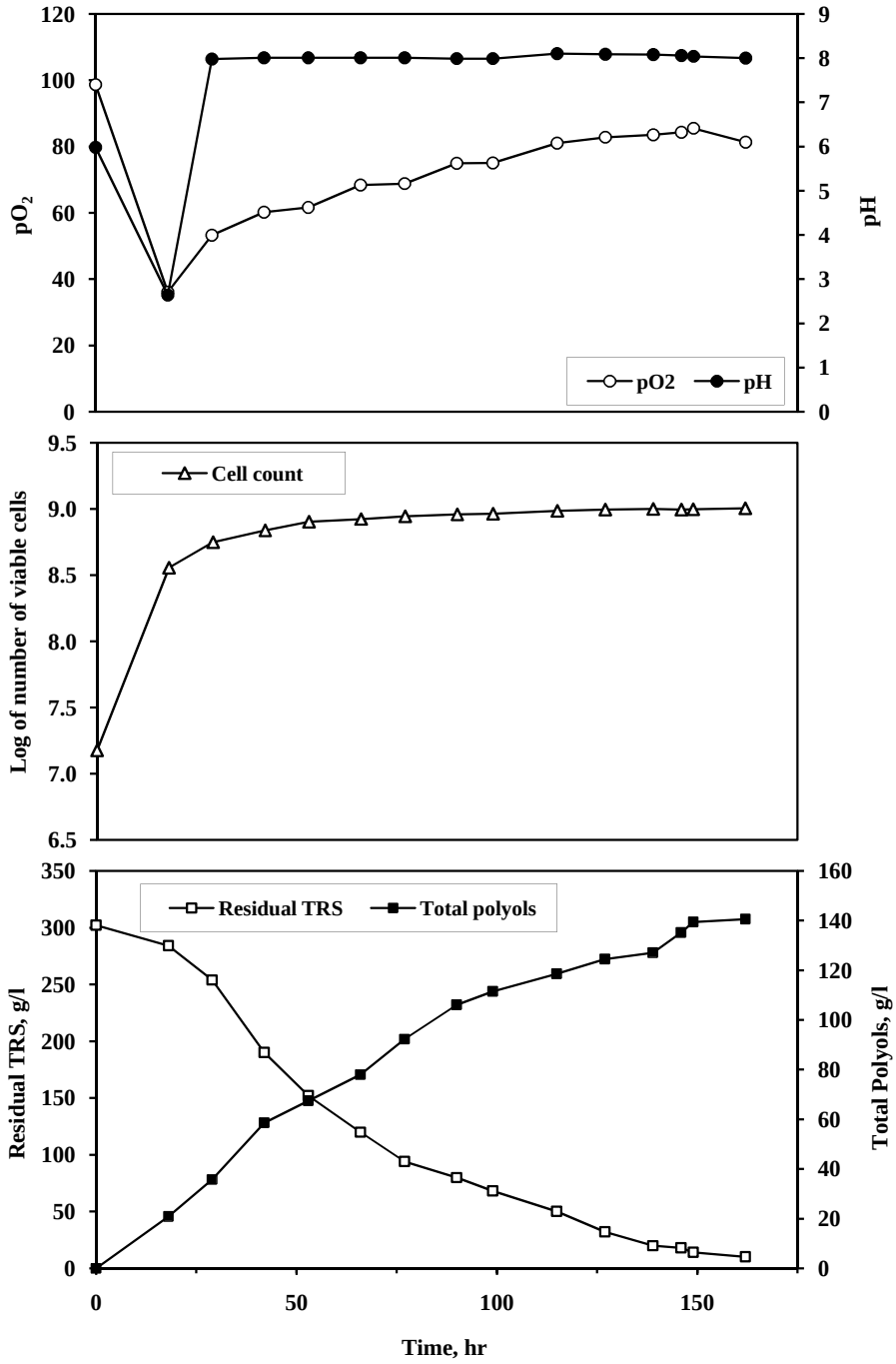


Fig. 5.5.2: Time course profile of a)Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH- 8.0 + Invertase + 32.5 ^{o}C

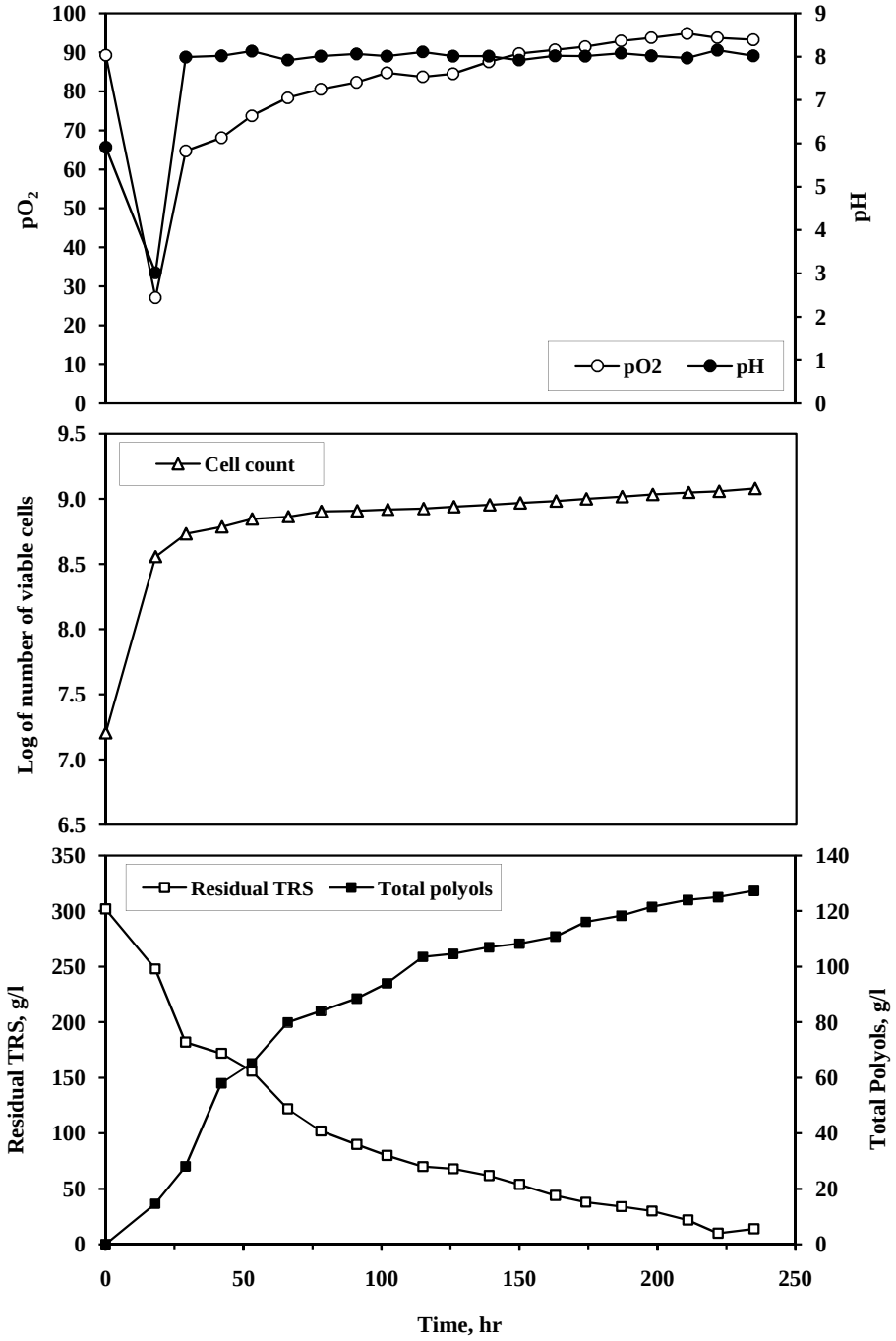


Fig. 5.5.3: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH 8.0 + Invertase + 35^{o}C

polyols concentration reached was 124.8 g/l and fermentation was completed in 138 hrs. The polyols yield based on sugar utilized (p/su) and the overall polyols yield based on total sugars (p/ts) was 41.67 % and 41.13 %, respectively. The sugar utilization rate was also on higher side at 2.174 g/l/hr. The oxygen % saturation (pO_2) was reduced to about 40.0 % in the first 18 hrs and then remained in between 56.0 to 78.0 % throughout the fermentation period, indicative of good oxygen uptake rate and moderate cell growth.

Temperature 32.5îC: After initial rapid growth for the first 18 hrs, there was further slow but steady growth up to about 66 hrs. The cell growth after 66 hrs remained slow throughout the fermentation and utilization of sugar required longer time (about 162 hrs) as compared to 138 hrs for fermentation at 30°C. The final polyols concentration reached was on higher side at 140.5 g/l as compared to that of 124.8 g/l for fermentation at temperature 30°C. The oxygen % saturation dropped to 36.10 % in first 18 hrs and then remained in between 60.0 to 80.0 % throughout the fermentation period, indicative of good oxygen uptake rate and moderate cell growth. The final polyols productivity (0.867 g/l/hr), cell mass productivity (0.061×10^8 cells/ml/hr) and sugar utilization rate (1.802 g/l/hr) were also on lower side as compared to that obtained at 30°C.

Temperature 35îC: Cell growth was rapid for the first 18 hrs and then increased slowly up to 29 hrs. Cell growth reduced substantially after 29 hrs as compared to growth at 30 and 32.5°C. Utilization of sugar was not complete even after 235 hrs of fermentation and the final polyols concentration reached was lower (127.3 g/l) than at 32.5°C (140.5 g/l). Oxygen % saturation value gradually increased and remained in between 70 to 90 % throughout the fermentation, indicating reduced oxygen up-take rate and cell growth.

Fig. 5.5.4 and Fig. 5.5.5 represents the relationship between a) Overall polyols yield, b) Cell mass productivity, c) Polyols productivity and d) Sugar utilization rate and time at different temperatures. It can be seen that overall polyols yield, polyols productivity and sugar utilization rate, most of the time during the course of fermentation, are slightly on higher side for temperature 32.5°C. Cell mass productivity is however, higher at temperature of 30°C.

Table 5.2 summarizes the final results of effect of temperature on polyols production by *H. anomala*. It can be seen that polyols productivity, cell mass

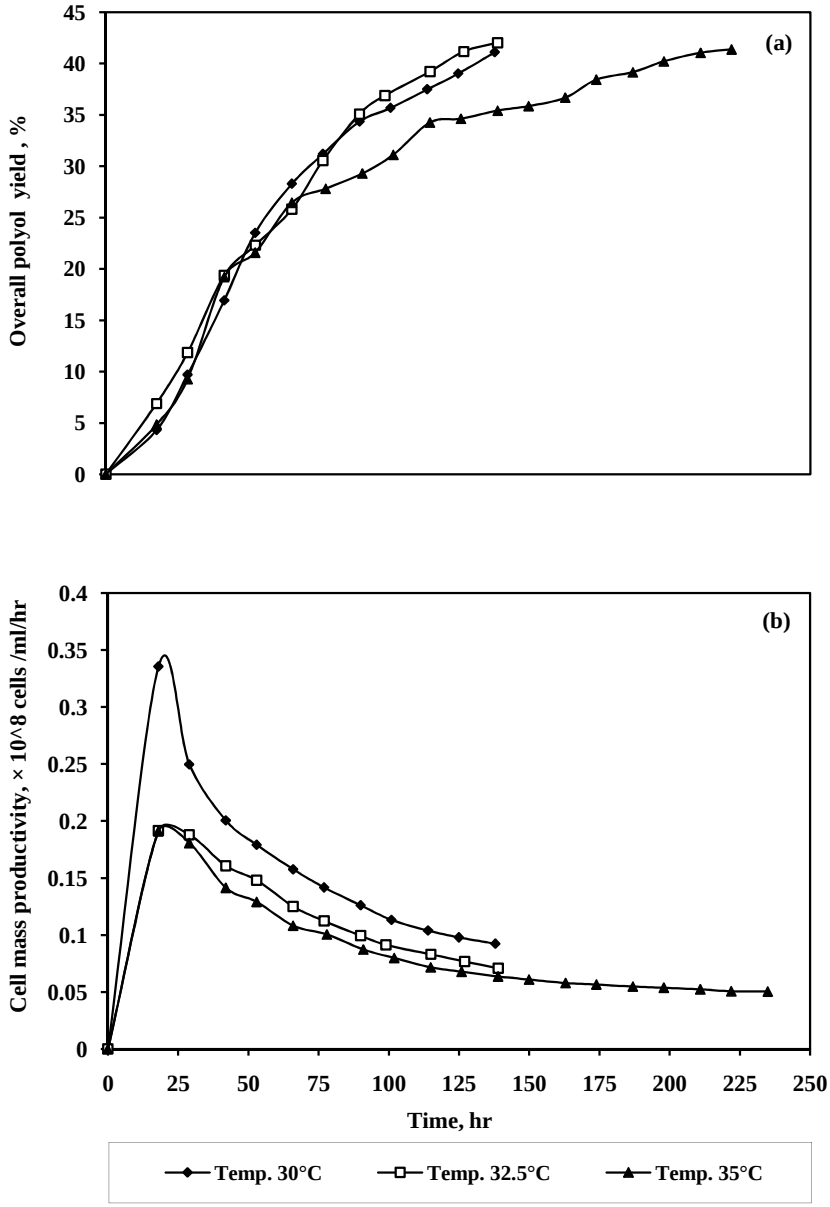


Fig. 5.5.4: Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for different temperatures

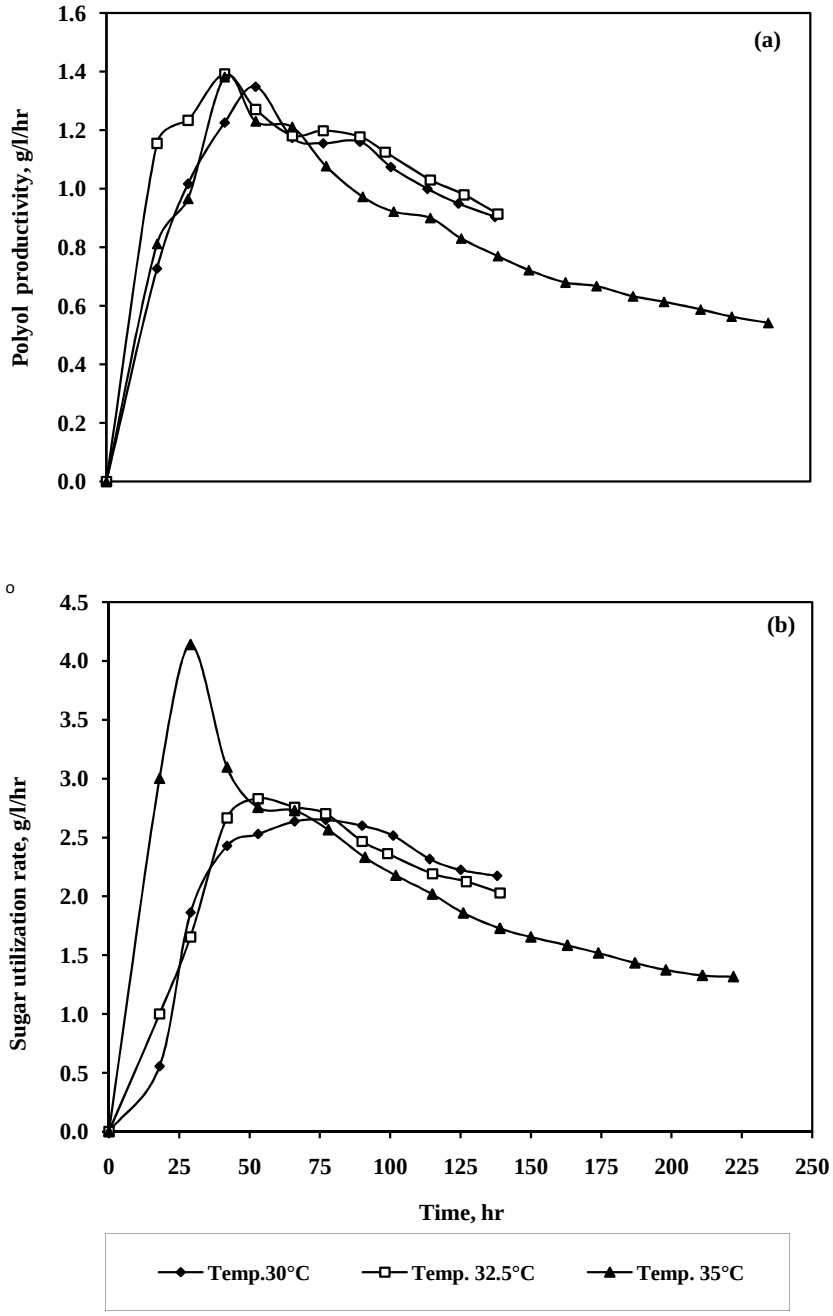


Fig. 5.5.5: Relationship between a) Polyol productivity and Time & b) Sugar utilization rate and Time for different temperatures

productivity and sugar utilization rates were maximum at 30°C resulting into reduced time of fermentation. The cell mass yield was also highest at 30°C. However, overall polyols yield was the lowest. At 32.5°C, the time required to complete the fermentation increased resulting in reduced rate of sugar utilization, polyols productivity and cell mass productivity. However, the overall polyols yield is maximum at this temperature. At 35.0°C, the time required to complete the fermentation increased further indicating increasing stress on the osmophilic yeast because of higher temperature.

Table 5.5.1: Comparative results for different temperatures

Temp. (îC)	Time (hr)	Overall yield based on total sugars		Polyols productivity (g/l/hr)	Cell mass productivity (x ×10^8cells/ ml/ hr)	Sugar utilization rate (g/l/hr)
		Polyols (p/ts), (%)	Cell mass (x_{cc}/ts) (%)			
30.0	138	41.13	4.19	0.904	0.092	2.174
32.5	162	46.52	3.29	0.876	0.061	1.800
35.0	235	42.15	3.92	0.541	0.050	1.220

5.6 EFFECT OF USE OF FRUCTOSE AS CARBON SOURCE

Most of the researchers working with osmophilic yeasts for polyols production have used glucose as carbon source. We encountered reduced polyols yield while working with sucrose as compared to our previous yields with glucose. In shake flask experiments, we tested different carbon sources for polyols production with our strain of *H. anomala*. Maximum yield and final polyols concentration was obtained with fructose as compared to glucose and sucrose.

It is reported in the literature that fructose exerts maximum osmotic pressure on yeast cell in liquid media (228). Certain osmophilic yeasts are also known to be fructophilic. In contrast to *Saccharomyces cerevisiae, Zygosaccharomyces rouxii* is a fructophilic yeast and consumes fructose faster than glucose (219).

To investigate this aspect, we conducted experiment with fructose as carbon source on 2.5 liter fermenter at pH 8.0 and 30°C temperature. The investigational data of

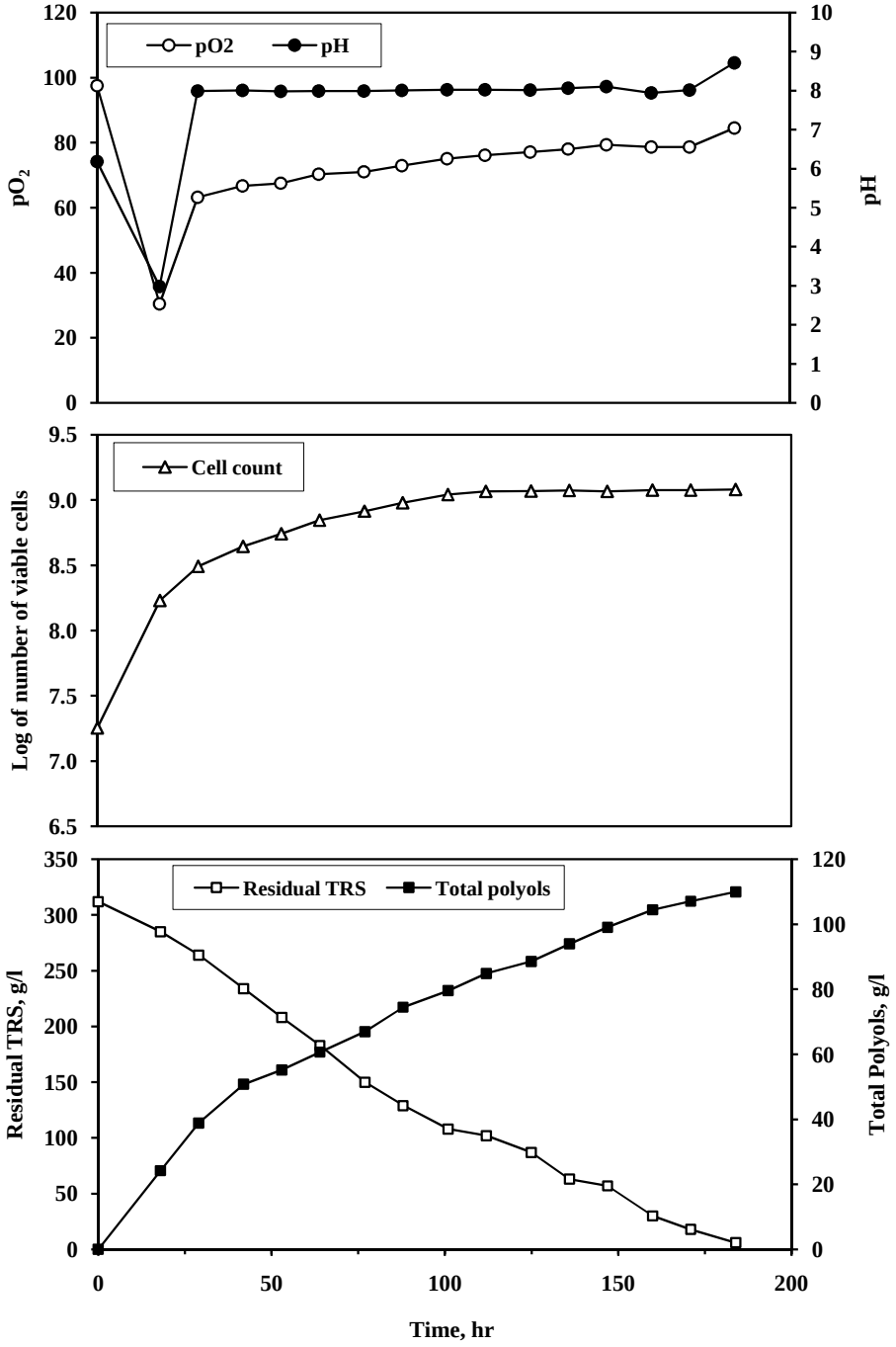


Fig. 5.6.1: Time course profile of a) Total polyol, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at pH- 8.0+ Fructose

this experiment is presented in the form of the time course profile of a) Total polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig.5.6.1.

RESULTS AND DISCUSSION

On fructose media, after the initial rapid growth for 18 hrs of *H. anomala*, further moderate growth was observed for about 101 hrs. At the end of 101 hrs the organism entered into a stationary phase. It is known that osmophilic yeasts continue to produce polyols in stationary phase also. Therefore, polyols production and sugar utilization continued even during the stationary phase. Though sugar was consumed completely, it took almost 184 hrs to complete the fermentation. The final polyols concentration reached was 110.0 g/l at the end of 184 hrs.

The fermentation performance of experiment with fructose as carbon source is compared with a) Experiment run with sucrose as carbon source at pH 8.0 without invertase and b) Experiment run with sucrose as carbon source at pH 8.0 with invertase in Fig 5.6.2 and Fig. 5.6.3.

Table 5.6.1: Comparative results for different sugars

Substrate at pH 8.0	**Time**	**Overall yield based on total sugars**		**Polyols productivity**	**Cell mass productivity**	**Sugar utilization rate**
		Polyols	**Cell mass**			
	(hr)	**(p/ts), (%)**	**(x_{cc}/ts) (%)**	**(g/l/hr)**	**(x × 10^8 cells/ml/ hr)**	**(g/l/hr)**
Sucrose (without invertase)	132 (50% sucrose was not utilized)	21.83	0.48	0.490	0.011	1.210
Fructose	184	35.26	3.78	0.597	0.064	1.663
Sucrose (with invertase)	138	41.13	4.19	0.904	0.092	2.174

Overall polyols yield, polyos productivity, cell mass productivity and sugar utilization rate were all on higher side for sucrose with invertase. The results with

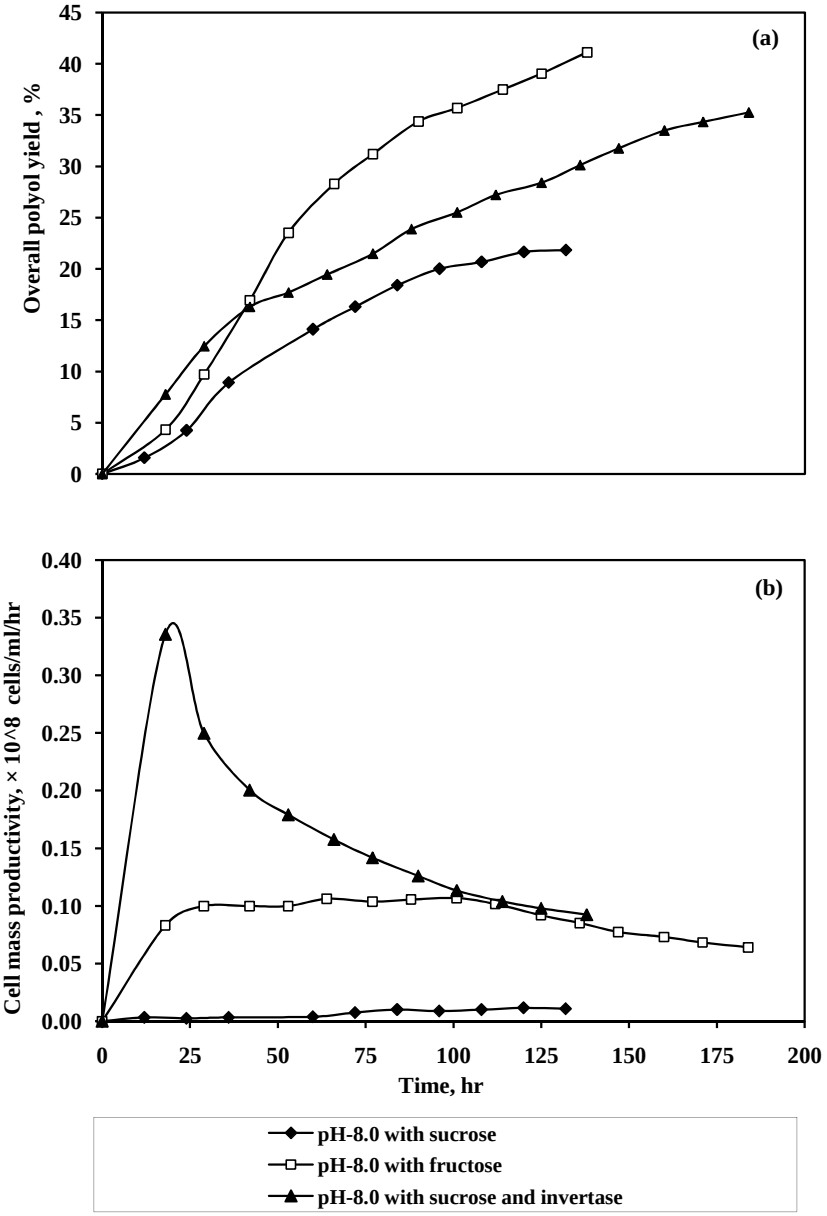


Fig. 5.6.2: Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for fructose &sucrose without and with invertase

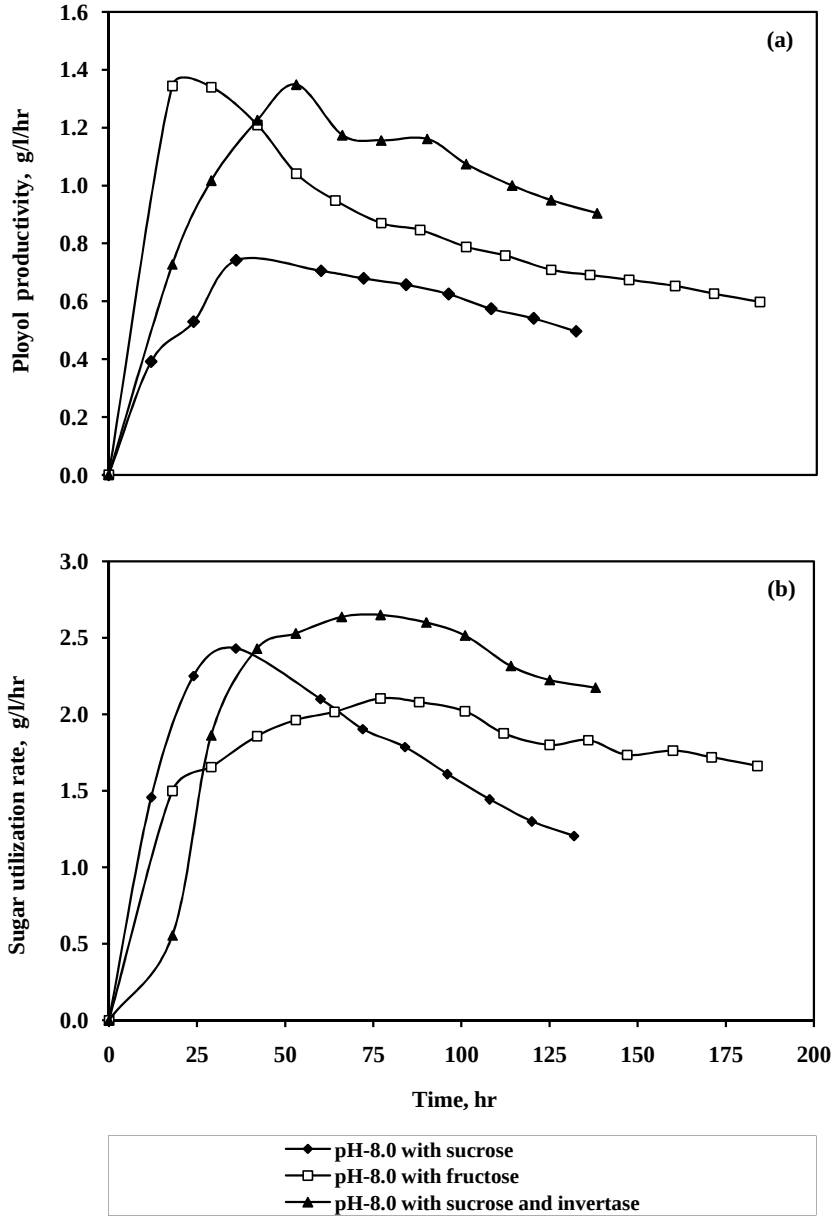


Fig. 5.6.3: Relationship between a) Polyol productivity and Time & b) Sugar utilization rate and Time for for fructose & sucrose without and with invertase

fructose at pH 8.0 were better than sucrose at pH 8.0 (without invertase). Thus, fructose is readily utilized by our strain of *H. anomala* as compared to sucrose. However, if sucrose is hydrolysed to glucose and fructose by addition of external invertase at pH 8.0, the performance improves and results are better than with fructose as carbon source. It is not clearly understood why the results with sucrose plus invertase are better than with fructose as substrate.

Final comparative results are also given on page 138 in the Table 5.6.1.

5.7 EFFECT OF INOCULUM SIZE WITHOUT pO_2 CONTROL

Production of polyols by osmophilic yeasts is an aerobic growth associated process. A very critical factor in production of polyols is the rate of oxygen supply per unit biomass, which determines the percentage conversion of sugar to polyols (8). This is directly related to the number of viable cells present in the medium. Under given environmental conditions and availability of nutrients, the number of cells present at any time in the medium is again determined by the quantity or size of inoculum used.

The quantity of inoculum normally used is between 3 to 10 % of the medium volume. Relatively large inoculum is used to minimize the length of the lag phase and to generate the maximum biomass in the production fermenter as rapidly as possible, increasing fermenter productivity. Thus starting from a stock culture, the inoculum must be built up in a number of stages to produce sufficient biomass to inoculate the main fermenter (222). Though inoculum size was optimized in shake flask experiments, the oxygen transfer efficiency of shake flasks is different than that of an aerated and agitated fermenter. Therefore, optimization of inoculum size on fermenter scale is an important aspect from process development point of view.

The inoculum size optimization experiments were conducted at fixed air flow rate of 0.56 vvm, 600 rpm agitation rate, 30°C temperature, pH 8.0, 0.5 g/l of invertase and at 2.5 liter medium volume. Investigations were carried out at inoculum size of 5 %, 10%, 15%, 20% and 30%. The inoculum was prepared, centrifuged and transferred to the fermenter as explained earlier in the inoculum development part.

The investigational data of individual experiments are presented in the form of the time course profiles of a) Total polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig. 5.7.1 to 5.7.5.

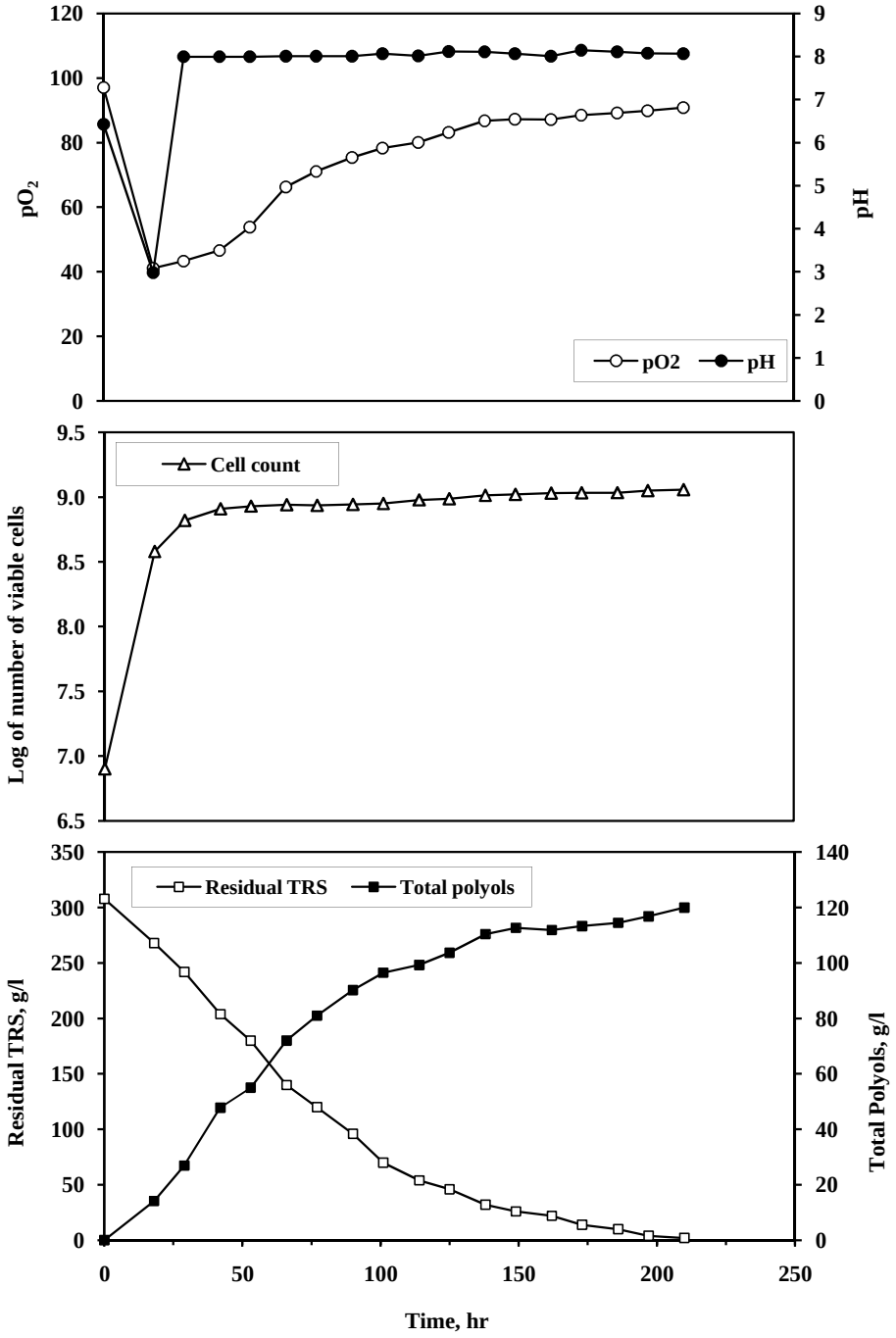


Fig. 5.7.1: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 5 % Inoculum

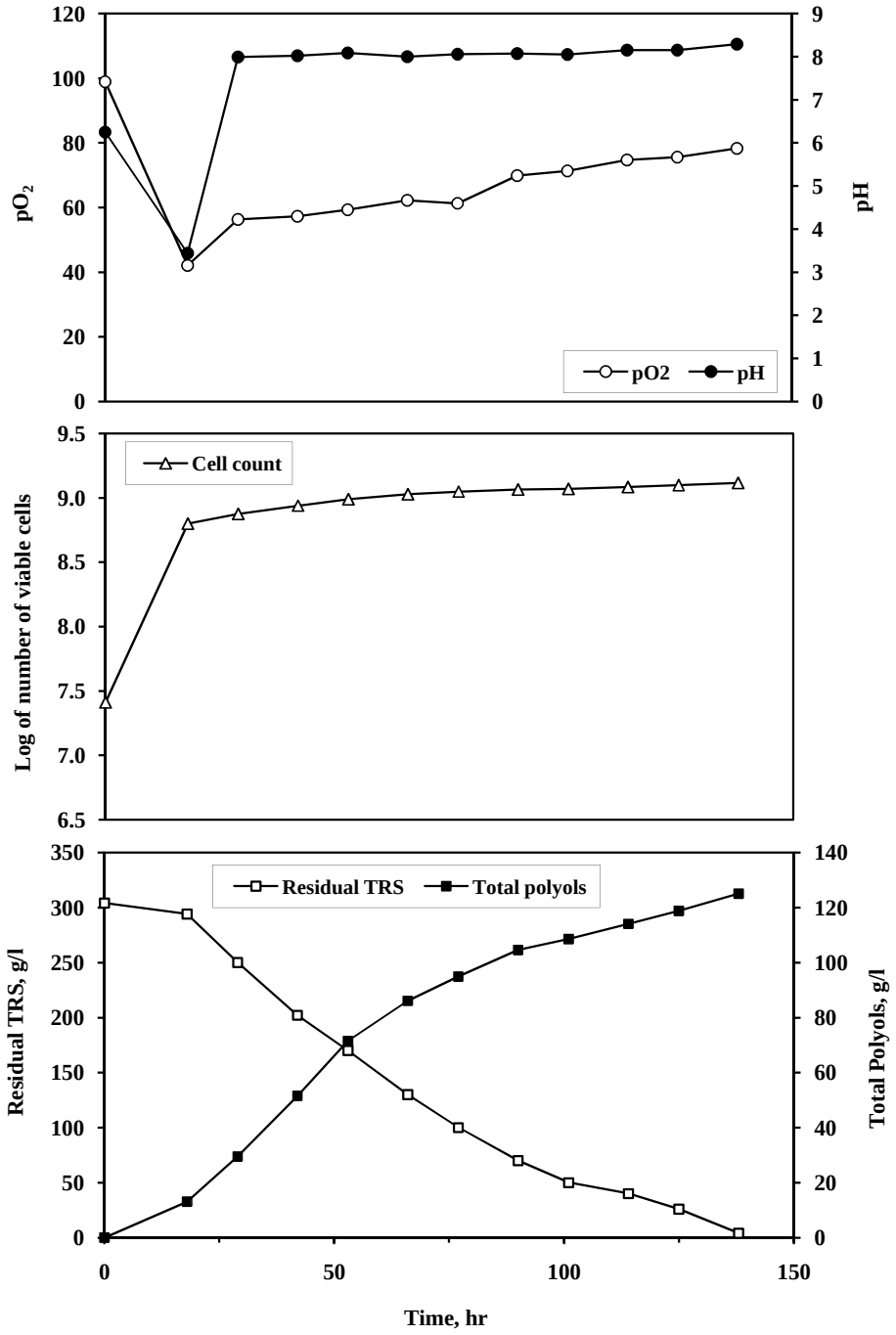


Fig. 5.7.2: Time course profile of a) Total polyols, b)Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 10 % Inoculum

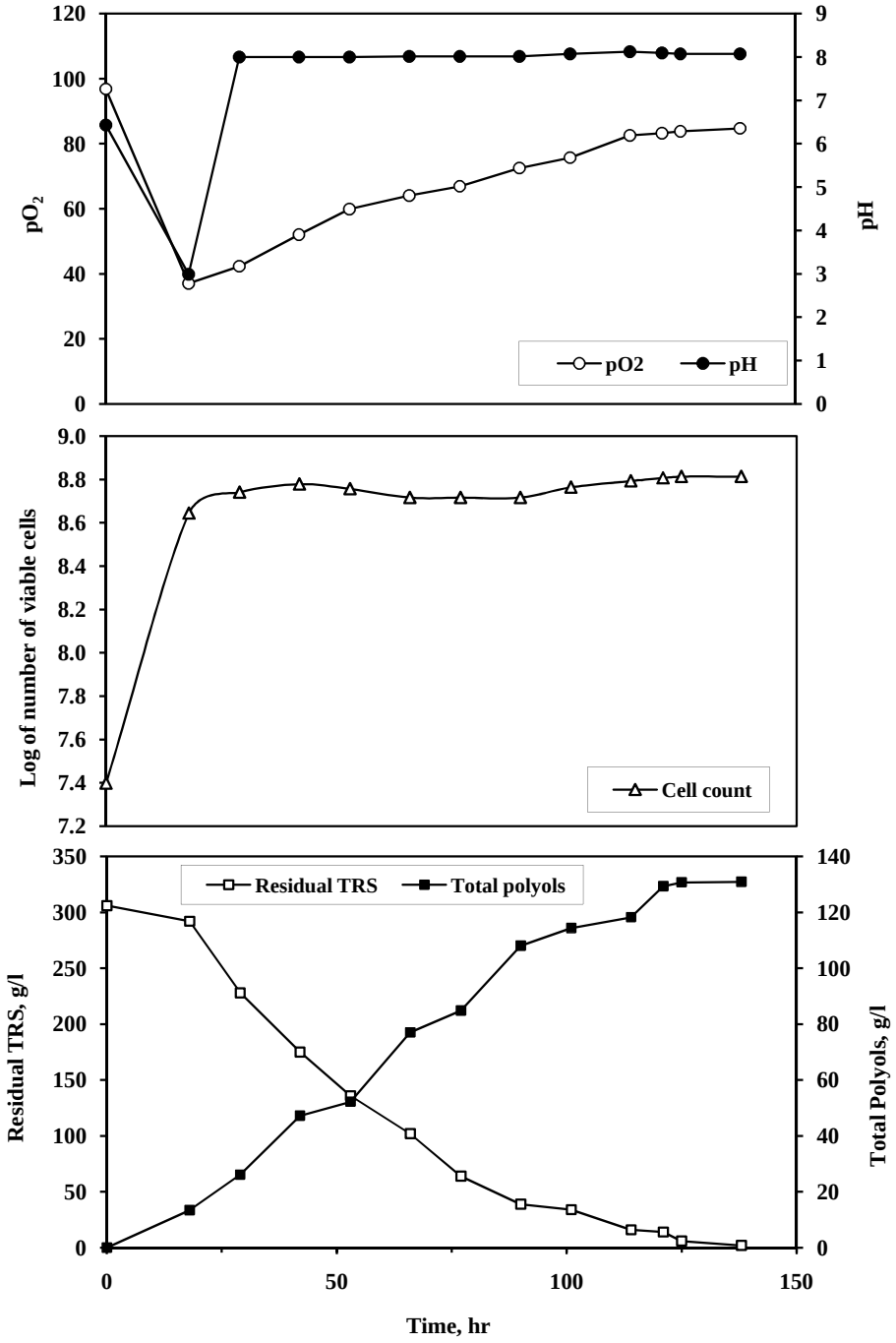


Fig. 5.7.3: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 15 % Inoculum

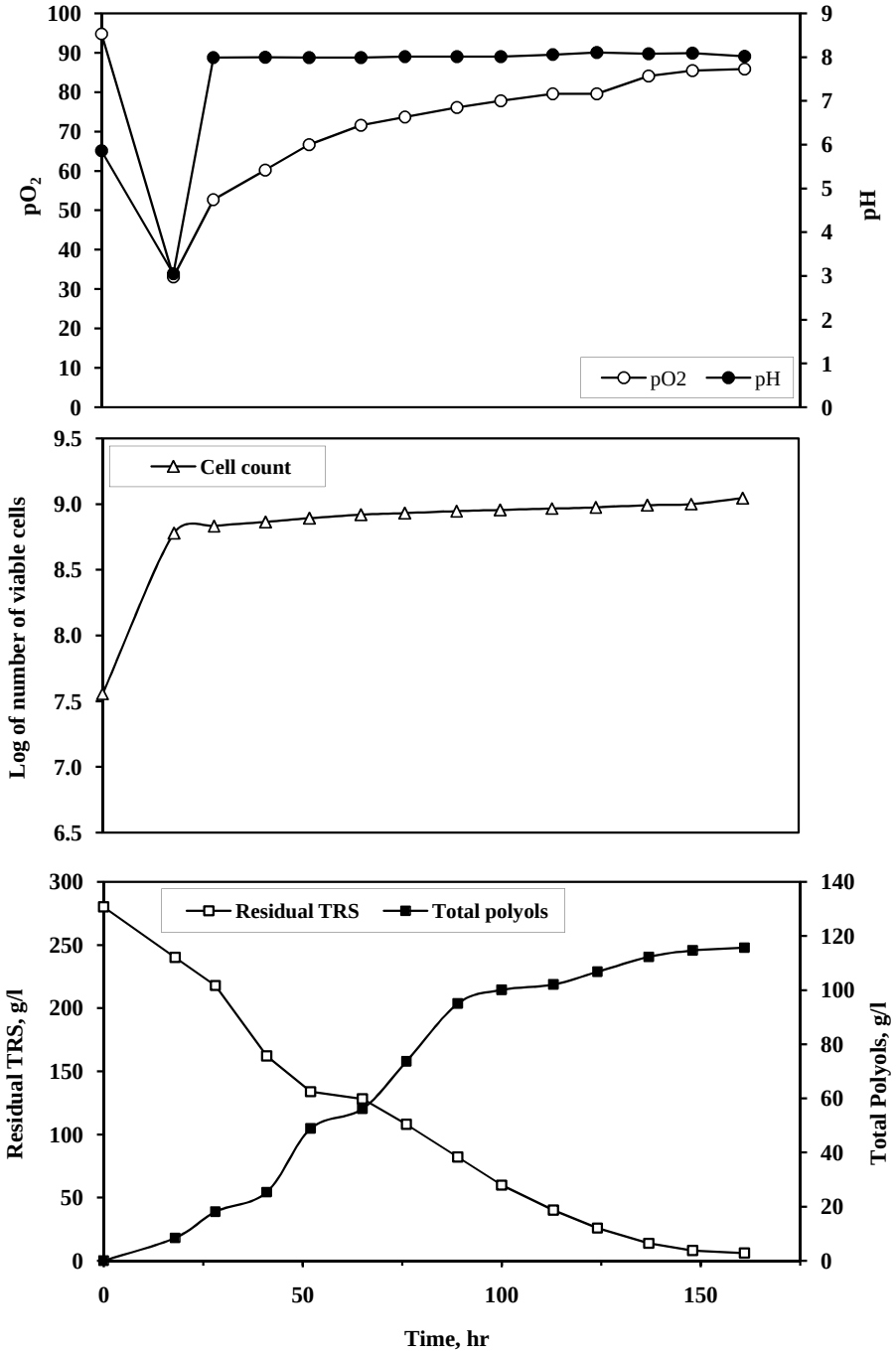


Fig. 5.7.4: Time course profile of a) Total polyols, b)Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 20 % Inoculum

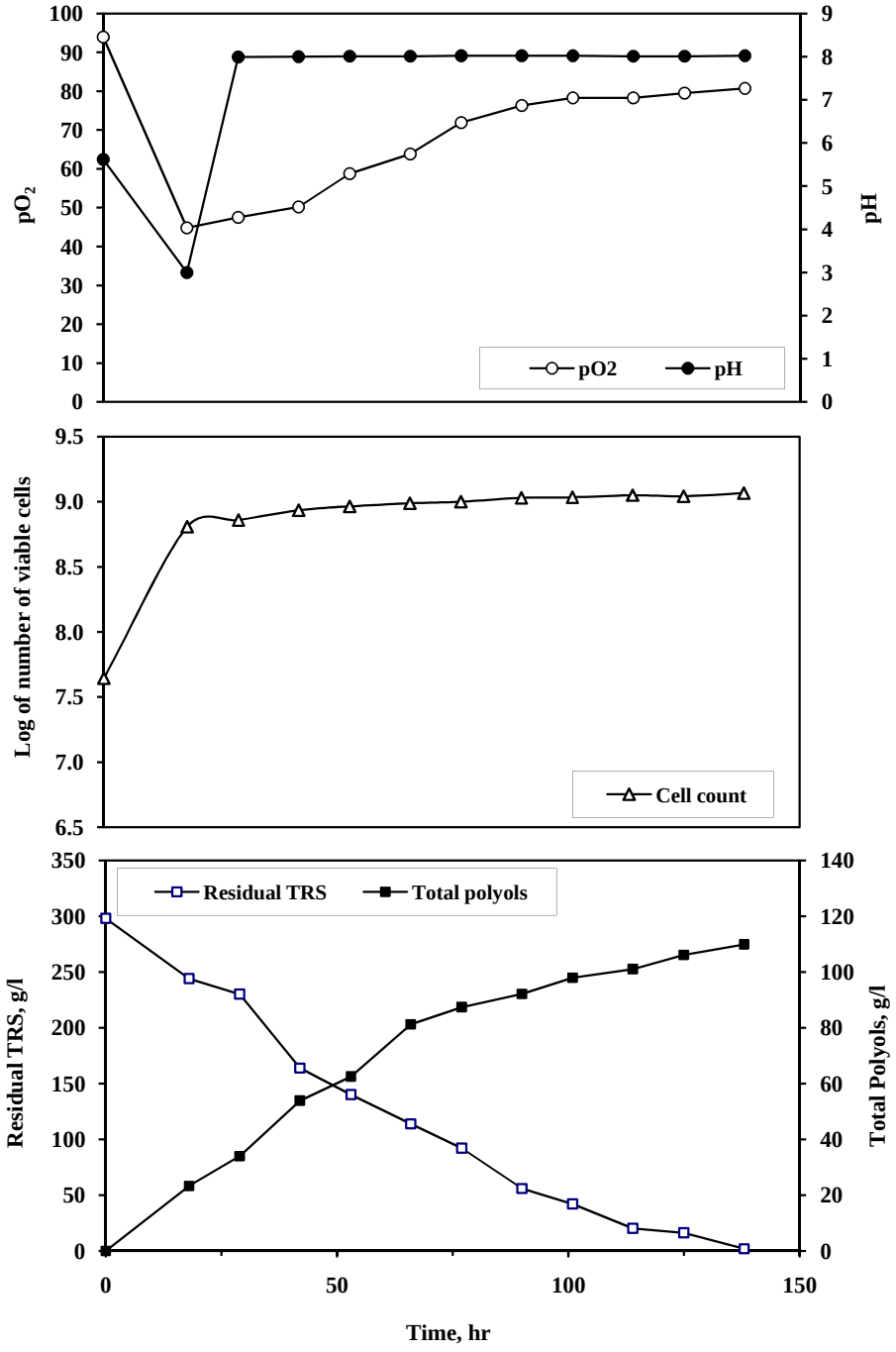


Fig. 5.7.5: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 30 % Inoculum

RESULTS AND DISCUSSION

5 % inoculum: The cell growth shows a typical pattern of rapid initial growth in first about 18 to 24 hrs followed by a phase of very slow growth or stationary phase as seen in Fig.5.7.1. Sucrose is rapidly consumed in the first 100 hrs and then the sucrose utilization rate declines. Percentage oxygen saturation (pO_2) value dropped down to about 40 % in the first 18 hrs and then gradually increased up to about 78 % in first 100 hrs. In between 100 to 210 hrs, the pO_2 value remained in between 80 to 90% indicating very slow oxygen uptake rate. Polyols are also produced at a fast rate in the first 100 hrs and then the rate of polyols production declines. Probably because of lower starting population of *H. anomala*, the time required to complete the fermentation was almost 210 hrs. The final total polyols concentration reached was 120.0 g/l. The final overall yield of polyols based on total sucrose used was 38.96 % at a polyols productivity rate of 0.571 g/l/hr.

10 % inoculum: Similar trend of rapid cell growth followed by slow growth was also observed with 10 % inoculum size experiment. However, the growth rate in the second phase was faster than with 5 % inoculum. Sucrose was almost consumed in 138 hrs. The final total polyols concentration also improved to 124.8 g/l. The final overall yield of polyols improved to 41.13 %. The yield of cell mass also improved to 4.19×10^8 cells/ml per 100 g of sugar used.

15 % inoculum: Similar trend of rapid growth followed by second phase of slow growth was noticed with 15 % inoculum size. The sugar was almost consumed in 138 hrs. The overall yield of polyols based on total sugars used improved further to 42.77 % with polyols productivity of about 0.948 g/l/hr. The final total polyols concentration also improved to 130.9 g/l. However, the cell mass yield was reduced to 2.042×10^8 cells/ml per 100 g of sucrose utilized. Oxygen % saturation value after initial drop to about 37 %, started rising gradually to about 80 % in the later part of fermentation indicating fast oxygen uptake rate.

20 % inoculum: After initial fast growth for about 18 hrs, the growth in the second part was very slow like an ideal stationary phase. Complete utilization of sucrose required almost 161 hrs as compared to 138 hrs with 10 % and 15 % inoculum size. The final total polyols concentration dropped down to 115.7 g/l. The polyols productivity was reduced to 0.718 g/l/hr and final overall yield of polyols reduced to 41.32 %. The cell mass yield once again improved to 3.84 %.

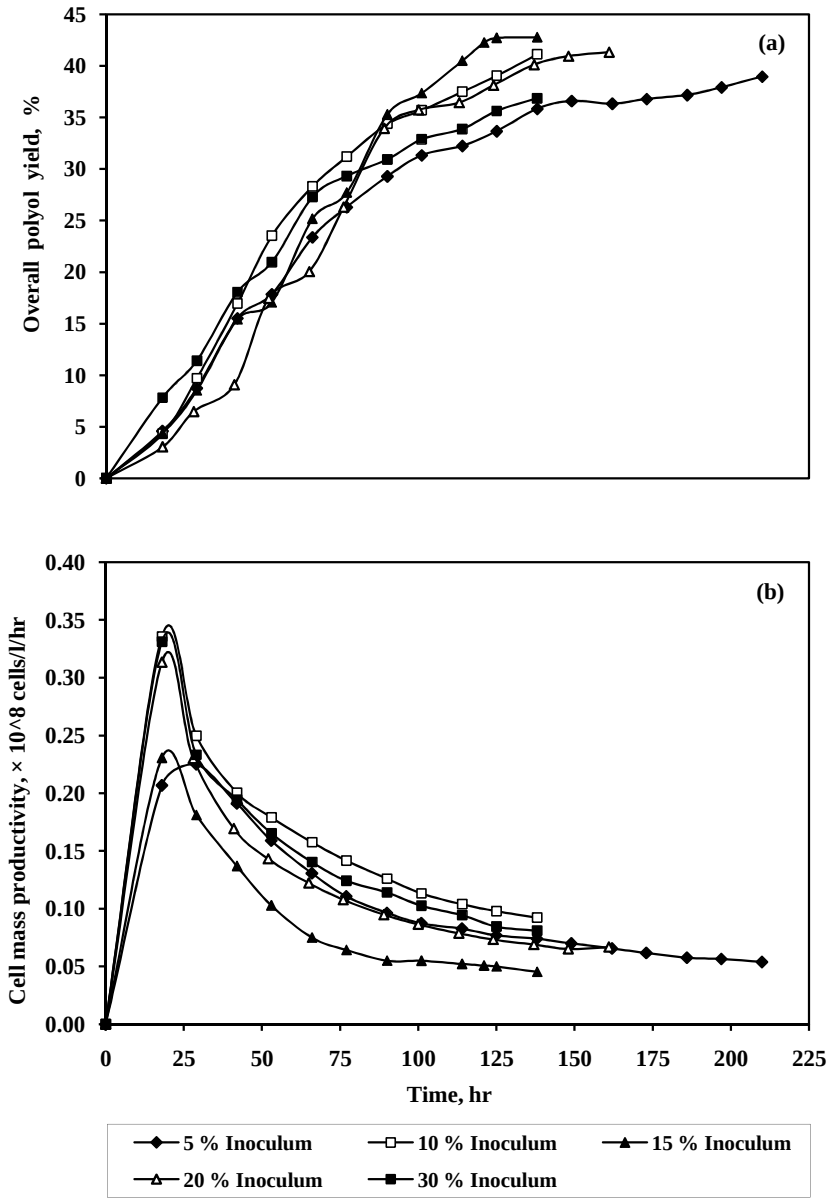


Fig. 5.7.6: Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for different inoculum size without pO_2 control

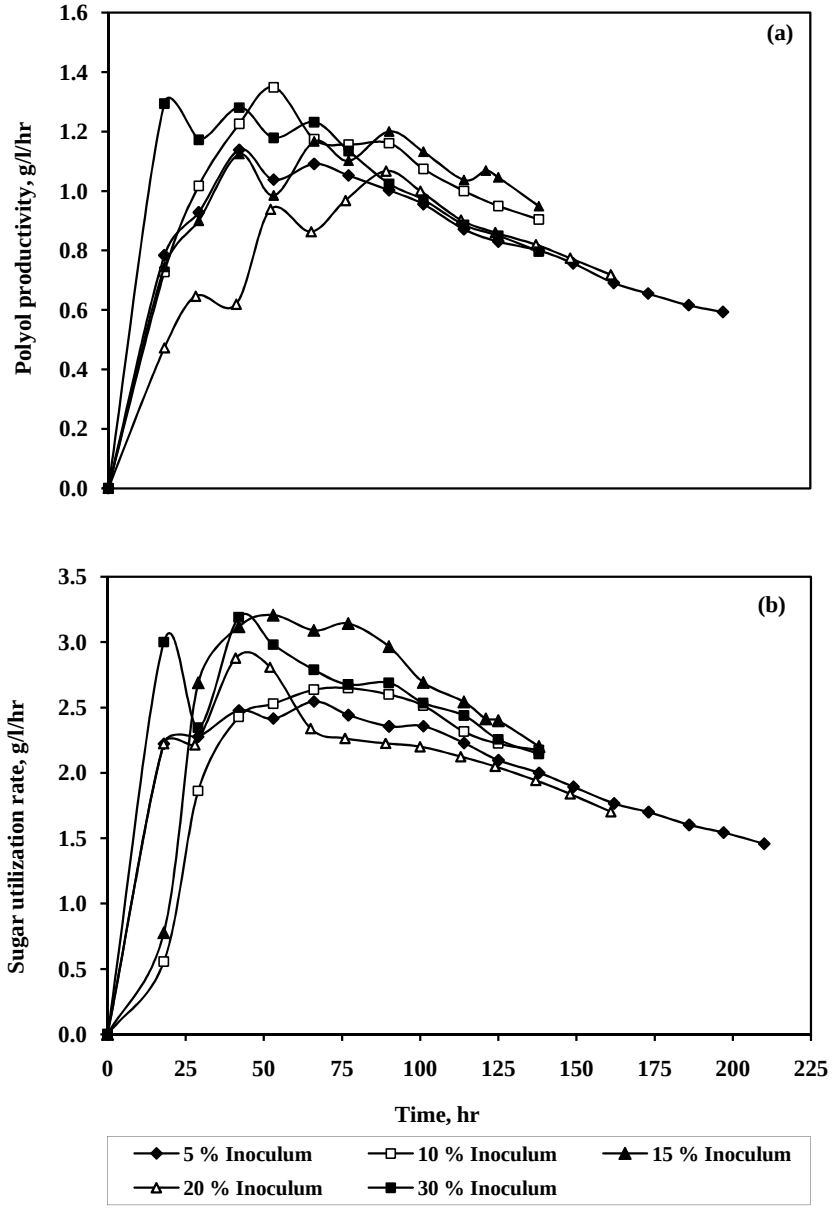


Fig. 5.7.7: Relationship between a) Polyol productivity and Time & b) Sugar utilization rate and Time for different inoculum size without pO_2 control

30 % inoculum: Similar trend of cell growth with rapid growth in the early part of fermentation followed by stationary growth in the later part. Though sucrose consumption rate was quite fast, the overall polyols yield (p/ts) and final polyols concentration were further reduced to 36.87 % and 109.9 g/l, respectively. At the same time the final cell mass yield also remained high at 3.75×10^8 cells/ml per 100 g of sucrose that was used.

Above observations indicate that 15 % inoculum at 30°C temperature, 0.54 vvm air flow rate, 600 rpm agitation, pH 8.0 with externally added invertase seems to be the optimum conditions for polyols production by *H. anomala*.

Fig. 5.7.6 and Fig. 5.7.7 illustrate the relationship between a) Overall polyols yield, b) Cell mass productivity, c) Polyols productivity, and d) Sugar utilization rate and time at different inoclum size. It indicates that overall polyols yield, polyols productivity and sugar utiliztation rate are all higher side in experiment with 15 % inoculum. However, the cell mass yield is minimum with 15 % inoculum experiment suggesting that controlled growth under aerobic conditions is necessary to maximise polyols production even though it is a growth associated product formation case. The final comparative results of variation of inoculum size are also given in Table 5.7.1

Table 5.7.1: Comparative results of variation of inoculum size

Inoculum Size	**Time**	**Overall yield based on total sugars**		**Polyols productivity**	**Cell mass productivity**	**Sugar utilization rate**
		Polyols (p/ts), (%)	**Cell mass (x_{cc}/ts) (%)**			
(%)	**(hr)**			**(g/l/hr)**	**(x × 10^8 cells/ml/ hr)**	**(g/l/hr)**
5.0	210	38.96	3.68	0.571	0.054	1.547
10.0	138	41.13	4.19	0.904	0.092	2.174
15.0	138	42.77	2.04	0.948	0.045	2.260
20.0	161	41.32	3.84	0.718	0.067	1.701
30.0	138	36.87	3.75	0.796	0.081	2.144

The Table above indicates that polyols production will require controlled growth conditions. 15 % inoculum size under the prevailing conditions of aerobic growth seems to offer maximum polyols yield.

5.8 EFFECT OF CONTROLLING OXYGEN PERCENT SATURATION (pO_2)

The majorities of fermentation processes are aerobic and therefore, require the provision of oxygen. If the stoichiometry of respiration is considered, then the oxidation of glucose may be represented as,

$C_6H_{12}O_6 + 6O_2 = 6H_2O + 6CO_2$

Thus, 192 grams of oxygen are required for the complete oxidation of 180 grams of glucose. However, both components must be in solution before they are available to the microorganisms and oxygen is 6000 times less soluble in water than is glucose. Thus, it is not possible to provide a microbial culture with all the oxygen it will need for the complete oxidation of the glucose (or any other carbon source) in one addition. Therefore, a microbial culture must be supplied with oxygen during the growth at a rate sufficient to satisfy the organisms demand. Cultures demand for oxygen is very much dependent on the source of carbon in the medium. Thus, the more reduced the carbon source, the greater will be the oxygen demand. Maximum biomass production may be achieved by satisfying the organism's maximum specific oxygen demand by maintaining the dissolved oxygen concentration greater than the critical level. If the dissolved oxygen concentration were to fall below the critical level then the cells may alter its metabolism.

Liu et al. have applied oxygen limitation strategy to enhance glycerol production by *C. krusei* (95). The availability of oxygen has been suggested to be the most important factor controlling the growth, glucose uptake and yield of glycerol (76). Dissolved oxygen concentration is one of the most important fermentation variables in aerobic production of polyols by osmophilic yeasts. The effect of the aeration on the production of polyols and ethanol has been examined since 1956 by Spencer and Sallans (73) followed by Hajny and coworkers (71). Onishi (33) has confirmed the positive effect of aeration on polyols production. Spencer (7) has suggested that aeration is needed, but should not be excessive to achieve maximum glycerol production. Working with the osmophilic yeast *Moniliella tomentosa var pollinis*, Burschäpers et al. (74) have concluded that if dissolved oxygen concentration decreases below 19 % saturation, ethanol is formed. Vijaikishor and Karanth (229), working with *Pichia farinose* on glucose based media, have shown that aeration and agitation should be maintained above a critical value to give a dissolved oxygen of 40 % saturation for enhanced glycerol and negligible ethanol production.

CHEMAP fermenter used in these studies has a facility to control dissolved oxygen % saturation of fermentation medium by controlling the air flow rate through an air flow control value. Signal from pO_2 electrode is given to the air control valve whenever the oxygen % saturation falls below the set point and vice versa. Therefore, it was possible to control dissolved oxygen % saturation values at different levels during the fermentation. A strategy of adjusting the pO_2 value to the desired level at 18 hrs was employed to allow the growth to take place in the early phase of fermentation.

The effect of controlling oxygen % saturation (pO_2) on polyols production by *H. anomala* was studied at 30 %, 40 %, 50 %, 60 % and 70 % values. Fermentations were carried out at 15 % inoculum and 32.5°C temperature based on our findings reported before. Agitation rate was fixed at 600 rpm and pH was controlled at 8.0 by automatic addition of Na_2CO_3. Invertase was used at a dose of 0.5 g/l. The investigational data of individual experiments are presented in the form of the time course profiles of a) Total polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig. 5.8.1 to 5.8.5.

RESULTS AND DISCUSSION

pO_2 Control at 30 % saturation:

It can be seen from time course profile of pO_2 in Fig. 5.8.1 that the pO_2 value falls rapidly in the first 18 hrs because of the rapid growth of the organism. At 18 hrs, the pH was adjusted to 8.0 with aseptic addition of Na_2CO_3. At the same time, the pO_2 value was also set to the desired level. It was observed that as soon as the pH is adjusted to near neutral or alkaline conditions, there is a sudden increase in the pO_2 value. This probably happens due to increased solubility of oxygen in alkaline conditions as compared to acidic conditions. After the rise in pO_2 value at 18 hrs, the pO_2 level remained constant at 30 % saturation value as required.

The cell growth showed typical rapid growth in the initial phase followed by a near stationary phase throughout the fermentation. The sugar utilization rate was reduced and it took almost 186 hrs to consume all fermentable sugars. The overall polyols yield based on total sugars reached to a value of 38.44 % with cell mass yield of 1.08×10^8 cells/ml per 100 g of sucrose consumed. The final total polyols concentration obtained was 115.3 g/l.

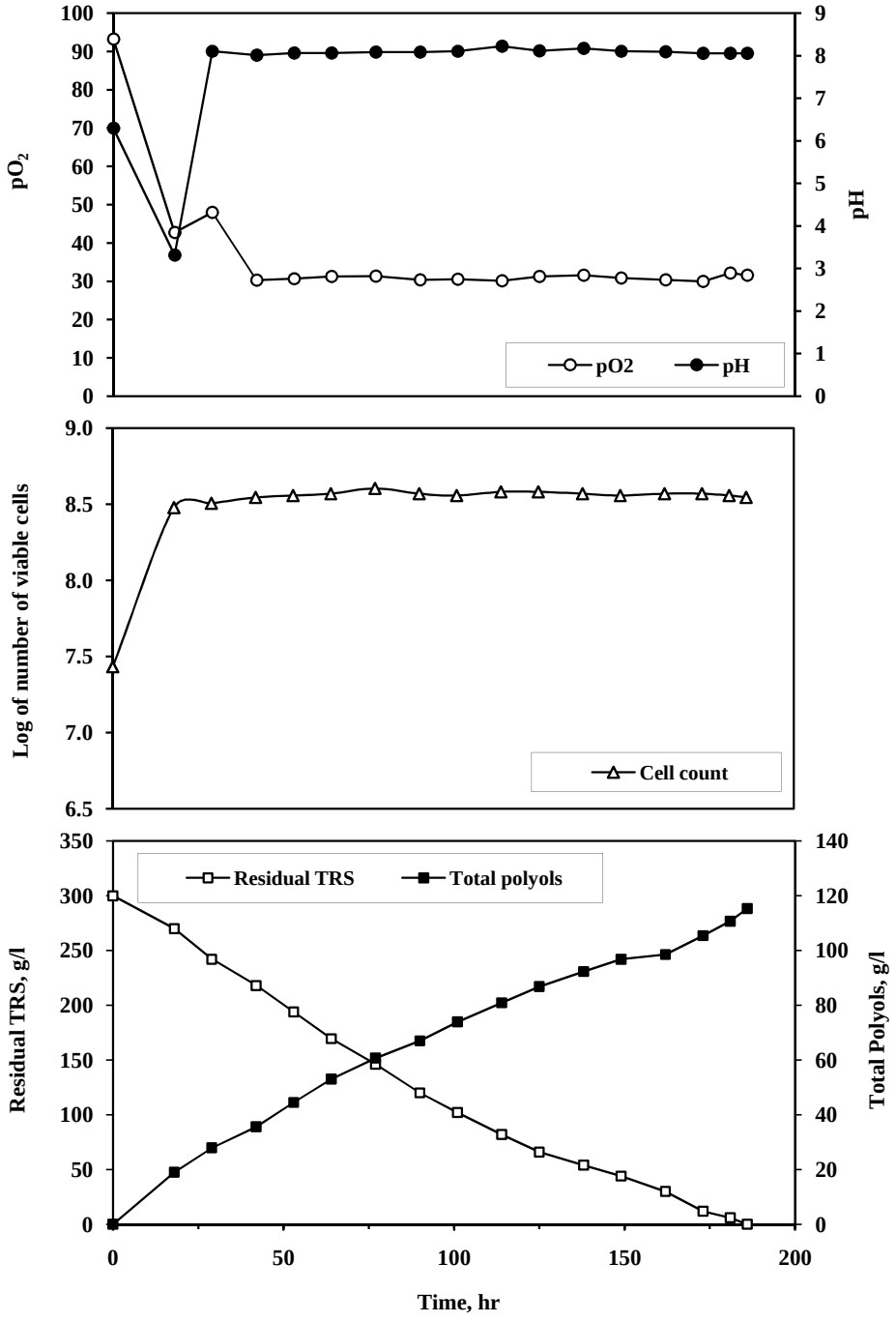


Fig. 5.8.1: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 30 % + pH 8+32.5^{o}C

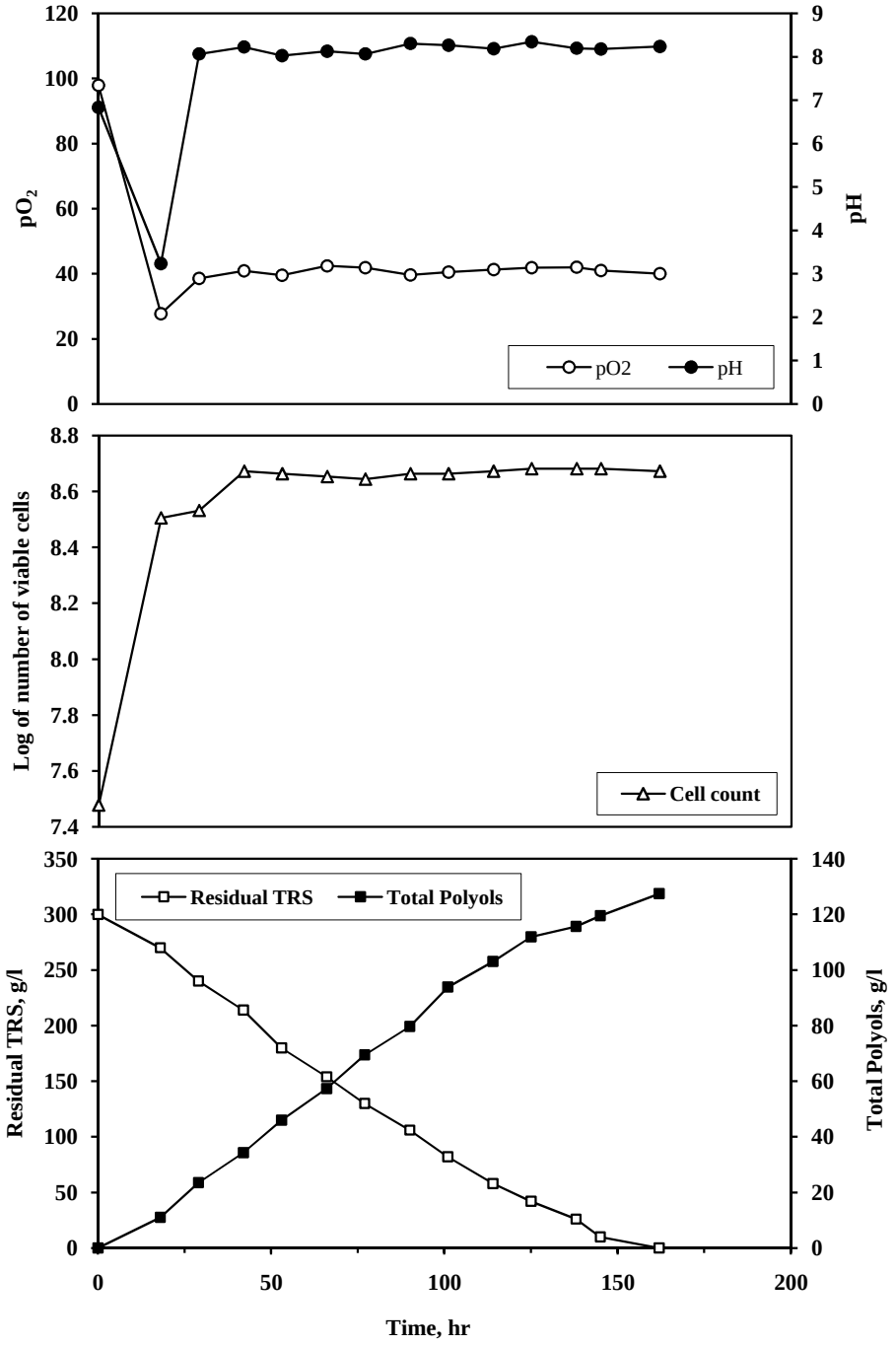


Fig. 5.8.2: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 40 % + pH 8+32.5^{o}C

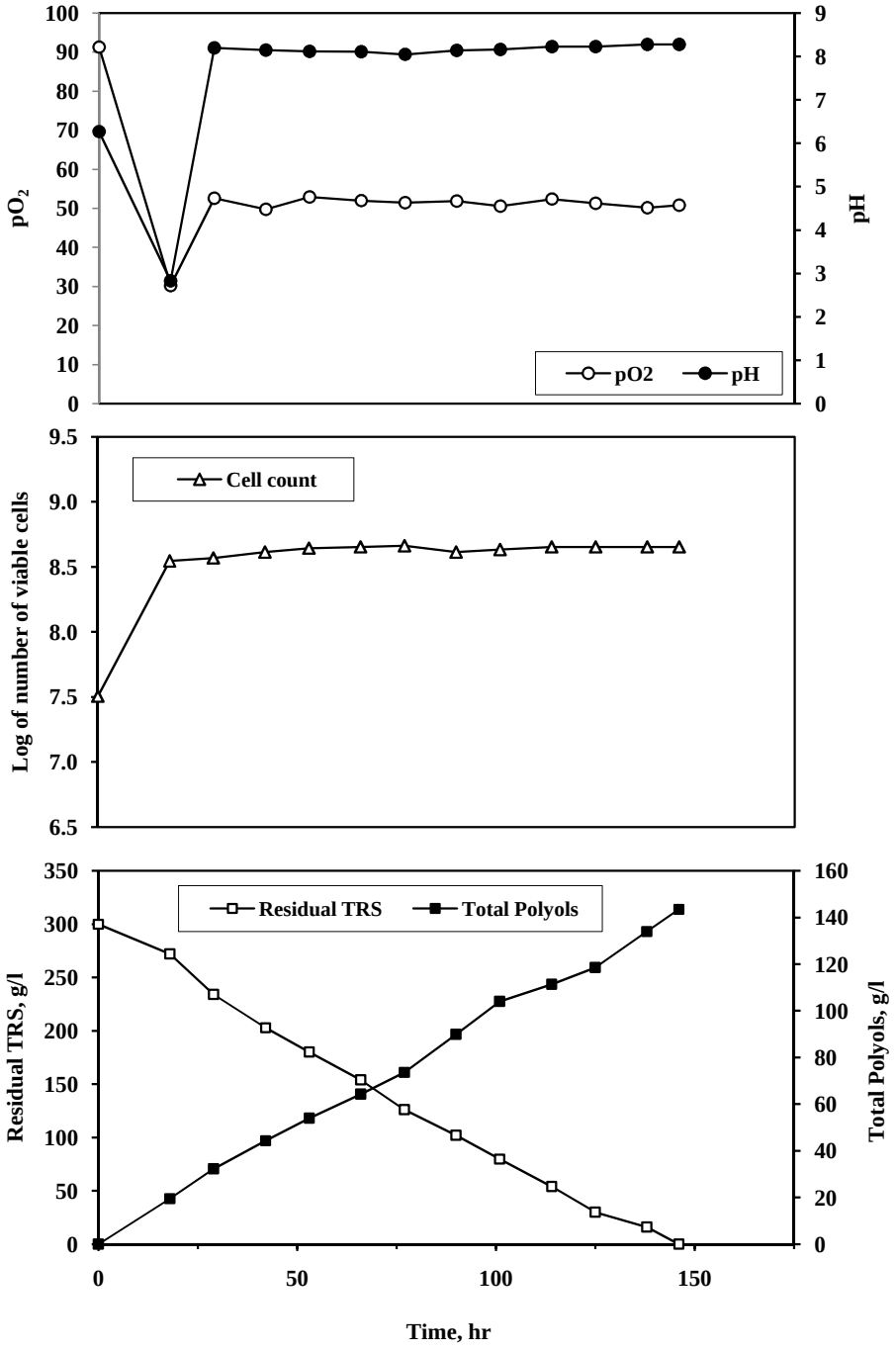


Fig. 5.8.3: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 50 % + pH 8+32.5^{o}C

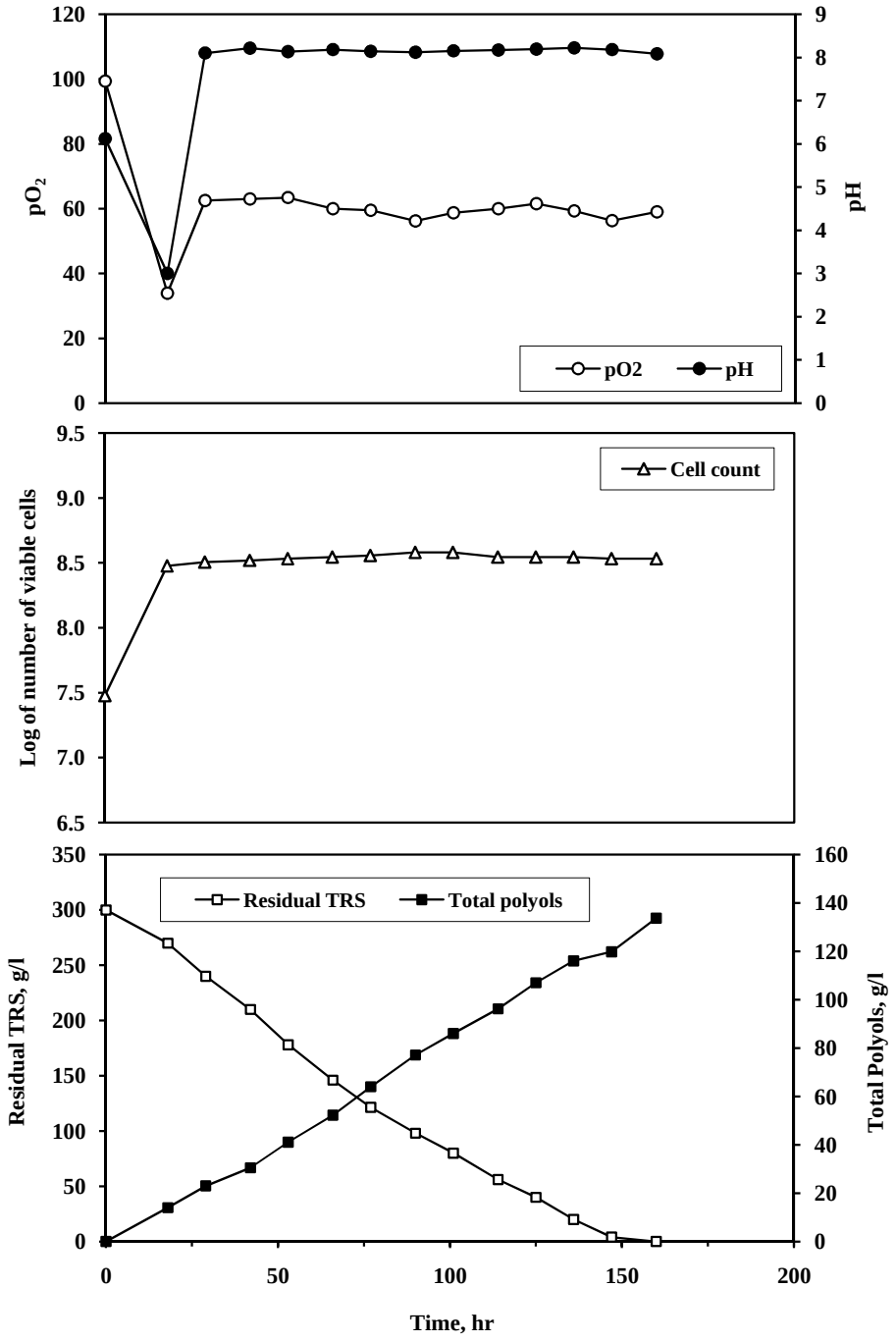


Fig. 5.8.4: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO2 at 60 % + pH 8+32.5^{o}C

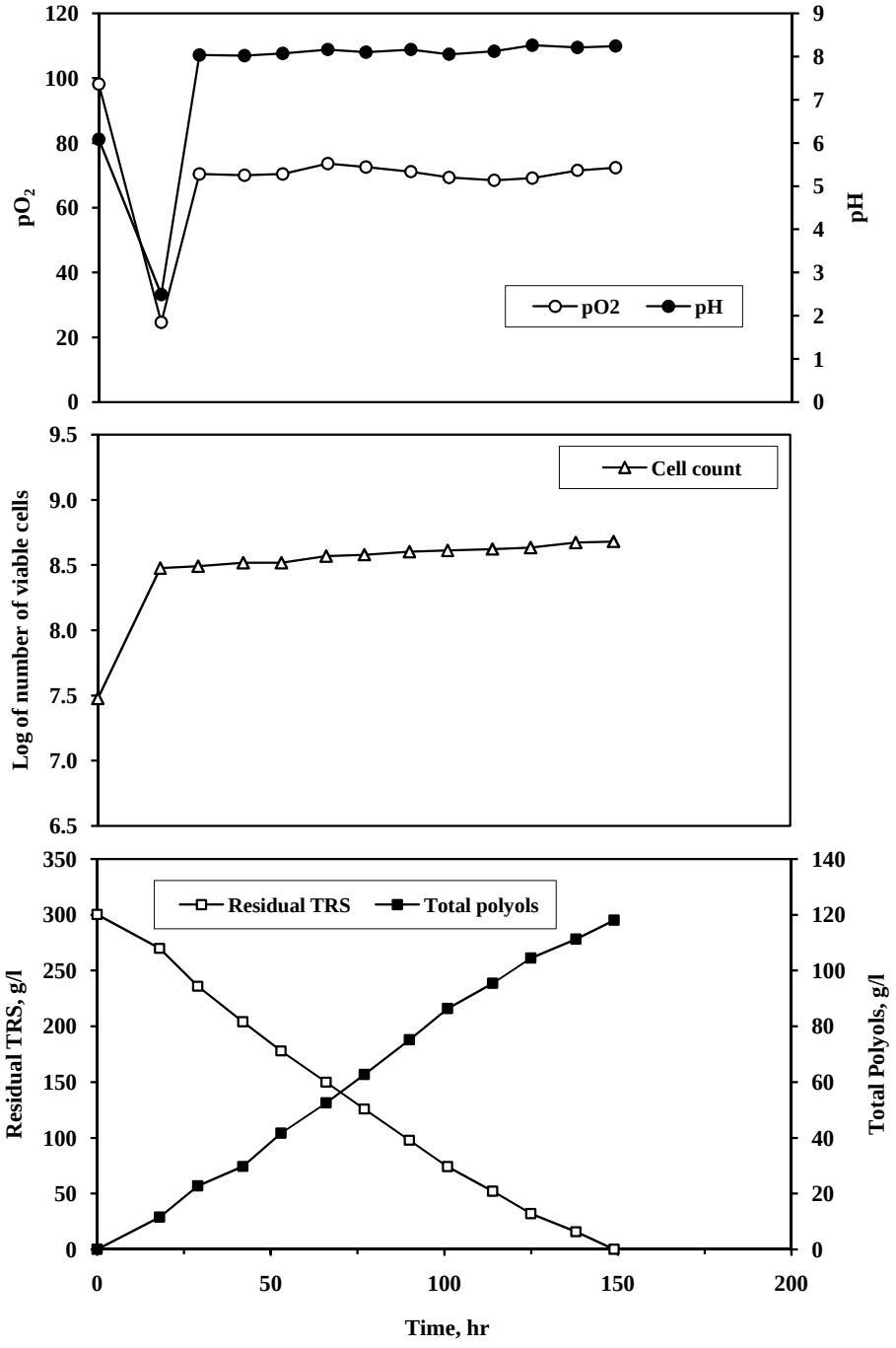


Fig. 5.8.5: Time course profile of a)Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 70 % + pH 8+32.5^{o}C

pO_2 control at 40 % saturation:

Similar trends of pO_2 and pH values were noted for pO_2 controlled at 40 % saturation level. Cell mass growth was also rapid in the early phase and entered in to stationary phase in the later part as seen in Fig. 5.8.2. The consumption of sugar was completed in about 162 hrs with an overall polyols yield of 42.47 %. The cell mass yield, however, improved to 1.467×10^8 cells/ml per 100 g of sugar utilized indicating that 40 % pO_2 value enhances cell growth as well as polyols production as compared to that achieved at 30 % pO_2 value. The final total polyols concentration also improved to 127.5 g/l.

pO_2 control at 50 % saturation:

Cell mass growth, variation in pO_2 and pH profile, all show similar trends as at 30 % and 40 % pO_2 values. However, sugar utilization was completed within 148 hrs resulting into maximum final polyols concentration of 143.5 g/l. The overall polyols yield based on sugar utilized (p/ts) improved to 47.84 %. However, cell mass yield was slightly reduced to 1.39×10^8 cells/ml per 100 g of sucrose consumed as compared to maximum cell growth at 40 % pO_2 control value. This indicates that oxygen supply to the organism has to be critically adjusted so as to control the cell growth and maximize the polyols production. The polyols productivity and sugar utilization rate reached the maximum value of 0.865 g/l/hr and 2.048 g/l/hr, respectively.

pO_2 control at 60 % saturation:

Almost identical trends were obtained for the cell mass growth, pH and pO_2. However, complete sugar utilization took longer time (160 hr) resulting into slightly reduced final polyols concentration of 133.7 g/l. Overall yield of polyols was slightly reduced to 44.57 %. The final cell mass yield at this value of oxygen % saturation was also reduced to 1.03×10^8 cells/ml per 100 g of sucrose utilized.

pO_2 control at 70 % saturation:

Though similar trends were obtained for cell mass, pO_2 and pH, the overall polyols yield further reduced down to 39.36 %. The overall yield of cell mass, however, increased to 1.50×10^8 cells/ml per 100 g of sucrose consumed. The time required to complete the fermentation batch was also reduced to 149 hrs as compared to 160 hrs required at 60 % pO_2 control experiment. The reason for increase in overall cell mass yield as well as reduction in fermentation batch duration is not clearly understood.

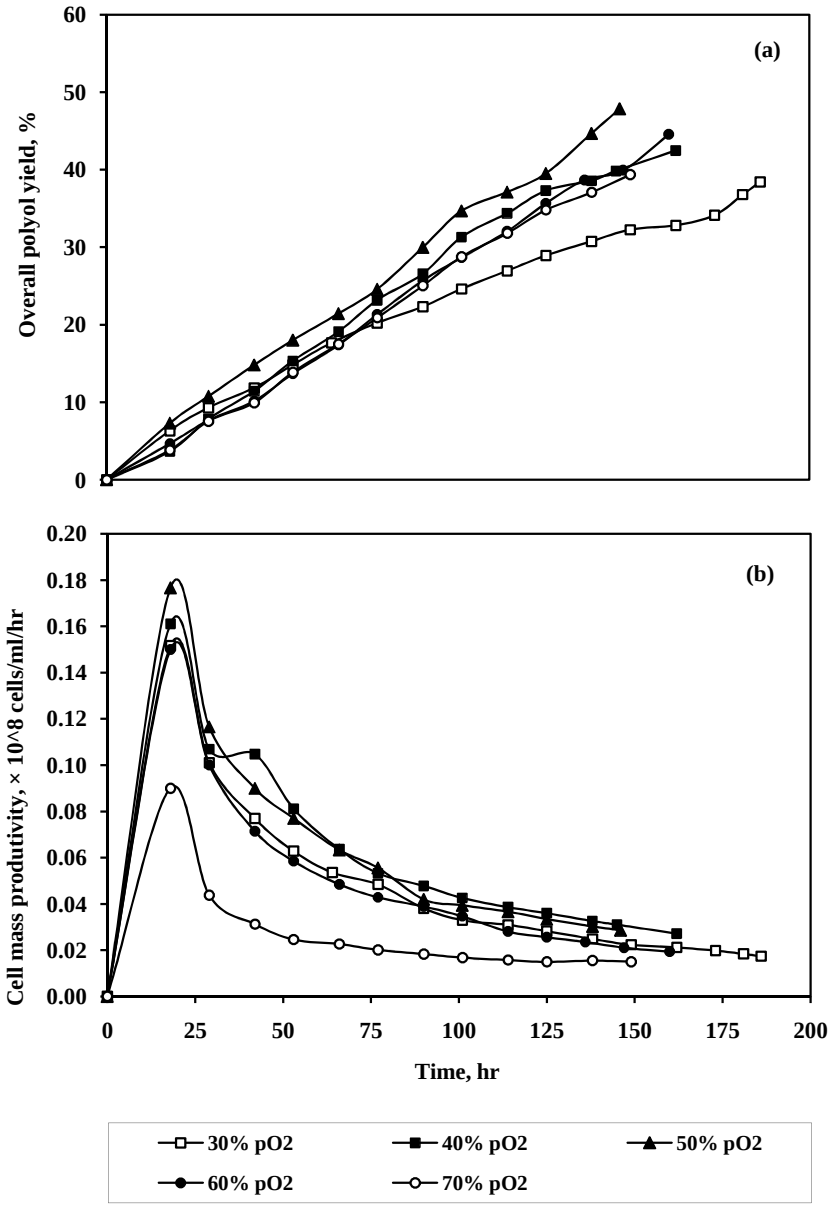


Fig. 5.8.6: Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for different percentage pO_2 control

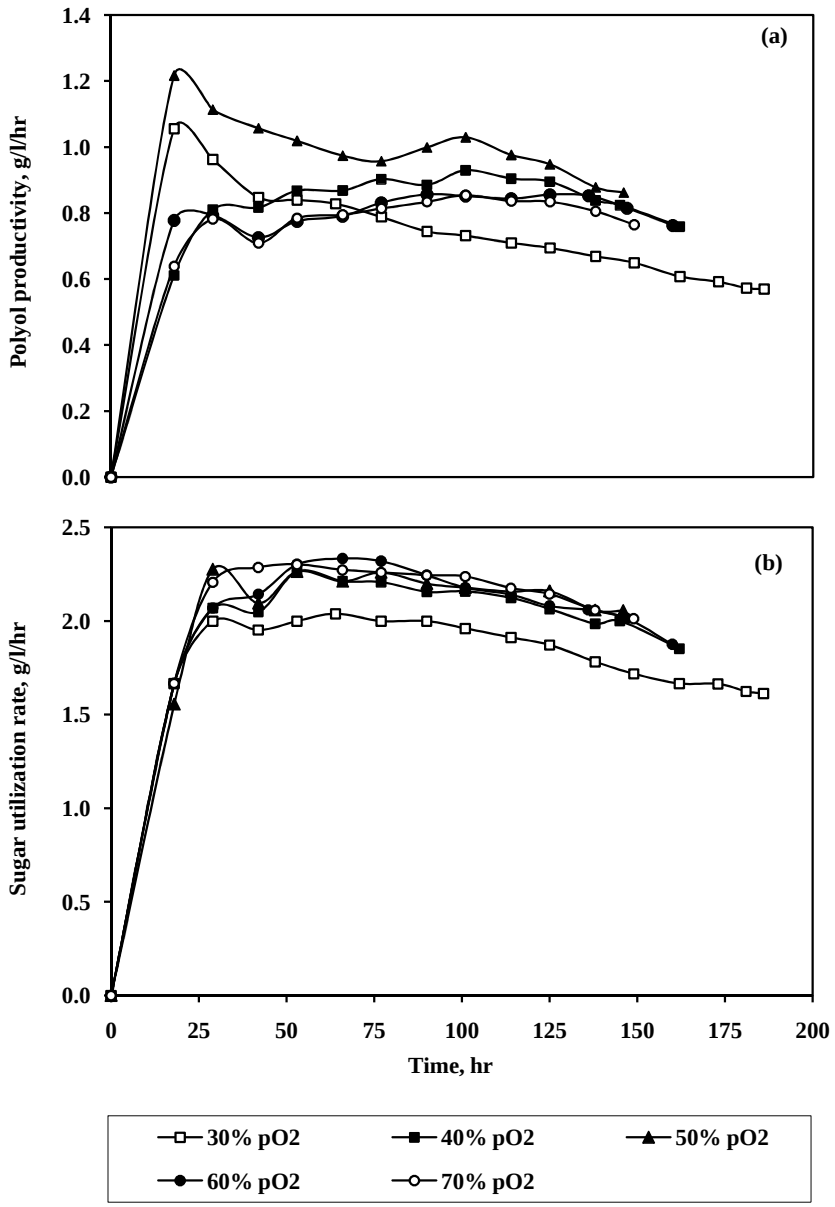


Fig. 5.8.7: Relationship between a) Polyol productivity and Time & b) Sugar utilization rate and Time for different percentage pO_2 control

The relationship between a) Overall polyols yield, b) Cell mass productivity c) Polyols productivity and d) Sugar utilization rate and time at different pO_2 control values is given in Fig. 5.8.6 and Fig 5.8.7.

Overall polyols yield for 30 % and 70 % pO_2 control experiments was substantially lower. 40 % and 60 % pO_2 control experiments resulted in improvement in polyols yield. However, the most promising results were achieved in 50 % pO_2 control experiment as seen from the trends given in Fig. 5.8.6. Similarly, polyols productivity and sugar utilization rates were also on higher side with 50 % pO_2 control value. Cell mass productivity was slightly higher at 40 % pO_2 control as compared to that of 50 % pO_2 control. The final comparative results of effect of variation of oxygen % saturation (pO_2) are also given in Table 5.8.1.

Table 5.8.1: Comparative results of effect of variation of pO_2

pO_2 Control Value (%)	**Time** (hr)	**Overall yield based on total sugars**		**Polyols productivity** (g/l/hr)	**Cell mass productivity** ($x \times 10^8$ cells/ml/ hr)	**Sugar utilization rate** (g/l/hr)
		Polyols (p/ts), (%)	**Cell mass (x_{cc}/ts) (%)**			
30	186	38.44	1.08	0.570	0.017	1.163
40	162	42.47	1.47	0.759	0.027	1.852
50	146	47.84	1.39	0.865	0.029	2.048
60	160	44.57	1.03	0.763	0.019	1.875
70	149	39.36	1.50	0.765	0.015	2.013

The above Table clearly indicates that pO_2 controlled at 50 % saturation value provides the optimum conditions for polyols production at 15 % inoculum, 32.5°C temperature and 600 rpm agitation rate.

5.9 SOLUBILITY OF OXYGEN IN FERMENTATION MEDIUM

It is known that oxygen % saturation value measured by pO_2 electrode based on Clark principle gives readings in terms of partial pressure of oxygen (pO_2) in equilibrium with the dissolved oxygen. According to Henry's law the dissolved oxygen concentration is proportional to its partial pressure,

$$\mathbf{C_L = pO_2 \times a}$$

Where '**a**' is the solubility factor

The solubility factor 'a' is strongly influenced by the temperature and also by the other dissolved solutes in the fermentation medium. In high sugar containing fermentation medium it is obvious that the solubility of dissolved oxygen at 100 % saturation will be much lower than the distilled water, which is 7.13 mg/l at 30°C and 710 mm Hg. In view of the above, it is of utmost importance that the relationship between the sugar concentration in the fermentation medium and the saturation value of dissolved oxygen concentration should be known.

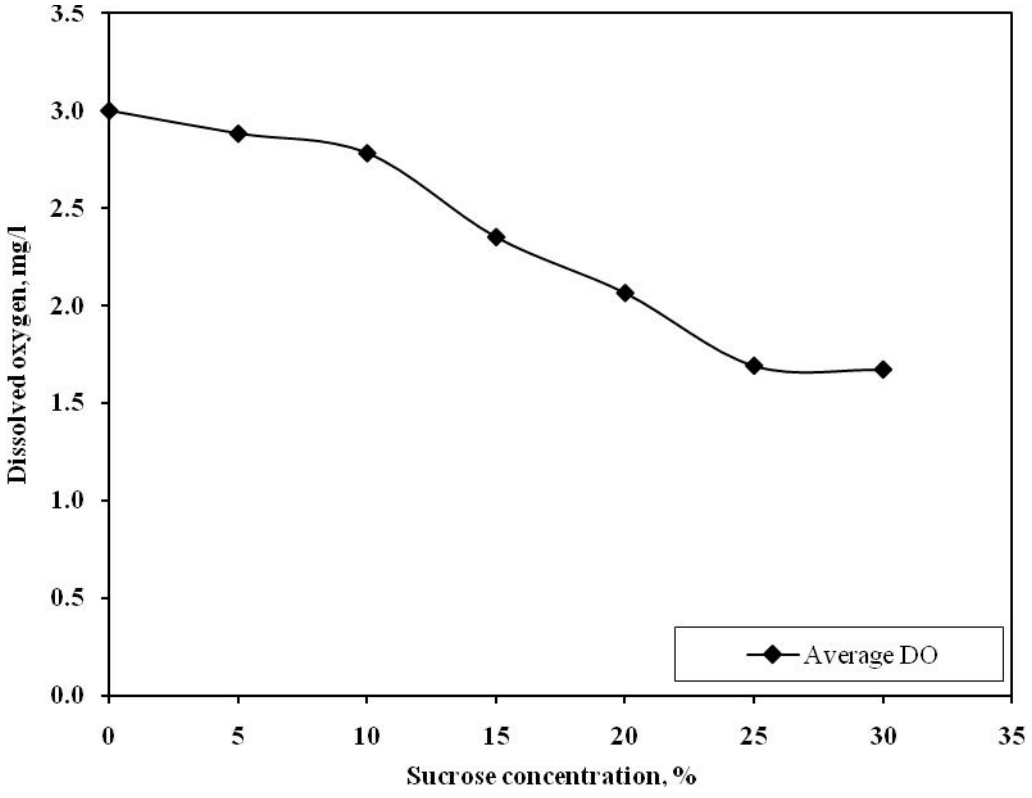


Fig. 5.9.1: Maximum oxygen solubility at various sugar concentrations

In case of fermentative production of polyols by osmophilic yeasts, sugar concentration as high as 40 % has been employed. Under these conditions it is imperative to take into consideration the effect of sugar concentration on oxygen solubility. Therefore, experiments were conducted to relate the solubility or dissolved oxygen concentration at 100 % saturation of oxygen with varying concentration of sugar. Other nutrients were also added to the medium to make it to resemble like a true fermentation medium with a pH of 5.5 – 6.0. The concentration of sucrose was varied between 0 to 30 % while concentration of other nutrients (Yeast extract = 0.125 %, Urea = 0.1 % and $MgSO_4 \cdot 7H_2O$ = 0.025 %) remained the same. The dissolved oxygen estimation was carried out by the Azid modification of the Wikler's titrimetric method based on the oxidizing property of dissolved oxygen (170, 230) as given in Chater-2.

The solubility estimation experiment was carried out in the fermenter vessel itself at 32.5°C. After filling the required quantity of fermentation medium (without inoculation) with varying concentration of sugar, aeration and agitation rates were kept at 1 vvm and 600 rpm, respectively. Samples were withdrawn when the pO_2 electrode indicated a consistent 100 % saturation value. For each sugar concentration three samples were withdrawn and average of this is taken in the plot of dissolved oxygen concentration as given in Fig. 5.9.1.

The results indicate that the oxygen 100 % saturation value in the early phase of fermentation is about 1.67 mg/l and goes on increasing to maximum 3.00 ppm as the sugar is consumed during the fermentation.

5.10 EFFECT OF INOCULUM SIZE WITH pO_2 CONTROL

As mentioned previously, the most critical factor in aerobic growth associated production of polyols is the rate of oxygen supply per unit of biomass, which determines the percentage conversion of sugars to polyols.

After optimizing the % saturation of oxygen (pO_2), we decided to once again optimize the inoculum size at fermentation temperature of 32.5°C. Therefore, experiments were conducted at fixed pO_2 value of 50 %, 32.5°C fermentation temperature, 600 rpm agitation rate. The pH was controlled at 8.0 after 18 hrs by automatic addition of Na_2CO_3. Invertase was used at a dose of 0.5 g/l. The effect of inoculum size was investigated at 10 %, 15 % and 20 %. The investigational data of individual experiments are presented in the form of the time course profiles of a) Total

polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig. 5.10.1 to 5.10.3.

RESULTS AND DISCUSSION

Effect of 10 % inoculum at 50 % pO_2:

After initial rapid growth of 18 hrs, the pH was adjusted to 8.0. At the same time pO_2 value was set to 50 % control. The cell growth shows typical rapid growth in the first part followed by stationary growth in the later part of fermentation. Complete utilization of sugar required almost 160 hrs resulting into final polyols concentration of 140.3 g/l. The overall polyols yield (p/ts) obtained was 46.46 % with a cell mass yield (x_{cc}/ts) of 1.16×10^8 cells/ml per 100 g of sugar used. The polyols productivity and sugar utilization rates achieved were 0.886 g/l/hr and 1.893 g/l/hr.

Effect of 15 % inoculum at 50 % pO_2:

Almost similar trends of cell growth, pO_2 and pH as seen in other experiments were obtained. The final polyols concentration marginally improved to 143.5 g/l. However, time required to complete the fermentation was slightly less (146 hr). The overall polyols yield obtained was slightly better at 47.84 % with polyols productivity of 0.983 g/l/hr. The sugar utilization rates achieved was 2.055 g/l/hr.

Effect of 20 % inoculum at 50 % pO_2:

Almost similar trends of cell growth, pO_2 and pH as seen in other experiments were obtained. However, time required to consume sugar completely, increased to 171 hrs. The final polyols concentration was less (126.5 g/l) as compared to experiments with 10 % and 15 % inoculum size. The overall yield based on total sugars was reduced to 42.18 % with increase in final cell mass yield at 1.98×10^8 cells/ml per 100 g of sugar consumed.

The relationship between a) Overall polyols yield, b) Cell mass productivity, c) Polyols productivity and d) Sugar utilization rate and time at different inoculum size is given in Fig.5.10.4 and Fig. 5.10.5. Cell mass productivity was higher for 20 % inoculum experiment. However, overall polyols yield and polyols productivity was higher for 15 % inoculum size experiment, particularly in the second half of fermentation time. The difference between the results of 10 % and 15 % inoculum is marginal.

Above observations confirm that 15 % inoculum size is optimum at 32.5°C temperature and 50 % pO_2 controlled conditions.

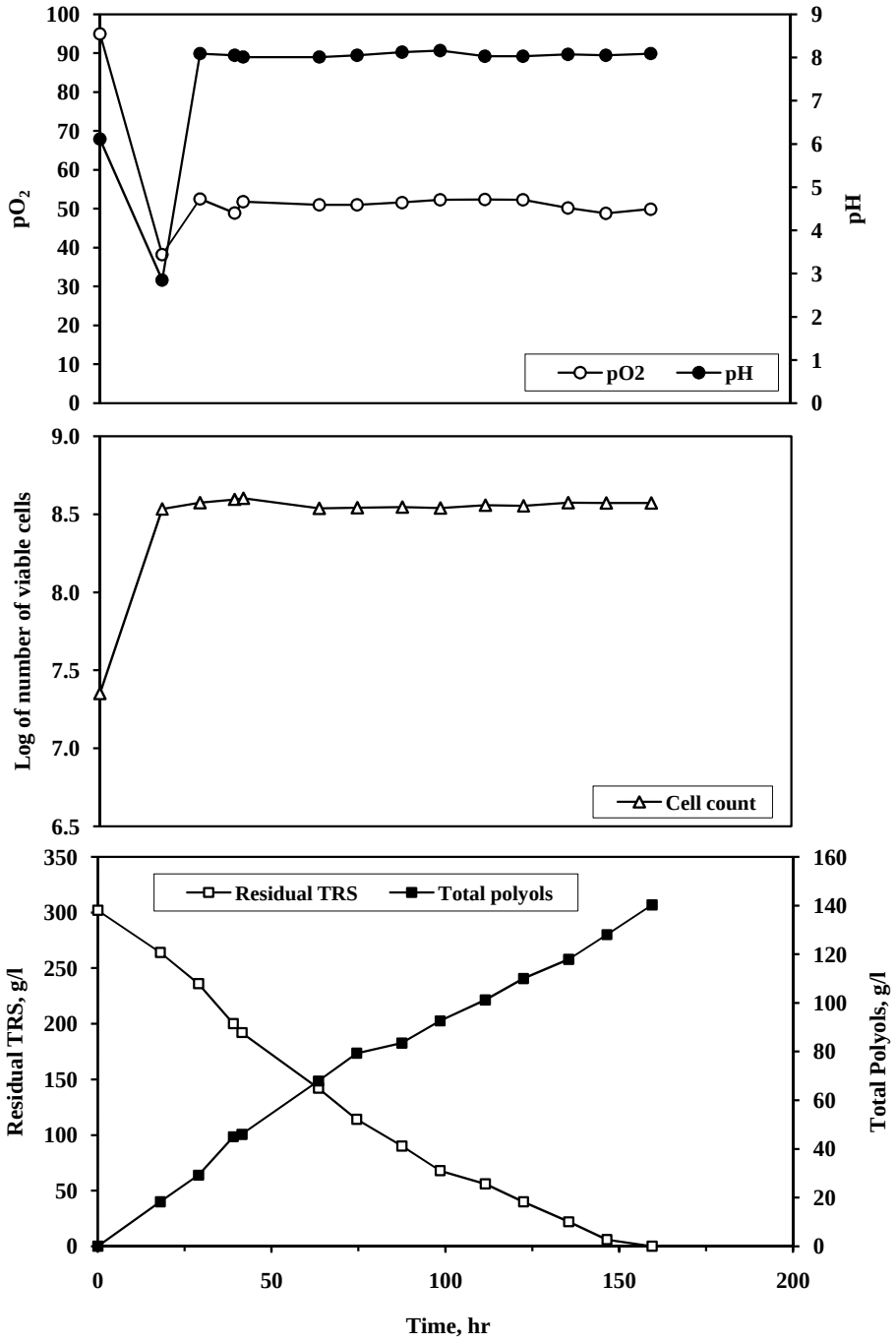


Fig. 5.10.1: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 10 % inoculum + pO_2 50 % + pH 8+32.5^{o}C

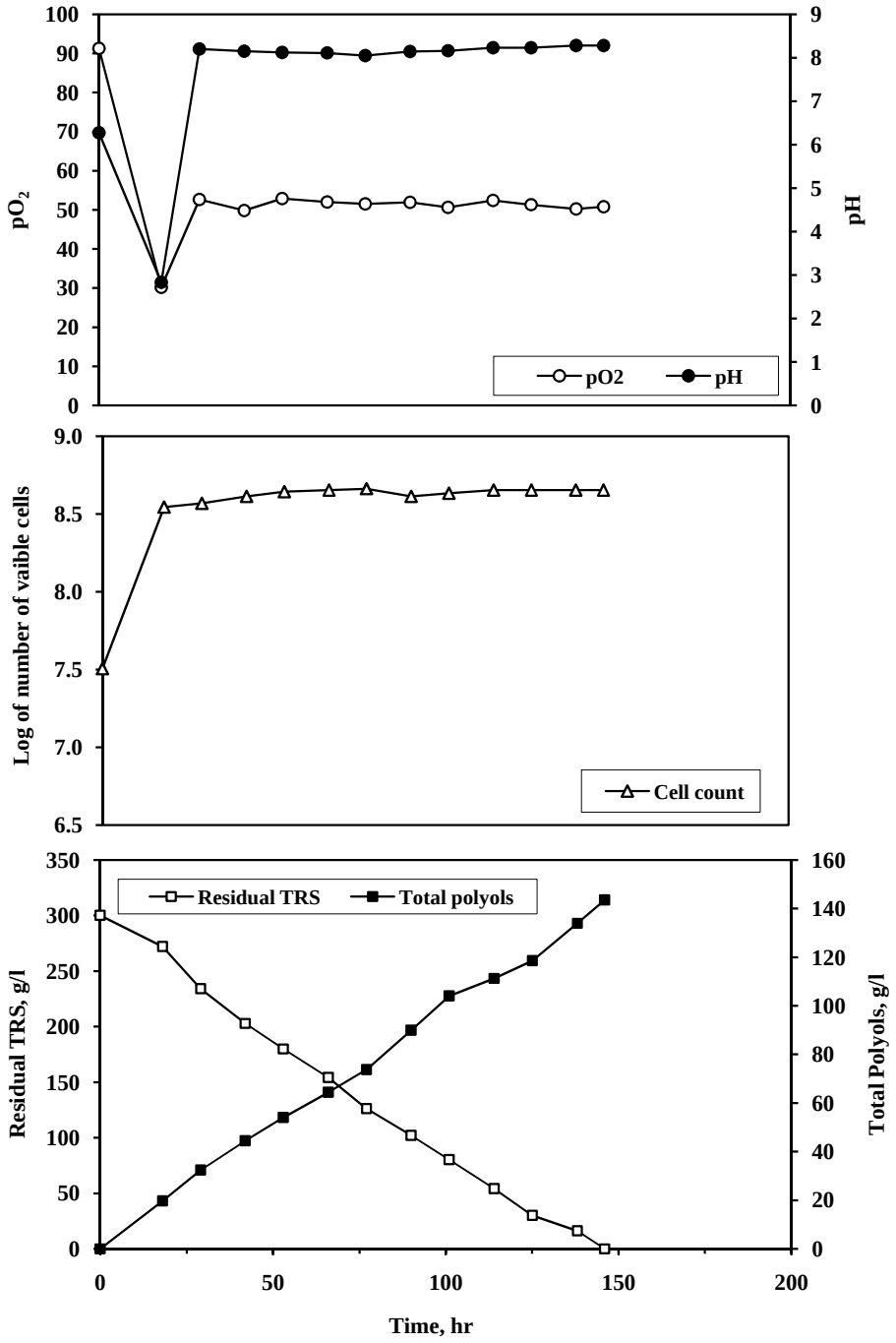


Fig. 5.10.2: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 15 % inoculum + pO_2 50 %+ pH 8.0 +32.5^{o}C

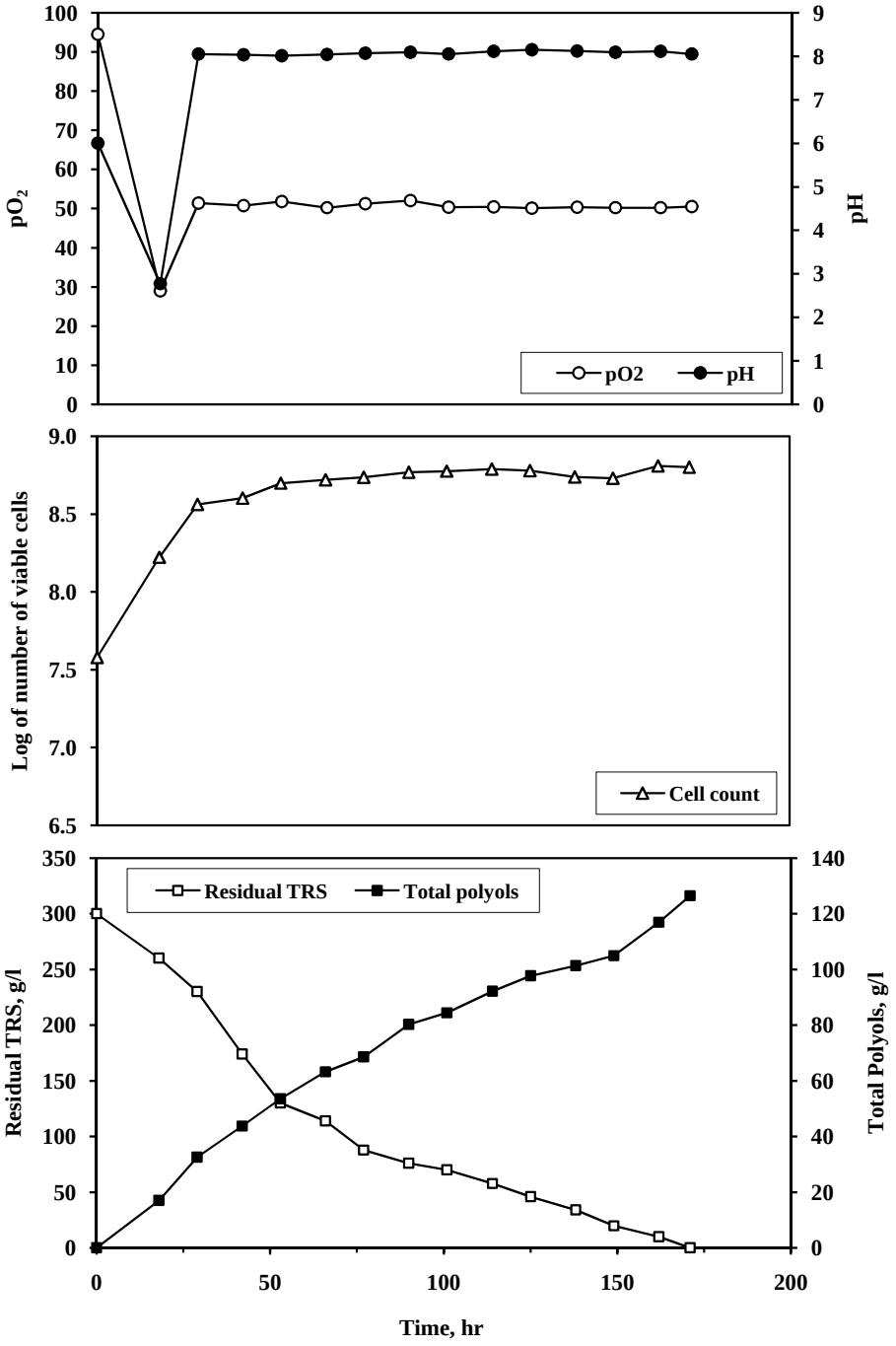


Fig. 5.10.3: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 20 % inoculum + pO_2 50 % + pH 8.0 + 32.5^{0}C

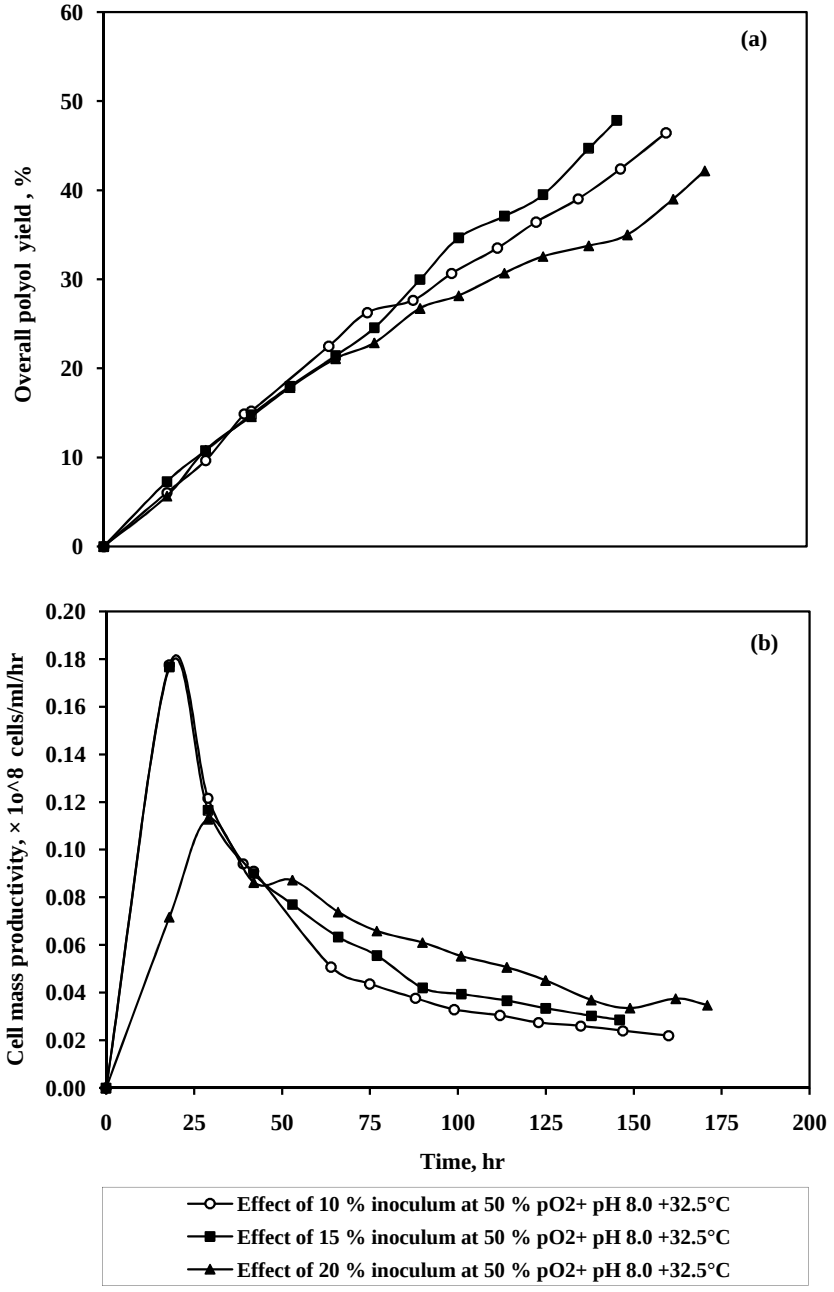


Fig. 5.10.4: Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for different percentage of inoculum at 32.5^{o}C

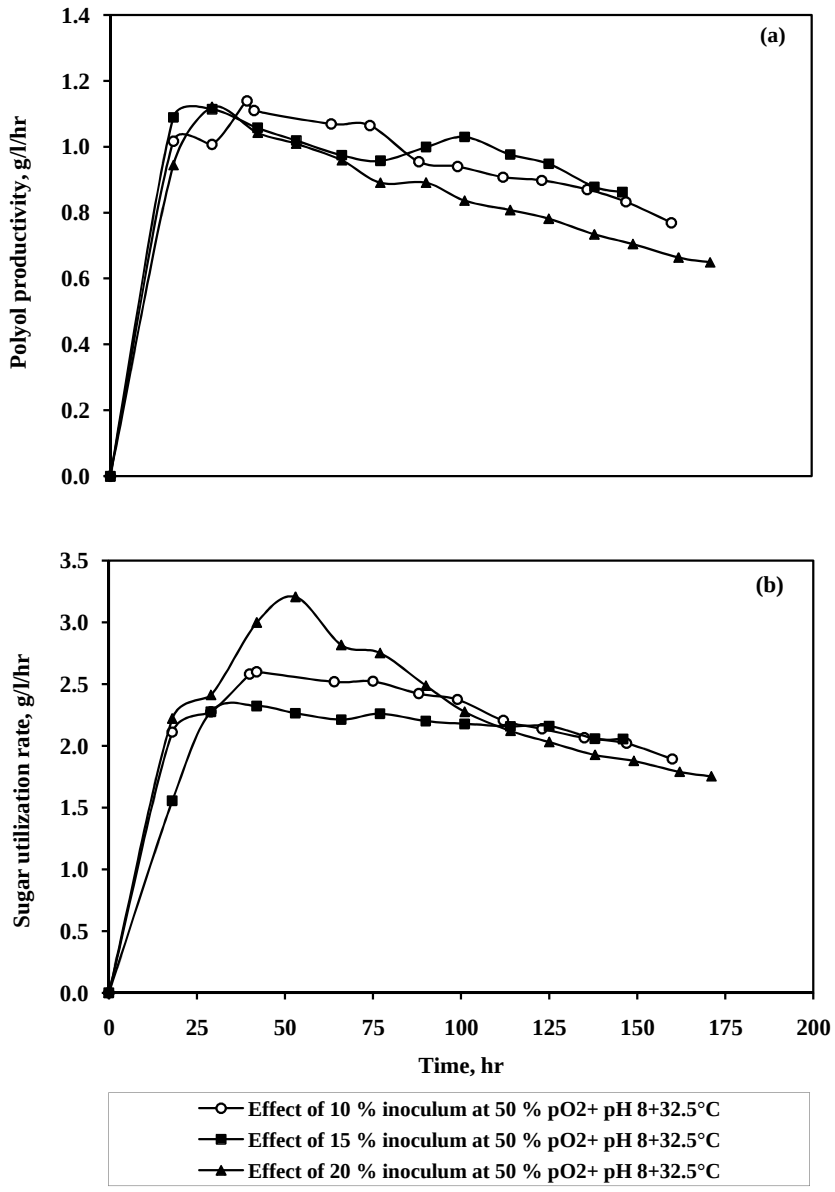


Fig. 5.10.5: Relationship between b) Polyol productivity and Time & b) Sugar utilization rate and Time for different percentages of inoculum at 32.5^{o}C

5.11 EFFECT OF AGITATION RATE WITH pO_2 CONTROL

The degree of agitation has a profound effect on the oxygen transfer efficiency of an agitated fermenter. Agitation influences the oxygen transfer by dispersing the air in the form of small air bubbles, delays the escape of bubbles from the liquid, prevents coalescence of air bubbles and decreases the thickness of the liquid film at the gas liquid interface.

In addition, agitation maintains the microbial population in suspension, controls the lump size and reduces the inter clump resistance.

In the present investigation, the effect of agitation was studied at 400, 500, 600 and 700 rpm at 50 % pO_2 control, 32.5°C temperature, 15 % inoculum, pH 8.0 and in the presence of externally added invertase. The investigational data of individual experiments are presented in the form of the time course profiles of a) Total polyols concentration, b) Residual total reducing sugars (TRS) concentration, c) Log of number of viable cells, d) pH and e) pO_2 as given in Fig. 5.11.1 to 5.11.4.

RESULTS AND DISCUSSION

Agitation rate of 400 rpm:

Typical profiles of cell growth, pO_2 and pH were seen with 400 rpm agitation rate. The fermentation was completed in about 162 hrs with final polyols concentration reaching to a value of 118.0 g/l. The overall yield of polyols based on total sugars (p/ts) was lower at 39.33 %. However, the cell mass yield was on higher side at 1.51 x 10^8 cells/ml per 100 g of sugar used. The polyols productivity and sugar utilization rates were 0.728 g/l/hr and 1.852 g/l/hr, respectively.

Agitation rate of 500 rpm:

Complete utilization of sugar under these conditions required slightly more time (162 hrs) with final polyols concentration reaching to a value of 133.3 g/l. The overall polyols yield improved to 44.42 %. However, cell mass yield was reduced ($1.35 \times 10_8$ cells/ml per 100 g of sugar consumed) as compared to cell mass yield at 400 rpm agitation rate. The polyols productivity and sugar utilization rates were 0.823 g/l/hr and 1.852 g/l/hr, respectively.

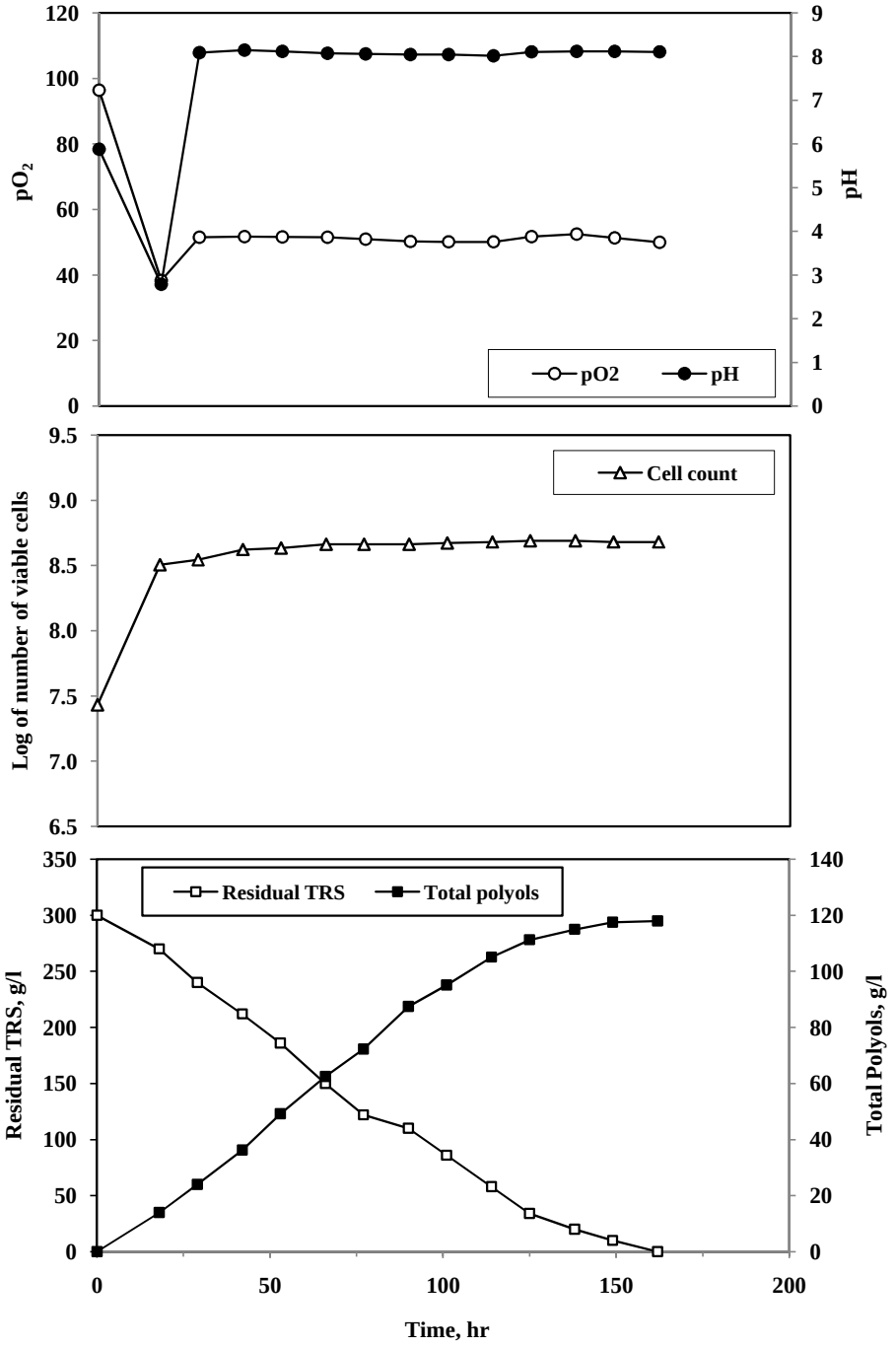


Fig. 5.11.1: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 400 rpm + pO_2 50 % + pH 8+32.5^{o}C

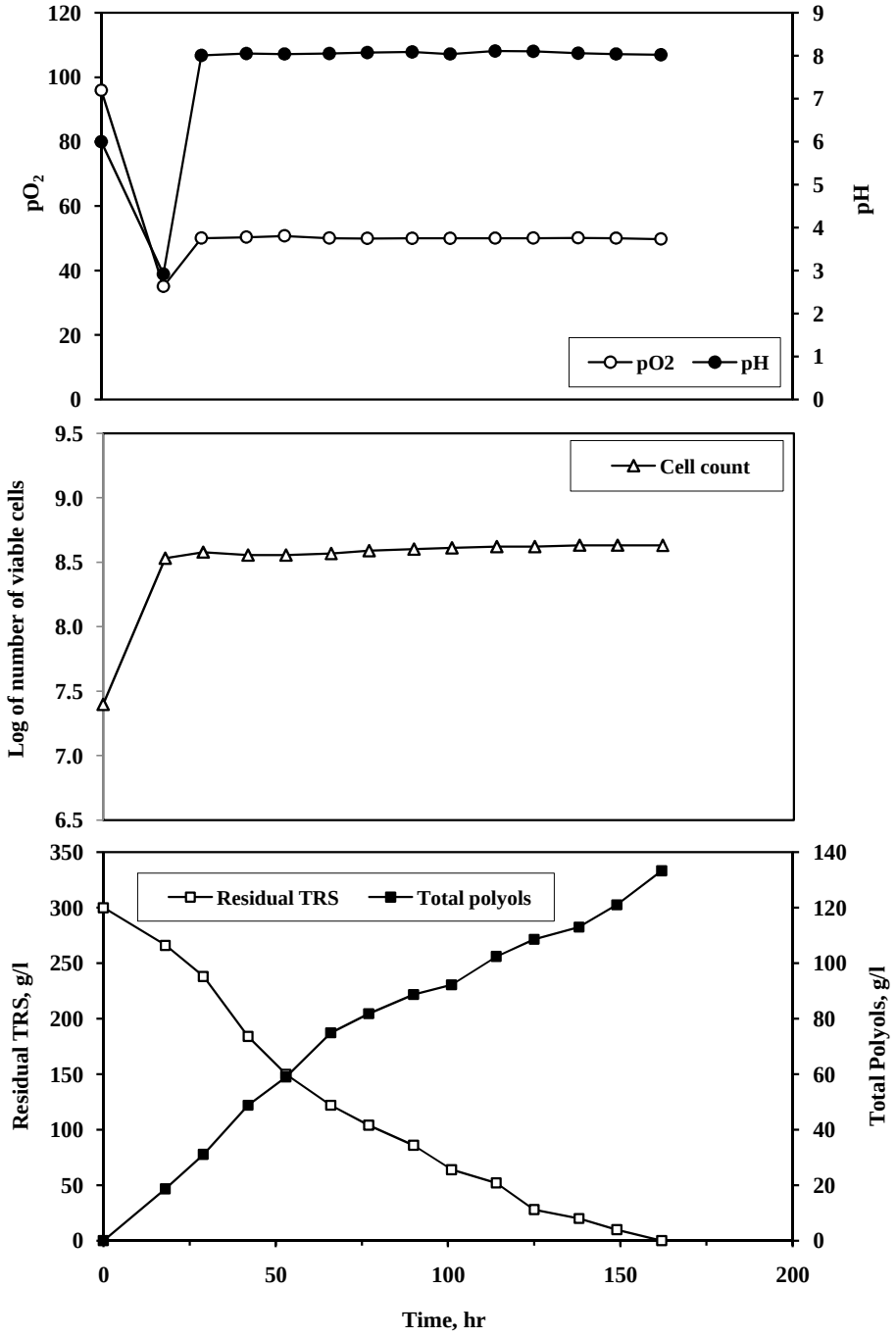


Fig. 5.11.2: Time course profile of a)Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 500 rpm + pO_2 50 % + pH 8+32.5^{o}C

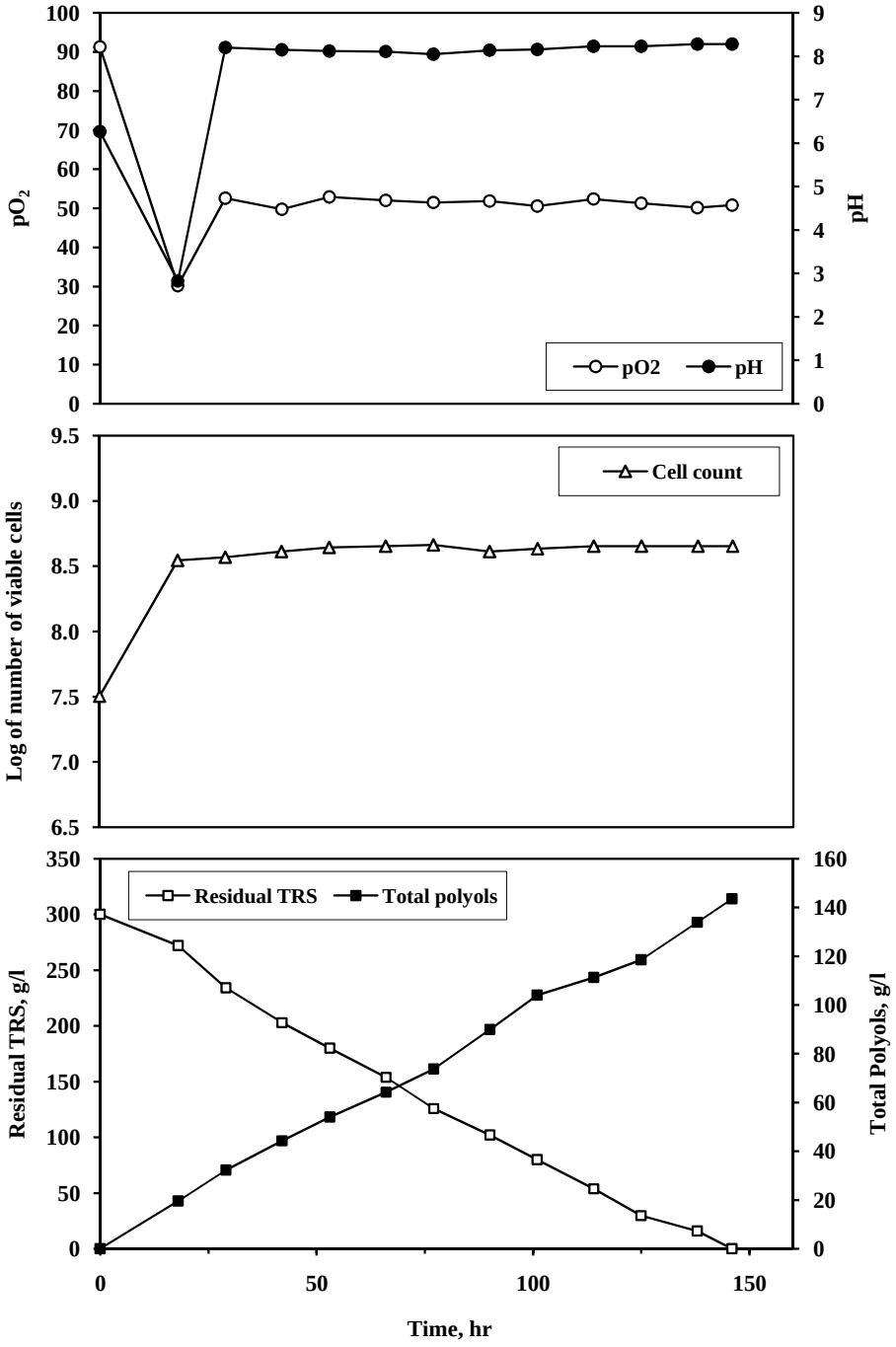


Fig. 5.11.3: Time course profile of a) Total polyols, b)Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 600 rpm + pO_2 50 % + pH 8.0 +32.5^{o}C

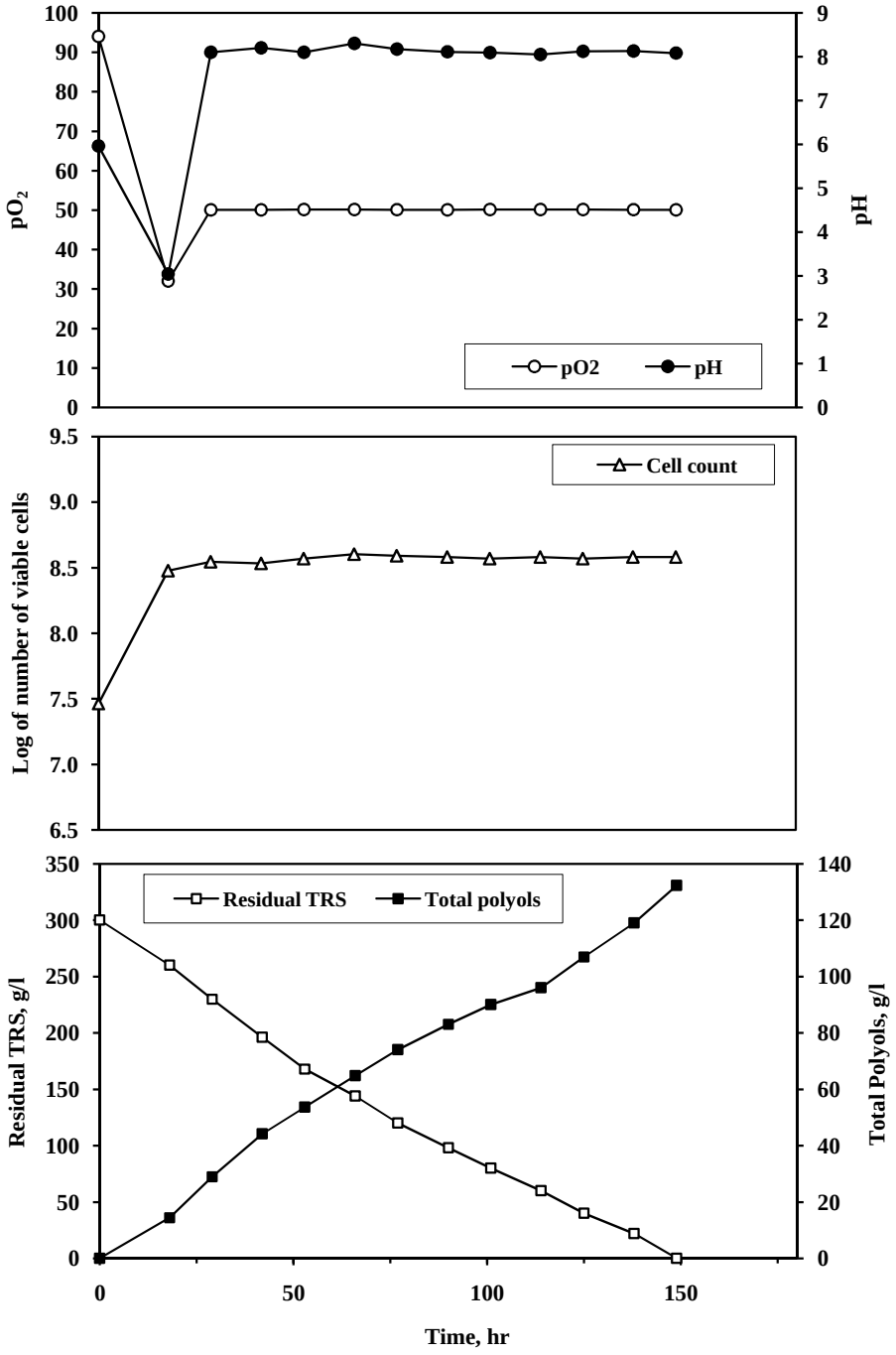


Fig. 5.11.4: Time course profile of a) Total polyols, b) Residual TRS, c) Log of number of viable cells, d) pH and e) pO_2 at 700 rpm + pO_2 50 % + pH 8.0 +32.5^{o}C

Agitation rate of 600 rpm:

Maximum final polyols concentration of 143.5 g/l was achieved in a period of 146 hrs of fermentation. Maximum polyols productivity (0.983 g/l/hr) and maximum sugar utilization rate (2.055 g/l/hr) were achieved at 600 rpm agitation rate. The overall polyols yield obtained was also maximum (47.84 %) with cell mass yield of 1.39×10^8 cells/ml per 100 g of sugar utilized.

Agitation rate of 700 rpm:

Similar trends of cell mass growth, pO_2 and pH were also seen with 700 rpm agitation rate. Completion of fermentation required 149 hrs and final polyols concentration reached a value of 132.3 g/l. Overall yield of polyols was reduced slightly to 44.08 % as compared to 47.84 % at 600 rpm The polyols productivity and sugar utilization rates were reduced to 0.888 g/l/hr and 2.013 g/l/hr, respectively. The cell mass yield was also reduced slightly.

The relationship between a) Overall polyols yield, b) Cell mass productivity, c) Polyols productivity and d) Sugar utilization rate and time at different agitation rates are given in Fig. 5.11.5 and Fig. 5.11.6. Agitation rates between 400 to 700 rpm have not affected the polyols productivity and cell mass productivity significantly, as seen from the Fig. 5.11.5. However, most optimum results of polyols yield were obtained with 600 rpm agitation rate.

Based on the investigations carried out on 2.5 liter fermenter scale, the optimum conditions for production of polyols using sucrose as carbon source are,

Inoculum size = 15 %, pO_2 = 50 % saturation, Agitation rate = 600 rpm, Temperature = 32.5°C, pH = 8.0, Invertase dose = 0.5 g/l

The Gas chromatographic analysis used for qualitative estimates indicated 4:1 ratio of glycerol to arabitol in the final fermentation broth sample.

5.12 FED-BATCH FERMENTATION

INTRODUCTION

Glycerol and other polyols produced by osmophilic yeasts are non-toxic to the organism and, therefore, can be accumulated to a high concentration level in batch fermentation. However, to maintain optimum productivity of polyols as well as optimum sugar utilization rate, it is necessary to maintain 30 % initial sucrose concentration in batch fermentation. It is reported that in batch fermentation, the high initial sugar

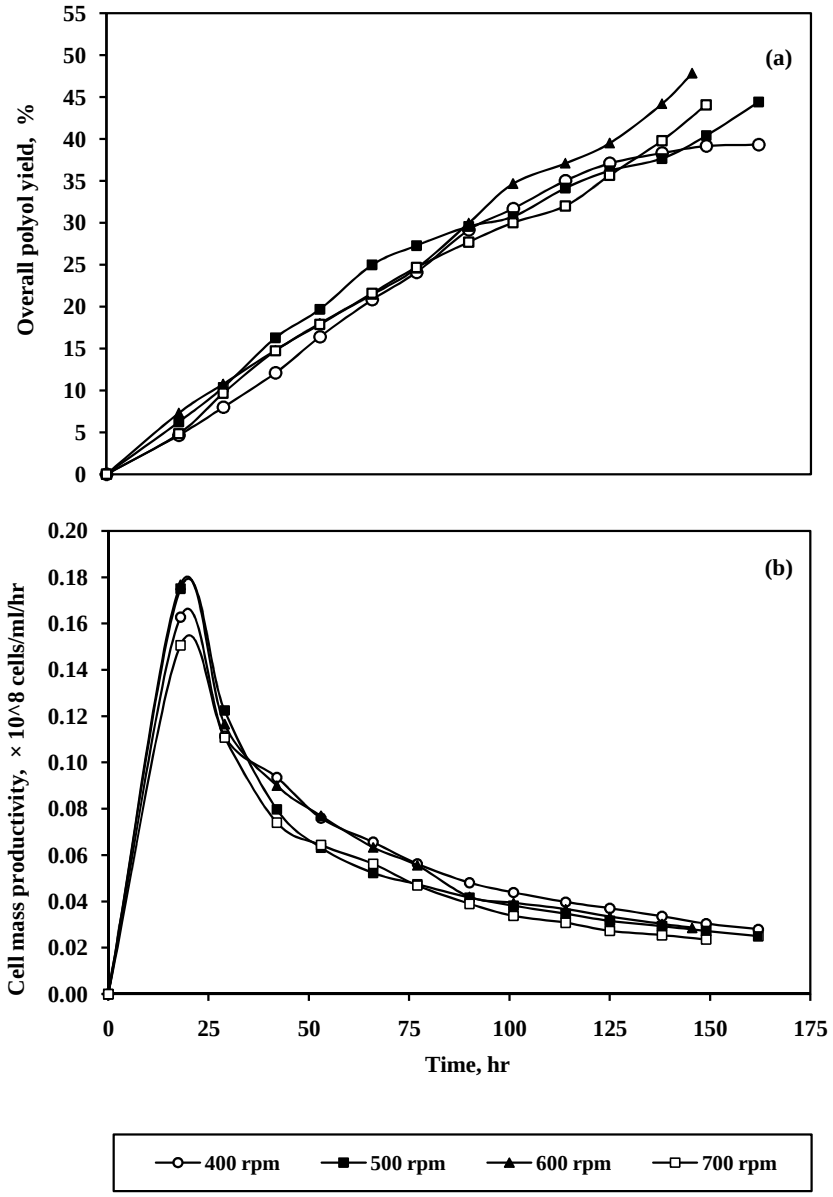


Fig. 5.11.5: Relationship between a) Overall polyol yield and Time & b) Cell mass productivity and Time for different agitation rates

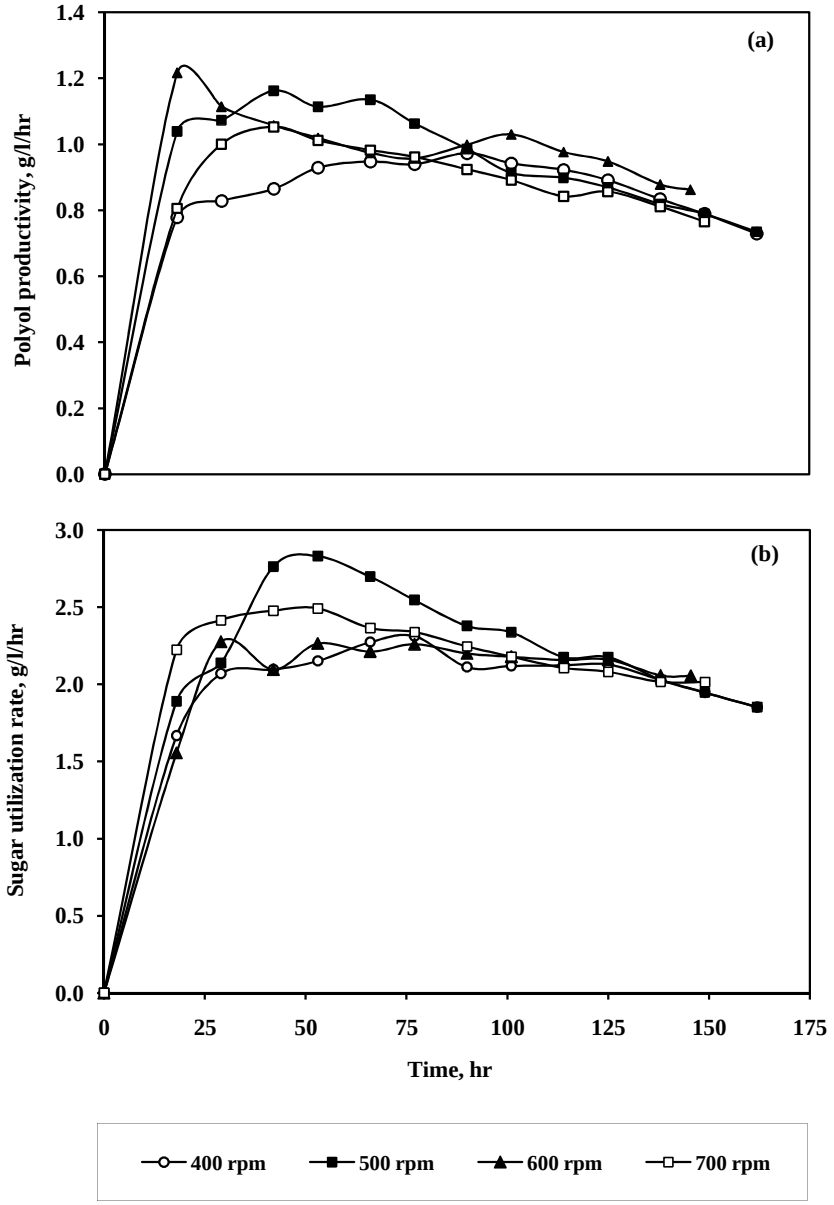


Fig. 5.11.6: Relationship between a) Polyol productivity and Time & b) Sugar utilization rate and Time for different agitation rates

concentration leads to significant inhibition of cell growth while low initial sugar concentration resulted in reduced glycerol productivity (231, 232). High initial sugar concentration in batch fermentation would also require very high air flow rates to maintain the oxygen transfer efficiency and can lead to excessive foaming problem in fermentation.

Therefore, fed-batch fermentation seems to be an attractive option for polyols production by osmophilic yeasts. With suitable aeration rate and fed-batch approach, Burschäpers et al. (74) have reported better results as compared to batch fermentation. Under optimal conditions of fermentation, they could achieve high erythritol concentration (up to 170 g/l) and high polyols concentration (up to 188 g/l). For production of glycerol in fed-batch fermentation, Sun (233) and Yang (234) have reported that glucose and corn steep liquor (the main phosphorus source) were the two essential nutrients to be fed. They proposed that dry glucose powder should be fed in pulse form at every certain time interval to maintain glucose concentration within certain range, while corn steep liquor should be fed just based on their experience. Using fed-batch fermentation approach, Button et al. (104) have reported a final glycerol concentration of 17 % at the end of 240 hrs of fermentation. In their process, the desired cell population was rapidly grown in the first phase of the fermentation and the terminal cell population was maintained in the proper phosphate deficient condition. The substrate was then periodically added as the conversion of glucose to glycerol was catalysed by the stationary cell population.

Vijaikishor (81) has also used fed-batch mode for conversion of glucose to glycerol under alkaline (pH 8.2) fermentation conditions. He has used glucose and salts in powder form. Glucose level in fermentation was continuously monitored and as soon as it reached 2 %, additional glucose along with salts were added directly so that the glucose level is brought back to about 10 %. In a period of 192 hrs, he could achieve a final glycerol concentration of 30 %. This is probably the highest glycerol concentration reported anywhere so far for glycerol production by fermentation.

EXPERIMENTAL

Apart from feed control strategy, the effect of oxygen transfer, inoculum size and temperature would also play an important role in fed-batch fermentation. Burschäpers et al. (74) have concluded that it is necessary to maintain oxygen concentration above 19 %

saturation in fed-batch fermentation to produce maximum polyols. Xie et al. (235) have proposed a model based approach for optimization of temperature and feed-control strategies for glycerol production by fed-batch culture of osmophilic yeast *Candida krusei*.

Therefore, it was decided to investigate the fed-batch fermentation aspect in two sets of experiments. In the first set of experiments, 10 % inoculum was used at 30°C fermentation temperature without pO_2 control. Under these conditions, the feed control strategy was investigated as described in three (5.12.1 to 5.12.3) different experiments. In the second set 15 % inoculum was used at 32.5°C fermentation temperature and pO_2 controlled at 50 %. Under the conditions of second set, the feed control strategy was investigated as described in three (5.12.4 to 5.12.6) different experiments. Except in one experiment, sucrose was used in powder form whereas all other nutrients were dissolved in distilled water and sterilized prior to addition during fed-batch fermentation.

All fed-batch fermentations were initiated as described for the batch fermentation experiments. The starting volume of fermentation medium was 2.5 lit. Experiments were conducted in 7.0 liter capacity CHEMAP fermenter, which allowed for increase in the fermentation broth volume. Invertase (Sucrose L 300 from Biocon India Ltd.) at a dose of 0.5 g/l of fermentation broth was added only once during the start of fermentation. The strategy of addition of sugar and nutrients in each experiment is described under the individual experiments.

RESULTS AND DISCUSSION

Set-I : Fed-batch experiments with 10 % inoculum, 30îC fermentation temperature without pO_2 control at 8.00 pH and in the presence of externally added invertase.

5.12.1: Refined sugar (sucrose) was used for direct addition during the fermentation. Sucrose was added at 76 and 161 hrs when the residual TRS was reduced to 92 g/l and 72 g/l, respectively. All nutrients were used as given in Table 5.3.1 (fermentation medium composition). However, the dose of nutrients was proportionally reduced depending on the quantity of sucrose used. The experimental data generated for this fed-batch experiment is given in Annexure-I (Table A-1). The time course profile of a) Residual TRS and total polyols concentration, b) Cell count and optical density of

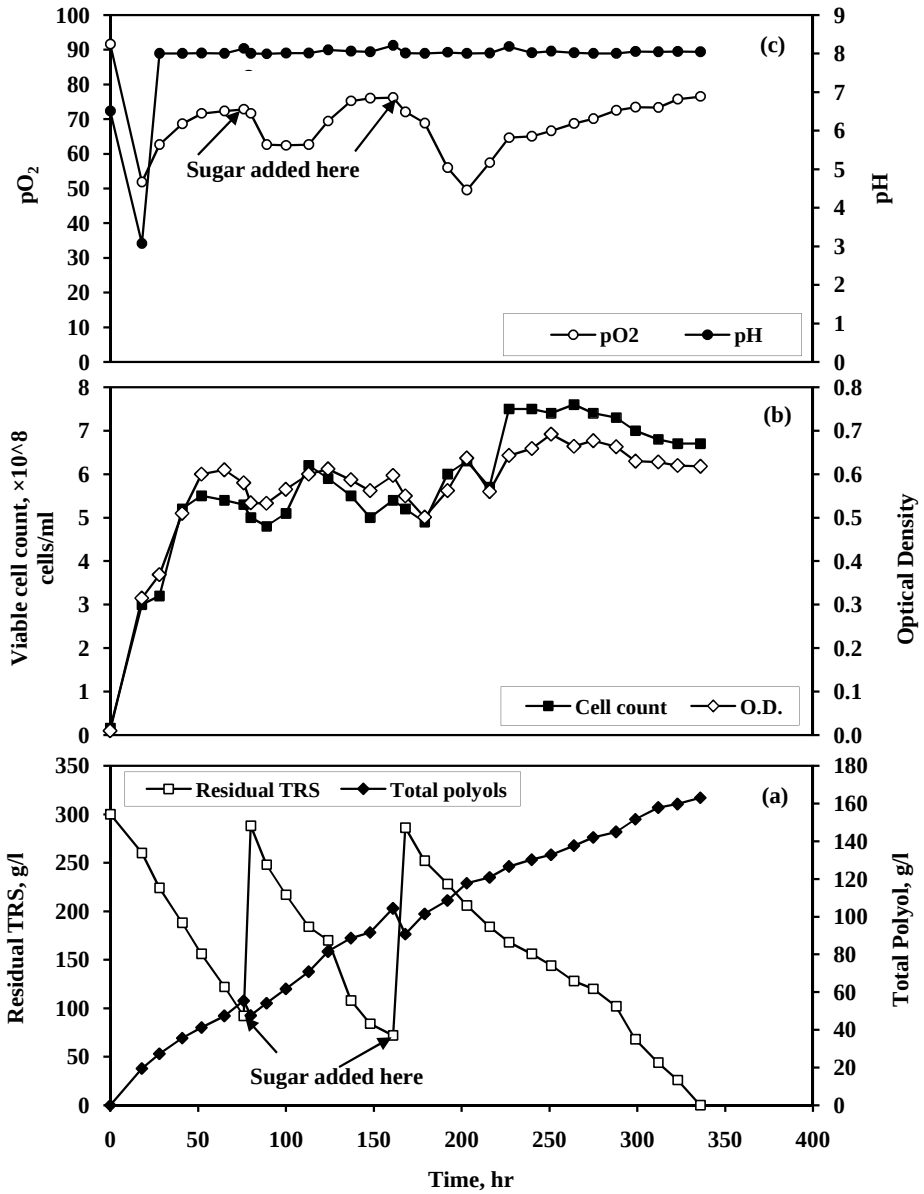


Fig. 5.12.1: Time course profile of a) Residual TRS and Total polyols, b) Cell count and Optical density of fermentation broth and c) pO_2 and pH during fed-batch fermentation of experiment 5.12.1

fermentation broth and c) pO_2 and pH during this fed-batch experiment is shown in Fig. 5.12.1. It can be seen from Table A-1 (Annexure-I) that two lots of sucrose was added. The quantity of nutrients based on sucrose used worked-out to be a) For 1st lot: Yeast extract: 2.333 g, Urea: 2.053 g and $MgSO_4 \cdot 7H_2O$: 0.478 g. and b) For 2nd Lot: Yeast extract: 2.225 g, Urea: 1.958 g and $MgSO_4 \cdot 7H_2O$: 0.456 g. The sucrose was added directly taking maximum precaution to maintain aseptic conditions. The nutrients were mixed in 100 ml distilled water and sterilized in an autoclave at 1.1 bar pressure for 15 minutes. The sterilized and cooled solution of nutrients was added immediately after adding the sucrose crystals.

Due to addition of sugar and nutrients as well as Na_2CO_3 solution used for controlling the pH, the volume of fermenter was increasing gradually. At the same time, due to heavy aeration rate employed, there was also some loss of moisture through the vent. It was not possible to measure the fermenter volume because of entrapped air and presence of foam. Therefore, the volume of the fermenter was measured only after termination of fermentation batch. In Table A-1 (Annexure-I) the final fermenter volume is given against the last sample drawn. All the results calculated are based on the total sucrose used, residual TRS, polyols concentration at the end of fermentation and final volume of fermentation broth.

It can be seen from Fig. 5.12.1 that oxygen % saturation (pO_2) value drops rapidly during the first 18 hrs because of rapid cell growth. At 18 hrs, the pH is adjusted to 8.0. The pO_2 value starts increasing, probably because of alkaline conditions in the fermenter and also because the cells enter the stationary phase or slow-growth phase. When sugar and nutrients are added, the pO_2 value drops probably because of increase in volume and increase in the solute concentration of the fermentation broth. This phenomenon was observed with all fed-batch experiments. In this experiment the pO_2 remained in between 65 % to 75 % during the second phase of fermentation.

In Fig. 5.12.1, the increase in cell count is also plotted against time. As mentioned previously in Chapter 2, we have monitored the cell growth by taking direct cell count. It can be seen that the cell count fluctuates during the fed-batch fermentation because of intermittent addition of sugar plus nutrients and Na_2CO_3 used for pH adjustment. We have also monitored the cell growth by measuring optical density of fermentation broth sample after appropriate dilution at 660 nm in a spectrophotometer. The trend of optical density is also plotted along with the trend of increase in cell count.

Both the trends exhibit similar behaviour suggesting that after the initial rapid growth there is a stationary kind of slow-growth phase during which also polyols are produced.

The plot of residual TRS and total polyols against time indicates the typical fed-batch pattern. Total 1843.8 g of sugar was consumed in a period of 336 hrs generating polyols at a final concentration of 163 g/l.

5.12.2: Refined sugar (sucrose) was used for direct addition during the fed-batch fermentation. Sucrose was added at a time when the residual sugar concentration was reduced to about 10-13 %. In this experiment, the dose of nutrients was reduced to 50 % of the original nutrients concentrations used to start the fermentation. Therefore, only 50 % of the nutrients were added based on sucrose to be used. The dose of nutrients was reduced with the anticipation that some of the nutrients added during the start of fermentation would still be available in the medium and excess availability of nutrients may promote cell growth at the expense of product of formation.

The data generated for this fed-batch experiment is given in Table A-2 (Annexure-I). It can be seen that sucrose was added three times during the fermentation period of 402 hrs. The nutrients at a reduced concentration of 50 % (based on sucrose used) were also added after dissolving the same in 100 ml distilled water, sterilization and cooling to room temperature. The time course profile of a) Residual TRS and total polyols concentration, b) Cell count and optical density of fermentation broth and c) pO_2 and pH during fermentation is illustrated in Fig. 5.12.2.

Sucrose plus nutrients were added at 77, 149 and 258 hrs and the final fermentation broth volume recorded was 3.07 liters. Similar trend of pO_2 variation is seen in Fig. 5.12.2 as it is described for the previous experiment. Cell count variations run exactly parallel to the changes in the optical density reading. This proves that our approach of measuring the cell number is valid and accurate. These trends again indicate that after the initial exponential growth, there is a second phase of slow growth or stationary like growth in which the products are produced throughout the fed-batch fermentation.

The plot of residual sugar and polyols against time indicates the typical fed-batch pattern in which almost 2192.5 g of sucrose was consumed. The total time required for this fed-batch experiment was 402 hrs and final polyols concentration reached was 23.0 %. This indicates that there is no inhibition of fermentation from the products

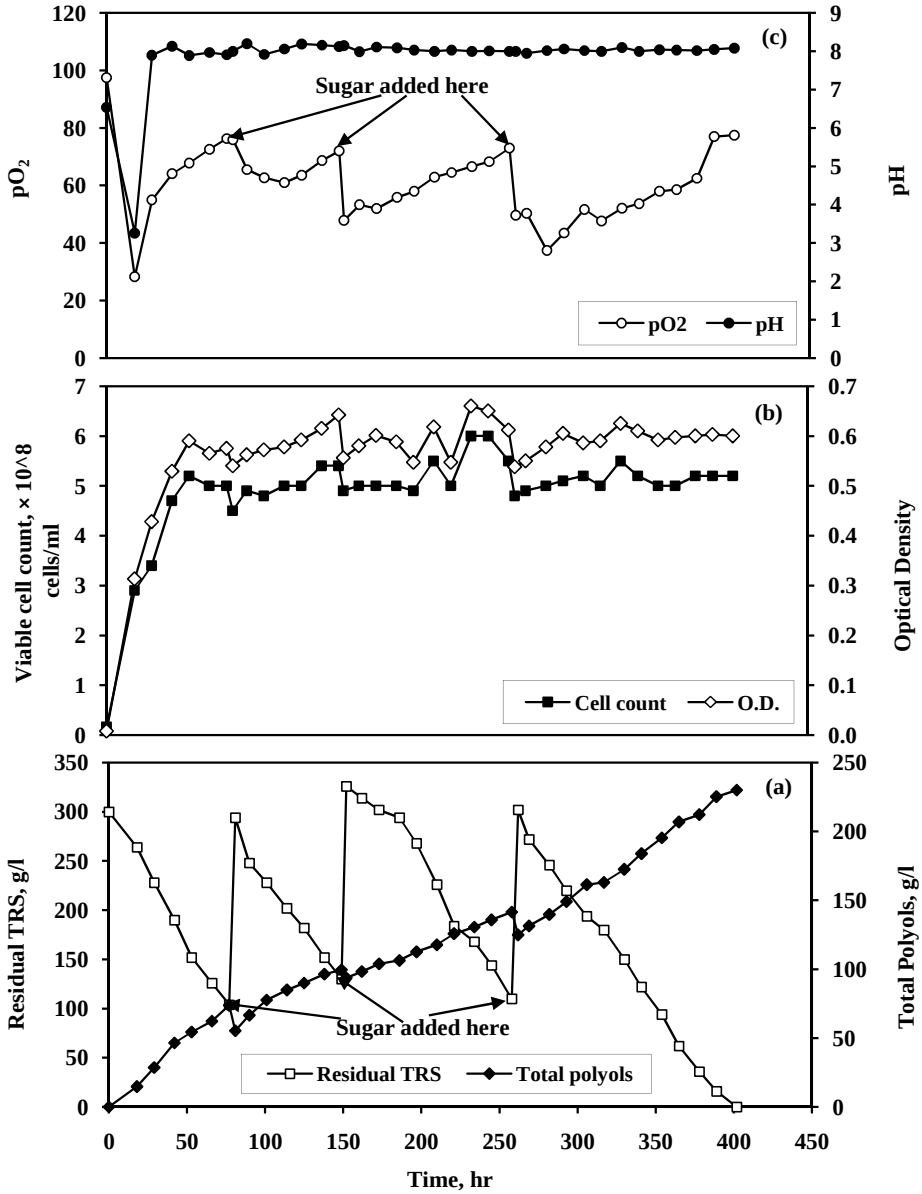


Fig. 5.12.2: Time course profile of a) Residual TRS and Total polyols, b) Cell count and Optical density of fermentation broth and c) pO_2 and pH during fed-batch fermentation of experiment 5.12.2

(polyols) produced by this osmophilic yeast. Why the final fermentation broth volume was reduced to 3.07 liters as compared to 3.55 liters in the previous experiment is not clear. This reduction in volume may be because of prolonged fermentation time and consequent evaporation losses.

5.12.3: In this experiment, the dose of nutrients was further reduced to 25 % of the initial dose. Refined sugar (sucrose) was used for direct addition. Sucrose was added only once when the residual TRS concentration was reduced to 10 %. Nutrients were sterilized separately and added along with the solid sucrose taking precautions to maintain aseptic conditions as much as possible.

The data generated for this fed-batch experiment is given in Table A-3 (Annexure-I). Sucrose was added slightly after 90 hrs. The time course profile of a) Residual TRS and total polyols concentration, b) Cell count and optical density of fermentation broth and c) pO_2 and pH during this fermentation is shown in Fig. 5.12.3.

The pO_2 dropped rapidly in the early phase but then remained at around 35 % to 55 % throughout the fermentation. The reason for this drop in pO_2 is not clear. The cell growth indicates drop in viable cell count in the last few hours of fermentation probably indicating insufficient availability of nutrients. This is also supported by the fact that sucrose was consumed at a reduced rate in the later part of fermentation. At the end of 258 hrs, more than 13 % of sugars remained unutilized. The organism still continued to produce polyols though at a very low rate. The maximum polyols concentration reached to 131.6 g/L in 258 hrs. The batch was terminated because of very slow rate of sugar consumption and microscopic detection of few contaminants in the fermenter. The final volume of fermented broth recorded was 2.375 liters.

The comparative results of first three fed-batch experiments are given in Table 5.12.1. It can be seen that optimum results are achieved in fed-batch experiment where the dose of nutrients is reduced to 50 % of the initial dose. With this dose of nutrients the overall polyols yield obtained was 32.2 %. The cell mass yield, defined as number of viable cells generated per 100 grams of total sugars, was less than in experiment 5.12.1 and higher than in experiment 5.12.3. This indicates that limited/controlled growth is required to achieve maximum polyols yield. In experiment 5.12.1, maximum cell mass yield is obtained at the expense of final polyols. In experiment 5.12.3, lower cell mass yield has resulted into lowest overall polyols yield. Maximum polyol productivity (0.572 g/l/hr) and maximum sugar utilization rate is obtained in experiment 5.12.2 (50 %

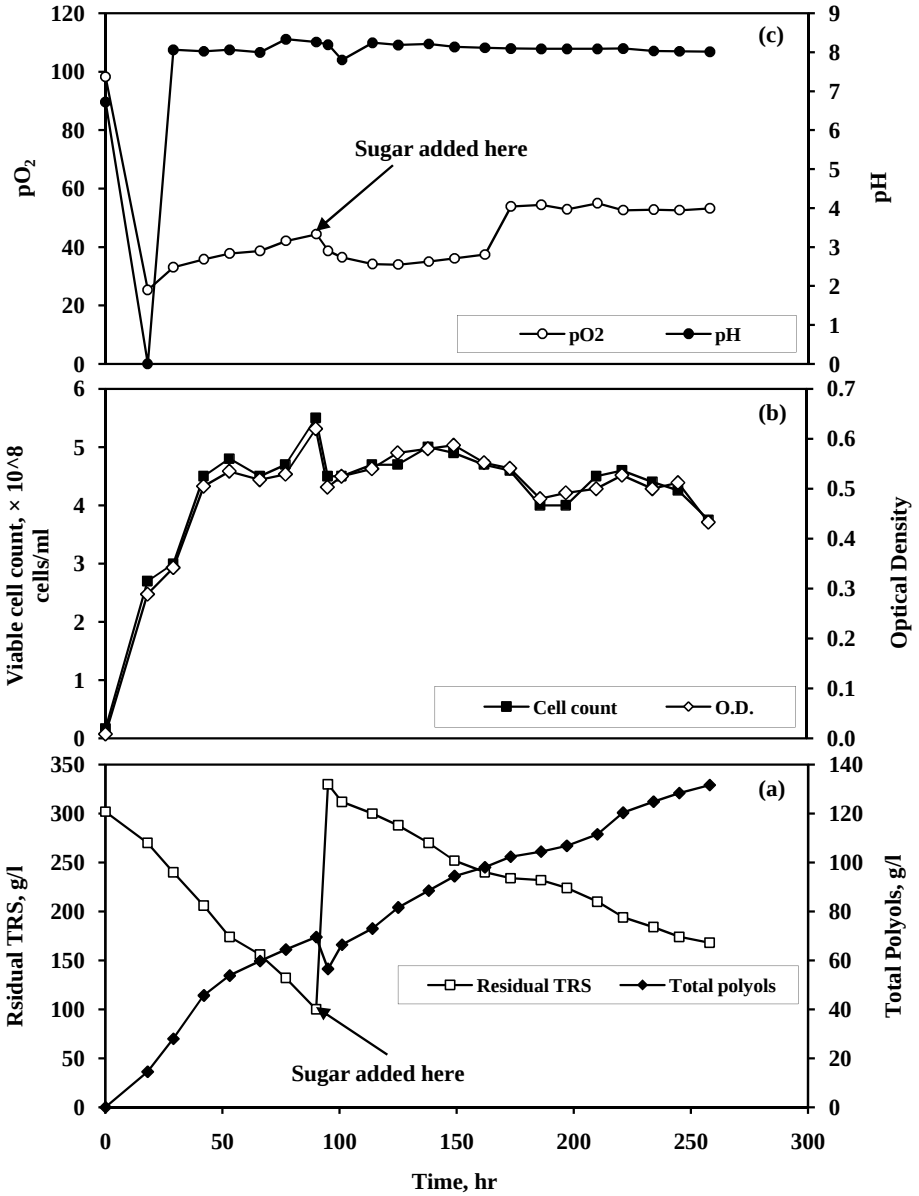


Fig. 5.12.3: Time course profile of a) Residual TRS and Total oolyols, b) Cell count and Optical density of fermentation broth and c) pO_2 and pH during fed-batch fermentation of experiment 5.12.3

Table 5.12.1: Comparative results of fed-batch fermentation at 10 % inoculum, 30îC temperature and without pO_2 control.

Sr. No.	Fermentation Parameters/results	Fed-batch experiments		
		5.12.1	**5.12.2**	**5.12.3**
1.	No. of additional doses of sucrose	3	2	1
2.	Nutrients concentration (based on sucrose added)	As in the initial medium	50 % of the initial medium	25 % of the initial medium
3.	Time, hr	336	402	258
4.	Total sugar added, g	1843.8	2192.5	1275.0
5.	Final volume, l	3.550	3.070	2.375
6.	Sugar utilized, g	1843.8	2192.5	1275.0
7.	Unutilized sugar, g	0.0	0.0	399.0
8.	Final polyols concentration, g/l	163.0	230.0	131.6
9.	Final viable cell count ($\times 10^8$ cells/ml)	6.700	5.200	3.750
10.	Total polyols produced, g	578.7	706.1	312.6
11.	Overall polyols yield (p/ts), %	31.4	32.2	24.5
12.	Yield based on sugar utilized (p/su), %	31.4	32.2	35.7
13.	Cell mass yield (x/ts), % (No. of cells/100 g of total sugar)	1.289×10^{11}	7.281×10^{10}	6.971×10^{10}
14.	Polyols productivity, g/l/hr	0.485	0.572	0.510
15.	Sugar utilization rate, g/l/hr	1.546	1.776	1.433

nutrients dose). However, the overall polyols yield based on total sugars of 32.2 % is substantially less than 47.84 % achieved in batch fermentation experiments at controlled pO_2 of 50 %.

Set-II: Fed-batch experiment with 15 % inoculum, pO_2 controlled at 50 %, temperature of 32.5îC, pH 8.0 and in the presence of externally added invertase.

5.12.4: Commercial grade plantation white sugar (table sugar) was used for direct addition during this experiment of fed-batch fermentation. Sucrose was added when the residual TRS concentration dropped down to just below 10 %. Nutrients dose along with the additional sucrose used was the same as that used in the preparation of initial fermentation medium. The data generated for this experiment is given in Table A-4 (Annexure-I). The total sugar used in this experiment was 2086.0 grams in a period of 330 hrs. The sugar was added in three lots as given in the Table A-4. The time course profile of a) Residual sugar and total polyols concentration, b) Cell count and optical

density of fermentation broth and c) pO_2 and pH during this fed-batch experiment is shown in Fig. 5.12.4.

From pO_2 profile it can be seen that in spite of controlling pO_2 at 50 % level, the actual values dropped down due to addition of sucrose three times. However, pO_2 remained in between 45 to 55 % level throughout the fermentation. The cell count increase was exponential in the first 40 hrs and then the increase was at slower rate. Total viable count actually dropped down at the end of fermentation. The optical density trend also supports the data generated by measuring the cell count. The residual sugar and polyols trends exhibit typical fed-batch fermentation pattern. At the end of 330 hrs, the polyols concentration remained almost constant though almost 10.6 % of sugar remained unutilized. Under microscope, few contaminants were seen and therefore, this experiment was terminated at 330 hrs.

5.12.5: In this experiment, commercial grade plantation white sugar was used by making concentrated solution, which was sterilized separately. The sterilized sugar solution was added at a fixed interval of 24 hrs so as to increase the sugar concentration to about 25-26 % concentration. The nutrients were sterilized separately in minimum quantity of water and dosed along with the concentrated sugar solution under aseptic conditions.

The nutrients were dosed at a concentration equal to the initial nutrients concentration and on the basis of sucrose used. It can be seen that sucrose along with nutrients were added five times during the course of fermentation. The data generated for this experiment is given in Table A-5 (Annexure-I). The total sugar added in 306 hrs of fermentation was 2474.0 gram. The time course profile of a) Residual sugars and total polyols concentration, b) Cell count and optical density and c) pO_2 and pH during this fed batch experiment is illustrated in Fig.5.12.5.

It can be seen that the pO_2 value fluctuated after addition of sugar and nutrients solution but remained around 50 % level throughout the fermentation. pH was also maintained at around 8.0. The viable cell count trend shows somewhat linear growth for about 220 hrs and then the viable count is reduced. This may be due to excessive increase in fermentation broth volume to almost double the initial volume resulting in excessive foaming and uneven distribution of cell mass in the fermenter.

The polyols concentration remained constant in the last 50 hrs of fermentation though substantial amount of sucrose (5.8 %) remained unutilized. The maximum

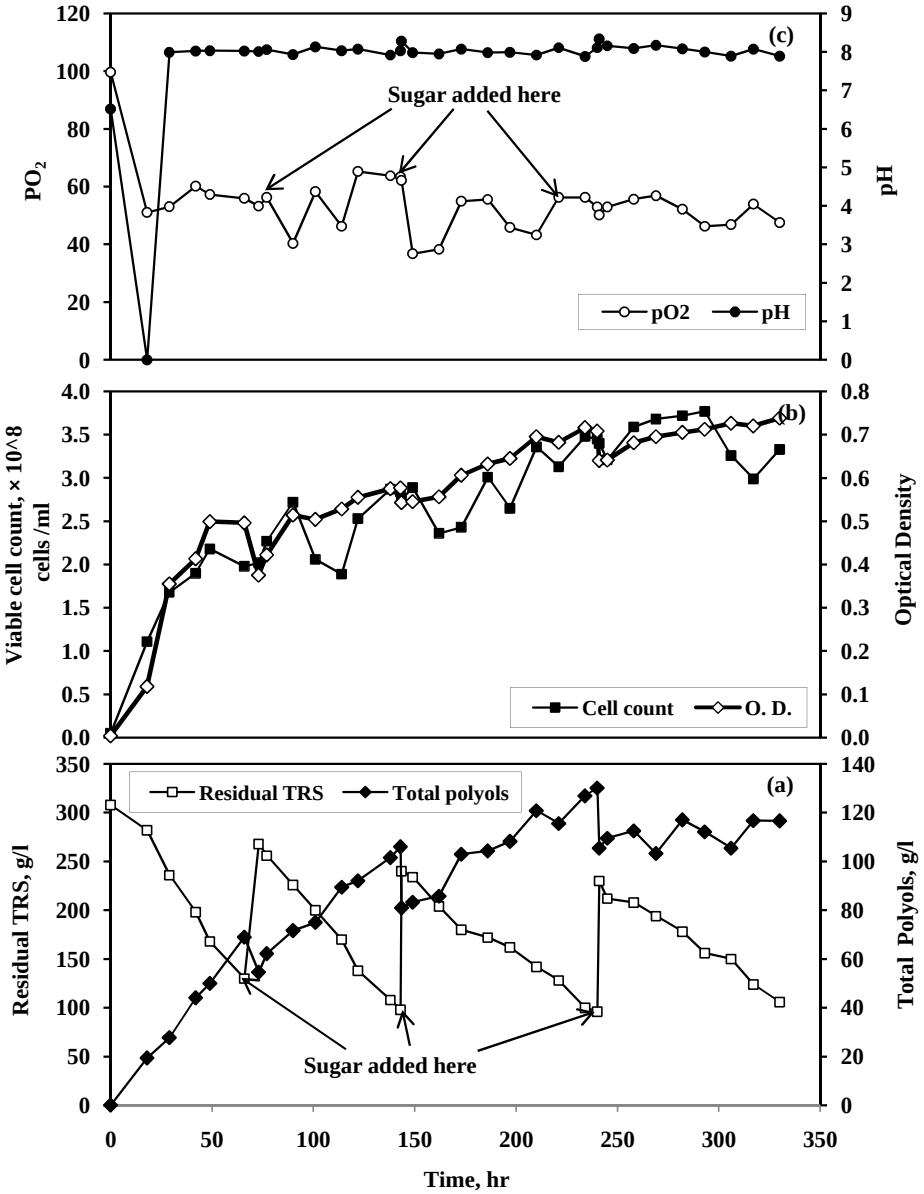


Fig. 5.12.4: Time course profile of a) Residual TRS and Total polyols, b) Cell count and Optical density of fermentation broth and c) pO_2 and pH during fed-batch fermentation of experiment 5.12.4

polyols concentration reached was 141.6 g/l at the end of 282 hrs, which then reduced down to 130.1 g/l at the end of 306 hrs. This indicates that the organism started utilizing the polyols produced as carbon source instead of using sucrose. Therefore, fermentation was terminated at this stage.

5.12.6: In this experiment, commercial grade sucrose was added directly along with sterilized nutrients solution. However, the nutrients were used at a dose of 50 % of the initial concentration (based on the sucrose used). Sucrose and nutrients were added at a fixed interval of about 48 hrs irrespective of residual TRS concentration in the fermenter. The data generated in this experiment is given in Table A-6 (Annexure-I).

It can be seen that sucrose plus nutrients were added four times at an interval of about 48 hrs. Thus, the total sucrose used in this experiment was 2400.9 gram. The time course profile of a) Residual sugars and total polyols concentration, b) Cell count and optical density and c) pO_2 and pH during this experiment is shown in Fig.5.12.6.

pO_2 remained almost constant at around 50 % in spite of fluctuations caused by addition of sugar at intervals. The cell mass growth was rapid in the first 40 hrs and then increased at a slow rate. The fluctuations seen in cell growth can be due to changes in the fermenter broth volume due to addition of sugar plus nutrients and Na_2CO_3 for controlling the pH. The final volume of fermented broth collected was 3.734 liters. The polyols concentration reached a maximum level of 152.4 g/l at the end of 366 hrs and then decreased to 142.9 g/l at 378 hrs. The sugar remained unutilized at 122 g/l concentration and therefore the batch was terminated at 378 hrs. Few contaminants were also seen at the end of fermentation under microscope.

The comparative results of the second set of fed-batch experiments are given in Table 5.12.2. It can be seen that maximum polyols concentration achieved in the second set is 14.29 % as compared to 23.00 % achieved in the first set. The overall yield based on total sugars (p/ts) as well as the yield based on sugar utilized (p/su) in this set is maximum in experiment 5.12.5 i.e. liquid sucrose feed and nutrients at a concentration equal to the concentration in initial fermentation medium. The maximum overall polyols yield of this set (27.55 %) is less than that obtained in the first set (32.20 %). However, overall yields of both the sets are substantially less than that obtained in batch fermentation (47.84 %). Similarly, the polyols productivity and sugar utilization rates in fed-batch fermentations are lower than those obtained under optimum conditions in batch fermentations.

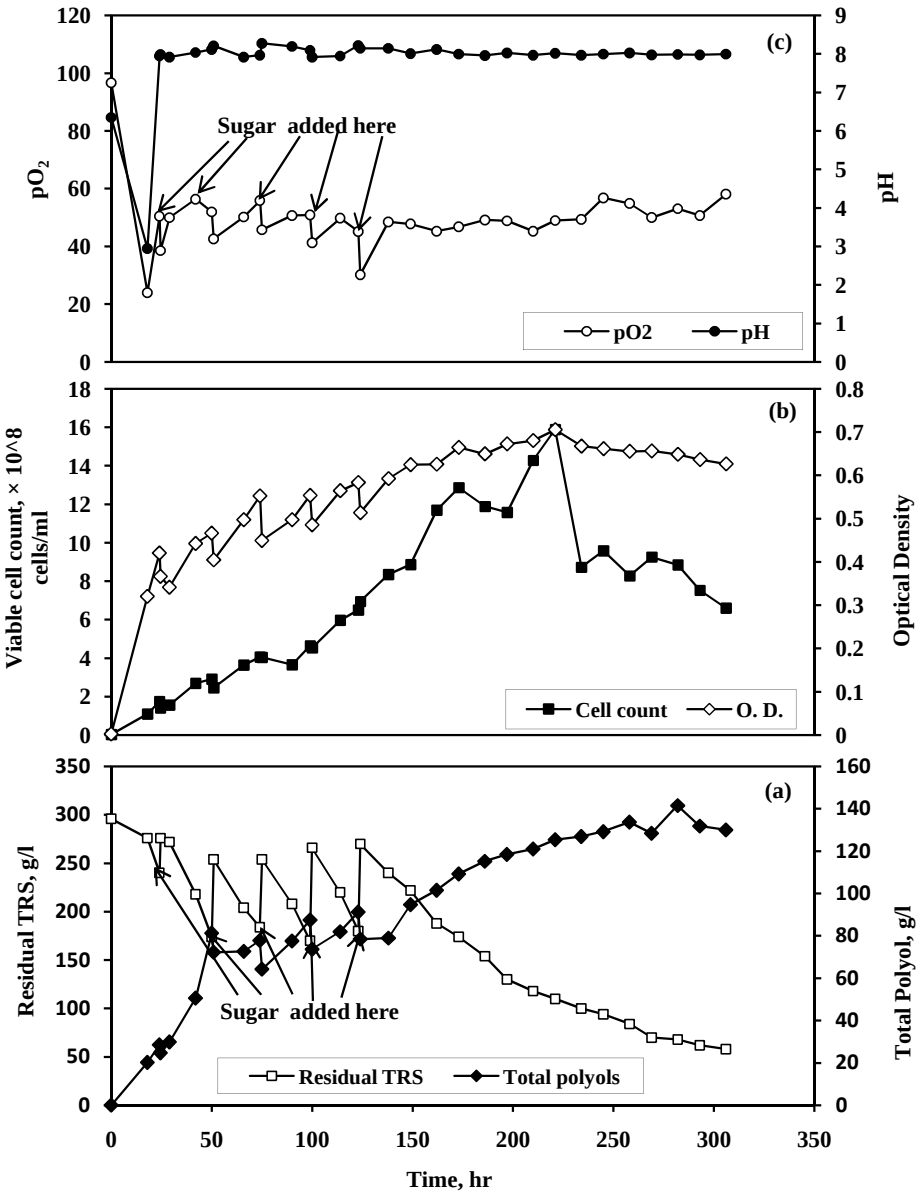


Fig. 5.12.5: Time course profile of a) Residual TRS and Total polyols, b) Cell count and Optical density of fermentation broth and c) pO_2 and pH during fed-batch fermentation of experiment 5.12.5

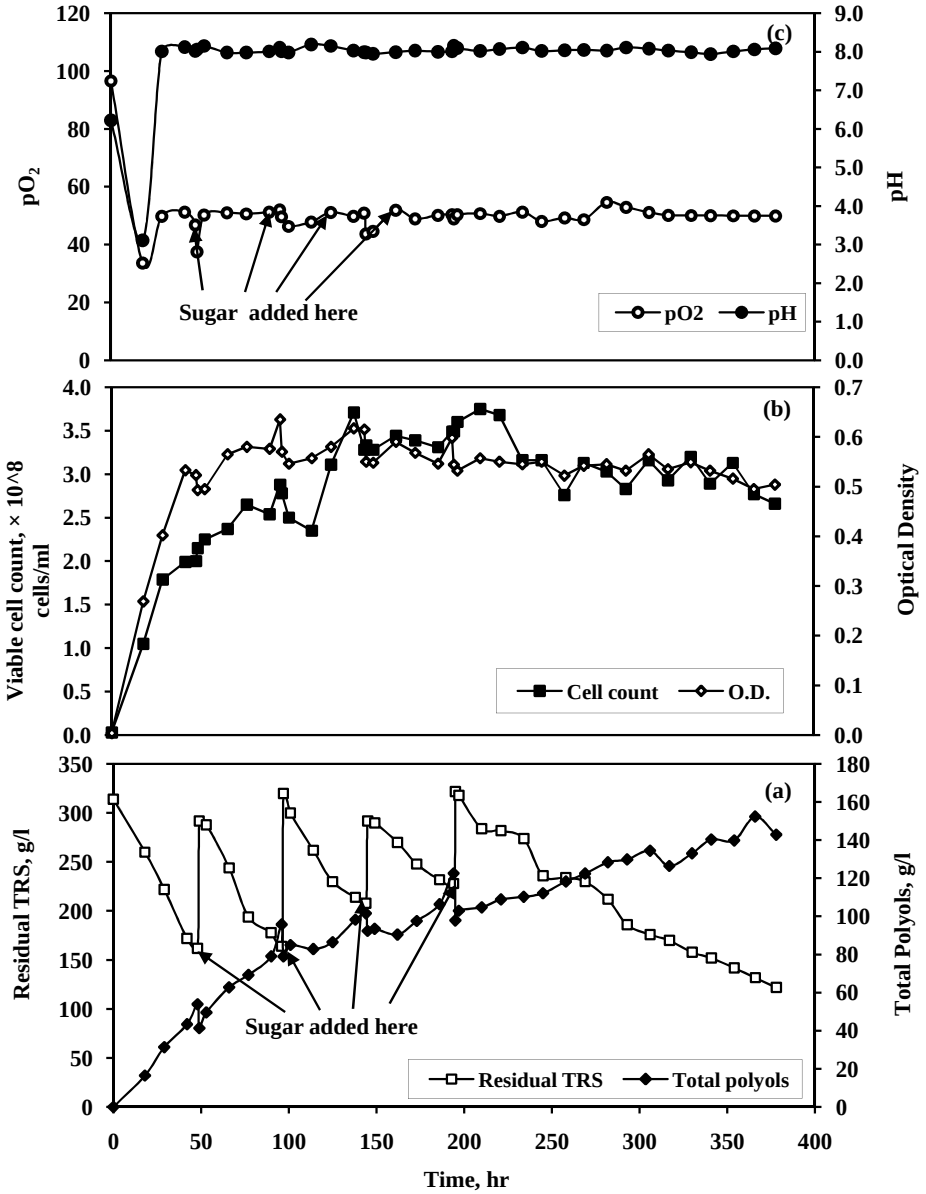


Fig. 5.12.6: Time course profile of a) Residual TRS and Total polyols, b) Cell count and Optical density of fermentation broth and c) pO_2 and pH during fed-batch fermentation of experiment 5.12.6

Table 5.12.2: Comparative results of fed-batch fermentations at 15 % inoculum, 32.5îC temperature and pO_2 controlled at 50 %.

Sr. No.	Fermentation Parameters/results	Fed-batch experiments		
		5.12.4	**5.12.5**	**5.12.6**
1.	Additional sucrose	Solid form	Concentrated liquid after sterilization	Solid form
2.	Sucrose addition at	After reduction of TRS to 10 %	After every 24 hours	After every 48 hours
3.	Nutrients	At the same concentration as initial dose	At the same concentration as initial dose	At 50 % concentration of the initial dose
4.	No. of additional doses of sucrose	3	5	4
5.	Time, hr	330	306	378
6.	Final volume, l	3.550	5.238	3.734
7.	Total sugar added, g	2086.0	2474.0	2400.9
8.	Final residual sugar, g/l	106.0	58.0	122.0
9.	Actual sugar consumed, g	1709.7	2170.2	1945.4
10.	Final polyols concentration, g/l	116.7	130.1	142.9
11.	Total polyols produced, g	414.3	681.5	533.6
12.	Overall polyols yield (p/ts), %	19.86	27.55	22.22
13.	Yield based on sugar utilized (p/su), %	24.23	31.40	27.43
14.	Final cell count, ($\times 10^{12}$ cells/ml)	2.66	6.61	3.33
15.	Total cell count in the fermenter	9.443×10^{15}	3.462×10^{16}	1.243×10^{16}
16.	Cell mass yield (x/ts), %	4.527×10^{12}	1.399×10^{13}	5.179×10^{12}
17.	Polyols productivity, g/l/hr	0.354	0.425	0.378
18	Sugar utilization rate, g/l/hr	1.459	1.354	1.378

The lower yield of polyols in the second set as compared to the first set of fed-batch fermentations suggests that the optimum conditions for fed-batch fermentations with respect to inoculum size, temperature of fermentation, optimum pO_2 level can be different than the optimum conditions of batch fermentation process. Therefore, it would be necessary to adjust these parameters as well as sucrose and nutrients feed strategy carefully to achieve maximum yield and productivity of polyols.

Longer fermentation cycles of fed-batch fermentations are also prone to get contaminated because of frequent dosing of sugar and nutrients.

In the best experiment of fed-batch fermentation it was possible to achieve 23.0 % polyols concentration at an overall yield of 32.20 %, polyols productivity of 0.552 g/l and sugar utilization rate of 1.776 g/l/hr.

5.13 DEVELOPEMT OF KINETIC MODEL FOR OPTIMUM POLYOLS PRODUCTION THROUGH DISSOLVED OXYGEN CONTROL IN BATCH FERMENTATION

INTRODUCTION

Batch fermentations were carried out to produce polyols (glycerol and arabitol) using sucrose based media and osmophilic yeast, *Hansenula anomala*. The fermentor used was well stirred vessel with air sparging arrangement. In order to maximize the yield of the polyols, the effect of different parameters were investigated one at a time. Starting with pH, it was observed that the maximum yield of polyols is obtained at pH 8.0 in the presence of externally added invertase. Similarly, the other parameters to obtain the maximum product yield are: Temperature of 32.5°C, inoculum size of 15 % and stirrer speed of 600 rpm. Finally, the effect of pO_2 was studied which is a very important factor as far as the final polyols yield is concerned.

Few kinetics models are reported for production of polyols using osmophilic yeast by batch fermentation (236) as well as for gluconic acid production by *Aspergillus niger* (237). But none of the models represents effect of dissolved oxygen or pO_2 on production of polyols. An attempt is made here to model polyols batch fermentation for different pO_2 values. A kinetic model is developed from numerical analysis by fitting experimental data. The simulation study results in obtaining various parameters. Some of the parameters show significant effect on the polyols formation by *Hansenula anomala*. Therefore, it is important to study the effect of these parameters to further optimize the yield of the products.

KINETIC MODEL

The model employs rate equations for Biomass (X), Total polyols (P), and Substrate-Total reducing sugars (S) to describe fermentation process at constant percentage oxygen saturation (pO_2). .

The rate of biomass is described by equation [1] where μ is specific growth rate

of cell and governed by Contois model (238). The yeast death and autolysis phase must be also considered in the bioprocess model since the significant decreases in cell concentration have been observed in the late stage (236). Hence K_d represents specific death rate of cells.

$$\frac{dX}{dt} = (\mu - K_d)X \quad [1]$$

Where $\boldsymbol{\mu}$ is specific growth rate given by

$$\mu = \mu_m \frac{S}{(K_S X + S)} X \quad [2]$$

Where $\boldsymbol{\mu_m}$ is maximum specific growth rate.

The kinetics of polyols production is based on Luedeking-Piret equation originally developed for the fermentation of lactic acid (239). It contains growth and non-growth associated factors for product formation. The product formation depends on both growth ***dx/dt*** and instantaneous biomass concentration *X* in a linear way.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad [3]$$

Where $\boldsymbol{\alpha}$ and $\boldsymbol{\beta}$ are Luedeking-Piret constants for growth and non-growth associated product formation, respectively.

Substrate consumption depends on the number of cell formation, product formation and cell maintenance. The rate equation is described by

$$\frac{dS}{dt} = -\frac{1}{Y_{XS}} \frac{dX}{dt} - \frac{1}{Y_{PS}} \frac{dP}{dt} - m_S X \quad [4]$$

Where $\boldsymbol{Y_{XS}}$ and $\boldsymbol{Y_{PS}}$ are yield coefficients of biomass and product respectively and m_S is the specific maintenance coefficient.

Substituting equation [3] in equation [4]

$$\frac{dS}{dt} = -\gamma \frac{dX}{dt} - \lambda X \quad [5]$$

Where $\boldsymbol{\gamma}$ and $\boldsymbol{\lambda}$ are constants for growth and non-growth associated substrate consumption respectively, defined as

$$\gamma = \left(\frac{\alpha}{Y_{PS}} + \frac{1}{Y_{XS}}\right) \quad [6]$$

$$\lambda = \left(m_S + \frac{\beta}{Y_{PS}}\right) \quad [7]$$

RESULTS AND DISUCSSION

The fermentation with osmophilic yeast was carried out in stirred type batch fermenter. The fermenter was having volume of 2.5 liter in which air was sparged through sparger from the bottom. The air was controlled automatically by measuring percentage oxygen saturation in the solution at temperature of 32.5°C. The experiments were carried out for different oxygen percentage saturation (pO_2) i.e. for 30 %, 40 %, 50 %, 60 % and 70 %. All other parameters were kept constant such as pH=8, Inoculum size = 15% and speed of stirrer=600 rpm.

The rate equations [1], [3] and [5] are coupled non-linear differential equations. There are total seven unknown parameters namely μ_m, K_d, K_S, α, β, γ and λ. In order to simulate the process, the unknowns are to be determined by minimizing the sum of squares between model predicted values and experimental values. For this purpose, the experimentally derived values of substrate (TRS, g/l), product (Total polyols, g/l) and cell mass (Dry Cell Weight, g/l) in pO_2 controlled batch fermentations were considered. The non-linear differential equations are solved in advance mathematical software MATLAB 7.5 by using 'fmincon' function of nonlinear optimization solver.

The predicted values are compared with experimental values for biomass, polyols and substrate as shown in figures 5.13.1 to 5.13.5. From these figures, it can be seen that the predicted values are very closely matching with the experimental values. This indicates that the proposed model satisfactorily describes fermentation process of polyols production for different pO_2 values.

The unknowns are determined by simulation for each pO_2 value. The unknown parameter values are given in Table 5.13.1. The effect of each parameter is discussed briefly below.

The parameters $\boldsymbol{\mu_m}$, $\boldsymbol{K_d}$ and $\boldsymbol{K_S}$ together determine rate of cell formation. From equations [1] and [2], the rate of cell formation is directly proportional to maximum

specific rate, μ_m while inversely proportional to death rate factor, K_d and mass transfer coefficient, K_S. Therefore, it is observed that the rate of cell formation is higher for the cases of pO_2 = 50 % and pO_2 = 70 % where μ_m is higher and K_d is lower. Because of higher specific growth rate and lower cell death rate, rate of cell formation is higher for these two cases. However, for pO_2 = 40 %, the rate of cell formation is higher due to lower ***Ks*** value. It is difficult to predict the optimized rate of cell formation from these three parameters. However, these parameters are not important for maximizing the yield of the polyols.

The next parameters are α and β which determine the rate of polyols formation. The parameter β is the most important factor as the contribution of α is lower towards polyols formation. Also β is the contribution of cell towards polyols formation and cell maintenance. Higher values of both α and β for pO_2 = 50 % indicates higher rate of product formation as compared to other pO_2 values. For pO_2 = 30 %, the rate of product formation is slowest due to lower β value. Since α and β determine the rate of polyols formation, for cases other than pO_2 = 50 %, the overall yield of polyols is lower. It is observed that higher pO_2 values (>50 %) lead to more foaming and entrapped bubble formation in the fermenter resulting in poor mass transfer of dissolved oxygen to the cells. Hence maximum product formation occurs at pO_2=50%.

The parameters γ and λ determine the rate of sucrose utilisation. From Table 5.13.1, it is found that the value of γ is more or less in the same range for different pO_2 values. Also the contribution of γ towards sugar utilisation is very small as compared to λ value. Therefore, higher λ value indicates higher rate of sugar utilisation. In this case, for pO_2 = 50%, maximum rate of sugar utilization takes place. This ultimately results in higher yield of polyols.

CONCLUSION

Modeling of batch fermentation to produce polyols by controlling pO_2 is successfully attempted. The values of α and β mainly determine rate of polyols formation. Also high λ value indicates higher rate of sugar utilisation. At higher rate of sugar utilization, the rate of polyols formation is also on higher side. These parameters (α, β and λ) at particular pO_2 value give maximum yield of polyols. Therefore, the maximum polyols formation was observed at pO_2 = 50%.

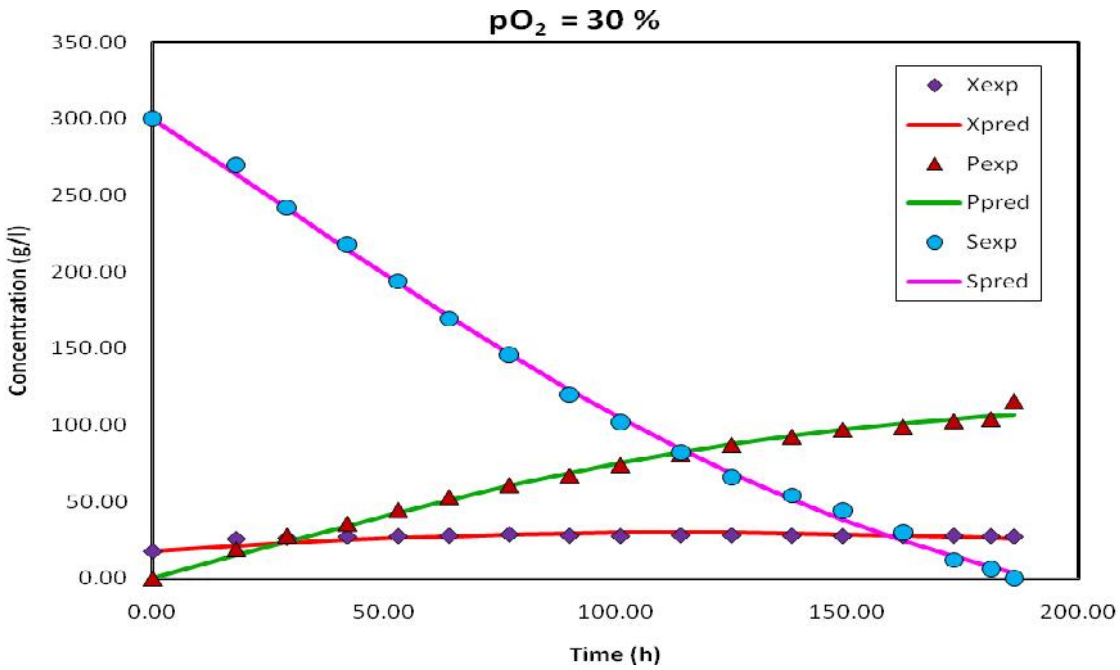


Figure 5.13.1: Comparison between experimental data (◆ Cell mass, ▲ Total Polyols, ● Residual TRS) and model predicted values (— Cell mass, — Total Polyols, — Residual TRS) at 30 % pO_2.

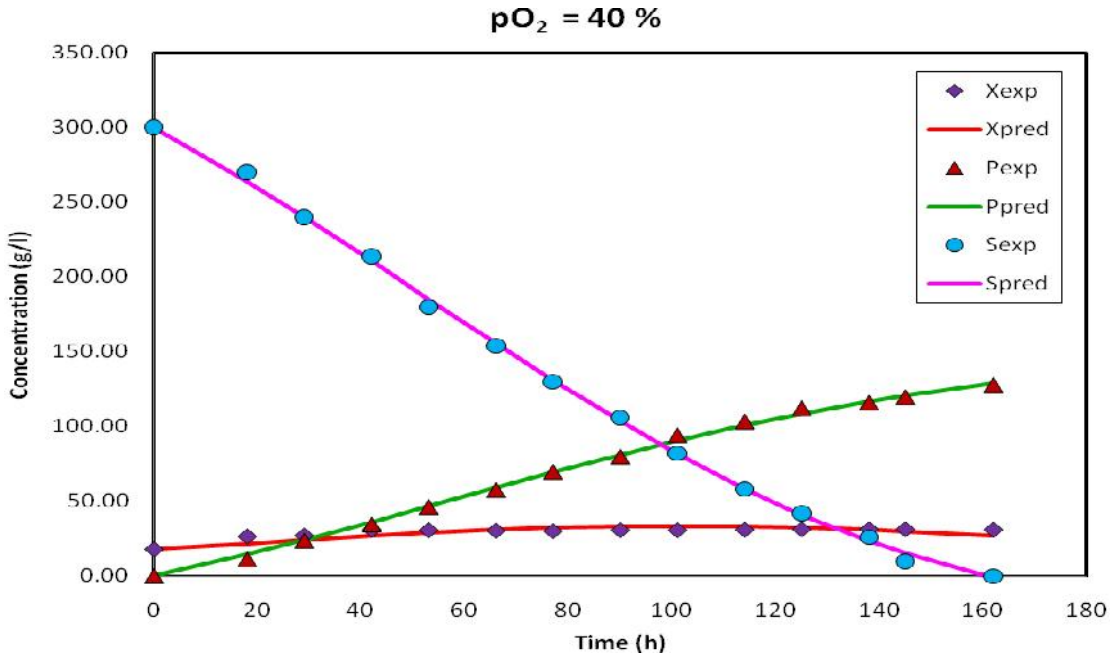


Figure 5.13.2: Comparison between experimental data (◆ Cell mass, ▲ Total Polyols, ● Residual TRS) and model predicted values (— Cell mass, — Total Polyols, — Residual TRS) at 40 % pO_2.

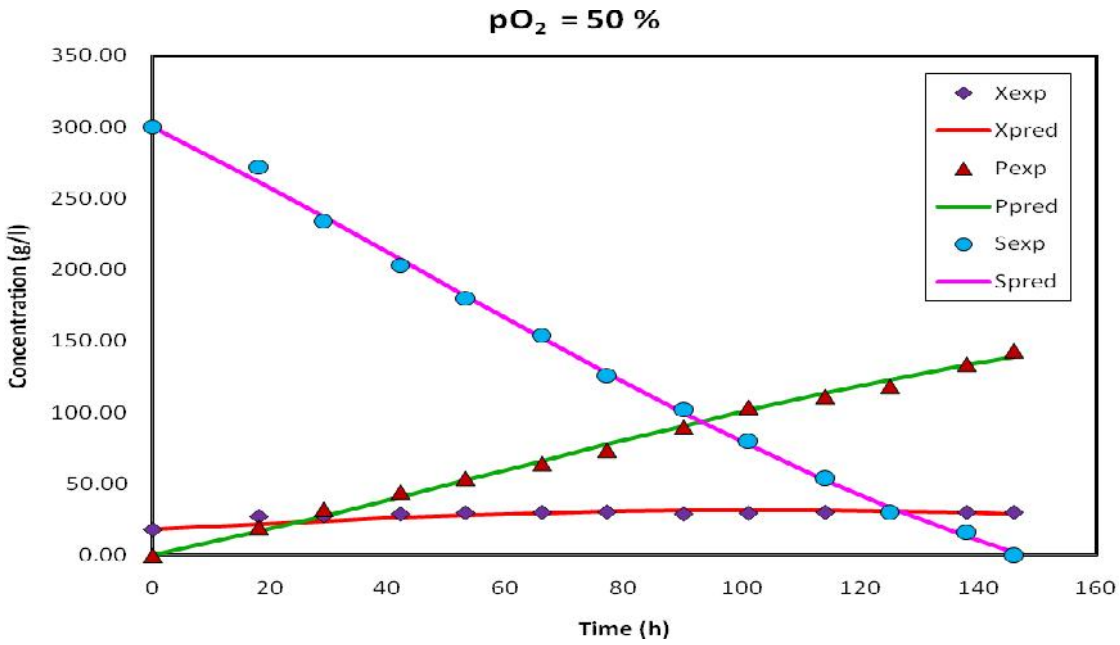


Figure 5.13.3: Comparison between experimental data (◆ Cell mass, ▲Total Polyols, ● Residual TRS) and model predicted values (— Cell mass, — Total Polyols, — Residual TRS) at 50 % pO_2.

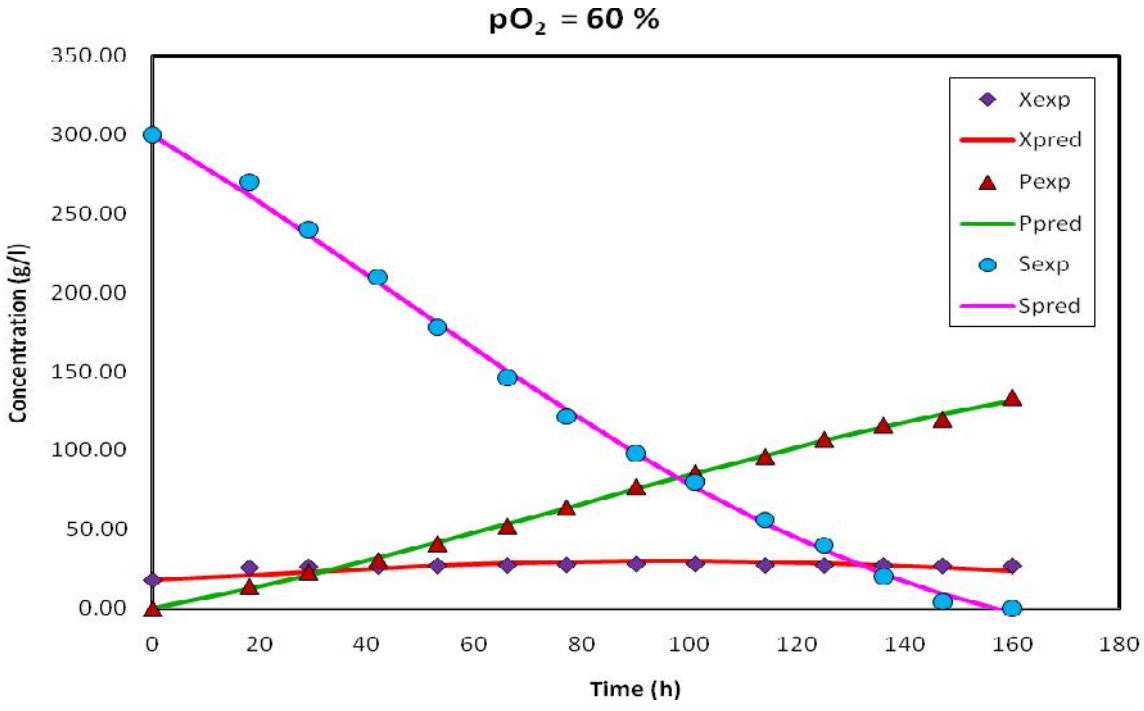


Figure 5.13.4: Comparison between experimental data (◆ Cell mass, ▲Total Polyols, ● Residual TRS) and model predicted values (— Cell mass, — Total Polyols, — Residual TRS) at 60 % pO_2.

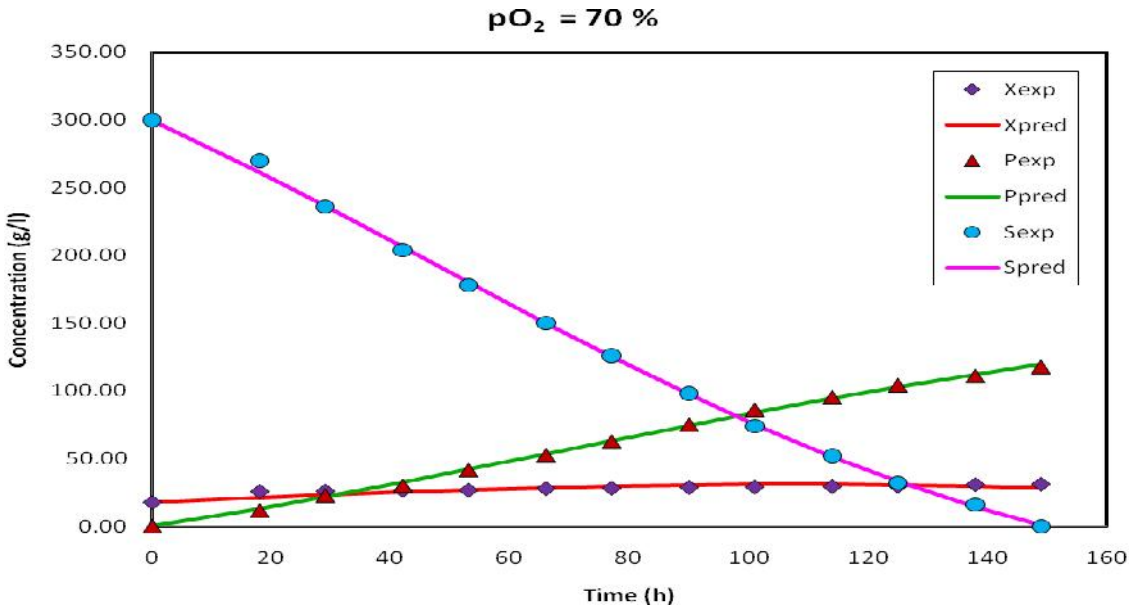


Figure 5.13.5: Comparison between experimental data (◆ Cell mass, ▲ Total Polyols, ● Residual TRS) and model predicted values (— Cell mass, — Total Polyols, — Residual TRS) at 70 % pO_2.

Table 5.13.1: Predicted value of unknown parameters for different pO_2 values.

Parameters	pO_2				
	30 %	**40 %**	**50 %**	**60 %**	**70 %**
μ_m	0.032	0.027	**0.036**	0.026	0.0225
K_s	20.00	7.55	20.00	8.32	9.70
K_d	0.0040	0.0065	**0.0040**	0.0066	0.0040
α	2.630	1.420	**1.485**	0.568	1.135
β	0.007	0.015	**0.024**	0.027	0.022
γ	5.57	3.98	4.00	4.52	5.23
λ	0.028	0.030	**0.046**	0.036	0.039

CHAPTER-6

SUMMARY AND CONCLUSIONS

CHAPTER-6

SUMMARY AND CONCLUSIONS

6.1 OBJECTIVES

The necessity of undertaking this work originated because of the following facts,

1) Increasing stocks of sugar (sucrose) in the world as well as in the country have resulted in decline in sugar prices and profitability of sugar mills. Sugar mills are required to pay higher price for the sugar cane to the sugar cane growers. Therefore, it has become necessary for the sugar industry to diversify and produce value added products apart from conventional products such as sugar, cogen power and ethanol.
2) Increasing prices of petroleum feed–stock which has made it mandatory to look for alternate sources of energy as well as parallel chemistry for downstream petro-chemicals.
3) Increasing prices of oil-seeds and replacement of soaps with detergents resulting in making availability of natural glycerol difficult in India.

The solution to these problems is to develop alternate products based on sugar or sugarcane juice or molasses. Use of such, renewable feed-stocks for producing value added chemicals is possible because of the latest developments taking place in yeast biotechnology as well as in downstream separation technologies.

Polyols production using osmophilic yeasts and renewable feed-stocks has gained renewed interest and deserves reconsideration. Though glycerol is now also available as a by-product of bio-diesel industry apart from the other routes of its production, it is still being imported in the country. Few Chinese Universities and research institutions have done pioneering work in the last decade for the development of technologies for production of glycerol and other polyols. It is reported that some of these technologies have reached commercial scale production.

Glycerol has wide industrial applications and is one of the vital chemicals of international trade. Other polyols produced by osmophilic yeasts are also being used principally in food and pharmaceutical applications. The market potential of these polyols will further develop if they are produced cheaply at a large scale.

6.2 ANALYTICAL METHODS

Various analytical methods reported in the literature for monitoring osmophilic yeast fermentation were tested and standardized in our laboratory. High performance liquid chromatography (HPLC) could have been the best possible method for monitoring these fermentations. However, HPLC facility was not available for routine use. Therefore, attempts were made to develop a method for simultaneous estimation of sugars and polyols in fermentation broth samples using Gas Chromatography. This involved centrifugation of fermentation broth samples, filtration through 0.2 µm Millipore membrane filter and derivatization of sugars and polyols to their trimethylsilyl ethers followed by gas chromatographic separation and estimation using two internal standards. The advantage of this method is requirement of very small sample quantity (5 µl), high sensitivity and fast speed.

Fermentation broth is a complex mixture consisting of inorganic salts, suspended impurities and yeast cells, residual substrates, products and unknown by-products of fermentation. It is, therefore, necessary to remove the interferences due to these impurities. Mannitol and lactose have been used as internal standards to estimate concentrations of polyols and sucrose, respectively. The conditions of derivatisation of samples viz. reagent concentration, heating time, temperature, heating block design etc. were arrived at after evaluating several combinations of these to ensure proper derivatisation.

No simultaneous estimation of sugars and polyols in such media has been reported earlier. For standard mixtures, the results obtained were in good agreement with those obtained by conventional methods.

Though the method developed gave excellent resolution of individual components with good peak characteristics, it was found after repeated investigations that it lacked consistency and reproducibility of results with fermentation broth samples. This may be due to the interference of salts, nutrients and other unknown by-products produced by the osmophilic yeast and present at varying concentrations in the fermentation broth samples. Therefore, this method was used mostly for qualitative estimation of polyols. It is necessary to make an in-depth investigation of the reasons for inconsistency and lack of reproducibility. With the latest methods of sample pretreatment and preparation available now, it may be possible to improve the method in future.

Many groups working on osmophilic yeasts for glycerol and other polyols production have used spectrophotometric method based on periodate oxidation for quantitative estimation of total polyols. This method estimates quite accurately the total polyols in presence of sugars in fermentation broth. Therefore, periodate oxidation method was used for quantitative estimation of total polyols (glycerol + arabitol) throughout this investigation. Necessary corrections for the presence of sugars were applied in expressing the final results.

Total reducing sugars, which is sum total of sucrose and inverted sugars (glucose + fructose), was estimated after acid inversion of fermentation broth samples by the spectrophotometric method of arsenomolybdate.

Ethanol concentrations in fermentation broth samples were estimated by spectrophotometric method of dichromate reduction. Under the conditions of fermentation employed in our investigation on shake flask and fermenter scale experiments, ethanol was never detected or detected at minute concentrations and therefore, it has not been accounted quantitatively as the final product of fermentation.

In case of our strain of *H. anomala*, the viable and dead cells could be easily examined and counted in suspension (45 × magnification) under microscope. Therefore, a method of methylene blue staining for viable cell count measurement was standardized and used routinely. We have also measured and collected data on dry cell weight and optical density of fermentation broths. However, for most of the experiments, cell mass growth results are expressed in terms of viable cell count. Cell count measurement method was found to be fast and fairly accurate.

For measurement of dissolved oxygen concentration in fermentation broth samples the Winkler's titration method was used.

6.3 SIMULATION STUDIES

In our previous study, an osmophilic yeast species, *Hansenula anomala* (NCIM-3341) was selected after screening several yeast cultures for polyols production. The initial work was carried out using glucose as substrate.

Brief review of our previous work and results on media optimization, oxygen transfer, kinetics of glucose utilization and effect of pH is given. The maximum overall yield on the basis of total initial sugar under optimum conditions was 51 % with polyols productivity of 1.11 g/l/hr using glucose as substrate.

The data generated in our previous study was used in the present investigation to undertake simulation studies using “Lazy Learning” technique of robust regression. The version of lazy learning method employed in this work is memory-based local learning requiring the storage of training data in the memory.

Experimental data generated in 39 experiments was used for training purpose. Randomly selected 8 query points were used to test the efficacy of the algorithms. The performance metric in this case was root mean squared method (RMSE). The final prediction errors reported are based on average values of 100 simulation experiments in the example that is considered. The polyols yield prediction problem is solved with lazy learning. In this case, local quadratic model with 2.212 % RMSE is found to be performing better than kernel principal component regression (KPCR) and kernel partial least squares (KPLS) with 4.009 % and 4.417 % RMSE, respectively. Local combination of models achieved the same RMSE as local quadratic model.

The case example as illustrated involved the use of laboratory experimental data. For real world application, it would be necessary to have sufficient amount of carefully monitored historical database. Lazy learning tool can then be directly incorporated into the monitoring system. The technique is especially useful in instances where fast training and online updation of process models is required. These situations arise as, for example, in incipient fault detection and diagnosis.

The method employs a memory based local learning approach, and processes the data only when a query is posed. The relevant neighborhood is estimated by a simple distance function. The adoption of recursive algorithm along with the model selection criterion makes lazy learning distinct from the other local learning techniques. Employment of local weighted regression for parametric identification and PRESS statistic to assess a local model in cross-validation for structural identification, are the two unique features of the lazy learning. Local weighted regression is a fast parametric technique to determine in a single step, the best local parameters. The PRESS statistic is a well-founded and easiest way to validate the local model. It is this judicious use of linear techniques for parametric and structural identification that makes lazy learning an attractive alternative to the contemporary methods. Even with the simple metric used in the study, the algorithm performed better than some of the state-of art approaches.

6.4 SHAKE FLASK EXPERIMENTS: SUCROSE BASED MEDIA OPTIMIZATION USING EVOLUTIONARY ALGORITHM

One of our objectives was to develop a process of polyols production using sucrose or sucrose containing raw materials. In our preliminary investigations, it was found that with sucrose as substrate, the organism gave substantially less yield as compared to the yield with glucose as substrate. It is known that in *S. cerevisiae* and osmophilic yeasts, the yeast generates invertase endogenously and liberates it outside the cell in response to the presence of disaccharide sugar, sucrose. The inversion of sucrose occurs outside the cell and the inverted products, glucose and fructose, are carried inside the cell by an active transport mechanism. It is also reported in the literature that *H. anomala* has invertase activity. It was not clear why this strain of *H. anomala* was not able to use sucrose efficiently.

Therefore, it was decided to reinvestigate and optimize substrate and nutrients composition of fermentation media based on sucrose as carbon source on shake flask level. It was decided to use the conventional approach of one-variable-at-a time (OV approach) as well as recent genetic algorithm based approach (GA approach) for optimization purpose and to compare the results.

The composition of optimized media obtained by the conventional approach was (g/l), Sucrose: 300, yeast extract: 2.5, Urea: 1.0, Casein hydrolysate: 1.0 and $MgSO_4$: 0.25 and was identical to that obtained with glucose as substrate (except the sugar in question). However, the yield with sucrose was substantially less.

A new real coded evolutionary algorithm was developed for optimization purpose. By iteratively employing the nature-inspired techniques of selection, crossover and mutation for a fixed number of generations, the algorithm obtains the optimum values of important process parameters, such as inoculum size and concentrations of medium components. The composition of optimized media obtained by the genetic algorithm approach was (g/l), Sucrose: 300.5, yeast extract: 1.25, Urea: 1.10, and $MgSO_4$: 0.256 and was slightly different from that obtained with conventional approach. The final concentration as well as the yield of polyols, particularly during early period of fermentation was higher with medium optimized by the GA approach. Maximum polyols yield on the basis of sucrose utilized was found to be 76.43 % and final polyols yield on the basis of total initial sugars was 30.23 %.

Genetic algorithms are stochastic search technique that mimics the process of natural selection. They have the inherent ability of simple representation to encode highly complicated structures and to use simple transformation to improve such structures to reach optimal solutions. This approach is efficient in locating a true optimum when interaction effects are present.

Comparison of yields obtained between GA optimized media and OV approach optimized media indicated that yields obtained with GA optimized media were substantially higher, particularly during the early phase of fermentation.

In shake flask experiments it was found that fructose as substrate resulted in maximum final polyols concentration with *H. anomala* followed by glucose and sucrose. This may be due to the fact that fructose is supposed to exert more osmotic pressure than that of glucose and sucrose. Use of sucrose as substrate resulted in lower final polyols concentration. However, addition of external invertase resulted in improved final polyols concentration with sucrose as substrate. Other explanation of this aspect can be the fructophilicity of osmophilic yeasts as it has been demonstrated for *S. rouxii*.

The lower final polyols concentration with sucrose may be due to weak invertase activity of *H. anomala* under the conditions of fermentation in shake flasks.

It was also found that inoculum and yeast extract concentration above a certain level reduces polyols formation. Initial sucrose concentration of 30 % in fermentation media resulted in maximum polyols concentration. Urea and phosphate concentrations should be maintained at minimum to improve the polyols concentration and probably to eliminate the formation of ethanol. Magnesium sulfate at low level is also found to be necessary.

6.5 BATCH FERMENTATION: SCALE-UP STUDIES AND PROCESS OPTIMIZATION

Media optimized by GA approach was used for scale-up and further process optimization studies in an automated fermenter at 2.5 liter capacity. The fermenter has an in-situ sterilization facility with automatic temperature, pH, pO_2 and foam control systems and facilities to monitor agitation and aeration rates.

In our previous study with glucose as substrate on the effect of pH on polyols production, it was observed that maintaining the pH from the beginning of the experiment had an adverse effect on the course of fermentation. Sugar utilization rate

was very low and there was no polyols formation. Therefore, during all experiments on fermenter scale, the pH of the fermenter was brought to the required level at the end of 18 hrs. During this early phase, the pH of fermenter was reduced from initial value of 6.0 to about 2.9 to 3.0. It was also observed that in this period the cell growth was exponential resulting in rapid consumption of dissolved oxygen and thus, a sharp fall in % pO_2 value.

In most of the experiments, it was observed that the cell entered a stationary or very slow growth phase after the initial exponential growth phase. The yield of polyols on the basis of sugar utilized as well the polyols productivity was maximum in the initial exponential growth phase. It was found that the polyols production also continued during the second phase of stationary growth though at a reduced rate. This observation clearly indicates that polyols production with osmophilic yeast is a growth associated phenomenon.

All results are expressed in terms of 1) Overall polyols yield based on total initial sugars used (p/ts, %) 2) Polyols yield based on actual sugar utilized (p/su, %). The cell growth of *H. anomala* is expressed as 3) Overall cell count yield (x_{cc}/ts) as $x \times 10^8$ cells/ml per 100 g of total initial sugars and 4) Cell count yield (x_{cc}/su) as $x \times 10^8$ cells/ml per 100 g of actual sugar consumed. From economic and process development point of view, overall polyols yield (p/ts) based on total initial sugars used is an important criterion. Productivity of polyols formation is calculated as grams of polyols formed per liter per hour (g/l/hr). Productivity of cell mass (or cell count) is calculated and expressed as $x \times 10^8$ cell/ml/hr. In all the results related to cell count, x stands for number of cells. Sugar utilization rate is also calculated and expressed in g/l/hr.

The effect of pH was studied at uncontrolled, 4.0, 5.0, 6.0, 7.0, 8.0 and 8.5 pH. Experiments were also conducted to study the effect of addition of an external invertase (β-D-fructofuransidase, EC 3.2.1.26). As compared to other pH, the polyols yield was maximum at pH 8.0 indicating that moderate alkaline conditions favour polyols production . This probably results from fixing of acetaldehyde by Na_2CO_3 used, resulting in two molecules of acetaldehyde to undergo Cannizero's reaction and form acetic acid and ethanol in molecular proportion of 1:1. Acetic acid formation is accompanied by formation of NADH, which must be balanced by formation of glycerol by the altered Embden Mayerhof pathway. Between pH 6.0 to pH 8.0,

sucrose was never utilized completely and in the later phase, the *H. anomala* shifted its metabolism towards consuming the polyols produced in the earlier phase. This was contrary to our observation with glucose as substrate where the organism shifted over to polyols consumption only after all sugar was utilized. At pH 8.0 and after addition of invertase the yeast growth rate improved significantly. Sugar was almost completely consumed in 138 hrs. Polyols productivity improved to 0.904 g/l/hr and overall polyols yield increased to 41.13 %. This indicated that pH 8.0 with external invertase is the optimum condition for polyols production by *H. anomala* with sucrose as substrate. The mode of controlling the pH after 18 hrs of growth and use of external invertase with sucrose as carbon source is probably reported first time in polyols production with osmophilic yeast.

The effect of temperature on polyols production by *H. anomala* was investigated at 30, 32.5 and 35.0°C. At 32.5°C, the final polyols concentration reached was 140.5 g/l as compared to that of 124.8 g/l and 127.3 g/l for fermentation at temperature 30 and 35°C, respectively. Polyols yield (46.52 %), polyols productivity (0.867 g/l/hr) and sugar utilization rate (1.802 g/l/hr) are all on slightly higher side for temperature 32.5°C as compared to other temperatures. However, cell mass productivity was higher at temperature of 30°C. This probably indicates that for maximum polyols production by *H. anomala* a controlled or limited growth is necessary. It was also found in shake flask experiments that temperature tolerance of this organism is higher (35°C) with fructose and glucose.

It was found in shake-flask studies that fructose as a substrate gave maximum polyols concentration as compared to glucose and fructose. We wanted to verify this observation on fermenter scale at pH 8.0. Our results indicated that fructose is certainly a better substrate as compared to sucrose. However, after addition of invertase to the sucrose medium, the final polyols yield was slightly better than with fructose. This may be due to the active transport mechanism employed by yeast cells for the inverted products i.e. glucose and fructose.

Though the effect of inoculum size was studied and optimized on shake flask level, the oxygen transfer efficiency of shake flasks is different than that of an aerated and agitated fermenter, Therefore, optimization of inoculum size on fermenter scale is an important aspect from process development point of view. Production of polyols by osmophilic yeasts is an aerobic growth associated process. A very critical factor in

production of polyols is the rate of oxygen supply per unit biomass, which determines the percentage conversion of sugar to polyols. This is directly related to the number of viable cells present in the medium.

The inoculum size experiments were conducted at fixed air flow rate of 0.56 vvm, 600 rpm agitation rate, 30°C temperature, pH 8.0, 0.5 g/l of invertase and at 2.5 liter medium volume. Investigations were carried out at inoculum size of 5 %, 10 %, 15 %, 20 % and 30 %. At 15 % inoculum size, the sugar was almost consumed in 138 hrs. The overall yield of polyols based on total sugars used was maximum at 42.77 % with polyols productivity of about 0.949 g/l/hr. Therefore, 15 % inoculum size under the prevailing conditions of aerobic growth seems to offer maximum polyols yield.

The availability of oxygen has been suggested to be the most important factor controlling the growth, sugar uptake and yield of polyols. Maximum cell growth can be achieved by satisfying the organisms maximum specific oxygen demand by maintaining the dissolved oxygen concentration greater than the critical level. If the dissolved oxygen concentration is allowed to fall below the critical level, the cells may alter its metabolism. Oxygen limitation strategy has been used to enhance glycerol production with osmophilic yeast.

Using automated fermenter, it was possible to control the dissolved oxygen % saturation values at different levels during the fermentation. A strategy of adjusting the pO_2 value to the desired level at 18 hrs was employed to allow for the exponential growth to take place in the early phase of fermentation.

The effect of controlling oxygen % saturation (pO_2) on polyols production by *H. anomala* was studied at 30 %, 40 %, 50 %, 60 % and 70 % values. Fermentations were carried out at 15 % inoculum, 600 rpm agitation rate and temperature of 32.5°C. At 50 % pO_2, sugar utilization was completed within 148 hrs resulting into final polyols concentration of 143.5 g/l. The overall polyols yield based on sugar utilized improved to 47.84 %. However, the cell mass yield was slightly reduced to 1.39×10^8 cells/ml per 100 g of sucrose consumed compared to the maximum cell growth achieved at 40 % pO_2 control value. This indicates that oxygen supply to the organisms has to be critically adjusted so as to control the growth and maximize the polyols production.

Experiments were also conducted on establishing the relationship between the sugar concentration in the fermentation medium and the saturation value of dissolved

oxygen concentration. The results indicate that the oxygen 100 % saturation value in the early phase of fermentation is about 1.68 ppm and goes on increasing to maximum 3.00 ppm as the sugar is consumed during the fermentation.

After optimizing the % saturation of oxygen (pO_2), the effect of inoculum size (at 10 %, 15 % and 20 %) was reinvestigated at controlled pO_2 value of 50 %. It confirmed that 15 % inoculum size is optimum at 32.5°C temperature and 50 % pO_2 controlled conditions.

The effect of agitation was studied at 400, 500, 600 and 700 rpm. Maximum yield of 47.84 % was again obtained at optimum agitation rate of 600 rpm

Based on the investigations carried out on 2.5 liter fermenter scale, the optimum conditions for production of polyols using sucrose as substrate in batch fermentation mode are Inoculum siz:15 %, pO_2 control at: 50 % saturation, Agitation rate: 600 rpm, Temperature: 32.5 °C, pH: 8.0, Invertase dose: 0.5 g/l

The Gas chromatographic analysis used for qualitative estimates indicated 4:1 ratio of glycerol to arabitol in the final fermentation broth sample.

6.6 FED-BATCH FERMENTATION

Fed-batch fermentation aspect was investigated in two sets of experiments. In the first set of experiments, 10 % inoculum was used at 30°C fermentation temperature without pO_2 control. In the second set, 15 % inoculum was used at 32.5°C fermentation temperature and pO_2 controlled at 50 %.

In the first set of fed-batch experiments, optimum results were achieved where the dose of nutrients was reduced to 50 % of the initial dose with maximum overall polyols yield of 32.2 %. In the second set of fed-batch experiments the maximum polyols concentration achieved is 142.9 g/l as compared to the maximum 230.0 g/l achieved in the first set. The maximum overall polyols yield of second set (27.55 %) is less than that obtained in the first set (32.20 %). However, overall yields of both the sets are considerably less than that obtained in batch fermentation (47.84 %). Similarly, the polyols productivity and sugar utilization rates in fed-batch fermentations are lower than those obtained under optimum conditions in batch fermentations. These observations suggest that the optimum conditions for fed-batch fermentation with respect to inoculum size, temperature of fermentation, optimum pO_2 level can be different than the optimum conditions of batch fermentation process.

Therefore, it would be necessary to adjust these parameters as well as sucrose and nutrients feed strategy carefully to achieve maximum yield and productivity of polyols in fed-batch experiments.

The maximum overall polyols yield obtained in batch fermentation with sucrose was 47.84 % as compared to the yield of 51.00 % reported earlier with glucose as substrate. Our work has clearly established that *H. anomala* strain used in this work can produce maximum polyols under alkaline condition (pH 8.0). However, under alkaline conditions, the invertase activity of this strain is seriously reduced making it unable to consume sucrose as efficiently as glucose. Addition of external invertase has helped in improving the sucrose utilization rate (2.055 g/l/hr), polyols productivity (0.983 g/l/hr) and overall polyols yield (47.84 %).

The maximum reported polyols yield by the Chinese researchers is 63 % with polyols productivity of 1.333 g/l/hr. Therefore, there is further scope to improve the process before it can be tested on pilot scale.

Though it was possible to achieve 23.0 % polyols concentration in fed-batch fermentation, the overall polyols yield was substantially reduced as compared to the yield achieved in batch fermentation experiments. This indicates that further investigations are also required for improving the performance of fed-batch fermentation system.

6.7 DEVELOPMENT OF KINETIC MODEL

Modeling of batch fermentation to produce polyols by controlling pO_2 is successfully attempted.

The kinetic model is based on minimizing the sum of square method. The coupled non-linear differential equations are solved by using METLAB software tool. Different unknown parameters are determined to investigate their effects on polyols yield.

The major contributing parameters towards yield of polyols are α, β and λ. It is observed that higher value of these parameters results in higher yield of polyols.

The kinetic model developed for the batch fermentation system can be used for predicting the yield of polyols on higher scale of operation as well as for design of fermentation process.

6.8 RECOMMENDATION FOR FUTURE WORK

Following factors can improve the performance and economic viability of the process.

1. Cell recycle system for repeated batch fermentations.
2. Simulation studies for fed-batch fermentation, which can lead to better polyols yield.
3. Use of cheaper raw materials such as sugarcane juice or molasses.
4. To avoid the increase in the volume of the broth due to addition of alkali, solid Na_2CO_3 can be tested.
5. Further scale-up to pilot plant level.
6. Downstream separation techniques, such as ultrafilteration or reverse osmosis can be tried for efficient product recovery.
7. Interest in fermentative production of other polyols is also increasing.

6.9 PUBLICATIONS/PRESENTATIONS

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ANNEXURE-1

Annexure-1

Set-I **Expt: 5.12.1** **Table: A-1**

Fed Batch1: Without pO_2 conrol, 10% Inoculum, 600 rpm, 30^{o}C

Time (hr)	pH	pO2 (%)	Polyols (g/l)	Residual TRS (g/l)	O.D.	Cells/ml × 10^{8}	Added Solid sucrose (g)	Volume (ml)
0	6.51	91.60	0	300	0.010	0.16	750.0	2500
18	3.07/8.01	51.8/58.6	19.5	260	0.315	3.00		
28	8.00	62.60	27.3	224	0.369	3.20		
41	8.00	68.60	35.6	188	0.510	5.20		
52	8.01	71.60	41.2	156	0.600	5.50		
65	8.00	72.30	47.4	122	0.610	5.40		
76	8.13	76.80	55.4	92	0.580	5.30	559.8	
80	8.00	71.60	47.6	288	0.534	5.00		
89	7.99	62.60	54.1	248	0.533	4.80		
100	8.01	62.40	61.7	217	0.565	5.10		
113	8.01	62.60	70.8	184	0.600	6.20		
124	8.09	69.40	81.5	170	0.612	5.90		
137	8.06	75.20	88.6	108	0.587	5.50		
148	8.04	76.00	91.6	84	0.562	5.00		
161	8.21	81.30	104.4	72	0.597	5.40	534.0	
168	8.01	72.00	90.7	286	0.550	5.20		
179	8.00	68.80	101.4	252	0.501	4.90		
192	8.03	56.00	108.6	228	0.562	6.00		
203	8.00	49.50	117.7	206	0.637	6.30		
216	8.01	57.40	120.8	184	0.560	5.70		
227	8.18	64.60	126.6	168	0.643	7.50		
240	8.02	65.00	130.2	156	0.659	7.50		
251	8.06	66.60	132.8	144	0.692	7.40		
264	8.02	68.70	137.6	128	0.664	7.60		
275	8.00	70.10	142	120	0.677	7.40		
288	8.00	72.50	144.9	102	0.663	7.30		
299	8.05	73.40	151.6	68	0.630	7.00		
312	8.04	73.30	157.8	44	0.628	6.80		
323	8.05	75.70	159.7	26	0.620	6.70		
336	8.04	76.50	163.0	0	0.618	6.70	1843.8	3550 ml

Medium composition, g/l :Sucrose-300; Yeast extract-1.25; Urea-1.0;$MgSO_4.7H_2O$-0.25

RPM: 600, Temperature:32.5^{0} C, Aeration rate:0.54vvm, Initial Fermenter volume 2.5L

Invertase: 0.5g/l, Inoculum: 10%

Set-I Expt: 5.12.2			Table: A-2					
	Fed Batch 2: Without pO_2 conrol, 10% Inoculum, 600 rpm, 30^{o}C							
Time,h	**pH**	**pO_2**	**Polyols**	**Residual TRS**	**O.D.**	**Cells/ml**	**Added Solid sucose**	**Volume**
(hr)		**(%)**	**(g/l)**	**(g/l)**		**$\times 10^8$**	**g**	
0	6.54	97.5	0	300.0	0.008	0.2	750.0	2500.0
18	3.26/8.09	28.3	14.80	264.0	0.313	2.9		
29	7.90	55.0	28.60	228.0	0.428	3.4		
42	8.13	64.1	46.50	190.0	0.529	4.7		
53	7.89	67.8	54.40	152.0	0.590	5.2		
66	7.97	72.6	62.20	126.0	0.565	5.0		
77	7.91	76.3	73.80	104.0	0.575	5.0	525.0	
81	8.00	75.9	55.30	294.0	0.540	4.5		
90	8.20	65.5	66.60	248.0	0.562	4.9		
101	7.92	62.7	77.60	228.0	0.572	4.8		
114	8.06	61.0	84.90	202.0	0.578	5.0		
125	8.19	63.6	89.90	182.0	0.592	5.0		
138	8.16	68.7	96.40	152.0	0.615	5.4		
149	8.12	72.0	99.50	130.0	0.642	5.4	459.0	
152	8.15	47.9	93.70	326.0	0.556	4.9		
162	7.99	53.3	98.30	314.0	0.580	5.0		
173	8.11	52.0	103.90	302.0	0.601	5.0		
186	8.09	55.9	106.30	294.0	0.588	5.0		
197	8.03	58.0	112.60	268.0	0.547	4.9		
210	8.00	62.9	117.70	226.0	0.618	5.5		
221	8.03	64.5	125.80	184.0	0.547	5.0		
234	8.00	66.6	130.60	168.0	0.660	6.0		
245	8.01	68.3	135.80	144.0	0.650	6.0		
258	8.00	73.0	141.50	110.0	0.612	5.5	458.5	
262	8.00	49.7	124.90	302.0	0.538	4.8		
269	7.95	50.3	131.40	272.0	0.550	4.9		
282	8.02	37.4	139.70	246.0	0.578	5.0		
293	8.06	43.5	149.00	220.0	0.605	5.1		
306	8.02	51.7	161.30	194.0	0.586	5.2		
317	8.00	47.7	163.00	180.0	0.590	5.0		
330	8.10	52.1	172.50	150.0	0.625	5.5		
341	8.00	53.7	183.90	122.0	0.610	5.2		
354	8.04	58.0	195.30	94.0	0.592	5.0		
365	8.03	58.6	206.90	62.0	0.597	5.0		
378	8.02	62.5	212.20	36.0	0.600	5.2		
389	8.05	77.0	225.20	16.0	0.603	5.2		
402	8.08	77.5	230.00	0.0	0.600	5.2	2192.5	3070 ml
Medium composition, g/l :Sucrose-300; Yeast extract-1.25; Urea-1.0;$MgSO_4.7H_2O$-0.25								
RPM: 600, Temperature:32.5^{0} C, Aeration rate:0.54vvm, Initial Fermenter volume 2.5L								
Invertase: 0.5g/l,		Inoculum: 10%						

Set-I Expt: F-FB-3			Table: A-3					
Fed Batch3: Without pO_2 conrol, 10% Inoculum, 600 rpm, 30^{0}C								
Time	**pH**	**pO_2**	**Polyols**	**Residual TRS**	**O.D.**	**Cells/ml**	**Added Solid sucrose**	**Volume**
(h)		**(%)**	**(g/l)**	**(g/l)**		**$\times 10^8$**	**(g)**	**(ml)**
0	6.72	98.2	0.00	302	0.009	0.170	750.0	2500
18	3.30/8.10	25.3	14.50	270	0.289	2.700		
29	8.06	33.1	28.00	240	0.342	3.000		
42	8.02	35.8	45.70	206	0.505	4.500		
53	8.06	37.8	53.80	174	0.535	4.800		
66	7.99	38.7	59.70	156	0.518	4.500		
77	8.33	42.1	64.40	132	0.529	4.700		
90	8.26	44.4	69.50	100	0.620	5.500	525.0	
95	8.19	38.7	56.50	330	0.503	4.500		
101	7.80	36.5	66.40	312	0.525	4.500		
114	8.24	34.2	73.00	300	0.540	4.700		
125	8.18	34.0	81.60	288	0.572	4.700		
138	8.21	35.0	88.50	270	0.580	5.000		
149	8.13	36.1	94.40	252	0.587	4.900		
162	8.11	37.4	98.00	240	0.552	4.700		
173	8.09	53.9	102.30	234	0.541	4.600		
186	8.08	54.4	104.40	232	0.480	4.000		
197	8.08	52.9	106.80	224	0.492	4.000		
210	8.08	55.0	111.50	210	0.500	4.500		
221	8.09	52.6	120.30	194	0.527	4.600		
234	8.03	52.8	124.80	184	0.500	4.400		
245	8.02	52.6	128.30	174	0.512	4.260		
258	8.01	53.2	131.6	168	0.433	3.750	1275	2375.0
Medium composition, g/l :Sucrose-300; Yeast extract-1.25; Urea-1.0;$MgSO_4.7H_2O$-0.25								
RPM: 600, Temperature:32.5^0 C, Aeration rate:0.54vvm, Initial Fermenter volume 2.5L								
Invertase: 0.5g/l,	Inoculum: 10%							
Batch was terminated because few contaminates were seen at 258 hrs.								

Set-II Expt: 5.12.4			Table: A-4					
	Fed Batch 4: With pO_2 conrol at 50 %, 15% Inoculum, 32.5^{0}C, pH-8.0, 600 rpm							
Time,h	**pH**	**pO_2**	**Polyols**	**Residual TRS**	**O.D.**	**Cells/ml**	**Added Solid sucose**	**Volume**
(hr)		**(%)**	**(g/l)**	**(g/l)**	**660 nm**	**$\times 10^{12}$**	**g**	**(ml)**
0	6.52	99.7	0.0	308	0.004	0.00005	750.0	2500.0
18	2.87/8.01	51.1	19.4	282	0.118	1.11		
29	7.99	53.1	27.7	236	0.355	1.68		
42	8.02	60.2	44.1	198	0.413	1.90		
49	8.03	57.3	50.0	168	0.499	2.18		
66	8.02	56.0	69.0	130	0.496	1.98	444.5	
73	8.01	53.3	54.6	268	0.375	2.02		
77	8.06	56.3	62.2	256	0.422	2.27		
90	7.93	40.4	71.7	226	0.514	2.72		
101	8.13	58.3	75.0	200	0.504	2.06		
114	8.03	46.3	89.5	170	0.528	1.89		
122	8.07	65.3	92.1	138	0.555	2.53		
138	7.92	63.8	101.6	108	0.575	2.87		
143	8.03	63.5	106.1	98	0.577	2.84	447.5	
144	8.28	62.2	81.0	240	0.543	2.75		
149	7.98	36.8	83.3	234	0.545	2.89		
162	7.95	38.3	85.8	204	0.556	2.36		
173	8.07	55.0	103.0	180	0.606	2.43		
186	7.98	55.6	104.4	172	0.632	3.01		
197	7.99	45.9	108.3	162	0.645	2.65		
210	7.92	43.3	120.9	142	0.695	3.36		
221	8.11	56.3	115.6	128	0.682	3.13		
234	7.88	56.3	127.0	100	0.716	3.48		
240	8.11	53.0	130.2	96	0.708	3.45	444.0	
241	8.34	50.2	105.5	230	0.640	3.40		
245	8.16	53.0	109.6	212	0.641	3.21		
258	8.09	55.7	112.6	208	0.681	3.59		
269	8.17	56.9	103.3	194	0.695	3.68		
282	8.08	52.2	117.1	178	0.705	3.72		
293	8.00	46.3	112.2	156	0.712	3.77		
306	7.89	46.9	105.5	150	0.726	3.26		
317	8.07	54.0	116.8	124	0.720	2.99		
330	7.89	47.6	116.7	106	0.738	3.33	2086.0	3550
Medium composition, g/l :Sucrose-300; Yeast extract-1.25; Urea-1.0;$MgSO_4.7H_2O$-0.25								
RPM: 600, Temperature:32.5^0 C, pH8.0, Initial Fermenter volume 2.5L								
Invertase: 0.5g/l,		Inoculum: 15 %						
Batch was terminated because few contaminates were seen from 293 hrs onwards.								

Set-II Expt: 5.12.5			Table: A-5					
	Fed Batch 5: With pO_2 conrol at 50 %, 15% Inoculum, 32.5^{0}C, pH-8.0, 600 rpm							
Time,h	**pH**	**pO_2**	**Polyols**	**Residual TRS**	**O.D.**	**Cells/ml**	**Added Liquid sucose**	**Volume**
(hr)		**(%)**	**(g/l)**	**(g/l)**	**660 nm**	**$\times 10^{12}$**	**g**	**(ml)**
0	6.35	96.7	0.0	296	0.003	0.00004	750.0	2500.0
18	2.94/7.99	24.0/61.0	20.2	276	0.321	1.10		
24	7.95	38.6	28.5	240	0.421	1.75	153.0	
25	7.99	50.3	24.7	276	0.367	1.42		
29	7.92	50.0	29.9	272	0.342	1.57		
42	8.04	56.4	50.6	218	0.443	2.70		
50	8.12	52.0	81.3	174	0.467	2.91	270.6	
51	8.21	42.6	72.3	254	0.405	2.47		
66	9.92	50.2	72.7	204	0.498	3.65		
74	7.97	55.9	77.8	184	0.553	4.06	394.4	
75	8.28	45.8	64.3	254	0.45	4.05		
90	8.20	50.7	77.6	208	0.498	3.66		
99	8.10	50.9	87.5	170	0.554	4.64	446.0	
100	7.92	41.3	73.7	266	0.486	4.54		
114	7.95	49.8	82.0	220	0.565	5.98		
123	8.22	45.2	91.3	180	0.584	6.51	460.0	
124	8.15	20.2	78.5	270	0.514	6.95		
138	8.15	48.5	79.0	240	0.593	8.35		
149	8.01	47.8	94.8	222	0.625	8.87		
162	8.12	45.3	101.6	188	0.626	11.70		
173	8.00	46.8	109.3	174	0.665	12.87		
186	7.96	49.2	115.3	154	0.650	11.89		
197	8.03	48.9	118.5	130	0.673	11.58		
210	7.97	45.3	121.1	118	0.681	14.28		
221	8.02	49.0	125.5	110	0.706	15.89		
234	7.97	49.4	127.0	100	0.668	8.73		
245	8.00	56.8	129.3	94	0.662	9.58		
258	8.03	54.9	133.8	84	0.656	8.28		
269	7.98	46.2	128.5	70	0.657	9.26		
282	7.99	53.1	141.6	68	0.649	8.85		
293	7.98	50.7	131.9	62	0.637	7.53		
306	8.00	58.1	130.1	58	0.627	6.61	2474.0	5238
Medium composition, g/l :Sucrose-300; Yeast extract-1.25; Urea-1.0;$MgSO_4.7H_2O$-0.25								
RPM: 600, Temperature:32.5^0 C, pH8.0, Initial Fermenter volume 2.5L								
Invertase: 0.5g/l,	Inoculum: 15 %							
Batch was terminated because of increase in broth volume and drop of viable cell count/OD.								

Set-II	Expt: 5.12.6		Table: A-6					
	Fed Batch 5: With pO_2 conrol at 50 %, 15% Inoculum, 32.5^{0}C, pH-8.0, 600 rpm							
Time,h	pH	pO_2	Polyols	Residual TRS	O.D.	Cells/ml	Added Solid sucose	Volume
(hr)		(%)	(g/l)	(g/l)	660 nm	$\times 10^{12}$	g	(ml)
0	6.22	96.6	0.0	314	0.004	0.00005	781.00	2487.0
18	3.11/7.97	33.6/51.7	16.5	260	0.269	1.05		
29	8.01	49.8	31.4	222	0.402	1.79		
42	8.12	51.2	43.4	172	0.533	1.99		
48	8.02	46.8	53.9	162	0.523	2.00	433.83	
49	8.06	37.5	41.4	292	0.493	2.15		
53	8.15	50.2	49.6	288	0.495	2.25		
66	7.98	51.0	62.8	244	0.565	2.37		
77	7.98	50.6	69.3	194	0.580	2.65		
90	8.01	51.2	79.1	178	0.576	2.54		
96	8.11	52.0	95.8	164	0.635	2.88	477.47	
97	8.01	49.6	79.0	320	0.570	2.78		
101	7.98	46.3	85.0	300	0.546	2.50		
114	8.19	47.8	82.9	262	0.557	2.35		
125	8.15	51.1	86.5	230	0.580	3.11		
138	8.03	49.8	98.3	214	0.617	3.71		
144	7.99	50.9	101.6	208	0.615	3.28	344.35	
145	7.98	43.7	92.4	292	0.550	3.33		
149	7.95	44.6	93.5	290	0.548	3.28		
162	7.99	51.9	90.5	270	0.590	3.44		
173	8.03	48.9	97.6	248	0.568	3.39		
186	8.00	50.1	106.4	232	0.546	3.31		
194	8.01	50.3	122.6	228	0.598	3.49	364.24	
195	8.16	48.9	97.9	322	0.544	3.49		
197	8.10	50.3	103.0	318	0.532	3.60		
210	8.02	50.7	104.8	284	0.557	3.75		
221	8.07	49.8	108.9	282	0.550	3.68		
234	8.11	51.2	110.3	274	0.545	2.16		
245	8.02	48.0	112.2	236	0.550	3.16		
258	8.04	49.2	118.3	234	0.522	2.76		
269	8.05	48.6	122.5	230	0.542	3.13		
282	8.03	54.6	128.4	212	0.545	3.03		
293	8.11	52.9	129.9	186	0.532	2.83		
306	8.08	51.1	134.5	176	0.565	3.16		
317	8.03	50.1	126.5	170	0.535	2.93		
330	7.99	50.0	133.1	158	0.549	3.20		
341	7.94	50.0	140.4	152	0.532	2.89		
354	8.01	50.0	139.9	142	0.516	3.13		
366	8.06	49.9	152.4	132	0.495	2.77		
378	8.09	49.9	142.9	122	0.504	2.66	2400.90	3734.0
Medium composition, g/l :Sucrose-300; Yeast extract-1.25; Urea-1.0;$MgSO_4.7H_2O$-0.25								
RPM: 600, Temperature:32.5^{0}C, pH8.0, Initial Fermenter volume 2.5L								
Invertase: 0.5g/l,	Inoculum: 15 %							
Batch was terminated because of increasing growth of contaminants after 234hrs.								