STUDIES ON IMMOBILIZATION OF BACTERIA

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IN
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by

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CERTIFICATE

Certified that the work presented in this thesis is based on the bona fide work done by Mr. Mohandass. C, under my guidance in the Centre for Biotechnology, Cochin University of Science and Technology and that no part thereof has been included in any other thesis submitted for the award of any degree.

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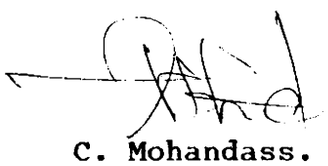
DECLARATION

I hereby declare that the work presented in this thesis is based on the original work done by me under the guidance of Dr. M. Chandrasekaran, Head, Centre for Biotechnology, Cochin University of Science and Technology and that no part of this thesis has been included in any other thesis submitted previously for the award of any degree.



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CONTENTS

INTRODUCTION

1.1. PREFACE :

Cell immobilization technology is a rapidly expanding area in the endeavour of microbial fermentation. During the last 15 years several processes have been developed and more are in developmental stage of approaching commercial utilization. Although cell immobilization is just now receiving wide spread attention, Immobilized cells have been successfully exploited for many years. One of the earliest example for use of immobilzed cell is vineger production. In quick vinegar process the microbial film which develop on wood shavings convert ethanol to acetic acid. Immobilized cells are the principle components of the trickling filter in waste treatment system and they are responsible for leaching low grade mineral ores where Chemoautotrophic bacteria attatched to the mineral surfaces brings about the leaching through the oxidation of mineral sulphites. Other natural habitates such as soil, marine environment, and even dental plaque are important ecosystem where immobilized microbes can play an important role. The term immobilization should not be restricted to the cells attached to solid surface. It can be considered as a physical confinement or localization of microbial cells. The clustered cells function as a heterogeneous catalyst in practice. This permits the economical reuse of the

microorganism. Immobilized microorganisms may display properties quite different from those of free suspended cells. These altered properties offer advantages to biological process that cannot be attained with conventional fermentation systems or with immobilized enzymes. The immobilized enzyme system so successfully applied on large scale operation to single step reaction and non cofactor linked reactions, because the continuous generation of cofactor is an unsolved problem.

The immobilized cell system offer highly accelerated reaction rates due to increase in cell density, facilitates operation at high dilution rate without wash out of the cells, and higher productivity. Immobilized cell system does not necessiate neither extration nor purification of enzymes from microbial cells. The most obviate benefit is capable to operate continuously. In continuous process the immobilized cells are not affected by the inhibited compounds and nutrient depletion. Further more the continuous immobilized cell reactor can be operated using nongrowing cells, which may be advantages for the production of some metabolites. Since dense cell population can be maintained in the system without causing rheological problems the mixing and mass transfer properties are improved.

The added beneficial feature of immobilized cell systems are the inert solid support, in dilute media may concentrate nutrients at a liquid solid interface and cells

attached to these supports are exposed to higher nutrient concentrations than exist in the bulk liquid, higher cell population, faster growth rate and rapid production may be achieved.

Inspite of the potential advantage offered by immobilized cell technology, cell immobilization do have certain inherent disadvantages and hence call for precautions. Thus in the process of immobilization the chances of few key enzymes being inactivated due to the presence of various polymeric compounds the desirable end product formation is greatly affected. In some cases adsorption of reaction products on to the supporting matrix may result in the decrease in productivity because of their toxicity. In large scale operation the reactor performance greatly depend upon several chemical engineering problems such as pressure drop, void volume, bed shrinkage, bed fouling associated with biological problems like contaminations and rapid degeneration of metabolic efficiency of the cell.

However the advantages offered by cell immobilization studies out weighs the drawbacks and hence the interest to develop novel immobilized cell system is continued in the research community.

Large number of techniques are available for immobilization on different supports. Most of the techniques used for immobilization of soluble enzymes have also been found suitable for cells and organelles. Selection of

techniques for immobilization depends on the nature of cells and number of chemical compounds and its ultimate applications in a proper reactor geometry. More gentle methods are often required for the immobilization of viable cells as compared to non viable cell system (D'Souza 1989)

In principle four different types of immobilized methods are distinguished. These include entrapment, covalent binding, cross linking and adsorption. No single system can be applicable to all enzymes or cells in view of the differences in their composition and overall charged distribution. The substrate concentration may also influence these methods.

Among the techniques the most extensively studied method so far in cell immobilization is entrapment of microbial cells in polymer matrix. The matrix used were Agar, Alginate, Carrageenan, Acrylamids, Agarose, Hen egg white, Collagen, Cellulose, Epoxy resin, polyster, polystyrene and polyurethene (Tampion & Tampion 1987).

Alginate has been extensively investigated for the preparation of immobilised system whereas acrylamide polymers were used after treating with or by radiation for preparation of immobilized non viable system (D'Souza & Nadkarni, 1980;Despande etal 1987).

The ability of alpha-amylase to cause mild changes in random degradation of starch has been of vital importance in baking, brewing, textile and paper manufacturing and starch liquefaction. Many important advantages for using alpha amylase includes use as supplement in the improvement of bread colour, flavour, texture and shelf-life.

In the food industries starch is used for the production of starch syrups like glucose syrup, maltose syrups and fructose syrups for the production of alcoholic beverages and in baking industries. In the non food industries starch is widely used in the paper and textile industries as a thickener; as adhesive and in chemical and pharmaceutical industries. Even starch is found to be used in the mining and oil exploration.

Hence in the light of the overwhelming significance of immobilized cell system mentioned above it was planned to apply the process of immobilization of viable cells of alpha-amylases producing *B. polymyxa*, isolated from Cochin University of Science and Technology campus, with a multilateral approach of maximizing alpha amylase production by batch and continuous process, starch degradation, reducing sugar production and coimmobilization along with yeast for the direct conversion of starch to alcohol using various starch sources employing alginate entrapment techniques.

1.2. Review of Literature.

1.2.1 Techniques of Immobilization.

Attention is paid only to the entrapment technique among the various techniques of immobilization while reviewing the available literature as other techniques are out of scope of the present study.

a) Polyacrylamide

Polyacrylamide gels have been used by several workers to entrap bacteria. Thus polyacrylamide entrapped cells were used for the production of L - citruline from L-Arginine by Streptococcus faecalis (Franks, 1972): glutamic acid from glucose by Corynebacterium glutamicum (Slowinski & Charm, 1973); L-aspartic acid from ammonium fumarate and 5 hydroxy L-Tryptophan from 5-hydroxy indole and Serene using E. coli (Chibata et al., 1974); L-lysine from 2,6-Diaminipimelic acid by Microbacterium ammoniaphilum (kanamitsu, 1975) L-Sorbosone from L-Sarbose by Gluconobacter melanogenus (Martin and Perlman,1976); benzene degradation by Pseudomonas putida(Somerville et al.,1977) production of alpha amylase by Bacillus subtilis (Kokubu et al.,1978); preparation of glucose 6- phosphate by Achromobacter butyri (Murata et al.,1979); productin of L-Citrulline (Kolot, 1981); accumulation of divalent metal cation by citrobacter (Macaskio and Dean, 1985); debittering of citrus juice by Corynebacterium fascians (Hosogawa et al., 1985); production of L-Sorbose by Gluconobacter suboxydans (Stofanova et al ., 1987); urocanic acid production by Achromobacter liquidium (Larsson & Mattiason, 1988) and synthesis of L-Dopa by E.Intermedia (Para and Baratli, 1988).

b) Alginate

Microbial cells have been reported to be immobilized in Alginate beads for the denitrification of water

by Pseudomonas denitrificans (Nilsson et al., 1980, Godbole et al., 1984); production of Vitamin B 12 by Propionibacterium sp. (Youngsmith and Chutima, 1983); ethanol production by Zymomonas mobilis (Margaritis et al., 1981); conversion of glycerol to dihydroxyacetone by Glucobobacter oxydans (Aldercreutz et al 1985) ; stereo selective reduction of oxo-acid esters by Thermo anaerobium brockii (Sonnleitner & Fiech ter, 1986) degradation of n-Valiric acid by Alcaligenes denitrificans (Caunt and Chase, 1987) production of propine oxide by Mycobacterium sp (Brink and Trempr 1987); protease production by Serratia marcescens and Myxococcus Xanthus (vuillomard et al 1988) apha amylease producton by Bacillus subtilis (Oriol, 1988) Bacillus coagulans (Nandakumar & Chandrasekaran, 1990); and B. Polymyxa (Mohandass and Chandrasekaran, 1992); production of Pepsin (Chitra and Gowrichandrakasan, 1992); and continuous synthesis of thermostable alpha- amylase by Bacillus (Jamuna and Ramakrishna, 1992).

c) K. Carrageenan.

Kappa-Carrageenan beads have been employed for immobilization of E coli for the production of L-Aspartic acid (Nishida et al., 1979); Pseudomonas flavum for L-Malic acid production (Tekata et al., 1980 & 1984). Brevibacterium decunhae for the production of L-Aspartic acid (Furni and Yamashita, 1983); Clostridium thermoaceticumfor acetic acid production (Wang and Wang, 1983) Penicillium chrysogenum for the production of penicillin-G (Deo & Gaucher, 1983); E coli for the degradation of aromatic rings of xenobiotics (Dhulster et al., 1984); protoplast of Doucus carota viability studies (Linsefors and Brodelius, 1985); Scendesmous actus and S. obliques for waste water nutrient removal; (Chevalier et al., 1987); Protease vulgaris for depolymerization of Chondroitin C-sulphate (Saito et al., 1986); Rhodospirillum ruburum for H2 production (Hirayama et al., 1986) →,

→ Brevibacterium flavum (protoplast) for preparation of L-Glutamate (karubē et al., - 1987); Myxococcus xantus for the production of extra_cellular protein (Younes et al., 1987) Streptomyces glavuligerus for the basic studies of O2 uptake (Scott et al., 1988); and Fungal mycelium in Kappa carrageenan for continuous synthesis of gluco amylase (Emilia Abraham et al., 1991).

d) Agar.

Microbial cells have also been entrapped in agar for glucose production from Lactose (Toda, 1975) by E. coli;

for the production of glucose and fructose by Saccharomyces pastorianus (Toda and Shoda, 1975); for hydrogen producton by Clostridium butyricum (Suzuki et al., 1980); for the degradation of Caffine by Pseudomonas putida (Middle Kovine and Bakker, -1982); for phenol degradation by Methenogenic bacteria (Dwyer et al.,1986); and for hydrogen production by Rhodospirillum ruburm (Hirayama, 1986).

e) Agarose

Agarose beads were used for immobilzing B. subtilis and E.coli containing plastid encoding rat pro insuline for the production on insulin (Mosbach et al., 1983) , and Thermoanaerobium brockii for stereo selective reduction of acid extract (Sonnleitner and Fiechitor, 1986).

1.2.2. Physoiology and Characterization of immobilized cells

The understanding of the relationship between the catalytic behaviour of the fixed cells is inadequate inspite of many observations. Primarily the change in the behaviour was attributed by the availability of nutrients (Brodelius et al 1979). During fermentation under immobilized state enhanced glucose transport led to increased rate of ethanol formation inspite of the fact that the substrate transportation is intercepted by support surface due to anchorage of the part of the cells (Von Steveninck and Rothstein, 1965 and Van uden, 1967).The increased metabolic rate of alcohol formation also suggested that it is due to the damage caused to the immmobilized cells, consequently resulting in the cell permeability and fast entry of glucose (Suzuki, 1972).

Penek (1963) while studying the cell cycle of S. cerevisiae under immobilized conditions observed the exclusion of buds following the degradation of cell storage in the period of polysyntehsis. Hartwell et al (1974) , Mitchison (1972), Barsel-Hahn-Hageradal (1989) and Sonal Vora (1986) have also studied the growth cycle of yeast.

The attatchment of microbial cells on carriers alters the permeability of cell membrane (Atkinson and Fowler, 1974 Marshall and Fletcher, 1982) and led to a short term increase in the respiration rate at the surface. (Navaroo and Durand, 1977). It has also been reported that the clustered yeast cells have vast change in generation time (Ghose and Bandhopadhyay, 1980). It was demonstrated that yeast cells appeared in the samples and followed a cyclic fashion and released periodically (RamaKrishna and Jamuna, 1990). Shimuzuet al (1979) observed that conditions of optimal growth rate for immobilization are often different from free cells. Ghose and Bandhopadhyay (1980) observed substantially higher product yields than those of free cells while Doran and Bailey (1984) reported that the product formation in immobilized cells are uncoupled with growth. Nishida et al (1979) indicated that mechanical strength and operation stability of the beads are due to hardening treatment with glutaraldehyde. Vorlop et al. (1981) indicated that drying of immobilized spheres has greately increased the compression strength. Martinson et al (1989) has reported that the gel forming capacity of the alginate

can be correlated with the proportion to L-glucuronic acid in the polymeric reaction. Ogbonna et al (1989) studied the various factors that affect the Ca-alginate gel beads. Martinson et al (1989) have correlated the composition sequence, structure and the physical properties of the gel beads.

Matteau & Saddler (1982) studied the half life of Trichoderma viridiae in the packed bed reactor column. Margaritis and Bajpai (1981) reported that higher cell loading will reduce the gel strength. Brink & Tramper (1986) reported that the internal pore diffusion of the limiting substrate affects mass transfer systems in Mycobacterium.

1.2.3 Immobilized Bioreactors.

The choice of an appropriate design of immobilized cell reactor is fundamental to the success of a process. In general, the type of reactor chosen determines the type of immobilized cell preparations which can be used and vice versa. The common reactors which are applied include fluid bed reactor (FBR), stirred tank reactor (STR), air lift reactor (ALR), column reactor etc.

Studies on immobilized Fluid Bed Reactors are rather limited and concerned with use of FBR at a pilot plant scale for the waste water denitrification (Stephenson and Murphy, 1980); decolourization of Kraft paper mill effluent (Royer et al., 1983); aerobic treatment of waste water (Bull et al., 1983) and development of fluidized bed

with sand as a biofilm support material in n-butanol and isopropanol production by calcium alginate immobilized Clostridium butyricum (Kronwel al 1980), continuous production of ethanol by Z. mobilis. (Strandberg, 1982; Margaritis & wallace,1982); and in a three stage inverted cone fluidized bed reactor system for Z mobilis (Klein & Kressdr-of, 1983).

Stirred tank reactors were reported to be used for maintaining stable condition (Humphrey & Millis, 1973), volumetric productivity of ethnol (Margaritis & Bajpai, 1981), isopropanol-butanol-ethanol (Krouwel et al., 1983) and acetone and butanol (Pierrot et al., 1986).

Column reactors were reported to be used for phenol degradation by Candida tropicalis (Klein et al, 1979), seperation of different phases of organic wastes to methane (Merrig, 1982), continuous synthesis of glucoamylase (Anitha, 1989; Emily Abraham, 1991) and alpha amylase (Jamuna & Ramakrishna 1992). Air lift reactors were used for the transformation on hydrocortisone by alignate immobilized Arthrobactor, simplex. (Kloosterman & Lilly, 1986).

1.2.4 Co - immobilization.

Co-immobilization involves the immobilization of two biocatalysts either simultaneously or one after another on a support by one or more methods. In the case of cells which do not contain all the components of enzymes necessary for carrying out a specific conversion, the cells are co-immobilized along wth an anzyme. Binding of the deficient

enzyme from an external source to free or immobilized microorganisms leads to co-immobilizates which combine the biocatalytic properties of the cells and of additional enzyme from another source (Hartmeir, 1983, 1984, 1985; D'Souza 1989).

Martin & Perlman (1976), used co-immobilized cells to convert sorbose into 2-keto guloconic acid. Trampler (1978) co-immobilized Adenate Kinase and Acetate Kinase in polyacrylamide gel and used to regenerate ATP/or ADP. D'Souza and Nadkarni (1980) prepared co-immobilizates by binding the deficient glucoenzymes to carbohydrate rich cell wall of yeast using coñ. Hartimer et., al (1984).co-immobilized S. cerevisiae with beta galactosidase directly to ferment lactose in whey. D'Souza and Melo (1989) developed a method for obtaining co-immobilizates by the simultaneous binding of enzyme to yeast cells and enzyme bound cells through adhesion to glass or cellulosic fibres by imparting anion exchanger property to yeast cells using polyethylenimine. Co-immobilizates were also obtained by immobilization of mixed cultures (Godbole et al, 1983 ; Tanaka and Murakami, 1986).

Co-immobilized enzyme/cell systems have successfully been used for the hydrolysis of lactose in milk under minimal microbial contamination by E. coli and glucose oxidase (Kaul et al 1984, 1986), sucrose to gluconic acid and fructose by Saccharomyces cerevisiae and glucose oxidase. (D'Souza and Nadkarni, 1980, & D'Souza, (1981); cellulose fermentation by S. cerevisiae and Pepsin (Hartmeir, 1981);

cellulose fermentation by S. cerevisiae and beta glucosidase (Hahn Hagerdal, 1983); lactose fermentation by S. cerevisiae and B.galactosidase (Hartmeir et al 1984) and oxygen removal from beer by Aspergillus niger and glucoamylase (Hartmeir 1981). Behavoiur of algae and bacteria co-immobilized in carrageenan in a fluid bed reactor was also observed by Chovalier (1988).

1.2.5 Bacillus and their Amylases.

Amylolytic enzymes are widely distributed in plants, animals and in microorganisms (Boyer & Ingle, 1972). Among them the microbial amylases have drawn the attention of many investigators in the recent years (Robyt & Ackerman, 1971; Shinke, 1975).

Among the many candidates of bacteria, Bacillus is an acknowledged source of amylase for various applications.(Coleman & Elliot, 1962; Welker & Campbell, 1963; Shinke, 1975; Anderson, 1985). Bacillus sp. is known to produce both alpha and beta amylases. Species of Bacillus subtilis, B. coagulans, B.stearothermophilus, B. licheniformis and B. amyloliquifaciens have been reported to produce alpha amylase (Welker & Campbell, 1963; Saito, 1973; Medda & Chandra, 1980; Pinches et al, 1985; Shah, 1989). While Bacillus cereus, B polymyxa, B.megaterium and B. circulans were reported to produce beta amylase (Marshall, 1974; Shinke, 1975; Takasaki, 1976; Taniguch, 1983; Kawazu, 1987). Recently these strains are reported to produce alphaamylase besides beta amylases (Yoshigi et al, 1988; Uozumi 1989).

Alpha amylase production in B. amyloliquifaciens and B.subtilis repressed by glucose (Boyer & Ingle, 1972; Tsuchiya, 1975; Saito & Yamamoto, 1975 and Siddhartha et al., 1989) and induced by maltose (Coleman et al., 1962; Yamamoto, 1975; Siddhartha et al 1989; Yoon et al, 1989.

Thermostable alpha amylase production was reported in B. stearothermophilus (Hartman, 1955; Pfueller & Elliot, 1969; Ogasahara, 1970) B. acidocaldreus (Bunoocore et al., 1976) B. licheniformis (Saito, 1973; Madsen et al., 1973; Chiang et al., 1979; Medda & Chandra, 1980; Morgan & Priest, 1981; Yankov et al., 1986; Bajpai & Promod, 1989). B. subtilis; Lin & King, 1988). B. amyloliquifaciens (Remesh & Lonsane, 1989) B. coagulans campbell, 1954; Medda & Chandra 1989 ; (NANDAKUMAR & CHANDRASEKHARAN (1990))

1.2.6 Immobilization of Bacillus and Amylases.

Species of Bacillus has drawn the attention of several researchers working on immobilization, Bacillus sp. has largely been immobilized for the production of alpha amylase. Bacillus subtilis (Argiravco, 1978; Kokobu et al, 1978, 1981; Castro et al, 1992) B amyloliquifaciens (Shinmyo, 1982; Argivakos, 1992) B. stearothermophiles (Beddows et al, 1984), B. coagulans (Nandakumar & Chandasekaran, 1990) B. licheniformis (Hu et al, 1991) and B. polymyxa (Mohandas & Chandrasekaran, 1992) and Bacillus sp; (Suzuki & Karube, 1979; Shinmyo et al., 1982; Anderson etal, 1985; Takaya et al, 1988; Jayima & Ramakrishna, 1992) have been immobilized. Bacillus sp have also been reported to produce bacitracin (Yasushi et al 1980). While immobilized

Bacillus spores have been used for the biological control of mosquitoes (Joshi et al, 1992). B.polymyxa were immobilized and tested for water purification in biofilters (Mohandas & Chandrasekaran, 1990). Reports on immobilization of alpha amylases are rather limited. Alpha amylases were used for the production of glucose and maltose after immobilizing on cyanogen bromide activated carboxymethyl cellulose and on Deolite DS 73141 for the treatment of paper mill effluent (Linko et al 1975) and on millipore filters for continous hydrolysis of starch (Similey et al 1975). Reports are also available on the immobilization of alpha amylase in soluble polymer dextran (Charles et al 1974; Kumakura et al 1977) on 2-hydroxy ethyl methacrylate and in magnetic iron oxide on cellulose (Kennedy et al, 1977; Kennedy and White, 1979), on cellulose (Kucera et al 1980) and in polystyrene (Fischer et al, 1978).

1.3. Objective of the present study

In the present programme it was planned to develop an optimized process for the immobilization of alpha amylase producing Bacillus polymyxa (CBTB 25) an isolate obtained from Cochin University campus primarily for the production of alpha-amylase. From the review of literature presented in the foregoing section it is very clear that research work carried out on alpha amylase production using immobilized cell systems are limited to one or two occasional reports and detailed study on the optimization of process parameters and technique of immobilization is lacking.

Main objective of the present study include the following:

(a) - To optimize support concentration, cell concentration, enzyme concentration, bead size, CaCl2 concentration and the curing time.

(b) - To characterise the immobilized Bacillus polymyxa for maximum enzyme production with reference to different temperature, pH, activation time, retention time, substrate concenteration, leaching and half life.

(c) - To compare the rate of enzyme synthesis by free and immobilized viable cells.

(d) - To study the continuous synthesis of amylase by immobilized viable cells in a fluid bed reactor.

(e) - To study the ethanol production from starch by coimmobilized Bacillus polymyxa and yeast cells of Saccharomyces cerevisiae.

Materials and Methods

2.1. Organism and source

In the present study immobilization studies were conducted using Bacillus polymyxa (CBTB-25) producing alpha amylase.

2.1.1 Source

Bacillus polymyxa (CBTB- 25) used in the present study was isolated from soil environments of Cochin University campus during the course of an investigation conducted during 1987-'89, and is available as a stock culture in the culture collection of the Centre for Biotechnology.

2. 1.2 Cultivation media

Two different types of media were used for the cultivation of B. polymyxa,

(a) Nutrient broth

Nutriet broth used in the present study was procured from Hi-Media laboratories (Pvt) Ltd., Bombay

(b) Mineral salts - starch medium (MSSM) The Mineral salts - starch medium (MSSM) contained the following (Nandakumar, 1991):

K2H P04	0.1%
KH2 P04	0.1%
MgS04 7H20	0.5%
CaC12	0.05%
(NH4)2 S04	0.1%
Soluble starch	1%(unless otherwise specified)
pH	7.0 + or - 0.2
Distilled water	100 ml

2.2 Cultivation of Alpha amylase producing B. polymyxa

2.2.1 Culture Medium

Nutrient broth [Hi- Media(India)] was used for cultivation of alpha amylase producing B. polymyxa whenever cells were required in large quantity for the preparation of immobilized beads as well as inoculation purposes during the course of study.

2.2.2 Preparation of Inoculam

i) A loopful of 18 hrs. old slope culture was transferred aspectically to 10 ml of autoclaved nutrient broth and incubated for 18 hrs at room temperature (28 + or - 2°C).

ii) This culture was transferred as such to 100 ml of N.B.medium and incubated on a rotary shaker (150 rpm) at room temperature (28 + or - 2°C) for a period of 18 hrs.

iii) Cells were harvested by centrifugation at 10,000 rpm for 30 min.; in a refrigerated centrifuge (Kubota 6700 model) at 5°C; washed with physiological saline and suspended in 10 ml of the same saline.

The prepared cell suspension was adjusted to 1.0 OD and used as inoculam.

2.2.3 Culture conditions

One litre of Nutrient broth was autoclaved and used for large scale preparation of cells. The medium was inoculated using the prepared inoculam(1% level). Cultivation was carried out in a Eyela Mini Fermenter (1.5 l capacity) at 30° C, pH 7.0, 200 rpm, for 18 hrs.

2.2.4 Harvest of Cells

Cells were harvested by centrifugation at 10,000 rpm for 30 min. at 5°C in a refrigerated centrifuge (Kubota 6700 model).

2.2.5 Maintenance

The harvested cells were repeatedly washed with physiological saline and suspended in 100ml of the same saline and maintained at 4 o C in a store cool, Walk in cooler (Blue star India), until used.

2.3. Alpha amylase production by free cells of

B.polymyxa

2.3.1 Enzyme production medium

Mineral salts starch medium was used for the production of amylase by the free cells. (Composition of the media is given under section 2.1.2). Different starch substrates (soluble starch, wheat starch, rice starch and potato starch) were used at 1% level. The prepared medium was autoclaved and used.

2.3.2 Preparation of Inoculam

Initially a loopful of 18 hrs. old NA slope culture was transferred to 10 ml sterilized nutrient broth and grown for 18 hrs at room temperature 28 + 2 o C.

ii) The grown culture was harvested by centrifugation at 10,000 rpm for 30 min. at 5°C and after repeated washing in physiological saline asceptically transferred into 100 ml of the enzyme production medium (MSSM) and incubated for 18 hrs on the rotary shaker (150 rpm) at room temperature (28 + 2 or -2°C)

iii) Cells were harvested by centrifugation (Kubota 6700 model Japan) at 10,000 rpm for 30 minutes at 5 oC.

iv) The harvested cells were made upto 10 ml• volume

using physiological saline (0.85% NaCL) after repeated washing with the same.

v) The prepared cell suspension was used as inoculam.

2.3.3 Inoculation and incubation procedure

1000 ml of MSSM taken in Eyela Mini Fermenter was inoculated using the prepared inoculam at (1% level) and inculbated at 30°C, 200 rpm for 18 hrs.

2.3.4 Harvest of enzyme.

Alpha amylase produced by the cells in the MSSM was separated as cell free extract after centrifugation at 10,000 rpm for 30 minutes at 5°C (Kubota 6700 model).

2.3.5 Purification and maintenance.

The crude enzyme preparation (cell free extract) was partially purified by $(NH4)_2 SO_4$ (Sisco enzyme grade) fractionation followed by dialysis using phosphate buffer (pH 8).

i) The crude enzyme fraction was subjected to $(NH4)_2SO_4$

precipitation from 30 to 80% saturation by increasing slowly the (NH4) 2SO 4 concentration along with continuous stirring using a magnetic stirrer at 4°C in cold room (Blue star store cool)

ii) The precipitate obtained at each saturation was removed by centrifugation and dissolved in 10 ml of phosphate buffer pH 8.0.

iii) The precipitate obtained after ammonium sulphate fractionation and dissolved in the phosphate buffer pH 8 was dialysed extensively against the same buffer for 24 hrs at 4°C.

iv) The dialysate was redissolved in the same buffer and used for further studies.

2.4 Analytical procedures

2.4.1. Cell protein : Cell protein was estimated employing Lowrys method. (1951)

1. 1 ml of cell suspension was taken in a test tube.

2. The contents were added with 1 ml of 3N NaOH in a screwcapped test tube.

3. Heated in a boiling water bath at 100°C for 5 minutes for extracting the cell protein.

4. After cooling at room temperature (28 ± 2 o C) the samples were added with 5 ml of alkaline reagent. (A -2% solution of Na2 CO3 in 0.1 N NaOH,B-0.5%. solution of CuSO 4 5H2O in a 1% solution of potassium sodium tartarate mixture of 1 ml reagent B and 50 ml reagent A.)

5. The contents were thoroughly mixed and Kept for 10 minutes.

6. To each tube 0.5 ml of folin's reagent diluted with an equal volume of water was added.

7. After 40 minutes absorbance of the sample was measured at 750 nm in a UV Visible spectrophotometer (Shimadzu 160 A)

8. Bovine serum albumin was used as standard.

9. Cell protein is expressed as mg/ml.

2.4.2. Enzyme protein

Enzyme protein was also estimated employing Lowrys method (1951).

1. 1 ml of partially purified enzyme was added with 5 ml of alkaline reagent.
2. Mixed throughly and left for 10 minutes.
3. 0.5 ml of folin's reagent diluted with an equal volume of water was added to each tube.
4. After 40 minutes absorbance was measured at 750 nm in a UV visible spectrophotometer.
5. Bovine serum albumin was used as standard.
6. Enzyme protein was expressed as mg/ml.

2.4.3 Starch .

The starch was estimated according to the methods suggested by Medda & Chandra (1980).

1. One ml of starch solution was added with 0.1 ml of I_2 - KI reagent (0.3% Iodine + 3% pottasium iodide was added).
2. Contents were made upto 15 ml with distilled water.
3. The blue colour developed was then measured at 650 nm in a UV/Visible spectrophotometer.
4. The standard curve was prepared using soluble starch (Qualigens sq. India) following the procedures mentioned above.
5. Starch is expressed as mg/ml.

2.4.4. Total sugar

Total sugar was estimated by phenol sulphuric acid method (Dubois et al, 1956). One ml of appropriately diluted sample was added with 1 ml of 5% phenol solution and 5 ml of concentrated sulphuric acid and the absorbance was measured at 490 nm. The results were computed from a standard curve prepared with glucose. The total sugar is expressed as mg/ml.

2.4.5 Reducing sugar

Reducing sugar was estimated by dinitrosalicyclic acid method (Miller 1959 modified by Jones & Grainger 1983). One ml of the appropriately diluted culture supernatent was added with 0.5 ml of dinitrosalicyclic acid reagent, heated at 100°C for 10 minutes in a water bath, cooled, added with 4 ml of distilled water and absorbance measured at 500 nm in a UV visible spectrophotometer.

Standard curve prepared with glucose was used for quantifying the concentration of reducing sugar present in the MSB broth and the values are expressed as mg/ml .

2.4.6 Alcohol

Alcohol was estimated by cerric ammonium nitrate method (Reid & Salmon 1955). 5 ml of sample was treated with 2 ml (20 gm in 100 ml of 4 N HNO 3) cerric ammonium nitrate and the optical density was measured at 486 nm. Absolute alcohol 99% (James Burrough (F.A.D) Ltd (UK) was used as standard.

2.4.7 Enzyme activity

Enzyme activity was assayed according to Medda & Chamdra (1980)

1. The reaction mixture contained 1 ml of cell

free extract/partially purified enzyme,1 ml of phosphate buffer pH 8 (unless otherwise stated) and 1 ml of 1% soluble starch prepared in distilled water.

2. After incubation at 40 degree centigrade for 30 minutes 1 ml of

I_2-KI (0.3% I and 3% KI in distilled water) solution was added.

3. Contents were made upto 15 ml with distilled water.

4. The blue colour developed in the solution was measured at 650 nm.

5. One unit of enzyme activity is defined as the amount of enzyme required to convert 0.1mg of soluble starch at 40°C within 30min.of incubation.

2.4.8 Measurment of cell growth in Immobilized beads.

The cell growth inside the immobilized beads were estimated as cell protein according to Jones etal (1984) with some modifications.

About 1gm (wet weight) of immobilized cell beads (15 beads) were dissolved in 3-5 ml of phosphate buffer pH8 in which the beads got dissolved after 1 hr of incubation. The liberated cells were extracted with 1 ml of 3N NaOH ,after removing the precipitated alginate by centrifugation at 10000 rpm, 30 min ,at 5°C .

2.5. Immobilization studies

2.5.1 Methods employed

Although several methods are available to effect immobilization such as entrapment, crosslinking,covalent binding and physical adsorption in the present study entrapment technique was employed for immobilization of cells and enzymes.Cross linking technique was employed, in addition to entrapment for immobilizing amylase only.

2.5.2 Supports

Sodium Alginate (CDH- India) was used as support for gel entrapment of cells and enzymes. Glutavaldehyde (Ferak Laborat GMBH Berlin West)was used as cross linking agent for immobilizing amylases.

2.5.3. Preparation of Immobilized cell beads.

2.5.3.1. Cells

Cell suspension prepared as mentioned under section 2.2 was used for the preparation of immobilized cell beads.

2.5.3.2 Preparation of support material and beads

1. 4 g of dry powder of sodium alginate (CDH Laboratory reagents) unless otherwise stated was slowly added to 100 ml of distilled water while being continuously stirred.

2. The stirring was continued for a further period of one hour and warmed at 60 degree centegrade to ensure complete dissolution.

3. The solution was then left to stand for about an hour to allow the air bubbles to escape.

4. Under sterile condition, 20 ml of the prepared cell slurry was mixed with 40 ml of sodium alginate solution (4% w/v).

2.5.3.3 Preparation of beads

The prepared sodium alginate cell slurry was then extruded dropwise through 10 ml syringe from a height of about 10 cms in to an excess of 0.2m CaCl2 solution (unless otherwise specified).

Beads of calcium alginate entraped (cells mean diameter 3 mm unless or otherwise specified) were maintained in the CaCl2 solution to be hardened for 30 minutes. After washing with physiological saline, three times, the beads were used further studies.

2.5.3.4 Maintanance of beads

The immobilized beads were cured in 0.2m CaCl2 solution for20 hrs (unless or otherwise specified)and preserved in normal saline at 4°C whenever, they were not used (D'Souza 1990).

2.5.4 Preparation of immobilized enzyme beads

1. The sodium alginate solution prepared as mentioned in the previous section (2.5.3.2) was mixed thoroughly with 5 ml of 5% Glutaraldehyde solution prepared in phosphate buffer pH 7. (Nandakumar 1991)

2. Twenty ml of the partially purified amylase from B.polymyxa (30-40 u/ml) was added separately to the sodium alginate glutaraldehyde mixture and mixed thoroughly.

3. The final mixture was then extruded dropwise through a 10 ml syringe from a height of about 10 cm into an excess of CaCl2 (0.2 m solution).

4. The beads with mean diameter of 3 mm were allowed to harden by leaving them as such in CaCl2 solution for 30 minutes. The prepared beads were then stored then in physiological saline (4°C) under refrigeration.

2.5.5 Activation of Immobilized viable cell and enzyme

The prepared beads were suspended in 1% starch solution having a pH 8 in a conical flask and incubated at room temperature 28 + or - 2°C for 24 hrs. (Unless otherwise specified)the activated beads were then removed and washed with fresh ,sterile saline solution and used for further studies.

2.5.6 Assay of the amylase activity of immobilized cells and enzymes

The amylase activity of the activated immobilized cells was tested according to Sen and Chakravarthy (1987), With slight modification.1 gm wet weight of activated beads were incubated with 1 ml of 1% soluble starch and 1 ml of phosphate buffer pH 8(0.2M) for enzyme activity and incubated for 30 minutes at 40°C.The reaction was arrested by the addition of 0.1 ml of 1N

HCl and the decanted supernatent was tested for residual starch as per the procedure detailed under section 2.4.3). Enzyme Activity was computed and expressed as mentioned under section 2.4.7.

2.6. Optimization of immobilization of cells and enzymes

Optimal concentration of support, cell, enzyme,and, CaCl2; curing time and size of the beads that could promote maximal activity of the immobilized cells were determined as detailed below:. Activity of immobilized cells was determined in terms of amylase activity, residual starch and reducing sugar which were estimated as per the procedures mentioned under sections 2.4.(7,3,5) respectively.

2.6.1 Support concentrations

Optimal support concentration required for the preparation of beads with maximal activity was determined using sodium alginate as support. Sodium alginate solutions with 2%, 3%, 4% and 5% were prepared by the slow addition of the required quantity of alginate to the distilled water under constant stirring. The solution were stirred for a further period of one hour and warmed at 60° C to ensure complete dissolution of the support. The solution were then left to stand for about an hour to allow the air bubbles to escape. Using these solutions immobilized beads were prepared and tested for their activity as mentioned under section 2.5

2.6.2. Cell concentration

Immobilized cell beads were prepared using batches of 50 mg,100 mg, 150 mg, 200 mg, and 250 mg wet weight of the cells and that activity was tested as mentioned under section 2.5

2.6.3 Enzyme concentration

Optimal enzyme concentration for the preparation of beads with maximal activity was carried out using different concentrations of partially purified enzyme obtained from bacteria (5 ml, 10 ml, 15 ml,20 ml, 25 ml, 30 ml of enzyme solutions section 2.3). Immobilized enzyme beads were prepared as mentioned under section 2.5.3, and after activation tested as mentioned under section 2.5.6

2.6.4. Bead size

Optimal size of the immobilized bead that could attribute maximal activity was determined by preparing beads of varying sizes (1mm - 5 mm) using syringes with different pore sizes. The immobilized beads were prepared under activity tested as mentioned under section 2.5

2.6.5 Concentration of $CaCl_2$

Concentration of calcium chloride required for preparing stable immobilized bead was optimized by preparing the beads using $CaCl_2$ (BDH) at different molar concentrations (0.01.0.05,0.1,0.15,0.2.0.25,0.3.0.4 and 0.5 M). Beads were prepared and their activity was tested as mentioned under section 2.5

2.6.6 Curing time

Optimal curing time for obtaining stable immobilized beads were determined by allowing the beads formed in the 0.2M calcium chloride solution to remain for varying periods of curing, namely 1 hr, 2 hr, 3 hr, 4 hr and 5 hrs. The beads were treated with starch solution, after draining the CaCl2 and the activity of the beads was tested as mentioned under section 2.5.6.

2.6.7 Temperature

Optimal incubation temperature for maximal activity of the immobilized beads was determined by incubations the beads was determined by incubating the beads along with substrate at 30°C, 40°C and 50°C and testing their activity a mentioned under section 2.5.6.

2.6.8 pH

Optimal pH for maximal activity of the immobilized beads was estimated by adjusting the pH of the starch substrate solution to pH 4 - 11. Activity of the beads was tested as mentioned under section 2.5.6.

2.6.9 Activation time

Optimal Activation time required for maximal activity was determined by conducting the immobilized cell beads with soluble starch solution for varying time intervals of 1,6,12,24,48 hrs and testing their activity as mentioned under section 2.5.6.

2.6.10 Retention time

Optimal Retention time required for maximal activity was estimated by incubating the immobilized beads with soluble starch solution (1%) after activation, for varying periods and tested their activity as mentioned under section 2.5.6.

2.6.11 Leaching of immobilised cells from the beads

Rate of leaching of cells from the immobilized cell beads prepared using sodium alginate (4%) and sodium alginate crosslinked with glutaraldehyde was individually monitored as mentioned below. 100 beads of immobilized cells were maintained in 100 ml of 1% starch solution for a total period of 360 minutes and at regular intervals of 30 min., samples were drawn and the quantity of cells leached out was estimated as cell protein as per the procedures mentioned under section 2.4.1.

2.6.12 Substrate concentration

Optimal substrate concentration for maximal yield of amylase by immobilized cells was determined as detailed below: Prepared beads of immobilized cells were treated with different substrate concentrations of 0.1%, 0.5%, 1% and 1.5% of soluble starch, Rice starch and wheat starch at 40°C, pH 8.0 for 60 minutes. Enzyme production was assayed as per the procedure mentioned under section 2.4.7.

2.6.13 Rate of substrate diffusion into the Beads

Rate of diffusion of soluble starch into the immobilised beads was estimated as follows:

1. Immobilised beads (3 mm) were prepared using *B. polymyxa* cells in 4% sodium alginate beads, in 1:2 ratio as mentioned under section 2.5.3.3 This formed the set A.

2. Another set beads (3mm) were prepared using sodium alginate (4%) without bacterial cells as control beads as per the same procedure. This formed the set B.

3. Batches of 100 beads (pre weighed) of both A and B sets were separately taken in 10 ml test tubes, as many as required.

4. At regular intervals of 30 minutes, one test tube each from A and B sets were drawn for analyses.

5. The starch solution was drained out completely leaving behind the beads using pipetting devices (Pipetman - variable pipetes)

6. Samples were analysed for residual volume, and residual starch, reducing sugar, total sugar as mentioned under section 2.4.

7. The beads remaining in the test tube were weighed to find out the increase in weight owing to diffusion of liquid into the bead.

8. Rate of diffusion of substrate was calculated and expressed in terms of mg/g of starch of bead/hr.

2.6.14 Rate of enzyme synthesis by immobilized cells

Alpha amylase synthesised by *B. polymyxa* cells under immobilised condition was monitored at regular intervals to compare the rate of synthesis with free cells. Immobilised cell beads were prepared as detailed under section 2.5 and contacted with starch solution as follows (100 mg of cells/100 beads) were taken in a 500 conical flask

and contacted with 100 ml of 1% starch solution for a total period of 96 hours, At regular intervals of 12 hrs samples were drawn out and assayed for amylase activity, cell protein, growth were assayed as per the methods outlined under sections 2.4.7, 2.4.1, respectively. Control experiments were run using free cells (100 mg of cells/100 ml of starch solution). Rate of enzyme synthesis is expressed as enzyme units /gm of beads.

2.7. Production of alpha amylase by Free Versus Immobilized Cells

Alpha amylase production by free cells and immobilized cells was compared with reference to various levels of pH (7,8 and 9); at different temperatures (30 ,40 and 50°C) and at different substrate concentrations (Viz. 0.1,0.5,1.0 and 1.5%) as per the methods mentioned under sections 2.6.8,2.6.7,2.6.12 respectively.

2.8. Continuous production of alpha amylase by immobilized cells

Continuous production of alpha-amylase by immobilized *B. Polymyxa* cells was monitored in a fluid bed column reactor.The effect of flow rate of the substrate solution into the reactor, residence time, half life period and the cumulative production of amylase and the performance of the reactors were determined.

2.8.1 Fluid bed reactor

The cylindrical glass column of 80 mm diameter, 150 mm length was used as the reactor, The bottom of the column, which was hemispherical in shape with 5 mm diameter inlet, was packed with small amount of glass wool and glass beads of 4 mm diameter upto 20 mm height. A sieve plate with 1 mm perforations were placed over the glass beads and immobilized beads were packed up to 100 mm height. A sieve plate was placed over the beads and fixed to the top of the reactor,

The column was provided with a side tube of 5 mm diameter at the top section, through which the sample broth was withdrawn. The reactor was fed from the bottom by peristallic pump (Mcclins India Ltd.) and the effluent was removed from the top.

2.8.2 Activation of the immobilized reaactor

The immobilized bioreactor was activated using 1% soluble starch solution (pH8) for a period of 24 hrs before commencing the experiment.

2.8.3 Estimation of void volume

The voidage of the packed bed reactor was determined by measuring the volume of the liquid actually occupied in the reactor. The reactor was packed with spherical gel beads to the desired level and bed depth was noted. Physiological saline was filled from the bottom with the help of the peristallic pump till the liquid level reached to the top layer of the gel beads. The liquid was then slowly withdrawn from the column till its level reached

the bottom layer of the bead and the quantity of water thus collected was measured. The procedure was repeated thrice and average value was taken.

2.8.4 Flow rate

Effect of flow rate of substrate solution on the continuous synthesis of alpha amylase by the immobilized cells and the performance of the immobilized reactor was determined by monitoring the rate of alpha amylase production at different flow rates.

2.8.5 Half life period

Half life period of the immobilized fluid bed reactor was estimated by observing the rate of soluble starch conversion by the immobilized cells in the fluid bed reactor continuously for a period of 50 days.

2. 9. Co-immobilization

Co-immobilization of Bacteria and Yeast

B. polymyxa and *Saccharomyces cerevisiae* were coimmobilized in calcium alginate beads and their efficiency to convert star ch to alcohol was determined.

B. polymyxa was cultured in MSSM and harvested as mentioned under the section 2.3.4. Yeast isolated from grapes in the laboratory and identified as *Saccharomyces cerevisiae* were cultured in a growth medium containing 0.3% malt extract,

0.3% yeast extract, 0.5% peptone 1% glucose with pH 5.0 in tap water. Cells were harvested after centrifugation at 10,000 rpm, washed with physiological saline and maintained in the same.

Both *B. polymyxa* and *S. cerevisiae* cell suspensions were mixed at various proportions V/V 1:1, 1.5:1, 1:2, 2:1, 1:1.5. The prepared culture mix was later added to the sodium alginate solution and the beads were prepared as per the procedures outlined under section 2.5.3.

The immobilized beads were treated with 1% soluble starch solution, after activation for 24 hrs, for total period of 20 hrs. Samples were collected at regular intervals of 4 hrs and analysed for residual starch, reducing sugar and alcohol as mentioned under sections 2.4.3, 2.4.5 and 2.4.6 respectively

2.10. Statistical Analysis

The variables used for statistical analysis included incubation time, pH, temperature, starch, reducing sugar, total sugar, enzyme activity, etc.

2.10.1 Correlation co-effecient analysis

To test whether the numerical defferences of the variables studied were significant or not, a pearson correlation coefficient 'r' was calculated by applying the microstate computer programmes (HCL India Ltd.)

2.10.2 Graphics

All the graphs drawn in this studies were made by the computer using HPG programm.

3. RESULTS

3.1 Optimization of process parameters

3.1.1 Support concentration :

Optimal concentration of support material that attributes stability and maximal activity to the immobilized cell beads was determined using different concentrations of sodium aliginate as support and estimation of amylase production. Results presented in Fig. 1 indicates that maximal activity of the beads in terms of enzyme production takes place at 2% support concentration and an increase support concentration leads to decrease in activity. Although maximal enzyme production (28u/gm of beads) was noted at 2 % support concentration, the beads were not stable and recorded rapid cell leaching after about 6 hrs and appeared spongy whereas 3 - 4 % concentration of support, though resulted in relatively moderate production of enzyme (25 - 20 u/g of beads) attributed stability to the beads which remain intact with out significant leaching of cells.

Reduction in activity of beads along with raise in support concentration from 2 - 5 % of sodium alginate was recorded at all pH levels (pH7, 8, & 9) tested. However maximal enzyme production was observed at pH9 (25.3 - 36.8 u/g of beads) followed by pH8 (17.9 - 27.6 u/g of beads) and pH7 (11.3 - 20.3 u/g of beads).

Of the three starch substrates, viz. soluble starch, rice starch and wheat starch. Rice starch could promote more activity in the immobilized beads at all concentrations of support tested when compared to the other two substrates (Table.1). Especially at pH 8 & 9 the enzyme production was significant at 2 - 4% sodium aliginate concentrations. Invariably at pH9 rice starch promoted similar levels of activity in beads at all concentrations of support tested. Wheat starch could promote significant level of activity only at pH 9, at 2 - 3 % sodium alginate concenration. soluble starch did not influence the level of activity in the beads with reference to change in support concentration.

3.1.2 Cell concentration.

Initial level of cell concentration required to prepare stable beads with significant activity was optimized using various levels of cell concentraion ranging from 50 mg - 250 mg of the cells (wet weight). Impact of cell concentrations on the activity of the beads was estimated by analysing % of starch conversion, production of enzyme and reducing sugar using soluble starch as substrate. Results obtained in this experiment (Fig 2a & b) suggest that initial cell concentration well above 100 mg/100 gm of beads is lead to a rapid decline in the percentage of starch concentration, enzyme production and reducing sugar production in a linear fashion along with raise in cell concentrations. Maximal starch conversion an production of

enzymes and reducing sugar were observed only after 100 mg of beads / hr. Data presented in Table 2 suggest that the residence time required for maximal activity on starch and enzyme production varied for differnt initial cell concentration in the bead. Thus both 100 mg & 200 mg of cell concentration recorded maximal activity at 4 hrs of residence time while higher concentration of initial celll levels viz. 150 mg, 250 mg, could show higher activity at 1 hour residence time. In all the cases it was noted that long hours of residence time (6 hrs) reduce the activity of the beads

3.1.3 Enzyme concentration

Optimal level of initial concentration of amylase required to prepare an Immobilized enzyme that is stable and active in converting starch rapidly was determined by preparing the immobilized beads with various concentrations of amylase isolated from B. Polymyxa and estimating the percentage of starch conversion and reducing sugar. Results presented in Fig 3a & b clearly indicate that 25 ml of initial level of enzyme would attribute maximal activity to the beads (99.28%) While concentrations above and less resulted in a decline in the activity as well as reducing sugar formation. In general enzyme concentration varying from 20 - 30 ml contributed 83 % of starch conversion

When the activity of the immobilized enzyme was monitored regularly at intervals of 10 minutes upto a period of 60 minutes of residence time it was observed that % of

starch conversion and level of reducing sugar increase progressively along with the increase in residence time as well as enzyme concentration in the bead (Table 3). Interestingly at 25 ml and 30 ml concentration of enzyme, the activity profile of the bead remained constant irrespective of the duration of residence time (99.3% & 86.1% of starch conversion at 25 ml and 30 ml of enzyme concentration respectively). A similar picture was also noted for reducing sugar at 25 ml and 30 ml of enzyme concentration. It was observed that low levels of initial enzyme concentration required longer residence time to score maximal starch conversion compared to higher levels of initial enzyme concentration which required shorter residence time to do the same.

3.1.4 Bead size

Right size of the immobilized bead that could promote maximal stability and activity was determined by prepa ring the immobilized cell beads of varying diameters (viz. 1, 2, 3, 4 & 5 mm.) and estimating the production of amylase on reducing sugar and percentage of starch conversion. Results presented in Fig. 4 indicate that at lower size range 1mm - 4mm bead size do not influence the performance of the immobilized cells as similar levels of % starch conversions (75 - 81.8 %). The production of amylase (44.1 - 48.1) and reducing sugar (0.2849 - 0.3087) were recorded. Whereas when the bead size was 5mm there was a marginal decline in the activity of the beads.

3.1.5 Concentration of $CaCl_2$

Optimal concentration of $CaCl_2$ required to prepare stable immobilized cell beads using sodium aliginate support was determined by preparing the beads using different concentration of $CaCl_2$ and estimating the activity of the beads. The results are presented in Fig. 5. Maximal activity of beads, in terms of percentage of starch conversion and production of amylase and reducing sugar occured when $CaCl_2$ concentration were 0.01M - 0.05M. However at concentration of .1 - .2 M the activity profile of the beads almost remained stable recording 16.67 - 16.60 u/gm of beads, 37.59 -37.51 of starch conversion and 0.4867 - 0.4847 mg of reducing sugar /g of bead. Further rise in concentration of $CaCl_2$ lead to a decline in the activity profile. Moreover the beads prepared at concentrations of 0.01M - 0.10M appeared swollen and spongy while $CaCl_2$ concentration above 0.1M yielded hardened beads. From this experiment it was infered that 0.2M $CaCl_2$ could be the optimal $CaCl_2$ concentration to achieve stable beads with significant activity.

3.1.6 Curing time in $CaCl_2$

Optimal time required for the curing of the prepared immobilized cell beads with maximal activity and stability was determined by maintaining the beads formed immediately after the addition of sodium alginate in $CaCl_2$ solution for varying periods and estimating the activity of

the beads in terms of enzyme production and percentage of starch conversion and reducing sugar production. From the results presented in Fig.6 it is evident that a minimum of 2 hrs of curing time is required to have significant levels of enzyme production, starch conversion and production of reducing sugar. Incubation over 2 hrs till 6 hrs, tested during the studies, although showed enhancement in the values of the parameters tested they were relatively not so high.

When tested for the impact of curing time in $CaCl_2$ on the residence time required for maximal activity of the bead, it was observed that irrespective of the period of curing in $CaCl_2$ the immobilized beads showed similar levels of activity during the residence time of 1 - 6 hrs. Difference among the beads prepared at different curing time could be noticed only at a residence time of 6 hrs. where, maximal activity in terms of higher enzyme yield (35.08 - 55.8 units/gm of beads), starch conversion (58.5 - 95.0) and reducing sugar (0.5172 - 1.257 mg/gm of bead) were recorded for the beads prepared after curing of 2 to 5 hrs in $CaCl_2$. Whereas 1 hr. of curing showed comparatively lower levels of activity (Table - 4).

3.2 Characterisation of the immobilized bacteria

3.2.1 Effect of temperature on the activity of the immobilized cells

Effect of incubation temperature on the activity and performance of the immobilized cell beads was

tested by incubating the immobilized beads at various temperatures, and estimating the % of starch conversion, production of amylase and reducing sugar. From the results presented in Table - 5, it is evident that the tested temperatures did not influence the performance of the beads since 81.4% - 88.4% of starch converion, 41.4 - 45.6 u/gm of beads of amylase and 1.109 - 1.208 mg/gm of beads reducing sugar were recorded. Comparatively at 40°C the performance of the beads were maximum than at 50°C. Although at 30°C the activity was lesser than at 40°C, it was significant.

3.2.2 Effect of pH on the activity of immobilized Cells

Effect of pH on the acticity of immobilized cells of B. polymyXa was determined by subjecting them to various pH and estimating their activity in terms of production of amylase and reducing sugar and percentage of starch conversion. Results obtained in this study (Fig 7 a & b) indicated that maximal pecentage of starch conversion (59.75) and production of amylase (45.8 u/gm of bead) and reducing sugar (0.3297 mg/g of bead) was promoted in the beads at pH8. However both at pH7 and pH9 the immobilized beads recorded significant levels of activity. In general, at acid pH levels (pH4 and 5) and at alkaline pH (11) there was minimal activity in the beads compared to that at pH6 and pH10.

3.2.3 Activation Time

Impact of activation time on the performance of the immobilized cells with reference to their efficiency in starch conversion,and production of reducing sugar and amylase was determined by activating the immobilized cell beads with starch solution for varying periods. From the data presented in Fig.8 it is clear that performance of the beads enhanced along with the rise in activation period till 24 hrs where maximal production of amylase (37.2 u/g of bead) and total sugars (2.430 mg/gm of bead) where recorded. Although percentage of starch conversion continued to rise even after 24 hrs, amylase production and both total sugar and reducing sugar levels declined after 24 hrs.

3.2.4 Residence time

Optimal residence time requird for the substrate solution to pass through the packed column reactor containing immobilized cell beads for obtaining maximal starch conversion and amylase production was determined by allowing the substrate solution to reside in the column for various timings. Mean values obtained for 6 experiments are presented in the Table .6. Results indicated that significant levels of starch conversion (69.45%) along with maximal enzyme (40.75 u/g of beads) could be obtained at 60 minutes of residence time though maximal percentage of starch conversion (73.84) was recorded at 300 minutes of

residence time. Enzyme production did show a decrease along with increase in residence time. Relatively 60-120 minutes of residence time proved to be ideal for obtaining maximal enzyme production along with significant percentage of starch conversion.

3.2.5 Effect of buffers on leaching

Effect of buffers on leaching out of cells from the immobilized beads was tested using various buffers, which included acetate buffer (pH 5.6),citrate buffer (pH6.2), citrate phosphate buffer (pH 7), phosphate buffer (pH 7 and 8) and glycine buffers (pH 9 and 10). Leached out cells were estimated in terms of cell protein and the % of leaching was calculated. From the results presented in the Table 7 it is evident that phosphate buffer pH 8 is the only buffer among the buffers tested that could leach out all the cells within 90 minutes. All the other buffers could record only meagre levels of cell leaching which varied from 10.5 - 48.3% even after 120 minutes. Relatively buffers with pH in the range of 5.6 - 7.0 excepting phosphate buffer pH7 recorded reduced levels of leaching than glycine buffers with alkaline pH. This experiment suggested that phosphate buffer pH8 could be used for disintegration of the beads to recover the cells.

3.2.6 Effect of initial cell concentration on leaching

Effect of initial cell concentration on the leaching of the cells from the immobilised beads were

studied using three different initial concentrations of 50, 100 and 150 mg/100 gm of beads over a contact period of 6 hours. Results presented in the table 8 indicate that in general leaching of cells from the beads at all levels of initial concentrations of cells varied from 0.4 - 0.96% for the various concentrations tested. Among the three concentrations tested, 50 mg concentrations of cells recorded higher percentage of leaching when compared to other concentrations. However the difference in the levels of leaching observed for the three concentrations were insignificant.

3.2.7 Effect of cross linking of cells on leaching

Effect of cross linking of cells with glutaraldehyde on the leaching of cells was monitored during a contact period of 6 hours at an initial cell concentration of 100 mg/100 gm of beads. Results presented in Table 9 suggest that leaching of cells are arrested in cross linked alignate beads after 60 minutes.

3.2.8 Effect of substrate concentration on Amylase Production by immobilized cells

Effect of substrate concentration on immobilization was studied by using various concentrations of (0.1% - 1.5%) soluble starch,rice starch and wheat starch.It is evident from the Fig.9 that lower parcentage of substrate

concentrations promote maximum production of amylase than higher concentrations which lead to reduced production of amylase .Thus 0.1% of starch effected amylase production in the range of 47-51 u/gm of beads and further increase in the concentrations of the substrate contributed a decrease in the level of enzyme production,irrespective of the type of starch substrate used. Among the three starches used wheat starch (51 u/gm) followed by rice starch (49 u/gm) and soluble starch (41 u/gm) promoted enhanced yield of amylase.

3.2.9 Rate of enzyme synthesis by immobilized cell beads.

Synthesis of amylase by the immobilized beads during the course of incubation with starch substrates was monitored for a total period of 96 hrs. Growth of cells inside the beads were also estimated at regular intervals along with enzyme production to find out the relation between the two .Results presented in Fig.10,a&b evidence that rate of enzyme synthesis is rapid during the early hours of incubation till 36 hrs.When maximal enzyme (52.1 u/g of beads) along with maximal growth(2.483 gm/ml of protein) was recorded. Later both enzyme synthesis and growth declined progressively. In general the rate of amylase synthesis was observed as much as 52.1u/gm of beads during the period of active synthesis (upto 36 hrs.).While it was only 29.7 u/gm of bead in the next 36 hrs (36-72 hrs).

Further results presented in Fig. 12b indicate that despite a slow rate of growth and enzymes synthesis, in

immobilized cells compared to free cells, during the first 24 hrs, the rate of enzyme synthesis and growth improved significantly during the later periods of study.

3.2.10 Rate of substrate diffusion into the beads

Rate of diffusion of starch into the immobilized beads was monitored by estimating the residual starch, redusing sugar and total sugar in the solution outside the beads and inside the bead at various time intervals. Results presented in Fig.11. indicate that the residual starch inside the beads were low and the levels of redusing sugar and total sugar were high indicating conversion of diffused starch. Data presented in Table-10. shows that the initial level of starch drastically got redused in the substrate solution outside the beads and total sugar level accumulated at moderate levels. Whereas redusing sugar levels remained almost unchanged. This suggest that starch rapidly diffused into the beads and suggested to amylase action by the immobilised cells.

3.3. Comparative studies of alpha- amylase by free cells Vs immobilized cells of B. polymyxa.

Synthesis of alpha- amylase by free cells Vs immobilised cells of B.polymyxa utilizing different starch substrates at various environmental conditions were compared. Results obtained for the different experiments are depicted in Table 11.

An overall assessment of the data obtained for the various studies conducted denotes that immobilized cells synthesize alpha-amylase at comparable rates with free cells and produce reducing sugars at a higher level than free cells.

Among the four different starch substrates used wheat starch followed by potato starch,soluble starch and rice starch favoured maximal synthesis of alpha-amylase and reducing sugar by both immobilized and free cells with marginal variations.

Studies on the effect of temperature on immobilized and free cells showed that at all the temperatures tested immobilized cells were capable of synthesizing enhanced level of alpha amylase than free cells. Maximal sysnthesis was recorded at 40°C in all the cases.

Maximal amylase synthesis was recorded by both free and immobilized cells at pH8.Free cells recorded amylase at a higher level than immobilized cells using wheat starch unlike other starches.Similarly immobilized cells produced higher level of alpha-amylase using rice starch than free cells.

Among the four concentration of the substrates tested while free cells synthesized amylase at higher levels at substrate concentrations of 0.1,1 and 1.5% immobilized cells could do the same only at 0.5% irrespective of the starch substrates provided.

Reducing sugar produced by the immobilized and free cells of Bacillus polymyxa was studied and the experimental data are presented in the table 11.Immobilzed cells showed more reducing sugar production than the free cells at all incubation temperatures tested (fig. 14).Among the three temperatures tested (30°C,40°C,50°C) 40°C promoted more production than the others. The highest level of reducing sugar was observed with wheat starch (1.443 mg/gm of bead) followed by rice starch (1.389 mg/ g of bead), potato starch (1.3365 mg/g of beads) and soluble starch (1.3365 mg/g of bead).

pH did not influence the reducing sugar production since the difference in the levels of reducing sugar between free and immobilized cells was meagre. Notable decline of reducing sugar was observed in soluble starch at alkaline pH9 (0.9712 -0.9207) in both the cases. Wheat starch recorded higher yield of reducing sugar followed by rice starch, potato starch and soluble starch.

Free cell showed more production of reducing sugar than immobilized cells at all the substrate concentration tested .Lowest concentration (0.5 - 0.1%) of starches showed higher production of reducing sugar.Whereas higher concentration observed an inverse relation with the reducing sugar . Nature of starch substrate did not effect any significant role in reducing sugar production.

3.4. Continuous synthesis of alpha-amylase by immobilized Bacillus polymyxa

The rate of amylase synthesis, percentage of starch conversion and levels of reducing sugar remained stable without much fluctuations during the period of study (Table 12). Maximal enzyme synthesis (40.4 - 41.2 u/g of bead) along with 96.67 - 98.1% starch conversion and 2.749 - 2.8036 mg/g of bead reducing sugar production could be recorded at the flow rate of 20 ml/hr. Whereas at 30 ml/hr flow rate percentage of starch conversion (71.7 - 77.32), enzyme synthesis (30.0 - 30.2 u/ml) and reducing sugar (2.014 - 2.238 mg/ml) drastically declined. At flow rates of 45 ml/hr, the percentage of starch conversion, rate of enzyme synthesis and reducing sugar production got almost reduced to half of the values obtained for 20 ml flow rates.

Data presented in Fig.13 demonstrate the impact of various flow rates on the cumulative synthesis of alpha-amylase by the immobilized cells. Maximal amylase (204.5) could be produced using minimal starch substrates (100 ml) in 5 hrs. at a flow rate of 20 ml/hr. Quantitatively amylase production declined progressively along with increase in the flow rate and total quantity of substrate solution used within the total period of 5 hrs. of analysis. Thus 157.3 u/g, 108.88 u/g, 74.6 u/g, 59.59 u/g and 49.7 units of amylase/g of bead was obtained using 150, 225, 300, 600 and 750 ml of starch solution in 5 hrs at 30 ml/hr, 45 ml/hr, 60 ml/hr, 120 ml/hr and 150 ml/hr flow rate respectively.

Rate of amylase synthesis during continuous operation of the immobilized reactors with reference to time and quantity of source solution is presented in Table A.8. Maximal rate of amylase synthesis was recorded as 0.6817 u/min. at 20 ml/hr flow rate followed by 30 ml/hr (0.5243) and 45 ml/hr (0.362) flow rates. Rate of amylase synthesis per ml varied from a maximum of 2.045 u/ml at 20 ml/hr flow rate, to 0.4839 u/ml at 45 ml/hr. At higher flow rates the rate of amylase synthesis was very less. From the data presented in the Table 14 it is infered that the relative synthesis of amylase at various flow rates was highly marked such that increase in the flow rates has led to many fold synthesis of alpha amylase. Thus the ratio of amylase synthesis with reference to units\ml between the flow rates increased along with the increase in the flow rates. The ratio between 20 ml/hr and 150 ml/hr was 1:31.5 while the ratio between 30 ml/hr and 150 ml/hr was 1:16.16. Similarly the ratio between 45 ml and 150 ml was 1:7.46. The ratio between 20 ml/hr and 120 ml/hr was recorded as 1:20.59 between 30 and 120 1:10.56.

The ratio of amylase synthesis with reference to units/ by the immobilized cells also exhibited a similar trend of increase in the ratio of production along with the increase in the flow rate (Table 14 b).
Thus the ratio between 20 ml/hr and 150 ml/hr was 1:4.2 20 ml/hr and 120 ml/hr was 1:3.43

30 ml/hr and 150 ml/hr was 1:3.2 and 45ml/hr and 150 ml/hr was 1:2.24.

Half life period

Half life period of the immobilized beads was computed based on the performance of beads in a fluid bed reactor packed with immobilized cells over a period of 50 days. From the data presented in Fig. 14. it could be deduced that the half life of the immobilized cells is 25 days. Although a sharp decline in the activity was shown by the beads during the initial 10 days, in the later period of study the performance of the cells declined in a regular fashion.

3.5 Co-Immobilization.

B. Polymyxa and S.Cerevisiae were coimmobilized in Calcium-Alginate at various proportions simultaniously and their efficiencies to produce alcohol from starch was studied. Results presented in Fig.15 clearly indicates that alcohol is produced directly from starch if both Bacillus polymyxa and S. Cerevisiae coimmobilized and subjected to starch treatement. In general the yield of alcohol varied from 12.2% to 14.8% per gram of starch used as a substrate and varied from 4.88% to 5.92% per 100 gm of beads used. Among the proportions studied the ratio of 1:2 and 1:1.5 of bacteria versus yeast showed a higher yield of alcohol compared to other combinations. When the bacterial concentrations were higher (2:1 and 1.5:1) in the beads compared to yeast cell concentrations, alcohol yield was declined. When they were at similar concentrations the alcohol yield was very much ignificant for the proportions.

Statistical Analysis

Pearson correlation coefficient analysis was carried out to find out whether there exist any positive or negative correlation between the variables studied. Results presented in Table 16 clearly indicates that there was a significant positive correlation between percent starch conversion and enzyme production, reducing sugar, total sugar, activation time & residence time; between enzyme production and reducing sugar,total sugar,activation time & residence time; and between total sugar and activation time.On the other hand a significant negative correlation existed between percent of starch conversion and bead size, concentration of sodium alginate, concentration of $CaCl_2$ and alcohol; enzyme and concentration of sodium alginate , bead size & concentration of $CaCl_2$; reducing sugar and concentration of $CaCl_2$.

Table - 1

Optimization of support concentration for the preparation of immobilized beads

Concentration of aliginate (%)	pH	E P units in soluble starch (SS)	E P units in Rice starch (RS)	Wheat starch (WS)
2*	7	25.3	26.6	9.0
	8	28.2	34.5	20.1
	9	30.0	40.8	39.6
3*	7	23.0	22.0	5.8
	8	26.3	34.4	19.4
	9	28.0	35.0	37.0
4*	7	18.8	17.4	6.0
	8	24.0	27.9	8.8
	9	25.3	35.7	2.0
5*	7	15.0	12.6	5.8
	8	22.0	26.0	5.8
	9	24.0	32.5	19.4

* Mean of 5 values.

E.P Enzyme Production.

Table - 2

Optimization of cell concentration for the preparation of immobilized beads

Cell concentration (mg/100g of beads)	Retention time in hrs	% of starch conversion	Enzyme production in units/gm of beads	Reduced sugar mg/gm of beads
50	1	70.0	41.1	1.984
	2	74.3	43.7	1.867
	3	73.6	43.2	1.867
	4	78.9	46.4	1.984
	5	69.6	40.9	1.758
	6	66.0	38.8	1.698
100	1	83.5	49.0	2.094
	2	76.2	44.8	1.924
	3	76.0	44.2	1.924
	4	87.3	51.3	2.207
	5	65.7	38.6	1.641
	6	73.2	43.0	1.839
150	1	79.4	46.7	2.179
	2	76.8	45.1	2.009
	3	69.7	41.0	1.758
	4	67.2	39.5	1.698
	5	73.8	43.4	1.867
	6	60.4	35.5	1.528
200	1	75.3	44.2	1.924
	2	75.3	44.2	1.924
	3	69.8	41.1	1.754

	4	65.2	38.3	1.698
	5	58.8	34.5	1.584
	6	55.0	32.2	1.471
	1	71.4	42.0	1.839
250	2	70.1	41.2	1.754
	3	63.2	37.1	1.556
	4	60.8	35.7	1.471
	5	54.2	31.8	1.301
	6	49.8	29.2	1.160

Table 3

Optimization of enzyme concentration for the prepation of immobilized beads

Enzyme concentration (ml)	Retention time (in minutes)	% of starch conversion	Reducing sugar (mg/gm of beads)
5	10	60.0	0.4150
	20	61.6	0.4260
	30	62.7	0.4336
	40	62.7	0.4336
	50	64.4	0.4454
	60	65.5	0.4530
10	10	68.8	0.4758
	20	70.3	0.4862
	30	79.1	0.4779
	40	79.5	0.4807
	50	70.3	0.4862
	60	81.1	0.4919
15	10	72.5	0.5014
	20	71.8	0.4966
	30	72.8	0.5035
	40	75.6	0.5229
	50	74.4	0.5146
	60	75.0	0.5187
20	10	83.4	0.5768
	20	82.8	0.5727
	30	85.0	0.5879
	40	85.6	0.5920
	50	86.3	0.5969
	60	85.6	0.5920

25	10	99.3	0.6868
	20	99.3	0.6868
	30	99.3	0.6868
	40	99.3	0.6868
	50	99.2	0.6864
	60	99.2	0.6864
30	10	86.1	0.5955
	20	86.1	0.5955
	30	86.1	0.5955
	40	86.1	0.5955
	50	86.1	0.5955
	60	86.1	0.5955

Table 4

Optimization of curing time for the preparation of immobilized beads

Curing time (in hrs)	Residence time in hrs	Enzyme activity in units/g.b.	% of starch converted	Reducing sugar production mg/gm of beads
	1	31.4	53.5	0.472
	2	34.4	58.5	0.517
1 hr	3	34.2	58.2	0.517
	4	36.4	61.9	0.818
	5	39.0	66.3	0.878
	6	35.0	58.5	0.517
	1	32.9	56.0	0.494
	2	33.9	57.0	0.509
2 hr	3	38.1	64.8	0.573
	4	35.2	59.9	0.792
	5	33.8	64.4	0.852
	6	55.2	94.0	1.254
	1	27.0	46.0	0.409
	2	34.5	58.7	0.520
3 hr	3	36.6	62.3	0.550
	4	37.1	63.2	0.837
	1	32.5	55.4	0.4912
	2	32.2	54.9	0.4874
4 hr	3	39.6	67.4	0.5954
	4	36.0	61.3	0.8112
	5	37.7	64.2	0.8484
	6	55.8	95.0	1.2570

	1	31.7	54.3	0.4800
	2	33.8	57.7	0.5098
5 hr	3	42.4	72.2	0.6400
	4	36.2	61.7	0.8186
	5	37.5	63.9	0.8447
	6	55.4	94.2	1.2420

Table 5

Effect of temperature on the activity of immobilized cells

Temp. °C	% of starch conversion	Enzyme production u/g of beads	Reduced sugar mg/gm of beads
30	87.6	44.6	1.195
40	88.4	45.6	1.208
50	81.4	41.4	1.109

Table 6

Effect of residence time on the activity of immobilized cells

Residence time (min.)	Percentage of starch conversion	Enzyme production(u/g of beads)
60	69.45	40.75
120	69.73	39.65
180	70.69	38.68
240	72.55	37.32
300	73.84	35.65
360	60.88	35.00

Table - 7

Effect of buffers on leaching

Time (min.)	Acetate buffer pH 5.6	Citrate buffer pH 6.2	Phosphate buffer pH 7.0	Citrate phophate buffer pH 7.0	Phosphate buffer pH 8.0	Glycine NaOH pH 9.0	Glycine NaOH pH 10.0
15	0	0	2.1	0	5	0	0
30	0	0	4.4	1.2	30.5	9.3	2.4
45	5.4	5.7	12.4	6.8	64.8	19.4	8.6
60	9.7	10.4	20.8	11.4	80	20	17
90	10.2	11.1	32.7	13.8	100	20.2	20.5
120	10.5	13.8	48.3	14.4	100	20.6	21.6

Table - 8

Effect of initial cell concentration on leaching

Time (hours)	50mg	100mg	150 mg
1	0.45	0.41	0.40
2	0.53	0.48	0.49
3	0.62	0.53	0.51
4	0.68	0.64	0.54
5	0.72	0.66	0.58
6	0.96	0.70	0.64

* % of leaching were expressed in mg of protein /gm of beads

Table - 9

Effect of cross linking on leaching of cells from the immobilized beads

Time in minutes	Entrapped aliginate	cross linked aliginate
0	0	0
30	0.28	0.26
60	0.41	0.28
90	0.44	NIL
120	0.50	NIL
180	0.58	NIL
240	0.69	NIL
300	0.72	NIL
360	0.76	NIL

* Leaching expressed in % of cell protein / gm of bead

Table 10 (a)

Rate of Starch Diffusion into the immobilized beads

Hours	Rate of Starch diffusion rate	
	Cells + Alginate mg/gm of beads	Alginate mg/gm of beads
0	0	0
1	0.1212	0.1894
2	0.1537	0.1920
3	0.1241	0.1697
4	0.1202	0.1523
5	0.1212	0.1598
6	0.1472	0.1453
7	0.1327	0.1453

Table 10 (b)

Sugar Conversion

Hours	Inside the bead		Outside the bead	
	Reducing sugar mg/gm of bead	Total Sugar mg/gm of bead	Reducing Sugar	Total Sugar mg/gm of bead
0	0	0	0	0
1	0.0134	0.1275	0.1671	1.845
2	0.0170	0.1380	0.1819	1.387
3	0.0137	0.1095	0.1793	1.746
4	0.0178	0.1035	0.1970	1.572
5	0.0134	0.378	0.1658	1.396
6	0.0163	0.051	0.1969	1.635
7	0.0214	0.152	0.1847	2.011

Table - 11

Comparative studies of alpha amylase production in free Vs immobilized cells of B. Polymyxa

Temp oC	Rice starch		Soluble starch		Potato starch		Wheat starch	
	immobilized	free	immobilized	free	immobilized	free	immobilized	free
30	45.7	43.5	44.6	43.3	44.0	41.6	48.6	47.2
40	46.8	43.7	45.0	44.2	45.8	42.3	48.6	47.4
50	43.9	42.7	41.4	42.5	43.8	41.4	44.8	46.7
pH								
7	43.9	42.3	43.0	45.0	40.8	43.0	46.8	49.5
8	46.8	43.0	45.0	45.2	45.0	42.3	46.6	41.8
9	40.5	40.0	43.8	41.0	44.8	41.4	47.0	44.2
Substrate concentration %								
0.1	49	52	47	56	45	51	51	58
0.5	46	43	45	45	44	42	48	47
1.0	28	39	21	30	32	36	24	33
1.5	14	20	12	18	19	24	16	20

Reducing sugar production

Temp oC	Rice starch immobilized	Rice starch free	Soluble starch immobilized	Soluble starch free	Potato starch immobilized	Potato starch free	Wheat starch immobilized	Wheat starch free
30	1.359	1.291	1.324	1.312	1.3068	1.2563	1.443	1.401
40	1.389	1.297	1.336	1.286	1.360	1.235	1.443	1.407
50	1.303	1.268	1.229	1.267	1.300	1.229	1.330	1.386
pH								
7	1.290	1.198	1.0835	1.033	1.225	1.10	1.334	1.298
8	1.358	1.266	1.2958	1.2453	1.2958	1.1708	1.443	1.407
9	1.295	1.203	0.9712	0.9207	1.083	0.958	1.258	1.222
Substrate concentration %								
0.1	1.103	1.395	1.0575	1.26	1.0125	1.1475	1.1475	1.53
0.5	1.035	0.9675	1.0125	1.0125	0.990	0.945	1.08	1.0575
1.0	0.630	0.8775	0.4725	0.675	0.720	0.81	0.54	0.4425
1.5	0.315	0.450	0.270	0.405	0.4275	0.54	0.36	0.45

Table - 12

Continuous synthesis of alpha amylase

Flow rate	Residence time (in min.)	Samples collected (Hrs)	% of starch convertion	Enzyme Synthesis in units Mg/g of beads	Reduced sugar mg/g of beads
150 ml/hr	16	1	24.40	10.20	0.6941
		2	25.12	10.50	0.7145
		3	22.44	9.38	0.6383
		4	23.25	9.72	0.6614
		5	21.70	8.90	0.6056
120 ml/hr	20	1	29.45	12.31	0.8378
		2	29.00	12.14	0.8261
		3	29.57	12.36	0.8410
		4	28.47	11.90	0.8097
		5	26.03	10.88	0.7403
60 ml/hr	40	1	35.36	14.78	1.0050
		2	36.51	15.26	1.0380
		3	36.63	15.31	1.0418
		4	35.65	14.90	1.0139
		5	34.33	14.35	0.9765
45 ml/hr	53.3	1	43.74	18.28	1.2439
		2	48.57	20.30	1.3814
		3	55.99	23.40	1.5923
		4	59.34	24.80	1.6876
		5	52.88	22.10	1.5039
30 ml/hr	80	1	78.72	32.90	2.2380
		2	78.72	32.90	2.2380
		3	78.48	32.80	2.2380
		4	71.7	30.0	2.0415

		5	68.67	28.7	1.9530
20 ml/hr	120	1	96.67	40.4	2.7492
		2	97.87	40.9	2.7832
		3	98.10	41.0	2.7900
		4	98.10	41.0	2.7900
		5	98.10	41.2	2.8036

Table 13

Cumulative production of alpha - amylase

During continuou synthesis of alpha amylase by immobilized fluid bed reactor

Total volume in 5 hrs ml	Enzyme U/g	Total time min	Rate of enzyme production U/min.
750	48.7	80 mts	48.7/min.
600	59.6	133 mts	36.67/80 min.
300	74.6	200 mts	29.84/80 min.
225	108.8	267 mts	32.62/80 min.
150	157.3	400 mts	31.46/80 min.
100	204.5	600 mts	27.27/80 min.

Table - 14

Comparative Ratios Between The Flow Rates (U/ml)

Flow Rate	30	45	60	120	150
20 ml	1.95	4.2	8.22	20.59	31.51
30 ml		2.17	4.22	10.56	16.16
45 ml			1.95	4.87	7.46
60 ml				2.5	3.83
120 ml					1.53
150 ml					

Table - 14 (b)

Comparative Ratios Between The Flow Rates (U/min)

Flow Rate Ratio	B	C	D	E	F
20 ml(A)	1:1.3	1:1.8	1:2.74	1:3.43	1:4.2
30 ml(B)		1:1.44	1:2.11	1:2.64	1:3.2
45 ml(C)			1:1.46	1:1.83	1:2.24
60 ml(D)				1:1.25	1:1.53
120 ml(E)					1:1.22

Table - 15

Immobilization of Bacillus polymyxa and Saccharomyces cerevisae for the production of alcohol

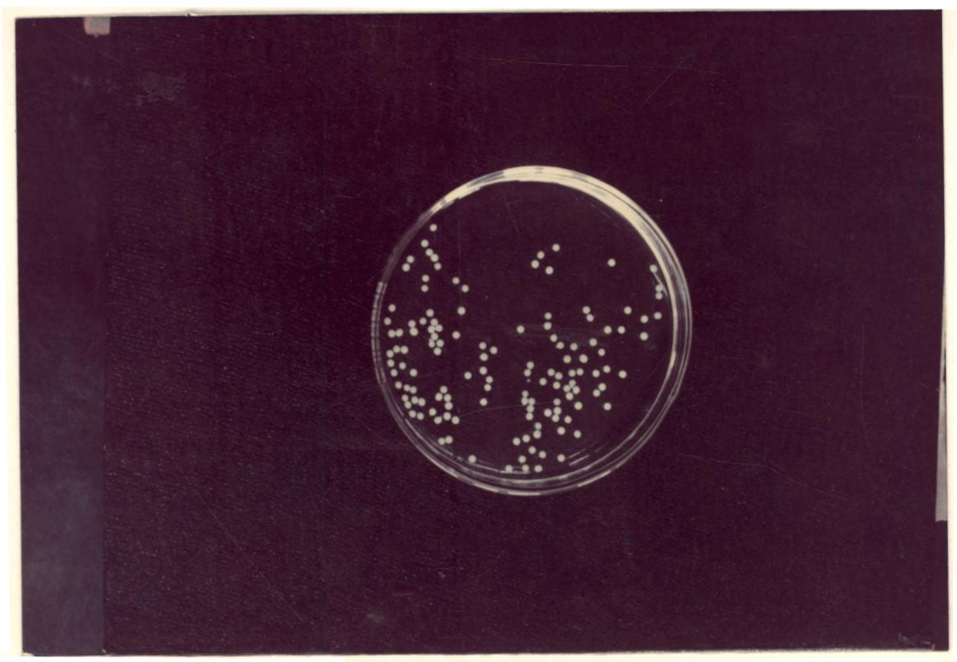
Ratio of B.polymyxa and Scerevisiae conc. in the beads	% of conversion of starch	% of production of reducing sugar	Yield of alcohol in %
1:1	66.8	35.15	14.6
1:2	66.3	33.57	14.8
2:1	77.5	34.2	12.3
1.5:1.0	77.6	30.15	12.2
1.0: 1.5	65.6	26.6	14.6

TABLE- 16

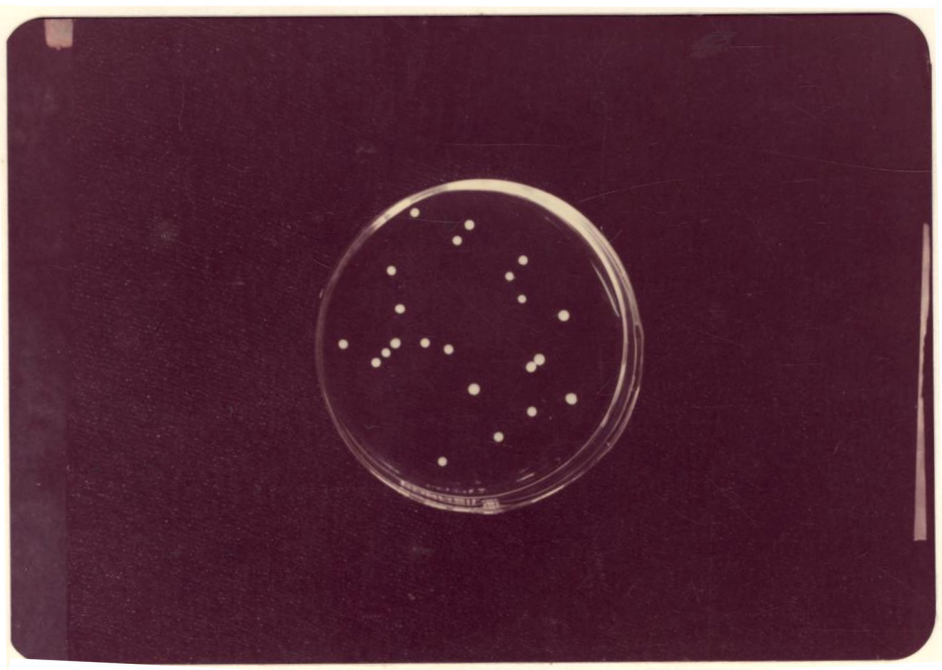
STATISTICAL ANALYSIS

	Enzyme	Reducing sugar	Total sugar	Activa-tion time	Bead size	Con. of alginate	Con. of $CaCl_2$	Residence time	Alcochol
% of Starch Conversion	0.9984	0.8822	0.9387	-0.7278	0.8158	-0.9497	-0.9640	0.8167	-0.0822
Enzyme	..	0.9998	0.9371	-0.7069	0.8039	-0.9497	-0.9437	0.8159	..
Reducing sugar	..	..	0.9697	0.8107	0.8395	..	-0.9686	0.8119	-0.3214
Bead size	..	..	..	0.7293	..	..	..	..	..

PLATE-I- CALCIUM ALGINATE IMMOBILIZED BEADS

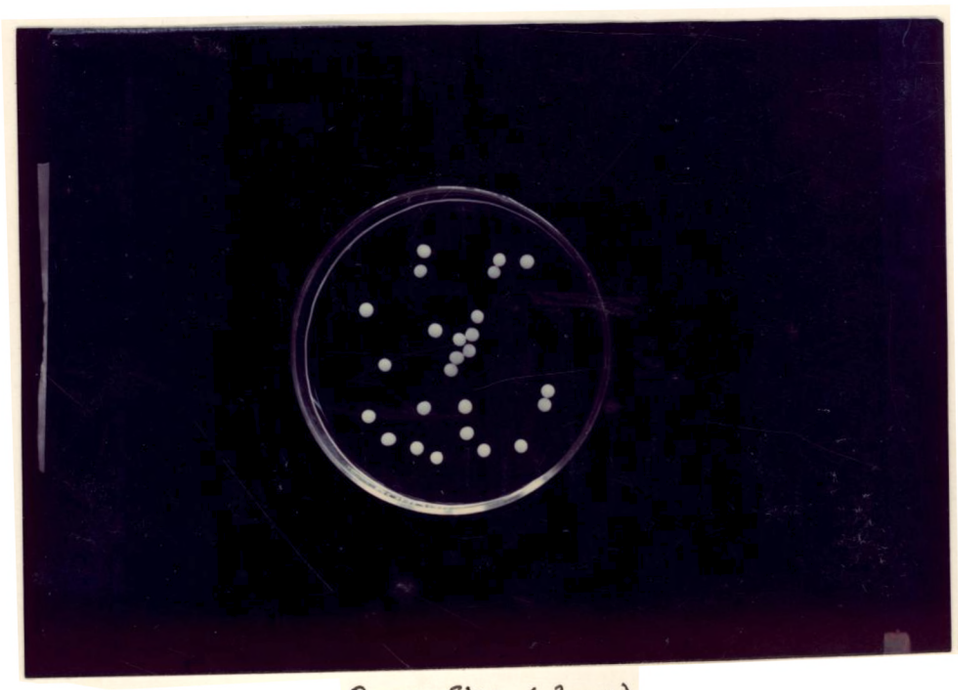


BEAD SIZE (1mm)

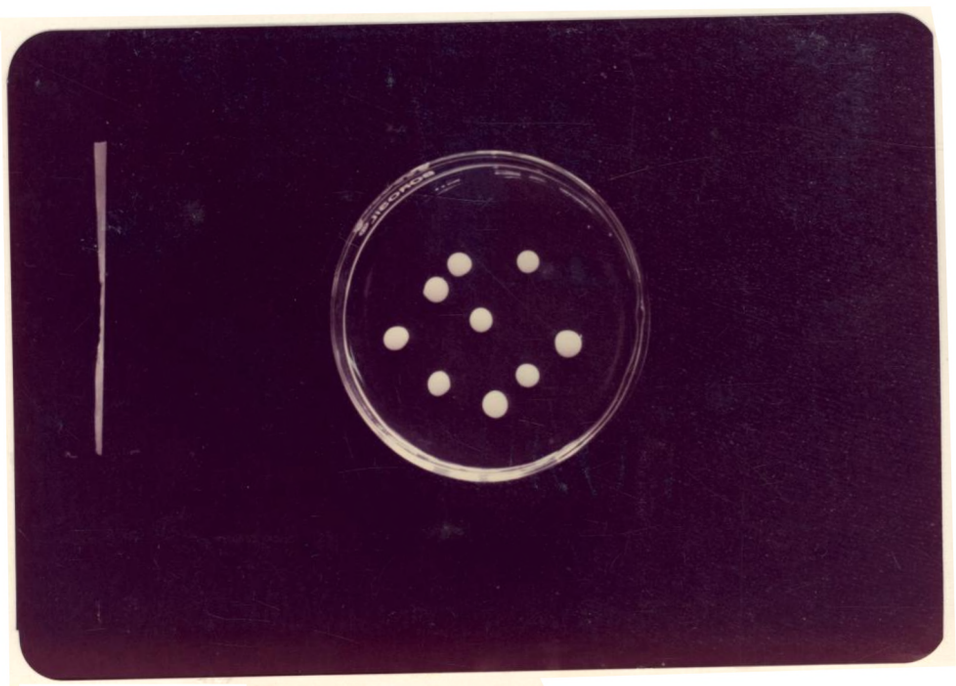


BEAD SIZE (2mm)

PLATE - II: CALCIUM ALGINATE IMMOBILIZED BEADS

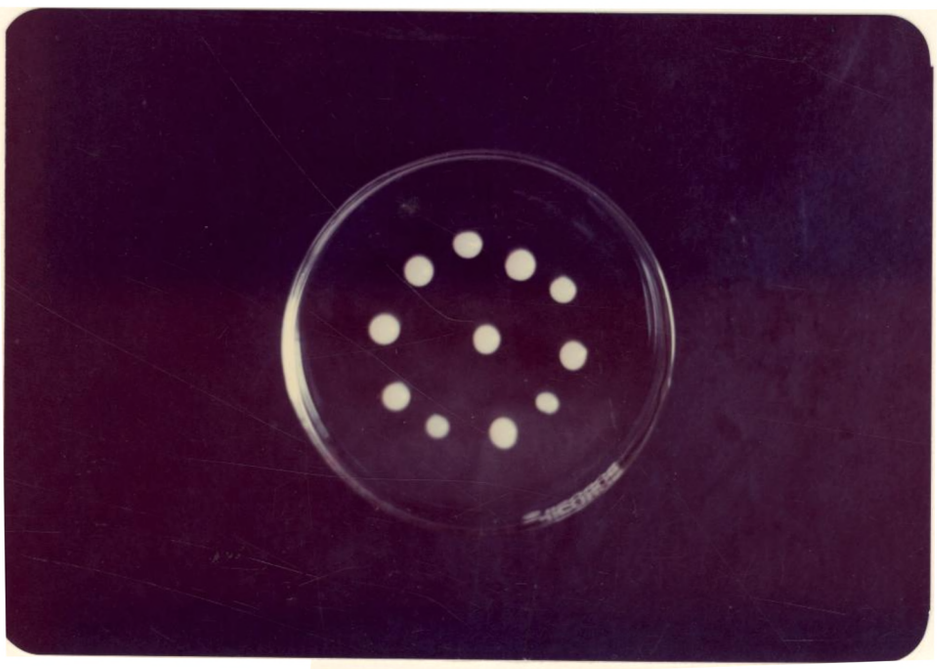


BEAD SIZE (3mm)



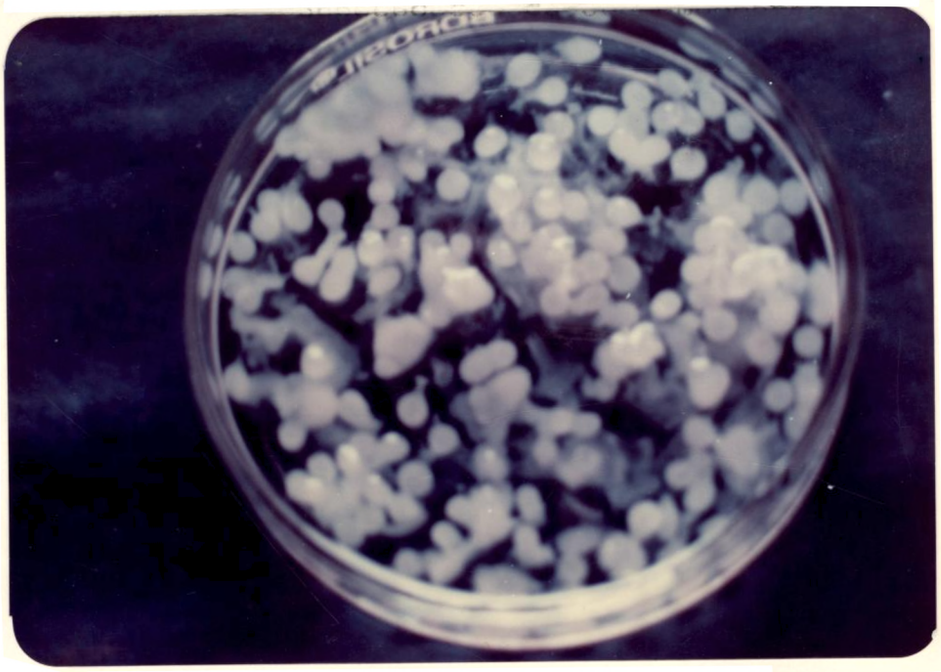
BEAD SIZE (4mm)

PLATE - III CALCIUM ALGINATE IMMOBILIZED BEADS

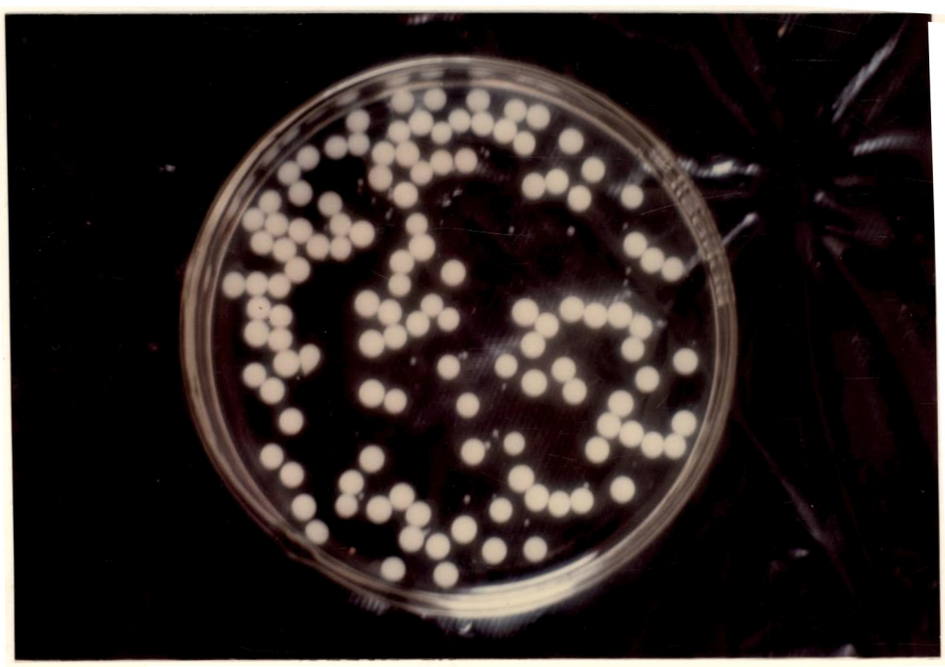


BEAD SIZE (5mm)

PLATE - IV Effect of $CaCl_2$ Con. on Bead Preparation

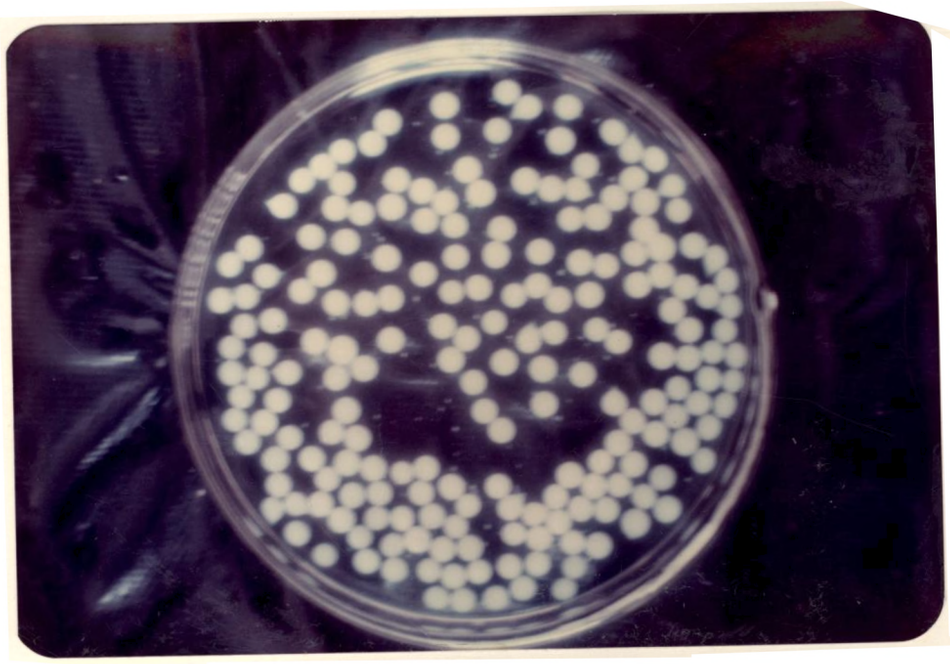


Bead Formation in 0.01 M $CaCl_2$ Con.

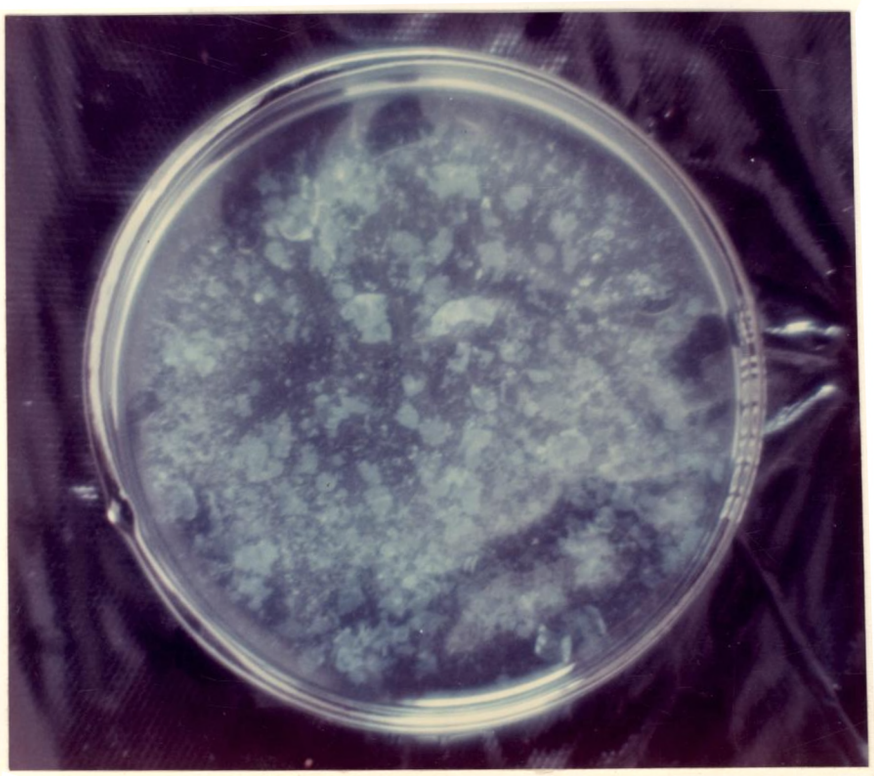


Bead Formation in 0.2 M $CaCl_2$ Con.

PLATE - V Effect of Buffers on Immobilized Beads

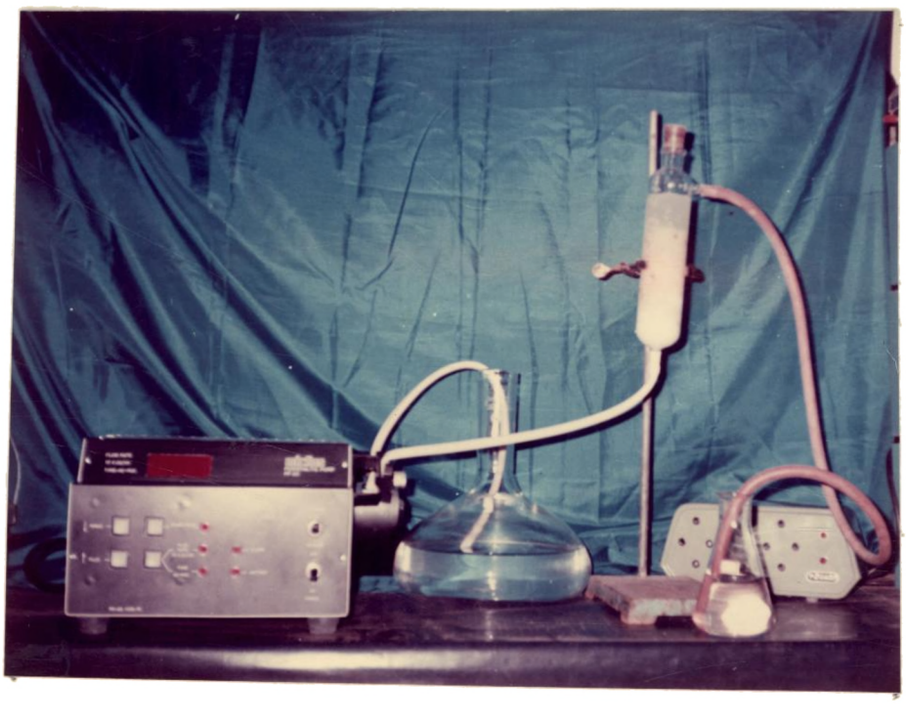


Normal Immobilized Beads



Disintegrated Beads in Phosphate Buffer

PLATE-VI IMMOBILIZED FLUID BED REACTOR



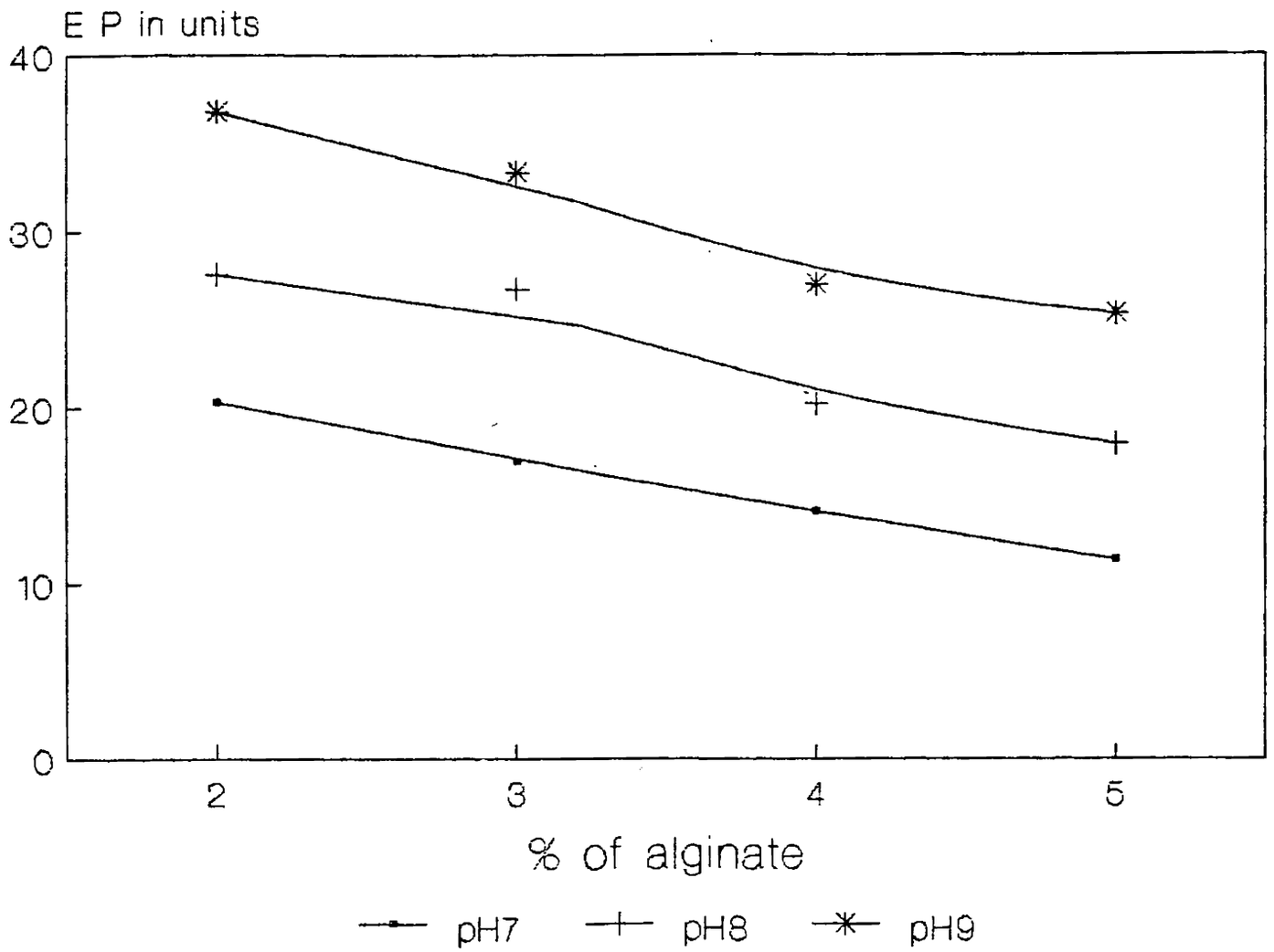


Fig.1. Optimization of Support Concentration for the preparation of immobilized beads.

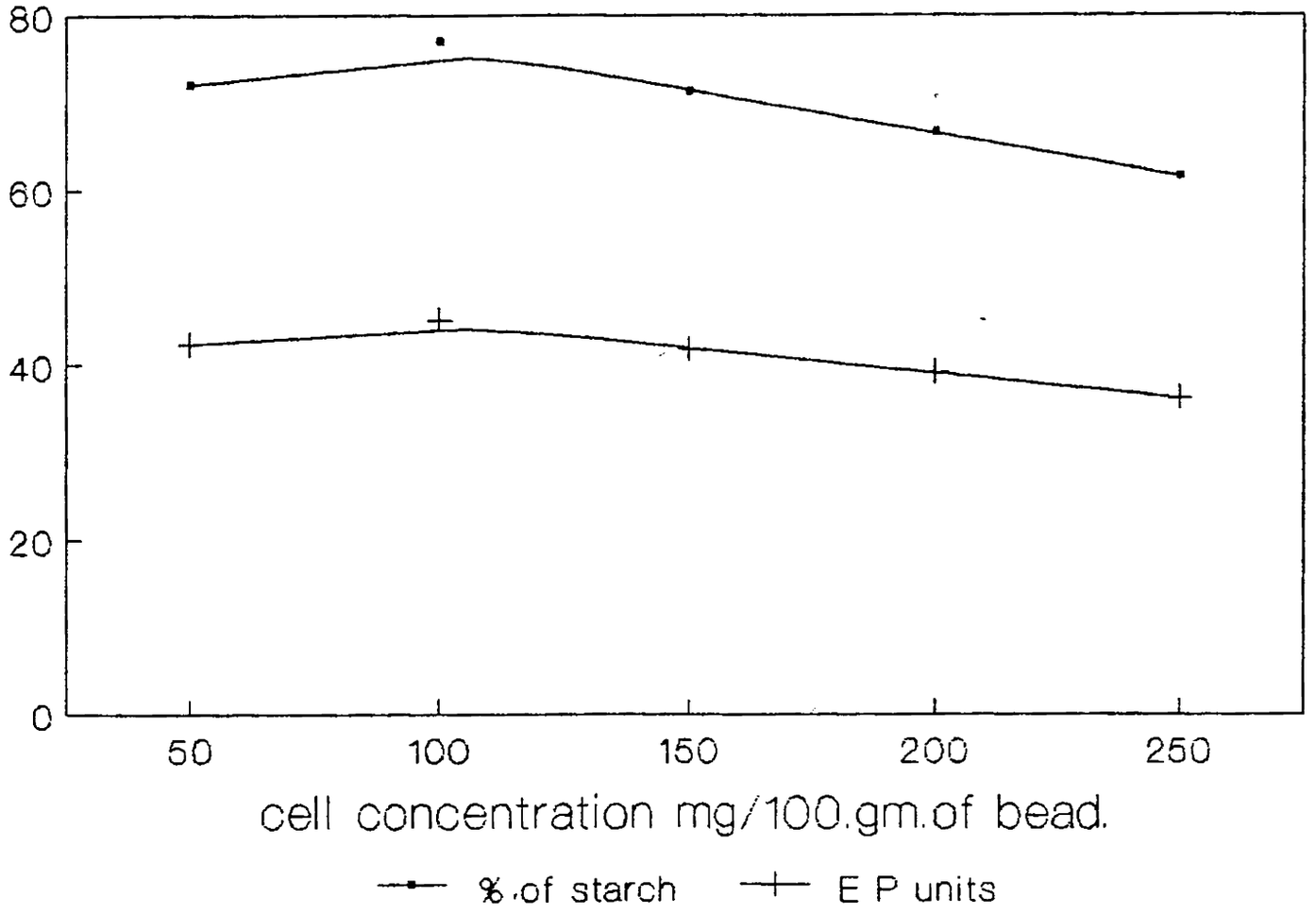


Fig.2.a. Optimization of Cell Concentration for the preparation of immobilized beads.

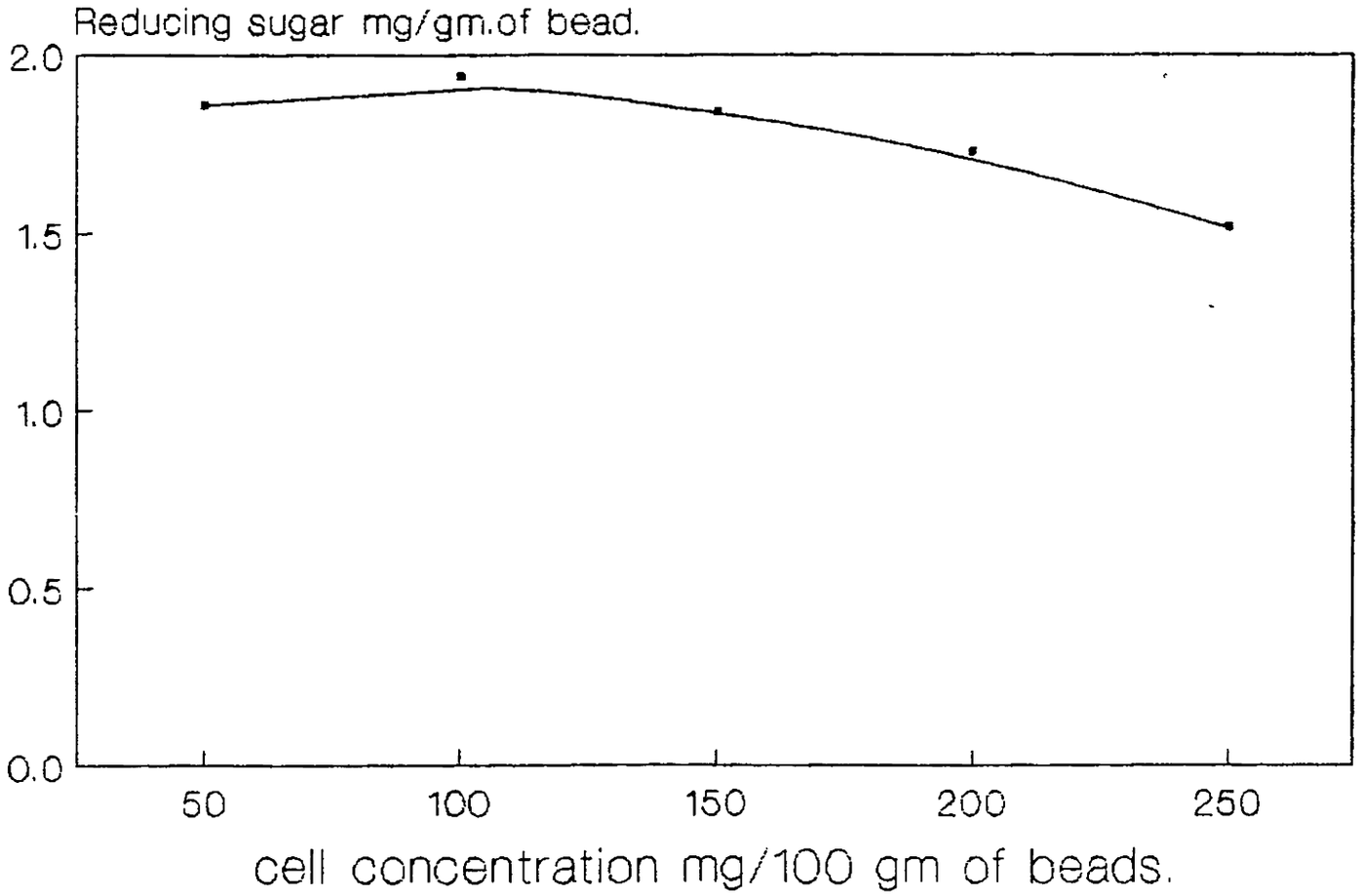


Fig.2.b. Optimization of Cell Concentration for the preparation of immobilized beads.

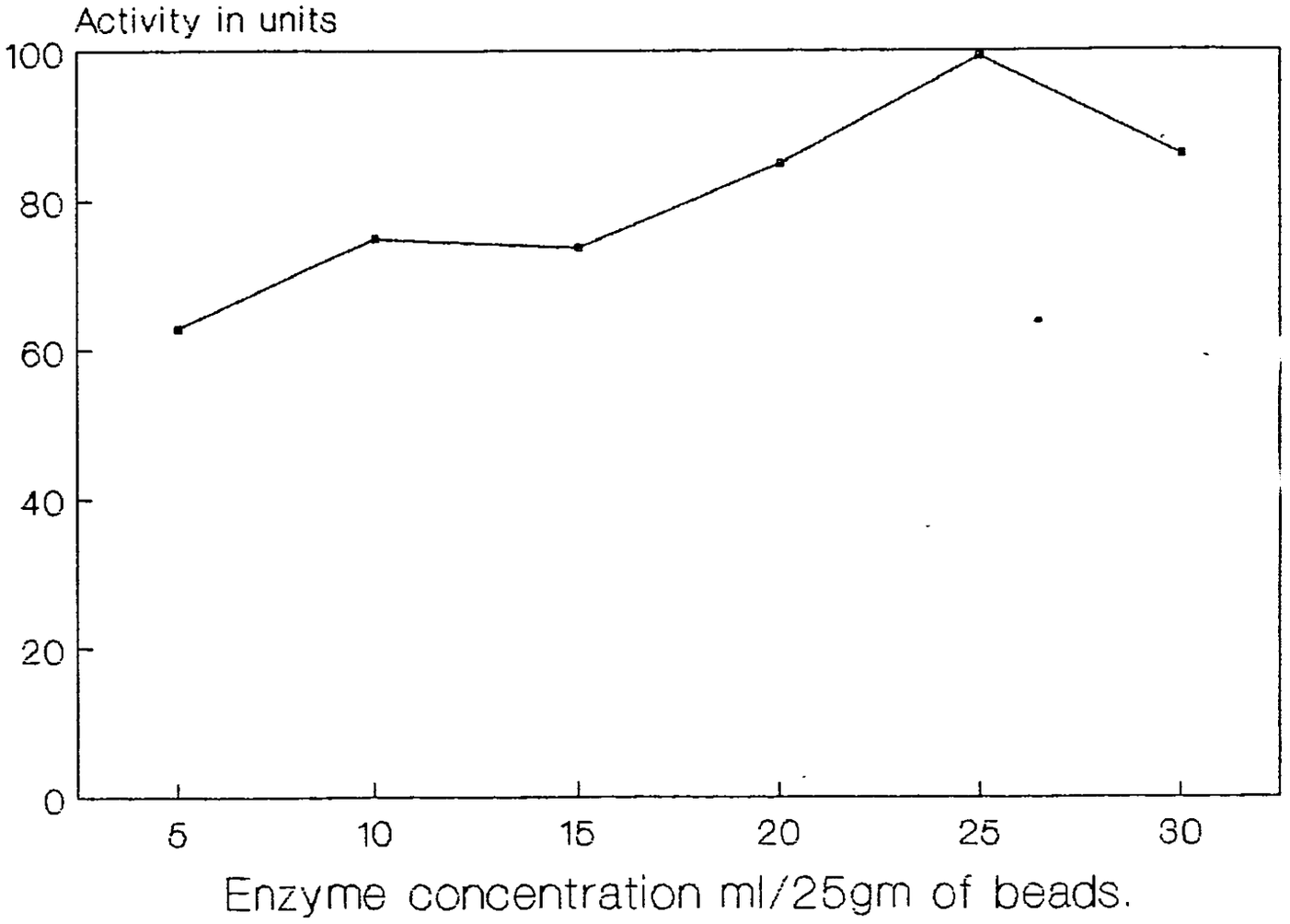


Fig.3.a. Optimization of Enzyme Concentration for the preparation of immobilized beads.

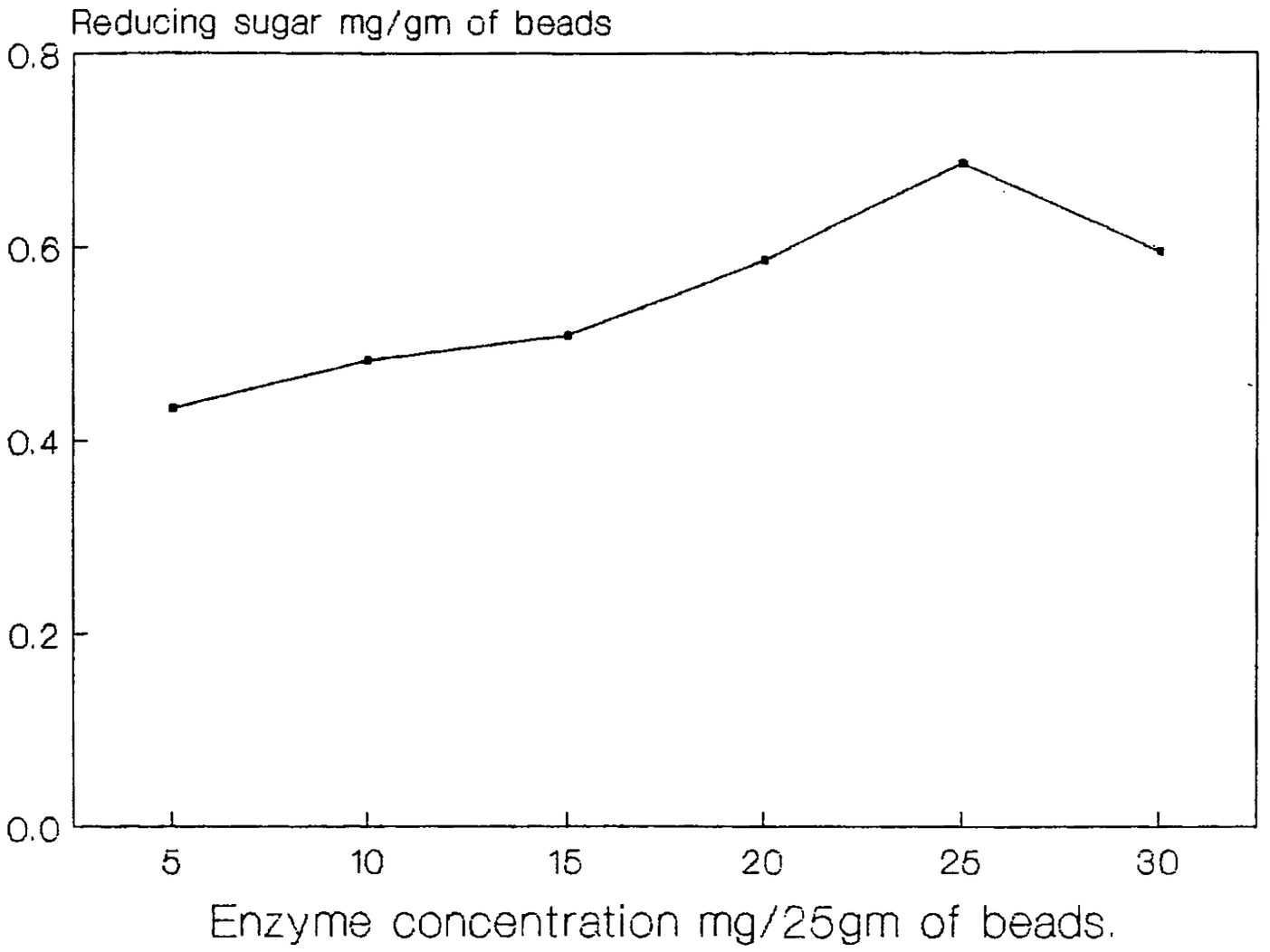


Fig.3.b. Optimization of Enzyme Concentration for the preparation of immobilized beads.

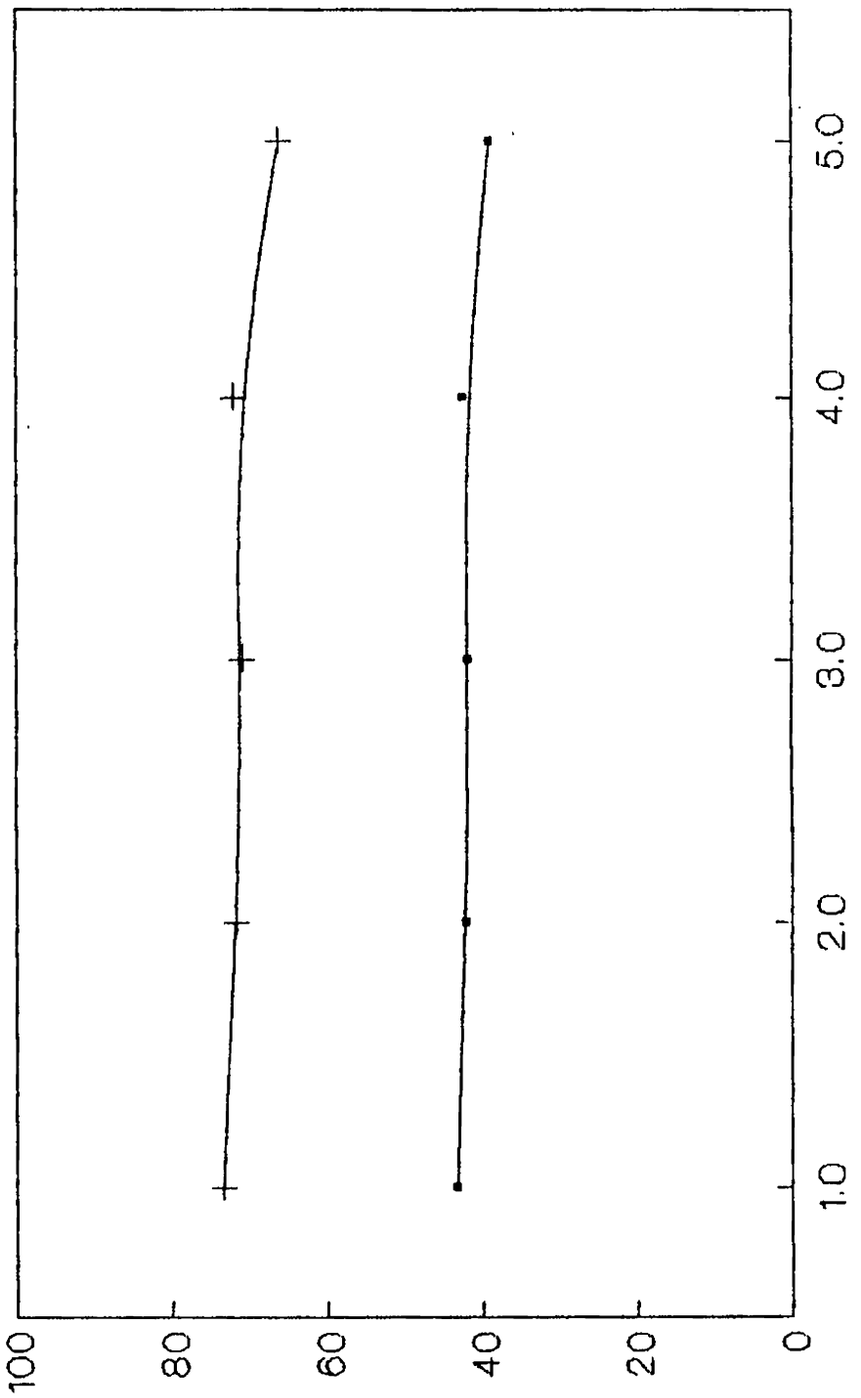


Fig.4.a. Optimization of Bead Size for immobilization.

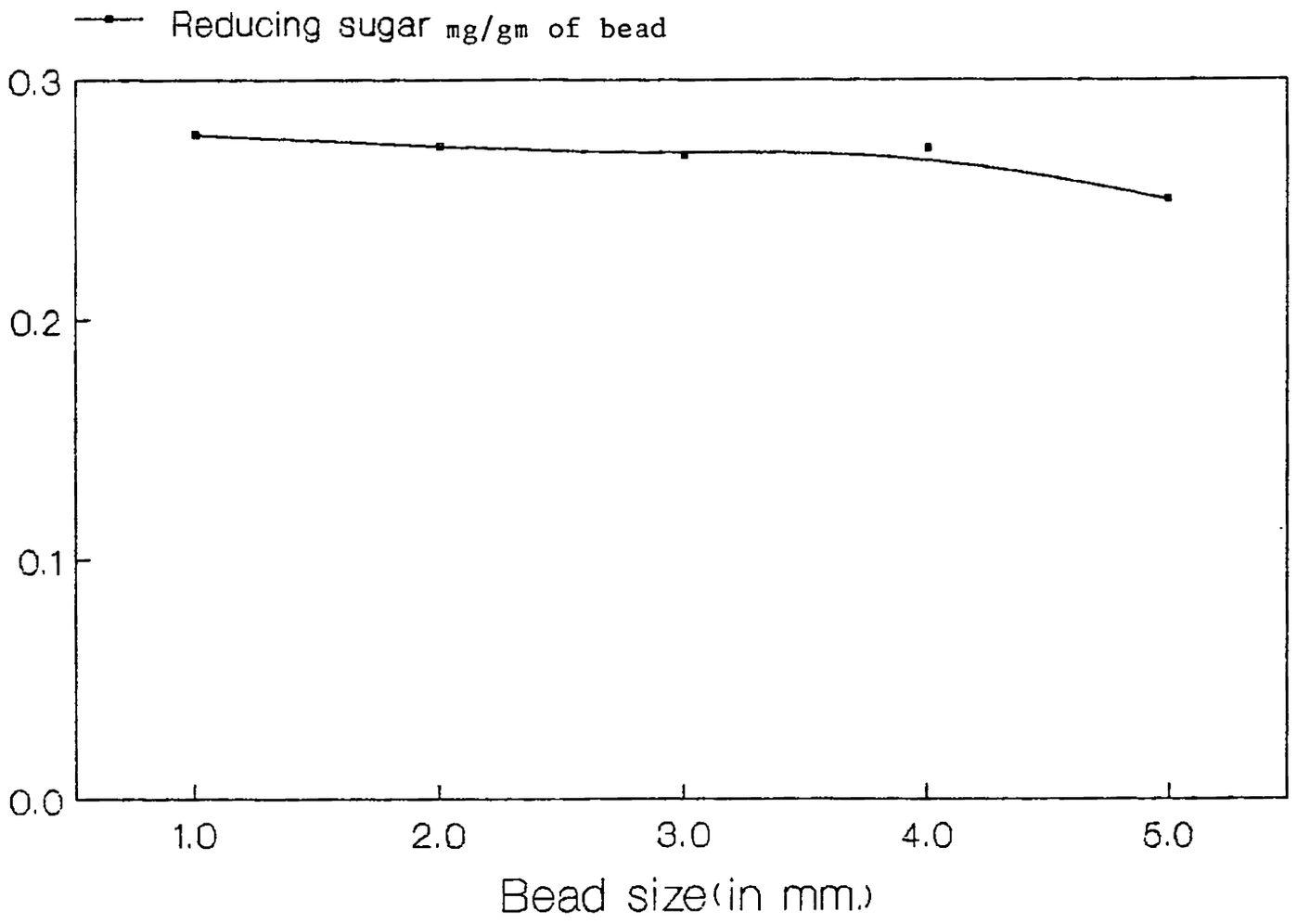


Fig.4.b. Optimization of Bead Size for immobilization.

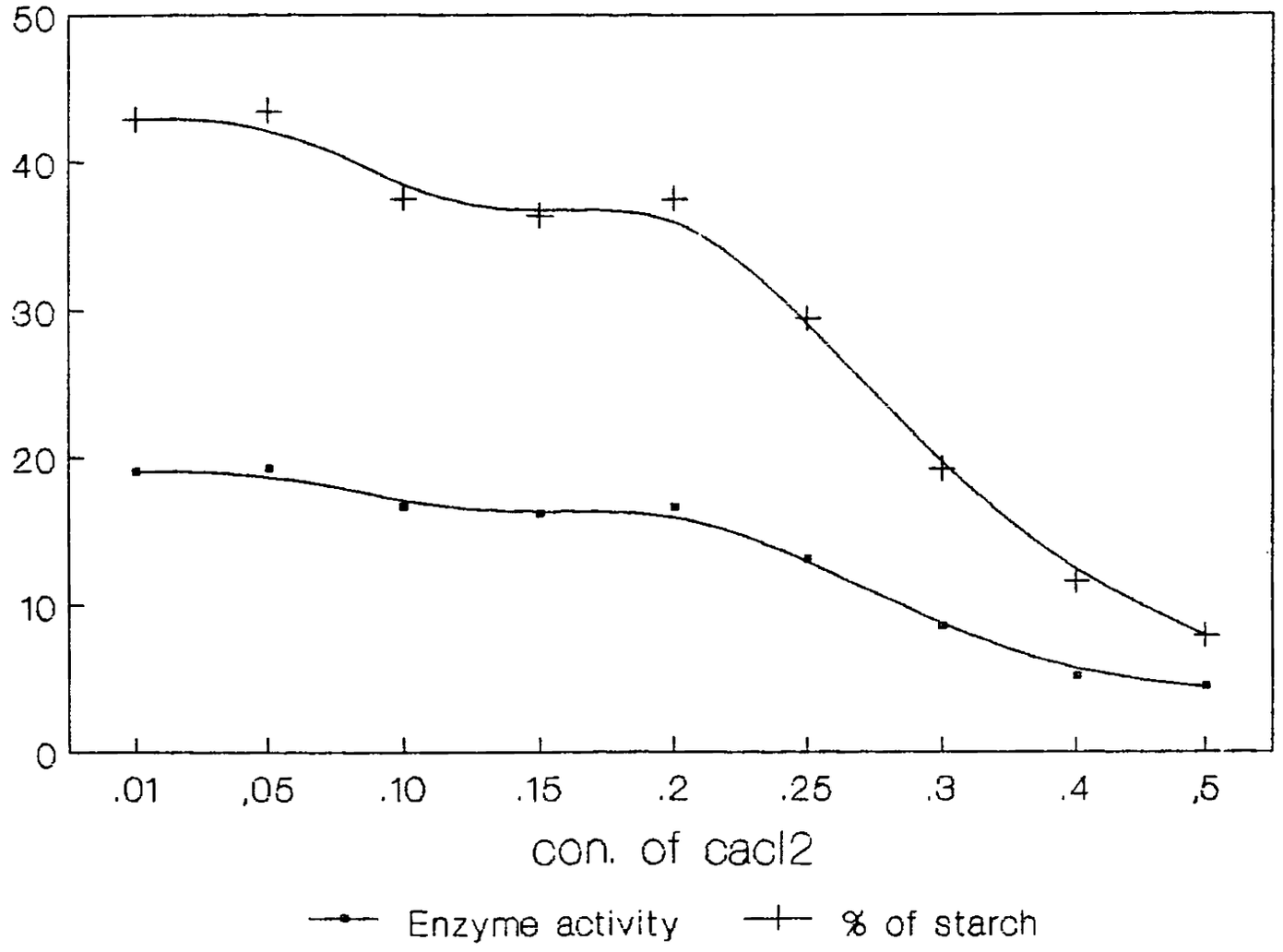


Fig.5.a. Optimization of $CaCl_2$ for the preparation of immobilized beads.

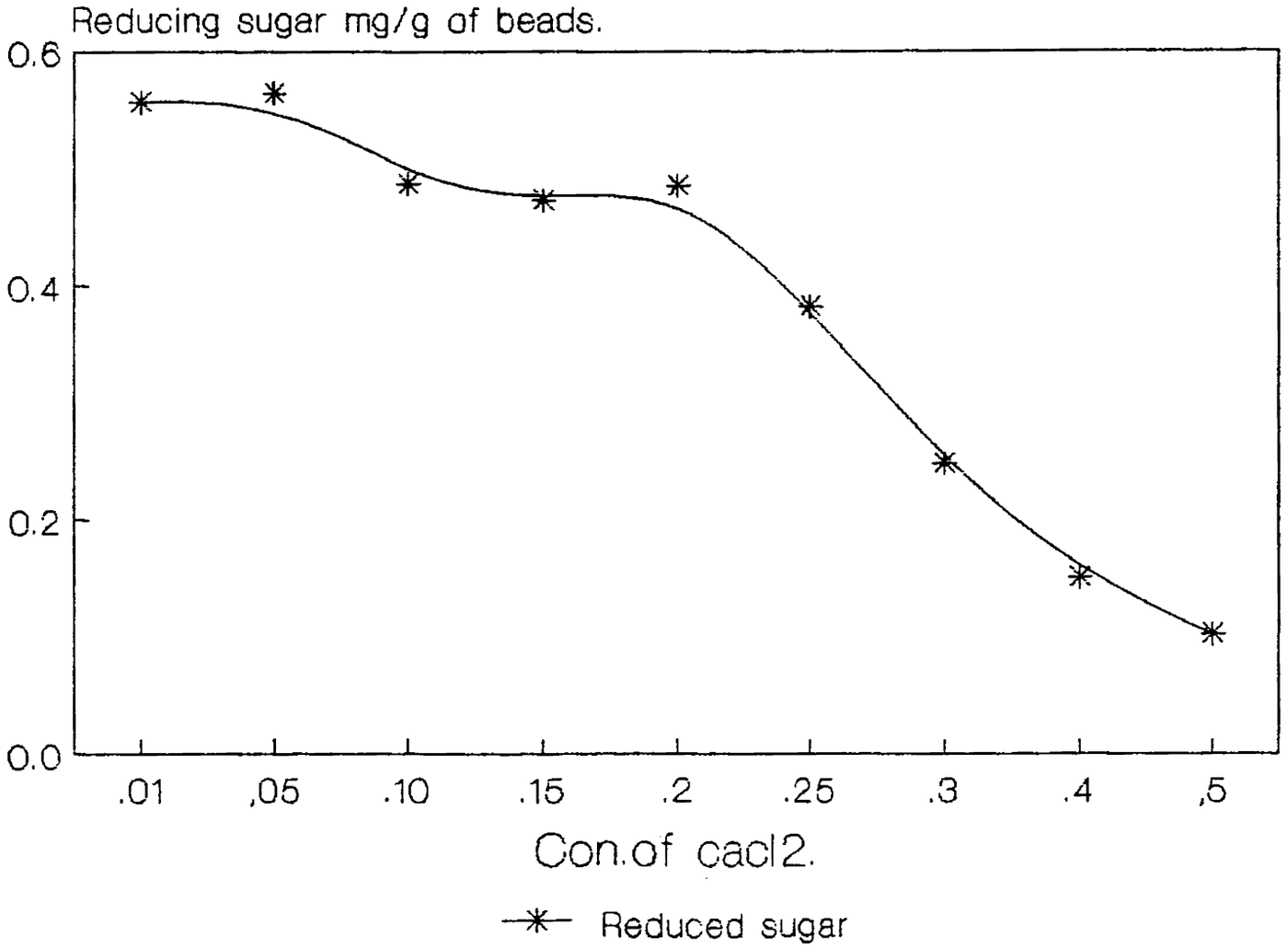


Fig.5.b. Optimization of $CaCl_2$ for the preparation of immobilized beads.

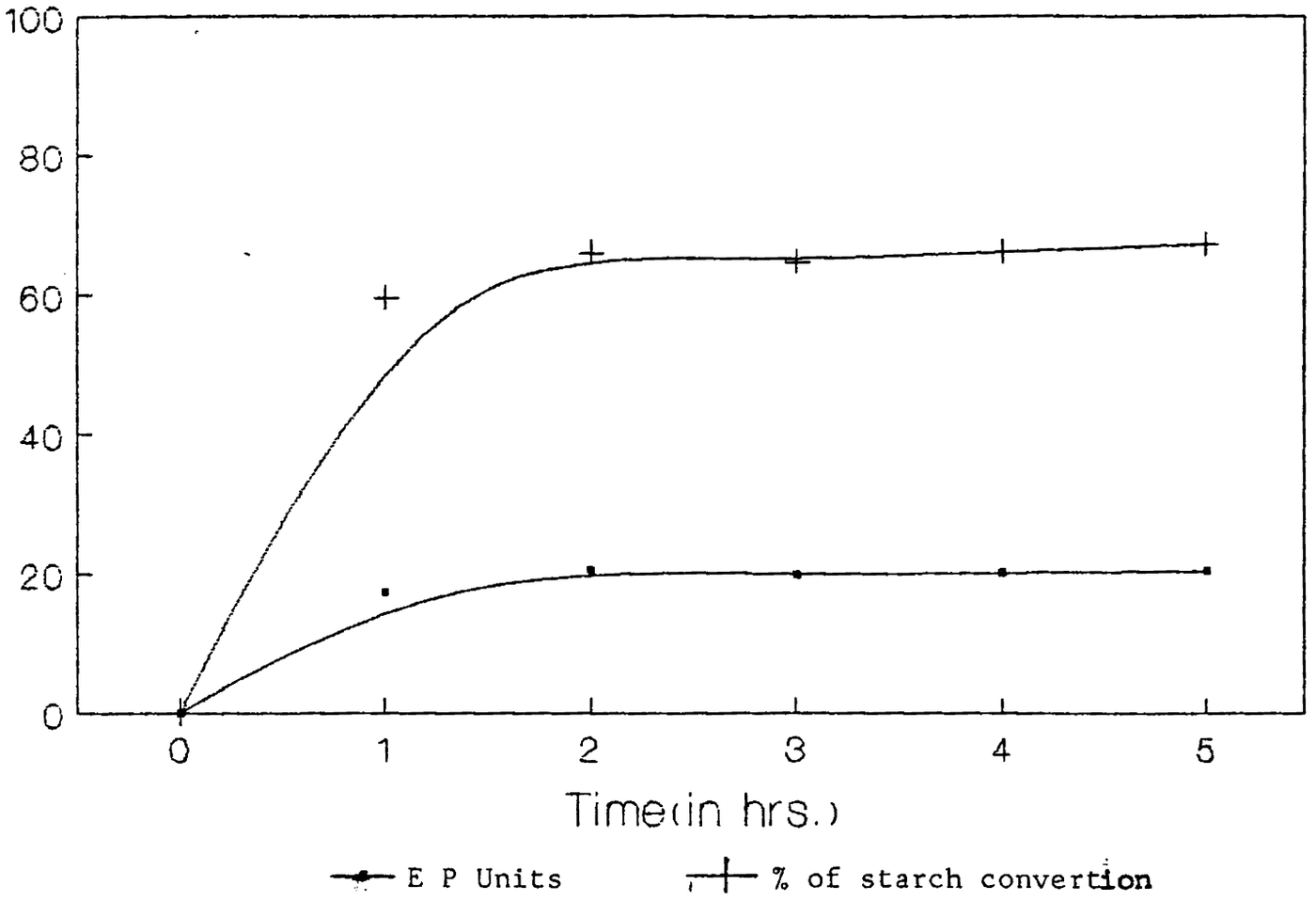


Fig.6.a. Optimization of Curing Time for the preparation of immobilized beads.

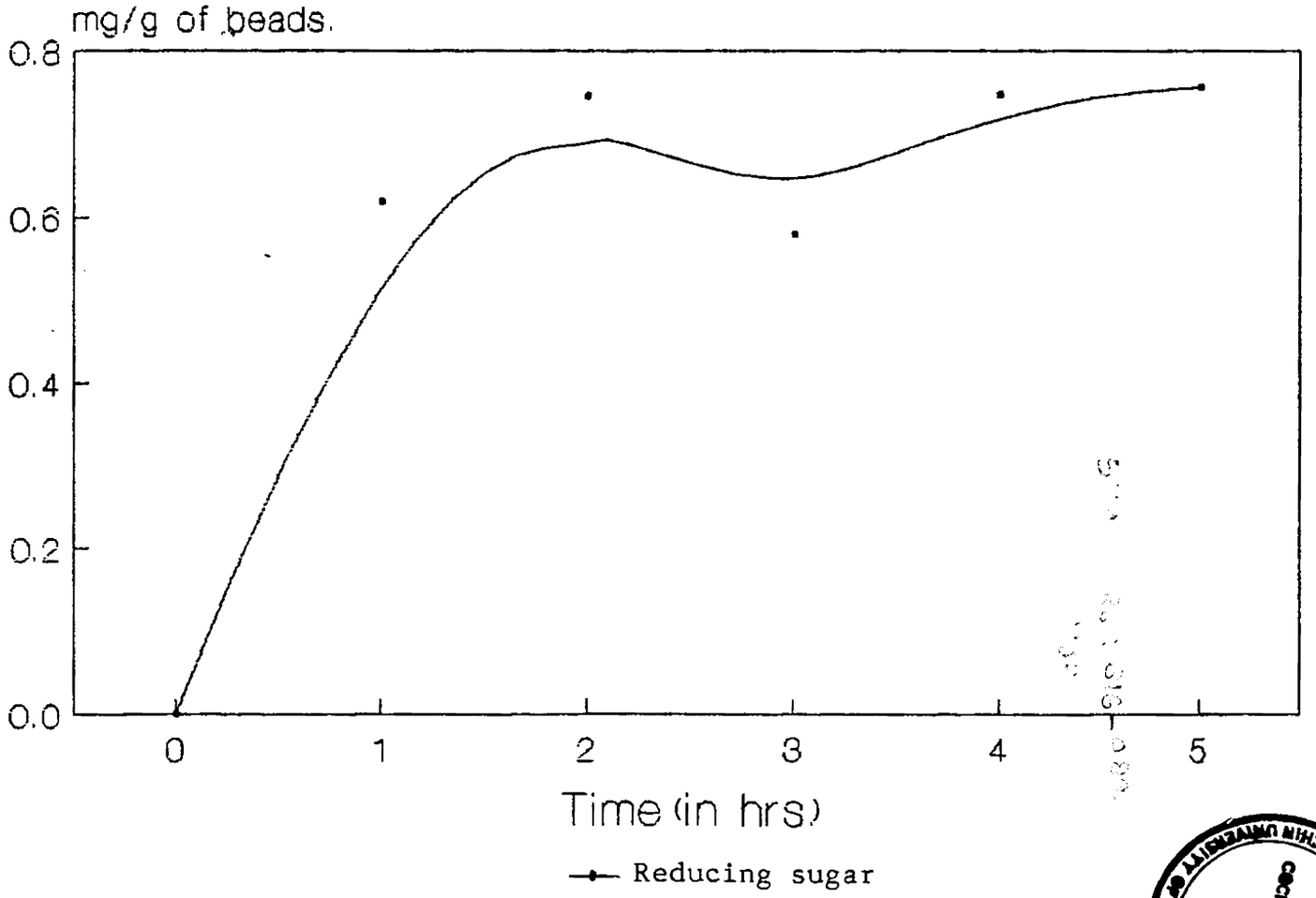


Fig.6.b. Optimization of Curing Time for the preparation of immobilized beads.

Effect of buffers on the activity of Immobilized beads.

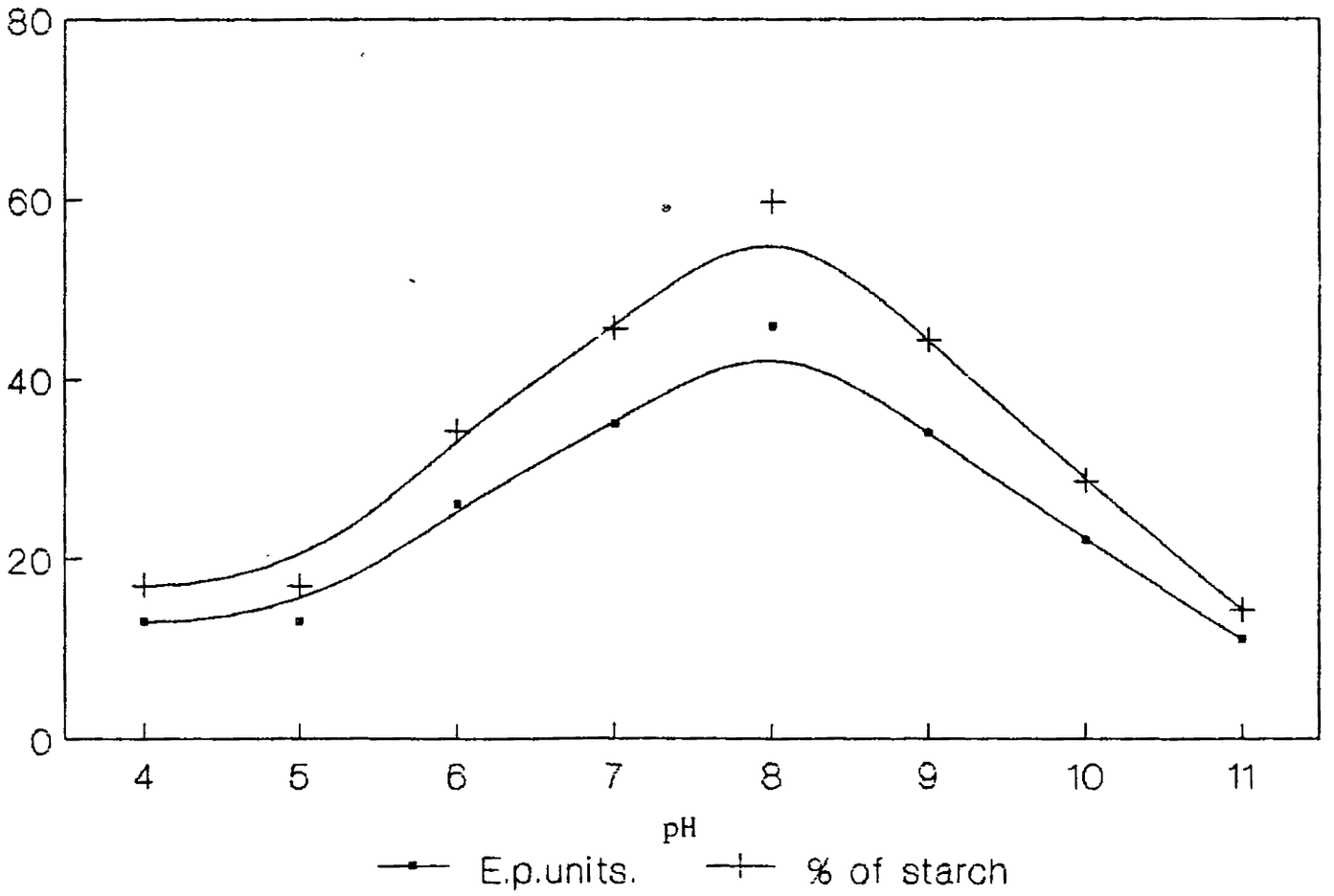


Fig.7.a.

Effect of buffers on the activity of Immobilized beads.

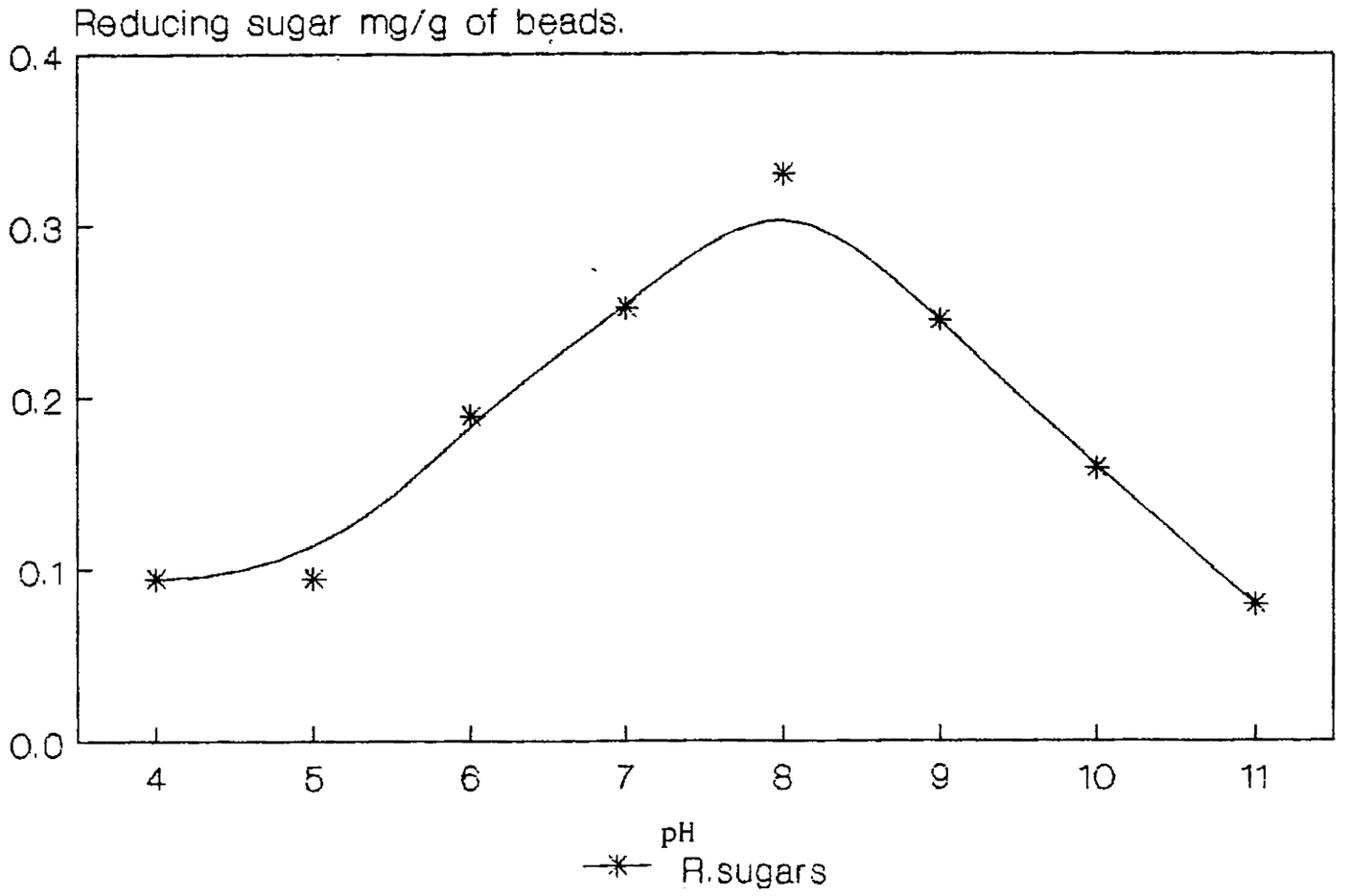


Fig.7.b.

Optimization of activation time for immobilization.

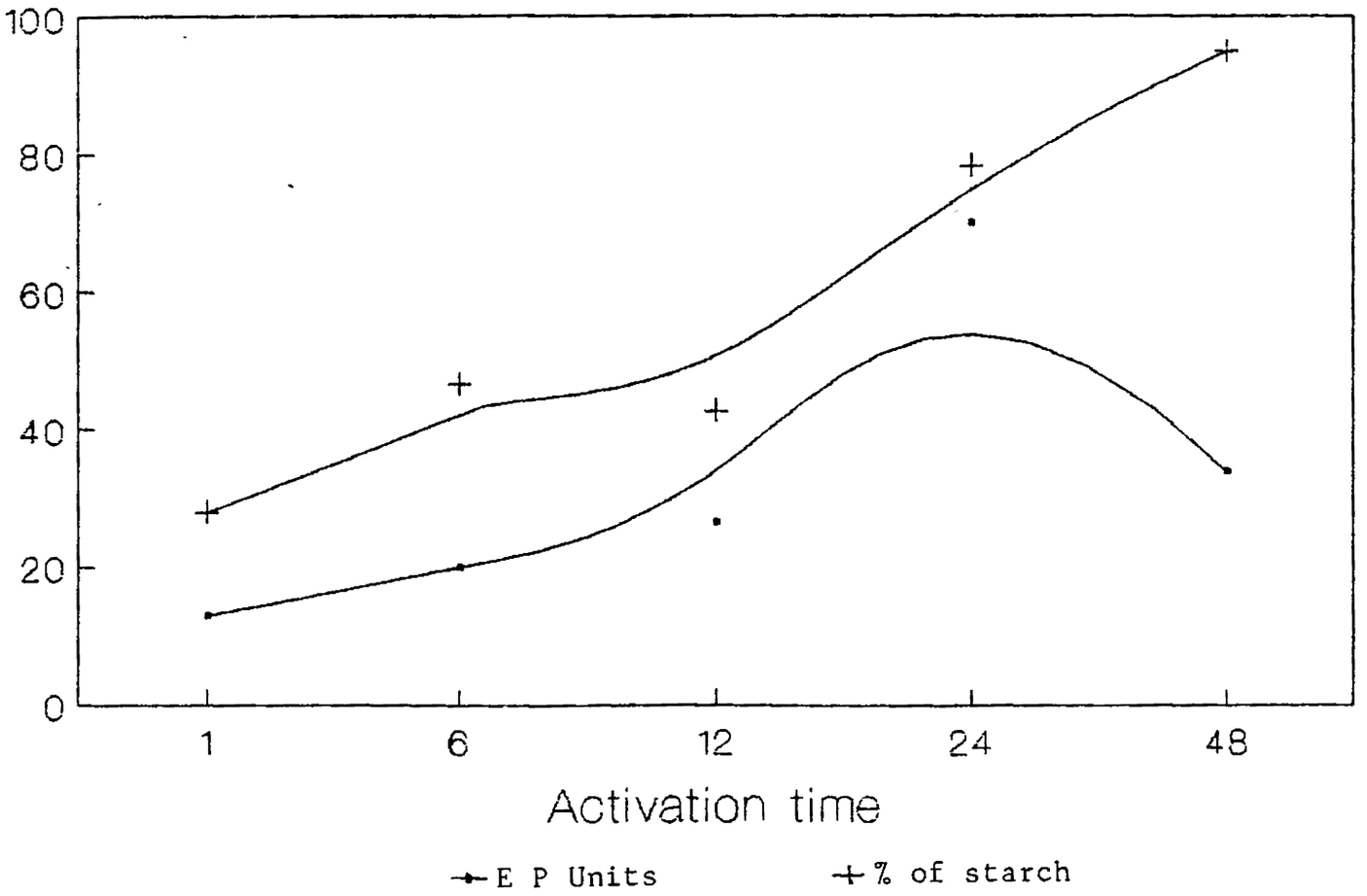


Fig.8.a.

Optimization of activation time for immobilization

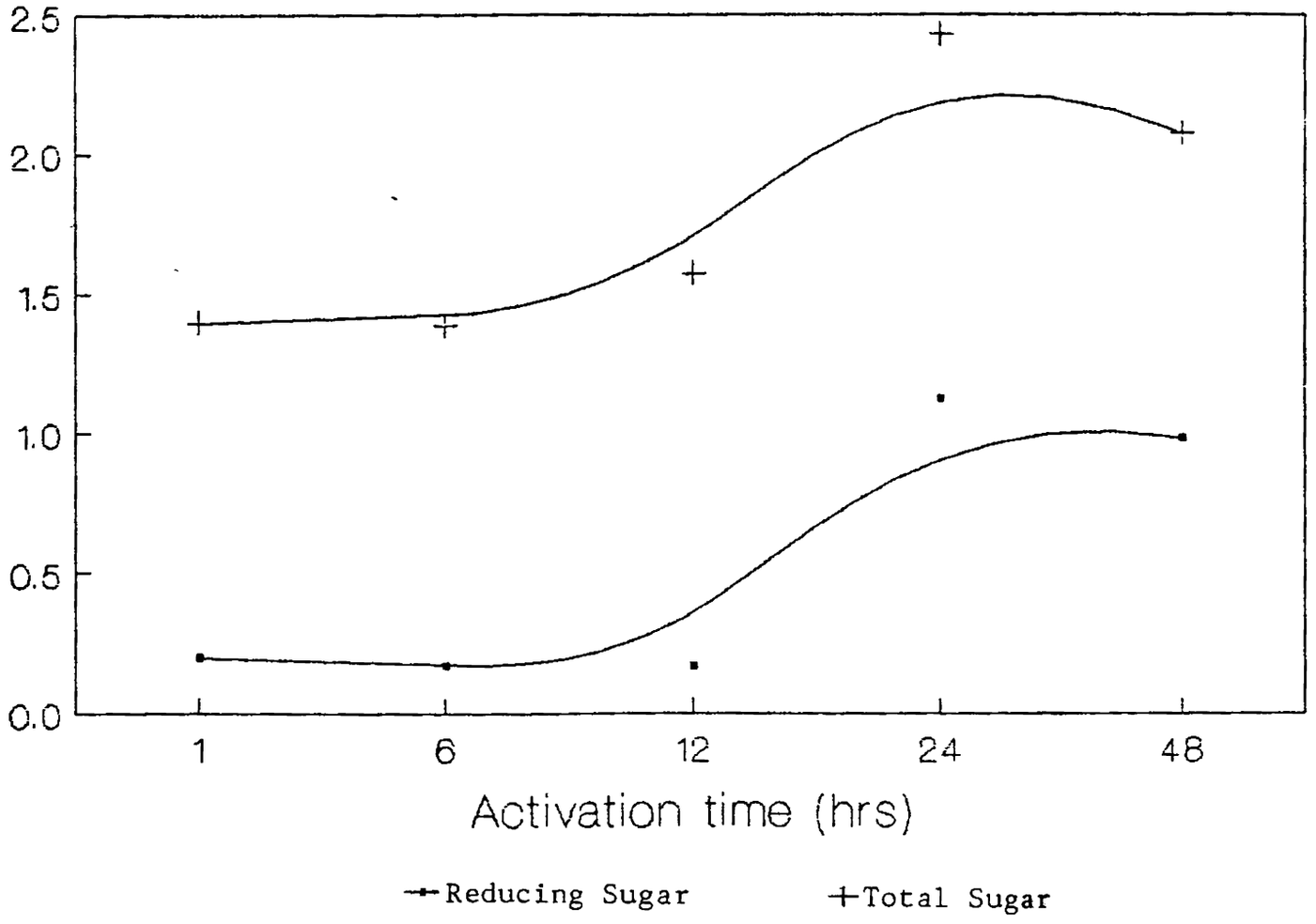


Fig.8.b.

Effect of substrate concentration on activity

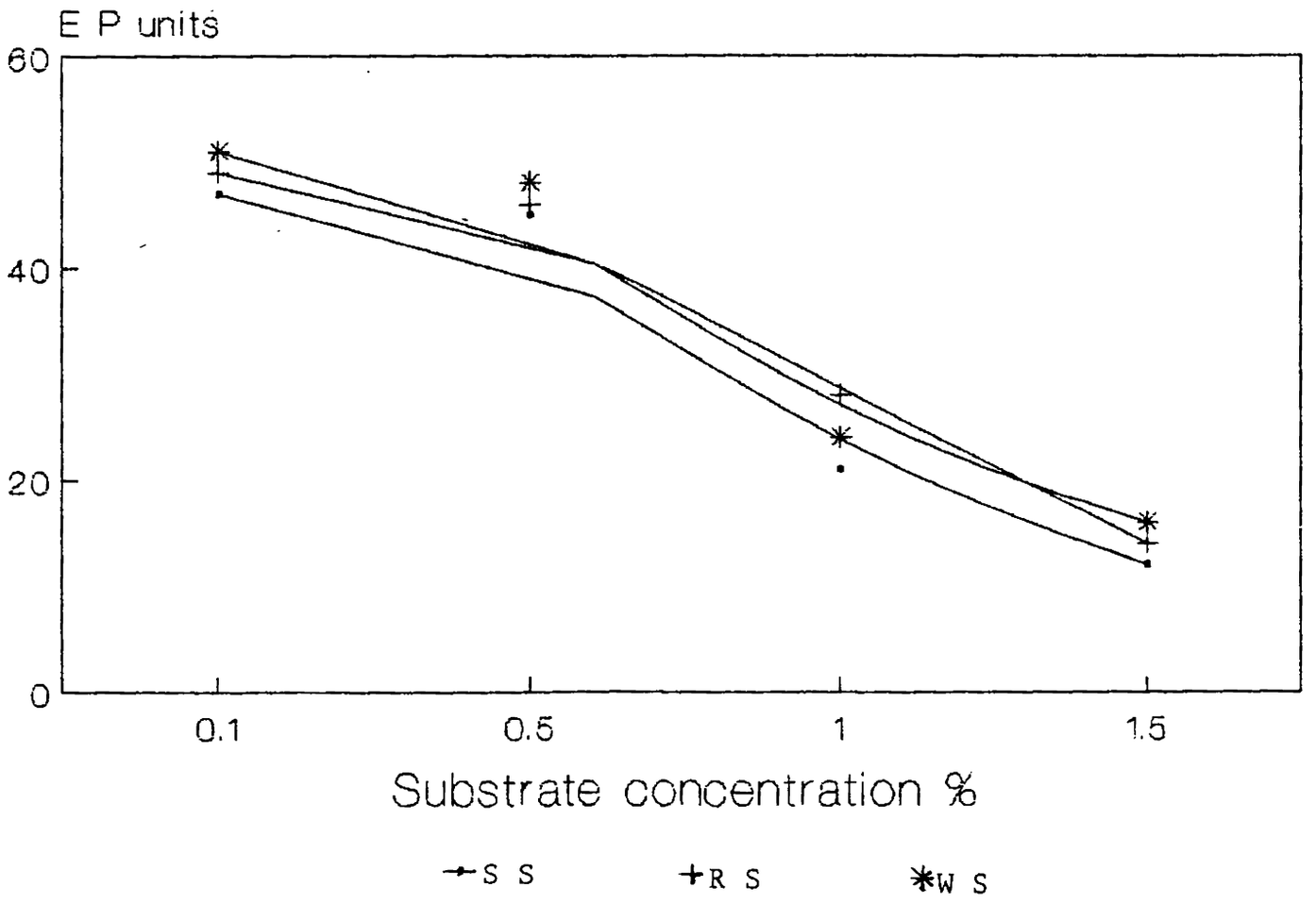


Fig.9.

Rate of growth
by Free&Immobilized cells

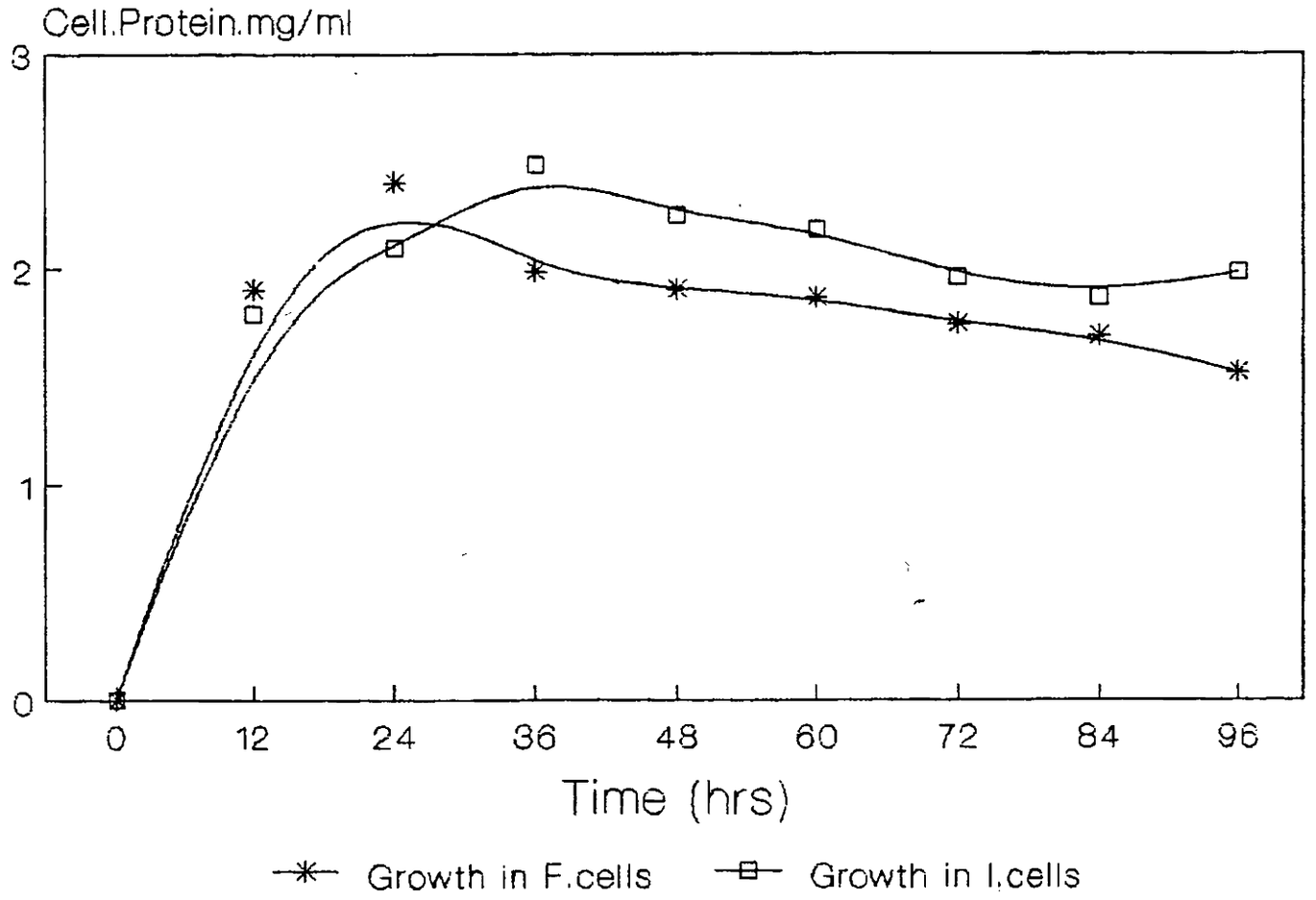


Fig.10.a.

Rate of Enzyme synthesis by Free&Immobilized cells

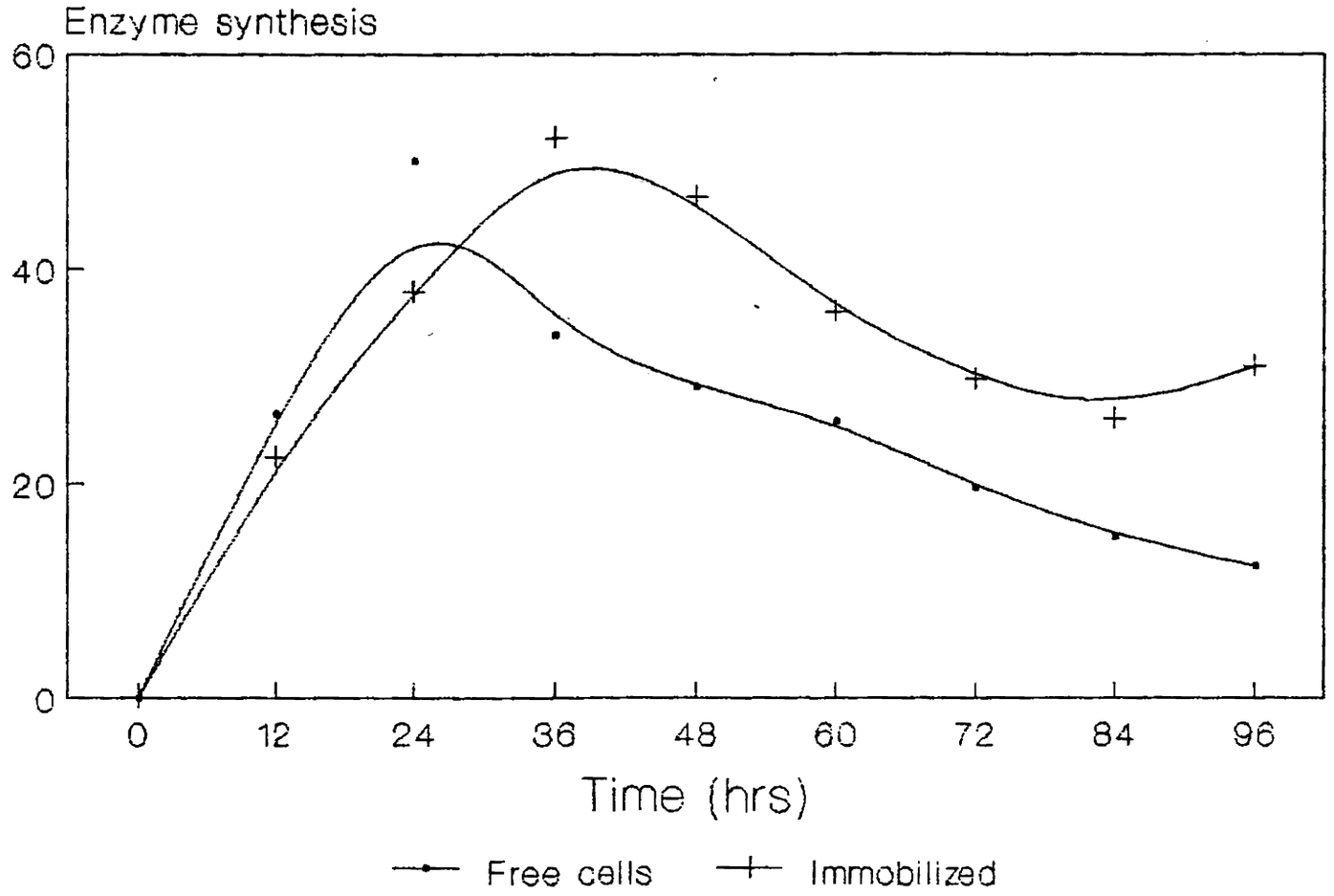


Fig.10.b.

Rate of starch diffusion in to the beads.

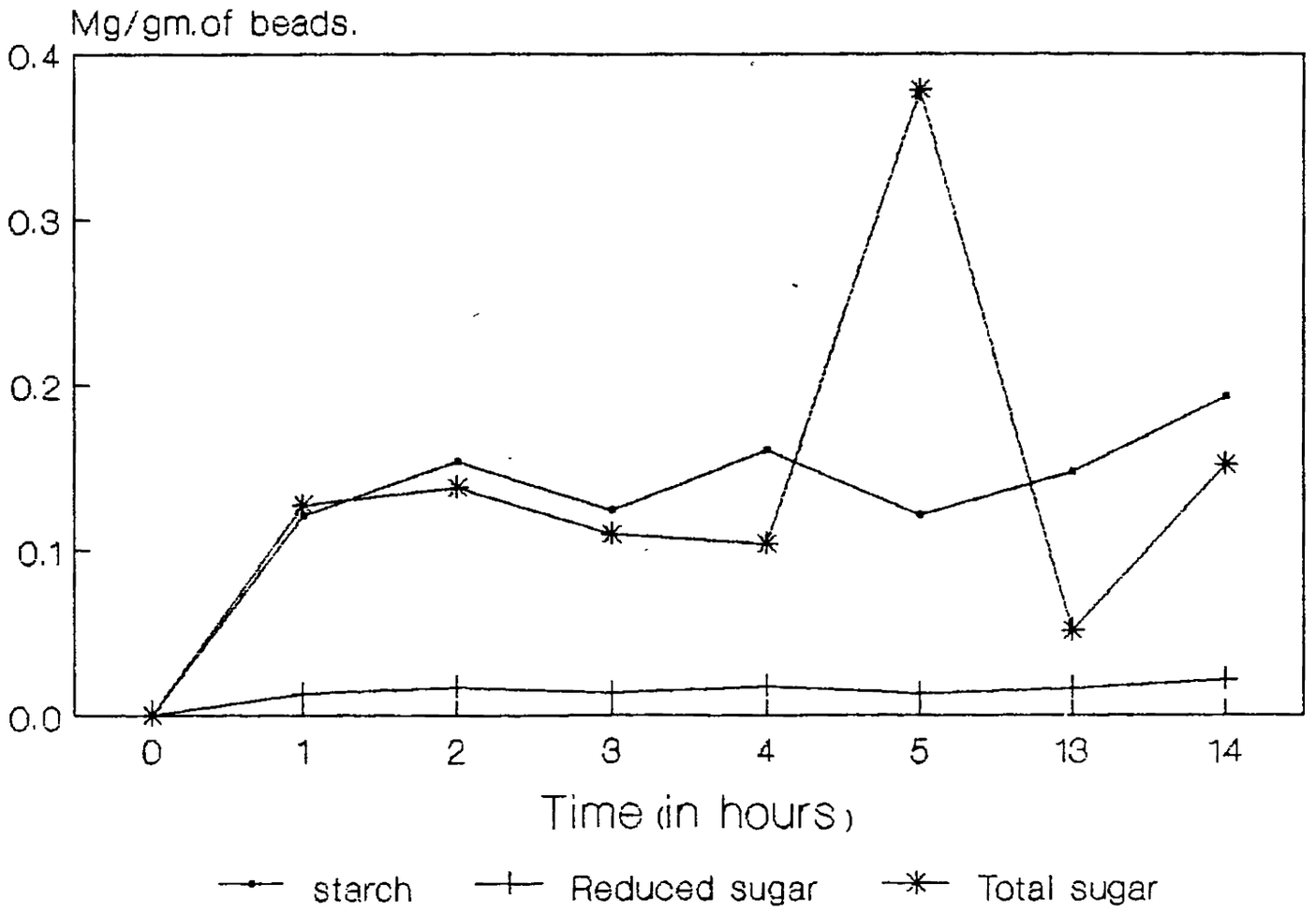


Fig.11.

Continuous synthesis of alpha Amylase

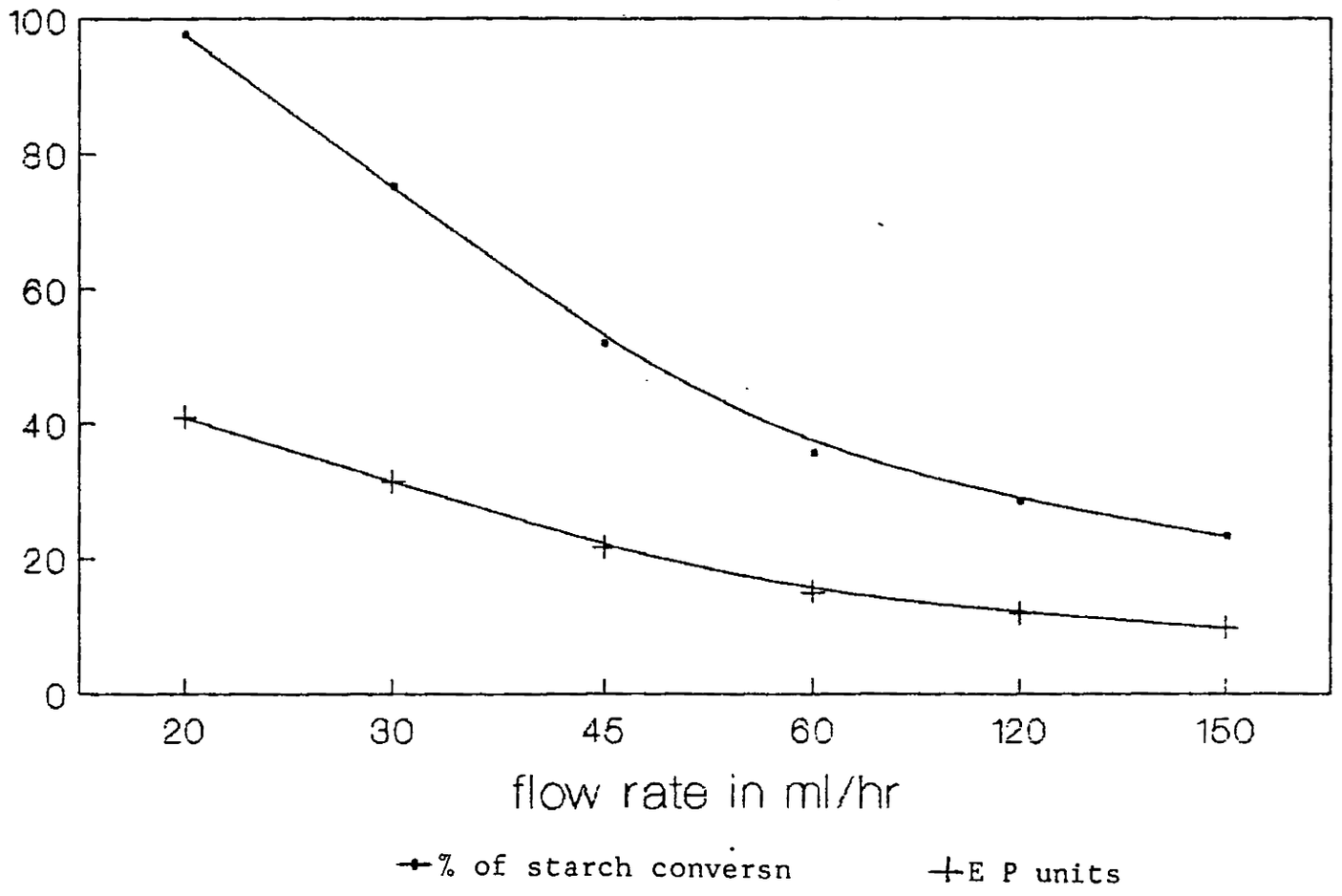


Fig.12.

Continuous synthesis of alpha amylase (cumulative production)

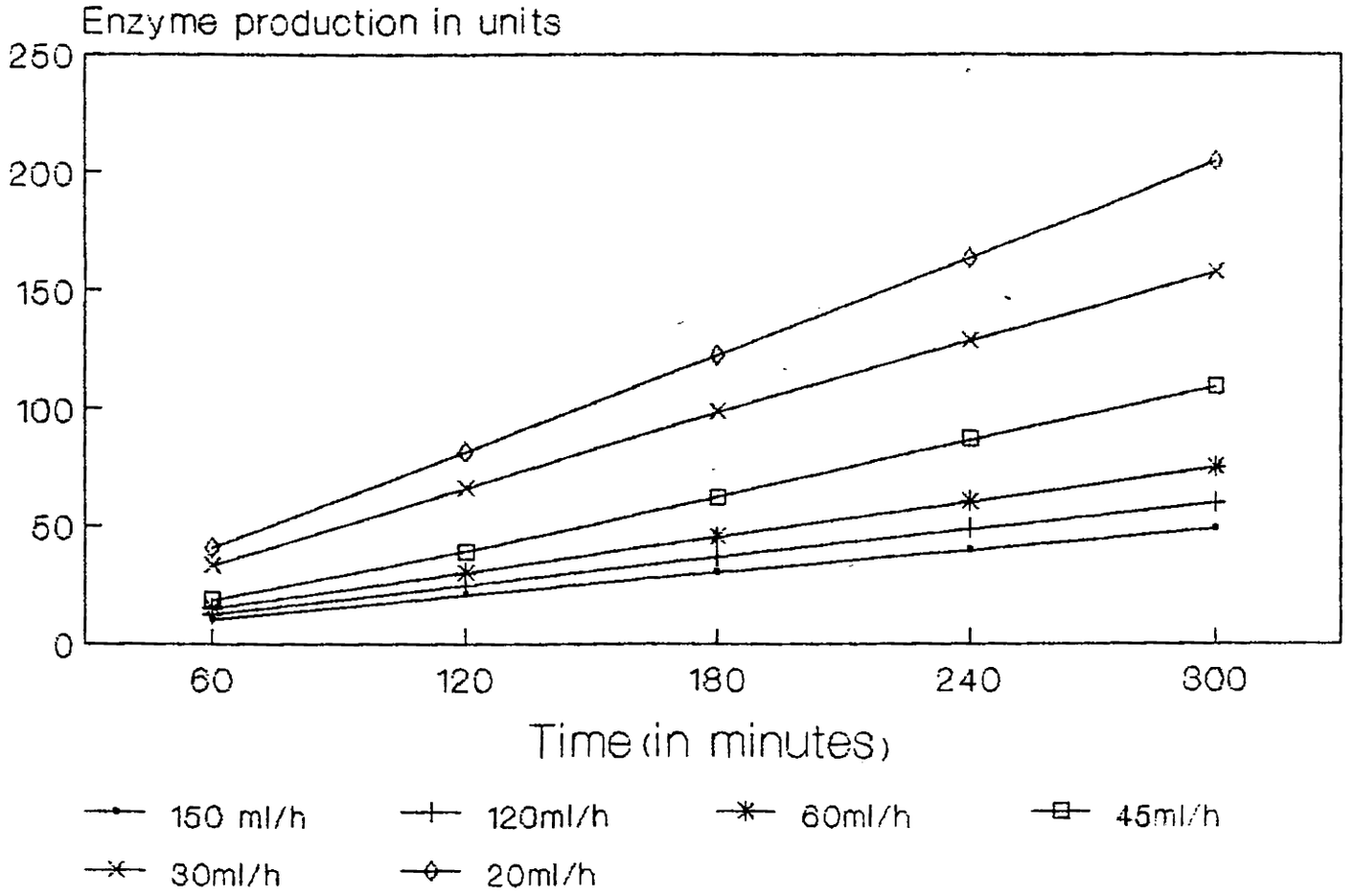


Fig.13.

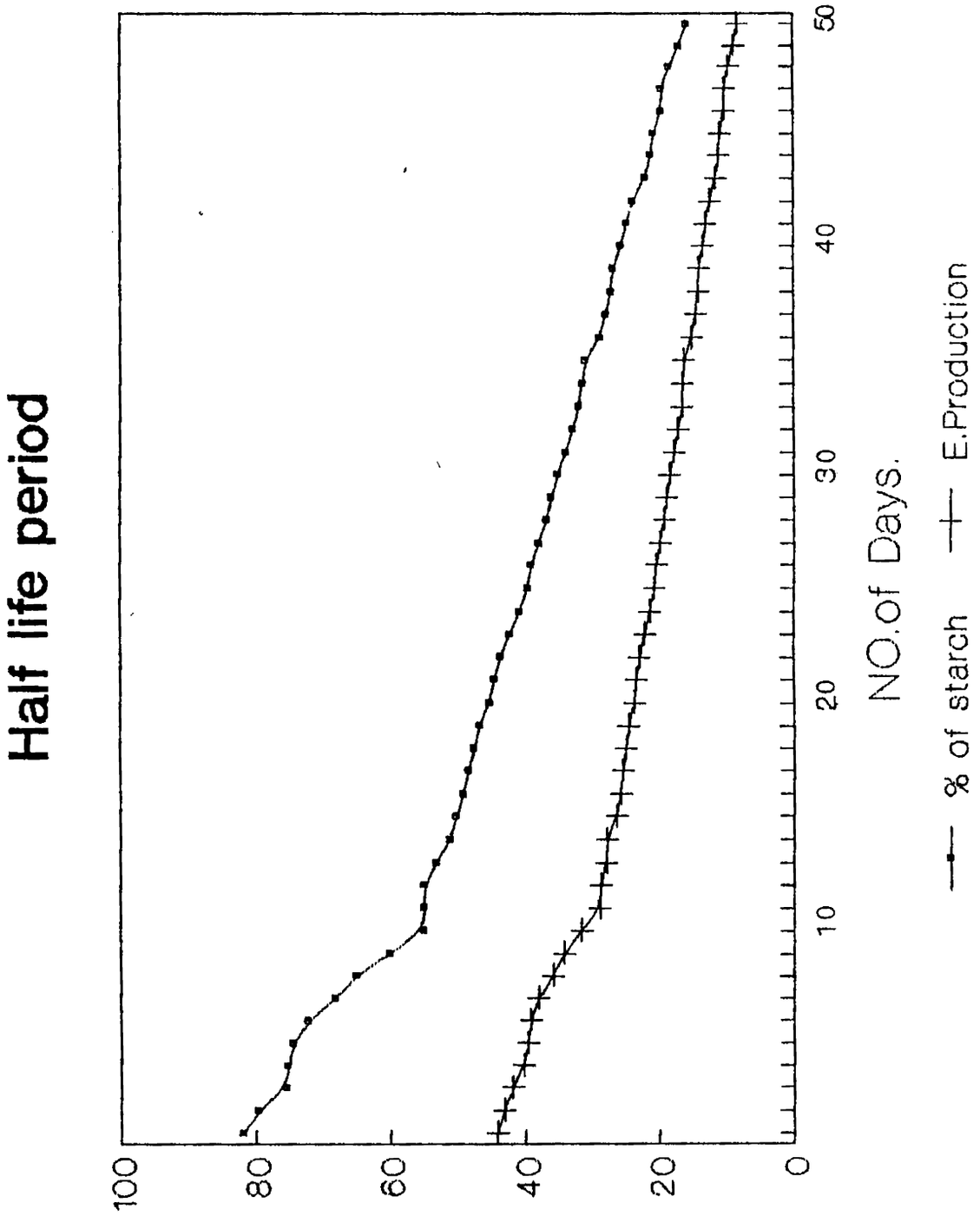

Half life period
100
80
60
40
20
0
10
20
30
40
50
NO.of Days.
% of starch
E.Production

Fig.14.

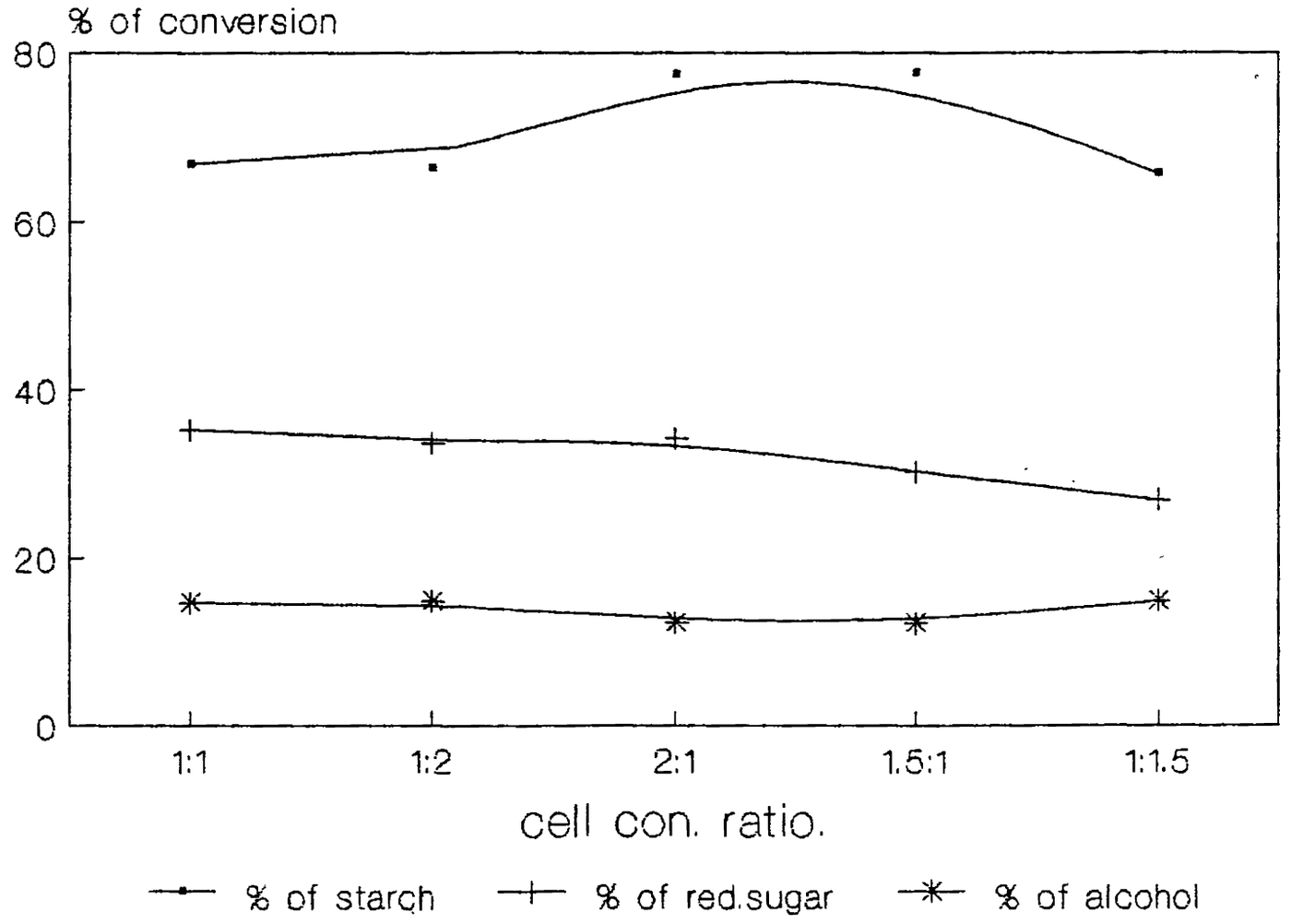

Production of alcohol by
co-immobilized B.polymyxa&S.cerevisiae
% of conversion
80
60
40
20
0
1:1
1:2
2:1
1.5:1
1:1.5
cell con. ratio.
% of starch
% of red.sugar
% of alcohol

Fig.15.

4. DISCUSSIONS

Use of immobilized cells obviate the need to isolate and purify the required enzyme. Enzymes being in their natural environments their operational denaturation is reduced to large extent besides facilitating co-factor regeneration which can take place under suitable conditions.This extends the range of enzyme that can be exploited and allows the operation of synthetic and operative process.Moreover multistep enzymatic reactions are possible.

In the presence of monovalent cation polysaccharide forms a viscous solution even at low concentration. In contrast in the presence of divalent cations especially calcium gelation occurs.Since gel formation can takes place under mild conditions entrapment in this matrix is very suitable for the viable cells.In fact it is in this context it has found most extensive application. Ofcourse, the most studied method of iontropic gelation of alginic acid is by multivalent cations in particular by calcium ions. This mild method retains cell viability but is affected by the use of chelating agent such as phosphate and citrate buffer which can disrupt the beads. The weak gel allows cell leakage particularly in entrapment (Tampion and Tampion 1987).

In the present study the various process parameters that influence the alginate entrapment of alpha amylase producing B.polymyxa were optimized for obtaining maximal alpha amylase production and starch conversion.

While optimizing the concentration of sodium alginate support it was observed that 2% level of sodium alginate yielded maximum enzyme when compared to other concentrations. In fact the raise in the concentration led to a decline in the activity of beads.Whereas when stability of the bead is concerned at 2% level of sodium alginate the beads were not stable and became spongy with excess cell leaching. On the other hand 3 to 4% of support concentration in furtherance to significant level of production of amylase attributed stability to the beads with less cell leaching. Kiersten and Goughnan (1985) observed that when the concentration of alginate sulution was raised a certain extent on addition to a CaCl2 solution resulted in more tighly cross linked gel.According to them high concentration is difficult to work and 4% w/v is satisfactory for more applications. Martinson et al (1985) indicated that sodium alginate concentration increased the enhancement of the strength of the bead.Hence in the present study based on the observation made on enzyme production,stability and conversion of starch 4% w/v of sodium alginate was used in the later experiments.

With reference to pH it was observed that an alkaline pH promoted maximal enzyme production at all the support concentrations tested. B polymyxa require an alkaline pH for maximal enzyme pruduction (Nandakumar 1990).

Rice starch promoted higher activity in the immobilized beads irrespective of the support concentrations in soluble starch and wheat starch at pH9.Where as wheat starch influenced significant level of activity only when the sodium alginate concentrations were 2 to 3% .This response of B.Polymyxa in yielding various levels of enzymes with refrence to support concentrations and type of starch substrates could be attributed to the viscosity caused by wheat starch than rice starch and consequent limitations in the transportation of the starch molecules into the beads.

Margaritis and Bajpai (1981) reported that high cell loading led to significant decrease in gel strength.In the present study it is inferred that B polymyxa cells could show maximal enzyme production when their initial cell concentrations were high in the range of 100-250 mg/100 beads whereas when the inintial concentrations were less, enzyme production was more only after three hours of residence time.However irrespective of the residence time low level of cell concentrations (100 mg/100 beads) were observed to be the optimal level of cell concentrations that could promote maximal enzyme activity.It was further observed that the initial high cell concentration resulted in excessive cell leaching and therby decreasing the activity of immobilized beads, whereas when the initial concentration was low such a phenomena was not incured.

According to Hagerdal (1982) release of cells to the surrounding medium from gel beads, once the matrix space is occupied, might be the reason for excess leaching of cell.It is also important to avoid overloading the matrix when binding the molecules, since overloading leads to over crowding and consequent reduced activity. According to Hackel (1989) location of cells determines to what degree of oxygen limitation they are exposed at a bead diameter of 4 mm and a cell loading of 50.6% w/w. Only cells in the close vicinity were oxygenated whereas in the beads diameter of 1mm. and a cell loading of 5.6% w/w,oxygen should be available throughout the bead.

Concentration of CaCl2 is ekremely important to obtain high strength gel beads.Martinson et al (1985) reported that the load bearing capacity of the beads increased upto nearly 0.2 M $CaCl_2$ and for higher concentration of $CaCl_2$ the gel strength is constant.According to Sonal Vora (1989) the effect of $CaCl_2$ on gel strength is significant and extremely stable beads could be obtained by increasing $CaCl_2$ concentration. Ogbona (1989) found that about 22 hrs. of curing time was found to be the optimum. Inthe present study although maximal activity of the beads, starch convesion and reducing sugar formation could be noted when the CaCl2 concentration was 0.01M - 0.05M The beads appeared swollen and spongy. Whereas, when the concentration of CaCl2 was more than 0.05M the beads were hard and 0.2M CaCl2 promises to be the optimal CaCl2 concentration for the preparation of the desirable beads.

Sonal Vora (1989) further observed that 50 hrs of curing time was optimum for yeast immobilization. Martinson and Ogabona etal (1989) found that about 22 hrs. of curing time is optimum for gel stability. Whereas in the present study B.polymyxa showed an optimal requirment of 2 -5 hrs of curing time at 0.2M CaCl2 concentration for maximal activity. As there is lack of uniformity in the use of concentration of Sodium alginate, concentration of CaCl2, cell loading and curing time by several workers it will be difficult to assess the realative merits of modified procedures.

Margaritis,Bajpai and Wallace (1981) used a more conventional alginate bead system with 1mm diameter and very high cell density for alcohol production using Zymomonas mobilis. Klein&Kressdorf(1983) in their studies on Z.mobilis used beads of sizes between 0.5 3mm and observed that beads of 0.8mm average diameter were good because of their diffusional characteristics.Ramakrishna & Jamuna (1990) reported that the smaller bead diameters surface reaction is rate limiting and at larger beads diameters diffusion limits the overall reaction.Whereas in the present study it was observed that bead size did not influence the performance of the beads and irrespective of the bead size significant levels of activity could be obtained.

According to Tampion and Tampion (1987) in the practical studies of immobilized enzymes and cell reactors the biocalytic activities usually expressed in terms of percentage relating to the activity of the free enzymes of

cell preparations and both assays are carried out under similar conditions of temperature and pH.

In the present study immobilized B polymyxa cells were able to demonstrate significant levels of biocatalytic activity in starch conversion, reducing sugar production and amylase production at 40°C, pH8 and substrate concentration of 0.1% as do their free cell counter parts.This observation indicates that immobilization of B.polymyxa performed at their optimized conditions do not affect drastically the normal performance.

According to Woodward (1989) at lower subastrate concentration the rate of reaction will be faster, substrate concentrations promoted higher enzyme production than higher concentration which led to reduced production of amylase.

According to Ohlson (1978) activaton means increase in the measured total activity of specific enzymatic steps in micro organism.There were several explanations offered with regard to activation mechanism,such as lysis of the immobilized cells leading to facilitated transport of reactants and products ,growth of immobilized micro organism taking p lace inside the gel matrix or preferential cellular matrix one or two (induction).Chua et al (1979) recorded activation in medium as a result of microbial growth and observed growth of enterobacter aerogens both inside the gel and on the outer surface .According to them it is better to immobilise a

small amount of cells rather than large amount so that the gel can contain more active cells. If few cells are immobilized there will be much free space in the gel and the cells can be made to grow by activation. If many cells are immobilized ,not much space will be available for further growth of cells, They have further observed that activation of immobilised cells of 24 hrs resulted in the maximum production of 2 - 3 butane diole. A similar observation was made with B. polymyxa in that 24 hrs of activation time was required for maximal amylase production.

On treatment with various buffers, cells leached rapidly and subsequently the beads disintegrated in response to phosphate buffer followed by citrate than others. These results are in agreement with the statement of Tampion and Tampion (1987). Further it was also observed that when the cells were cross linked with glutaldehyde cell leaching was arrested.

Shimizu et al (1979) reported that the condition under immobilized state were rather different from those of free cells. Robin (1985) observed reduced growth rate ,generation time and respiration rate in immobilized conditions. According to Tampion and Tampion (1987) the free cells loosely entraped in the matrix may proliferate to very high density and these may be higher per unit of volume than can be obtained in free culture in a fermentor. They further stated that even a high cell density was acheived during immobilized state which might be advantageous to react with or without cell division. In the present study though

significant growth of the cells inside the beads was observed,on comparison with free cells the time required for maximal growth and enzyme synthesis varied .This particular observation may raise doubt on the utility of immobilized process for the production of amylase.However when reusability of the immobilized biocatalyst is of interest, there is ample scope for continuous synthesis of amylase at concentrated titeres and subsequent higher productivity compared to free cells that involves batch process.

An attempt was made to compare the performance of free cells and immobilized cells entraped in aliginate beads for their efficency in the production of alpha-amylase with reference to varying temperature, pH and various substrate concentrations. Results obtained for the experiments clearly indicated the fact that even under immobilized conditions the B polymyxa was efficent at comparable levels with free cells at similar environmental conditions. Despite the physical stress imposed by the bead particles on the intact cells inside the bead, cells could elaborate amylase as they could do under free state. Although their response to the nature of starch substrate in terms of quantum of amylase synthesis in general the efficency of cells were not restricted. Based on the result it may be suggested that the immobilized process may be preffered for the production of amylase then the free cells in fermentors when commercial exploitation is of intreast.

Compared to free cells immobilized cells can be advantageous when used in continuous process owing to the fact that the reactor volume can be comparatively smaller for the unit production of enzyme, cell concentration is very high in the immobilized bio reactors. According to Tampion & Tampion (1987) immobilized cell process can also be operated in a batch mode generally though it is commercial to use them in continuous process. Jamuna & Ramakrishna (1992) have stated that immobilization of whole cells for extra celular enzyme offer many advantages such as the ability to seperate cell mass from the bulk liquid for possible reuse facilitating continuous operations over a prolonged period and enhance reactor productivity. They observed that bacillus sp showed maximum amylase activity at low dilution rate which gradually decreased with increased dilution rate. They also observed that the productivity of amylase in a packed bed reactor for a period of 40 hrs declined gradually recording 90 u/ml upto 120 hrs. They further observed that the enzyme yield on continuous operation was 10 u/gm compared to free cells. Where as in the present study maximal enzyme synthesis (41.2 u/g) along with maximal starch conversion (98.1%) could be recorded at a flow rate of 20 ml / hr (dilution rate 0.5 hr) and productivity of amylase declined with the increase in flow rate as well as the dilution rate.

Enzyme productivity which represents the system was calculated by multiplying the dilution rate by the activity (Jamuna and Ramakrishna 1992). Productivity increased to 205

u/g of bead at the dilution rate of 0.5 hrs/gm of beads and declined when the dilution rate was enhanced. Jamuna and Ramakrishna (1992) observed an increase in productivity with increase in dilution rate upto 2.0 which decreased later. Whereas in the present study B.polymyka showed a progressive decline in the productivity along with increase in dilution rate.

An interesting observation made in the present study (Table 12) with continuous production of alpha amylase is that using minimal substrate solution maximum enzyme could be produced under immobilized state on continuous operation.

These cells may not contain all the components of enzymes necessary for carrying out the specific conversions. One of the practical alternatives currently explored to obtain such enzyme, cell system is preparation of co-immobilizates. Binding of the deficient enzyme from external source, free or immobilized micro-organism leads to co-immobilizates which combine the bio-catalytic properties of the cells and of additional enzymes from another source (D'souza, 1989). Whereas in the present study an attempt was made to co-immobilize an amylolytic B.polymyka and ethanol producing S.cerevisiae in a single bead in order to compliment the two enzyme system necessary for converting starch into alcohol directly without conducting two seperate batch process. The effort yielded fruitful result and significant levels of alcohol could be obtained by the co-immobilized beads even at a ratio of 1:1 . This particular result has much scope for their commercialization of producing alcohol from starch wastes.

Concluding Remarks

B. polymyxa isolated from Cochin University environment which has a short generation time capable of producing alpha amylase rapidly has a larger scope in the fermentation industry for the production of alpha amylase which has a wide commercial application and alcohol production in combination with yeast using enormous starch waste available. It is suggested that immobilized process is a right choice for starch utilization.

The statistical analyses carriedout in the present study confirms the observations made on the nature of relationship that exist between the variables under immobilized condition and their impact on the activity of the beads.

SUMMARY

1. Bacillus polymyxa producing alpha amylase, isolated from soil of Cochin University Campus, was used for studying the immobilizataion of cells in gel entrapped beads.

2. Initially the process parameters that influence the preparation of efficient immobilized beads were optimized. Thus concentrations of support material - alginate, calcium chloride, cells, Enzyme, Bead size, curing time, and activation time were optimized.

3. It was observed that 4% of sodium alginate favoured maximal gel stability, starch conversion and alpha amylase production.

4. Immobilized beads prepared using 100 mg/100 g beads of cell concentration yielded maximal alpha amylase synthesis along with starch conversion on incubation for minimal residence time.

5. When alpha amylase was immobilised in alginate beads, at a concentration of 25 ml/25 gm of bead maximal starch conversion was observed.

6. Alginate Immobilized beads, varying in size from 1 mm - 4 mm did not influence the performance of the beads while beads of size 5 mm and above led to decline in the rate of enzyme synthesis by the beads.

7. At concentrations of 0.1 m - 0.2 m, CaCl2 attributed stability to the immobilized beads infurtherance to maximal activity.

8. Maximal alpha amylase production was recorded when the beads were maintained in CaCl2 for curing a minimum period of two hours.

9. Optimum temperature for maximal enzyme synthesis and starch conversion by immobilized cells of B. polymyxa was observed at 40°C.

10. Immobilized cells of B.polymyxa did prefer pH of 8 and 9 for their maximal synthesis of alpha amylase and starch conversion as do their free cells counter parts.

11. It was observed that 24 hrs. of activation of immobilized cell beads was necessary for obtaining maximal activity by the immobilized biocatalysts.

12. A minimum of 60 minutes of residence time was required for maximal yield of enzyme and maximal conversion of starch.

13. Results indicated that both phosphate and citrate buffers could be used for disrupting the immobilized beads since they enforced maximal release of cells through leaching from the beads within one hour.

14. Variation in the initial cell concentration did not influence much on the rate of cell leaching from the beads which varied from 0.4 to 0.96% during a period of 6 hrs.

15. Cross linking of cells with glutaraldehyde (5%) arrested leaching of cells from the beads.

16. At substrate concentrations varying from 0.1 - 0.5, immobilized beads recorded maximal levels of alpha amylase than at higher concentrations.

17. Rate of alpha amylase synthesis by immobilized cells were rapid until 36 hrs (52 u/g of beads) and declined later.

18. Although residual starch was minimal inside the beads significant levels of reducing sugar and total sugar could be recorded inside the beads indicating a maximal conversion of the starch inside the bead by the cells. The starch level in the solution outside the bead drastically got reduced indicating a rapid diffusion of starch into the beads which is evinced by a meagre level of reducing sugar outside the bead.

19. On comparative analysis it was observed that immobilized cells could synthesize alpha amylase at similar levels with free cells of B. polymyxa.

20 Among the various starch substrates tested wheat starch favoured higher levels of alpha amylase followed by rice starch, potato starch and soluble starch, by both immobilized and free cells.

21. On continuous operation of the fluidized bed reactor packed with immobilized cell beads, at a flow rate of 20 ml/hour, maximal alpha amylase was recorded than at higher flow rates.

22. The rate of enzyme synthesis on continuous operation was 0.6118 u/min and 2.045 u/ml at 20 ml/hour flow rate.

23. Half life of the immobilized cell beads was estimated to be 25 days.

24. On co-immobilization of *B. polymyxa* with *S.cerevisiae*, the co-immobilizate beads could effeciently convert starch directly to ethanol with a yield of 14.8% at 1 : 2 ratio.

REFERENCES

1. Adlercreutz P., Holst,o and Mathiasson, B (1985) Appl. Microbiol. Biotechnol.,22 :1

2. Anderson, E., Aun, C.J., Barbel, H.H. (1985) Enz. Microbiol. Technol., 7 .(7) : 333

3. Anitha C. J. (1989), M Sc Project, Cochin University of Science & Tech; Cochin.

4. Argirako, S. G. (1978) M.Phil Thesis, University of Birmingham, U.K

5. Argirako, K., Thayanithy and D.A John Wase (1992) J. Chem. Technol. & Biotechnol.,53:33

6. Atkinson, B. & Fowler, H.W; (1974) Adv. Biochem. Eng. :3, : 221

7. Bajpai, P., Promod, K B. (1989) Biotech. Bioengg.,33 : 72

8. Bedows, C.B., Gil H.G, & Guthrie J. T. (1984) Biotechnol. & Bioeng, 27

9. Boyer, E.M & Ingel, M. B. (1972), J Bacteriol;110 (3) : 992.

10. Brink, L. E. S. & TramPer, J. (1986 a) Enz. M.icrobiol. Technol,8 : 281

11. Brink L.E.S & Tramper J. (1986 b) Enz. Microbial Technol,8 : 334

12. Brodelius, P., Deus,B., Mosbach, K., & Zenk, M.H (1979) FEBS lett. 103 : 93

13. Bull, M.P., Sterritt, R.M & Lester, J.N. (1983) Biotechnol. lett.5:333

14. Bunoorcore, V., Carlo, C., Mario, De Rosa, Agata, G (1976) J. Bacteriol.,128 (2) : 515

15. Caunt, P & Chase, H.A (1987) Appl. Microbiol. Biotechnol.25:453.

16. Campbell, L.L. (1954) J. Am Chem. Soc.76 : 5256.

17. Castroital, (1992) Biotechnol. lett.,14 (1):49

18. Chandrasekaran M. & M. P. Nandakumar (1990) paper presented in the National Symposium on current trends in Biotechnology held during Nov. 28 - 30,1990 at Cochin.

19. Charles ,(1974) Biotechnol. Bioeng.16:1553.

20. Chen, H. C & Lall, R.R. (1982) Process Biochem;17 (1);20.

21. Chevalier, P. & Dela Naue, J. (1987) Enz,Microb. Technol.,9

22. Chevalier P.& De La Naue,J.,(1988) Enz. Microbiol. Technology 19-23.

23. Chiang, J. P., Alter , J.E.,& Sterenberg, E.M (1979) Die Starke, 31 :86.

24. Chibata,I. ,Tosa T. , & Sato T. ,(1974) Appl. Microbiol. 27:870.

25. Chibata ,I. Kakimoto ,T. & Nabe, K.,Japanese Patent 74, 81590 (1974).

26. Chitra S. &Gowrichandrakasan,(1992) Paper presented at 32nd Annual Conference of Association of Microbiologists of India.

27. Chotani ,G. K & Constatinidus ,A.(1984) Biotechnol. Bioieng. 26:217.

28. Coleman, G., Elliot, W.H.(1962) Biochem. J.,83:256.

29. Doe, V.M. & Gaucher G. M. (1983) Biotechnol. lett. 5 ;125.

30. Dhulster, P., Barbotin ,J. N. & Thomas D. (1984) Appl. Microbiol, Biotechnol, 20:87.

31. Doran, P. M. , Bailey ,J. E., (984) Proc. vii Australian Biotechnol, Conf. Brisbane.

32. D'Souza, S. F. & Nadkarni, G. B. (1980). Biotechnol. Bioeng. 22 :2179.

33. D'Souza S. F. & Melo J. S.(1989) Proc. of symposium on "Recent Advances in Biochemical engineering ". Banaras, Feb ,16-18.

34. D'Souza,S.F (1989) Indian J. Microb.29 :33

35. D'Souza S. F. (1990) F.Biotechnol. 4:373

36. Dubois, M., Giller. K. A, Hamilton J. K ,Rebors P. A & Smith F. (1956) Anal. chem, 28:350.

37. Dwyer D. F , Krummo M L ,Bovd S A &Tiedge J. M. (1986) Appl. Environ.Microbiol.52:345.

38. Elkmeirer, H &Techm H. J(1984) Appl. Microbiol. Biotechnol. 20:365.

39. Emilia Abraham T; Jamuna R ;Vijay Bansilal C & Ramakrishna S. V. , Trivandrum (India).(1991) VCH Verlagszellischaft mbh ,D-6940, Weinteim.

40. Fischer J., Uebrich, R; Schellenberg, A. (1978) Acla. Biol. Med. Ger. 37:1413.

41. Fletcher, M. , Marshall,K. C. ,(1982) Adv. Microb. Ecol, 6:199

42. Furni ,M. & Yamashita,K.,(1983) J. Ferment, Technol. 61 :587.

43. Furni M. & Yamashita K. ,(1983). J.Ferment , Technol. 61 :593.

44. Ghose T. K. , Bandyopadhyay ,K. K. ,(1980). Biotechnol. Bioeng. 22:1489.

45. Godbole ,S. S. , D'Souza, S F. & Nadkarni G. B. (1983) Enzyme Microb. Technol. 5 :125.

46. Godbole S. S. ,D'Souza S. F. , Kaul,R. & Nadkarni G. B. (1984) Proc. DAE Symp. on Newer Approches to Biological Applications, Baroda,P.62.

47. Hartman, A. P. , Ralph, W. Jr.Tetrault , P. A. (1955) Appl. Microbiol.3:7.

48. Hartmeir W. (1981), Current Development in Yeast Research, Strevart &Russel (Eds) pergmon press Toronto p.105.

49. Hartmeier, W. & Doppne ,T. (1983) Biotechol, Letters, 5 :743.

50. Hartwell ,L. H. , Culotti ,J., Pringle, J. R. ,Reid B. J (1974). Science : 183:46

51. Hirayama , O; Vya K., Hiramatsu Y., Yamada H, & Moriwaki K. (1986).Agric.Biol. Chem. 50 : 891

52. Hosogauea S; Vander cook, C.E ,Choi, G. Y:Hormanz & Oup (1985) J. Food Sci . 50 :330

53. Jamuna , R & Ramakrishna,S.R (1992),Enz.Microb. Technol., 14:36.

54. Jones K.L., Grainger ,J. M (1983) Eur. J.Appl. microbiol Biotechnol. 18;181

55. Jones, A., Razeniewska, T.,Lesser ,B.H., Siqueira ,R., Berk ,D.,Beibie, L.A, Gaucher, G.M.(1984) Can. J. Microbiol . 30 :475.

56. Kanamitsu, O; (1975), Japanese Patent 75, 132181.

57. Karube , I; Wang , Y; Tamiya , E.& Kawari , M.(1987). J, Biotechnol. 6:1.

58. Kaul R , D. Souza S.F & Nadkarni G.B (1986). J. Micro biol,Biotechnol.1:12

59. Kaul,R, D'souza S.F & Nadkarni G.B (1984). Biotechnol. Bioeng. 26 : 901.

60. Kawazu, T; Nakanishi. Y; Vozumi V ; Sasaik. T; Yamagatatt; Tsukagoshi. W; & Udaka .S.(1987),J. Bacterio. 169;1564.

61. Klein, J., Hackel,V & Wagner ,F .(1979), Immobilized microbial cells, Acs Symposium series 106, ed . K. Venkata Subramanian, 101.

62. Klein , J. & Kressdorf, B.(1983) Biotech. Lett.5; 497

63. Kloosterman, J . & Lilly , M. D . (1986) Biotech. Lett. 7:25.

64. Kokubu, T ; Taso K ; & Shuichi ; S (1978) Appl. Microbiol, Biotechnol;5 : 233

65. Kokubu T. Karube,I & Suzuki S (1981) Biotechnol. Bioeng. 23: 29

66. Krouwel , P. G ; Van Der Laan, W.F.M & Kossen , N.W.F. (1980) Biotech. Lett.,2: 253

67. Krouwel, P. G ; Groot , W.J. & Kossen, N.W.F. (1983) Biotechnol. & Bioeng.25: 281

68. Kacera ,J & Kuminkova, M .(1980) Collect. Czech . Comm.45: 298.

69. Kumaleura ,M. , Yoshida M, Assanol ,N & Kaet sa ,I (1977) J. Solid phase Biochem.2: 279

70. Laesson M. & Mattiasson B , (1988) Biotechnol. Bioeng31: 979.

71. Lin , Li ; Kang J. 1988 Shipin Kokcie (Beijing),5(9) : 104

72. Link , O.V; Saarinen, P ; Linko , M. (1975) Biotech. Bioeng.17: 153

73. Linsefors , L .& Brodeline F. (1985) Plant Cell reports 4: 23 Lowry , O. H. , Roseburough , N.S, Farr , A .

74. L.,Randall, R.Y.(1951) J.Biol .chem., 193 : 265

75. Makaskid , L.E. & Dean A.C.R. (1985) Biotechnol . Lett. 7 : 457

76. Margaritis, A ; Bajpai, P. K. & Wallace , J.B (1981).Biotechnol. Lett3 : 613.

77. Margaritis , A & Bajpai, P. (1982) Proc. Fourth Bioenergy R & D Seminar (Nrc, Ottava) 548.

78. Margiritis.A. & Wallace J. B ; (1982) In Biotechnology & Bioengineering Symposium 12 ed E.L Gaden Jr.PP. 147.

79. Mashall , J.J. (1974) FEBS, Lett , 46 : 265

80. Martin, C.K.A. & Perlman, D. (1976) Biotechnol, Bioeng- 18 : 217.

81. Martinsen , A. Skiak - Dracle .G, & Smidsnodo.Biotechnol, Bioeng. 33 : 79

82. Matteau, P.P. & Saddler , J. N (1982) Biotechnol. Lett. 4: 513

83. Medda, S ; Chandra A . K. (1980) J. Appl. Bacteriol. ;48(1) : 47

84. Messing , R.A. (1982) Biotechnol.Bio engg.24:1115

85. Middelhovel, W.J & Bakker C.M (1982) Eur. J. Appl. Microbiol. biotechnol.

86. Mitchison, J. H; (1972) M.sc,project work , Cambridge University.

87. Mohandass,C.& Chandrasekaran ,M.(1990) paper presented in the National Symposium on "current trends in Biotechnology" held during Nov 28-30,1990 at Cochin.

88. Mohandass C. & Chandrasekaran. M (1992) Paper presented at 32nd Annual conference of Association of microbilologists of India held during Jan 10-12,1992 at Madurai.

89. Morgan, F.J & Priest, F.G (1981) J. Appl. Bactriol, 50(1) :107.

90. Moshach,K.,Birnabaum,S., Hardy K; Davios J. & Buloue L. (1983). Nature 302 : 543.

91. Murata;K; . ,Uchida T; Tani,K; Kato J. & Chibata I (1979), Eur. J. Appl Microbiol. 7:45.

92. Nadkarni. G.B.& D'souza S.F. (1981) Indian J. Microbiol. 21:244.

93. Nandakumar. M.P. (1991) Ph. D., Thesis, Cochin University of Science and Technology, Cochin.

94. Nararro, J.M; & Durand. G; (1977) Eur. J. Appl. Micr obiol: 4:243 .

95. Nilssan I; Ohlson. S Haggstorm L; Molin N. & Mosbach K. (1980). Eur. J. Appl. Microbiol. 10:261.

96. Nishida, Y; Sato, T.T & Chibata, I.(1979), Enz. Microbiol. Technol. 1:95.

97. Oriol,P; (1988) Enz. Miicrobiol. Technol. 10: 158.

98. Para , G .& Bartti , J ; (1988). Appl. Microbiol. Biotechnol. 28 : 222

99. Panek,A. (1963). Arch. Biochem. Biophys; 100:422.

100. Paul, F & Vinais, P.M (1980). Enz. Microbiol. Technol. 2:281.

101. Perlman, D, (1977) Annual Reports On. Fermentation process Vol.1.,
I. Acadamic Press. London.205.

102. Ptuller, B. & Elliott, W.H; (1969).J. Biol. Chem; 40:244.

103. Pinches, A. Louue, M.E; Watson, T.G. (1985). Biotechnol, Lett., 7(9) : 621.

104. Purrot P; Fick M. & Engasser J.M. (1986).Biotechnol. lett. 8:253.

105. Ramakrishna, S.V; Sreedharan, V.P & Prema, P. (1988) Bioreoactor immobilized enzymes & cells, Elsevier Publ.,.

106. Ramakrishna, S.V.& Jamuna, R (1990),National Symposium Cum Workshop held during August 6-14 at Trivandrum.

107. Ramesh. M.V. & Lonsane, B.K (1989), Biotechnol. lett; 11(I) :49.

108. Ramesh , M.V;Lonsane, B.K,(1989),Proc. Biochem, October:176.

109. Reop; and Salmon (1955), Methods in Microbiology, Acadamic Press, London.

110. Robyt, J.& Ackerman, R.J (1971) Biophys. 145:105.

111. Royer G. Livernoche, D; Desroocher,M; Jurasek,L; Rouloace, D. & Mayer, R.C. (1983) Biotech. Lett. 5:321.

112. Rocileace, D. &Mayer, R.C. (1983), Biotech. Lett.5:321.

113. Saito, N.(1973). Arch. Biochem. Biophys. 155:290.

114. Saito, N & Yamamoto,K. (1975). J. Bacteriol, 12:848.

115. Saito, N; Murata K. & Kimura A. (1986). Biotechnol. Bioeng. 28:1707.

116. Sato N, Murata,K and Kimura, A (1986). Biotechnol. Bioeng. 28:1707.

117. Scott, R.J; Wills S.T & Bucke,C. (1988).Enz. Microbiol. Technol. 10:151.

118. Sidhartha, R; ,Satish, J.P; William, A.W. (1989).Biotechnol.Bioeng; 33:197.

119. Shah, V.K., Vehete,P.N., Shah, V.D., Kothari, R.M. (1989) J. Biotech. 11:67.

120. Shimizu, S; Tani Y; Yamada Y; (1979).ACS Symp. Ser. 106:87.

121. Shinke,R,Yuji, K;Hiroshi, N. (1975) Technol; 53 (10) :693.

122. Shinke, R;Yuji, K; Hiroshi, N. (1975) J Fermentn. Technol, 53(10);687.

123.Shinmyo, A; Hiroyike, K; Hirosuki,O. (1982).Eur.J. Appl. Microbiol. Biotech.; 14:7.

124. Siddhartha, R; Satish J.P; William A.W. (1989) Biotech. Bioengg; 33:197.

125. Slowinski, W & Charm, S.E. (1973). Biotechnol. Bioengg. 15:973.

126. Smiley,K.L; Hotreiter, B.T; Boundy, J.A, Rogorin, S.P; (1975).Proceedings of the International Symposium on Biodegradation, 1001 Applied Science.

127. Somervill; M.J. Mason J.R. & Ruffell R.N; (1977).Eur. J. Appl. Microbiol. Biotechnol. 4:75.

128. Sonal vora (1989) ,M.Sc. Project, Cochin University of Science & Technology.

129. Sonnleitner, B. & Fiechter, A. (1986).Appl. Microbiol. Biotechnol. 23:424.

130. Stofanova, S.Kosova M; Topavicharova, I & Boschkov, V (1987).Biotechnol. Lett. 9:475.

131. Strandberg,G.W; Donaldson T.L & Arcuri E.J (1982) . Biotechnol. Lett. 4:347.

132. Sutar II. Vartak, H.G; Srinivasan, M.C. & Sivaraman H. (1986). Enz.Microb. Technol. 8:632.

133. Sen, S., Chakraborthy. S.L (1987) Enz. Microbiol. Technol., 9:112.

134. Suzuki, S. & Karbe, I (1979).

Inimmoobilized microbial cells, Acs Symposium Series, 10:221.

135. Suzuki & Karube (1979).

In immobilized cell, ACS Symposium Series 106, ed.K. Venkatasbramanian. P.P 59-72, Washington, D.C American Chemical Society.

136. Suzuki, S; Karubo, I; Matsunaga, T; Kuriyama, S; Suzuki, N Shiragami, T ;

Takemura. T (1980) J.Bio. chem. 62:353.

137. Suzuki, Y & Marumo, S., (1887) Tet. Letter .

138. Takasaki, Y. (1986). Agri. Biol. Chem 40(8) : 1515.

139. Takata, I; Yamamota K; Mossa, T. & Chibata I, 1980. Enz. Microbiol. Technol.

2:30.

140. Takata I, Tosa T. & Chibata I, (1984). Appl. Microbiol. Biotechnol; 19:85.

141. Takaya, Hayashi; Terubileo, Akuba & Koki, Horikoshi (1988). Appl. Microbilol. Bioteochnol. 28:281.

142. Tampion. J. & M.D Tampion (1987). Immobilized cells: principles and application. Cambridge University Press, Cambridge.

143. Tanaka,H; & Murakami,K. 1986, Biotechnol. Bioengg. 28: 1761.

144. Taniguchi, H; ,Fumihiko, O; Makoto, I ; Yoshiharu, M;Michionori, N. (1983).Agric. Biol. Chem; 46 (8): 2107.

145. Toda, K. & Shoda M; (1975) Biotechnol. Bioengg. 17:481.

146. Thoshinori,Kokubu, Isao,Karube & Shuichi,Suzuki (1978) Eur.J. Appl. Microbiol. Biotechnol. 5:233.

147. Tsuchya, K; Akihisa, B; Koore, S; Mitosuo, O; Yoshiharah, M. (1975) J. Fermebtn. Technol; 53 (4) : 199.

148. Tyagi, R.D & Ghose T.K; (1982), Biotechnol . Bioengg. 4:781.

149. Vozumi, N., Kyoko, S. Takuji, S., Shiro, T., Hideo, Y., Nirihiro,
T., Shigezo, U. (1989) J. Bacteriol., 171 (I) : 375.

150. Van Stenaninckw, J; & Rothostein, A; (1965).J. Gen. Physiol.: 49 : 235.

151. Van Uden, N; (1967) Arch. Microbiol: 58:155.

152. Vorlop, K.D & Klein J; (1981) Biotechnol. Lett. 3:9.

153. Vuillomard, J.C., Torro.S Bonoit.S & Amiot J (1988) Appl. Microbiol. Biotechnol 27:423.

154. Wang, G. & Wang, D.I.C (1983), Appl. Biochem. Biotechnol. 8:491.

155. Welker , N.E; & Campbell, L.C (1963).J. Bacteriol; 86(4) :681.

156. Welkar, N.E; & Campbell, L.L. (1963).J. Bacteriol; 86 (4):687.

157. Yankov, D; E; Beseikov, V; Emanuilova, E. (1986). Enz. Microrbiol. Technol; 8 (11) : 665.

158. Yoon, M; Yoo, Y; Cadman, T.(1989) Biotech. Lett; 11 (1) ;57.

159. Yoshigi, N; & Kanimura, M. (1988),Agric. Biol. Chem; 52 (9) : 2365.

160. Younys, G. Bretona, M. & Guespin- Michel J; 1987, Appl. Microbiol . Biotechnol 25 :507.

161. Youngsmith, B; & Chutima, K; (1983). J. Fermentn. Technol. 61:593.

APPENDIX

Table A.1

Optimization of Bead size for Immobilization

Bead size (in mm)	Retention time (hrs)	Enzyme produced in units/gm of beads	% of starch conversion	Reducing sugar mg/gm of bead
1	1	35.4	60.2	0.2267
	2	45.3	77	0.2914
	3	40.7	69.2	0.2612
	4	46.5	79.2	0.3001
	5	48.1	81.8	0.3087
2	1	35.2	60.8	0.2310
	2	42.8	72.9	0.2763
	3	43.4	73.7	0.2785
	4	44.8	76.3	0.2893
	5	44.1	75.0	0.2849
3	1	38.5	65.5	0.2482
	2	38.5	65.5	0.2482
	3	42.8	72.5	0.2741
	4	43.1	73.3	0.2763
	5	45.9	78.1	0.2957
4	1	37.0	62.9	0.2374
	2	42.6	72.5	0.2741
	3	40.7	69.2	0.2612
	4	43.3	73.7	0.2785
	5	48.1	81.8	0.3087

5	1	33	56.1	0.2115
	2	37	62.9	0.2374
	3	35	59.6	0.2245
	4	46.1	78.5	0.2979
	5	43.7	74.4	0.2806

Table - A.2

Optimization of CaCl2 for the preparation of immobilized beads

Concentration of CaCl2 in moles/litre	Enzyme activity units/gm of bead	% of starch conversion	Reducing sugar mg/g of bead
0.01	19.07	42.90	0.5568
0.05	19.30	43.49	0.5640
0.10	16.67	37.59	0.4867
0.15	16.18	36.39	0.4724
0.20	16.60	37.50	0.4847
0.25	13.10	29.46	0.3825
0.30	8.50	19.20	0.2482
0.40	5.15	11.60	0.1503
0.50	4.40	9.90	0.1020

Table A.3

Effect of pH on the Activity of Immobilized *B. Polymyxa*

pH	Enzyme production units/gm of beads	% of starch conversion	Reducing sugar (mg/gm of beads)
4	13	17	0.0948
5	13	17	0.0948
6	26	34.2	0.1891
7	35	45.6	0.2520
8	45.8	59.65	0.3297
9	34	44.34	0.2448
10	22	28.6	0.1584
11	11	14.3	0.0792

Table A.4

Effect of activation time on the activity of immobilized cells

Activation time (in hours)	Enzyme production in units	% of starch conversion	Reduced sugar mg/gm of beads	Total sugar mg/gm of beads
1	13.0	27.9	0.1970	1.3965
6	22.0	46.6	0.1671	1.3875
12	20.5	42.7	0.1658	1.5720
24	37.2	78.3	1.1220	2.4300
48	30.1	64.9	0.9815	2.0760

Table - A.5

Effect of substrate concentration on enzyme production

Substrate concentration %	Enzyme production in units/gm of beads (soluble starch) SS	(Rice starch) RS	(wheat starch) WS
0.1	47	49	51
0.5	45	46	48
1.0	21	28	24
1.5	12	14	16

Table A.6

Rate of cell Leaching from the beads

Time in minutes	Leached out cell mg protein/ml	% of cell leaching
00	000	Nil
30	0.00079	0.44%
60	0.00089	0.49%
90	0.00078	0.43%
120	0.00090	0.50%
180	0.00092	0.51%
240	0.00098	0.54%
300	0.00094	0.52%
360	0.00094	0.52%

Table - A.7

Rate of enzyme synthesis at different timings

Hours (hrs)	Growth in free cells mg protein /ml	Enzyme synthesis in free cells(v/ml)	Growth in immobilised beads cell protein (mg/ml)	Enzyme synthesis (v/gm of beads)
0	0.0	0.0	0.0	0.0
12	1.905	26.5	1.791	22.4
24	2.401	50.0	2.097	37.8
36	1.987	33.8	2.483	52.1
48	1.907	29.0	2.248	46.7
60	1.867	25.8	2.185	36.0
72	1.743	19.6	1.962	29.7
84	1.690	15.0	1.867	26.0
96	1.521	12.3	1.985	30.9

Table A.8

Continuous Synthesis of alpha amylase in fluid bed reactor

	Flow rate (ml/hr)	Enzyme Synthesis U / minute	V / ml
A	20	0.6817	2.045
B	30	0.5243	1.0487
C	45	0.3629	0.4839
D	60	0.2486	0.2487
E	120	0.1986	0.0993
F	150	0.1623	0.0649

Table A.9

Half Life Period

No.of days	% of starch conversion	EP units/gm of beads
1	81.90	44.15
2	79.60	43.10
3	76.46	41.89
4	75.23	40.24
5	74.46	39.59
6	72.30	39.18
7	68.12	38.05
8	65.08	35.85
9	60.12	34.25
10	55.05	31.60
11	54.98	28.90
12	54.96	28.80
13	53.12	27.90
14	51.08	27.80
15	50.20	26.42
16	49.12	25.80
17	48.40	25.47
18	47.50	25.00
19	46.80	24.60
20	45.30	23.80
21	44.60	23.47
22	43.70	23.00
23	42.30	22.20
24	40.80	21.40
25	39.60	20.80

Half Life Period

No.of days	% of starch conversion	EP units / gm of beads
26	39.10	20.50
27	37.90	19.94
28	36.81	19.37
29	36.02	18.90
30	35.12	18.90
31	33.90	17.80
32	32.80	17.20
33	31.90	16.50
34	31.40	16.50
35	30.90	16.20
36	28.80	15.20
37	27.90	14.60
38	27.10	14.20
39	26.80	14.10
40	25.60	13.47
41	24.80	13.05
42	23.90	12.50
43	22.00	11.57
44	21.20	11.15
45	20.80	10.94
46	19.60	10.31
47	19.60	10.31
48	18.45	9.71
49	16.90	8.89
50	15.80	8.31