MICROBIAL ENZYMES: IMMOBILIZED WHOLE CELL SYSTEMS IN FERMENTATION

A THESIS

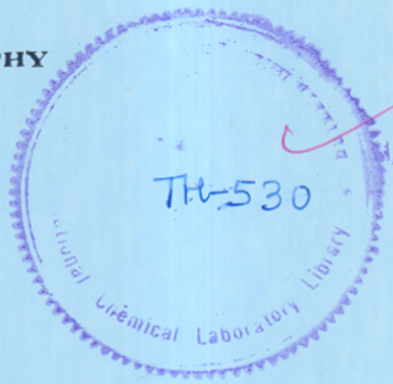
SUBMITTED TO THE

UNIVERSITY OF POONA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(IN BIOCHEMISTRY)



BY

B. SEETARAMARAO

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE 411 008 (INDIA)

JULY 1987

CONTENTS

DECLARATION

Certified that the work incorporated in the thesis entitled: **"MICROBIAL ENZYMES: IMMOBILIZED WHOLE CELLS SYSTEMS IN FERMENTATION"** submitted by Shri Bommaraju SeetaRama Rao for the Ph.D. degree was carried out by the candidate under my general supervision. Such material as has been obtained from other sources has been duly acknowledged in this thesis.

C. SivaRaman
Research Guide

ACKNOWLEDGEMENTS

The author is deeply indebted to Dr. C. SivaRaman and wishes to express his profound gratitude for his valuable guidance, encouragement and sustained interest during the course of these investigations.

The author is grateful to Dr. (Mrs.) H. SivaRaman, Mrs. A.V. Pundle and Mrs. A.A. Prabhune for the invaluable help for carrying out various studies and microbiological analysis.

The author wishes to thank Dr. V. Shankar for the great help in the studies on SeaPlaque agarose immobilization, and he is grateful to Mr. D.V. Gokhale for the screening and isolation of fermentative isolates and to Dr. S. SivaRamakrishnan for the help in the studies on yeast enzymes. Technical assistance rendered by Mr. S.M. Kotwal is gratefully acknowledged.

He also wishes to thank Dr. C.S. Dorai of analytical group of the Inorganic division for the analysis of inorganic salts in cane molasses.

He is grateful to Mr. S.T. Dhume and Mr. M.N. Kamthe for the excellent maintenance and help on special equipment. He would also like to thank Drs. S. Sainkar and Mrs. A. Mitra for the scanning electron micrographs.

Support received for the project under the United

Nations Development Programme is also gratefully acknowledged.

He also wishes to express his gratitude to the Director, National Chemical Laboratory, Poona, for permission to submit this work in the form of a thesis.

LIST OF ABBREVIATIONS

ADH	:	Alcholdehydrogenase
CFUS	:	Colony forming units
DNSA	:	3,5-Dinitrosalicylic acid
HPLC	:	High performance liquid chromatography
SDS	:	Sodium dodecyl sulphate
TCA	:	Trichloroacetic acid
TEMED	:	N,N,N'N'Tetramethylethylene diamine
PDC	:	Pyruvate decarboxylase
SEM	:	Scanning electron micrograph

SYNOPSIS OF THE THESIS

Ethanol is a key product in the conversion of sugars and starches to liquid fuel and chemical feedstock. Ethanol production in India is exclusively through traditional fermentation of sugarcane molasses, a by-product of the sugar industry. Essential requirements for the development of more cost effective processes in such a route of molasses utilization are enhanced productivity and high conversion efficiencies at an adequately high product concentration. Pre-requisites for such improvements are the availability of microbial strains suitable for the fermentation of molasses sugars having ethanol- and osmo-tolerances and high substrate transformation rates in continuous fermentation systems which operate at high cell concentrations. With these objectives in view, studies were carried out to:

(a) Characterize substrate- and ethanol- tolerant yeasts from among available strains and isolates;

(b) Develop novel open-pore matrices for the entrapment of yeast cells so as to minimise diffusional restrictions; and

(c) Establish the usefulness of the immobilized yeast systems for the long term continuous fermentation of cane molasses to ethanol at conversion efficiencies of about 95% and volumetric productivities of about

15 $gl^{-1}h^{-1}$ at about 6% w/v ethanol concentration. The original findings which contribute to new knowledge in this field are embodied in the present thesis.

The thesis is set out in four parts.

PART I: GENERAL INTRODUCTION

This part comprises a literature survey, mainly of ethanol production by biotechnology; the general methods used for the immobilization of microbial whole cells and an account of such systems used hitherto for the production of ethanol from fermentable sugars.

PART II: YEAST STRAIN SELECTION FOR ETHANOLIC FERMENTATION

Characterization of substrate- and ethanol-tolerant Saccharomyces cerevisiae isolate, designated as Y-10 and comparison with standard brewing strains such as Saccharomyces uvarum ATCC 26602 and other isolates in batch mode fermentation with free cells.

Levels and behaviour of 3 key enzymes involved in ethanolic fermentation of molasses, namely; invertase (EC 3.2.1.26), alcohol dehydrogenase (EC 1.1.1.1) and pyruvate decarboxylase (EC 4.1.1.1) in the ethanol-tolerant yeast are compared with those of the corresponding enzymes in the standard strain.

Specific ethanol productivities of the ethanol tolerant isolate S. cerevisiae Y-10 at high molasses concentrations have been shown to be markedly higher

than those of the S. uvarum. Alcohol dehydrogenase activity is 40% to 100% higher in the isolate than in the S. uvarum. The alcohol dehydrogenase isozyme in the isolate also differs electrophoretically from that of the standard strain. In the case of invertase and pyruvate decarboxylase activities no marked differences are observed between the strains.

PART III: MATRICES FOR YEAST CELL ENTRAPMENT

Several novel open-pore gel matrices are described for the immobilization of yeast cells.

Gelatin. Open-pore gelatin pellets with entrapped yeast cells are obtained by selective leaching out of calcium alginate from a composite matrix obtained by dropping a suspension of the cells in a solution containing gelatin and sodium alginate into an aqueous solution of calcium chloride followed by crosslinking with glutaraldehyde. S. uvarum immobilized in the porous carrier shows maximum ethanol productivity of about 67 $gl^{-1}h^{-1}$ on total reactor volume basis when monitored in packed bed reactors at 35°C with continuous cane molasses feed containing 10% fermentable sugars.

Immobilization and conversion conditions have been standardized.

Agar. The use of open pore gelatin as a carrier for the entrapment of yeast cells has posed problems of bead erosion from the interior when used over prolonged

periods of 2 - 3 months in the continuous fermentation of cane molasses to ethanol. Agar, a more stable matrix, has therefore been substituted for gelatin and a procedure is described for obtaining open-pore agar beads with entrapped yeast cells. The porous agar matrix, unlike unprocessed agar, has been shown to be suitable for use in reactions that involve cell growth and gas formation. Beads of the porous agar with entrapped yeast cells have been used for the continuous fermentation of sugarcane molasses to ethanol without apparent bead rupture even after prolonged periods of about three months of continuous use.

SeaPlaque agarose. The high temperatures of about 45°C required for melting standard agar gels can cause yeast cell inactivation. A commercially available low gelling temperature agarose, namely SeaPlaque agarose, has therefore been used in place of agar and the system has been applied for continuous ethanol fermentation.

The ethanol productivities of these and other open-pore matrix immobilized yeast cell systems have been monitored in packed-bed reactors. The performances of the reactors are consistent over a period of about 4 to 5 weeks after which a gradual loss in activity is observed on further extended use in the case of all the matrices that have been investigated.

PART IV: EFFECT OF FEED STOCK CONSTITUENTS

Studies on the possible cause (or causes) for the lowered conversion efficiencies in prolonged continuous conversion have been investigated using the usual calcium alginate immobilized standard strain of S. uvarum. Controlled aeration and co-immobilization of ergosterol have been shown to be without effect in prolonging the period of consistent reactor performance. Both sucrose and deionized molasses feedstocks when supplemented with requisite amount of yeast extract or cornsteep liquor caused no observable decrease in conversion efficiencies and volumetric productivities over a period of 3 months through which the continuous conversions were carried. Supplementation of cane molasses with adequate amount of yeast extract showed no enhancement in the period of full conversion and reactors performed consistently only upto about 30 days of continuous operation. Supplementation of the sucrose-yeast extract medium with KCl, NaCl, and $CaCl_2$ levels simulating those present in cane molasses caused no adverse effect on reactor performances indicating that constituents of cane molasses other than these are inhibitory.

LIST OF PUBLICATIONS

1. Continuous ethanol production by yeast cells immobilized in open pore gelatin matrix
   H. SivaRaman, B. SeetaRamaRao, A.V. Pundle and C. SivaRaman
   Biotechnology Letters 4, 359-364, 1982.

2. Yeast cells entrapped in low-gelling temperature agarose for the continuous production of ethanol
   V. Shankar, S.M. Kotwal and B. SeetaRamaRao
   Biotechnology Letters 7, 615- 618, 1985.

3. Ethanol production by yeast cells immobilized in open-pore agar
   B.S. Rao, A.V. Pundle, A.A. Prabhune, V. Shankar and H. SivaRaman
   Appl. Biochem. Biotechnol. 12, 17, 1986.

4. Alcohol dehydrogenase and invertase activities in ethanol tolerant yeasts
   D.V. Gokhale, B.S. Rao, and S. Sivaramakrishnan
   Enzyme and Microbial Technology 8, 623 - 626, 1986.

PART I

GENERAL INTRODUCTION

Industrial Perspective

Ethanol is an important industrial solvent and a chemical feedstock in the synthesis of a wide range of other solvents, pharmaceuticals, adhesives, detergents, plastics, plastisizers and a host of other industrially important compounds. Further, potable ethanol has high economic value in the beverages industry. Ethanol is also an energy feedstock and can serve as a fuel in internal combustion engines. In Brazil, which has land and water resources for large scale sugarcane cultivation, fuel-grade ethanol obtained from the fermentation of cane juice and molasses is displacing petrol under their "proalcool" programme and the annual production was 2.5 billion US gal in 1984 (Anderson, 1985). It has been estimated that only 2% of the cultivable land in that country is needed to provide the cane juice and molasses required for the ethanol to substitute their entire petroleum imports (Suomalainen, 1981).

The current routes used for the commercial production of ethanol are either through chemical synthesis from ethylene, derived from petroleum, or through fermentation either of sugars obtained from sugar crops such as sugarcane, sugarbeet, hybrid sorghum or of starch or starch hydrolysates derived from cereal crops such as maize (corn), wheat, barley, grain sorghum and root and tuber crops such as cassava (topioca, manioc), potato and Jerusalem

artichoke (wild sunflower). The awareness of the increasingly high current rates of depletion of available non-renewable fossil resources and the possibility of a resurgence in the cost of petroleum and petroleum products have resulted in the establishment of fermentation plants for the production of fuel additive and chemical feedstock-grade ethanol from starch-base raw materials even in countries such as the USA where earlier production had been exclusively through chemical synthesis.

The production of ethanol in India is exclusively from the fermentation of cane molasses on account of the low statutory price of the substrate. The annual production during 1982 - 1983 was 576.2 x 10^6L ethanol of which 70% and 30% were for industrial and potable end uses, respectively; and the fermentations were reportedly at conversion efficiencies ranging from 72% - 88% of the available sugars in molasses (Rajan and Ayyangar, 1985). The cane production during 1986 - 1987 is estimated at 180 x 10^6T (Financial Express, January 12, 1987) compared to 177.4 x 10^6T during 1982 - 1983 (Rajan and Ayyangar, 1985). The total production is insufficient to meet even marginally the fuel feedstock requirements of the country

Improvements in fermentation processes for the production of ethanol are warranted, particularly on account of the industrial importance of ethanol and the need to obtain this key compound from renewable resources.

Such efforts have focussed mainly on the following problems:

* Isolation or genetic construction of suitable microbial strains with high specific productivities and requisite tolerances to substrates, product and temperature.

* Development of continuous fermentation technology capable of high conversion efficiencies and high productivities at relatively high ethanol levels in the product stream.

* Efficient heat recovery in the distillation step.

* Cost effective effluent treatment through either biomethanation or incineration procedures.

The present introduction summarizes briefly the extensive literature on the first two aspects; namely, the microorganisms used and the fermentation processes for ethanol production.

I.1 MICROORGANISMS FOR ETHANOLIC FERMENTATION

I.1.1. Yeasts

Current commercial scale ethanol production uses only yeasts. The main advantages of yeasts is the highly selective ethanol production from glucose and other fermentable hexoses with only traces of acidic or other undesirable by-products. The organisms are more hardy and are also easier to separate than bacteria. Yeast strains which are generally used for glucose, sucrose and molasses fermentations are Saccharomyces cerevisiae, S. ellypsoideus, S. uvarum (carlsbergensis), S. fragilis and Schizosaccharomyces pombe. Strains are choosen for high fermentation rates, high ethanol yields, osmotolerance, substrate tolerance, low optimal pH for fermentation and stability under temperature stresses and resistance to physical and chemical stresses. Osmotolerance is particularly important where cane and other black strap molasses constitute the raw materials. Osmotolerance would also permit stillage recycle as in the Biostil process. Low optimal pH for the fermentation contributes to exclusion of contamination by more pH sensitive organisms. Industrial strains are also selected for catabolite repression of oxidative pathways viz. Crabtree effect. At sugar concentration ranging from 3 to 30 gl^{-1} the production of enzymes of the oxidative pathway is inhibited in such strains, the sugar level varying with the strain (Moss et al.,1971;

DeDeken, 1966). Such strains exhibiting marked Crabtree effect are forced to use the glycolytic pathway even in the presence of oxygen which is needed for poly-unsaturated lipid and sterol biosyntheses, without leading to aerobic sugar metabolism. Anaerobically cultured yeasts are known to have nutritional requirements for ergosterol and unsaturated lipids (Hossack and Rose, 1976; Nes et al. 1978; Proudlock et al. 1968).

A property that is being selected for, more recently, is the killer character first reported by Bevan and Makower, (1963) and Bendova (1986). In Saccharomyces killer strains, an extracellular protein toxin is produced and this depends on the presence of virus like protein encapsulated ds-RNA (Tipper and Bortrain, 1984). The killer activity contributes to protection against contamination from sensitive yeasts (Bendova, 1986).

I.1.1(i) Nutrient requirements. Besides sugar source for ethanol production, secondary nutrient supplements are necessary for cell maintainence, growth and fermentation (Jones et al., 1981; Jones and Greenfield, 1984). These include nitrogen sources such as NH_4^+ ions, urea etc. required for protein and nucleic acid syntheses, growth factors (vitamins etc.), minerals, phosphorus. Thiamine has been reported to increase ethanol tolerance (Rahn, 1952). Complex substrates such as black strap molasses normally require supplementation with ammonium sulfate or urea

only (Hodge and Hilderbrandt, 1954).

Several cations also act as inhibitors of growth and fermentation at varied concentrations (Jones and Greenfield, 1984).

I.1.1(ii) Effect of sugars. Hexoses such as glucose, fructose, galactose and maltose are primary substrates in the fermentation by Saccharomyces strains.

At substrate concentration lower than 3 gl^{-1} these starve resulting in decreased productivity (Levenspiel, 1980). Optimal rates of ethanol production are generally at the saturation limit of 150 gl^{-1} concentration of sugar. At higher levels catabolic inhibition of yeast growth as well as inhibition of the enzymes of the glycolytic pathway cause lowering in conversion rates (Holzer, 1968; Holcberg and Margalith, 1981; Wang et al. 1979; Hahn-Hägerdal et al. 1982).

I.1.1(iii) Effect of ethanol. Ethanol is toxic to yeasts and high ethanol tolerance is a requirement for industrial strains of brewers yeast (Casey and Ingledew, 1985). The inhibitory effect is generally negligible at ethanol concentrations lower than 20 gl^{-1} (Bazua and Wilke, 1977) but is generally marked at concentrations above 110 gl^{-1}. Slow fermenting yeasts such as S. sake tolerate ethanol concentrations of 160 gl^{-1} at relatively low temperatures (Hayashida and Ohta, 1981). Ethanol inhibits growth and viability on the one hand and fermentation on the

other, the former being complex and more marked than the latter (Brown et al. 1981).

Improved tolerance to the toxic effects of ethanol has been reported when the medium is supplemented with complex nutrients such as unsaturated lipids (Ingram and Buttke, 1984), soy flour, yeast extract, Aspergillus oryzae proteolipid, sterol-lipid-protein complex, skim milk powder (Damiano and Wang, 1985; Hayshida et al. 1976; Ohta and Hayshida, 1983; Patil et al. 1986).

The property of ethanol tolerance in yeast is under polygenic control (Ismail and Ali, 1971) and ethanol tolerant mutants are not easily obtainable by conventional screening and selection techniques. Tolerance however is enhanced by nutritional as well as environmental factors. Magnesium-supplemented cultures have been shown to increase cell mass as well as ethanol accumulation suggestive of the metal being the active component of the complex protective agents (Dombek and Ingram, 1986a). Heat shock treatment has been reported to increase markedly ethanol tolerance in several Sacch. strains (Watson and Caviechioli, 1983). Loss of viability of S. cerevisiae cells in ethanol containing buffers was less rapid when these organisms contained plasma membrane enriched in linoleyl rather than oleyl residues or enriched in ergosterol or stigmasterol rather than campestrol or cholesterol (Thomas et al. 1978). Ethanol tolerant yeasts have also

been shown to have higher percentage of unsaturated fatty acids in their plasma membranes compared to less tolerant strains (Chen, 1981). The toxic effect of ethanol is attributed to inhibition and denaturation of key glycolytic enzymes as well as modification of cell membrane (Millar et al. 1982; Rose and Beavan 1981). Glucose, lysine and arginine uptake rates are markedly lowered by 0.5M ethanol (Thomas and Rose, 1979), probably by resultant cell membrane changes. It was believed earlier that during fermentation, intracellular accumulation of ethanol occurs in yeasts to levels markedly higher than in the fermented beer (Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978; Panchal and Stewart, 1980). More recent studies using improved procedures for assay of intracellular ethanol, however, indicate that such an accumulation of ethanol against concentration gradient does not occur in yeast (Guijjaro and Legunas, 1984; Dombek and Ingram, 1986b). Exogenously added ethanol however is less inhibitory than equivalent levels produced endogenously during fermentation (Novak et al., 1981).

I.1.1(iv) Effect of temperature. Most distillery yeasts have temperature growth optimum between 30°C to 35°C (Gray et al., 1942). The optimum temperature for fermentation however is about 39°C (Brown and Oliver, 1982; Krouwel, 1979).

The storage of yeasts is conveniently done at about 0°C - 5°C, the stored cells being easily revived (Stark,1954).

I.1.1(v) Starch fermentation. Yeast fermentation of starchy materials is generally preceded by hydrolysis to maltose or glucose. However, several amylase-producing yeasts utilize starch directly without the requirement for a separate pre-fermentation hydrolysis. In the 'Symba' process of the Swedish Sugar Company developed during 1960's a mixed culture of Endocopsis fibuliger, an amylase producing yeast and Candida utilis has been used for the production of single cell protein from potato waste (Skogman, 1976), Schwanniomyces alluvius ferments soluble starch to ethanol at conversion efficiencies greater than 95%, with only trace amounts of side products (Calleja et al. 1982). Saccharomyces diastaticus and S. castellii also ferment dextrin or soluble starch directly to ethanol (Amin et al., 1980).

Inulin from Jerusalem artichoke tuber extracts has been fermented to ethanol with strains of inulinase-containing yeasts Kluyveromyces marxianus and Debaryomyces polymorphus (Margaritis and Bajpai, 1982a, 1982b; Margaritis et al., 1983).

I.1.1(vi) Pentoses. D-Xylose and L-arabinose, the constituent pentoses of hemicellulosic materials, have been considered traditionally as non-fermentable by yeasts.

Many yeasts including S. cerevisiae readily ferment pentuloses such as D-xylulose to ethanol and at ethanol yield of about 90% of the theoretical of 2 moles ethanol per mole of sugar (Gong et al., 1983). The ability to produce ethanol from D-xylose under semi-aerobic conditions has been shown in some yeasts such as Pachysolan tannophilus (Schenider et al., 1983) and several Candida sp. (Jeffries,1981).

1.1.2. Bacteria

Major disadvantages with most of the bacterial strains used for ethanol production are the production of undesirable by-products such as acids in the beer, the narrow range of pH optimum and their small size which makes the pre-distillation cell separation generally more difficult than with the larger yeast cells. The latter problem, however, can be overcome by using flocculent strains. Bacteria are also highly sensitive to presence of oxygen.

The advantages of using bacteria are the possibility of extreme thermophily which contributes to easier downstream processing for ethanol recovery as well as prevention of contamination, the intrinsic ease for genetic manipulation and the wide range of substrates which are fermented viz. both hexoses and pentoses including delignified biopolymers in some cases. Among the thermophilic anaerobic bacteria that have been studied are Clostridium thermocellum, C. thermohydrosulfuricans, C. thermosaccharolyticum and

Thermoanaerobium brockii (Zeikus et al., 1979). All strains have an operative Embden-Meyerhof glycolytic pathway (Lamad and Zeikus, 1980). Thermoanaerobacter ethanolicus has been shown to have temperature optimum of 69°C and to produce 1.7 to 1.9 moles of ethanol per mole of glucose at an initial medium pH 7.5 to 8.0 which is higher than that observed in mono-cultures of other thermophilic organisms (Kannan and Muthurasan, 1985). The ethanol tolerances of all these bacteria are poor.

I.1.2(i) Zymomonas mobilis. The most intensively studied of ethanol producing bacteria is Z. mobilis, the organism responsible for traditional fermentation of palm and agave saps (Rogers et al., 1982). Lactate is a typical by-product of Z. mobilis fermentations compared to glycerol which is a characteristic yeast product (Amin et al., 1983).

Z. mobilis uses the Entner-Doudoroff pathway (Gibbs and DeMoss, 1951) of glucose metabolism producing 1 mole of ATP per mole of glucose utilized (Gibbs and DeMoss, 1954; Swings and DeLey, 1977). S. cerevisiae in contrast uses the Embden-Meyerhof glycolytic pathway, the overall reaction producing 2 moles each of ethanol, CO_2 and ATP per every mole of glucose consumed.

Z. mobilis : D-Glucose $\longrightarrow 2C_2H_5OH + 2CO_2 + ATP$

S. cerevisiae: D-Glucose $\longrightarrow 2C_2H_5OH + 2CO_2 + 2ATP$

Assuming that the requirement of ATP for biomass synthesis

from glucose is approximately the same in Z. mobilis and S. cerevisiae, the first organism would produce half the biomass as the second from the same amount of glucose. This makes Z. mobilis theoretically a better ethanol producer. Z. mobilis also produces 1.9 mole ethanol per mole glucose utilized under anaerobic conditions.

The fermentation of fructose by Z. mobilis is less efficient, wasteful products such as glycerol, dihydroxyacetone and acetaldehyde being formed (Swings and DeLey, 1977). Efficiency of sucrose conversion to ethanol is also generally affected by levan formation from sucrose but not from fructose, glucose or the mixture of the two (Ribbons et al., 1962; Dawes et al., 1966). Levan formation by the enzyme levansucrase however is suppressed in Z. mobilis at high initial pH (Lynesse and Doelle, 1983) and elevated temperature (Lyness and Doelle, 1980). A highly adapted strain of Z. mobilis has been reported to ferment sucrose, sugarcane juice and syrup at hydrolysis efficiency of 94% - 98% and ethanol conversion efficiency of 95% - 98% (Doelle and Greenfield, 1985). Unlike the other bacteria, Zymomonas strains have been improved for high ethanol tolerance. Highly flocculent mutants of the organism have also been isolated (Rogers et al., 1982).

Zymomonas mobilis does not utilize lactose, maltose, cellobiose and pentose sugars.

I.1.3. Fungi

Some mycelial fungi such as Fusarium, Mucor and Monilia ferment pentoses to produce ethanol although at impractically slow rates (Ueng and Gong, 1982; Gong et al. 1981; Srinivasan et al., 1986). Direct fermentation of cellulose to ethanol has also been reported in a species of Monilia (Gong et al., 1981) and by Neurospora crassa (Rao et al., 1983).

A novel fungus Paecilomyces sp. has recently been reported to ferment all the major sugars derived from hydrolysis of plant biomass to ethanol (Wu et al., 1986). These include cellobiose, fructose, glucose, galactose, mannose, starch, xylose, L-arabinose, lactose, maltose and D-ribose. The "Pekilo" process developed in Finland uses Paecilomyces varioti for microbial biomass production from stripped sulphite waste liquor (Romantschuk and Lentomaki, 1978).

I.2 RAW MATERIALS FOR ETHANOL PRODUCTION

Currently used raw materials for the industrial production of ethanol by fermentation fall under two types: (i) saccharine and (ii) starchy materials.

I.2.1 Saccharine substrates. The usual saccharine raw materials are sugar stalk crops such as sugarcane (Saccharum species) and sweet or hybrid sorghum (Sorghum bicolor Moench), both of which exhibit C_4 photosynthetic pathway and sugar and fodder beets, the latter being obtained through crossing of sugar beets with marigold (Earl and Brown, 1979). The advantages of the beet crops are their tolerance to a wide range of soil and climatic conditions and the resistance of their sugars to degradation over prolonged periods of storage. In these crops, black strap molasses (Hodge and Hilderbrandt, 1954) which represent the non-crystallizable residues remaining after sucrose isolation constitute the main raw materials for the fermentation processes. However, in South American countries like Brazil, sugarcane juice is also directly used as substrate and in Gautamala solid-phase Ex-Ferm fermentation process employs fresh or dried sugarcane pieces as raw substrate (Rolz, 1981). Kirby and Mordon (1980) have used the Ex-Ferm procedure without water addition for sugar beets as raw material. Fruit canning wastes and whey which is produced as a by-product in cheese manufacture have also been used

as substrate for ethanol production mainly for reducing the BOD of effluent wastes.

I.2.2 Starchy materials. The starchy raw materials comprise cereal grains mainly corn, wheat, rice, barley and grain sorghum and tuber and root plants such as potato, Jerusalem artichoke, manoic . Among the starchy materials, corn is a major grain used for ethanolic fermentation in the USA. Cleaned and dehulled grain is generally gelatinised by cooking, cooled and hydrolysed with barley malt before fermentation (Stark, 1954). Continuous hydrolysis processes have also been used and mold bran has been used in place of barley malt. Although cellulosic raw materials have also been suggested as future sources for fuel grade ethanol production, pretreatment costs make these economically unacceptable for industrial application.

I.3 ETHANOLIC FERMENTATION PROCESSES

I.3.1 Industrial Processes

A brief account is given below of representative current fermentation processes used for industrial ethanol production. The volumetric productivities of conventional batch fermentations is about 2 $gl^{-1}h^{-1}$ and of continuous processes about 7 $gl^{-1}h^{-1}$ (Cysewski and Wilke, 1978).

I.3.1(i) Conventional batch fermentation (Maiorella, 1985). The batch fermentation involves the building of an active yeast inoculum generally in a medium similar to the fermentation mash. The ethanol production rate is a product of the concentration of the cells and the specific productivity. In the initial phase during which the temperature is optimally maintained at 30° - 35°C, air is sparged for obtaining high cell densities. The fermentation with yeast is generally optimal at pH 4.0 to 4.5 depending on the yeast strain and subsequent ethanol production after the initial growth phase is carried out optimally at temperatures of 35°C - 38°C. The period of fermentation varies from 36 to 72 h depending on yeast strain and substrate.

I.3.1(ii) Mellé-Boinot fermentation process (Lagomasino, 1949). Fermentation time is reduced in this process through recycle of yeast to initial cell densities of about 8×10^{10} cell l^{-1} which is approximately equivalent

to that at the completion of conventional batch fermentation. Overall fermentation time is reduced to approximately half to two thirds and the volumetric productivity enhanced to about 6 $gl^{-1}h^{-1}$.

I.3.1(iii) Continuous fermentation (Maiorella, 1985; Guidoboni, 1984; Moirella et al., 1984). Productivity is enhanced significantly in these processes by yeast cell recycle. Conventionally, centrifuges are used to separate yeasts from the product stream and the concentrated yeast cream is recycle through the fermentor. The culture is made viable through the controlled bleeding of the cells (Del Rosario et al., 1979; Pirt and Kurowski, 1970).

Since capital and maintainance costs of centrifuges are high, alternatives to cell recycle have been suggested for cell retention in the fermentor, including temporary thermal shock to arrest CO_2 evolution (Walsh and Bungay 1971) and the use of flocculent yeast strains in specially designed fermentors with an inclined side arm (Stephanopoulos et al., 1985).

I.3.1(iv) Biostil process (Alpha Laval, 1982). The main features of this continuous process with yeast recycle is that the fermentation and distillation are coupled, the beer being continually cycled after initial removal of yeast and subsequent stripping of ethanol

in a vaporizer. The ethanol concentration in the fermentor is maintained low at about 5% (w/v) to minimise product inhibition effects. Concentrated feed stocks are used for replenishing the substrate in the fermentor. This becomes possible because of large liquid recycle. Less water is therefore consumed and more concentrated stillage results. The Biostil process is limited by the build up of inhibitory non-fermentable feed components and fermentation by-products. An osmotolerant strain of Schizosaccharomyces pombe is used. With sugar cane juice, stillages at 0.4 L per L of ethanol and solid content of 55% (w/v) are obtained while the corresponding value for cane molasses is reported to be about 5 L of stillage per L of ethanol compared to about 12 L stillage per L ethanol in the conventional processes.

The Biostil stillage, with little or no additional evaporation, reportedly sustains combustion and is capable of providing energy by burning of their waste without an external fuel source.

A commercial scale 12 000 L ethanol per day Biostil plant had been commissioned in Australia in 1982 and a 100 000 L per day plant was commissioned in Brazil in the same year.

I.3.1(v) Vogelbusch cascade Process. This includes a combination of batch and continuous process. Plants ranging in size from 30 - 600 000 L/day alcohol have been in operation since 1975 (Guidoboni, 1984). Between 5 and 7 production fermentors are operated in series, the first tank being aerated and the others having CO_2 mixing devices. Beer from the last fermentor is centrifuged for yeast cell separation and the acid treated cream is recycled back to the first fermentor in the series.

I.3.1.2 Ethanol recovery step. Ethanol recovery from fermentation beer is energy intensive and a cost centre in the overall process. The approaches tried for effective recovery and the separation problems are not discussed except to state that the rectification energy requirement is high at ethanol levels of less than about 6% (w/v) and that no energy reduction except for small savings in lower volumes of heat, results from increased feed beer concentration above 7% (w/v).

I.3.1.3 Fermentation waste treatment. Molasses stillages (vinasse) is low in nitrogen and high in salt and is not suited for cattle feed supplementation except in limited amounts. Biomethanation systems and other stillage disposal processes are outside the scope of the present introduction and are not discussed.

I.3.2. Experimental Systems

I.3.2(i) Tower fermentor. Gas lift, tower fermentors have been used with highly flocculent yeast strains for production of beverage beers (Hough et al., 1977; Greensheilds and Smith, 1971) although large scale industrial ethanol production using the system has not been reported (Maiorella, 1985). The major drawback is the long time required to build up the desired high cell densities and the deflocculating effect of high salt-containing media such as molasses. Experimental systems have been described for glucose feeds by Cysewiski and Wilke, 1977; Ghose and Tyagi, 1979; Del Rosario et al., (1979); BuLock et al. (1984a; 1984b); Comberbach and Bu'Lock (1984); Kuriyama et al. (1985), Prince and Barfort (1982), Prince and Barfort (1982b).

I.3.2.(ii) Vacuum flash fermentation with cell recycle. Volumetric ethanol productivities as high as 83 $gl^{-1}h^{-1}$ have been achieved in the laboratory through selective removal of ethanol in vacuum (Cysewski, 1977). Continuous conversions were run in fermentors under sufficient vacuum to boil the ethanol off at temperatures conducive to yeast growth. A laboratory scale improvement has been the flash-ferm process with the ethanol removal by flashing in an auxillary flash vessel to reduce the energy consumption and to avoid sophisticated fermentor

design (Maiorella et al., 1981; 1983). The high cost of the process however is unlikely to lead to commercial application.

I.3.2(iii) Membrane bioreactors. Selective and continuous removal of ethanol from the fermentation broth by pervaporation through a membrane filter has been suggested for overcoming product inhibition (Mulder and Smolder, 1986).

I.3.2(iv) Extractive fermentation. Removal of ethanol either by selective extraction with immiscible solvents such as dibutyl phthalate (Mena et al., 1980), dedecanol (Minier and Goma, 1981) or two-phase systems based on polyethylene glycol and dextran (Kuhn, 1980) have also been suggested for overcoming product inhibition.

I.3.2(iv) Immobilized yeast cell continuous fermentation

Among the new trends, the most promising is the application of immobilized cells for continuous ethanolic fermentation. Many laboratory scale systems and their advantages are described in the following section (I.4). The present account relates to a system which has been used on 2.4 KL ethanol per day scale for continuous conversion of cane molasses to ethanol under the auspices of the Ministry of International Trade and Industry (MITI) of the Japanese Government (1984).

Saccharomyces cells entrapped in calcium alginate

gel have been used in a pilot plant with two series of fluidized bed, column reactors at the Kyowa Hakko's Hofu Plant. Productivities of more than 20 $gl^{-1}h^{-1}$ are claimed at final ethanol concentrations of about 6.8 - 7.2% (w/v). Reactors could be operated 3 to 6 months, the longer periods requiring entrapment of sterols or unsaturated fatty acids into the gel beads and suitable aeration into the reactor for enhancing the viability of yeast. The type of cane molasses used in the work has not been described, particularly whether the high-test black strap variety has been used as raw material.

I.4 IMMOBILIZED MICROBIAL WHOLE CELL SYSTEMS

It will be apparent from the preceding Section that immobilized yeast cell systems offer the attractive features of high volumetric productivities and other advantages of continuous ethanolic fermentation without the requirements for high cost and complex equipment such as centrifuges for cell recovery or vacuum systems for flash evaporation. Such immobilized systems permit high cell loading and high dilution rates without the attendant problem of cell washout or of deflocculation as in the case of highly flocculent microbial strains in presence of high-salt containing cane molasses.

I.4.1. Catalysis of multistep transformations

An alternative to microbial whole cell immobilization is the immobilization of isolated enzyme systems for catalyzing a desired transformation. Immobilized microbial whole cell systems, however, have distinct advantages over immobilized isolated enzymes where a sequence of enzyme reactions, particularly those involving cofactors, is required for bringing about the overall conversion of a substrate to the desired product. Besides the saving in tedious enzyme isolation procedures, cofactor regeneration is readily achieved through the use of living cell systems. This is particularly true in the fermentation of glucose or other

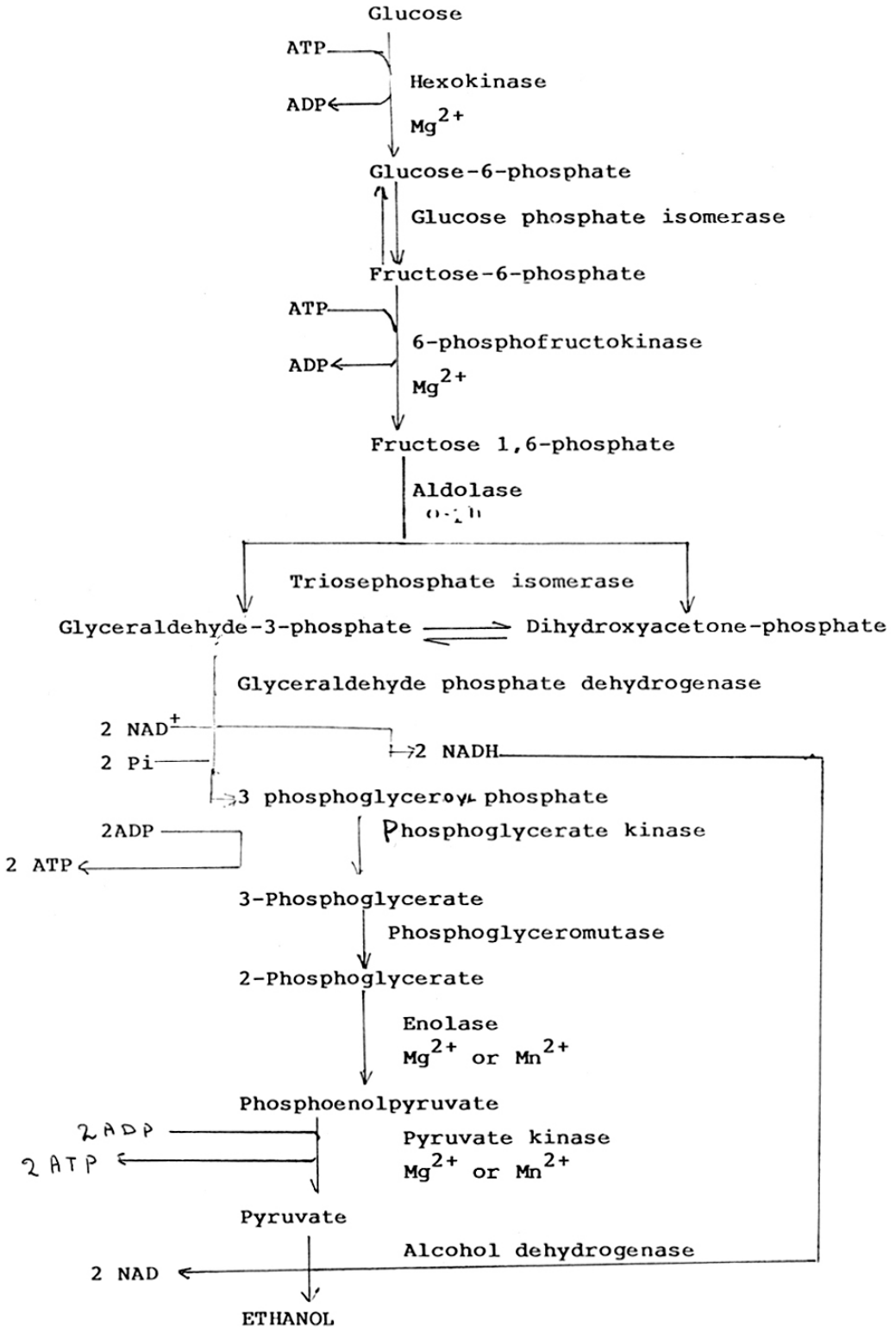
fermentable sugars to ethanol which involves metabolic sequences such as of glycolysis.

The conversion of glucose to ethanol by Saccharomyces spp which use the normal Embden- Meyerhof glycolysis, involves eleven enzymes and ATP/ADP - NAD/NADH cofactors in the sequence of reactions as shown in Figure I.1. While individual enzymes and cofactors regenerating systems have been immobilized and shown to convert glucose to ethanol (Luca and Kricka, 1983), the large scale use of such coimmobilized multienzymes are clearly impracticable. In contrast, living yeast cells suitably immobilized perform effectively in the efficient conversion of glucose and other fermentable sugars to ethanol without the need to supplement cofactor requirements extraneously.

I.4.2. Growth and metabolism of immobilized cells

Although immobilization does not alter grossly the biochemistry of cell function and growth, several experimental evidences indicate a wide range of modifications in the metabolism of entrapped cells as compared to those of free cells in suspension. Among these are differences in conditions for optimal growth (Williams and Munnecke, 1981), altered product yields (Navarro and Durand, 1977; Tyagi and Ghose, 1982), altered lag times (Hattori and Furusaka, 1959), changes in cell morphology (Jirku et al., 1980) and higher specific

FIG I.1 : EMBDEN MEYERHOF GLYCOLYTIC PATHWAY OF ETHANOLIC FERMENTATION



rates of product synthesis or substrate uptake (Navarro and Durand, 1977; Vijayalakshmi et al., 1979; Marcipar et al., 1979). Factors such as changes in liquid micro-environment and cell permeability have been suggested as possible reasons for the observed alterations. However, these suggestions do not explain the observation that cells detached from the support and resuspended in solution exhibit in certain cases the same altered properties as in thoseimmobilized (Hattori and Furusaka, 1960).

A careful examination has been made recently by Doran and Bailey (1986a) of some of the alterations in cell metabolism caused by immobilization. S. cerevisiae cells immobilized in glutaraldehyde cross-linked gelatin were shown by these authors to have higher ploidy and a lower frequency of budding compared to cell suspensions, despite the continuous synthesis of DNA and new cell wall materials. The immobilized cells were found to overproduce DNA and to have enhanced glucose uptake and ethanol production rates. The specific growth rate and biomass production, however, were reduced significantly in the case of the immobilized cells. Immobilized cells were also observed to store larger quantities of reserve carbohydrates.

I.4.3 Effect of Inhibitors of Cell Growth

An interesting approach has been used to artificially arrest immobilized yeast cells in a "no-growth" state through the use of metabolic inhibitors. This was expected to prevent the wasteful diversion of the substrate to the synthesis of biomass and to route it entirely towards the more desirable transformation reactions viz. of fermentation to ethanol. Trevelyan et al. (1952) have shown that in presence of azide, bakers yeast quantitatively converts glucose to CO_2 and ethanol while in the absence of the inhibitor the conversion was only 70% of theory. Quantitative conversions were shown to occur only with azide in the presence of the complete growth medium and oxygen (Hahn-Hagerdal and Mattiasson, 1982a). Similar results were observed with Ca-alginate immobilized cells of S. cerevisiae, theoretical yields of CO_2 and ethanol being obtained with azide only when supplemented with complete growth medium, while with plain glucose and azide, ethanol production was suppressed and ceased totally within 8 days (Hahn-Hagerdal and Mattiasson, 1982b). In addition to azide, dinitrophenol and arsenate have been used by Amin et al. (1984) with the three yeasts: S. bayanus, Schizosaccharomyces pombe and S. diastaticus. Both dinitrophenol and azide were effective in increasing the conversion of glucose to ethanol, while arsenate was without effect at the

concentration used. In immobilized systems, dinitrophenol or azide inclusion enhanced the steady state ethanol production from a 15% glucose medium.

Hydroxyurea, a preferential inhibitor of DNA synthesis, has been used by Doran and Bailey (1986b) to arrest yeast cell growth. S. cerevisiae cells immobilized in glutaraldehyde cross-linked gelatin were observed to accumulate as doublets and the specific rates of ethanol production was observed to increase by an average of 24% in presence of hydroxyurea, compared to a 3-fold increase in the corresponding rate in the case of cells in suspension. Immobilized cell ethanol yields were, however, lowered by 16% compared to an average lowering by 50% in the suspended cells. Hydroxyurea caused 30% increase in polysaccharide content of the immobilized cells, while in the suspended cells the content was reduced by 65%. These observations confirmed further the earlier findings that cell metabolism is altered as a consequence of immobilization.

I.4.4 General methods of microbial whole cell immobilization

The different methods used for cell immobilization, in particular of yeasts for ethanolic fermentation, are discussed briefly in the rest of this Section.

Several reviews on immobilization of microbial cells have appeared in the literature (Chibata, 1978;

Chibata et al., 1977; Chibata et al., 1986; Chibata and Wingard Jr., 1983; Fukui and Tanaka, 1982; Mattiasson, 1983; Koscheenko, 1981; Kierstein and Coughlan, 1985; Kennedy and Cabral, 1985; Linko and Linko, 1984; Brodelius, 1978; Linko and Linko, 1983; Vojtisek and Jirku, 1983).

The suitability of many methods described in the literature for an individual application has to be determined by experimentation as none of these are ideal generally.

The main methods used for microbial whole cell immobilization fall into the following categories:

* Physical adsorption or attachment on carriers
* Covalent attachment to carriers
* Cross-linking of cells
* Entrapment in gels
* Encapsulation

A few general examples and some specific applications relating to the immobilization of yeast cells for ethanol production are listed under each of these categories.

I.4.4(i) Physical adsorption/attachment on carriers.

Physical adsorption has been used extensively for immobilization of microbial whole cells. The earliest of such systems is in acetic acid production from ethanol by Acetobacter sp. adhering to beach wood shavings in trickle filter reactors, a process which has been used

over the last 100 Y in the manufacture of vinegar.

Atkinson et al. (1979) have used steel wire cages as support for the attachment of mycelial fungi such as Aspergillus foetoidus, a citric acid producer and for the attachment of mixed cultures for biomethanation. The mechanism responsible for microbial attachment in these cases is the natural adhesive character of the microbes and/or their ability to form flocs. Surface active supports like carbon have been used for the immobilization of Pseudomonas sp. for denitrification (Scott and Hancer, 1976). Hattori and Furusaka (1961) have used E. coli and Azotobacter agile cells adsorbed on Dowex-1 for succinate oxidation, the electrostatic interaction between the charged cell and charged support contributing to adhesiveness. Table I.1 includes some of the yeast cells adsorbed systems used for ethanol production. The destabilising effects of environmental factors such as pH, presence of high levels of salts limit the application of the absorbed systems particularly where substrates like molasses are used.

I.4.4(ii) Covalent attachment to the carrier. Applications of this method of immobilization of whole cells are limited on account of the toxic effects of the coupling reagents. In this procedure, cells are retained on activated carriers through suitable covalent coupling.

TABLE I.1 : IMMOBILIZATION OF YEAST CELLS BY ADSORPTION AND THEIR APPLICATION IN ETHANOL PRODUCTION

CULTURE Matrix	Productivity $gl^{-1}h^{-1}$	Reference
S. cerevisiae		
Carrier A	24.9	Ghose and Bandyopadhyay (1980)
Raschig rings	7.4	Sitton and Gaddy (1980)
Ion exchange resins	62 (liquid volume basis)	Daugulis et al. (1981)
Bricks of PVC		Navarro et al. (1976)
Wood chips	$6.06(10^{-3})$ $Kg/m^3/s$	Robinson et al. (1986)
Wood chips	21	Ghose and Bandyopadhyay (1979)
Wood chips	21.2-83.7 (liquid volume basis)	Gencer and Mutharasan (1983)
Wood chips	4.3	Ryu et al. (1982)
Sugarcane bagasse	14.2	Cheung et al. (1986)
Porous glass		Navarro and Durand (1977)
Agar beads		Day and Sarkar (1982)
Steel wire mesh		Atkinson et al. (1979)
Crosslinked gelatin coated on glass		Doran and Bailey (1985)
Gelatin coated on distillation column and crosslinked		Griffith and Compere (1978)
Cotton cloth		Joshi and Yamajaki (1984)

S. carlbergensis cells have been covalently coupled to the glutaraldehyde derivative of alkyl amine porous silica carriers (Navarro and Durand, 1977). Cells of Serratia marcescens and S. amurcae have been coupled to isocyanate derivatives of glass and zirconiam (Messing et al., 1979). Micrococcus luteus cells have been immobilized on carbodiimide derivatives of carboxymethylcellulose (Jack and Zajic, 1977). The immobilized cells in this example however were not viable. Arthrobacter globuformis cells have been attached to silica gels activated by $TiCl_3$, cells in this case retaining their viability (Arigasarova and Koshcheenko, 1980).

Kluveromyces lactis cells have been immobilized on the aldehyde derivative obtained by treating polyphenylene oxide with glutaraldehyde (Jirku et al., 1980). S. cerevisiae has been immobilized on glutaraldehyde treated tetramethylenediamine-modified hydroxymethacrylate gel (Jirku et al., 1980).

I.4.4(iii) Cross-linking. Bifunctional reagents such as glutaraldehyde, toluene di-isocyanate have been used for cross-linking E. coli cells (Chibata et al., 1974). Glucose isomerase-active, homogenized Bacillus coagulans cell mass has been cross-linked with glutaraldehyde and pelleted thereafter through extruders for commercial production of high-fructose syrups (Amotz et al., 1976). An interesting example of the use of

metal-link processes for immobilization of microbial cells is the procedure described by Kennedy and his group. Viable S. cerevisiae, Acetobacter and E. coli cells have been obtained through the use of titanium (IV) chloride or zirconium (IV) chloride for achieving metal chelation between cells (Kennedy et al., 1976; Kennedy and Cabral, 1985). S. cerevisiae cells cross-linked by such metal chelation processes were shown to have 30% higher oxygen uptake rate compared to corresponding population of free cells. Kennedy et al. (1976) have cross-linked Acetobacter sp. cells with Ti metal linkages for acetic acid production from ethanol.

I.4.4(iv) Gel entrapment. Entrapment of microbial cells in gel supports represents the most widely used of immobilization procedures. The reasons for the extensive applications are the high cell loading capacity and the mild conditions under which several such hydrophilic gels can be formed. Amongst the gels that have been extensively used are those obtained from proteins and protein derivatives (gelatin, egg white, albumin, collagen), naturally occurring polysaccharides (agar, agarose, alginate, κ-carrageenan, pectin) and synthetic polymers like polyacrylamide, hydroxymethyacrylate and polyurethane, epoxy resins and photo cross-linkable resins.

Two approaches are available for obtaining immobilized

cells suitable for bioconversions:

* Through entrapment of pre-grown cells at high initial cell loading, and

* Through incorporation of cell inoculum within the gel followed by incubation in a growth medium for building up adequate cells density within the gel.

On account of the diffusional limitations, gel immobilization restricts applications to relatively low molecular weight substrates and products.

A brief account of a few selected examples of gel entraped microbial systems follows:

(a) Gelatin

Ceramic Raschig rings dip-coated in 25% gelatin and subsequently sprayed with glutaraldehyde for cross-linking of gelatin gel have been used by Gaddy and Sitton (1980) for the entrapment of S. cerevisiae and the immobilized cell reactor has been used for ethanol production from glucose.

(b) Hen egg white

Hen egg white cross-linked with glutaraldehyde has been used for the immobilization of E. coli and S. cerevisiae (D'Souza and Nadkarni, 1981; D'Souza et al., 1982). Hen egg white matrix has been reported to confer protection from infection on account of lytic

activity of endogenous lysozyme (Kaul et al., 1983).

(c) Bovine albumin

Petre et al. (1978) have immobilized E. coli cells in porous sponge like particles and membranes obtained from bovine serum albumin.

(d) Cellulose beads

Several microorganisms have been immobilized by Linko et al. (1979) within α-cellulose fibres and beads. The cellulose dissolved in N-ethyl-pyridinium chloride and dimethyl formamide are treated with microbial cells and extruded into water.

(e) Agar

Agar has been used for entrapment of E. coli for β-glucosidase activity (Toda, 1975); yeast for its invertase activity (Banerjee et al., 1984). Cells of S. pastorianus have been immobilized in agar by dropping of the cell suspension into toluene or tetrachloroethylene (Toda and Shoda, 1975).

(f) Alginate

Alginic acid which is obtained from seaweeds is a linear copolymer of D-manuronic acid and L-guluronic acid. The polymer contains blocks of manuronic acid, blocks of guluronic acid as well as alternating manuronic-guluronic acid blocks. The proportions between these acids vary with the type of seaweed and the environmental factors.

The alginates find use in food and pharmaceutical industry besides their use as an attractive gel for whole cells immobilization. The polymer can be gelled by cross-linking in presence of bi- or multivalent ions such as calcium and aluminium. An exception is magnesium alginate which is water soluble. Calcium is the preferred metal ion for whole cell immobilization. The presence of phosphates and citrates in the medium disrupts the calcium alginate gel. Suspensions of cells are dropped into an agitated solution of salts like calcium chloride to obtain beads of diameter ranging from 0.1 to 5.0 mm (Kierstan and Bucke, 1977). The gel has been used for immobilising a wide range of microbial cells including Arthrobacter simplex for the production of prednisolone from cortisol (Ohalsson, Larsson and Mosbach, 1979), S. cerevisiae for the fermentation of glucose to ethanol (Veliky and Williams, 1981; Kalot, 1980; Cheetam et Larsson and Mosbach, 1979; Takata et al., 1971; White and Portno, 1978).

Several devices have been described for obtaining uniform size gel beads of Ca-alginate. These include also the recently described procedure that employs vibrating nozzle for the rapid preparation of such beaded gel material (Hulst et al., 1985). Besides such externally gelled beads of calcium alginate of any desired size, internal gelation methods has been used (Flink and

Johansen, 1985; Johansen and Flink, 1986). In this procedure gelation is initiated by addition of tricalcium citrate suspension followed by addition of a solution of glucono-δ-lactone and gelling in a mould. Alginate with high guluronic acid content (70%) yield stronger gels. Alginate gel strength has also been enhanced with glutaraldehyde cross-linked polyethyleneimine (Velky and Williams, 1981; Mohammed and Salleh, 1982) and epoxy resin (Klein and Eng, 1979) or with divalent metals such as Sr^{2+} and Ba^{2+} and trivalent metals like Al^{3+} (Klein and Manecke, 1982).

(g) κ-Carrageenan

κ-Carrageenan which is a polysaccharide containing repeating disaccharide units made up of β-galactose-4-sulfate and 3,6-anhydro-α-galactose is obtained from red algae. κ-Carrageenan gels on cooling or in presence of gel-inducing agents such as K^+, NH_4^+, Cu^{2+}, Ca^{2+}, Mg^{2+} and Fe^{2+}, amines or water miscible organic solvents (Tosa et al., 1979). κ-Carrageenan readily and most strongly gels in presence of K^+. The presence of gel inducing metal ions is required in the system for maintaining gel integrity. Sodium chloride solutions on the other hand enhance gel disintegration.

(h) Polyacrylamide.

Polyacrylamide gel has been extensively used for immobilization of microorganisms. The acrylamide monomer polymerizes in the presence of cells held in suspension. Among the organisms immmobilized are Escherichia coli (Wada et al. 1979), Acetobacter suboxydans (Scharr et al. 1977), Clostridium butyricum (Karube et al. 1976).

Table I.2 includes some examples of gel entrapped yeast cell systems which have been reported in the literature for ethanol production.

TABLE I.2 : IMMOBILIZATION OF YEAST CELLS BY GEL ENTRAPMENT AND THEIR APPLICATION IN ETHANOL PRODUCTION

ORGANISM Matrix	Productivity ($gl^{-1}h^{-1}$)	Reference
S. cerevisiae		
κ-Carrageenan	43.6	Wada et al. (1981)
κ-Carrageenan + Tricalcium phosphate	2.8 mg./ 10^8 $cell^{-1}h^{-1}$	Wang and Hettwer (1982)
κ-Carrageenan	15	Go'dia et al. (1985)
Agar, Ca-alginate, κ-Carrageenan and polyacrylamide		Holcberg and Margalith (1981)
κ-Carrageenan	$63(l.gel)^{-1}h^{-1}$	Mitani et al. (1984)

ORGANISM Matrix	Productivity ($gl^{-1}h^{-1}$)	Reference
S. cerevisiae		
κ-Carrageenan	15	DelRosario and Pamatong (1985)
Prepolymerized Hydrazide polyacrylamide crosslinked		Pines and Freeman (1985)
Ca-alginate		Kierstan and Bucke (1977)
Ca-alginate	53.8	Williams and Munnecke (1981)
Ca-alginate	21.3	Linko and Linko (1981b)
Ca-alginate		Nagashima et al. (1984)
Ca-alginate		Veliky and Williams (1981)
Ca-alginate	37.8	Lee et al. (1983)
K. fragilis		
Ca-alginate	6.3	King and Zall (1983)
Ca-alginate		Linko et al. (1981)
Pachysolan tannophilis		
Ca-alginate	2.1 - 2.2	Slinger et al. (1982)
K. marxianus		
Ca-alginate	104 (Void volume basis)	Margaritis and Bajpai (1982)
Debaryomyces polymorphus		
Ca-alginate		Margaritis et al. (1983)

I.4.4(v) Encapsulation/membrane reactors

Encapsulation of E. coli cells within cellulose triacetate fibres has been reported by Dinelli (1972). The cells were used for 6-aminopenicillanic acid preparation from benzylpenicillin.

An approach which resembles the fibre entrapped system is the membrane bioreactor for ethanol production in which S. cerevisiae cells are immobilized within hollow fibres or on their shell side while the nutrient or glucose feed is pumped through the fibre lumen or shell volume (Inoles et al., 1983; Mehaia and Cheryan, 1984). Productivity of 10 $gl^{-1}h^{-1}$ has been reported by Mehaia and Cheryan (1984) at 85% utilization of 10% glucose feed using a hollow fibre reactor. In a variant of this approach, the membrane module is coupled to the fermentor, the beer being allowed to flow continuously through a cross flow membrane filtration unit (Cheryan and Mehaia, 1984; Muolder and Smolders, 1986). Cherayan and Mehaia (1984) report an ethanol productivity of 100 $gl^{-1}h^{-1}$ for complete utilization of a 10% glucose feed in this membrane bioreactor.

PART II

YEAST STRAIN SELECTION FOR ETHANOLIC FERMENTATION

SUMMARY

Two isolates of Saccharomyces cerevisiae designated as strains Y-7 and Y-10 have been shown to have markedly higher initial specific ethanol productivities at high sugarcane molasses concentration of 30% dry solids content compared to that of some selected brewers yeast strains, the isolates showing values of 0.58 to 0.60 $gl^{-1}h^{-1}$ compared to 0.44 $gl^{-1}h^{-1}$ for Saccharomyces uvarum ATCC 26602, the most efficient among the standard strains tested. The high specific ethanol productivity values reflect higher osmotolerance of the isolates compared to the standard strains.

S. cerevisiae Y-10 also has better ethanol-tolerance in molasses fermentation than the S. uvarum, the ethanol level in the fermented beer obtained with an inoculum of 1.25% dry weight equivalent of cells being 82 gl^{-1} in 24 h fermentation of 180 gl^{-1} glucose equivalent of molasses fermentable sugars at 30°C and 95 gl^{-1} product from 225 gl^{-1} fermentable sugars, compared to corresponding values of 66 gl^{-1} and 82 gl^{-1}, respectively, with the S. uvarum under similar conditions.

The isolate S. cerevisiae Y-10 has adequate temperature tolerance. The final product concentrations in batch mode fermentations using 12.5 g dry weight equivalent of cells per litre of molasses containing 135 gl^{-1} glucose

equivalent of fermentable sugars being 64 gl^{-1} at 30°C, the levels attained at higher temperatures upto 40°C being only slightly lower, the corresponding values being 60 gl^{-1} and 58.5 gl^{-1} at 35°C and 40°C, respectively. Inhibition was severe at 45°C.

A study of the comparative kinetics of the S. cerevisiae isolates Y-7 and Y-10 and the standard S. uvarum ATCC 26602 strain in batch cultures on 157 gl^{-1} molasses total sugars at 30°C have shown that the isolates have more desirable characteristics than the standard strain for ethanol production from this source.

In comparative studies on some key enzymes involved in the ethanolic fermentation of molasses, invertase activity of S. cerevisiae Y-10 cells has been shown to be markedly lower than in the S. uvarum when grown either on sucrose or molasses. No differences were observed in pyruvate decarboxylase of cells of S. cerevisiae Y-10 and S. uvarum grown either aerobically or in stationary cultures. No inactivation of the decarboxylase activity was observed when crude cell extracts were incubated for 30 min at 30°C with added KCl (2%), ethanol (20% w/v) or a combination of KCl (2%) and ethanol (10% w/v). The alcohol dehydrogenase (ADH) activities in S. cerevisiae Y-7 and Y-10 cells were 40% - 100% higher than in cells of S. uvarum ATCC 26602, the percentage difference varying with growth conditions. The ADH isoenzyme pattern in

both the isolates was similar but distinct from that in S. uvarum.

The lack of any simple correlation between the enzyme activities studied and osmotolerance, ethanol-tolerance and temperature-tolerance of Saccharomyces spp. is discussed.

INTRODUCTION

The multi-purpose industrial usefulness of ethanol as liquid fuel, enhancer of octane value in premium grade lead-free petrol and as a chemical feedstock and the long range need to obtain this key compound from renewable resources has led to extensive researches on its production by efficient and improved fermentation processes. The main source for the fermentative production of ethanol in cane growing regions of the world like Brazil and India is cane molasses and the commercial production of ethanol from this source utilizes yeasts.

A prerequisite for the development of more cost effective and efficient processes than the conventional low-yield, batch fermentations is the availability of suitable microorganisms with high specific ethanol productivities and adequate tolerances towards substrate, product and temperature. The high level of salts present in cane molasses makes osmotolerance in the yeast strain an essential requirement for rapid and efficient fermentation and a combination of this trait with ethanol-tolerance is essential for obtaining adequate product concentrations from this source. A further desirable trait is temperature-tolerance, particularly for industrial fermentations in regions with high ambient temperatures. Ethanol-inhibition, osmotolerance and temperature-tolerance are complex phenomena and literature reports on some aspects of these problems have been described in the foregoing **General Introduction.**

Two isolates of Saccharomyces cerevisiae strains designated as S. cerevisiae Y-7 and S. cerevisiae Y-10 obtained by us from fermenting sugarcane juice showed high ethanol productivities at relatively high substrate concentrations. The performances of these isolates in cane molasses fermentation in batch culture are compared in this Chapter with those of standard strains of Saccharomyces. Also presented are comparative studies on three key enzymes involved in ethanolic fermentation of molasses, namely invertase (EC 3.2.1.26), pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1).

MATERIALS AND METHODS

Materials

Chemicals used were generally of analytical grade or of high purity reagent grade available commercially.

NAD, crystalline bovine serum albumin, phenozine methosulfate, nitroblue tetrazolium, thiamine pyrophosphate chloride (cocarboxylase) and Trizma base were from Sigma Chemical Co., USA; Glox reagent was obtained from Kabi Diagnostica, Sweden; acrylamide monomer, N,N'-methylene bisacrylamide and N,N,N',N'-tetramethylethylenediamine were of electrophoresis grade and were obtained from Eastman Organic Chemicals, USA.

Yeast extract was obtained from Oxoid Ltd., England; Bacto-agar and Bacto-peptone were from Difco Laboratories, USA; malt extract was from Loba Chem., Bombay.

The absolute ethanol used as standard was puriss p.a. grade from Fluka AG, Switzerland and standard glucose was prepared from the guaranteed reagent grade chemical from E. Merck, Germany. 3,5-Dinitrosalicylic acid was obtained from Ridel-DEHAEN AG, West Germany. Acetonitrile (HPLC grade) was purchased from Spectrochem, Bombay.

Sugarcane molasses. Cane molasses used for ethanol production was obtained from local sugar factories.

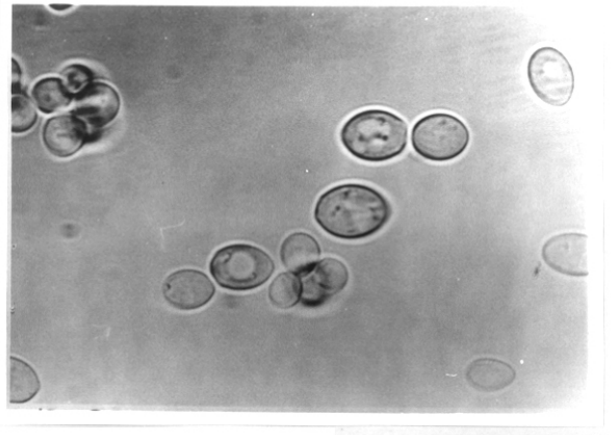
Organisms. Standard yeast cultures used in the experiments were obtained from the National Collection of Industrial Microorganisms (NCIM), Pune.

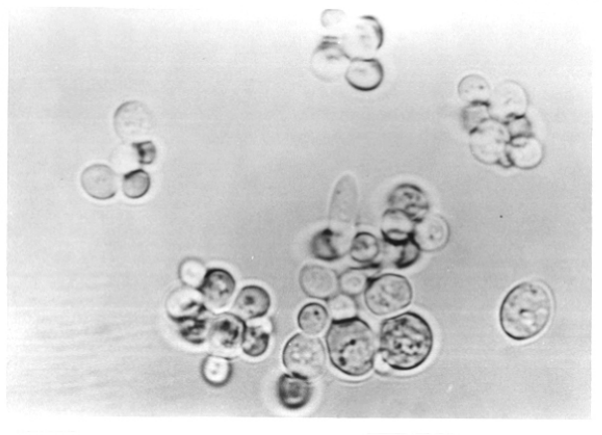
The stock cultures were maintained on the following MYPG-medium containing malt extract (M), yeast extract (Y), peptone (P) and glucose (G) and of the following composition (gl^{-1}); malt extract, 3; yeast extract, 3; peptone, 5; glucose, 100; pH adjusted to 5.8.

Cultures for cane molasses fermentation were adapted to growth in media containing increasing concentrations of the molasses and transferred finally to a MYPM-medium containing in gl^{-1}: malt extract, 3; yeast extract, 3; molasses total sugars (M) in glucose equivalent, 200. The sample of molasses used in these studies contained 78% w/w non-volatile solids (dry solids); 52% w/w fermentable sugars and 6% w/w non-fermentabe sugars.

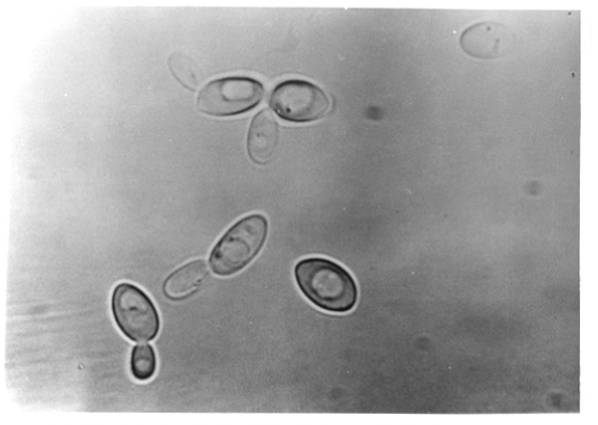
Yeast isolates. Two yeast cultures identified and designated as S. cerevisiae strains Y-7 and Y-10 were obtained from fermentating sugarcane juice. The identification of the isolates as Saccharomyces cerevisiae strains was confirmed by the National Collection of Yeast Cultures (NCYC), London, UK. The isolate Y-7 was morphologically found to be round to long oval, occurring singly and in clumps (Fig. II.1A) whereas S. cerevisiae Y-10 was found to be long, oval to elongated, occurring only singly (Fig. II.1B). S. cerevisiae Y-7 fermented galactose rapidly whereas Y-10 did not utilize this sugar. Both strains fermented glucose, sucrose and maltose rapidly. Raffinose was also utilised by both the strains. The isolates were maintained on agar slants of the MYPM-medium described above.

(A)





(B)



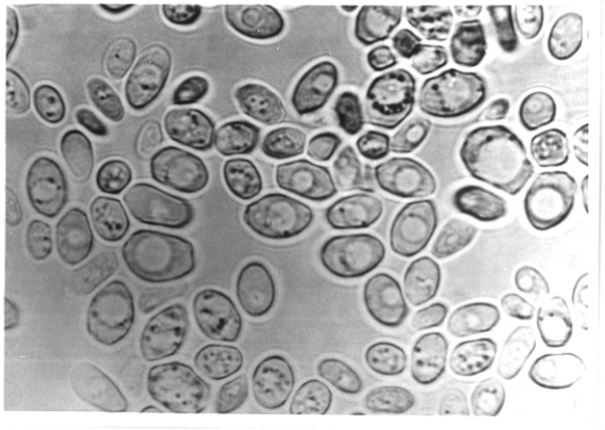


Fig. II. 1 : Microphotographs of cells of *Saccharomyces cerevisiae* isolates

(A) *S. cerevisiae* Y 7

(B) *S. cerevisiae* Y 10

Methods

Analysis of cane molasses. The total dry solids (DS) content was estimated by drying in an air oven initially at 80°C and finally to constant weight at 100°C.

Sugars. Total sugars were estimated as the total reducing substances obtained after inversion with HCl. Inversion of sucrose in molasses was done by addition of HCl to a final concentration of 0.55 N followed by heating of the acidified sample for 15 min in a boiling water bath after which the sample was cooled, neutralized with NaOH and aliquots were taken for assay of reducing substances. Reducing sugars were routinely estimated by 3,5-dinitrosalicylic acid (DNSA) method (Fischer and Stein, 1961).

Total reducing substances by dinitrosalicylic acid (DNSA) method. DNSA reagent: 3,5-dinitrosalicylic acid (5g) was dissolved in water (100 ml) by dropwise addition of NaOH (8 g dissolved in 75 ml H_2O). Sodium potassium tartarate (150 g) was added, stirred till it was dissolved completely and the solution made up to volume (500 ml) with water. The solution was filtered through a glass sintered funnel and stored in the dark.

Estimation of total reducing substances. 1 ml DNSA reagent was added to 1 ml of diluted sample of inverted molasses taken in a test tube, the tube stoppered loosely and kept in a boiling water bath for 5 min. After cooling,

10 ml water was added and the absorbance was read at 540 nm. Reagent blanks and standards of glucose solutions were run simultaneously. The content of total sugars in molasses was calculated in glucose equivalents.

Determination of fermentable and non-fermentable sugars. Estimation of fermentable sugars was carried out essentially according to the procedure described in the Official Methods of Analysis of the Association of Official Analytical Chemists, (AOAC) USA (1975).

S. uvarum cells were pre-grown, harvested by centrifugation and washed repeatedly with normal saline till washings were free from reducing sugars. Washed cells (1.25% dry weight equivalent) were added to diluted cane molasses and left at 30°C till fermentation was complete. Residual reducing substances estimated in glucose equivalents after inversion was taken as the non-fermentable sugars component and the difference between total sugars and the non-fermentable component as the fermentable sugars in the molasses sample.

Estimation of molasses sugars by High Performance Liquid Chromatography (HPLC). The amount of fructose, glucose and sucrose present in cane molasses was analyzed according to the procedure described by Damon and Pettitt (1980). A Hewlett Packard Model 1082B instrument with computing integrator accessory and a LiChrosorb-NH_2 200mm column was used. Acetonitrile + water (80:20) solvent system was used, the solvent being degassed and filtered

through a 0.45 µm filter before use. The degassing and filtration steps were routinely done with all samples.

Standard sugars. D-Glucose, D-fructose and sucrose standard solutions were analyzed individually as well as in mixture. Maltose was selected as internal standard for estimations with cane molasses. The standard stock solution was prepared to contain glucose, fructose and sucrose in concentrations approximately equal to that of each of the sugars in the molasses sample and was treated and diluted in the same manner as the molasses.

The sugars eluted in the order fructose, glucose sucrose and maltose with retention times of ca 2.07, 2.75, 3.90 and 4.52 min, respectively (Fig. II.2). Values for molasses sugars derived from the internal standard and from individual standard curves were in agreement.

Cane molasses sugars. The cane molasses was appropriately diluted and then treated with a mixture of cation (Dowex 50) and anion (IRA 400) exchange resins. The sample was degassed and filtered through a 0.45 µm filter before injection into the column.

Growth of yeast cells. Cells required for batch fermentation studies were obtained by growth in shake flasks on a rotary shaker at 150 rpm and 30°C. Cells were grown on either MYPG- or MYPM-medium containing 100 gl^{-1} glucose or its equivalent of total molasses sugars. 200 ml medium were taken in 1 litre flasks and

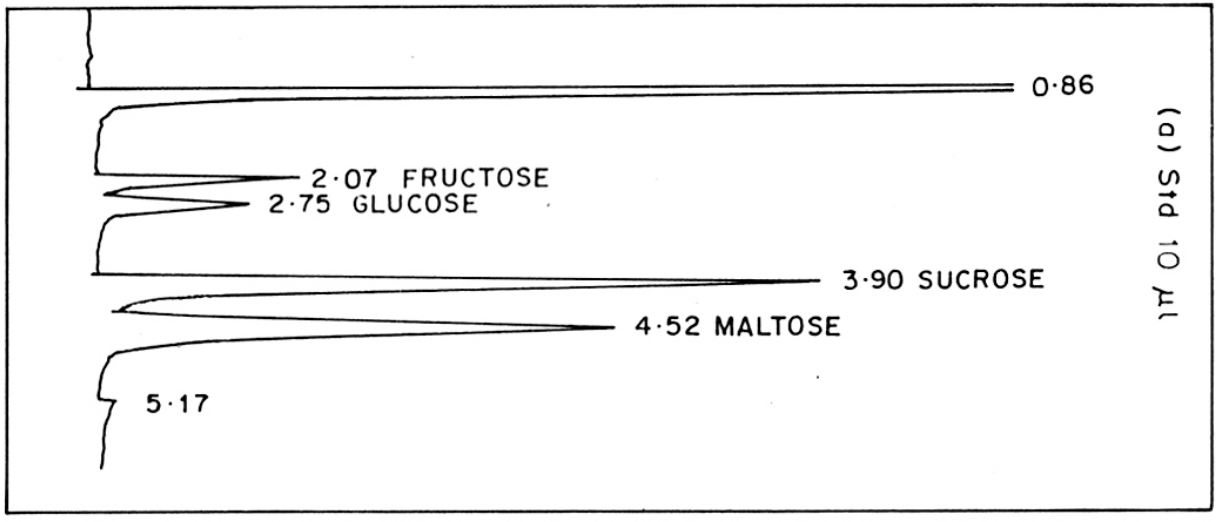

0·86
2·07 FRUCTOSE
2·75 GLUCOSE
3·90 SUCROSE
4·52 MALTOSE
5·17
(a) Std 10 µl

FIG. II.2 : Liquid chromatogram of standard sugars

sterilization was done by autoclaving at 121°C for 20 min.

Batch fermentations. Unless otherwise stated, batch fermentations with free cells were carried out in 150 ml conical flasks containing wet cells (1.25 g dry weight equivalent) in 100 ml of the molasses medium. The medium contained varying concentrations of molasses and was supplemented with 0.1% urea and the fermentation was carried out at 30°C and pH 4.5. Fermentations were under stationary conditions.

Specific ethanol productivity was calculated during the initial phase of fermentation before the ethanol concentration reached inhibitory levels.

Kinetic analysis of batch cultures. A New Brunswick controlled environment incubator shaker was used for the growth and fermentation. Cultures were initially adapted for growth in MYPM-molasses medium as described above. Inocula (20 ml) were grown at 30°C and 150 rpm in molasses medium of the following composition (gl^{-1}) : cane molasses, 50 (glucose equivalent of total sugars); yeast extract, 3; malt extract, 3; peptone, 5.0; pH 6.0.

The uptake of sugars and the production of biomass under non-aerated fermentation conditions were studied at 30°C and under mild agitation at 50 rpm which was adequate for the uniform suspension of the cells.

The fermentation medium (18[illegible] ml) contained (gl^{-1}): cane molasses, 170 [illegible] glucose equivalent of total sugars; yeast extract, 3; malt extract, 3; peptone, 5.0; pH 5.0. Inocula grown for 16 h at 30°C and 150 rpm were transferred to the fermentation medium. The uptake of sugar and the production of biomass and ethanol were monitored in samples (8 ml) withdrawn aseptically at intervals between 0 h and 48 h required for completion of fermentation.

The data were analyzed in terms of various kinetic parameters such as specific growth rate (μ), growth yield ($Y_{x/s}$), specific sugar uptake rate (q_s), specific ethanol production rate (q_p) and ethanol yield ($Y_{p/s}$).

A brief description of the analysis of data is given below:

Specific growth rate (μ). The data were analyzed during the exponential phase of growth. The mathematical relationships are:

$$dx = \mu.dt$$

where dx is increase in biomass in the small time interval dt,

x the amount of biomass present and

μ is the constant and termed the specific growth rate

$$\mu = \frac{1}{t} \ln(x/x_o)$$

where x_o and x are the initial and final amounts of biomass.

Growth yield ($Y_{x/s}$). $Y_{x/s}$ remains constant during growth-associated fermentation

$$Y_{x/s} = -dx/ds$$

$$\text{and } Y_{x/s} = (x-x_o)/(s_o-s)$$

where x_o and s_o are the initial biomass and substrate concentrations , and x and s are the corresponding concentrations during the growth of the culture

Product yield ($Y_{p/s}$)

$$Y_{p/s} = (p-p_o)/(s_o-s)$$

where p and p_o are final and initial product concentrations, respectively.

Metabolic quotients.

Specific substrate uptake rate (q_s)

$$ds = -q_s.dt$$

From above relationships $q_s = (1/Y_{x/s})\mu$

Specific ethanol production rate (q_p)

$$d_p = q_p.dt$$

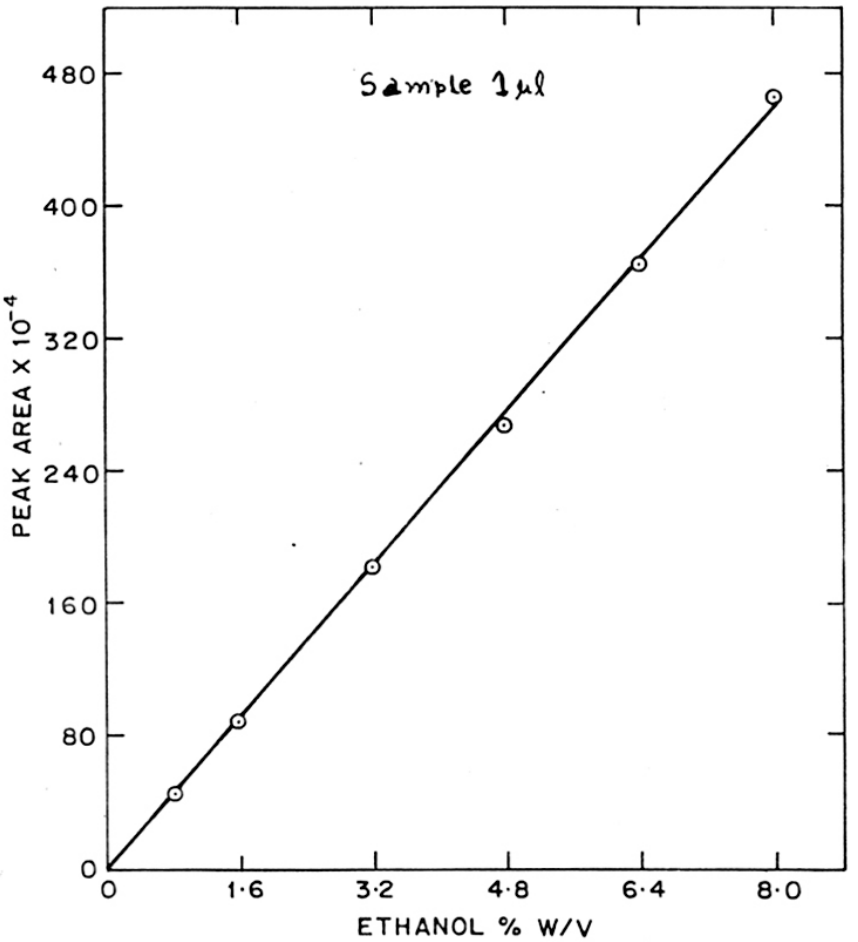
As above $q_p = (Y_{p/x})\mu$

Analysis of fermentation broth. Estimation of ethanol. Ethanol was assayed either by gas chromatography or by the chemical method of Reid and Truelove (1954). The methods were standardized with absolute alcohol made up to volume with water double distilled in glass.

Ethanol in the fermentation broth was initially recovered by distillation, essentially as described in the Official Methods of Analysis of the Association of Official Analytical Chemists, USA (1975). One volume of the fermentation broth was mixed with 0.5 volume water and submitted to distillation in an all glass system and one volume of distillate was collected. The distillate was made up to volume and the ethanol estimated either by gas chromatography or by colorimetry. The recovery of ethanol by the distillation procedure was checked with standards recovered under identical conditions and shown to be quantitative.

Gas chromatography. A Shimadzu GC RIA model instrument with computing integrator accessory and Poropak (or Chromosorb 101) 80 - 100 mesh 2m column was used. The column temperature was maintained at 150°C and the injection temperature was 180°C. The carrier gas was nitrogen. Flow rate of the carrier gas was 40 $ml.min^{-1}$ and a flame ionization detector was used. Estimations were carried out in replicates. A standard curve of average peak areas plotted against ethanol concentrations is shown in Fig. II.3.

Colorimetric estimation. Reagent blanks, standards and aliquots of the distillates were made up to volume. 2 ml of 0.36 N ceric ammonium nitrate reagent prepared in 4 N nitric acid was added to the samples. The colour


Sample 1 µl
480
400
320
240
160
80
0
PEAK AREA X 10^{-4}
0
1·6
3·2
4·8
6·4
8·0
ETHANOL % W/V

developed was read within 5 min at 486 nm. Determinations were carried out in replicate. A standard curve of the absorbance values plotted against ethanol concentrations is shown in Fig. II.4.

Enzymes. Extraction of cells. The enzymes were extracted from yeast cells which were grown 24 h either aerobically in shake-flasks as described above or under semi-anaerobic, static conditions with 100 ml medium taken in 150 ml conical flask. Cells were recovered by centrifugation and washed twice with normal saline.

Freshly grown yeast cells after the saline wash were frozen under liquid nitrogen. The frozen pellets were thawed in a pre-cooled mortar and ground well with sand. The disrupted cells were suspended either in Tris-HCl buffer (50 mM, pH 7.5) or in potassium phosphate buffer (150 mM, pH 6.0), 4 volumes g^{-1} wet cells in each case. The latter buffer was used for pyruvate decarboxylase activity assays. The suspension was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was filtered through glasswool and used for enzyme assays. Protein in the filtrates was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme assays. Invertase activity. This was assayed according to the method of Gascon and Lampen (1968). The reaction mixture contained in a total volume of 0.5 ml:

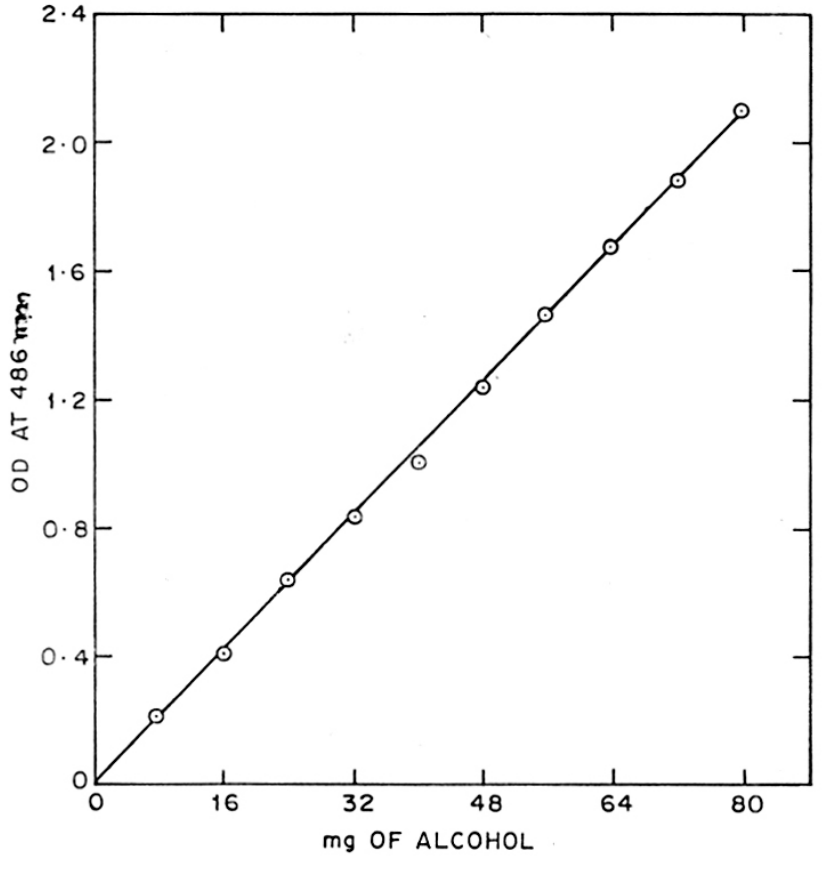

2·4
2·0
1·6
1·2
0·8
0·4
0
OD AT 486 mμ
0
16
32
48
64
80
mg OF ALCOHOL

FIG. II. 4 : Standard curve for colorimetric estimation of ethanol with cerric ammonium nitrate

sodium acetate buffer (pH 4.5), 40 mM; sucrose, 0.1 M. The reaction was started with the addition of the culture broth or cell extract and the mixture was incubated at 37°C for 10 min. The reaction was then stopped by adding 0.5 ml of 0.2 M K_2HPO_4 and heating immediately for 5 min in a boiling water bath. Suitable aliquots of the reaction mixture were taken for the estimation of D-glucose by the D-glucose oxidase method using Glox reagent.

One unit of enzyme activity corresponds to the liberation of 1 µmole glucose in 1 min.

Pyruvate decarboxylase. The activity was assayed by the method described by Reed and Willms (1966). The ferric ammonium sulfate-sodium dodecyl sulfate (SDS) reagent was prepared as follows: 1.7 g of ferric ammonium sulfate was dissolved in 10 ml distilled water, the solution filtered and the filtrate added to 20 ml of a solution containing 1.5 g of SDS in 20 ml water. To this solution, 25 ml of 85% phosphoric acid was added and the mixture was made to a final volume of 140 ml with water.

The test system was made up of : potassium phosphate buffer (1.0 M pH 6.0), 0.15 ml; thiamine pyrophosphate (2 mM), 0.1 ml; $MgSO_4$ (3 mM), 0.1 ml; potassium pyruvate (0.5 M), 0.1 ml; potassium ferricyanide (0.25 M), 0.1 ml; enzyme fraction to be assayed and water to make to a final volume of 1.4 ml. The control contained all reagents but no enzyme. The mixture was incubated for 30 min at 30°C. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA) and the precipitate formed was removed by centrifugation.

[illegible] was removed by centrifugation. To 0.1 ml or 0.2 ml aliquot of the supernatant was added 1 ml of 10% TCA; 0.1 ml of potassium ferricyanide and water to a final volume of 2.4 ml. To this 1 ml of 4% SDS and 0.5 ml of the ferric ammonium sulfate-SDS reagent were added. The mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 540 nm against the control. The standard curve was prepared with potassium ferrocyanide.

The unit of activity is defined as that amount required to produce 2 µmoles of ferrocyanide per h under the conditions described.

Alcohol dehydrogenase (ADH). The activity was assayed by a modification of the method described by Barron and Levine (1952). The assay mixture contained in a total volume of 3 ml: Tris-HCl buffer (pH 8.6), 20 mM; NAD, 0.1 mM; L-cysteine, 1.0 mM; ethanol, 0.6 M. The addition of cell extract was made before that of ethanol to start the reaction. The change in absorbance at 340 nm was monitored every 15 sec for 1 min at 25°C.

The unit of activity is defined as that amount which causes the reduction of 1 µmole NAD per min.

Gel electrophoresis. Both disc and slab gel electrophoresis were carried out in polyacrylamide gels (7%) with the Tris-glycine pH 8.3 buffer system of Davis (1964).

4 ma per tube or 20 ma per slab were used and runs were of 6 h duration at 4°C.

The gels were stained for ADH activity according to Lutstorf and Megnet (1968) except that Tris-HCl (60 mM, pH 8.6) was used instead of pyrophosphate buffer. Gels were incubated for 1 h at room temperature with a mixture containing ethanol, 0.5 M; NAD, 7 $mg.ml^{-1}$; phenazine methosulfate, 25 $\mu g.ml^{-1}$; nitroblue tetrazolium, 1$mg.ml^{-1}$ in 60 mM Tris-HCl buffer, pH 8.6.

RESULTS

Composition of cane molasses

The dissolved solids and the total and fermentable sugar contents varied within wide limits between the different samples used in the present studies. The composition of a typical batch used in the studies described in this part of the thesis is tabulated below, together with the range of values for the various samples analyzed shown in parenthesis (Table II.1)

TABLE II.1 : COMPOSITION OF A REPRESENTATIVE SAMPLE OF SUGARCANE MOLASSES

Constituents	% w/w	
Dissolved solids	78	
Total sugar	58	(50 - 58)
Fermentable sugar	52	
Non-fermentable	6	

Determination of molasses sugars by HPLC

Fructose, glucose and sucrose in the cane molasses sample were determined by HPLC as described under **Methods**. The analyses were carried out in duplicate and individual values were in agreement. The liquid chromatogram

FIG. II.5 : Liquid chromatography of molasses with maltose as internal standard.

of a typical analysis is shown in Fig. II.5 and the contents of the individual sugars and total sugars in the molasses sample determined by HPLC are summarized in Table II.2. The value for the fermentable sugars (Total sugars-non-fermentable sugars) estimated by the dinitrosalicylic acid method is also included for the sake of comparison.

TABLE II.2 : FRUCTOSE, GLUCOSE AND SUCROSE CONTENTS OF SUGARCANE MOLASSES BY HPLC ANALYSIS AND COMPARISON OF HPLC AND DINITROSALICYLIC ACID (DNSA) ANALYSES OF TOTAL FERMENTABLE SUGARS IN MOLASSES AND STANDARD SUCROSE SOLUTION

Sample	Content			Total Fermentable Sugars*	
	Fructose	Glucose	Sucrose	HPLC analysis	DNSA assay
	w/w	w/w	w/w	w/w	w/w
Cane molasses	8.3%	3.3%	37.6%	49%	52%
Standard sucrose	-	-	$121gl^{-1}$	$127.3gl^{-1}$	$127.0gl^{-1}$

*Expressed as glucose equivalent

The analytical values establish the presence of sucrose as the major sugar present in cane molasses. Standard sucrose solutions gave almost identical values whether analysed by HPLC or colorimetric method after inversion. However, the content of the total fermentable sugars of molasses (expressed in glucose equivalents) determined by the colorimetric procedure was about 6%

higher than that computed from HPLC analysis. Damon and Pettit (1980) have also reported such differences between the values for total sugars in cane molasses analysed by the HPLC method and by the Official AOAC procedure, the latter giving about 5% higher values for the total sugars. This would indicate that the simple and rapid dinitrosalicylic acid method is comparable to the Official method in precision for the assay of total sugars in molasses.

Batch fermentation of molasses

A preliminary screening was done of various standard yeast strains and the two isolates, S. cerevisiae Y-7 and S. cerevisiae Y-10, for ethanol production from molasses medium containing about 180 gl^{-1} of total sugars (calculated as glucose equivalent after inversion). In these studies the cells grown in 10 ml growth medium containing 5% total sugars were used as inoculum and transferred to 90 ml fermentation medium containing 20% molasses total sugars. The fermentation was carried out at 30°C under stationary conditions and samples were withdrawn at 24 h and 48 h periods for ethanol assays in the fermentation broth.

The values obtained in the preliminary studies are set out in Table II.3.

TABLE II.3 : ETHANOL PRODUCTION FROM CANE MOLASSES AT 30°C

10 ml inoculum (5% total sugars) added to 90 ml molasses medium containing 20% total sugars + 0.1% urea, pH 4.5.

Yeast strains	Ethanol % (w/v)	
	24 h	48 h
S. uvarum ATCC 26602	4.48	5.9
S. cerevisiae NCIM 3095	2.8	4.1
S. cerevisiae NCIM 3107	5.14	6.9
S. cerevisiae NCIM 3176	4.5	5.8
S. cerevisiae NCIM 3300	6.16	6.8
Schiz. pombe NCIM 3360	1.19	2.1
S. cerevisiae Y-7	5.12	7.1
S. cerevisiae Y-10	4.48	6.3

Of the strains tested, all except S. cerevisiae NCIM 3095 and Schiz. pombe NCIM 3360 showed ethanol production of more than 5.8% (w/v) at 48 h (5.8% to 7.1% w/v). No attempt was made to equalize yeast cell densities at the commencement of fermentations. This would give an approximate measure of a combination of the capacities for growth on molasses and for its fermentation to ethanol.

The more efficient cultures were further screened for assessing their specific ethanol productivities during

the initial phase of fermentation of high gravity sugar media at 30°C and pH 4.5 using cell desities of 1 g dry weight equivalent in 100 ml molasses or sucrose medium of 23% total sugar concentration (expressed in glucose equivalents after inversion). As stated under **Materials and Methods**, the productivities were calculated during the initial phase of fermentation before product inhibition was apparent. The values are set out in Table II.4.

TABLE II.4 : SPECIFIC ETHANOL PRODUCTIVITIES DURING THE INITIAL PHASE OF FERMENTATION OF SUCROSE AND OF CANE MOLASSES AT CONCENTRATIONS OF 23% GLUCOSE EQUIVALENT OF TOTAL SUGARS, 30°C AND pH 4.5. INOCULUM, 1% DRY WEIGHT EQUIVALENT.

Yeast strains	Specific ethanol productivity	
	Sucrose	Molasses
	$gg^{-1}h^{-1}$	$gg^{-1}h^{-1}$
S. uvarum ATCC 26602	0.5	0.44
S. cerevisiae NCIM 3107	0.59	0.31
S. cerevisiae NCIM 3176	0.58	0.32
S. cerevisiae NCIM 3300	0.65	0.36
S. cerevisiae Y-7	0.88	0.60
S. cerevisiae Y-10	0.65	0.58

From the data in Table II.4 it is clear that under the conditions used S. uvarum and the two isolates,

S. cerevisiae Y-7 and Y-10, show considerably higher specific ethanol productivities with molasses compared to those of the other strains tested. The isolate Y-7 gave consistently higher productivities than S. cerevisiae Y-10 both in sucrose and in molasses medium.

Although strain Y-7 had shown higher specific ethanol productivities, studies on continuous fermentation of cane molasses to ethanol which are reported in Part III of the thesis indicate better operational stability for both S. uvarum and S. cerevisiae Y-10, immobilized S. cerevisiae Y-7 showing poor performance over prolonged periods of continuous fermentation of molasses. Hence more detailed studies were carried out with S. cerevisiae Y-10 and S. uvarum only.

The specific ethanol productivities of S. uvarum and S. cerevisiae Y-10 were determined at molasses concentrations varying from 100 to 300 gl^{-1} total sugars expressed as glucose equivalents, corresponding to 13.5% - 40% dry solids (DS) content. The specific ethanol productivities again were determined during the initial phase of fermentation before the inhibitory effects of ethanol were apparent (Table II.5)

TABLE II.5 : SPECIFIC ETHANOL PRODUCTIVITIES DURING THE INITIAL PHASE OF FERMENTATION OF MOLASSES AT 30°C; INOCULA 1 g DRY WEIGHT EQUIVALENT PER 100 ml MEDIA

Cane molasses		Specific ethanol productivity	
R.S.*	D.S.+	S. uvarum ATCC 26602	S. cerevisiae Y-10
%	%	$gg^{-1}h^{-1}$	$gg^{-1}.h^{-1}$
10	13.5	0.56	0.68
15	20	0.57	0.70
20	27	0.40	0.68
25	33.5	0.32	0.58
30	40	0.19	0.35

*R.S. Reducing sugars after inversion

+ D.S. Dry solids content

It can be seen from Table II.5 that in ethanol production the isolate S. cerevisiae Y-10 shows greater tolerance than the S. uvarum towards high concentrations of cane molasses. The specific ethanol productivities of S. cerevisiae Y-10 were higher than those of S. uvarum at all the substrate concentrations tested. The differences were less marked at the lower concentrations of molasses, the values being 0.56 - 0.57 $gg^{-1}h^{-1}$ for S. uvarum at 13.5 and 20% DS content and 0.68 - 0.70 $gg^{-1}h^{-1}$ for S. cerevisiae Y-10 at the corresponding DS contents. The isolate however, showed markedly higher specific productivities at the higher levels of molasses compared

to the S. uvarum, which is severely inhibited at these substrate concentrations. Thus the value of 0.68 - 0.70 $gg^{-1}h^{-1}$ at 13.5 and 20% DS content remains unchanged in the case of S. cerevisiae Y-10 at 27% DS content and is lowered only by about 14 - 15% at 33.5% DS content compared to 30% and 44% reductions in the specific productivity value of 0.57 $gg^{-1}.h^{-1}$ at 20% DS content for the S. uvarum at 27% and 33.5% DS contents, respectively. At 40% DS content both cultures were inhibited markedly, the S. cerevisiae Y-10 showing 50% reduction and the S. uvarum a 67% reduction in their respective initial specific ethanol productivity values.

The higher specific productivities at high DS contents of molasses of the isolate reflect its better osmo-tolerance in the fermentative production of ethanol.

Effect of molasses concentrations on the production of ethanol

The effects of molasses concentration on ethanol production by S. uvarum and S. cerevisiae Y-10 used in batch mode under the same conditions are shown in Fig. II.6.

At all the concentrations tried, the conversion rates prior to completion of fermentation were faster at all stages with the S. cerevisiae Y-10 isolate than with the S. uvarum strain. Thus with molasses containing

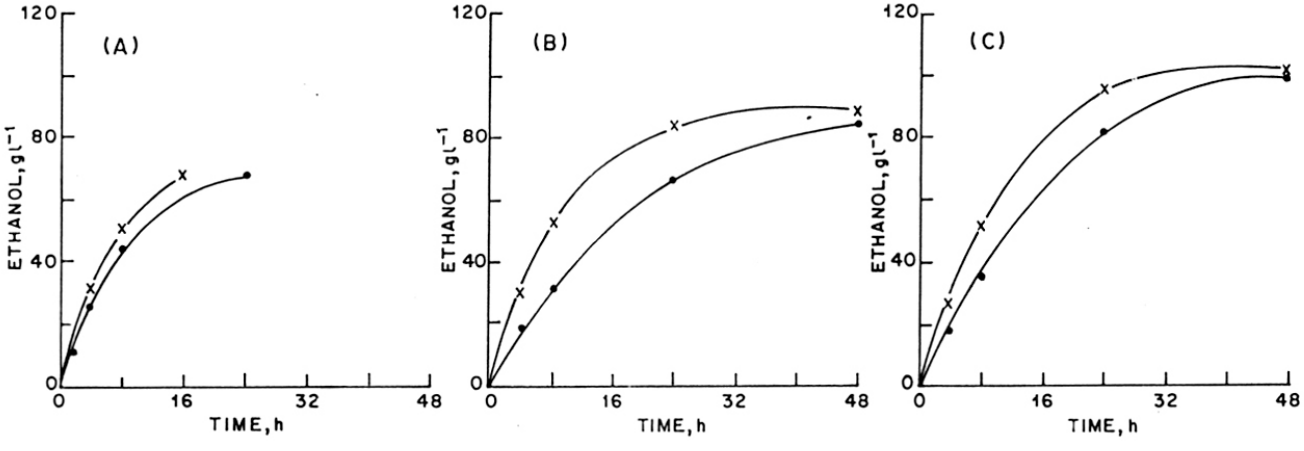

(A)
(B)
(C)
ETHANOL, g l⁻¹
TIME, h
0
40
80
120
16
32
48

FIG. II.6 : Batch fermentation of cane molasses by suspended cells of S. uvarum ATCC 26602 (-•-) and S. cerevisiae Y-10 (-x-). Molasses containing

(A) 150 gl^{-1} total sugars

(B) 200 gl^{-1} sugars, and

(C) 250 gl^{-1} total sugars

Details as in text.

15% total reducing sugars corresponding to 135 gl^{-1} glucose equivalent of fermentable sugars the initial specific ethanol productivities up to 30 gl^{-1} product concentration were about 0.66 $gg^{-1}.h^{-1}$ with S. cerevisiae Y-10 and about 0.52 $gg^{-1}\ h^{-1}$ with S. uvarum, in agreement with values reported in Table II.5. At this susbstrate concentration, about 66 gl^{-1} ethanol, corresponding to 95% yield of product, was obtained in 16 h in the case of the S. cerevisiae isolate, while the time taken for a comparable yield is about 24 h in the case of S. uvarum (Fig. II.6A). In the case of the isolate, the initial specific ethanol productivity values remained unchanged at the higher level of 20% total molasses sugars (180 gl^{-1} glucose equivalent of fermentable sugars) and an yield of about 95% of theory was attained in about 32 h at this substrate level. In comparison, the S. uvarum had an initial specific ethanol productivity of 0.36 $gg^{-1}\ h^{-1}$ at 20% total sugar concentration and even after 48 h of fermentation the final yield was only about 92% of theory (Fig. II. 6B), the specific ethanol productivity values again being in good agreement with those reported in Table II.5. At 25% total molasses sugars content (225 gl^{-1} glucose equivalent of fermentable sugars) the initial specific ethanol productivity values are 0.56 $gg^{-1}h^{-1}$ and 0.36 $gg^{-1}h^{-1}$ for the S. cerevisiae Y-10 and S. uvarum, respectively, while the ethanol concentrations at 24 h

are 96 gl^{-1} and 81 gl^{-1}, respectively.

The experiments described above clearly establish the superiority of the fermentative capacity of the isolate S. cerevisiae Y-10 in batch fermentation of cane molasses, particularly at high substrate concentrations. The temperature tolerance of the isolate has also been shown to be satisfactory, the optimum temperature being 30°C, and the performance being slightly lower at high ethanol values even at 35°C and 40°C.

Effect of inoculum size

The effect of inoculum size on the fermentation rates was studied at 30°C with S. cerevisiae Y-10 and the S. uvarum at molasses total sugars content of 22% corresponding to about 200 gl^{-1} glucose equivalent of fermentable sugars and cell concentrations of 0.25, 0.5, 1.0 and 1.25 g dry weight per 100 ml medium. Ethanol was monitored at regular intervals.

The inocula sizes ranging from 0.25 g to 1.25 g dry cell equivalent per 100 ml molasses medium had no effect on the initial specific productivities of S. cerevisiae Y-10 and the S. uvarum, the values being in the ranges of 0.68 to 0.70 $gg^{-1}h^{-1}$ for the S. cerevisiae and 0.36 to 0.46 $gg^{-1}h^{-1}$ for the S. uvarum. Inocula levels in this range of cell loadings apparently have no significant effect on the initial specific ethanol productivities.

Effect of pH

Both cultivars showed a pH optimum in the range pH 4.0 to pH 5.0 at 30°C, at all the molasses sugar concentrations tested. No changes in pH were recorded in the course of 72 h of fermentation.

Effect of temperature

The effects of temperatures in the range of 20°C to 45°C on fermentation of molasses at pH 4.5 and 20% total sugar concentrations by S. cerevisiae Y-10 are shown in Fig. II.7. The initial rates were maximum at 40°C and 45°C, the specific ethanol productivities being about 1 $gg^{-1}h^{-1}$ at both temperatures. The rates were similar till 4 h at which period the ethanol concentration at both temperatures of 40°C and 45°C was 30 gl^{-1}. However, between 4 and 8 h the fermentation rates at the higher temperature of 45°C decreased markedly and ethanol production ceased at the level of 40 gl^{-1}. The fermentation rate at 40°C was affected less markedly by the product. The initial fermentation rates were slowest at 20°C and rates increased with temperature in the range of 20°C to 40°C. Beyond about 30 gl^{-1} ethanol concentration, the rate was fastest at 30°C and decreased with temperature in the range 30°C to 40°C and an ethanol concentration of about 55 gl^{-1} was attained at the same period of ~16 h at all three temperatures of 30°C, 35°C and 40°C. The

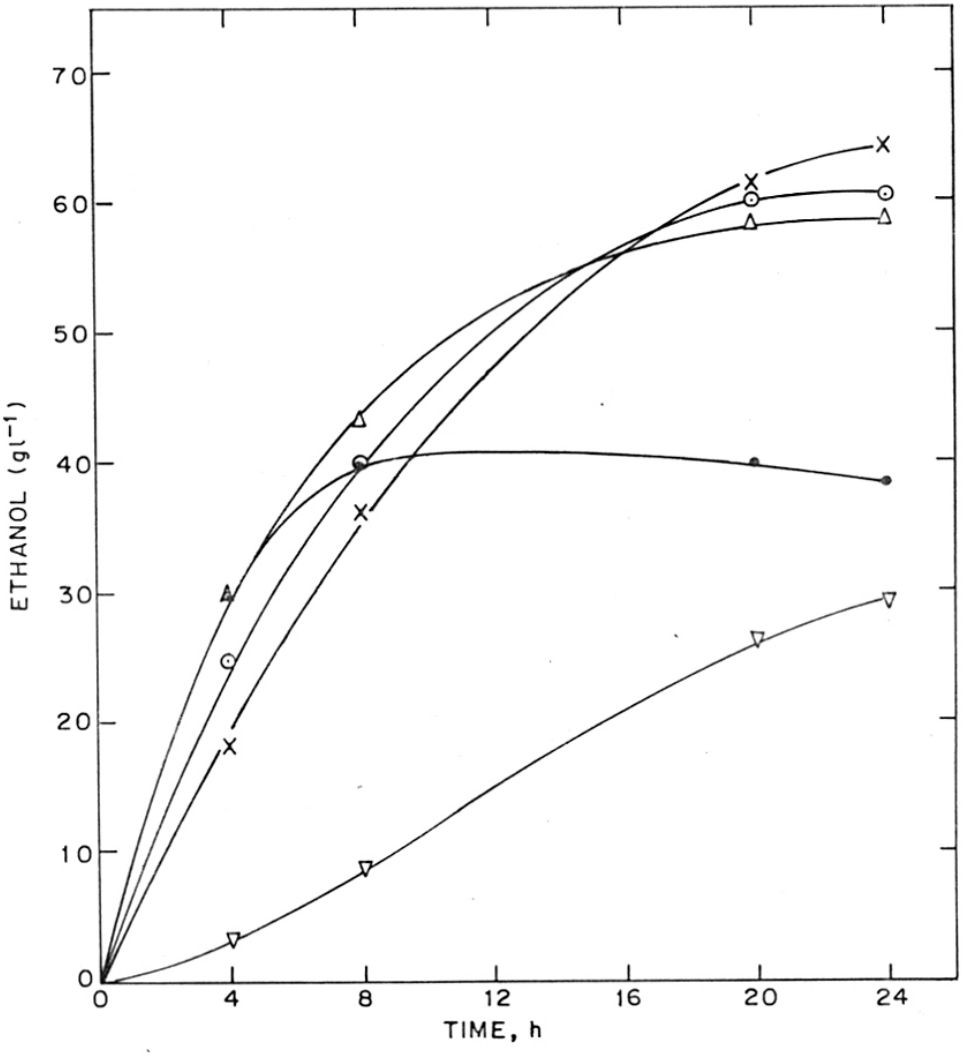

ETHANOL (gl⁻¹)
TIME, h
0
10
20
30
40
50
60
70
0
4
8
12
16
20
24

FIG. II.7 : Effect of temperature on batch fermentation of cane molasses by suspended cells of S. cerevisiae Y-10. Molasses containing 200 gl^{-1} toal sugars; pH 4.5

Temperatures, 45°C (-●-); 40°C (-△-); 35°C (-⊙-); 30°C (-✕-); 20°C (-▽-).

Details as in text.

ethanol concentration at 24 h was highest at 30°C, the yield, being progressively less with increase in temperature up to 40°C. The ethanol concentrations attained at 20°C were the lowest at all periods up to 24 h fermentation.

Kinetic parameters in batch cultures

The comparative kinetics of the 3 yeasts, S. cerevisiae isolates Y-7 and Y-10 and the standard S. uvarum strain in the growth-associated fermentation of cane molasses were studied in batch mode under similar nutrient and other environmental conditions. The conditions used for the growth of inocula and subsequent growth and fermentation of cane molasses have been described under **Materials and Methods**. The kinetic parameters were calculated from the exponential phase of growth-associated fermentation unless otherwise stated.

The uptake of sugars and the production of biomass and ethanol are shown in Fig. II.8 and the kinetic parameters are given in Table II.6.

From Fig. II.8 and Table II.6 it can be seen that the isolates have more desirable characteristics than the standard S. uvarum strain for the fermentation of cane molasses to ethanol in batch culture. Both specific ethanol production rates and specific sugar uptake rates are comparatively higher in the isolates than in the S. uvarum. Specific growth rates of the isolates are also higher than that of S. uvarum.

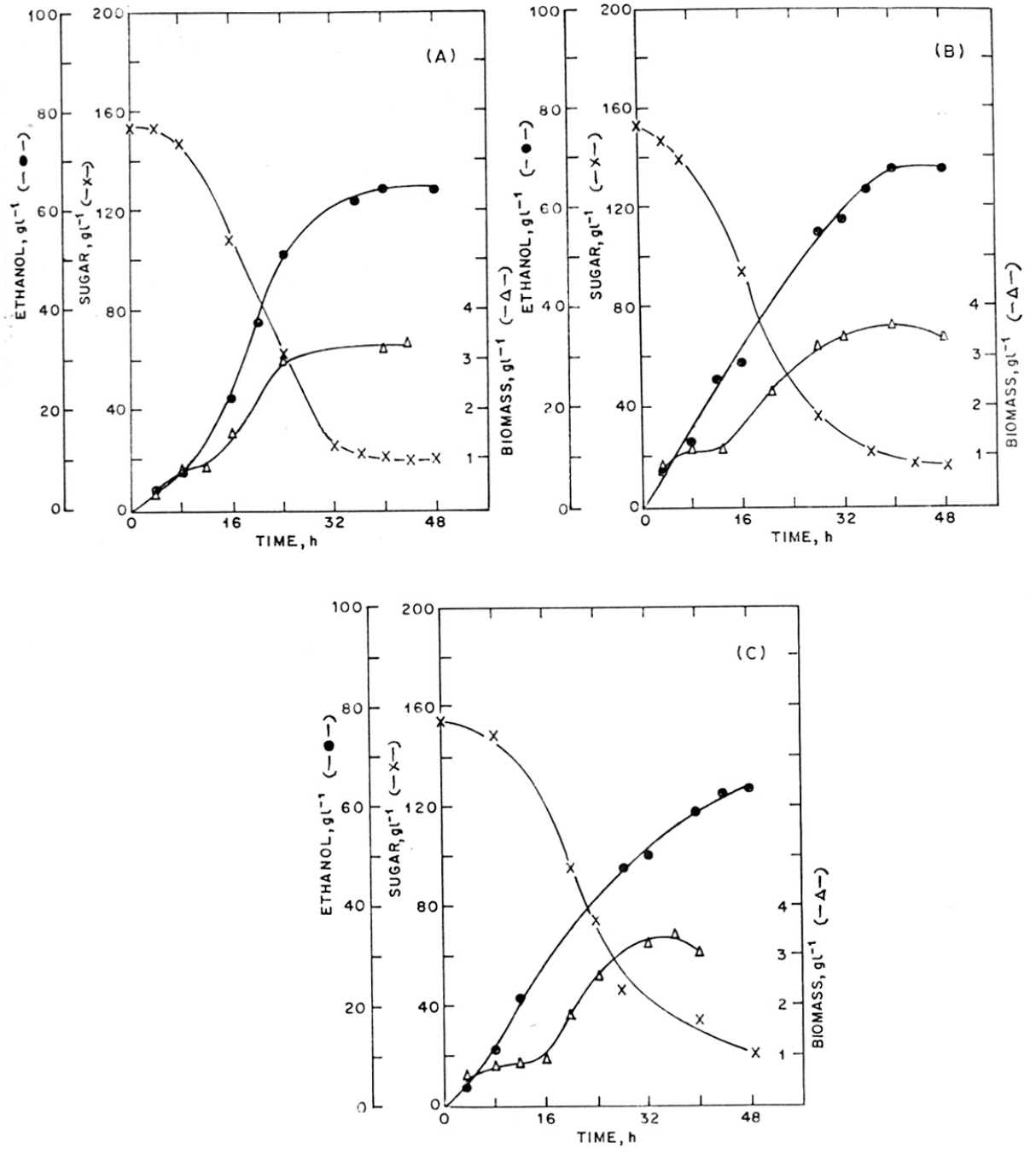
FIG. II.8 : Kinetics of batch fermentation of cane molasses by

(A) S. cerevisiae Y-10

(B) S. cerevisiae Y-7, and

(C) S. uvarum ATCC 26602

Details as in text.


(A)
(B)
(C)
ETHANOL, gl⁻¹ (—●—)
SUGAR, gl⁻¹ (—X—)
BIOMASS, gl⁻¹ (—Δ—)
TIME, h
0
16
32
48
20
40
60
80
100
120
160
200
1
2
3
4

The ethanol yields are about 93 - 96% of theoretical in the case of the isolates compared to 88.7% of theory for S. uvarum.

TABLE II.6 : COMPARISON OF KINETIC PARAMETERS IN BATCH CULTURE IN MOLASSES MEDIUM. INITIAL TOTAL SUGAR CONCENTRATION 157 gl^{-1} GLUCOSE EQUIVALENT; TEMPERATURE, 30°C; INITIAL pH 5.0, FINAL pH AT THE END OF 48 h, 4.3.

	S. cerevisiae Y-7	S. cerevisiae Y-10	S. uvarum ATCC 26602
Specific growth rate, μ (h^{-1})	0.092	0.08	0.06
Specific ethanol production rate, q_p ($gg^{-1}h^{-1}$)	1.7	1.6	1.1
Specific glucose uptake rate, q_s ($gg^{-1}h^{-1}$)	3.3	3.5	2.14
Overall biomass yield $Y_{x/s}$ (gg^{-1})	0.029	0.023	0.028
Final ethanol concentration (gl^{-1})	68	65	62
Overall ethanol yield $Y_{p/s}$ (gg^{-1})	0.489	0.474	0.453
Ethanol yield (% of theoretical)	95.9	93.0	88.7

Profile of key enzymes

The levels of invertase, pyruvate decarboxylase and alcohol dehydrogenase; enzymes which play key roles

in molasses utilization and its fermentation by the glycolytic pathway were determined in S. cerevisiae Y-7, S. cerevisiae Y-10 and S. uvarum ATCC 26602 under different conditions of growth and in the presence of different sugar substrates.

Invertase catalyses the first step in the utilization of sucrose by Saccharomyces spp., namely the hydrolysis of this sugar molecule to a molecule each of glucose and fructose, both substances being glycolysis substrates. Pyruvate decarboxylase catalyses the penultimate step yielding acetaldehyde and ADH catalyses the interconversion of acetaldehyde and ethanol, the final step of alcoholic fermentation by yeast (Fig. I.1).

Invertase

The data on invertase activity are summarized in Table II.7.

TABLE II.7 : COMPARISON OF INVERTASE ACTIVITY

	Invertase activity (units/mg protein)		
Sugar used	Saccharomyces uvarum	Saccharomyces cerevisiae Y-10	Saccharomyces cerevisiae Y-7
D-Glucose	2.74	0.49	1.64
Sucrose	3.60	0.99	4.22
Molasses	3.28	1.38	4.56

The values obtained are the average of at least three independent experiments.

The extracellular invertase activity was negligible in all the three strains. The intracellular invertase levels are markedly enhanced in presence of sucrose and molasses compared to glucose as the substrate. The data also suggest that invertase activity is inducible in certain strains of yeasts but the amount of constitutive enzyme activity present is probably adequate for the conversion of substrate needed for ethanol production.

Pyruvate decarboxylase (PDC)

Pyruvate decarboxylase activities of the S. uvarum and S. cerevisiae Y-10 grown aerobically on molasses and of the pre-grown cells transferred to 16% molasses sugar containing medium were assayed, the latter at the end of 24 h fermentation and the results are shown in Table II.8.

TABLE II.8 : COMPARISON OF PYRUVATE DECARBOXYLASE ACTIVITY UNDER AEROBIC AND ANAEROBIC CONDITIONS

Sugar used	Pyruvate decarboxylase activity (Units/mg protein)	
	Saccharomyces uvarum	Saccharomyces cerevisiae Y-10
Aerobic		
Molasses	2.5	2.2
Anaerobic		
Molasses	3.175	3.7

The pyruvate decarboxylase activity of the two cultures were in the range 2.2 and 2.5 U/mg protein in cells grown aerobically while the activity was significantly higher under fermentation condition, the values being 3.2 and 3.7 U/mg protein for the S. uvarum and the S. cerevisiae, respectively.

The sensitivity of enzymes of the two cultures to high levels of salt, ethanol and a combination of the two was examined by incubating the crude cell extracts for 30 min at 30°C with 2% KCl, 20% (w/v) ethanol or 2% KCl + 10% (w/v) ethanol. Under all the conditions used, no losses of pyruvate decarboxylase activity could be detected in the extracts from both the sources grown under fermentation conditions. These results are in contrast to the results obtained by Millar et al. (1982) with the purified enzymes from yeast. These authors have reported 90% loss in activity of purified yeast pyruvate decarboxylase activity at 19% (w/v) ethanol concentration and 50% loss at 17% w/v concentration of the reagent. The other purified enzymes of glycolytic pathway were less sensitive to ethanol denaturation than pyruvate decarboxylase. The differences in the results obtained in the present study might be due to the protective effects of other proteins and factors present in the cell extracts. The conclusion that enzyme denaturation is unlikely to play a direct part in ethanol tolerance is in keeping with that of

Millar et al. (1982) since even these authors observed only 10% or less losses in activity of the individual purified enzymes when exposed in vitro to 12% (w/v) ethanol concentration.

Alcohol dehydrogenase (ADH)

A comparison of the specific activities of alcohol dehydrogenase between S. uvarum and the isolates S. cerevisiae Y-10 and S. cerevisiae Y-7 under aerobic and anaerobic conditions is shown in Table II.9. The

TABLE II.9 : COMPARISON OF ALCOHOL DEHYDROGENASE ACTIVITY UNDER AEROBIC AND ANAEROBIC CONDITIONS

Condition	ADH activity (units/mg protein)		
Sugar used	Saccharomyces uvarum ATCC 26602	Saccharomyces cerevisiae Y-10	Saccharomyces cerevisiae Y-7
Aerobic			
D-Glucose	0.319	0.521	0.531
Sucrose	0.388	0.651	0.731
Molasses	0.309	0.493	0.633
Anaerobic			
Sucrose	0.520	0.814	0.897
Molasses	0.427	0.661	0.788

The values obtained are the average of three independent determinations

The percentage increase in enzyme activity of the isolates varies from 40 - 100% depending on the sugar used in growth media. S. cerevisiae Y-7 always shows a higher alcohol dehydrogenase activity than the other two strains. A slight inhibition is observed in molasses grown cells often masking the true alcohol dehydrogenase activity which can be attributed to the high salts and other impurities present in molasses.

TABLE II.10 : ALCOHOL DEHYDROGENASE ACTIVITY IN DIFFERENT STRAINS OF YEAST

Strain used (NCIM)	ADH activity (units/mg protein)	
	Sucrose	Molasses
Saccharomyces cerevisiae 3300	0.345	0.327
Saccharomyces cerevisiae 3107	0.326	0.273
Saccharomyces cerevisiae 3176	0.273	0.312
Saccharomyces cerevisiae 3095	0.319	0.299
Schizosaccharomyces pombe 3360	1.049	0.561

The data in Table II.10 shows that it is not possible to draw a direct correlation between high alcohol dehydrogenase activity and the increased ethanol productivity exhibited by certain strains of yeasts. For example, Schizosaccharomyces pombe shows a very high alcohol

dehydrogenase activity compared to other yeast strains but the ethanol production from molasses is found to be low (Table II.3). This suggests that the nature of the fermentation medium is also a contributing factor for higher ethanol productivity. In other words, Schizosaccharomyces pombe has the potential to give higher ethanol productivity based on the high alcohol dehydrogenase activity present provided the proper substrate is used. The same way it can be argued that the lower productivity obtained with different strains of S. cerevisiae tested (Table II.7) can be due to the presence of lower alcohol dehydrogenase activity (Table II.8). In the studies on vinification by several yeast strains, Singh and Kunkee (1976) could correlate the level of alcohol dehydrogenase with fusel oil production but not with ethanol. This is in contrast to the findings of Sharma and Tauro (1986) who claim that high PDC + ADH activities contribute towards rapid ethanol production. A simple and direct correlation between alcohol dehydrogenase and ethanol productivity seems to be difficult at present due to the complexity of the process and lack of knowledge of other influencing factors.

Isozymes of alcohol dehydrogenase

It is well established that yeast alcohol dehydrogenase has three isozymes, namely ADH I (cytosolic), ADH II (oxidative, cytosolic) and ADH III (mitochondrial with five bands) (Fowler et al., 1971; Wills and Phelps, 1975 and Wills and Jornwall, 1979) and they are also known to exhibit different kinetic characteristics (Wills, 1976). To get a better understanding of alcohol dehydrogenase in the isolates, the isozyme pattern was studied on polyacrylamide gels. Our results show that S. cerevisiae Y-10 and Y-7 have a similar isozyme pattern compared to S. uvarum (Fig. II .9). in the electrophoregram only one major band of alcohol dehydrogenase was seen which has lower mobility in both isolates compared to S. uvarum. The differences in the minor bands seen in the gels were not very obvious in our studies. Though the multiplicity of ADH isozymes has been shown by others (Singh and Kunkee, 1977) the kinetic characterization has not been carried out. The absence of multiplicity of ADH isozyme pattern in Sch.pombe (Wills and Phelps, 1975) in contrast to other S. cerevisiae species also suggests that the kinetic parameters like K_m and V_{max} are of more importance.

It is known that the glycerol production is enhanced in partial alcohol dehydrogenase mutants lacking the particular isozyme (Johansson and Sjostrom, 1984)


(−)
1 2 3 4
(+)

FIG. II.9 ; Polyacrylamide gel electrophoresis pattern of ADH isozymes. Tube gels were run and stained for ADH activity as described in Materials and Methods. About 50 µg supernatant protein was loaded in each tube

(1) Saccharomyces cerevisiae Y-10

(2) S. uvarum

(3) Mixture of S. cerevisiae Y-10 and S. uvarum

(4) S. cerevisiae Y-7

and the ethanol production is increased in petite mutants lacking mitochondrial isozymes of ADH (Moulir et al. 1981 and Easer et al., 1982). Hence a more detailed study on the different aspects of ADH and other important enzymes are needed for a better understanding of the complex process of ethanol fermentation.

DISCUSSION

The main raw material used indigenously for the production of fermentation ethanol is sugarcane molasses. The primary requirement for achieving process improvement in the industrial production of non-beverage alcohol is the selection of a suitable microbial strain, yeast in the case of cane molasses, and the establishment of appropriate environmental conditions to achieve the following objectives:

* high ethanol yield to maximise substrate utilization,
* high ethanol concentration in the fermented beer to reduce product recovery costs, and
* high ethanol productivity to reduce fermentation capital costs.

Ethanol tolerance

The main limiting factor in ethanol production is the inhibitory effect of the product on yeast growth and fermentation. As the ethanol concentration in the broth increases, growth rate decreases as an early manifestation of the inhibition (Troyer, 1963). Ethanol-tolerance is strain dependent, and the maximum ethanol concentration in the medium at which growth occurs is generally limited to 10% w/v, while ethanol production ceases at a higher level of about 20% w/v ethanol even in the highly ethanol-tolerant strains (Rose, 1980; 1983).

Several evidences indicate that the inhibition of growth of Saccharomyces spp. by ethanol is mainly the result of perturbations of the plasma membrane in presence of the solvent (Ingram and Buttke, 1984). Ethanol inhibits solute accumulation by the organism and this has been documented for a wide range of solutes including D-xylose (Leao and Van Uden, 1982), maltose (Loureiro-Dias and Peinado, 1982), ammonium ions (Leao and Van Uden, 1983), glucose (Thomas and Rose, 1979) and glycine and other amino acids dependent on the general amino acid permease system (Thomas and Rose, 1979; Leao and Van Uden, 1983). In all cases ethanol had no effect on the affinity of the transport system, K_m values being unaffected while V_{max} of the rates of solute uptake were lowered indicative of non-competitive inhibition. The assumption that the main target of ethanol inhibition is the plasma membrane is also supported by the observation that the membrane composition of ethanol-tolerant Saccharomyces strains show characteristic membrane fatty acyl composition. Thus enrichment of linoleyl compared to oleyl residues of plasma membrane lipids is associated with tolerance to high product concentrations (Thomas et al., 1978; Thomas and Rose, 1979). Beavan et al.,(1986) have reported that S. cerevisiae adapted to growth in the presence of ethanol shows dose-dependent increase in the content oleyl accompanied by a decrease in saturated residues of membrane phospholipids.

The recent observation by Cartwright et al. (1987) of the inhibition of Saccharomyces membrane ATPase by ethanol, indicates that the active transport process disruption could be the cause of product inhibition. The enzyme is required for most active transport processes that depend on transmembrane proton electrochemical gradient (ΔP). Inhibition of this enzyme by ethanol would contribute to ΔP dissipation and consequent lowering in solute uptake rates and accumulation.

The sensitivity of Saccharomyces to inhibition by ethanol is increased when other stresses such as of high substrate and salt and elevated temperature conditions are superimposed. These inhibitory effects are apparently synergistic although quantitative relationships have not been investigated. Only limited data are available on ethanolic fermentation by yeasts under high osmotic pressure conditions (Haraldson and Bjorling, 1981). Under solute stress, Saccharomyces cerevisiae accumulates glycerol, the only polyol produced by the organism (Jennings, 1984; Kenyon et al., 1986, Larsson and Gustafsson, 1987). Even under dilute environmental conditions, glycerol is formed as a byproduct during ethanolic fermentation at specific glycerol yields of about 0.03 to 0.05 gg^{-1} glucose (Oura, 1977).

In ethanolic fermentation by yeasts at relatively high temperatures of about 37°C to 40°C, the specific

rate of ethanol formation and the viability of cells are reduced, often severely (Van Uden, 1984). Thus even at 3% w/v ethanol the maximum temperature of growth is lowered.

Elevated growth temperatures have been shown to increase markedly the spontaneous mutation rate to respiratory-deficient, petite mutants in S. cerevisiae and other yeasts, suggestive of mitochondria being the main site of inactivation (Yeas, 1956; Sherman, 1959; Bulder, 1964). Ethanol causes a downward shift in the temperature profile (Van Uden and Duarte, 1981; Loereiro and Van Uden, 1982) and enhances the petite mutation rate (Zakharov and Bandas, 1979; Bandas and Zakharov, 1980).

In the present study, a comparison of the rates of ethanol production by the two S. cerevisiae isolates designated as Y-7 and Y-10 has shown that these are more tolerant to high cane molasses and ethanol concentrations than some standard strains of yeasts. Among the brewing strains, S. uvarum ATCC 26602 showed better performance than other standard strains tested. Saccharomyces cerevisiae Y-10 has also been shown to tolerate high incubation temperatures.

Tolerances to ethanol, solute and temperature stresses are complex and polygenic characteristics involving multiple enzymes and cell function. This would explain the lack of any simple correlation

between the levels of the key enzymes involved in sucrose fermentation and enhanced tolerances towards the substrate, product and temperature. A factor to consider in extraporating data on enzyme inactivation from studies on cell-free systems to the intact organism is that of the possible effects of the location of the enzyme within the cell. Thus the location of the enzyme in the cytoplasm membrane could provide it protection from solvent effects. Such a hypothesis has been proposed to explain the ability of a Zymomonas mobilis strain to grow in presence of 12% (w/v) ethanol while the isolated partially purified alcohol dehydrogenase from the source is inhibited to the extent of 90% even at 4.6% (w/v) ethanol (Hoppner and Doelle, 1983).

PART III

OPEN PORE GEL MATRICES FOR YEAST CELL ENTRAPMENT

SUMMARY

A procedure is described for obtaining open-pore gelatin pellets with entrapped yeast cells. The process involves selective leaching out of calcium-alginate from the composite beads of calcium alginate-gelatin followed by crosslinking with glutaraldehyde. The porous nature of the selectively leached matrix has been shown by scanning electron microscopy.

Yeast cells immobilized by entrapment in the porous gel have been shown to give higher specific ethanol productivities and better tolerance towards high salt concentrations in molasses than the free cell suspensions under batch fermentation conditions.

Yeast cells immobilized in the porous gelatin matrix gave higher ethanol productivities in continuous fermentation of cane molasses compared to that of the yeast strain entrapped on plain calcium-alginate matrix. The effects of gelatin concentration and initial cell loading on reactor performance are reported.

The continuous fermentation of high gravity molasses with the ethanol and osmotolerant S. cerevisiae Y-7 and Y-10 isolates have been investigated. The immobilized S. cerevisiae Y-10 showed high productivities and operational stability over an extended period of 5 to 6 weeks of continuous conversion, the values ranging from 22 to 26 $gl^{-1}h^{-1}$ at 95% conversion efficiency

of molasses containing 162 gl^{-1} fermentable sugars. The immobilized S. cerevisiae Y-7 in contrast gave comparable ethanol productivities only over a limited period of about 2 weeks.

Open-pore gel beads with entrapped yeast cells could also be obtained with other proteinous substances such as whole hen's egg by a modification of the process described for gelatin, glutaraldehyde crosslinking being done in this case during bead formation in calcium-chloride solution.

Open-pore gelatin entrapped yeast cells showed erosion from the interior of beads after periods of about 2 months of continuous fermentation.

Open-pore agar and open-pore agarose obtained by differential leaching of calcium alginate from a composite matrix containing alginate and agar or agarose has been shown to be suitable for the entrapment of microbial whole cells required for use in reactions that involve cell growth and gas evolution.

Beads of porous agar and of agarose with entrapped yeast cells have been used for the continuous fermentation of sugarcane molasses to ethanol, without apparent bead rupture even after 3 months of use. High volumetric ethanol productivities were obtained, comparable to those obtained with yeast cells entrapped in open-pore gelatin or whole egg beads. The agar and agarose gels however did not erode during prolonged operation, unlike the porous gelatin crosslinked with glutaraldehyde.

INTRODUCTION

As discussed in the **General Introduction** one of the most promising systems for continuous fermentations at high conversion efficiencies and high volumetric productivities is the application of immobilized microbial whole cells for this purpose. Such systems allow high cell loading and high dilution rates without the attendant problems of cell washout. Wasteful cell growth can also be controlled and growth and conversion conditions optimised separately.

The various techniques that have been used for microbial whole cell immobilization have already been discussed in Part I of the thesis. The most extensively used procedure is of microbial whole cells entrapment in hydrophilic gels since this permits higher cell loading than in the surface-adsorbed cell systems and the conditions used for effective entrapment are generally milder and less aggressive than those used for covalent attachment. Several polymer gel matrices, both synthetic and naturally occurring, have been applied for this purpose. The use of polyacrylamide, agar, calcium alginate, k-carrageenan and gelatin as single component matrices for microbial whole cell immobilization and their application in continuous ethanolic fermentation have also been described in the **General Introduction.**

The main limitation with gel carrier matrices is that of diffusional restrictions. This problem is alleviated if the gel carriers are rendered adequately porous. Klein and Wagner (1978) and Klein and Eng (1979) attempted this in epoxy polymer systems by initially preparing calcium alginate coated gel beads containing epoxy precursor, polyfunctional amines and microbial whole cells followed by cross-linking of the epoxy resin on drying and the subsequent leaching out of the calcium alginate component with phosphate buffer systems. The porous epoxy polymer system was used by them for the immobilization of penicillin acylase-containing Escherichia coli cells required for 6-amino-penicillanic production from benzylpenicillin. Loss in some cell activities, particularly cell division however were reported by Klein et al. (1978).

Novel open pore gel matrices have been obtained by us using the approach of differential leaching, somewhat similar to that used by Klein and his group for their epoxy polymer systems. Composite beads of calcium alginate and some proteinous gels were prepared initially and these were treated with phosphate buffer to disrupt the alginate component. The beads, after or before leaching out of the calcium alginate component were stabilized by chemical cross-linking with glutaraldehyde when proteinous matrices like gelatin

or whole hen's egg were used. Polysaccharide gel carriers like agar, agarose, and k-carrageenan did not require any additional strengthening. Yeast cells immobilized in such open-pore matrices retained their ability to divide and to ferment molasses sugars to ethanol.

This part of the thesis describes the preparation of open-pore proteinous systems of gelatin, whole egg and of open-pore polysaccharide gels of agar, and low melting SeaPlaque agarose. The application of such systems with yeast cells entrapped in the open-pore gel matrices for the continuous conversion of cane molasses to ethanol is described. The comparative evaluations of performances of immobilized S. cerevisiae Y-7 , Y-10 and S. uvarum ATCC 26602 in such systems are also described.

MATERIALS AND METHODS

Materials

Gelatin and hensegg were purchased from the local market. Agar and sodium alginate were obtained from Loba Chemical Co. SeaPlaque agarose and κ-carrageenan were generous gifts from the Marine Colloids Division, FMC Corporation, USA. Glutaraldehyde (50% in water) was obtained from Fluka AG, Switzerland. All other chemicals were of high purity reagent grade.

Methods

Yeast cultures. Cells of Saccharomyces cerevisiae isolates Y-7 and Y-10 and the standard strain Saccharomyces uvarum ATCC 26602 described in Part II of the thesis were used. The conditions for their maintenance and growth have been described in detail in Part II of the thesis.

Cells required in large amounts for immobilization were grown in a New Brunswick Labroferm batch fermentor of 14 litre capacity. The medium contained in gl^{-1}: cane molasses total sugars, 50; yeast extract, 3.0; malt extract, 3.0; and peptone, 5.0, pH 5.8. The medium (7 l) was sterilized in the fermentor at 121°C for 20 min. After cooling, the medium was inoculated with the culture (10% v/v), initially grown in shake flasks. The pH of the medium was maintained at 5.8, and air was sparged at one volume per min per volume of the

medium. The temperature was maintained at 30°C. After 24 h, the cells were harvested in an A-12 Sharples Supercentrifuge at 15,000 rpm.

Open pore cross-linked protein gels

Yeast cells entrapped in open pore gelatin beads. The composite gel beads containing alginate and gelatin were obtained by a modification of the procedure described by Brodelius and Nilsson (1980). In a typical experiment, 100 ml of an aqueous solution containing 20% (w/v) gelatin and 2% (w/v) sodium alginate was prepared by steaming or boiling the mixture till a clear opalescent solution was formed.

The solution was cooled to about 40°C and a known wet weight of yeast cells (usually 20 g) were added and mixed to give a uniform suspension of the cells in the gelatin-alginate solution. The resulting slurry was extruded dropwise through a hypodermic syringe needle connected to a peristaltic pump into a stirred solution of 0.07 M calcium chloride kept cooled at 10°C. Bead diameter was approximately 2 to 3 mm.

The alginate in the composite beads was leached out at about 10°C with 50 mM potassium phosphate buffer, pH 7.6. The beads were washed with the buffer till the washings were clear.

The porous beads were then crosslinked with 0.01 M

glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.6 for a period of 90 min at 10°C and then washed thoroughly with water. To determine the glutaraldehyde concentration to be used such that deleterious effects of the reagent on cell activity are avoided, the fermentation of cane molasses to ethanol by gelatin-entrapped cells and of free cells treated with varying concentrations of reagent was compared to that of untreated suspended cells in batch mode at 10% fermentable sugar concentration. When glutaraldehyde concentrations of 0.01 M to 0.015 M were used, the rate of ethanol production by immobilized cells was unaffected and was higher than that of the equivalent amount of free cells by about 30%. Such enhancement in fermentation rates of immobilized systems has been reported by earlier workers (Holcberg and Margalith, 1981). Glutaraldehyde concentrations above 0.015 M however markedly lowered fermentation rates.

The initial cell loading in the beads when 20% wet packed weight of yeast cells were used was 31 g dry weight in 1 litre bead volume.

Open-pore crosslinked whole egg beads with entrapped yeasts. Open-pore gel of crosslinked proteins in whole egg was obtained essentially as described above for gelatin. Whole egg substance was mixed uniformly with an equal volume of 4% sodium alginate solution. Packed yeast cells (20% g wet weight) were uniformly dispersed in the mixture of whole egg and sodium alginate

solution. The suspension was added dropwise through a nozzle into a stirred solution of 0.07 M $CaCl_2$ containing 0.01 M glutaraldehyde at 10°C. Beads (3 mm dia) were allowed to harden in the $CaCl_2$- glutaraldehyde solution for 90 min at 10°C. The beads were leached and washed as described for the gelatin matrix.

Open-pore agar entrapped yeast. Open-pore gel beads with the entrapped yeast cells were obtained by selective leaching of calcium alginate from an agar - calcium alginate composite matrix. The procedure was essentially similar to that used for obtaining open-pore gelatin beads, except that the glutaraldehyde treatment was omitted.

A slurry of known wet weight of packed yeast cells in 100 ml of an aqueous solution contining 2% agar and 2% sodium alginate at 45°C was dropped into a stirred solution of 2% calcium chloride and the beads removed immediately after the end of the addition. The diameters of the beads were about 3 mm. The calcium alginate in the composite beads was leached out by washing with 50 mM potassium phosphate buffer at pH 7.5 until the washing was clear. The resulting porous agar beads were left overnight in a known amount of fermentable sugar in cane molasses pH 4.5 before packing into a column for continuous fermentation.

Open-pore SeaPlaque agarose beads. SeaPlaque agarose has the unique property of gelling at a low temperature. A dilute solution of the material (1 - 2%) sets to a reasonably firm gel in 10 min at 25°C and once gelled does not remelt unless the temperature exceeds 65°C.

Entrapment of yeast cells in SeaPlaque agarose was carried out according to the procedure described for agar. A known amount of wet packed cells were mixed thoroughly with 2% (w/v) aqueous solution of SeaPlaque agarose and 2% (w/v) sodium alginate. The resulting suspension was maintained around 40°C and dropped into 2% calcium chloride solution at 20°C. The beads formed were then treated with 50 mM phosphate buffer, pH 7.5 to leach out the calcium alginate component of the beads.

ι-Carrageenan beads. A solution 3.5% (w/v) ι-carrageenan and 2% w/v sodium alginate was prepared by heating in a boiling water bath for about 3 min. After cooling to 40°C, 20% w/v packed yeast cells were added to the solution of ι-carrageenan and sodium alginate and the slurry was dropped into a solution containing 0.3 M KCl and 1% (w/v) $CaCl_2$. The beads thus formed were treated with 50 mM phosphate buffer, pH 7.5.

Calcium alginate beads. The conventional calcium alginate bead preparation was made essentially as described

by Kierstan and Bucke (1977). A 4% w/v sodium alginate solution was made in hot water, the solution was cooled to room temperature and packed yeast cells (20% by wet weight) were added and the slurry added dropwise to a stirred solution of 0.07 M $CaCl_2$. Beads were left to harden for 60 min and washed with molasses solution.

Yeast cell growth in beads. For obtaining the open-pore agar bead system with entrapped inoculum, 20 mL of Saccharomyces Y-10 cell suspension in the growth medium were mixed with 80 mI of a solution containing 2% agar + 2 g sodium alginate at 45°C. The growth medium contained in 1 litre: glucose, 50 g; yeast extract, 3 g; malt extract, 3 g; and peptone, 5 g. Beads were obtained as described above and the calcium alginate leached out with potassium phosphate buffer.

Calcium alginate beads with the yeast inoculum were prepared from a suspension obtained by adding 20 mI of the inoculum to 80 mL of an aqueous solution containing 4 g of sodium alginate at 30°C. The suspension was dropped into a stirred solution of 2% calcium chloride and the beads left 60 mins in the calcium chloride solution.

Beads in both cases were about 3 mm in diameter, and the yeast cell density was approximately 1×10^7 cells/g bead.

Scanning electron micrographs. The immobilized yeast was fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, then dried with increasing concentrations of acetone and finally in a stream of air. The sections were prepared, coated with gold and observed in a Cambridge Stereoscan Model 150 Electron Microscope.

Packed-bed immobilized cell bioreactors. The columns used in the continuous studies for the production of ethanol from cane molasses were made of glass with a jacket around for the circulation of water. These were prepared according to Krouwel et al. (1980). The dimensions of a typical reactor of 230 ml working volume were top 4.5 cm I.D.; bottom 3.5 I.D. and height 20 cm.

Volumetric productivities. The productivities were calculated both on total bioreactor volume basis as well as on void volume basis. The latter was calculated according to Margaritis et al. (1980) and the values are used only for comparisons with the available literature data expressed in these terms. The void volume (total reactor volume minus solid bead volume) was generally 30% of the reactor's total working volume.

Fermentation medium. The medium used in the continuous fermentation studies consisted of diluted cane molasses containing 0.1% (w/v) urea. The pH was 4.5 (adjusted with 1 N H_2SO_4 when required) which was the usual pH of the diluted molasses solutions.

RESULTS

Preliminary experiments had been carried out using inert supports such as wood chips, catalyst grade alumina and polyurethane sponge on which growing yeast cells were attached by adsorption. This was achieved by adding inocula and growing yeast cells *in situ* in packed bed reactors. Such reactors gave low productivities of about 4 $gl^{-1}h^{-1}$ after 10 to 16 days of continuous conversions at 90% utilization of feedstream of molasses containing 135 gl^{-1} fermentable sugars (Table III.1)

TABLE III.1 : ETHANOL PRODUCTIVITIES OF S. UVARUM CELLS IMMOBILIZED ON DIFFERENT INERT MATRICES BY ADSORPTION

Feed stream, molasses with 135 gl^{-1} fermentable sugars, 30°C, pH 4.5

Inert support	Period (days)	Ethanol productivity ($gl^{-1}h^{-1}$)
Wood chips	3	6.6
	16	3.7
	26	3.1
Alumina beads	3	6.6
	16	3.7
Polyurethane sponge	3	5.7
	9	4.4

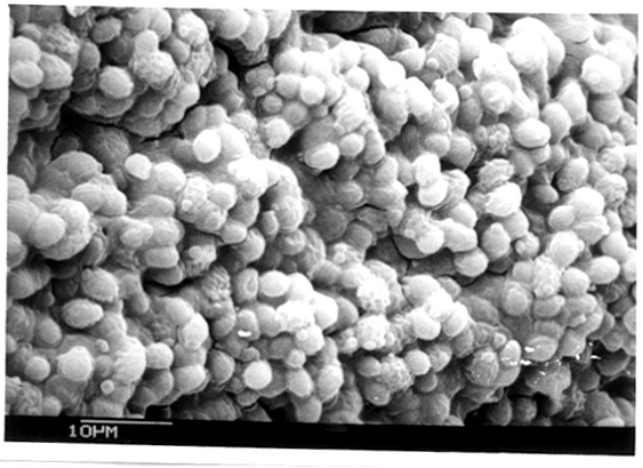
Studies on such adsorbed systems were therefore discontinued and yeast cells entrapped in gels were investigated.

Open-pore crosslinked protein gel matrices. The entrapment procedures described under Materials and Methods were used for obtaining the open-pore protein matrices.

Gelatin. The leaching effect of phosphate on the composite calcium alginate - gelatin beads is shown in scanning electron micrographs (SEM) (Fig. III.1). The open-pore structure of the gelatin matrix with the exposed yeast cells after leaching with the phosphate buffer and crosslinking with glutaraldehyde is shown in Figure III.1B compared to the covered appearance of the cells in the unleached composite matrix (Fig. III.1A). Estimation of calcium in beads indicated that approximately 40% was leached out on treatment with phosphate buffer.

Batch fermentation with yeast cells immobilized in open-pore gelatin matrix. Preliminary experiments were carried out in batch mode using S. uvarum ATCC 26602 and the S. cerevisiae Y-10 isolate immobilized in the open-pore 20% gelatin matrix. For the sake of comparison, equivalent amounts of free and immobilized cells at a concentration of 1.25% dry weight equivalent were used under identical conditions. The progress

(A)



(B)

FIG. III.1 ; SEMS of gelatin immobilized cells of S. cerevisiae NCIM 3107

(A) Cells entrapped in Ca-alginate gelatin composite beads

(B) Entrapped cells in matrix after leaching out of alginate and crosslinking with glutaraldehyde

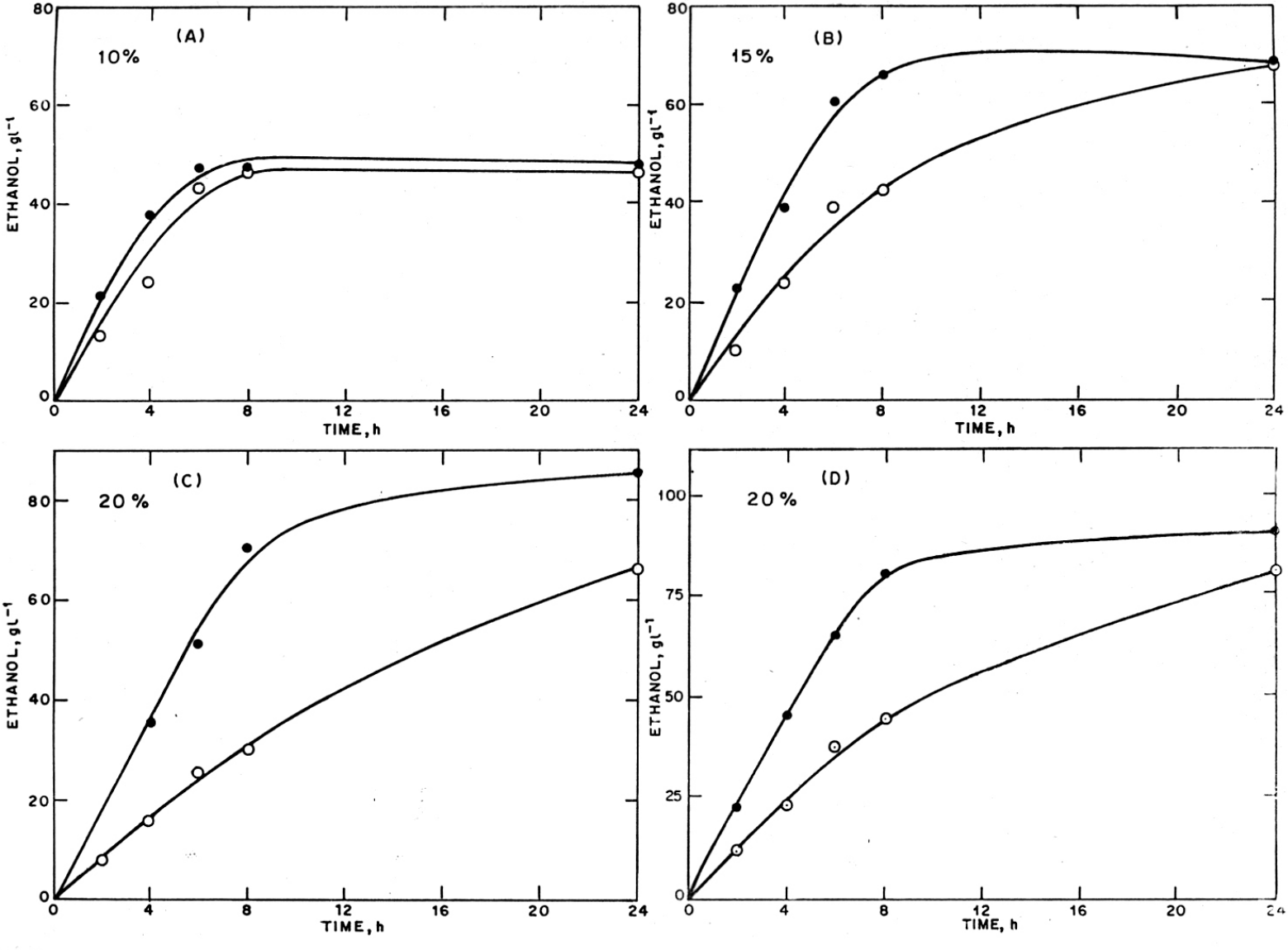

(A)
10%
ETHANOL, gl⁻¹
TIME, h
(B)
15%
ETHANOL, gl⁻¹
TIME, h
(C)
20%
ETHANOL, gl⁻¹
TIME, h
(D)
20%
ETHANOL, gl⁻¹
TIME, h

FIG. III.2 : Batch fermentation of molasses with suspended yeast cells (—⊙—) and open-pore gelatin immobilized cells (—•—).

(A) S. uvarum ATCC 26602 cells and molasses ($100\ gl^{-1}$ total sugars)

(B) S. uvarum cells and molasses ($150\ gl^{-1}$ total sugars)

(C) S. uvarum cells and molasses ($200\ gl^{-1}$ total sugars)

(D) S. cerevisiae Y-10 and molasses ($200\ gl^{-1}$ total sugars)

curves of fermentation are shown in Fig. III.2. The specific ethanol productivities in the initial phase of fermentation of molasses containing 100 to 200 gl^{-1} total sugars, before product inhibition is apparent, were calculated for S. uvarum (Fig. III.2A to C) and for S. cerevisiae Y-10 (Fig. III.2D) at the highest concentration used of 200 gl^{-1} glucose equivalent. The results are summarized in Table III.2.

TABLE III.2 : SPECIFIC ETHANOL PRODUCTIVITIES OF FREE AND IMMOBILIZED CELLS IN BATCH FERMENTATION OF MOLASSES

Open-pore gelating beads with entrapped cells and free suspended cells in molasses, pH 4.5, 30°C

Cane molasses Total sugars	Specific ethanol productivity			
	S. uvarum ATCC 26602		S. cerevisiae Y-10	
	Free cells	Immobilized cells	Free cells	Immobilized cells
gl^{-1}	$gg^{-1}h^{-1}$			
100	0.6	0.74		
150	0.5	0.8		
200	0.36	0.7	0.5	0.90

In all the cases the immobilized cells gave higher specific ethanol productivities than free cell suspensions under similar conditions. Such enhancement in fermentation rates has been reported by earlier workers

(Holcberg and Margalith, 1981). The immobilized S. cerevisiae Y-10 showed higher specific ethanol productivity compared to the immobilized S. uvarum at the highest sugar concentration tried (200 gl^{-1}).

Continuous fermentation of molasses using open pore gelatin immobilized S. uvarum cells. Fig. III.3 shows the relationship of ethanol productivity as a function of flow rates through the packed bed reactor column. The productivity with immobilized S. uvarum reaches a maximum of 76 $gl^{-1}h^{-1}$ on reactor volume basis at 35°C with 100 gl^{-1} total fermentable sugar concentration of the cane molasses substrate. This value was obtained at a dilution rate of 2.1 h^{-1} on reactor volume basis (7.1 h^{-1} on void volume basis) and 70% utilization of the fermentable sugars.

The maximum productivity obtained at 30°C with the immobilized S. uvarum was 67 $gl^{-1}h^{-1}$ with 100 gl^{-1} fermentable sugar concentration of the cane molasses. The bioreactor run continuously at 35°C for 30 days showed no decrease in the ethanol productivity and the gel beads showed no disruption. Cell loss through leaching during runs is apparently not significant. Entrapped cells examined microscopically showed evidence of budding. The presence of free cells in the effluent is explained on the basis of cell growth during the runs. Table III.3 summarizes the ethanol productivities

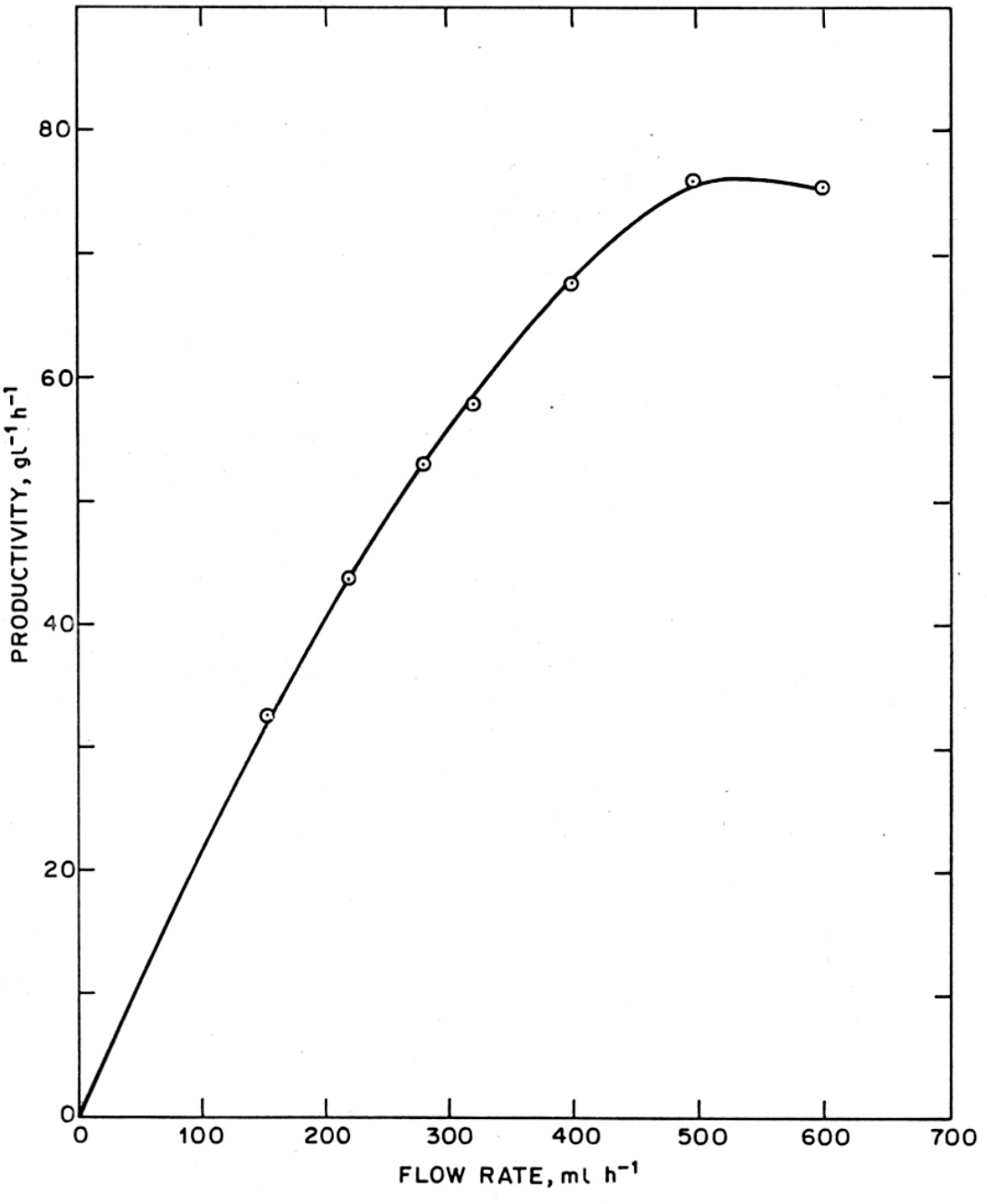

80
60
40
20
0
PRODUCTIVITY, gl⁻¹ h⁻¹
0
100
200
300
400
500
600
700
FLOW RATE, ml h⁻¹

FIG. III.3 : Effect of flow rate on ethanol productivity of reactor with open-pore gelatin immobilized *S. uvarum* cells using molasses containing 100 gl^{-1} fermentable sugars
Details as in text.

at various percentages of sugar utilization obtained with 100 gl^{-1} feed sugar concentration.

TABLE III.3 : ETHANOL PRODUCTIVITIES AT 35°C AND VARYING FLOW RATES OF MOLASSES CONTAINING 100 gl^{-1} FERMENTABLE SUGARS

Reactor working volume 230 ml; void volume 30% of reactor volume.

Flow rate	Utilization of feed sugar	Productivity	
		Reactor volume basis	Void volume basis
$ml^{-1}h^{-1}$	%	$gl^{-1}h^{-1}$	
150	100	32.6	107
220	95	44	144
320	85	58	190
500	70	76	250

The maximum ethanol productivity on void volume basis is 250 $gl^{-1}h^{-1}$ corresponding to 76 $gl^{-1}h^{-1}$ on reactor volume basis. Williams and Munnecke (1981) have reported a maximum ethanol productivity of 53 $gl^{-1}h^{-1}$ calculated on void volume basis using Ca-alginate immobilized S. cerevisiae systems at 30°C with 12.7% glucose as feed sugar and at 63% utilization.

Margaritis et al. (1981) have reported a maximum ethanol productivity of 102 $gl^{-1}h^{-1}$ for an inlet glucose concentration of 10% at 87% conversion using small calcium alginate beads of immobilized Zymomonas mobilis cells at 30°C. The markedly higher productivities obtained in the present investigation are apparently due to the open-pore structure of the gelatin matrix.

Effect of gelatin concentration. Gelatin concentrations of 10%, 15% and 20% were tried admixed with 2% sodium alginate and 20 % wet weight of S. uvarum cells. The relationship of ethanol productivity as a function of flow rate with reactor column packed with gelatin beads showed no differences with varying gelatin concentrations. The reactor performance over extended periods of use however showed that with 10% gelatin concentration, efficient conversion of 135 gl^{-1} molasses fermentable sugars was obtained only for a period of about 12 days, productivity dropping after this period of time. Gelatin at higher concentration of 15 and 20% however performed efficiently upto 35 to 40 days, with little change in volumetric productivities at about 95% conversion. The operational characteristics at 15% gelatin concentration are shown in Fig. III.4.

Effect of cell load. The effect of varying initial S. uvarum cell loading in the matrix on the conversion

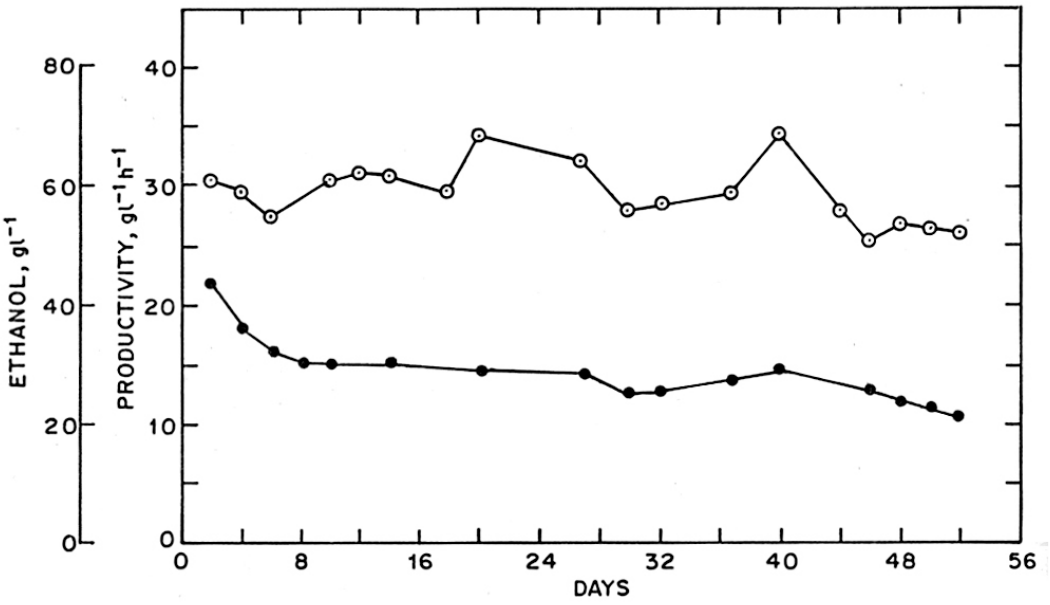

ETHANOL, gl⁻¹
PRODUCTIVITY, gl⁻¹h⁻¹
DAYS
0
20
40
60
80
0
10
20
30
40
0
8
16
24
32
40
48
56

FIG. III.4 : Continuous fermentation of molasses $135gl^{-1}$(fermentable sugars) in reactor with open-pore gelatin (15%) beads with entrapped S. uvarum cells. Ethanol concentration (—⊙—), Productivity (—●—).

of molasses sugars to ethanol was studied at 15,25 and 30 g wet packed cells in 100 ml of the gelatin + sodium alginate solution.

The beads after leaching out with potassium phosphate buffer (50 mM) and crosslinking with glutaraldehyde were washed with molasses solution and packed in reactors for continuous conversion. With varying cell loads, the period of time required for attainment of equilibrium increased with decreasing cell loads. The performances of the reactors after attainment of equilibrium,however, showed no differences with the different initial cell loading tried. The crosslinked gelatin beads could not be solubilized under mild conditions of treatment and only an approximate estimate of cell densities could be obtained after attainment of equilibrium by drying samples of beads (a) containing no yeast cells (b) immediately after yeast cell immobilization and (c) bead samples removed from reactors after the attainment of equilibrium. The estimation indicated that the cell weight within beads had approximately doubled in the case of beads initially loaded with 20% wet weight of S. uvarum cells.

Since S. cerevisiae isolates had shown comparatively higher productivities in batch fermentation than the S. uvarum at higher molasses concentration, these were investigated in continuous fermentations.

Continuous fermentations using open-pore gelatin immobilized S. cerevisiae isolates. With higher molasses sugar concentrations even at low dilution rates the S. uvarum immobilized in the various proteinous matrices did not give ethanol concentrations higher than about 70 gl^{-1}. S. cerevisiae Y-10 and Y-7, however, gave consistenly ethanol concentrations of about 75 - 80 gl^{-1} at 95% conversion efficiency under appropriate flow rate conditions.

Fig. III.5 shows the relationship of ethanol productivity as a function of flow rates using S. cerevisiae Y-10 cells immobilized in gelatin and molasses feed stream containing 162 gl^{-1} fermentable sugars at 30°C.

The maximum productivity obtained was 77 $gl^{-1}h^{-1}$ at a flow rate of 600 mlh^{-1} at 33% sugar utilization. Table III.4 summarizes the productivity data at various conversion efficiencies.

TABLE III.4 : ETHANOL PRODUCTIVITIES AT 30°C OF IMMOBILIZED S. CEREVISIAE Y-10 AND MOLASSES FEEDSTREAM CONTAINING 162.1 gl^{-1} FERMENTABLE SUGARS

Utilization of feed sugar	Productivity (reactor volume basis)
%	$gl^{-1}h^{-1}$
95	25
80	42
70	55
60	62
33	77

FLOW RATE, min-1
0
200
400
600
PRODUCTIVITY, gl-1h-1
0
20
40
60
80

FIG. III.5 : Effect of flow rate on ethanol productivity of reactor with open-pore gelatin entrapped S. cerevisiae Y-10 cells using molasses containing 162 gl^{-1} fermentable sugars

The productivity at 95% sugar utilization is 25 $gl^{-1}h^{-1}$ for the immobilized S. cerevisiae Y-10 with a fermentable sugar concentration of 162 gl^{-1} compared to a value of about 15 $gl^{-1}h^{-1}$ for the S. uvarum even at a lower feed stream fermentable sugar concentration of 135 gl^{-1} (Fig. III-4).

The bioreactor with S. cerevisiae Y-10 cells immobilized in gelatin and run continuously for 40 days with a feedstream containing 162 gl^{-1} fermentable sugars gave uniformly high productivities ranging from 26 to 22 $gl^{-1}h^{-1}$. The results are shown in Fig. III.6.

Table III.5 summarizes the ethanol productivities at various percentages of sugar utilization obtained with S. cerevisiae Y-7 at 171 gl^{-1} fermentable sugar concentration at 30°C. The productivity of 23 $gl^{-1}h^{-1}$ at 95% sugar utilization was about the same as that given by S. cerevisiae Y-10 cells under similar conditions.

TABLE III.5 : ETHANOL PRODUCTIVITIES OF IMMOBILIZED S. CEREVISIAE Y-7 AND MOLASSES FEEDSTREAM CONTAINING 171 gl^{-1} FERMENTABLE SUGARS TEMPERATURE 30°C
Reactor volume 220 ml

Utilization of feed sugar	Productivity
%	$gl^{-1}h^{-1}$
95	23
90	30
80	43.5
70	56.5
64	74

The bioreactor with S. cerevisiae Y-7 cells immobilized in open-pore gelatin when run continuously with a feed-stream containing 162 gl^{-1} molasses fermentable sugars at 95% sugar utilization gave high ethanol productivities of about 22 $gl^{-1}h^{-1}$ only for a limited period of 12 days after which the conversion efficiency and productivity values decreased significantly. This is in contrast with the uniformly stable performance of immobilized S. cerevisiae Y-10 (Fig. III.6).

Continuous fermentation with yeast cells immobilized in open-pore proteinous matrices. Table III.6 summarizes the volumetric ethanol productivity data at 95% substrate utilization of molasses sugars at 135 gl^{-1} concentration of fermentable sugars expressed in glucose equivalents and the S. uvarum cells immobilized in calcium alginate and in open pore gelatin beads. The values for 95% substrate utilization were calculated from plots of ethanol productivity versus dilution rate.

TABLE III.6 : CALCULATED VOLUMETRIC ETHANOL PRODUCTIVITIES FOR (95% UTILIZATION OF MOLASSES SUGARS (135 gl^{-1} FERMENTABLE SUGARS) AT 30°C, pH 4.5. S. UVARUM ENTRAPPED IN BEADS 3 mm dia

Matrix	Productivity
	$gl^{-1}h^{-1}$
Gelatin (20% w/v)	20
Alginate (4%)	16.5

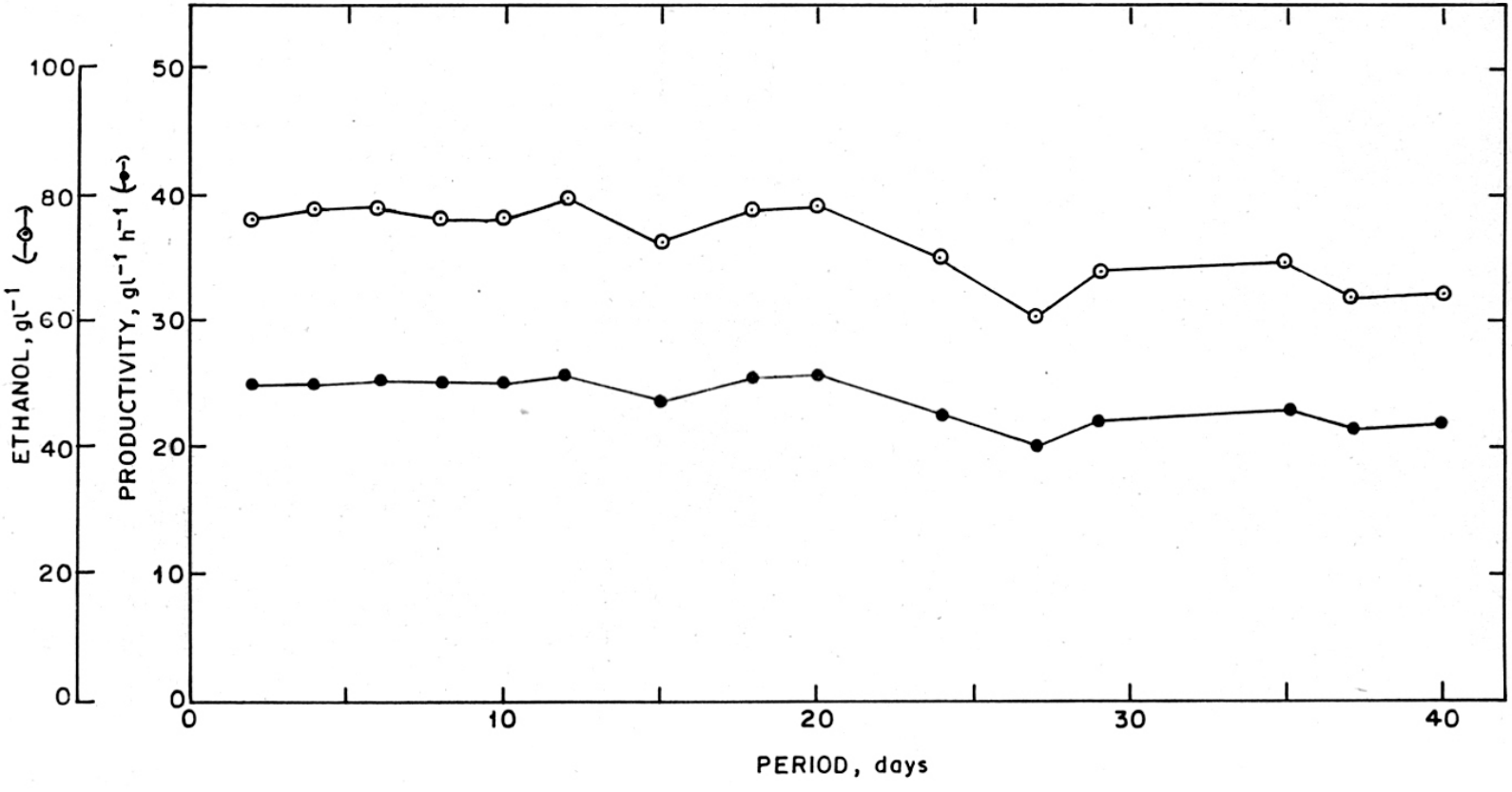

ETHANOL, gl⁻¹ (⊙)
PRODUCTIVITY, gl⁻¹ h⁻¹ (●)
100
80
60
40
20
0
50
40
30
20
10
0
0
10
20
30
40
PERIOD, days

FIG. III.6 : Operational stability of packed-bed bioreactor with open-pore gelatin entrapped S. cerevisiae Y-10 cells. Molasses concentration 162 gl^{-1} fermentable sugars; product concentration 75 gl^{-1} ethanol.

Calcium alginate gels without the gelatin component when used for entrapping S. uvarum cells have a productivity value of 16.5 $gl^{-1}h^{-1}$ at the same substrate concentration and at the same conversion efficiency. This confirms the better performances of an open-pore gelatin matrix compared to the calcium alginate system. The gelatin + alginate composite matrices gave ethanol productivities value of about 15 $gl^{-1}h^{-1}$.

Table III.7 summarizes ethanol productivity data for S. uvarum Y-10 cells immobilized in open-pore gelatin and open-pore whole hen's egg matrix and molasses feedstream containing 162 gl^{-1} fermentable sugars.

TABLE III.7 : CALCULATED VOLUMETRIC ETHANOL PRODUCTIVITIES FOR 95% UTILIZATION OF MOLASSES SUGARS (162 gl^{-1} FERMENTABLE SUGARS AT 30°C, pH 4.5 S. CEREVISAE Y-10) ENTRAPPED IN BEAD 3 mm DIA

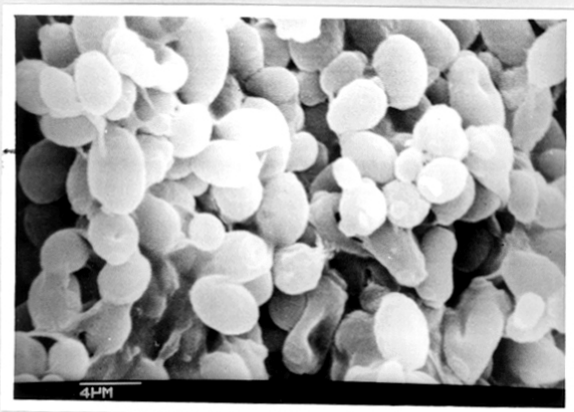
Matrix	Productivity
	$gl^{-1}h^{-1}$
Gelatin	24
Whole hen's egg	21

Open-pore gelatin entrapped S. cerevisiae Y-10 cells when used in the packed bed reactors for the continuous conversion of cane molasses to ethanol over extended periods of 2 to 3 months showed a decrease in the ethanol productivity after about 40 days and progressive loss of bead material through erosion from the interior of beads. The degradation was apparently caused by the utilization of the matrix material by the yeast cells

Open-pore polysaccharide matrices

Agar. An obvious alternative carrier that is resistant to microbial degradation in general is agar which finds extensive use as a solid support for the growth of cell cultures. Applications of agar for whole cell immmobilization have been described in the literature and these include the entrapment of Escherichia coli for its β-glucosidase activity (Toda, 1975; Bannerjee et al., 1982), yeast for its invertase activity (Toda and Shoda, 1975), Rhodospirillum rubrum for its hydrogenase activity (Weetall and Bennett, 1976) and plant cells for their secondary metabolites (Brodelius and Nilsson, 1980). A general disadvantage with agar as a carrier is that of diffusional limitations (Toda and Shoda, 1975). A specific problem in its use in reactions involving cell growth and gas production is its brittle nature and consequent rupture of the

(A)



(B)

FIG. III.7 : Scanning electron micrographs of pregrown y-10 yeast cells entrapped in

(A) Porous agar bead, and

(B) Calcium alginate-agar composite bead

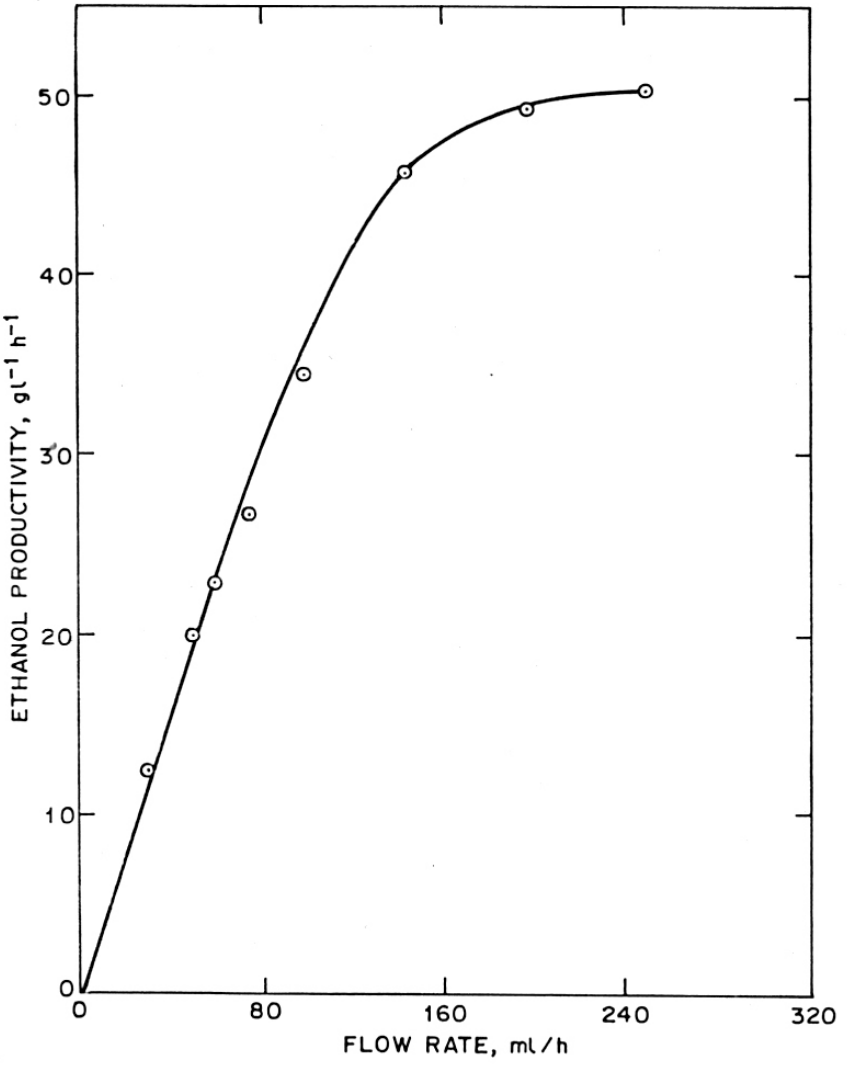
FIG. III.8 : Scanning electron micrographs showing growth of Saccharomyces Y-10 cells in
(A) Porous agar bead and
(B) Calcium alginate bead

carrier gel during such fermentations unless strengthened by incorporation of polymers like polyacrylamide (Kuu and Polack, 1983). These disadvantages however could be overcome by rendering the agar support porous through selective leaching of calcium alginate from a composite matrix containing calcium alginate and agar.

Scanning electron micrographs of gel entrapped cells. The effect of leaching the calcium alginate agar beads with potassium phosphate buffer, pH 7.5 can be seen from Fig. III.7 . The open-pore structure of the leached bead in which the entrapped cells are fully exposed (Fig. III.7A) is clearly distinct from the covered cells observed in the alginate composite matrix.

Fig. III.8 shows differences in cell growth from inoculum entrapped within the open-pore agar bead (Fig. III.8A)and plain calcium alginate bead system (Fig. III.8B). The cell density within the open-pore agar matrix is uniform and abundant throughout the bead, while the growth in the calcium alginate bead is markedly lower. The more porous nature of the former system could account for the enhanced availability within the beads of nutrients required for cell growth.

Continuous fermentation of molasses with open-pore agar entrapped yeast cells. Preliminary experiments carried out, using the composite agar-calcium alginate beads before leaching the calcium alginate with phosphate treatment, showed that beads rupture during fermentation. This however was not observed with the porous agar


ETHANOL PRODUCTIVITY, $gl^{-1}\,h^{-1}$
0
10
20
30
40
50
0
80
160
240
320
FLOW RATE, ml/h

beads obtained from leaching out of the calcium alginate component.

Fig. III.9 shows the relationship of ethanol productivities as a function of flow rates through a reactor column packed with open pore agar beads with entrapped, pregrown S. cerevisiae Y-10 cells. The ethanol productivity at various percentages of substrate utilization are summarized in Table III.8.

TABLE III.8 : ETHANOL PRODUCTIVITIES AT 30°C AND VARYING FLOW RATES OF MOLASSES CONTAINING 162 gl^{-1} FERMENTABLE SUGARS

Flow rate	Utilization of feed sugar	Productivity
ml^{-1}	%	$gl^{-1}h^{-1}$
50	100	20
60	95	23
73	85	26
145	80	46

The productivity of ethanol at 95% conversion efficiency and about 77 gl^{-1} product concentration is 23 $gl^{-1}h^{-1}$ which is comparable to that reported for calcium alginate gel beads immobilized system operated in fluidized bed reactors for continuous fermentation of cane molasses to ethanol (Nagashima et al. 1984). Values obtained

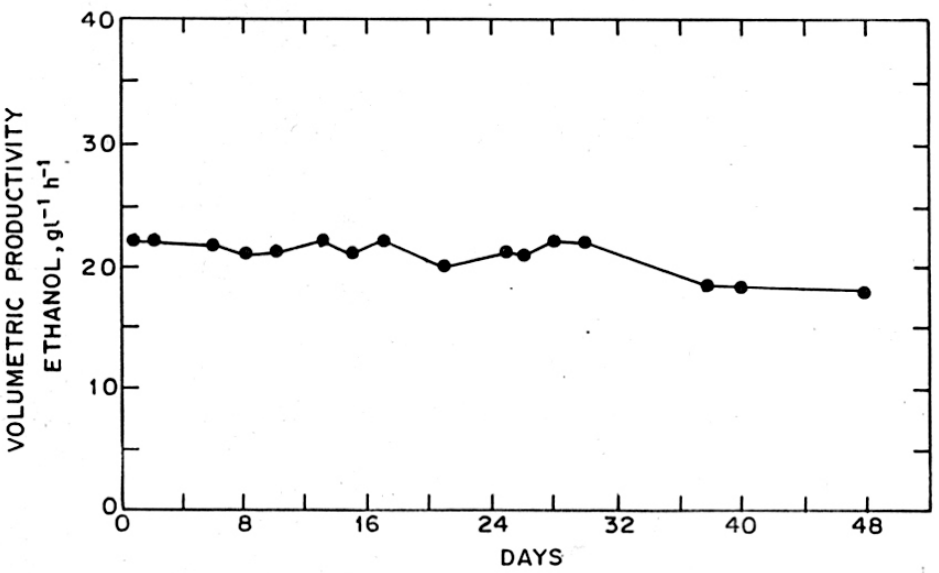

VOLUMETRIC PRODUCTIVITY
ETHANOL, $gl^{-1} h^{-1}$
40
30
20
10
0
0
8
16
24
32
40
48
DAYS

FIG. III.9 : Operation stability of packed-bed bio-reactor in the continuous fermentation of molasses to ethanol, using S. cerevisiae Y-10 immobilized in open-pore agar beads. Molasses feedstream, containing 160 gl^{-1} fermentable sugars. Initial product concentration 77 gl^{-1} ethanol.

by us, however, with the calcium alginate system and the sample of cane molasses used in our study was only about 15 $gl^{-1}h^{-1}$ under the conditions where the open-pore agar system gave a value of 23 $gl^{-1}h^{-1}$. Besides the yeast strains differences, the nature of the molasses used by Nagashima et al. (1984) is not specified. High test and other low salt containing molasses would give higher productivity than high ash containing molasses such as those available indigenously.

The operational stability of the packed bed reactor with open-pore beads used continuously for the fermentation of cane molasses to ethanol is shown in Fig. III.10. No decrease in ethanol productivity was observed upto a month of operation, after which the conversion efficiency dropped and the ethanol concentration in the effluent became limiting at about 58 gl^{-1} with consequent decrease in the volumetric ethanol productivity to about 17 - 18 gl^{-1}. No apparent change in bead structure could be observed even after periods of about 2 months of continuous use.

In addition to open-pore agar matrix, open-pore SeaPlaque agarose, which is commercially available and has unique property of gelling at low temperature was also used in place of standard agar. κ-Carrageenan bead with entrapped yeast cells were also investigated as a more permanent matrix in place of cross-linked proteinous gels.

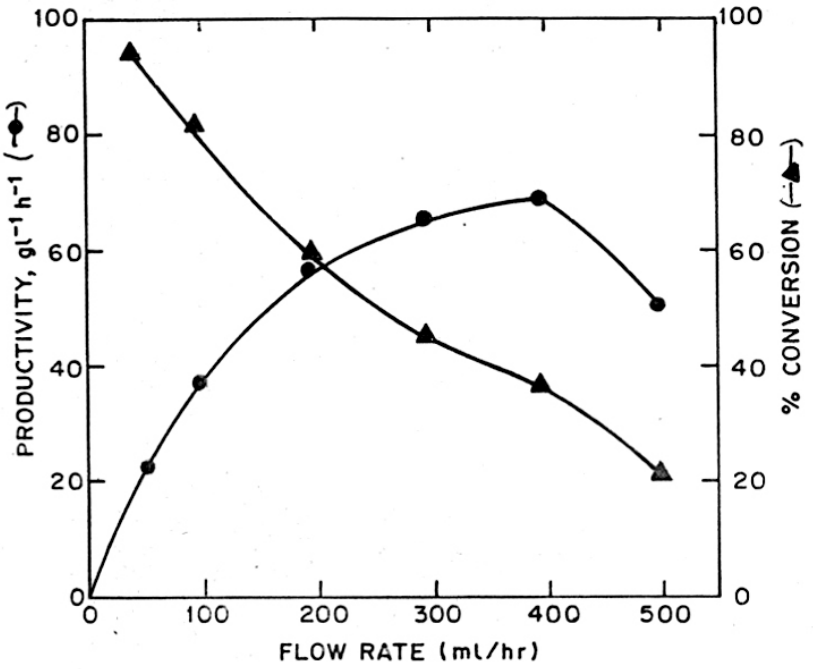

PRODUCTIVITY, gl⁻¹ h⁻¹ (●—)
% CONVERSION (▲—)
FLOW RATE (ml/hr)
0
20
40
60
80
100
0
100
200
300
400
500

FIG. III.1[illegible] : Effect of flow rate on ethanol productivity of S. uvarum cells immobilized in open-pore SeaPlaque agarose beads using cane molasses 135 gl^{-1} fermentable sugars.

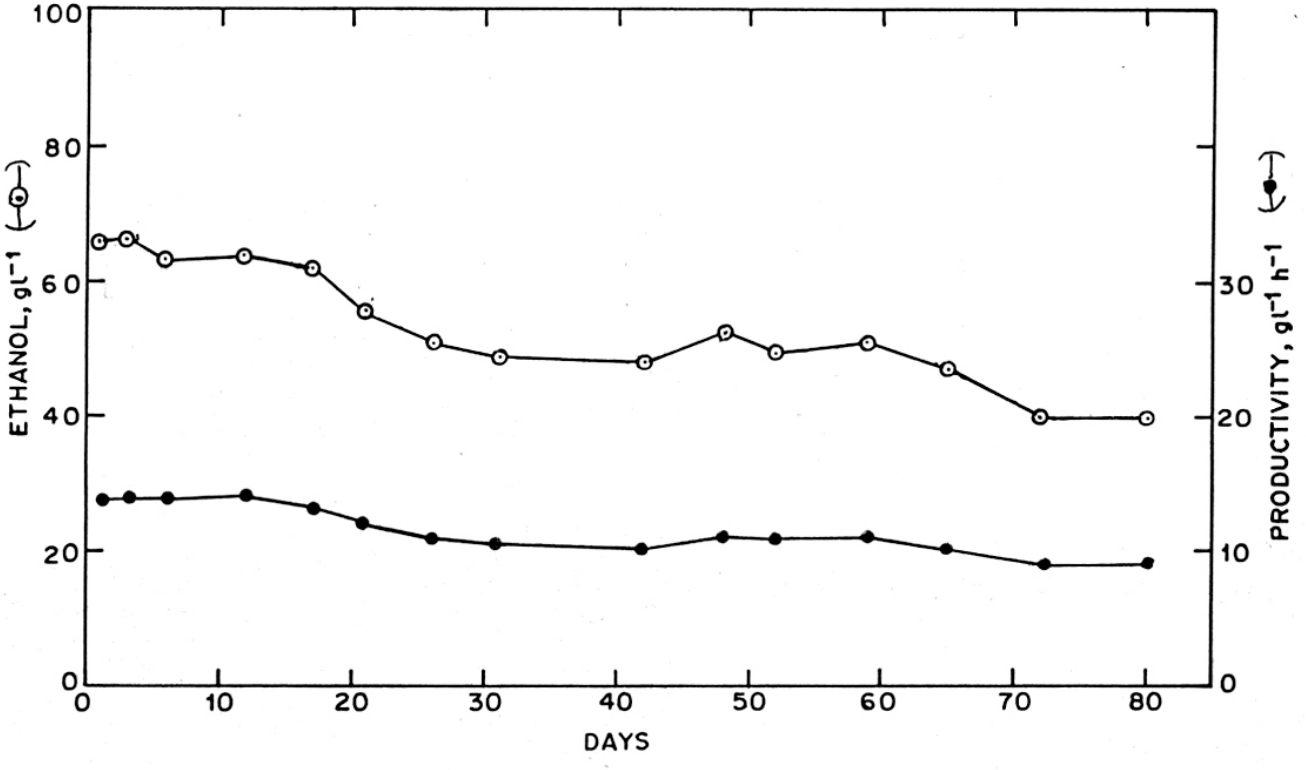

100
80
60
40
20
0
ETHANOL, gl⁻¹
30
20
10
0
PRODUCTIVITY, gl⁻¹ h⁻¹
0
10
20
30
40
50
60
70
80
DAYS

FIG. III.12 : Operational stability of packed-bed bioreactor in the continuous fermentation of cane molasses to ethanol, using S. uvarum immobilized open-pore SeaPlaque agarose beads. Cane-molasses feedstream, containing 135 gl^{-1} fermentable sugars. Initial product concentration was 64 gl^{-1} ethanol.

Open-pore SeaPlaque agarose. Open-pore SeaPlaque agarose system was carried out with S. uvarum ATCC 26602 in place of S. cerevisiae Y-10. Since S. uvarum has lower specific ethanol productivity at high substrate concentration the continuous fermentation studies were carried out with only 135 gl^{-1} molasses sugar expressed as glucose equivalents. Profiles of ethanol productivity with respect of flow rates are shown in Fig. III.11.

A productivity of 23 $gl^{-1}h^{-1}$ was obtained at a flow rate 50 $ml.h^{-1}$ at 95% sugar utilization. However, the maximum productivity of 68 $gl^{-1}h^{-1}$ was obtained at a flow rate of 400 $ml.h^{-1}$. Lowering of the productivity values at high flow rates is attributable to cell washout from bead surface under these conditions.

Fig. III.12 shows the operational stability of S. uvarum cells immobilized in SeaPlaque agarose matrix. The bioreactor was run continuously at a fixed flow rate of 30 $ml.h^{-1}$ using cane molasses as feed containing 135 gl^{-1} fermentable sugars at 95% utilization. The reactor retained 60% of its initial activity upto 80 days.

κ -Carrageenan. Scanning electron micrographs of yeast cells entrapped in κ -Carrageenan (3.5 w/v) plus alginate (2% w/v) and the composite matrix treated with potassium phosphate buffer are shown in Fig. III.13. The micrographs show no differences between the composite

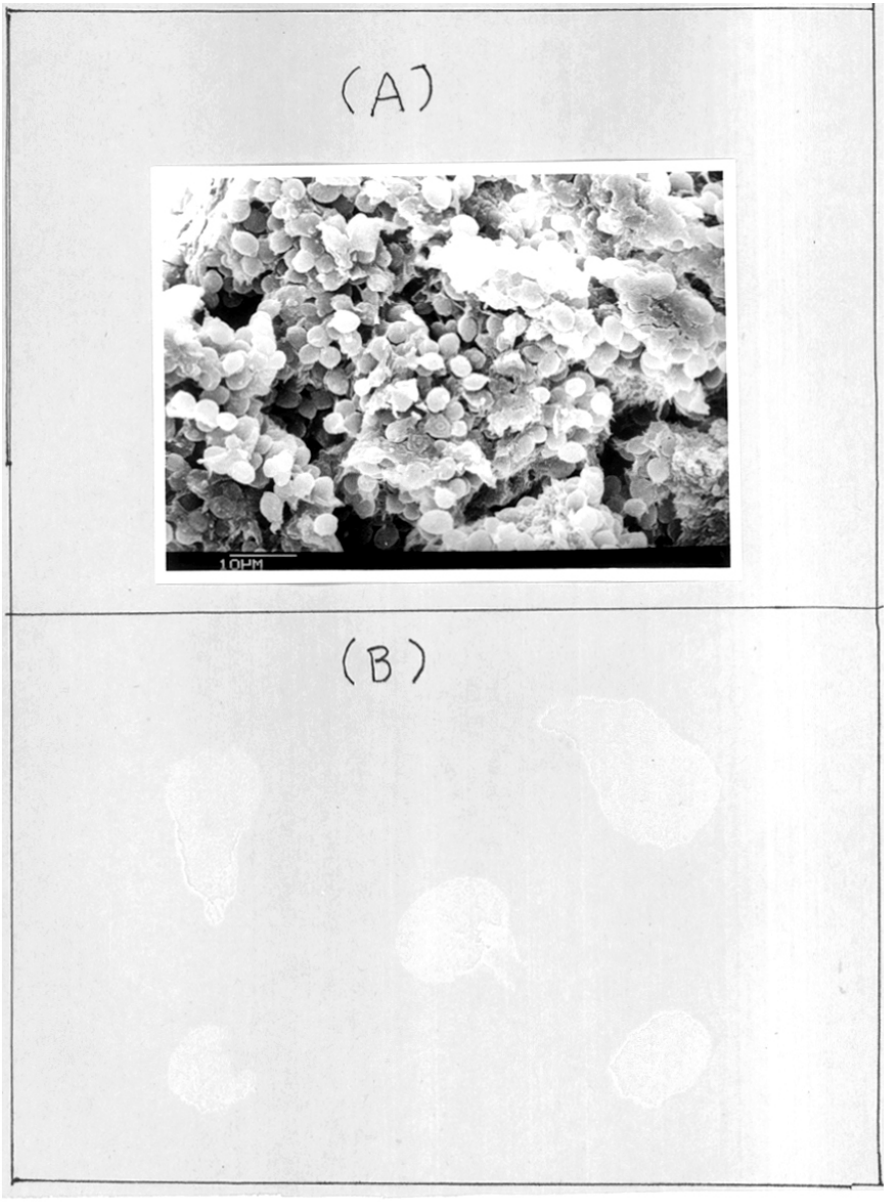


FIG. III.13 : Scanning electron micrographs of immobilized cells of S. uvarum

(A) Cells entrapped in Ca-κ -carrageenan -alginate bead treated with K-phosphate

(B) Cells in Ca-κ -carrageenan-alginate

and the phosphate treated systems.

κ -Carrageenan which contains β-D-galactose sulfate as a unit structure is known to gel by contact with aqueous solutions containing cations such as K^+ and Ca^{2+} (Tosa et al., 1979). Treatment with κ-phosphate buffer is unlikely to remove calcium from the Ca-κ -carrageenan. The lack of an apparent porous structure of the phosphate treated beads is reflected in the ethanol productivities of the system in continuous conversion of molasses to ethanol. The ethanol productivity at 95% utilization of molasses sugars containing 135 gl^{-1} fermentable sugars was only 14.0 $gl^{-1}h^{-1}$ with S. uvarum cells entrapped in the phosphate treated ι -carrageenan beads. Open-pore gelatin beads in contrast gave a corresponding productivity value of 20 $gl^{-1}h^{-1}$ (Table III.6) under similar conditions. The results will indicate that κ-phosphate buffer treatment of composite matrices containing Ca^{2+} κ-carrageenan does not leach out the alginate component unlike composite systems of alginate with gelatin or agar.

DISCUSSION

The obvious advantages of using immobilized whole cells for continuous bioconversions such as fermentation of sugars to ethanol are the ease of carrying out sequential reactions some of which require cofactors and the high productivities achieved by high cell loading without attendant problems of cell washout. A less apparent advantage is of modifications in metabolism of entrapped cells as compared to those of free cells in suspension. Some of these alterations reported in the literature have been reviewed in the **General Introduction.** In the present studies we have observed two significant effects of immobilization. The specific ethanol productivity of the cells immobilized in open-pore gelatin matrix have been found to be higher than that obtained with free cell suspensions in batch fermentation. Such enhancement in fermentation rates have also been reported by Holcberg and Margalith (1981). Our data also indicate that the osmotolerance of yeast is also enhanced when immobilized. The specific ethanol productivities of free cells suspension of strains such as S. uvarum ATCC 26602 which are markedly lowered in molasses at relatively high dry solids content do not show this lowering when the cells are immobilized and used in batch mode. The reasons for this effect are not obvious.

An advantage of a higher degree of porosity in gels used for entrapment of microbial whole cells is the obvious one of lower diffusional limitations. Hence enhanced bioconversion rates can be expected when transport of substrate across the cell membrane is not the limiting factor. The disadvantage in rendering gels porous, however, is the one of lowered gel strength. This would restrict the use of such systems to plug flow rather than fluidized bed reactor mode.

The higher efficiency of yeast cells entrapped in open-pore gels of gelatin and agar described in the present work have been established from higher productivities shown by the open-pore gel systems compared to the usual calcium alginate gel matrix. A further advantage of rendering gels such as agar and agarose more porous is their applicability to systems where cell growth and gas evolution are known to cause disruption and breakage when intact gels are used. The porous nature of these proteinous and polysaccharide gels obtained by the differential leaching of one component from a composite matrix is also apparent from the comparative scanning electron micrographs. The growth of inocula within such porous gels has also been shown to be comparatively more than in conventional unleached systems. The advantage of lowered resistance to diffusion in the open-pore gelatin system described

by us has been confirmed by Bajpai and Margaritis (1985). Kluyveromyces marxianus cells with inulinase activity when immobilized in open-pore gelatin pellets prepared as described by us have been shown by these authors to retain greater than 90% of the original activity when used for hydrolysis of inulin of Jerusalem artichoke compared with 67% for calcium-alginate entrapped cells and 76% with agar-entrapped cells. In this case the macromolecular substrate apparently permeates more readily through the porous matrix.

Crosslinked proteinous supports such as gelatin have been found by us to erode from the interior on prolonged use with entrapped yeast cells, obviously due to the slow degradation of support material through proteolytic activity of the entrapped cells. This difficulty could be overcome through the use of agar which is not susceptible to breakdown through the action of the enzyme released or present in yeast cells. The use of open-pore agar and agarose matrices over prolonged periods of 2 to 3 months have been shown by us to have little effect on the structural integrity of the matrix. However, the volumetric ethanol productivities of reactors with yeast cells immobilized in open-pore agar were consistently high only over a limited period of about 5 to 6 weeks of continuous conversion.

Whether this is caused by an intrinsic limitation of the yeast strains to continuously function within the gel matrix or is a problem caused by inhibitory components in the molasses samples has been investigated and the results are presented in Part IV of the thesis.

PART IV

EFFECT OF FEEDSTOCK CONSTITUENTS ON CONTINUOUS ETHANOLIC FERMENTATION IN IMMOBILIZED YEAST CELL REACTORS

SUMMARY

Continuous fermentation of sucrose and deionized cane molasses could be effectively carried out over extended periods at uniformly high productivities and conversion efficiencies using immobilized yeast cell systems. No detectable lowering in efficiency was observed with the former feedstock over periods upto 4 months of reactor operation. Such feedstocks, unlike cane molasses, however, require supplementation with yeast extract and essential inorganic salts in addition to a nitrogen source.

Supplementation of sucrose feedstream with KCl, NaCl and $CaCl_2$ to simulate the contents of the metal ions in clarified molasses did not lower reactor efficiencies, indicating that these are not causative factors for the shorter period of efficient reactor performance in continuous conversion of cane molasses.

The continuous conversion of cane molasses by immobilized yeast cell reactors was monitored for yeast cell counts in the effluent stream. The studies show that the free cell count decreases at the time of lowered reactor performance. The correlation of these findings and of the reported lowering of yeast cell density within the matrix during lowered reactor performance is discussed.

INTRODUCTION

In the two earlier sections of the thesis, the isolation of fermentative yeast strains tolerant to high-gravity cane molasses and to ethanol; and their application to continuous ethanolic fermentation in plug-flow reactors packed with the cells immobilized in open-pore proteinous and polysaccharide matrices have been described. While such immobilized systems gave high ethanol productivities initially at about 95% conversion efficiency, their operational stability was restricted to a period of about 4 to 6 weeks.

In this part of the thesis, data are presented which indicate that the operational stability of reactors is maintained over extended periods, when clean feedstocks such as sucrose and deionised cane molasses are used in place of clarified cane molasses. The possible causes for early reactor failure observed with cane molasses have also been examined.

In these studies, standard calcium alginate beads were used for immobilization, since this permits easy bead disruption for evaluating cell densities within the matrix. As pointed out earlier, crosslinked open-pore gelatin beads could not be disrupted under mild conditions, while agar and agarose beads required high temperatures at which cell death is unavoidable. Disruption of crosslinked gelatin beads with proteolytic enzymes was

ineffective, while the enzymatic degradation of agar beads was not attempted. Standard S. uvarum ATCC 26602 was used in these studies as the primary objectives were to find out whether immobilization affects cell viability adversely or if molasses feedstream constituents are toxic to cell growth and/or ethanolic fermentation.

MATERIALS AND METHODS

Materials

Sodium alginate was either from Loba, Bombay or was high (70%) guluronic acid-containing Protanal type LF120 from Protan AS, Norway. Sucrose was obtained from the local market. Deionized cane molasses (liquid sugar) was a gift from Thermax Pvt. Ltd., Pune, and was completely deionized to low conductivity and supplied as 80% w/w syrup. Ergosterol was provitamin D, pharmaceutical grade, obtained from the local market. A stock solution of ergosterol was prepared by dissolving 0.22 g in 22 ml Tween-80 (polyoxyethylene sorbitan monooleate, Sigma) + 30ml ethanol (95%).

Methods

Analysis of inorganic constituents of cane molasses. Ashing was done at 500°C after initial drying. K^+, Na^+ and Ca^{2+} were estimated by flame photometry and the analysis was done by the Inorganic Analysis Section of the Laboratory.

Immobilization of yeast cells in calcium alginate. Immobilization of yeast cells in calcium alginate beads was done as described in Part III of the thesis. 3% Protanal LF120 sodium alginate gave beads which performed similar to 4% Loba product in packed bed reactors.

When ergosterol supplementation was made, this was done during entrapment by incorporating 0.4 ml of its

stock solution in 100 ml Na alginate-yeast slurry.

Packed-bed immobilized cell bioreactors. Cylindrical water jacketed columns with feed inlet from the base were used. Two types of reactors were made use of, one for determining the operational stability of the calcium alginate immobilized yeast system with various feedstock and the other for monitoring yeast cell densities within the bead matrix. The former (reactor volume ~ 250 ml) had a height to diameter ratio of 2.5 to 1 and the later (reactor volume ~ 500 ml) of 5 to 1 in which the calcium alginate immobilized cells were distributed (~ 60 g each) in 5 separate stainless steel, wire-mesh baskets fitted snugly one above the other within the reactor column. The baskets could be removed after interruption of the feed to enable sampling of the beads in the various sections of the reactor.

Feed inlet was from the bottom of the reactor and not from the top as in the Krouwel et al. (1980) type reactors used in the work reported in the earlier sections

Feedstocks for continuous fermentation. Feedstreams of sucrose and liquid sugar solutions were used at about 140 gl^{-1} glucose equivalent and were supplemented with either yeast extract (0.5 to 3.0 gl^{-1}) or corn steep liquor (1 to 10 gl^{-1}). Sucrose and deionized molasses were also supplemented with KH_2PO_4 (500 mgl^{-1}) and

$MgSO_4.7H_2O$ (500 mgl^{-1}).

All feedstreams contained urea (1.0 gl^{-1}). $CaCl_2$ (200 mgl^{-1}) was also included to prevent disruption of the calcium alginate beads by phosphate.

Substrate and product analysis. Sugar and ethanol analyses were routinely carried out as described in **Part II** of the thesis.

Clarified cane molasses. Molasses sample was diluted to the required concentration of sugars and treated with H_2SO_4 to pH 2.0, steamed for 15 min and left at 4°C overnight. The clear supernatant was decanted and the pH readjusted to 4.5 with NaOH solution.

RESULTS

Inorganic constituents of cane molasses

The contents of the major inorganic constituents in the cane molasses sample is shown in Table IV.1.

TABLE IV. 1 : INORGANIC CONSTITUENTS OF CANE MOLASSES SAMPLE

Constituents	% w/w
Ash	15.5
Sodium (as Na_2O)	0.2
Potassium (as K_2O)	4.27
Calcium (as Ca)	0.67

Continuous fermentation of sucrose to ethanol

Preliminary experiments indicated that yeast extract supplementations at 1 to 3 gl^{-1} were adequate for continuous conversion over extended periods of 3 months. The yeast extract supplements could be effectively replaced by corn steep liquor at 5 gl^{-1}. Unlike complex media like cane molasses which require supplementation only with a nitrogen source (Hodge and Hildebrandt, 1954), media containing simple sugars require secondary nutrient supplements (Jones et al., 1981; Jones and Greenfield, 1984).

Reactors gave uniformly high productivities of 15 to 22 gl^{-1} over the test period of 3 months at about

65 gl^{-1} ethanol and about 95% conversion efficiency, the productivity value depending on the ratio of bead to reactor volume.

Fig. IV. 1 summarizes the performance of a packed-bed reactor with 60% by volume of beads containing entrapped S. uvarum ATCC 26602 cells. The feedstream of sucrose (140 gl^{-1} glucose equivalent + yeast extract, 3 gl^{-1}) after 50 days of continuous conversion was switched to the sucrose-yeast extract solution supplemented with KCl (8.8 gl^{-1}) + NaCl (0.5 gl^{-1}) + $CaCl_2.2H_2O$ (20.5 gl^{-1}). KCl and NaCl supplementations were made to levels simulating the amounts of these present in cane molasses of equivalent fermentable sugar concentration. The $CaCl_2$ addition was to simulate the levels of the salt present in the clarified molasses. These were in addition to the KH_2PO_4 and $MgSO_4$ supplements.

As can be seen from Fig. IV.1, the reactor performance was unaffected during the subsequent period of 60 days of operation with the high salt containing sucrose medium.

The results indicate that the relatively early reactor failure with cane molasses is probably not due to any inherent problems such as of differential release from the matrix of the newly formed daughter cells after division of the entrapped cells, leaving a population of more scarred and consequently less metabolically active mother cells in the matrix. Such an effect should then

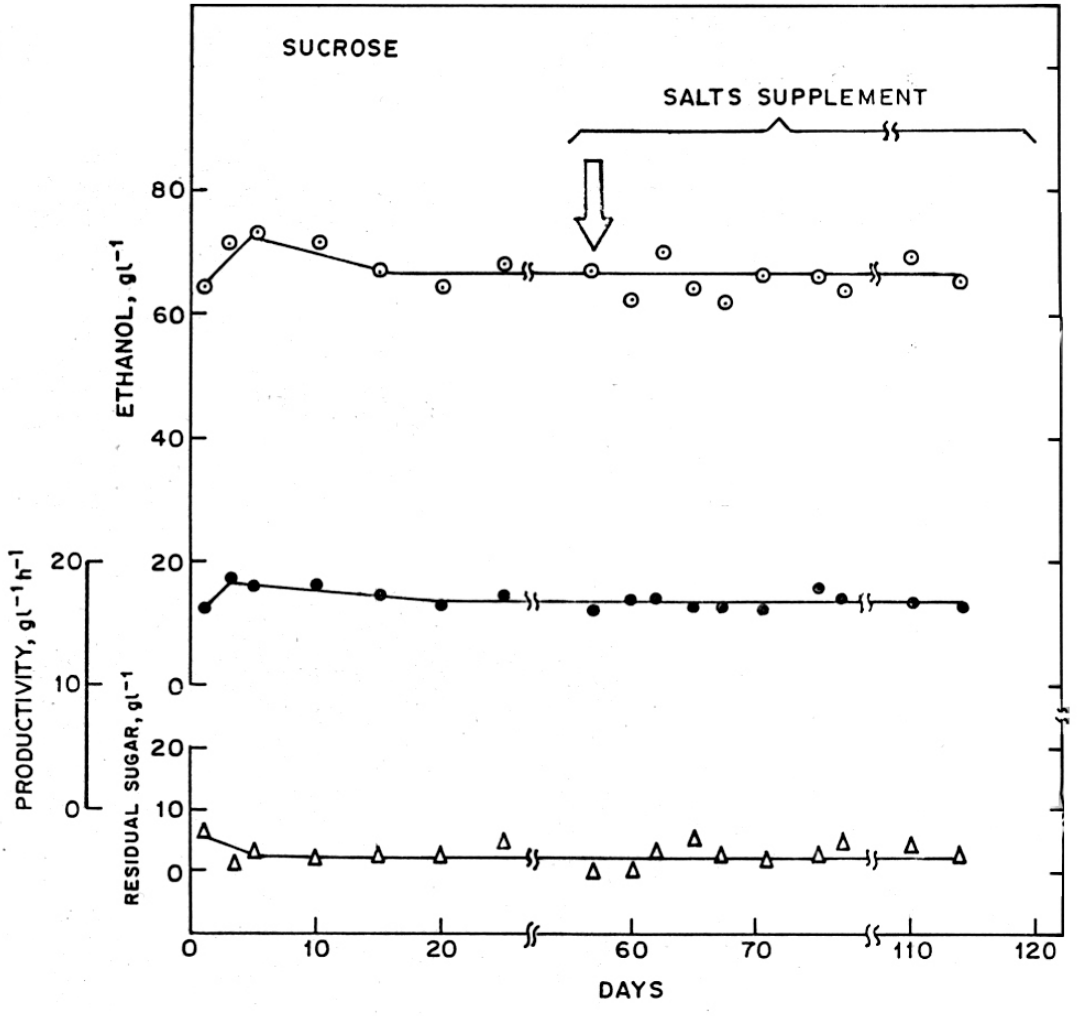

SUCROSE
SALTS SUPPLEMENT
ETHANOL, gl⁻¹
80
60
40
20
0
PRODUCTIVITY, gl⁻¹h⁻¹
20
10
0
RESIDUAL SUGAR, gl⁻¹
20
10
0
0
10
20
60
70
110
120
DAYS

FIG. IV.1 : Operational stability of packed bed bioreactor in the continuous fermention of sucrose to ethanol using S. uvarum immobilized in Ca-alginate beads. Salt supplementation made at stage indicated by arrow. Details as in text.

Ethanol concentration (—o—)

Productivity (—•—)

Residual sugars (—△—)

have occurred also with the sucrose feedstream and resulted in lowering the operational stability of the sucrose reactor. The data also indicate that the KCl, NaCl and $CaCl_2$ levels in molasses are not causative agents or inhibitors responsible for the early lowering of conversion efficiencies. Despite their supplementation to sucrose feedstock, fermentation proceeds at uniformly high productivity and unabated efficiency over a further period of more than 2 months of continuous operation.

Deionized molasses fermentation

Continuous fermentation of deionized molasses was carried out over a period of 60 days. This period was limited by the availability of the liquid sugar supplied. The results of continuous fermentation over this period were similar to those obtained with sucrose feedstock, the productivity being consistently about 15 to 17 $gl^{-1}h^{-1}$ at 65 gl^{-1} ethanol and about 95% conversion efficiency during the entire period of operation.

The data show that the neutral sugars in molasses do not adversely affect reactor performance over extended periods of fermentation.

Cane molasses fermentation

The continuous fermentation of cane molasses with daily monitoring of effluent yeast cell counts was carried out with a feedstream containing 135 to 150 gl^{-1} fermentable sugars, urea 1 gl^{-1} and $MgSO_4.7H_2O$ 0.5 gl^{-1}.

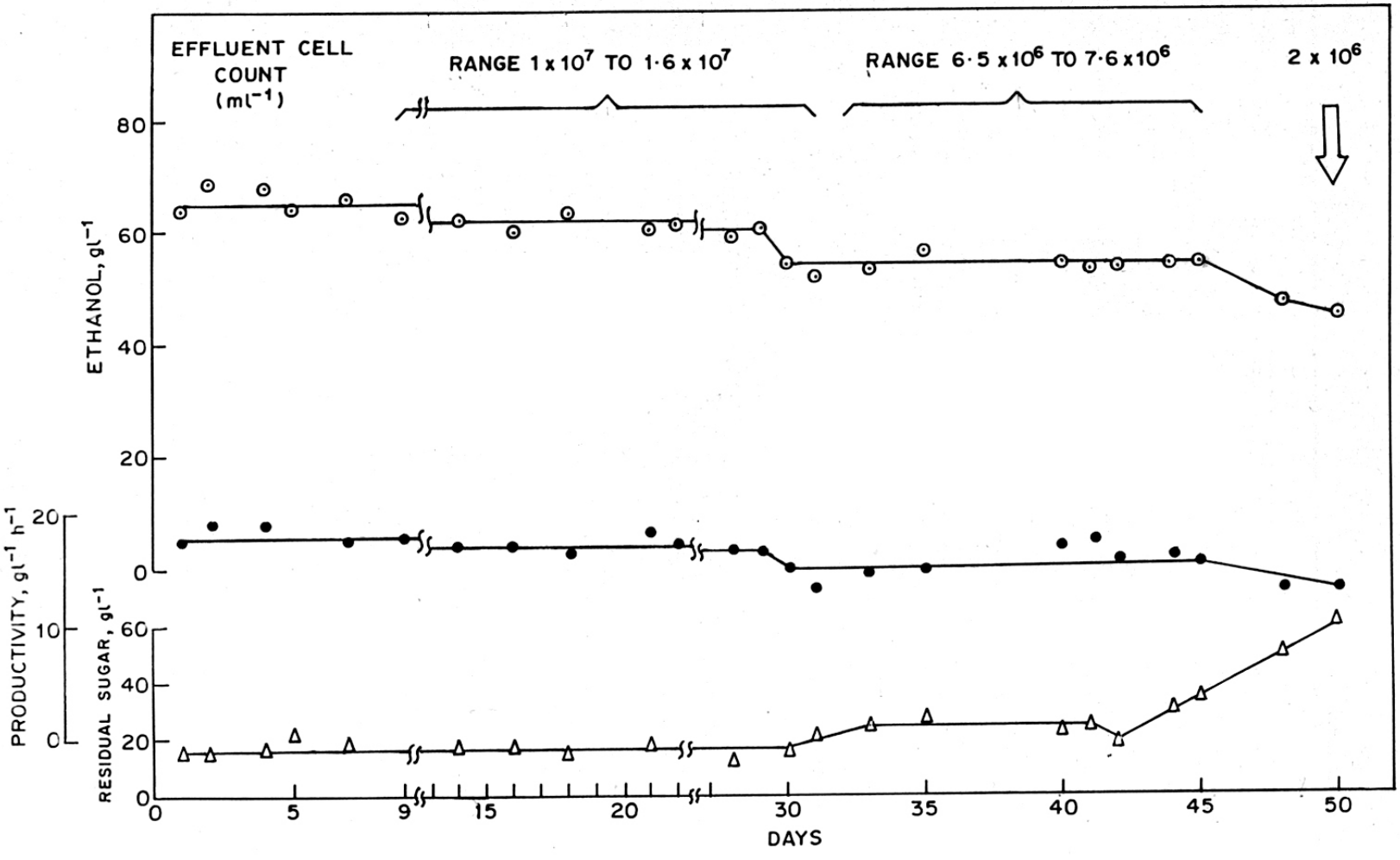

EFFLUENT CELL COUNT (ml⁻¹)
RANGE 1 x 10⁷ TO 1·6 x 10⁷
RANGE 6·5 x 10⁶ TO 7·6 x 10⁶
2 x 10⁶
ETHANOL, gl⁻¹
PRODUCTIVITY, gl⁻¹ h⁻¹
RESIDUAL SUGAR, gl⁻¹
DAYS

FIG. IV.2 : Operational stability of packed bed bioreacor in the continuous fermentation of cane molasses to ethanol using S. uvarum immobilized in Ca-alginate beads. Range of the cell counts in effluent are shown at top of the Figure. Arrow indicates the cell count on day 50. Details as in text.

Ethanol concentration (-o-)

Productivity (-•-)

Residual sugar (-△-)

Fig. IV.2 describes the performance of a reactor with calcium alginate beads containing S. uvarum cells in the continuous fermentation of clarified cane molasses containing 135 to 150 gl^{-1} fermentable sugars expressed as glucose equivalent. The reactor performance was stable during the first 25 days of continuous conversion to ethanol at product concentrations ranging from about 62 - 65 gl^{-1}. Fluctuations in the ethanol levels were between 60 to 69 gl^{-1} and the range reflects the changes in the feedstream sugar concentrations. The volumetric ethanol productivity values during this period ranged between 14 to 16 $gl^{-1}h^{-1}$ and the residual sugars about 16 to 18 gl^{-1}. The effluent monitored for free yeast cell count gave values in the range 1 - 1.6 x 10^7 cells.ml^{-1} after the attainment of equilibrium and upto about 31 days which includes the period of efficient performance of the reactor. The reactor performance from 31 days to about 45 days had dropped to yield lower ethanol concentrations of about 53 gl^{-1} at ethanol productivity of 15 $gl^{-1}h^{-1}$ and residual sugar concentration of 26 gl^{-1}. The effluent yeast cell count values during this period decreased and were in the range of 6.5 - 7.5 x 10^6 cells.ml^{-1}. Beyond the 42nd day of operation, the ethanol concentration and productivity values dropped more sharply with corresponding increase in residual sugar concentrations till at the 50th day, the ethanol concentration was 45 gl^{-1},

productivity about 13 $gl^{-1}h^{-1}$ and the residual sugar concentration 60 gl^{-1}. The effluent yeast cell count dropped sharply to 2 x 10^6 cells.ml^{-1} at this stage.

The data would indicate that the lowered efficiency of reactor performance is probably the result of cell depletion within the matrix which is reflected by a decrease in free cells released into the effluent stream. This was confirmed by monitoring of cell densities within beads. These findings are included in the **Discussion** of the present results.

Experiments on continuous molasses fermentation were also conducted with (i) yeast extract supplementation at 0.05 %, (ii) ergosterol supplements entrapped with yeast cells and (iii) with controlled aeration at 0.1 reactor volume.min^{-1}. In all cases no significant enhancement in the period of efficient reactor performance was achieved indicating that lack of secondary nutrient supplements and O_2 limitations are probably not the causes in the early drop observed in reactor performance with cane molasses feedstock.

DISCUSSION

Immobilized yeast cell systems had shown early failure in maintaining high ethanol productivities and high conversion efficiencies at product concentrations of about 60 gl^{-1} in the continuous fermentation of cane molasses. The present work would indicate that this failure is possibly caused by inhibitory substances present in cane molasses which decrease yeast cell growth and lower cell densities within the matrix. KCl, NaCl and $CaCl_2$ when added to sucrose feedstream to simulate their content in the clarified molasses feedstream caused no adverse effects on reactor performance even over a prolonged period of about 2 months under such conditions of continuous operation. These salts apparently are not the causative factors. That the sugars and other neutral constituents of cane molasses do not also contribute towards early failure in efficient reactor performance is indicated by the uniformly high efficiency in continuous fermentation of deionied cane molasses. The results are suggestive of non-fermentable organic charged compounds and/or heavy metals or other toxic trace elements present in cane molasses as the inhibitors leading to relatively early reactor failure, possibly through cumulative effects. A observed change associated with decrease in reactor efficiency has been the lowering in the free yeast cell count in the effluent stream. That such an effect is

also associated with a decrease in cell density within the bead matrix was confirmed from cell counts made after disruption of the Ca-alginate beads with sodium citrate solutions (A.A. Prabhune and H. SivaRaman, unpublished results). In these confirmatory studies, beads were sampled at various sections along the height of a reactor as described under **Materials and Methods**. The reactor after attainment of equilibrium showed relatively uniform matrix cell densities of $\sim 5 \times 10^9$ cells.g^{-1} wet weight bead matrix measured as colony forming units (CFUs). However, at the stage where product concentration had dropped from 60 gl^{-1} to below 50 gl^{-1}, the cell densities were uniformly about 1.9 - 2.2 x 10^9 cells.l^{-1} beads in the three middle sections and only 1.8 x 10^8 cells.g^{-1} at the topmost section near the outlet. Since the molasses feedstream in this case had not been clarified , the lowest section nearest the inlet had accumulated fine sediments and the cell count at this level was 1.3 x 10^9 cells.g^{-1}. These findings indicate that bead cell density is lowered at all stages of the reactor, particularly at the uppermost stage where ethanol concentration is the highest and fermentable sugars the lowest. This occurs when reactor efficiency drops.

The results support the following conclusions: Continuous ethanol fermentation by immobilized yeast cells over extended periods is sustained only by adequate

cell growth within the matrix. Under steady state conditions of reactor performance, live yeast cell distribution within the bead is apparently also at steady state levels and cell mass generation would atleast have to balance cell death. In positive balance, excess cells would be released into the effluent stream. In the prsent studies the effluent cell count was about 1 - 1.6 x 10^7 cell.ml^{-1} during efficient reactor performance. This corresponds to about 0.17 to 0.26 mg.ml^{-1} biomass on dry weight basis with the feedstream of molasses sugars at 135 - 150 mg.ml^{-1} concentration. The diversion of sugars to biomass generation is thus insignificantly low. Over extended periods of operation, any negative balance between biomass generation and cell death would result in depletion of yeast cell density within the bead matrix and a consequent lowered release of free cells into the effluent stream. Such a condition apparently exists when cane molasses feedstock is used at about 135 - 150 gl^{-1} sugar concentration as observed in the present studies. Sucrose and deionized molasses feedstocks apparently sustained requisite biomass generation to maintain a positive balance over longer periods than cane molasses.

An obvious strategy for overcoming the adverse effects of inhibitory substances in cane molasses would be through deionization. This however, would not be economically

feasible for a low cost, bulk chemical like ethanol. An alternative approach could be through the use of 2 or more bioreactors in series with rotation in their sequences in the series to enable periodic recovery of cell densities in the first stage. Such systems could not be conveniently operated in laboratory scale reactors and our attempts led to frequent infection and bacterial contamination. Such contaminations did occur, even in single stage reactors run continuous for periods exceeding about a month. Such occurrences in the single stage reactors, however, were infrequent and the reactors could be conveniently decontaminated with the addition of tetracycline to the feedstream (50 $mg.l^{-1}$), immediately on detection of bacteria in the effluent stream.

REFERENCES

Alfa Laval (1982) Biostil. Report PB-4-661E-8205 Alfa Laval Tumba, Sweden.

Amin, G., DeMote, R., Van dijek, K. and Verachtert, H. (1985) Appl. Microbiol. Biotechnol. 22, 237.

Amin, G., Van Den Eynde, E. & Verachtert, H. (1983) Eur. J. Appl. Microbiol. Biotechnol. 18, 1.

Amin, G., Standaert, P. and Verachtert, H. (1984) Appl. Microbiol. Biotechnol. 19, 91.

Anderson, E.V. (1985) Chem. Engg. News (April 22, 1985)

Aringasarova, A.Y. and Koshcheenko, K. (1980) Pirkl. Biokhim. Microbiol. 16, 854.

Atkinson, B., Black, G.M., Lewis, P.J.S. and Pinches, A. (1979) Biotechnol. Bioeng. 21, 193.

Amotz, S., Nielssen, T.K. and Thiesson, N.O. (1976) US Patent 3980521

Bandas, E.L. and Zakharov, I.A. (1980) Genetika 16, 787.

Bannarjee, M., Chakrabarty, A. and Majumdar, J.K. (1982) Biotechnol. Bioeng. 24, 1839.

Barron, E.S.G. and Levine, S. (1952) Arch. Biochem. Biophys. 41, 175.

Bazua, C.D. and Wilke, C.R. (1977) Biotechnol. Bioeng. Symp. 7, 105.

Bendova, O. (1986) Folia. Microbiol. 31, 422.

Beavan, M.J., Carpentier, C. and Rose, A.H. (1982) J. Gen. Microbiol. 128, 1447.

Beavan, E.A.and Makower, M. (1963) Proc. 11th Internat. Cong. Genetics. XI, 1, 202.

Brodelius, P. (1975) Adv. Biochem. Eng.

Brodelius, P. and Nilsson, K. (1980) FEBS Lett. 122, 312.

Brown, S.W. and Oliver, S.G. (1982) Eur. J. Appl. Microbiol. Biotechnol. 16, 119.

Brown, S.W., Oliver, S.G., Harrison, D.E.F. and Righelato,R.C. (1981) Eur. J. Appl. Microbiol. Biotechnol. 11, 151.

Bu'Lock, J.D., Comberback, D.M., Ghommidh, C. and Williams, P.D. (1984) Chem. Industr. 432.

Bu'Lock, J.D., Comberback, D.M. and Ghommidh, C. (1984) Chem. Eng. J. 29, B9.

Bulder, C.J.E.A. (1964) Antonie Van Leeuwenhoek 30, 442.

Casey, G.P. and Ingledew, W.M. (1985) Am. Soc. Brew. Chemists) 43, 75.

Calleja, G.B., Levy-Rick, Lusena, C.V., Nasim, A. and Moranelli (1982) Biotechnol. Lett. 4, 543.

Cartwright, C.P., Veazey, F.J. and Rose, A.H. (1987) J. Gen. Microbiol. 133, 857.

Cheetam, P.S.J., Blunt, K.W. and Bucke, C. (1979) Biotechnol. Bioeng. 21, 2155.

Cheung, I.H.S., Gischen, M., Ghosh, P. and Pamment (1986) Appl. Microbiol. Biotechnol. 23, 413.

Chen, E.C. (1981) J. Am. Soc. Brew. Chem. 39, 117.

Cheryan, M. and Mehaia, M.A. (1984) Process. Biochem. 19(6).

Chibata, I. (1978) Immobilized enzymes research and development, John Wiley and Sons Inc., New York.

Chibata, I. and Wingard, Jr. L.B. (1983) Immobilized microbial cells. Appl. Biochem. Bioeng. 4, 1.

Chibata, I., Tosa, T. and Sato, T. (1983) Immobilized cells in the preparation of fine chemicals. Adv. Biotechnol. Processes 10, 203.

Comberback, D.M. and Bu'Lock, J.D. (1984) Biotechnol. lett. 6, 129.

Cysewski, G.R. and C.R. Wilke (1977) Biotechnol. Bioeng. 19, 1125.

Cysewski, G. and Wilke, C. (1978) Biotech. Bioeng. 20, 1421.

Chibata, I., Tosa, T. and Sato, T. (1974) Appl.Microbiol. 27, 878.

Chibata, I., Tosa, T. and Sato, T. (1986) In "Industrial Microbiol. and Biotechnol. (Ed. Demain and Solomon), p. 217.

Damaino, D. and S.S. Wang (1985) Biotechnol. Lett. 7, 135.

Damon, C.E. and Pettitt, Jr., B.C. (1980) J. Assoc. Off. Anal. Chem. 63, 476.

Dawes, E.A., Ribbons, D.W., and Rees, D.A. (1986) Biochem. J. 98, 804.

Davis, B.J. (1946) Ann. N.Y. Acad. Sci. 121, 404.

Daugulis, A.J., Brown, N.M., Cluett, W.R. and Dunlop, D.B. (1981) Biotechnol. Lett. 3, 651.

Day, D.F. and Sarkar, D. (1982) Enzyme Engineering 6, 343.(Eds. Chibata, I., Fukui, S. and Wingard, L.B., Jr. Plenum Press, New York.

De Deken, R.H. (1966) J. Gen. Microbiol. 44, 149

del Rosario, E.J. and Pamatong, F.V. (1985) Biotechnol. Lett. 7, 819.

del Rosario, E.J., Lee, K.J. and Rogers, P.K. (1979) Biotechnol. Bioeng. 21, 1477.

Dinelli, D. (1972) Process. Biochem. 7, 9.

Doelle, H.W. and Greenfield, P.F. (1985) Appl. Microbiol. Biotechnol. 22, 405.

Dombek, K.M. and Ingram, L.O. (1986a) Appl. Environ. Microbiol. 51, 197.

Dombek, K.M. and Ingram, L.O. (1986b) Appl. Environ. Microbiol. 52, 975.

Doran, P.M. and Bailey, J.M. (1986a) Biotechnol. Bioeng. 28, 73.

Doran, P.M., and Bailey, J.E. (1986b) Biotechnol. Bioeng. 28, 1814.

D'Souza, S.F. and Nadkarni, G.B. (1980a) Biotech. Bioeng. 22, 2179.

D'Souza, S.F. and Nadkarni, G.B. (1980b) Biotech. Bioeng. 22, 2191.

D'Souza, S.F., Kaul, R. and Nadkarni, G.B. (1982) Biotech. Bioeng. 24, 1701.

D'Souza, S.F. and Nadkarni, G.B. (1981) Biotech. Bioeng. 23, 431.

Durand, G. and Navarro, J.M. (1978) Process Biochem. 13, 14.

Earl, W.B. and Brown, W.C. (1979) 3rd Int. Symp. on Alcohol fuel, California.

Esser, K., Schmidt, U. and Stahl, U. (1982) Eur. J. Microbiol. Biotechnol. 16, 161.

Fischer, E.H. and Stein, E.A. (1961) Biochem. Prep. 8, 27.

Flink, J.M. and Johansen, A. (1985) Biotechnol. Lett. 7, 767.

Fowler, OP.W., Ball, A.J.S. and Griffiths, D.E. (1971) Can. J. Biochem. 50, 35.

Fukui, S. and Tanaka, A./ (1982) Ann. Rev. Microbiol. 36, 145.

Gas'con, S. and Lampen, J.O. (1968) J. Biol. Chem. 243, 1567.

*Ghose, T.K. and Bandyopadhyay (1980) Biotech. Bioeng. 22, 1489.

Ghose, T.K. and Bandyopadhyay, M.M. (1979) In "Proc. Ist Natl. Seminar on Immobilized enzyme engineering (Ed. Mukherjee, R.N.) Jadhavpur University, Calcutta

Ghose, T.K. and Tyagi, R.D. (1979) Biotechnol. Bioeng. 21, 1401.

Ghose, T.K. and Tyagi, R.D. (1984) Biotech. Bioeng. 24, 781.

Gibbs, M. and DeMoss, R.J. (1951) Arch. Biochem. Biophys. 34, 478.

Gibbs, M. and DeMoss, R.J. (1954) J. Biol. Chem. 207, 689.

Godia, F., Casas, C. and Sola, C. (1985) J. Chem. Tech Biotechnol. 35B, 139.

Godbole, S.S., D'Souza, S.F. and Nadkarni, G.B. (1980) Enzyme Microbiol. Technol. 2, 10.

Gong, C.S., Chen, L.F., Tsao, G.T. and Flicinger, M.C. (1981) Adv. Biochem. Eng. 20, 93.

Gong, C.S., Claypool, T.A., MacCraken, L.D., Maun, C.M. Veng, P.P. and Tsao, G.T. (1983) Biotechnol. Bioeng. 25, 85.

Gong, G.S., Maun, C.M. and Tsao, G.T. (1981) Biotechnol. Lett. 3,

Gray, W.D., Stark, W.H. and Kolachov, P. (1942) J. Bacteriol. 43, 370.

*Gencer, M.A. and Mutharasan, R. (1983) Biotech. Bioeng. 25, 2243.

Greenshields, R.N. and Smith, E.L. (1971) Chem. Eng. (London) 249, 182.

Griffith, W.L. and Compere, A.L. (1978) US Patent 14,127,447.

Guidoboni, G.E. (1984) Enzyme and Microbiol. Technol. 6, 194.

Guijarro, J.M. and Lagunas, R. (1984) J. Bacteriol. 160, 874.

Hahn-Hagerdal, B. and Mattiasson, B. (1982a) Eur. J. Appl. Microbiol. Biotechnol. 14, 140.

Hahn-Hagerdal, B. and Mattiasson, B. (1982b) Biotechnol. Bioeng. Symp. 12, p.193.

Haraldson, A., Bjorling, T. (1981) Eur. J. Appl. Microbiol. Biotechnol. 13, 34.

Hattori, T. and Furusaka, C. (1961) J. Biochem. 50, 312.

Hatturi, T. and Furusaka, C. (1960) J. Biochem. 48, 831.

Hayshida, S. and Ohta, K. (1981) J. Inst. Brew. 87, 42.

Hayshida, S., Feng, D.D., Ohta, K. and Chaitiumvong, S. and Hongo, M. (1976) Agric. Biol. Chem. 40, 73.

Hodge, H.M. and Hilderbrandt, F.M. (1954) Alcholic fermentation of molasses: In Industrial Fermentation" (Ed. Underkolfer and Hicker, R.J.) 1, 73, Chemical Publication Company, New York.

Hahn-Hagerdal, B., Larsson, M. and Mattiasson, B.C. (1982) Biotechnol. Bioeng. Symp. 12, 199.

Holcberg, I.B. and Margalith, P. (1981)Eur. J. Appl. Microbiol. Biotechnol. 13, 133.

Holzer, H. (1968) In "Aspects of yeast metabolism" (Ed. Mills, A. and Krebbs, S.) p. 155, Blackwell, Oxford.

Hossack, J.A. and Rose, A.H. (1976) J. Bacteriol. 127, 67.

Hough, J.S., Gough, P.E. and Davis, A.D. (1977) Continuous culture in brewing "In Continuous Culture; Applications and new fields' (Ed. Dean, H. and Ellwood, D) p. 226, Holsted Press, London.

Hulst, A.C., Tramper, J., Var'triet, K. and Westerbeek, J.M.M. (1985) Biotechnol. Bioeng. 27, 870.

Ingram, L.O. and Buttke, T.M. (1984) Adv. Microbiol. Physiol. 25, 253.

Inloes, D.S., Talor, D.P., Cohen, S.N., Michaels, A.S. and Robertson, C.R. (1983). Appl. Environ. Microbiol. 46, 264.

Ismail, A.A. and Ali, A.M.M. (1971) Folia Microbiol. 16, 346.

Jack, T.R. and Zajic, J.E. (1977) Biotechnol. Bioeng. 19, 631.

Jeffries, T.W. (1981) Biotechol. Lett. 3, 213.

Jennings, D.H. (1984) Adv. Microbiol. Physiol. 25, 149.

Jirku, V., Turkova, J., Veruovic, and Kubanek, (1980) Biotechnol. Lett. 2, 451.

Jirku, V., Turkova, J. and Krumphanzi, V. (1980) Biotechnol. lett. 2, 509.

Johansen, A. and Flink, J.M. (1986) Biotechnol. Lett. 8, 121.

Johansen, A. and Flink, J.M. (1986) Enz. Microbiol. Technol. 8, 245.

Johanasson, M. and Sjostrom, J.E. (1984) Biotechnol. Lett. 6, 49.

Jones, R.P., Pamment, N. and Greenfield, P. (1981) Process biochem.16, 171.

Jones, R.P. and Greenfield, P.F. (1984) Process Biochem. 19, (April)48.

Joshi, S. and Yamazaki, H. (1984) Biotechnol. Lett. 6, 797.

Kannan, V. and Mutharasan, (1985) Enzyme Microbiol. Technol. 7, 87.

Karube, I., Kuriyama, S., Matsunaga, T. and Suzuki, S. (1980) Biotechnol. Bioeng. 22, 847.

Kaul, R., D'Souza, S.F. and Nadkarni, G.B. (1983) Biotechnol. Bioeng. 25, 887.

Kuhn, I. (1980) Biotechnol. Bioeng. 22, 2393.

Kennedy, J.F., Barker, S.A. and Humphreys, J. (1976) Nature 261, 242.

Kennedy, J.F. and Cabral , J.M.S. (1985) Aool. Biochem. and Bioeng. 4, 190.

Kierstan, M. and Bucke, C. (1977) Biotechnol. Bioeng. 19, 387.

Kierstein, M.P.J. and Coughlan, H.J. (1985) Immobilized cells and enzymes - Practival approach (Ed. Woodward,J)IRL Pre

King, V.A.E. and Zall, R.R. (1983) Process Biochem. 18, (Dec) 17.

Kirby, K.D. and Mordon, C.J. (1980) Biotechnol. Bioeng. 22, 2455.

Klein, J. and Eng, H. (1979) Biotechnol. Lett. 1, 171.

Klein, J. and Wagner, F. (1978) DeChema-Monogr. 82,142.

Klien, J.M. and Manecke, G. (1982) Enz. Eng. 6 181

Klein, J.M., Stock, J. and Vorlop, K.D. (1983) Eur. J. Appl. Microbial. Biotechnol. 18, 86.

Kolot, F.B. (1980) Process. Biochem. 15, 2.

Koshcheenko, K.A. (1981) Prikl. Biokhim. Mikrobiol. 17, 477.

Krouwel, I.P.G. (1979) Anton. Von. Leeuwen 45, 646.

Krouwel, P.G., Van Der Laan, W.F.M. and Kossen, N.W.F. (1980) Biotechnol. Lett. 2, 253.

Kuriyama, H., Seiko, Y., Murokami, T., Kobayashi, H. and S onoda, Y. (1985) J. Ferm. Technol. 63, 159.

Kuu, W.Y. and Polack, J.A. (1983) Biotechnol. Bioeng. 25, 1995.

Lamed, R., Zeikus, J.G. (1980) J. Bacteriol. 144, 569.

Lagomasino, J.M. (1949) Inst. Sugar. J. 61, 338.

Larsson, C. and Gustafsson, I. (1987) Arch. Microbiol. 147, 358.

Larsson, P.O. and Mosbach, K. (1979) Biotechnol. Lett. 501.

Lee, T.H., Ahn, J.C. and Ryu, D.D.Y. (1983) Enzyme Microbiol. Technol. 5, 41.

Leao, C. and Van Uden, N. (1982b) Biotechnol. Bioengg. 25, 2085

Levenspiel, O. (1980) Biotechnol. Bioeng. 22, 1671.

Leao, C. and Van Uden, N. (1983) Biotechnol. Bioeng. 25, 2085.

Linko, P. and Linko, Y.Y. (1983) Appl. Biochem. Bioeng. 4,

Linko, P. and Linko, Y.Y. (1984) Crit. Rev. Biotechnol. (Ed. Steward, G.G. and Russel,I.) CRC Press, Boca Raton, Florida, 1, pp. 289.

Linko, Y.Y.. Jalank, H. and Linko, P. (1981) Biotechnol. lett. 3, 263.

Linko, Y.Y., Poutanen, K., Weckstrom, L. and Linko, P. (1979) Enzyme and Microbial.Technol. 1,

Linko,Y.Y. and Linko, P. (1981) Biotechnol. Lett. 3, 21.

Loureiro-Dias, M.C., Peinado, J.M. (1982) Biotechnol. Lett. 4, 721.

Loureiro, V. and Van Uden, N. (1982) Biotechnol. Bioeng. 24, 1881.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.

Lutstorf, U. and Megnet, R. (1968) Arch. Biochem. Biophys. 126,933.

Lyness, E. and Doelle, H.W. (1980) Biotechnol. Lett. 2, 549.

Lyness, E. and Doelle, H.W. (1983) Biotechnol. Lett. 5,345.

Luca, M.D. and Krica, J.L. (1983) Arch. Biochem. Biophys. 226, 285.

Maiorella, B.L. (1985) Comprehensive Biotechnol. (Ed.in Chief Moo-Young, M.) 3. 861.

Maiorella, B.L., Wilky, C.R. and Blanch, H.W. (1981) Adv. Biochem. Eng. 20, 43.

Maiorella, B.L., Blanch, H.W. and Wilky, C.R. (1983) Biotechnol. Bioeng. 25, 103.

Maiorella, B.L., Blanch, H.W. and Wilke, C.R. (1984) Biotechnol. Bioeng. 1003, 1984.

Marcipar, A., Cochet, N., Blackenbridge, L. and Lebeault, (1979) Biotechnol. Lett. 1, 65.

Margaritis, A. and Bajpai, P. and Wallace, J.B. (1981) Biotechnol. Lett. 3, 613.

Margaratis, A. and Bajpai, P. (1982a) Biotechnol. Bioeng. 24, 941.

Margaritis, A. and Bajpai, P. (1982b) Biotechnol. Bioeng. 24, 1483.

Margaritis, A. and Bajpai, P. and Lachance, (1983_ J. Ferment. Technol. 61, 533.

Mattiason, B. (1983) Immobilized cells and organelles Chemical Rubber Co., Cleveland, Ohio.

Mehaia, M.A. and Cheryan, M. (1984) Appl. Microbiol. Biotechnol. 20, 100.

Messing, R., Oppermann, R.A. and Kolot, F.B. (1979) Pore dimensions for accumulating biomass in Immobilized microbial cells. ACS (Am. Chem. Soc.) Symp. Ser. 106, 13.

Mena, R.H., Ribaud, J.A. and Humphrey, (1980) Proc. VI Int. Ferm. Symp. F738 (P) London (Canada)

Millar, D.G., Griffiths-Smith, K., Algar, E. and Scopes, R.K. (1982) Biotechnol. Lett. 21, 601.

Miner, M. and Goma, G. (1981) Biotechnol. Lett. 3, 405.

Mitani, Y., Nishizawa, Y. and Nagai, S. (1984) J. Ferment. Technol. 62, 401.

Moss, F.J., Riccard, P.A.D., Bush, F.E., Caiger, P. (1971) Biotechnol. Bioeng. 13, 63.

Muolder, M.H. and Smolders, C.A. (1986) Process Biochem. 21, 35.

Moulir, G., Boze, H. and Galzy, P. (1981) Biotechnol. Lett. 3, 351.

Moreno, M. and Goma, G. (1979) Biotechnol. Lett. 1, 483.

Moo-Young, M., Lamptey, J. and Robinson, C.W. (1980) Biotechnol. Lett. 2, 541.

Nagashima, M., Azuma, M., Vouguchi, S., Inuzuka, K. and Samejima, H. (1984) Biotechnol. Bioeng. 26, 992.

Nagodawithana, T.W. and Steinkraus, K.H. (1976) Environ. Microbiol. 31, 158.

Navarro, J.M. and Durand, G(1977) Eur. J. Appl. Microbiol. Technol. 4, 243.

Navarro, J.M. et. al. (1976) Industr. Aliment. agricol. 6, 695.

Navarro, J.M. and Durand, G. (1978) Annals of Microbiol. (Paris) 129b, 215.

Nes, W.R., Sekula, B.C., Nes, W.D. and Adler, J.H. (1978) J. Biol. Chem. 253, 6218.

Novak, M., Strehaiano, P., Moreno, P. and Goma, G. (1981) Biotechnol. Bioeng. 23, 201.

Ohalson, S., Larsson, P.O. and Mosbach. K. (1979) Eur. J. Appl. Microbiol. Biotechnol. 8, 143.

Oura, E. (1977) Process Biochem. 12, 19.

Ohta, K. and Hayashida, S. (1983) Appl. Environ. Microbiol. 46, 821.

Panchal, C.J. and Stewart, G.G. (1980) J. Inst. Brew, 86, 207.

Patil, S.G., Gokhale, D.V. and Patil, B.G. (1986) Enz. Microbial. Technol. 8, 481.

Petre, D., Noel, C. and Thomas, D. (1978) Biotechnol. Bioeng. 20, 135.

Pines, G. and Freeman, A. (1982)Eur. J. Appl. Microbial. Biotechnol. 16, 75.

Pirt, S.J. and Kurowski, W. (1970) J. Gen. Microbiol. 63, 357.

Prince, I.G. and Barfort, J.P. (1982a) Biotechnol. Lett. 4, 525.

Prince, I.G. and Barfort, J.P. (1982b) Biotechnol. Lett. 4, 621.

Proudlock, J.W., Wheeldon, L.W., Jollow, D.J. and Linnane, A.W. (1968) Biochim. Biophys. Acta. 152, 434.

Rahn, O. (1952) Growth 16, 59.

Rajan, J.V., and Ayyangar, N.R. (1985) Maharashtra Sugars, Dec. 1985.

Rao, M., Deshpande, V.V., Keskar, S. and Srinivasan, M.C. (1983) Enz. and Microb. Technol. 5, 133.

Reid, V.W. and Truelove, R.K. (1952) Analyst 77, 325.

Reed, L.J. and Williams, C.R (1966) Meth. Enzymol. 9, 247.

Ribbons, D.W., Dawes, E.A. and Rees, D.A. (1962) Biochem. J. 82, 45P.

Robinson, C.W., Moo-Young, M. and Lampjey, J. (1980) Ethanol production by immobilized yeast cells in a packed reactor". Paper given at the 6th Int. Ferment. Symp., London, Canada.

Rogers, P.L., Lee, K.J., Skotnicki, M.L. and Tribe, D.E. (1980) Adv. Biochem. Eng. 23, 37.

Rolz, C. (1981) Enzyme. Microbiol. Technol. 3, 19.

Romantschuk, M. and Lehtomaki, (1978) Process Biochem. 13(3), 16.

Rose, A.H. (1980) In "Biology and Activities of Yeasts (Eds. Skinner, F.A., Passmore, S.M. and Davenport, R.R.) 103, London, Academic Press.

Rose, A.H. (1983) In "Proc. of the Royal Soc. of Canada: Int. Symp. on ethanol from Biomass (H.W. Duckworth and E.A. Thompson eds) p. 45 p., The Royal Soc. Canada, Ottawa, Canada.

Rose, A.H. and Beavan, M.J. (1981) In "Trends in the biology of fermentations for fuels and chemicals" (Eds. Hollander, A. and Robson, R.) Plenum publishing Corporation, New York.

Salleh, A.B. and Mohmed, S. (1982) Biotechnol. Lett. 4, 611.

Sharma, S. and Tauro, P. (1986) Biotechnol. Lett. 8, 735.

Schnarr, G.W., Szarek, W.A. and Jones, J.K.N. (1977) Appl. Microbiol. 33, 732.

Scenbider, H., Wang, P.Y., Chanzy, K. and Maleszka (1981) Biotechnol. Lett. 3, 89.

Scott, C.D. and Hancer, C.W. (1976) Biotechnol. Bioeng. 18, 1393.

Sitton, O.C. and Gaddy, J.L. (1980) Biotech. Bioeng. 22, 1735.

Singh, R. and Kunkee, R.E. (1976) Appl. Environ. Microbiol. 32, 666.

Sherman, F. (1959) Journal of Cellular and Comparative Physiology 54, 37.

Skogman, H. (1976) In "Food from Waste", (Eds. Birch, G.G., Parker, K.J. and Worgan, J.T.) Pub. ATPL, Sci. Lond, p. 167.

Slininger, P.J., Bothast, R.J., Black, L.T. and McChee, (1982), Biotechnol. Bioeng. 24, 2241.

Stark, W.H. (1954) In "Industrial fermentations" (Ed. Underrofler, L.A. and Hickey, R.J.) 1, 17, Chem. Pub. Co., New York.

Srinivasan, M.C., Prabhune, A.A. and SivaRaman, H. (1985) Current Sci.

Stephanopoulos, K., San, Y. and Davison, B.H. (1985) Biotechnol. Progress. 1, 250.

Suo malainen, H. (1981) In current development in yeast research, Advances in Biotechnology(Ed. Stewart G.G. and Russel, W.I., pp. 3 - 8, Pergamon Press, Toranto.

Swings, J. and DeLey, J. (1977) Bacterial. Rev. 41.

Takata, I., Tosa, T. and Chibata, I. (1977) J. Solidphase Biochem. 2, 225.

*

Thomas, D.S. and Rose, A.H. (1979) Arch. Microbiol. 122, 49.

Thomas, D.S., Hossack, J.A. and Rose, A.H. (1978) Arch. Microbiol. 117, 239.

Tipper, B.J., Bortrain, K.A. (1984) Microbiol. Rev. 48, 125.

Toda, K. (1975) Biotech. Bioeng. 17, 1729.

Toda, K. and Shoda, H. (1975) Biotechnol. Bioeng. 17, 481.

Tosa, T., Sato, T., Mori, J., Tamamoto, K., Takata, I., Nishida, Y. and Chibata, I. (1979) Biotechnol. Bioeng. 21, 1697.

Trevelyan, W.E., Grammon, J.N., Wiggins, E.H., Harrison, J.S. (1952) Biochem. J. 50, 303.

Troyer, J.R. (1953) Mycologia 45, 20.

*Takata, I., Yamamoto, K., Tosa, T., Chibata, I. (1980) Enzy. Microbiol. Technol. 2, 30.

Tyagi, R.D. and Ghose, T.K. (1982) Biotechnol. Bioeng. 24, 781.

Ueng, P.P. and Gong, C.S. (1962) Enzyme Microbiol. Technol. 4, 169.

Ueng, P.P., Hunter, C.A., Gong, C.S. and Tsao, G.T. (1981) Biotechnol. Lett. 6, 315.

Van Uden, N. (1984) Adv. Microbiol. Physiol. 25, 149.

Van Uden, N. and da Cruzduarte, H. (1981) Zeitschrift fur Allgemeine Mikrobiologie 21, 743.

Veliky, I.A. and Williams (1981) Biotechnol. Lett. 3, 275.

Veliky, I.A. and Williams, R.E. (1981) Biotechnol. Lett. 3, 275.

Vojtisek, V. and Jirku, V. (1983) Folia Microbiol. 28, 309.

Vijayalakshmi, M., Marcipar, A., Segard, E. and Broun, G.B. (1979) Ann. N.Y. Acad. Sci. 326, 249.

Wada, M., Kato, J. and Chibata, I. (1980) Eur. J. Appl. Microbiol. Biotechnol. 10, 275.

Wada, M., Kato, J. and Chibata, I. (1980) Eur. J. Appl. Microbiol. Technol. 11, 67.

Walsh, T.J. and Bungay, H.R(.1979) Biotechnol. Bioeng. 21, 1081.

Wang, H.Y. and Hettwer, D.J. (1982) Biotechnol. Bioeng. 24, 1827.

Wada, M., Kato, J. and Chibata, I. (1979) Biotechnol. Bioeng.

Watson, K. and Cavicchioli, R. (1983) Biotechnol. Lett. 5, 683.

Wang, D.I.C., Cooney, C.L., Demain, A.L., Dunnil, P. Humphrey, A.E. and Lily, M.D. (1979) Ferment. and Enzy. Technol. Wiley, New York.

Weetall, H.H. and Bennett, M.A. (1976) Abst. of papers from Int. Ferm. Symp. Berlin, 299.

White, F.H. and Portno, A.D., 1978, J. Inst. Brewing 84, 228.

Williams, D. and Munnecke (1981) Biotechnol. Bioeng. 23, 1813.

Willis, C. and Jornwall, H. (1979) Eur. J. Biochem. 99, 323.

Willis, C. and Phelps, J. (1975) Arch. Biochem. Biophys. 167, 627.

Wu, J.F., Lastick, S.M. and Updegraft, O.M. (1986) Nature(London) 321, 887.

Willis, C. (1976) Nature 261, 26.

Yeas, M. (1956) Experimental Cell Res. 10, 746.

Zakharov, I.A. and Bandas, E.L. (1979) Genetika 15, 927.

Zeikus, J.G. (1979) Enz. Microbiol. Technol. 1, 243.

Zeikus, J.G., Hegge, P.W. and Anderson, M.A. (1979) Arch. Microbiol. 122, 41.

Alcohol dehydrogenase and invertase activities in ethanol tolerant yeasts

D. V. Gokhale, B. S. Rao and S. Sivaramakrishnan*

Biochemistry Division, National Chemical Laboratory, Poona 411 008, India

(Received 7 May 1986)

Two ethanol tolerant yeast isolates of Saccharomyces cerevisiae *Y-10 and* Saccharomyces cerevisiae *Y-7 were compared for their invertase (EC 3.2.1.26, β-fructofuranoside fructohydrolase) and alcohol dehydrogenase (ADH, EC 1.1.1.1, alcohol:NAD oxidoreductase) activities as well as ethanol productivity. The isolates showed significantly higher ethanol productivities compared to the standard strain* Saccharomyces uvarum *and other yeast strains tested. The alcohol dehydrogenase activity was 40–100% higher in the isolates than* S. uvarum *and the percentage change varied depending on the growth conditions. However, this was not true for invertase activity. Both the isolates showed a similar ADH isozyme pattern in contrast to* S. uvarum. *The results suggested that a better correlation between ADH activity and ethanol productivity could be drawn only after extensive studies on the kinetic parameters of the individual isozymes.*

Keywords: Ethanol productivity; molasses fermentation; alcohol dehydrogenase (ADH); isozyme pattern

Introduction

The increasing need of fuel ethanol has prompted extensive investigations for ethanol production from various renewable resources and their efficient utilization by novel microorganisms. Though molasses is a prime source of ethanol, its fermentation efficiency is limited by the sugar and ethanol tolerant capacities of the yeasts. Ethanol inhibition of the fermentation processes is a complex phenomenon and many reports are available on different aspects of this problem.[1–6] Immobilization of yeast cells on solid matrices has been used to obtain higher ethanol productivities by partially overcoming the problem of ethanol inhibition compared to batchwise fermentation.[7,8] Attempts are being made to isolate ethanol and osmo-tolerant yeast strains having better fermentation characteristics from natural sources and by genetic manipulation.[9,10]

With a view to improving the efficiency of the fermentation processes, studies were initiated in our laboratory to isolate substrate and ethanol tolerant yeast strains. Two such isolates of *Saccharomyces cerevisiae* designated as Y-10 and Y-7 were obtained from fermenting sugar-cane juice which showed higher ethanol productivities compared to the standard strain *S. uvarum*.[11] The present paper reports the studies on invertase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase) and alcohol dehydrogenase (EC 1.1.1.1, alcohol:NAD oxidoreductase) levels in these two isolates which play key roles in sugar utilization and ethanol production respectively and their possible role in higher ethanol productivity is discussed.

Materials and methods

Ethanol tolerant yeasts were isolated from fermenting sugar-cane juice and were characterized as *Saccharomyces cerevisiae* at NCYC. They were designated as *S. cerevisiae* Y-10 and *S. cerevisiae* Y-7. *S. uvarum* ATCC 26602 and other yeast strains were obtained from National Collection of Industrial Microorganisms, Poona, India. Sugar-cane molasses obtained from the local sugar factory was analysed for total reducing sugar as described by SivaRaman *et al.*[12] The molasses sample contained 50% (w/v) reducing sugars. The cultures were routinely maintained on a medium containing 20% reducing sugar of cane molasses, yeast extract, 0.3%; malt extract, 0.3% and bacto-peptone, 0.5%. The growth medium (pH 6.5) consisted of either D-glucose, sucrose or reducing sugar of cane molasses at a concentration of 5% in addition to yeast extract, malt extract and bacto-peptone as mentioned above. The fermentation medium (pH 4.5) consisted of either sucrose or reducing sugar of cane molasses at 20–23% final concentration.

Batch fermentation

Cells grown in 10 ml growth medium containing 5% reducing sugar of molasses for 24 h at 30°C on a rotary shaker were transferred to 90 ml fermentation medium containing 20% molasses sugar. The fermentation was carried out at 30°C under stationary conditions. Samples were withdrawn at 24 and 48 h and ethanol was estimated by ceric ammonium nitrate as well as by gas chromato-

NCL Communication No. 3787
*Present Address: Radio Isotope Lab., ICRISAT, Patancheru P. O., A. P. 502 324, India

0141-0229/86/100623-04 $03.00

graphy using Chromosorb 101.[12]

Cells were grown at 30°C on a rotary shaker in growth medium containing the respective sugar. After 20 h cells were harvested by centrifugation and used for the determination of specific ethanol productivity and also for the assay of enzymes.

Specific ethanol productivity

About 1 g (dry weight equivalent) of cells were transferred to fermentation media containing 23% sucrose or reducing sugar of molasses. The flasks were incubated at 30°C and samples were withdrawn at different intervals of time. Ethanol was estimated as mentioned above. Specific ethanol productivity was expressed as ethanol produced per gram dry weight of cells per hour.

Enzyme assays

Yeast cells grown as above (aerobic) or under stationary conditions (anaerobic) were harvested by centrifugation, washed twice in saline and the pellets were frozen in liquid nitrogen. The pellets were slowly thawed and ground well with sand in a precooled mortar. The disrupted cells were suspended in 3 ml buffer (Tris 50 mM, pH 7.5) and the suspension was centrifuged at 12 000*g* for 20 min in a Sorvall RC 5B centrifuge. The supernatant was filtered through glass wool and used for enzyme assays. Invertase activity was assayed according to Gascon and Lampen.[13] The total reaction mixture of 0.5 ml contained 0.2 ml acetate buffer (0.1 M, pH 4.5), 0.1 ml sucrose (0.5 M) and 0.2 ml supernatant. The mixture was incubated at 37°C for 10 min and the reaction was stopped by the addition of 0.5 ml dibasic potassium phosphate (0.2 M) and placed in a boiling water bath for 5 min. Suitable aliquots were used for estimating the D-glucose by the D-glucose oxidase method using Glox reagent. One unit of enzyme activity corresponds to the liberation of one micromole of D-glucose per minute. Alcohol dehydrogenase activity was assayed by the method of Barron and Levine[14] with minor modification. The assay mixture contained Tris–HCl (20 mM, pH 8.6), NAD (1 mM), L-cysteine–HCl (1 mM) in a total volume of 2.8 ml. The reaction was started by the addition of supernatant followed by ethanol (0.6 M). The change in absorbance at 340 nm was monitored every 15 s for one minute. One unit of activity is defined as one micromole of NAD reduced per minute.

Gel electrophoresis

Both the disc and slab gel electrophoresis were carried out in polyacrylamide gels (7%) with the buffer systems of Davis.[15] The gels were stained for ADH activity according to Lutstorf and Megnet[16] except that Tris–HCl (60 mM, pH 8.6) was used instead of pyrophosphate buffer.

Protein was estimated by the method of Lowry *et al.*[17] with bovine serum albumin as standard. NAD, BSA and Tris base were obtained from Sigma Chemical Co., USA. Glox reagent was purchased from Kabi Diagnostica, Sweden. Yeast extract, malt extract and bacto-peptone were from Difco Laboratories, USA. All other chemicals used were of Analar grade.

Results and discussion

Ethanol production by various yeast strains was measured in cane molasses medium and values obtained are given in *Table 1*. Of the strains tested, since all except two showed similar ethanol production at 24 and 48 h these were further screened for their ethanol productivity. The results are given in *Table 2*. The specific ethanol productivities were calculated during the initial phase of fermentation before the ethanol concentration attained inhibitory levels. From the data it is clear that *S. uvarum* and the two isolates *S. cerevisiae* Y-10 and Y-7 show considerably higher ethanol productivities compared to other stains tested. The isolate Y-7 consistently gives higher productivities than *S. cerevisiae* Y-10 both in sucrose and in molasses media. The higher ethanol productivites obtained can be attributed to the greater tolerance towards high sugar and salts present in molasses.

Table 1 Ethanol production by various strains of yeast

Yeast strains	Ethanol [illegible], (w v)	
	24 h	48
Saccharomyces uvarum	4.48	5.9
Saccharomyces cerevisiae Y-10	4.48	6.3
Saccharomyces cerevisiae Y-7	5.12	7.1
Saccharomyces cerevisiae 3300	6.16	6.8
Saccharomyces cerevisiae 3107	5.14	6.9
Saccharomyces cerevisiae 3176	4.5	5.8
Saccharomyces cerevisiae 3095	2.8	4.1
Schizosaccharomyces pombe 3360	1.19	2.1

Table 2 Specific ethanol productivity during the initial phase of fermentation at 30°C

Yeast strains	Specific ethanol productivity ($g\,g^{-1}\,h^{-1}$)	
	Sucrose	Molasses
Saccharomyces uvarum	0.50	0.44
Saccharomyces cerevisiae Y-10	0.65	0.58
Saccharomyces cerevisiae Y-7	0.88	0.60
Saccharomyces cerevisiae 3300	0.65	0.36
Saccharomyces cerevisiae 3107	0.59	0.31
Saccharomyces cerevisiae 3176	0.58	0.32

The values obtained are the average of three independent determinations

Ethanol affects growth rate, cell viability and in turn the overall fermentation rate which is a complex response that can be considerably influenced both by genetic and environmental factors. The kinetics of ethanol inhibition are further complicated by the exact levels of both intra and extracellular ethanol present in the system.[3,4,6,18] In addition to the effect of ethanol on membrane composition its role on other factors like sugar transport and glycolytic enzymes also has been implicated in ethanol inhibition.[18–20] Though ethanol has been shown to affect several enzymes of the glycolytic pathways[20–22] the inhibition *per se* does not seem to be responsible for the slow fermentation observed. However, the actual levels of the key enzymes present in a cell and their role in the sugar utilization and ethanol production do merit consideration.

We selected the two enzymes namely invertase and alcohol dehydrogenase which play key roles in molasses fermentation and determined their levels in the two isolate

and the standard strain *S. uvarum*. The data in *Table 3* show that the invertase activity is higher in *S. uvarum* and *S. cerevisiae* Y-7 compared to *S. cerevisiae* Y-10. This difference in enzyme activity is observed irrespective of the growth medium used though the specific activity is found to vary with the medium.

The extracellular invertase activity is negligible in all the three strains (data not shown). This clearly indicates that invertase is not one of the rate limiting factors in yeast, unlike in the case of *Zymomonas mobilis* where the enzyme is required for sucrose fermentation more than levan sucrase.[23] The data also suggest that there can be a constitutive and an inducible invertase activity in certain strains of yeast (*Table 3*) but the amount of constitutive enzyme present is far in excess of the level essential for the conversion of substrate needed for ethanol production.

Table 3 Comparison of invertase activity

	Invertase activity (units/mg protein)		
Sugar used	*Saccharomyces uvarum*	*Saccharomyces cerevisiae* Y-10	*Saccharomyces cerevisiae* Y-7
D-Glucose	2.74	0.49	1.64
Sucrose	3.60	0.99	4.22
Molasses	3.28	1.38	4.56

The values obtained are the average of at least three independent experiments

Table 4 Comparison of ADH activity under aerobic and anaerobic conditions

	ADH activity (units/mg protein)		
Sugar used	*Saccharomyces uvarum*	*Saccharomyces cerevisiae* Y-10	*Saccharomyces cerevisiae* Y-7
Aerobic			
D-Glucose	0.319	0.521	0.531
Sucrose	0.388	0.651	0.731
Molasses	0.309	0.493	0.633
Anaerobic			
Sucrose	0.520	0.814	0.897
Molasses	0.427	0.661	0.788

The values obtained are the average of three independent determinations

Table 5 Alcohol dehydrogenase activity in different strains of yeast

	ADH activity (units/mg protein)	
Strain used	Sucrose	Molasses
Saccharomyces cerevisiae 3300	0.345	0.327
Saccharomyces cerevisiae 3107	0.326	0.273
Saccharomyces cerevisiae 3176	0.273	0.312
Saccharomyces cerevisiae 3095	0.319	0.299
Schizosaccharomyces pombe 3360	1.049	0.561

The comparison of specific activities of alcohol dehydrogenase from different strains tested is given in *Table 4*. Both the isolates, *S. cerevisiae* Y-10 and Y-7 show significantly higher alcohol dehydrogenase activity than *S. uvarum* under aerobic and anaerobic conditions. The percentage increase in enzyme activity of the isolates varies from 40–100% depending on the sugar used in growth media. *S. cerevisiae* Y-7 always shows a higher alcohol dehydrogenase activity than the other two strains. A slight inhibition is observed in molasses grown cells often masking the true alcohol dehydrogenase activity which can be attributed to the high salts and other impurities present in molasses.

The data in *Table 5* shows that it is not possible to draw a direct correlation between high alcohol dehydrogenase activity and the increased ethanol productivity exhibited by certain strains of yeasts. For example *Schizosaccharomyces pombe* shows a very high alcohol dehydrogenase activity compared to other yeast strains but the ethanol production from molasses is found to be low (*Table 1*). This suggests that the nature of the fermentation medium is also a contributing factor for higher ethanol productivity. In other words, *Sch. pombe* has the potential to give higher ethanol productivity based on the high alcohol dehydrogenase activity present provided the proper substrate is used. The same way it can be argued that the lower productivity obtained with different strains of *S. cerevisiae* tested (*Table 3*) can be due to the presence of lower alcohol dehydrogenase activity (*Table 4*). In the studies on vinification by several yeast strains Singh and Kunkee[24] could correlate the level of alcohol dehydrogenase with fusel oil production but not with ethanol. A simple and direct cor-

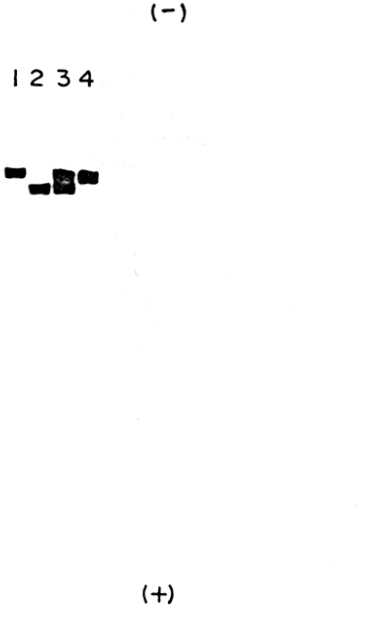


Figure 1 Gel electrophoresis pattern of ADH isozymes. Tube gels were run and stained for ADH activity as described in Materials and methods. About 50 μg supernatant protein was loaded in each tube. 1, *Saccharomyces cerevisiae* Y-10; 2, *S. uvarum*; 3, mixture of *S. cerevisiae* Y-10 and *S. uvarum*; 4, *S. cerevisiae* Y-7

relation between alcohol dehydrogenase and ethanol productivity seems to be difficult at present due to the complexity of the process and lack of knowledge of other influencing factors.

It is well estabilished that yeast alcohol dehydrogenase has three isozymes namely ADH I (cytosolic), ADH II (oxidative, cytosolic) and ADH III (mitochondrial, with five bands)[25-27] and they are also known to exhibit different kinetic characteristics.[28] To get a better understanding of alcohol dehydrogenase in the isolates the isozyme pattern was studied on polyacrylamide gels. Our results show that *S. cerevisiae* Y-10 and Y-7 have a similar isozyme pattern compared to *S. uvarum* (*Figure 1*). In the electrophoregram only one major band of alcohol dehydrogenase was seen which has lower mobility in both isolates compared to *S. uvarum*. The differences in the minor bands seen in the gels were not very obvious in our studies. Since the kinetic characteristics of the ADH isozymes are known to be different[29] it would be interesting to study the kinetics of the purified isozymes from these isolates. Though the multiplicity of ADH isozymes has been shown by others,[29] the kinetic characterization has not been carried out. The absence of multiplicity of ADH isozyme pattern in *Sch. pombe*[26] in contrast to other *S. cerevisiae* species also suggests that the kinetic parameters like K_m and V_{max} are of more importance.

It is known that the glycerol production is enhanced in partial alcohol dehydrogenase mutants lacking the particular isozyme[30] and the ethanol production is increased in petite mutants lacking mitochondrial isozymes of ADH.[31,32] Hence a more detailed study on the different aspects of ADH and other important enzymes are needed for a better understanding of the complex process of ethanol fermentation.

Acknowledgements

The authors are grateful to Dr V. Shankar for his useful suggestions and discussion in the preparation of the manuscript. The work received financial support under the United Nations Development Programme project of the Government of India DP/IND/80/003.

References

1 Aiba, S., Shoda, M. and Nagatani, M. *Biotechnol. Bioeng.* 1968, **10**, 845–864
2 Thomas, D. S. and Rose, A. H. *Arch. Microbiol.* 1979, **122**, 49–55
3 Brown, S. W., Oliver, S. G., Harrison, D. E. F. and Righeleto, R. C. *Eur. J. Appl. Microbiol. Biotechnol.* 1981, **11**, 151–155
4 Novak, M., Strehaiano, P., Moreno, P. and Gome, G. *Biotechnol. Bioeng.* 1981, **23**, 201–211
5 Hoppe, G. K. and Hansford, G. S. *Biotechnol. Lett.* 1982, **4**, 39–44
6 Dasari, G., Roddich, F. Connor, M. A. and Pamment, N. B. *Biotechnol. Lett.* 1983, **5**, 715–720
7 Wada, M., Kato, J. and Chibata, I. *Eur. J. Appl. Microbiol. Biotechnol.* 1968, **11**, 67–71
8 Ghose, T. K. and Bandyopadhyay, K. K. *Biotechnol. Bioeng.* 1980, **22**, 1489–1496
9 Panchal, C. J., Peacock, L. and Stewart, G. G. *Biotechnol. Lett.* 1982, **4**, 639–644
10 Brown, S. W. and Oliver, S. G. *Eur. J. Appl. Microbiol. Biotechnol.* 1982, **16**, 119–122
11 Gokhale, D. V., SivaRaman, H., Patil, S. G., Rao, B. S. and SivaRaman, C. unpublished results
12 SivaRaman, H., Rao, B. S., Pundale, A. V. and SivaRaman, C. *Biotechnol. Lett.* 1982, **4**, 359–364
13 Gas'con, S. and Lampen, J. O. *J. Biol. Chem.* 198, **243**, 1567–1577
14 Barron, E. S. G. and Levine, S. *Arch. Biochem. Biophys.* 1952, **41**, 175–187
15 Davis, B. J. *Ann. N. Y. Acad. Sci.* 1946, **121**, 404–427
16 Lutstorf, U. and Megnet, R. *Arch. Biochem. Biophys.* 1968, **126**, 933–944
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 1951, **193**, 265–275
18 Beaven, M. J., Charpentier, C. and Rose, A. H. *J. Gen. Microbiol.* 1982, **128**, 1447–1455
19 Thomas, D. S., Hossack, J. A. and Rose, A. H. *Arch. Microbiol.* 1978, **117**, 239–245
20 Nagodawithana, T. W., Witt, H. and Cutaia, A. J. *J. Am. Soc. Brew.* 1977, **35**, 179–193
21 Millar, O. C., Smith, K. G., Algar, E. and Scopes, R. K. *Biotechnol. Lett.* 1982, **9**, 601–606
22 Larue, F., Lafen-Lafourcade, S. and Gayon, R. R. *Biotechnol. Lett.* 1984, **6**, 687–692
23 Lee, K. J., Skotnicki, M. L., Tribe, D. E. and Rogers, P. L. *Biotechnol. Lett.* 1981, **3**, 207–212
24 Singh, R. and Kunkee, R. E. *Appl. Environ. Microbiol.* 1976, **32**, 666–670
25 Fowler, P. W., Ball, A. J. S. and Griffiths, D. E. *Can. J. Biochem.* 1971, **50**, 35–43
26 Wills, C. and Phelps, J. *Arch. Biochem. Biophys.* 1975, **167**, 627–637
27 Wills, C. and Jornwall, H. *Eur. J. Biochem.* 1979, **99**, 323–331
28 Wills, C. *Nature* 1976, **261**, 26–29
29 Singh, R. and Kunkee, R. E. *Arch. Microbiol.* 1977, **114**, 255–259
30 Johansson, M. and Sjostrom, J. E. *Biotechnol. Lett.* 1984, **6**, 49–54
31 Moulir, G., Boze, H. and Galzy, P. *Biotechnol. Lett.* 1981, **3**, 351–356
32 Esser, K., Schmidt, U. and Stahl, U. *Eur. J. Appl. Microbiol. Biotechnol.* 1982, **16**, 161–164

Biotechnology Letters Vol.4 No. 6 359-364 (198

CONTINUOUS ETHANOL PRODUCTION BY YEAST CELLS IMMOBILIZED IN OPEN PORE GELATIN MATRIX

H. SivaRaman, B. Seetarama Rao, A. V. Pundle and C. SivaRaman*

Biochemistry Division
National Chemical Laboratory,
Poona 411 008, India.

SUMMARY. Open pore gelatin pellets with entrapped yeast cells were obtained by selective leaching out of Ca alginate from the composite matrix followed by crosslinking with glutaraldehyde. *Saccharomyces uvarum* cells immobilized in the porous carrier showed high ethanol productivities with a maximum value of 250 g/l.h when monitored in packed bed reactors at 35°C with continuous cane molasses feedstock containing 10% fermentable sugars.

INTRODUCTION. Various techniques have been used for the immobilization of microbial whole cells, the most widely used being entrapment in hydrophilic gels since this permits high cell loading. Several polymer gel supports, both naturally occurring and synthetic, have been used for this purpose. Among these are polyacrylamide (Wada *et al.*, 1969), agar (Toda and Shoda, 1975), Ca alginate (Kierstan and Bucke, 1977), κ-carrageenan (Chibata, 1977) and gelatin (Gaddy and Sitton, 1981).

The main limitation with gel carrier systems is that of diffusional restrictions. High porosity of gel carriers is therefore a desirable feature. An open pore epoxy polymer carrier system has been obtained by Klein and Wagner (1978) and Klein and Eng (1979) by first preparing Ca alginate coated gel beads containing epoxy precursor, polyfunctional amines and microbial whole cells followed by crosslinking of the epoxy resin on drying and the subsequent leaching out of the Ca alginate component with phosphate buffer solution. Open pore gelatin pellets have been obtained by us using a similar approach wherein composite beads of Ca alginate - gelatin with entrapped yeast cells have been treated with phosphate buffer solution to disrupt the Ca alginate component, the residual gelatin then being crosslinked with glutaraldehyde. The preparation of such a system and the high productivities obtained for the conversion of cane molasses to ethanol are reported in the present paper.

MATERIALS AND METHODS *Saccharomyces cerevisiae* (NCIM 3107) and *Saccharomyces uvarum* (ATCC 26602) were obtained from the National Collection of Industrial Microorganisms, Poona, India. The cultures were grown in a New Brunswick LabroFerm batch fermentor of 14 ℓ capacity under standard conditions. Cells were harvested on the Sharples supercentrifuge and used without storage.

Cane molasses was obtained from a local sugar factory and analysed for total reducing sugars after inversion with HCl by the dinitrosalicylic acid method (Fischer and Stein, 1961). The molasses sample contained 58% w/w reducing sugars, 94% of which was fermentable.

Ethanol was analysed either by gas chromatography using Chromosorb 101 or chemically by the method of Reid and Truelove (1952). Values obtained by both procedures were in agreement.

Immobilization technique. Composite gel beads containing alginate and gelatin were obtained by a modification of the procedure described by Brodelius and Nilsson (1980). A slurry of 20 g wet packed yeast cells in 100 ml of an aqueous solution containing 20% w/v gelatin and 2% w/v sodium alginate at 40°C was dropped into a stirred solution of 0.07 M $CaCl_2$. The alginate in the composite beads was leached out by washing at about 10°C with 0.05 M potassium phosphate buffer (pH 7.6) till the washing was clear. The porous pellets were then crosslinked with 0.01 M glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.6) for a period of 1.5 h at about 10°C and were then washed thoroughly with water.

To determine whether the glutaraldehyde used for crosslinking of gelatin has any deleterious effect, the fermentation of cane molasses to ethanol by the immobilized cells was compared to that of suspended free cells in batch systems at 10% fermentable sugar concentration. When glutaraldehyde concentrations of 0.01 to 0.015 M were used, the rate of ethanol production by immobilized cells was higher than that of free cells by about 30%. Such enhancement in fermentation rates of immobilized systems has been reported by earlier workers (Holcberg

Margalith, 1981). Glutaraldehyde concentrations above 0.015 M, however, markedly lowered fermentation rates.

Packed bed immobilized cell bioreactors. Water jacketed glass columns of the type described by Krouwel *et al.*, (1980) were used (top 4.5 cm I.D.; bottom 3.5 cm I.D. and height 20 cm). The void volume (total reactor volume minus solid bead volume) was 30% of the working bioreactor volume of 230 ml. The immobilized cell concentration was 31 g dry weight/ℓ bead volume. The medium used for the continuous fermentation consisted of diluted cane molasses (pH 4.5) containing 0.1% urea. Ethanol productivity (g/ℓ.h) was determined on void volume basis as described by Margaritis *et al.*, (1981).

Scanning electron micrographs (SEM). The immobilized yeast was fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, then dried with increasing concentrations of acetone and finally in a stream of air. Thin sections were prepared, coated with gold and observed in a Cambridge Stereoscan Model 150 electron microscope.

RESULTS AND DISCUSSION. The leaching effect of phosphate on the composite Ca alginate - gelatin beads is apparent in the SEM (Fig. 1). The Open structure of the gelatin matrix with the exposed yeast cells after leaching with the phosphate buffer and crosslinking with glutaraldehyde can be seen in Fig. 1b.

Fig. 2 shows the relationship of ethanol productivity as a function of flow rates through the packed bed reactor column. The productivity with immobilized *S. uvarum* reaches a maximum of 250 g/ℓ.h at 35°C with 10% total fermentable sugar concentration of the cane molasses substrate. This value was obtained at a dilution rate of 7.1 h^{-1} and 70% utilization of the fermentable sugars.

The maximum productivity obtained at 30°C with the immobilized *S. uvarum* was 220 g/ℓ.h with 10% fermentable sugar concentration of the cane molasses. The bioreactor run continuously at 35°C for 30 days showed no decrease in the ethanol productivity and the gel beads

showed no disruption. Cell loss through leaching during runs is apparently not significant. Entrapped cells examined microscopically showed evidence of budding. The presence of some free cells in the effluent could probably be explained on the basis of cell growth during the runs.

Table 1 summarizes the ethanol productivities at various percentages of sugar utilization obtained with 10% feed sugar concentration.

TABLE 1

Ethanol productivities at 35°C and varying flow rates of molasses containing 10% fermentable sugars.

Flow rate (ml/h)	% utilization of feed sugar	Productivity (g/ℓ.h)
150	100	107
220	95	144
320	85	190
500	70	250

Williams and Munnecke (1981) have reported a maximum ethanol productivity of 53.8 g/ℓ.h calculated on void volume basis using Ca alginate immobilized *S. cerevisiae* systems at 30°C with 12.7% glucose as feed sugar and at 63% utilization. Margaritis *et al.*, (1981) have reported a maximum ethanol productivity of 102 g/ℓ.h for an inlet glucose concentration of 10% at 87% conversionlusing small Ca alginate beads of immobilized *Zymomonas mobilis* cells at 30°C. The markedly higher productivities obtained in the present investigation are apparently due to the open pore structure of the gelatin matrix.

ACKNOWLEDGEMENT We wish to thank Dr. S. R. Sainkar for taking the SEM. The work received support under the United Nations Development Programme project of the Government of India, IND/80/003.

Communication No. 2988 from the National Chemical Laboratory, Poona.

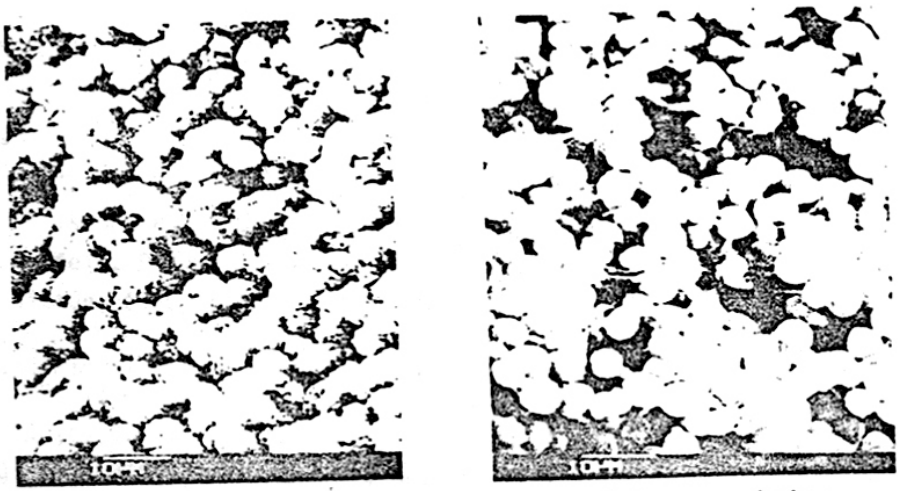


Fig. 1. SEM of immobilized cells of S.cerevisiae

(a)
Cells entrapped in Ca alginate-gelatin composite beads.

(b)
Entrapped cells in matrix after leaching out of alginate and crosslinking with glutaraldehyde.

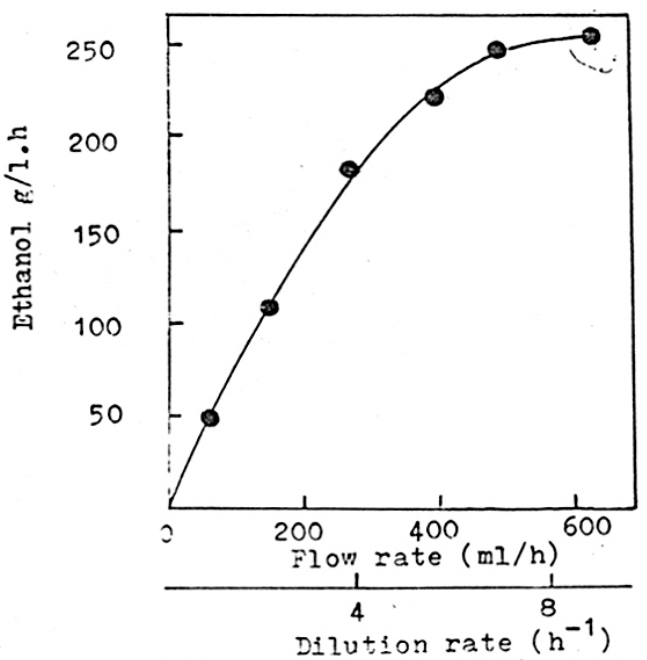


Fig. 2. Effect of dilution rate on productivity of immobilized yeast cells using molasses with 10% fermentable sugars.

REFERENCES

Brodelius, P. and Nilsson, K. (1980) *FEBS Lett.* 122, 312-316

Fischer, E. H. and Stein, E. A. (1961) *Biochem. Preparations* 8, 27-33

Gaddy, J. L. and Sitton, O. C. (1980) *Biotechnol. Bioeng.* 22 1735-1748.

Holcherg, I. B. and Margalith, P. (1981) *Eur. J. Appl. Microbiol. Biotechnol.* 13, 133-140.

Klerstan, M. and Bucke, C. (1977) *Biotechnol. Bioeng.* 19, 387-397

Klein, J. and Eng. H. (1979) *Biotechnology Letters* 1, 171-176

Klein, J. and Wagner, F. (1978) *DECHEMA-Monogr.* 82, 142-164

Krouwel, P. G., van der Laan, W. F. M. and Kossen, N. W. F. (1980) *Biotechnology Letters* 2, 253-258.

Margaritis, A., Bajpai, P. K. and Wallace, J. B. (1981). *Biotechnology Letters* 3, 613-618

Ohmiya, K., Hiroshi, O., Takashi, K. and Shimazu, S. (1977). *Appl. Environ. Microbiol.* 33,137-146.

Reid, V. W. and Truelove, R. K. (1952) *Analyst* 77, 325-328.

Toda, K. and Shoda, M. (1975). *Biotechnol. Bioeng.* 17, 481-497.

Wada, N., Kato, J. and Chibata, I. (1979). *Eur. J. Appl. Microbiol Biotechnol.* 8, 241-247

0273-2289/86/1201-0017$02.00

Ethanol Production by Yeast Cells Immobilized in Open-Pore Agar

B. S. RAO, A. V. PUNDLE, A. A. PRABHUNE, V. SHANKAR, AND H. SIVARAMAN*

Division of Biochemical Sciences, National Chemical Laboratory, Poona 411 008, India

Received July 25, 1985; Accepted October 29, 1985

ABSTRACT

An open-pore agar matrix has been shown to be suitable for the entrapment of microbial whole cells required for use in reactions that involve cell growth and gas evolution. Beads of porous agar with entrapped yeast cells have been used for the continuous fermentation of sugar cane molasses to ethanol, without apparent bead rupture, even after periods of 3 mo of use. The agar gel does not erode during prolonged operation, unlike porous gelatin cross-linked with glutaraldehyde.

Index Entries: Open-pore agar gel; carrier matrix, for *Saccharomyces sp.* immobilization; agar beads with entrapped yeast cells; packed bed reactor; sugarcane molasses, continuous fermentation; porous agar bead stability, in prolonged continuous fermentation.

INTRODUCTION

We had described earlier a procedure for obtaining open-pore gelatin beads as a carrier for the entrapment of yeast cells (*1*). Erosion from the interior of beads has been observed during a period of 2–3 mo when the gelatin with entrapped yeast cells had been used in packed-bed reactors for the continuous fermentation of sugar cane molasses to ethanol.

*Author to whom all correspondence and reprint requests should be addressed.

The degradation was apparently caused by microbial utilization of the matrix. An obvious alternative carrier that is resistant to microbial degradation, in general, is agar gel, which finds extensive use as a solid support for the growth of cell cultures. Applications of agar for whole-cell immobilization have been described in the literature, and these include the entrapment of *Escherichia coli* for its β-glucosidase activity (*2,3*), yeast for its invertase activity (*4*), *Rhodospirillum rubrum* for its hydrogenase activity (*5*), and plant cells for their secondary metabolites (*6*).

A general disadvantage with agar as a carrier is that of marked diffusional limitations (*4*). A specific problem in its use in reactions involving cell growth and gas production is its brittle nature and consequent rupture of the carrier gel during such fermentation, unless strengthened by incorporation of polymers, like polyacrylamide (*7*). The present paper demonstrates that these disadvantages can also be overcome by rendering the agar support porous through the selective leaching of calcium alginate from a composite matrix containing calcium alginate and agar. This approach had been used by us earlier for obtaining open-pore gelatin beads (*1*).

MATERIALS AND METHODS

Yeast

Saccharomyces sp, designated as Y-10, was used for the fermentation of cane molasses to ethanol. The isolate, which was substrate- and ethanol-tolerant, was obtained from sugar cane juice by Mr. D. V. Gokhale of our laboratory. Cells were grown in a New Brunswick Labroferm batch fermenter of 14 L capacity under standard conditions. Cell suspensions were either used directly for immobilization or harvested on the Sharples supercentrifuge and used without storage.

Molasses and Chemicals

The sample of sugar cane molasses was obtained from a local sugar factory and analyzed by the dinitrosalicylic acid method for total reducing sugars after inversion with HCl (*8*). The molasses sample contained 58% w/w sugars, 90% of which were fermentable. Agar and sodium alginate were from LOBA-Chemie Indoaustranal Co. Ethanol was analyzed by gas chromatography, using Chromosorb 101.

Immobilization Procedures

Agar Gel with Pregrown Yeast Cells

Open-pore gel beads, with the entrapped yeast cells, were obtained by selective leaching of calcium alginate from an agar-calcium alginate

composite matrix. The procedure was essentially similar to the earlier method described for obtaining open-pore gelatin beads (*1*), except that the glutaraldehyde treatment was omitted.

A slurry of 20 g of wet, packed, yeast cells in 100 mL of an aqueous solution containing 2% agar and 2% sodium alginate at 45°C was dropped into a stirred solution of 2% calcium chloride and the beads removed immediately after the end of the addition. The diameter of the beads was about 3 mm. The calcium alginate in the composite beads was leached out by washing with 0.05*M* potassium phosphate buffer at pH 7.5 until the washing was clear. The resulting porous agar beads were left overnight at pH 4.5 in the cane molasses diluted to 16% fermentable sugars, and packed the next day into columns for continuous fermentation.

Yeast Cell Growth Within the Bead Matrix

For obtaining the open-pore agar bead system with entrapped inoculum, 20 mL of *Saccharomyces* Y-10 cell suspension in the growth medium were mixed with 80 mL of a solution containing 2% agar + 2 g sodium alginate at 45°C. The growth medium contained in 1 L: glucose, 50 g; yeast extract, 3 g; malt extract, 3 g; and peptone, 5 g. Beads were obtained as described above and the calcium alginate leached out with potassium phosphate buffer.

Calcium alginate beads with the yeast inoculum were prepared from a suspension obtained by adding 20 mL of the inoculum to 80 mL of an aqueous solution containing 4 g of sodium alginate at 30°C. The suspension was dropped into a stirred solution of 2% calcium chloride and the beads left 1 h in the calcium chloride solution.

Beads in both cases were about 3 mm in diameter, and the yeast cell density was approximately 1×10^7 cells/g bead.

Packed-Bed Reactors

Water-jacketed columns of the type described by Krouwel et al. (*9*) were used (top, 5 cm id; bottom 3 cm id; and height, 18 cm). The bead vol was about 65% of the working bioreactor vol of 200 mL. The initial immobilized cell concentration was about 38 g dry wt/L bead vol. The feed stream was cane molasses containing 16% fermentable sugars supplemented with 0.1% urea and pH adjusted to 4.5. The column was operated at 30°C. Ethanol productivity (g/L/h) was determined on total reactor volume basis.

Scanning Electron Micrographs

The immobilized yeast was fixed, as described earlier (*1*), and sections observed in a Cambridge StereoScan model 150 Electron Microscope.

RESULTS AND DISCUSSION

Scanning Electron Micrographs of Gel-Entrapped Yeast Cells

The effect of leaching the calcium alginate–agar beads with potassium phosphate buffer at pH 7.5 can be seen in Fig. 1. The open-pore structure of the leached bead in which the entrapped cells are fully exposed (Fig. 1a) is clearly distinct from the covered cells observed in the unleached composite matrix (Fig. 1b). The effect of leaching on the carrier structure was similar to that observed with gelatin matrix (*1*).

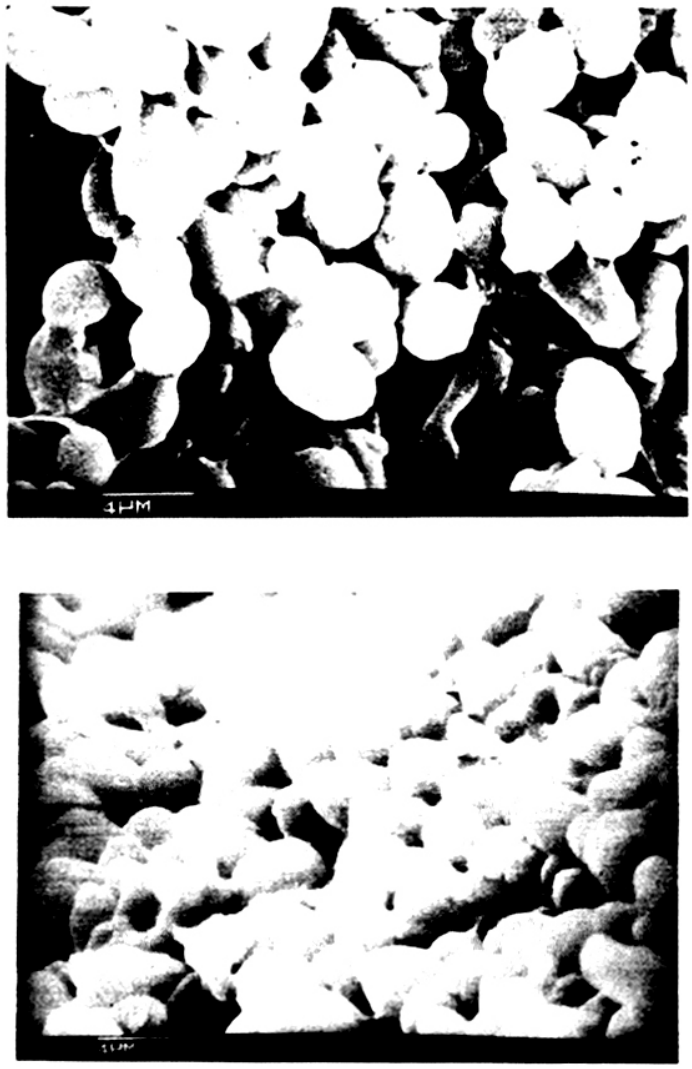


Fig. 1. Scanning electron micrographs of pregrown yeast cells entrapped in (a) porous agar bead and (b) calcium alginate–agar composite bead.

Figure 2 shows differences in cell growth from inoculum entrapped within the open-pore agar bead (Fig. 2a) and plain calcium alginate bead system (Fig. 2b). The cell density within the open-pore agar matrix is uniform and abundant throughout the bead, although the growth in the calcium alginate bead is markedly lower. The more porous nature of the former system could account for the enhanced availability within the beads of nutrients required for cell growth.

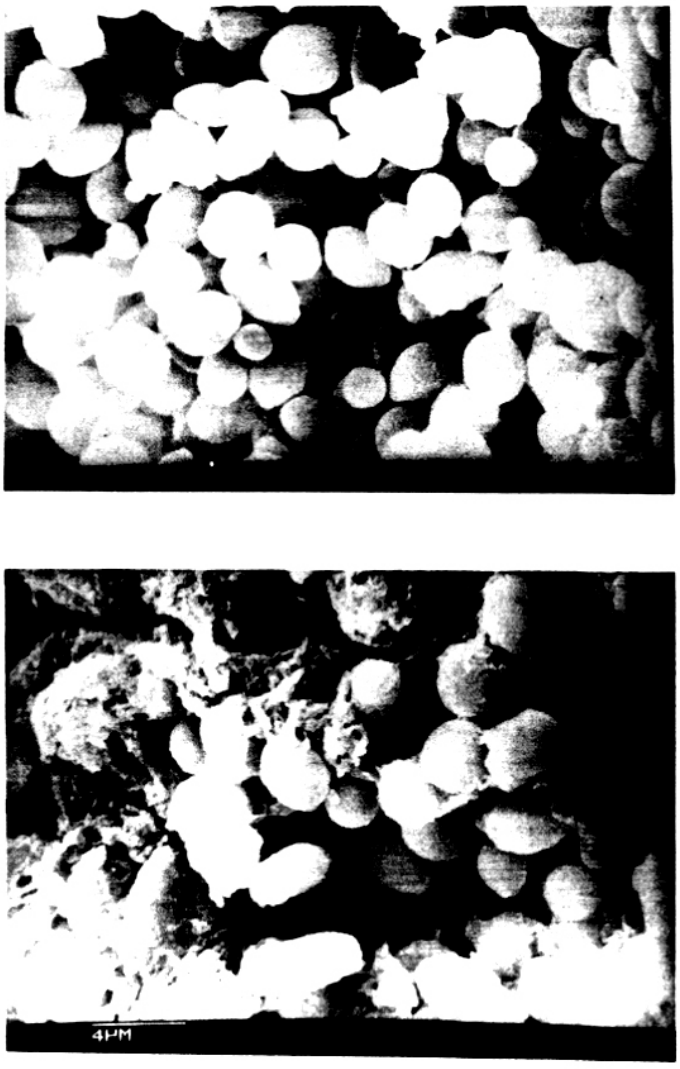


Fig. 2. Scanning electron micrographs showing growth of *Saccharomyces* Y-10 cells in (a) porous agar bead and (b) calcium alginate bead.

Continuous Fermentation of Cane Molasses to Ethanol

Preliminary experiments carried out, using the composite agar–calcium alginate beads before leaching the calcium alginate with phosphate treatment, showed that beads rupture during a period of 1–2

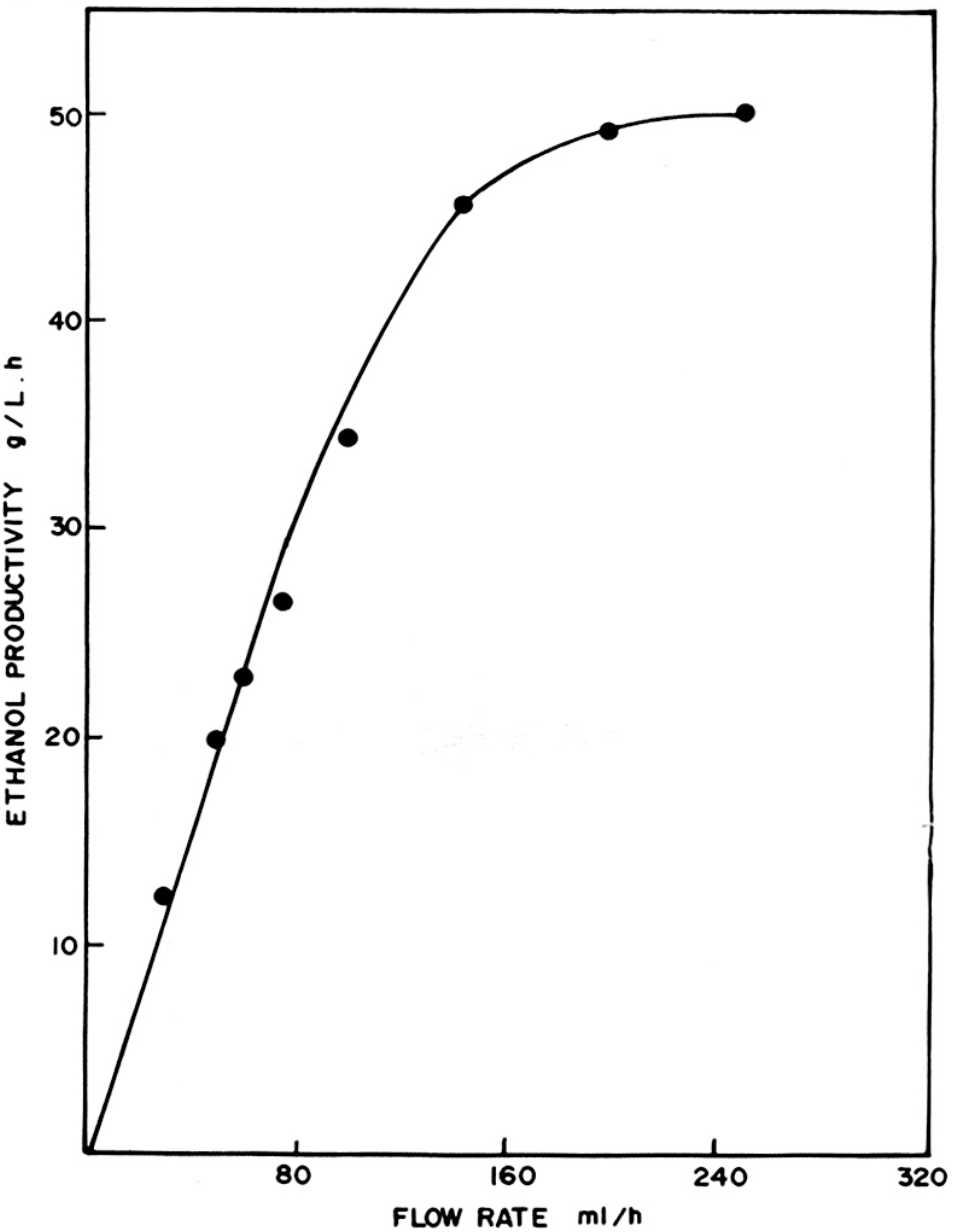


Fig. 3. Effect of flow rate on ethanol productivity of *Saccharomyces* Y-10 cells immobilized in open-pore agar beads, using cane molasses with 16% fermentable sugars.

TABLE 1
Ethanol Productivities at 30°C and Varying Flow Rates of Molasses Containing 16% Fermentable Sugars

Flow rate	% Utilization of feed sugar	Productivity, g/Lh
50	100	20
60	95	23
73	85	26
145	80	46

wk under the conditions of gas evolution and cell growth; this, however, was not observed with the porous agar beads obtained from leaching out of the calcium alginate component.

Figure 3 shows the relationship of ethanol productivity as a function of flow rates through a reactor column packed with open-pore agar beads with entrapped, pregrown *Saccharomyces* Y-10 cells. The ethanol productivities at various percentages of substrate utilization are summarized in Table 1.

The productivity of ethanol at 95% conversion efficiency and about 77 g/L product concentration is 23 g/L h which is comparable to that reported for calcium alginate gel bead immobilized system operated in fluidized bead reactors for continuous fermentation of cane molasses to ethanol (*10*).

The operational stability of the packed-bed reactor with open-pore beads used continuously for the fermentation of cane molasses to ethanol is shown in Fig. 4. No decrease in ethanol productivity was observed up to a month of operation, after which the conversion efficiency dropped

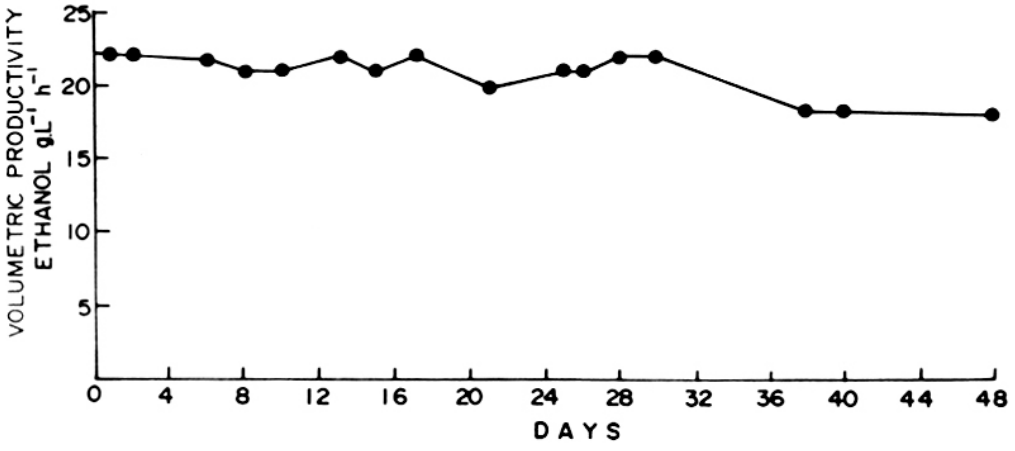


Fig. 4. Operational stability of packed-bed bioreactor in the continuous fermentation of cane molasses to ethanol, using *Sacchromyces* Y-10 immobilized in open-pore agar beads. Cane-molasses feed stream, containing 16% fermentable sugars; 30°C. Initial product concentration was 77 g/L ethanol.

and the ethanol concentration in the effluent became limiting at about 58 g/L, with a consequent decrease in the volumetric ethanol productivity to about 17–18 g/L/h. No apparent change in bead structure could be observed even after periods of about 2 mo of continuous use.

CONCLUSIONS

Open-pore agar beads can be obtained by leaching calcium alginate out of a composite matrix of agar and calcium alginate. The porous agar matrix is suitable for entrapment of yeast cells and is operationally stable even with gas evolution during the fermentation of molasses to ethanol.

ACKNOWLEDGMENTS

We thank Dr. C. SivaRaman for useful suggestions, discussions, and help in the preparation of this manuscript. Thanks are also due to Mr. S. M. Kotwal for technical assistance and Mrs. A. Mitra for taking the scanning-electron micrographs. This work received support under United Nations Development Project of the Government of India, IND/80/003.

REFERENCES

1. SivaRaman, H., Rao, B. S., Pundle, A. V., and SivaRaman, C. (1982), *Biotechnol. Lett.* **4**, 359.
2. Toda, K. (1975), *Biotechnol. Bioeng.* **17**, 1729.
3. Bannarjee, M., Chakrabarty, A., and Majumdar, J. K. (1982), *Biotechnol. Bioeng.* **24**, 1839.
4. Toda, K., and Shoda, B. (1975), *Biotechnol. Bioeng.* **17**, 481.
5. Weetall, H. H., and Bennett, M. A. (1976), Abst. of papers from *5th Int. Ferm. Symp.*, Berlin, 299.
6. Brodelius, P., and Nilsson, K. (1980), *FEBS Lett.* **122**, 312.
7. Kuu, W. Y., and Polack, J. A. (1983), *Biotechnol. Bioeng.* **25**, 1995.
8. Fisher, E. H., and Stein, E. A. (1961), *Biochem. Prep.* **8**, 27.
9. Krouwel, P. G., Van der Laan, W. F. M., and Kossen, N. W. F. (1980), *Biotechnol. Letts.* **2**, 253.
10. Nagashima, M., Azuma, M., Noguchi, S., Inuzuko, K., and Samejima, H. (1984), *Biotechnol. Bioeng.* **26**, 992.

Biotechnology Letters Vol 7 No 8 615-618 (1985)

YEAST CELLS ENTRAPPED IN LOW-GELLING TEMPERATURE AGAROSE FOR THE CONTINUOUS PRODUCTION OF ETHANOL

V. Shankar*, S. M. Kotwal and B. Seetarama Rao

Biochemistry Division
National Chemical Laboratory
Pune 411 008, India

SUMMARY

Continuous ethanol production by S. uvarum immobilized in a low-gelling temperature agarose namely SeaPlaque agarose was studied in a packed bed reactor at 30°C using sugarcane molasses containing 13.5% fermentable sugars as feed. The productivity at 95% conversion was 23 g/l.h (on reactor volume basis). The bioreactor was run continuously at a fixed dilution rate and it retained 60% of its initial activity upto 80 days.

INTRODUCTION

The advent of immobilized whole cell technology has led to increasing efforts to replace the conventional fermentative processes with immobilized systems since the latter offers many practical advantages. Among the various methods used for the immobilization of whole cells,entrapment in hydrophilic gels (Mosbach and Mosbach 1966, Toda and Shoda 1975, Kierstan and Bucke 1977, Wada et al. 1979, Gianfreda et al. 1980) is preferred since it is simple and permits high cell loading. Development of open pore gels (Klein and Kressdorf 1982, SivaRaman et al. 1982) has helped in overcoming the diffusional restrictions associated with the conventional gel systems. SeaPlaque agarose has the unique property of gelling at a low temperature. A dilute gel solution (1-2%) will set to a reasonably firm gel in 10 min. at 25°C and once gelled will not remelt unless the temperature exceeds 65°C. Since this property can be made use of to entrap cells under mild conditions, an attempt was made to immobilize yeast cells in SeaPlaque agarose and study the immobilized system for the continuous fermentation of cane molasses to ethanol.

MATERIALS AND METHODS

Saccharomyces uvarum (ATCC 26602) obtained from the National Collection of Industrial Microorganisms (NCIM), Pune, India was grown under standard

conditions and used immediately after harvesting.

Cane molasses obtained from a local sugar factory was diluted to 13.5% fermentable sugars with water, adjusted to pH 4.5 with concentrated sulfuric acid and autoclaved. Reducing sugar was determined by the dinitrosalicylic acid method (Fischer and Stein 1961). Before use, urea was added to a final concentration of 0.1%.

Ethanol was analyzed by GLC using chromosorb 101 and chemically according to the method of Reid and Truelove (1952).

SeaPlaque agarose was a generous gift from the Marine Colloids Division, FMC Corporation, USA.

Cell immobilization: Entrapment of yeast cells in SeaPlaque agarose was carried out according to the method of SivaRaman et al. (1982). 20 g of wet packed cells were mixed thoroughly with 100 ml of 2% (w/v) aqueous solution of SeaPlaque agarose and 2% (w/v) sodium alginate. The resulting suspension (maintained approximately at 40°C) was dropped into 2% calcium chloride solution (maintained around 20°C) under mild stirring. From the composite beads, alginate was leached out by treating the beads with 0.05 M potassium phosphate buffer (pH 7.5) till the washings were clear. The immobilized beads were then activated by incubating them with cane molasses containing 15% reducing sugars at 4°C overnight.

Immobilized cell bioreactor: Activated beads were packed in a water-jacketed glass column(top 4.5 cm I.D., Bottom 3.5 cm I.D. and height 27 cm) of the type described by Krouwel et al. (1980). The temperature of the bioreactor was maintained at 30°C. Cane molasses feed contained 13.5% fermentable sugars.

RESULTS AND DISCUSSION

In the continuous operation, steady states were established at substrate feed ranging from 0.35 h^{-1} to 3.5 h^{-1}. Profiles of ethanol productivity with respect to dilution rates are given in Fig. 1. A productivity of 23 g/l.h was obtained at a dilution rate of 0.35 h^{-1} with 13.5% fermentable sugars in the feed at 95% utilization. However, the maximum productivity of 68 g/l.h was obtained at a dilution rate of 2.8 h^{-1} at 35% utilization. The bioreactor was run continuously at a fixed dilution rate (0.21 h^{-1}) using cane molasses feed containing 13.5% fermentable sugars at 95% utilization. The reactor retained 60% of its initial activity upto 80 days. There was no significant bead disruption during the operation. Similar results were obtained with cells immobilized in 1% SeaPlaque

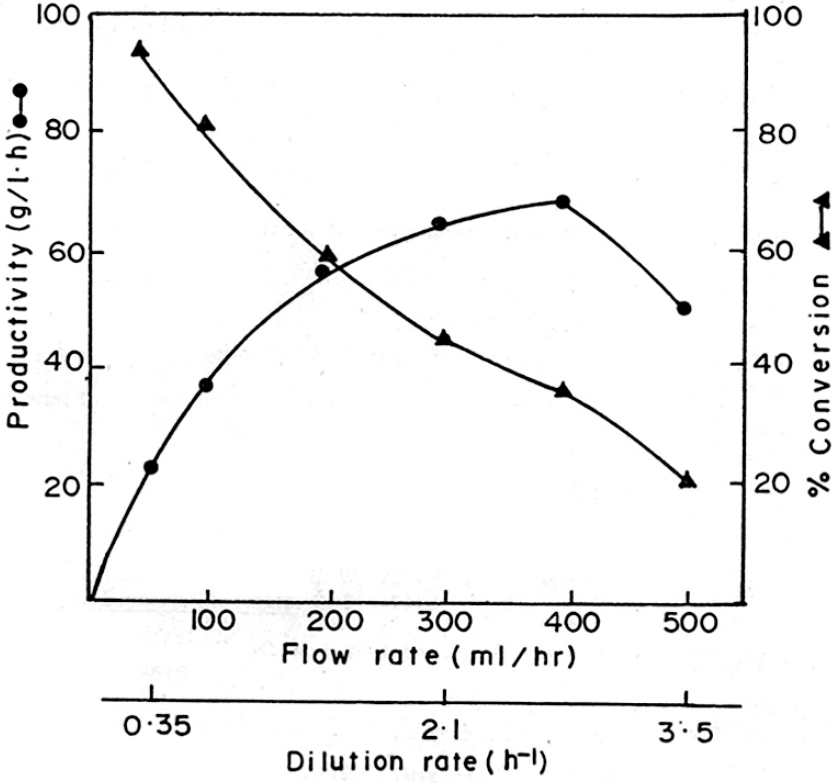


FIG. 1. EFFECT OF DILUTION RATE ON PRODUCTIVITY

agarose (data not shown), but 2% gels were more stable.

Due to its low-gelling temperature characteristics, SeaPlaque agarose can be used as a support for the entrapment of temperature sensitive microbial and plant cells by open pore method under mild conditions. Since the beads are stable, further hardening by covalent cross-linking (which affects the cell viability) can be avoided.

ACKNOWLEDGEMENT

We wish to thank Mrs. A. A. Prabhune for preparation of the cells. The work received support under the United Nations Development Programme of Government of India No. IND/80/003.

Communication No. 3785 from the National Chemical Laboratory, Pune.

REFERENCES

Fischer, E. H. and Stein, E. A. (1961) Biochem. Preparations, 8, 27-33.

Gianfreda, L., Parascandola, P. and Scardi, V. (1980) Eur. J. Appl. Microbiol. Biotechnol., 11, 6-7.

Kierstan, M. and Bucke, C. (1977) Biotechnol. Bioeng. 19, 387-397.

Klein, J. and Kressdorf, B. (1982) Biotechnol. Letts. 4, 375-380.

Krouwel, P. G., van der Laan, W. F. M. and Kossen, N. W. F. (1980) Biotechnol. Letts. 2, 253-258.

Margaritis, A., Bajpai, P. K. and Wallace, J. B. (1981) Biotechnol. Letts. 3, 613-618

Mosbach, K. and Mosbach, R. (1966) Acta Chem. Scand. 20, 2807-2810.

Reid, V. W. and Truelove, R. K. (1952) Analyst. 77, 325-328.

SivaRaman, H., Seetarama Rao, B. S., Pundle, A. V. and SivaRaman, C. (1982) Biotechnol. Letts. 4, 359-364.

Toda, K. and Shoda, M. (1975) Biotechnol. Bioeng. 17, 481-497.

Wada, M., Kato, J. and Chibata, I. (1979) Eur. J. Appl. Microbiol. Biotechnol. 8, 241-247.