FERMENTATION OF RICE AND RELATED PRODUCTS BY BACILLUS sp.

Thesis submitted to the
Cochin University of Science and Technology
in partial fulfilment of the requirements
of the Degree of
DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

By

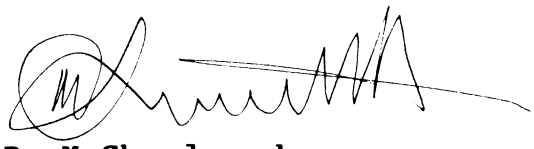
NANDAKUMAR M. P.

DEPARTMENT OF APPLIED CHEMISTRY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-682 022

JANUARY 1991

CERTIFICATE

Certified that the work presented in this thesis is based on the bona fide work done by Mr.Nandakumar, M.P. under my guidance in the Department of Applied Chemistry, Cochin University of Science and Technology and that no part thereof has been included in any other thesis submitted previously for the award of any degree.



Dr.M.Chandrasekaran
Lecturer in Microbiology
Dept. of Applied Chemistry
Cochin University of Science
and Technology

Cochin 682 022

17th January 1991

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, Lecturer in Microbiology, Department of Applied Chemistry, 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.

Cochin 682 022

17th January 1991

Nandakumar, M.P.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude and obligation to Dr.M.Chandrasekaran, Lecturer in Microbiology, Department of Applied Chemistry for suggesting the problem, valuable and patient guidance, discussions, critically going through the manuscript and endless support throughout my research career. His continued interest, constant encouragement and timely advice will always be remembered.

I am extremely thankful to Prof.K.L.Sebastian, Head, Department of Applied Chemistry and Prof.Paul A.Vatakencherry, former Head of the Department for their kind co-operation and encouragement. I am also expressing my sincere gratitude to all of the faculty members for their continued interest, co-operation and encouragement.

I am very much indebted to Dr.V.K.Griffin, Department of Food Science, University of Arkansas, USA and Dr.J.G. Zeikus, Department of Biochemistry and Microbiology, Michigan State University, USA for their stimulating suggestions and study materials.

I would like to extend my gratitude to Mrs.Prema, Scientist, Fermentation Section, RRL Trivandrum; Dr.Sridhar, Biochemistry Division, CMFRI Cochin and Dr.B.K.Lonsane, Fermentation Technology and Bioengineering discipline, CFTRI Mysore for their creative suggestions and encouragement.

I wish to thank the authorities of IISc Bangalore, RRL Trivandrum and CFTRI Mysore for their kindness in providing the library facilities.

The help extended by the authorities of NCL, Pune for providing the reference strains is gratefully acknowledged.

I would like to express my gratitude to my colleagues, Mr.T.Ravikumar, Mr.C.Mohandas and Miss V.K. Ananthalakshmy for their interest and constant encouragement. The endless support, affection and timely help extended by my friends are remembered with great appreciation and deep sense of gratitude.

I am very much indebted to Miss Renu. S. for her sincere and timely help and constant encouragement. Also I am expressing my thanks to Mr.Ajayakrishnan, Department of Electronics and Mr.P.G.Sankaran, Department of Mathematics and Statistics for their timely help for processing the data.

The financial assistance extended to me in the form of research fellowship (Dr.K.S.Krishnan Research Fellowship) by the Department of Atomic Energy, Government of India is gratefully acknowledged.

I wish to thank the authorities of Cochin University of Science and Technology for providing necessary facilities.

I am thankful to the administrative and technical staff of the Department of Applied Chemistry for their timely help and co-operation.

I would like to extend my gratitude to Mrs.V.K. Sumathy and Mr.K.P.Sibiraj for their excellent work of typing this thesis.

Finally, I am very much indebted to my beloved parents, brother and sister for their love, patience,interest and encouragement.

Nandakumar, M.P.

CONTENTS

1. INTRODUCTION

1.1 PREFACE

Starch plays an important role as a renewable raw material in many industries. A native starch can be modified or chemically derivatised by simple process technology. In addition starch is very succeptible to total or partial hydrolytic degradation by acid or enzymes yielding oligomeric or monomeric products which can be additionally modified or derivatised. Further potential arises from separation of starch into amylose and amylopectin (Rexen, 1984; Munk, 1987).

Starch is used either as such as a staple food or as a derivative after hydrolysis for other useful purposes. It is used in the food industries and non-food industries.

In the food industries starch is used for the production of starch syrups like glucose syrups, 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 thickner; as adhesive,

and in chemical and pharmaceutical industries. Even starch is found to be used in the mining and oil exploration.

The well-known biotechnological use of starch is in the production of amylases, viz., α-amylase, β-amylase and amyloglucosidases, which are widely used in the preparation of beverages, confectioneries, digestive aids and in the paper and textile industries. The starch hydrolyzed products maltose, glucose and maltodextrin are further used as starting materials in the production of alcohol, organic acids, aminoacids, single cell protein etc.

Fermentation is one of the oldest methods of food preparation and originated centuries ago. Generally traditional methods of preparing fermented foods are simple and inexpensive. However, the ancient methods of making such foods are changing rapidly through modern microbial technology.

Various fermentation industries are based on starch resources. Most of starch resources are obtained from cassava, potato, corn, wheat, maize etc. Corn starch is used extensively in the commercial preparation of sugar syrups. Cassava promises to be a potential raw material for microbial

fermentation and is widely used for the production of ethanol, single cell protein, sugar etc. However, this is awaiting for yet to be used in commercial preparations (Reade & Gregory, 1975). Among these resources rice is minimaly exploited as a raw material in the fermentation industry.

Fermentation processes have been highly developed in the recent years for the production of alcohol, biomass, enzymes, sugar syrups, secondary metabolites like aminoacids, vitamins and pharmaceutical compounds etc., employing both aerobic, and anaerobic microorganisms in an extensive manner all over the world. It is believed by many that the bioprocess technology may even substitute the chemical processes in future. Consequently an intensive search for cheaper as well as renewable raw materials have drawn the attention of many scientists towards economic production of products.

Recently recognition of immobilization technology for the rapid conversion of several substrates into metabolites and repeated reuse of the biocatalysts have drawn the attention of the fermentation scientists and technologists to try these new technologies for the rapid production of product and enhancement of the efficiencies of the systems.

Hence in the present study rice was selected as a substrate since it is a rich source of starch, available and cultivated throughout the year almost in all part of our country. Rice although known for its use as a staple food in many forms as rice, idli, dosai etc., has not been used in industry extensively. However, it is a potential resource for the production of alcohol, high protein food and for sugar and sugar syrups as it is evidenced by the few reports mentioned in the review of literature.

Of the several microorganisms available, Bacillus sp. is a known candidate for the production of amylases. Hence in the present study Bacillus sp. was desired for its known efficiencies in starch conversion.

1.2 REVIEW OF LITERATURE

1.2.1 Starch--a substrate for fermentation

The first major technological breakthrough was achieved in 1940, when Langois & Dale patented the use of commercially available enzymes and hydrolyzed starch.

The first significant step in the production of dextrose from starch was the introduction of enzymes produced

by Aspergillus niger capable of complete hydrolysis of starch and dextrins to glucose (Denault, 1963).

In the later years production of simple sugars and sugar syrups using starch as raw material employing microbial amylases found their applications in food industry especially in confectioneries, bakeries, brewing and soft drinks. Glucose syrups, dextrose syrups, high fructose syrups and high maltose syrups have been produced from starch (Martensson, 1974; Palmer, 1975; Saha & Zeikus, 1987, 1989; Hebeda et al., 1988).

Corn starch is widely exploited as a source of glucose and glucose syrups. Corn starch when treated with α-amylase at 80-90°C, brought about 15-25% of conversion into dextrose. When this was further subjected to gluco-amylase treatment, at 60°C, 98.5% of dextrose was obtained. (Sinclair, 1965). A pilot plant production of crystalline dextrose from corn starch, initially liquified by acid followed by saccharification by glucoamylase at 60°C, yielded 96% DE value is also reported (Kingma, 1969).

Raw corn starch was also used to obtain an initial high dextrose syrup and high fructose syrup followed by further isomerization with glucose isomerse in commercial scale (Harden, 1972, 1973). Continuous production of high glucose syrups and high fructose syrups from corn starch by immobilization (Oesterguard & Knudson, 1976; Hupkes & Van Telburg, 1976; Venkatasubramanian, 1978), and on the production of high fructose syrups from corn starch by liquifaction followed by a saccharification and isomerization using bacterial α-amylases, fungal glucoamylase and glucose isomerase (Kalevy, 1987) is also reported.

Cassava starch is a widely employed substrate for the production of glucose, glucose syrups and ethanol on a commercial basis. Available reports mainly dealt with preparation of high glucose syrup from cassava starch by a dual enzyme process (Park & Papini, 1970), production of 100 gm glucose/100 gm cassava starch by liquifaction of cassava starch by a thermostable bacterial amylase followed by a saccharification using fungal amyloglucosidase (Ana et al., 1978).

Ethanol was produced from cassava starch by saccharification with amylolytic enzymes or by employing Aspergillus usamii which produced glucoamylase followed by an ethanol fermentation using Saccharomyces cerevisiae (Reade & Gregory, 1975; Nakamura et al., 1978; Thammarutwasi, 1978). A one step process of fermentation which combined the conventional process of liquifaction, saccharification and alcohol fermentation using Aspergillus niger amylase and yeast is also reported (Ueda, 1981). Different aspects of ethanol production from cassava starch were also studied by many authors (Kunhi et al., 1981; Queiroz et al., 1982; Prema et al., 1986; Srikanta et al., 1987).

Solid state fermentation studies were carried out to bring about a simultaneous solid phase saccharification and fermentation of cassava fibrous residue employing glucoamylase and Saccharomyces cerevisiae (Jaleel et al., 1988), to produce 6780 u/ml glucoamylase by Aspergillus sp. after 6 days (Kinoshita, 1979) and a highly thermostable α-amylase by Bacillus sp. (Lonsane & Ramesh, 1990) through solid state fermentation with cassava starch as a substrate. An acidic food called 'gari' was attained by fermenting cassava starch employing a Lactobacillus sp. and Streptomyces sp. (Ngaba et al., 1979).

Starch syrups derived from potato starch are known years back (Heisler et al., 1952), production of a sweet glucose syrup from potato and maize starch by enzymatic hydrolysis (Dondero et al., 1978), and a starch syrup with a DE of 9.20 from potato starch by α-amylase liquifaction (Vance et al., 1972) are reported.

Reports on the production of high maltose syrup from waxy maize starch by enzymatic (β-amylase) hydrolysis (Hawling, 1973), and from potato starch by a thermostable maltogenic amylase of B. stearothermophilus (Slominska & Starogardska, 1986) are also available in the literature.

Ethanol production from potato starch using gluco-amylase of Aspergillus niger followed by alcoholic fermentation by baker's yeast (Ueda et al., 1981), or by alcoholic fermentation of raw sweet potato starch in a one step process employing glucoamylase of Rhizopus sp. followed by alcoholic fermentation of baker's yeast which yielded 94% alcohol (Hiroshi, 1982, Chua et al., 1984) was studied.

Whole ground barley was employed for the production of ethanol by liquifaction and saccharification followed by an alcoholic fermentation (Wayman, 1988).

1.2.2 Rice starch as a substrate

Rice (Oryza sativa) is known as a substrate for the preparation of traditional foods. In Japan starch is used for the preparation of sake--a fermented beverage containing 20-30% alcohol, Mirin--a sweet liquid containing about 35% glucose and 12% alcohol, Amasake etc. Further it is used to prepare Ia-chao in China and Tape in Indonesia (Ko, 1972; Cronk et al., 1977).

However, in India rice is used as a staple food and as a substrate along with other legumes for the preparation of traditionally fermented foods such as idli, dosai, appam etc., by natural fermentation. In rural areas, rice is used to prepare country arracks, which contains alcohol (Sakunthala & Shadeksharaswami, 1987).

Rice and black grams (Phaseolus mungulus) mixture is fermented in the proportion of 3 : 1 and 1 : 1 for the preparation of two South Indian foods--idli and dosai. Several reports are available on the methods of preparation, microbiology and nutritional value of idli (Desikacher, 1960; Rajalakshmi, 1967; Ramakrishna, 1979; Batra, 1981).

Microflora associated with the fermentation of idli mainly included bacterial species of Leuconostoc mesentroides, Lactobacillus corneyformis, L.fermentum, L.lactis, Pediococcus cerevisiae, B. subtilis, B. amyloliquifaciens and yeast species Candida fragicolor, Candida kefyr, Rhodotorula gramini, C. tropicalis and Torulopsis holmii (Batra, 1976; Lakshmi, 1978; Sandhu, 1984; Venkatasubbaiah, 1984; Sarasa & Nath, 1985).

Among bacteria species of Bacilli, like B. subtilis, B. amyloliquifaciens, B. polymyxa, Lactobacillus delbrueckii, Streptococcus facealis and yeast species including Oosporidium margaritiferum, Kluyveromyces maximonus, Candida kefyr, C.krusei were reported to ferment the batter produced for dosai preparation.

In general rice starch is minimally exploited in industries based on fermentation. Few reports are available on the use of rice starch in the fermentation production of alcoholic beverages and sugar syrups.

Available reports on alcoholic beverages mainly centered around the production of alcoholic chinease food

Ia-chao from rice with 2% alcohol after 45 hrs of incubation using on amylolytic filamentous fungi and an Endomycopsis sp. (Wang & Herseltine, 1970). Preparation of Tape--an indonesian delicacy with a sweet acid taste and mild alcoholic flabour by the fermentation of glutaneous rice using fungi like Chlamydomucor oryzae and Endomycopsis fibuliger for 5 days (Ko, 1972) and using fungi Amylomyces rouexii and Endomycopsis burotonii (Cronk et al., 1977), production of sake--a Japanese alcoholic beverage with an alcoholic content of 160 g/l which is the highest among the naturally fermented beverages not distilled, made from rice and water by the action of two organisms Aspergillus oryzae and Saccharomyces cerevisiae (Miyoshi et al., 1973; Fugita et al., 1984; Kondo, 1984; Sugimoto et al., 1986), on the development of sake fermentation processes (Miyoshi, 1973; Fugita et al., 1983; Sugimoto, 1984; Taneka et al., 1984), and on the single step ethanol fermentation from finely powdered rice employing glucoamylase preparations, Kojis, or Koji extract of Rhizopus strains with compressed baker's yeast (Elegado et al., 1986).

Reports on rice starch as a substrate for sugar production included the production of high dextrose syrups

from rice and other starches by amylolytic enzymes (Leach et al., 1975; Muller, 1978), high maltose syrups from rice, corn, potato, wheat, tapioca by an α-amylase of Streptomyces sp. and β-amylase of plant origin (Yamoto et al., 1976) and high fructose syrup (Chen & Chang 1984).

Rice starch is also reported to be a substrate for the production of high protein rice flour, childrens feed, from broken rice by partial enzyme digestion with α-amylase (Hansen et al., 1981; Chen & Chang, 1984).

1.2.3 Bacillus and their amylases

Amylolytic enzymes are widely distributed in plants, animals and in microorganisms (Boyer & Ingle, 1972). Among them microbial amylases has 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 α and β amylase. Species of Bacillus subtilis, B. coagulans, B. stearothermophilus, B. licheniformis and B. amyloliquifaciens have been reported to produce α-amylase (Welker & Campbell, 1963; Saito, 1973; Medda & Chandra, 1980; Pinches et al, 1985; Shah, 1989; Kochhar & Dua, 1990). While Bacillus cereus, B. polymyxa, B. megaterium and B. circulans were reported to produce β-amylase (Marshall, 1974; Shinke, 1975; Takasaki, 1976; Taniguchi, 1983; Kawasu, 1987). Recently these strains are reported to produce α-amylases besides β-amylases (Yoshigi et al., 1988; Uozumi, 1989).

Of the two types of amylases, α-amylases of Bacillus is used in numerous commercial processes including brewing, starch degradation and textile manufacturing (Rose, 1980; Wiseman, 1985).

Several aspects of amylase production by Bacillus has been studied in detail for various organisms. It was reported that in B. stearothermophilus there was an inverse relationship between growth and α-amylase synthesis and the enzyme was found to be induced by maltose and maltodextrin while inhibited by fructose and chloramphenicol (Welker & Campbell, 1963 a, b).

B. amyloliquifaciens showed increased rate of production of α-amylase in the post logarithmic phase when most of the enzymes were secreted, independent of carbon sources tested, fructose, maltose, starch which were present in the production medium (Coleman et al., 1962). But Boyer and Ingle (1972) and Tsuchiya (1975) have observed that glucose repressed α-amylase synthesis. Whereas during another study on the α-amylase synthesis by B. amyloliquifaciens it was observed that a four fold increase in α-amylase activity in the culture medium could be obtained with maltose than with glucose and changes in concentration of yeast extract in the medium influenced the formation of amylase in different phases of growth, Yoo et al., (1988) and Magee & Kosaric (1987) suggested that carbon and nitrogen sources in the medium influenced the metabolite formation in B. amyloliquifaciens which in turn had a modulating effect on enzyme synthesis by changing the pH of the system. When the enzyme production medium contained a higher concentration of yeast extract, a drastic change in pH from neutral to acidic range and a complete repression of α-amylase activity was observed (Alam et al., 1989). In another study, while higher cell growth with reduced amylase production was obtained with

glucose as the carbon source, higher levels of cell mass along with total and specific enzyme activities and enzyme production were obtained with maltose as carbon source (Siddhartha et al., 1989).

At the same time, while a high concentration of phosphate promoted maltose uptake and growth of microorganism, high maltose uptake rates repressed enzyme biosynthesis (Yoon et al., 1989).

B. subtilis was observed to synthesize a high amount of α-amylase in the presence of adenine and there was no repression by adenine (Tsuchiya et al., 1975). Inducive effect of starch and maltose and repressive effect of glucose on α-amylase synthesis was reported by Saito and Yamamoto (1975). Caesinate as a nitrogen source and hydrolyzed products of starch were found to repress the enzyme synthesis during growth in batch cultivation while promoting a two fold increase in enzyme production in fed batch cultivation (Pazlarova et al., 1984).

B. acidocaldreus was found to secrete a thermostable, thermoacidophilic α-amylase at maximum levels during the

stationary phase of growth and was induced by carbon sources such as glycogen, starch, maltose and maltotriose with an optimum pH of 3.5 and a temperature of 75°C (Buonocore et al., 1976).

β-amylases production by B. cereus was repressed by polysaccharides, glucose and maltose in the culture media. However, a high β-amylase activity was observed even in the absence of a carbon source for this species (Shinke et al., 1977).

β-amylase production by B. megaterium was not observed in the absence or in the presence of carbon sources other than starch suggesting that β-amylase production by this species is only by induction (Yamane & Tsukano, 1977).

B. coagulans is also known to secrete a α-amylase when induced by starch than by other carbon sources tested (Babu & Satyanarayana, 1990).

Thermostable α-amylases have had many commercial applications for several decades. These enzymes are used in

textile and paper industries, starch liquifaction, food, adhesive and sugar production (Bajpai & Bajpai 1989).

Buchanan & Gibbons (1974) described 48 species of Bacillus of which 32 are reported to produce α-amylase but only a few among them are capable of secreting thermostable enzymes.

Among the many Bacillus sp. that have been tried as the sources of thermostable α-amylase, B. stearothermophilus is shown to produce enzymes that are active at 90°C even after 12 hrs (Hartman, 1955). Pfueller & Elliott (1969) and Ogasahara (1970) purified and characterized this enzyme and found that addition of polyols and dimethyl formacide in the media increased thermostability and half life.

B. licheniformis is also known for the production of thermostable enzyme. Saito (1973) observed that the α-amylase of this species after purification exhibited an optimum temperature of 76°C and a pH of 9. In another study, activity of the enzyme was observed at 110°C and at narrow pH range of 6-7 (Madson et al., 1973; Chiang et al., 1979).

Whereas, Morgan & Priest (1981) observed optimum temperature of 70-90°C at pH 7 and maximal activities at pH 7 and 10 for the α-amylase. Kinetic studies of starch hydrolysis by α-amylase from B. licheniformis revealed that maximum conversion rate was observed at 100°C, with pH 7 and a substrate concentration of 300 gl^{-1}. This enzyme gave a higher rate of hydrolysis and a higher dextrose equivalent within a shorter period of time (Yankov et al., 1986). Bajpaiand Bajpai (1989) tried to liquify corn starch (30%) with a α-amylase from B. licheniformis which is active upto 100°C with an optimum at 90°C and a pH range of 5.5-10. They found that presence of Ca^{++} and Na^{++} in the reaction mixture, while repressing starch hydrolysis, enhanced reducing sugar production.

B. subtilis was found to produce a calcium requiring thermostable liquifying α-amylase at an optimum temperature of 70°C and with a broad pH range of 5.9-9.5 (Mosely & Keay, 1970). Lin and Kang,(1988) found that when the starch substrate concentration was increased upto 50% there was a decrease in thermostability of B. subtilis amylase. Whereas, presence of calcium along with the substrate effected only a secondary effect on thermostability.

An α-amylase with pH optima at two pH levels of 6.5 and 7.0 and an optimum temperature of 90°C was produced by B. amyloliquifaciens by means of solid state fermentation (Ramesh & Lonsane, 1989). Another α-amylase with a molecular weight of 68,000 with temperature optimum of 65°C and a pH of 6 was isolated from this species by Kochhar & Dua (1990).

A facultative, thermophilic B. coagulans was reported to produce α-amylase which maintained its thermostability even after crystallization and retained 90% of its activity after 1 hr at 90°C (Campbell, 1954).

Medda & Chandra (1980) described two species of Bacillus B. coagulans and B. licheniformis producing thermostable α-amylase active at alkaline pH. The enzymes from B. licheniformis showed a wide range of temperature for activity with optimum at 91°C and was stable for 1 hr at this temperature. It was active over a wide range of pH of 4-10 with optimum at 9.5. Enzymes of B. coagulans showed activity upto 90°C with optimum at 85°C and had a wide pH range with optimum at 7.5. A thermostable alkaline α-amylase active at a wide range of pH and temperature from a strain of B. coagulans ACMN 1 is reported by Nandakumar & Chandrasekaran, (1989).

Pure cultures of microorganisms are inherently variable in their growth characteristics and metabolic activities. Therefore, the initial activity of the microbiologist is to minimize the genetic variability of the microorganisms by selecting out stable and genetically uniform isolates which produces a minimum number of unwanted metabolites and copious amounts of the desired component. Mutagenesis followed by the subsequent selection, and purification of superior strains represents the most important activity in improving the yield of a fermentation product. This led to several mutation studies in Bacillus sp towards strain improvement to get enhanced enzyme yield.

B. subtilis is used widely as a model system for studies on regulation and synthesis of α-amylases. There are many reports on the expression of cloned α-amylase genes on plasmids on B. subtilis (Palva, 1982); Aiba et al., 1983); Ortlepp et al., 1983; Joyet et al., 1984; Corfield, 1984). Yoneda (1982) reviewed the reports on the regulatory genes of amylase which demonstrated synergestic effect on the production of α-amylase by B. subtilis. Absence of glucose catabolite repression of α-amylase genes in the recombinant

B. subtilis was reported (Pinches et al., 1985). A high yielding stable mutant of B. subtilis which secrete 5 fold more α-amylase activity was isolated by successive exposures to N-methyl-N-nitro-N-Nitrosoguanidine and UV radiation (Shah et al., 1989).

B. cereus NY-14 mutants were found capable of producing α-amylase in the presence of glucose (Yoshigi et al., 1988).

B. polymyxa amylase was shown to be synthesized as a precursor protein with β and α-amylases activities and that give rise to β and α-amylase (Uozumi et al., 1989).

1.2.4 Application of Bacillus amylases in industry

A substantial percentage of bacterial enzymes produced in the industry is accounted by the amylases and proteases of Bacillus sp. The main amylolytic enzymes used for the production of glucose, maltose, and maltosaccharides namely α-amylase, β-amylase and glucoamylase, are now commercially derived from Bacillus sp., fungal sp., and used in various other starch based industries (Shinke, 1975; Anderson, 1985).

α-amylases of Bacillus sp. and fungal amyloglucosidases are used for the production of high dextrose syrups from starch substrates (Leach et al., 1975; Takasaki & Takahara, 1976). A liquifying α-amylase from Bacillus sp. and glucoamylase from Aspergillus sp. were employed to produce glucose from cassava starch (Ana et al., 1978). An alkalophilic maltohexose forming Bacillus sp. H 167 from soil was found to be able to hydrolyze soluble starch to produce 25-30% maltohexose (Hayashi, 1988).

Thermostable amylases derived from B. amyloliquifaciens have been in use for many years. However, a more heat stable (upto 110°C) α-amylase from B. licheniformis (Madson et al., 1973) was found and introduced to commercial application after its discovery. The α-amylase from B. licheniformis was highly active and sufficiently stable to allow gelatinization of the starch at 105°C without any significant loss of enzyme activity and are widely used for starch liquifaction (Anon, 1984), a preparation of starch liquifying α-amylase from B. amyloliquifaciens was able to degrade raw corn starch rapidly and produced maltooligosaccharides and glucose from starch (Bergman, 1988).

β-amylase from B. polymyxa was used for the production of high maltose syrups with DE 58 and maltose content 60% (Armbruster & Jacaway, 1970). High maltose syrups with DE 50 and maltose content 75% and very high fermentables 90% was prepared by using pullulanase enzyme and B. polymyxa β-amylases (Heady & Armbruster, 1970).

Takasaki (1976) and Takasaki & Takahara (1976) patented a B. cereus var mycoides that produce an extracellular pullulanase and β-amylase simultaneously and employed for the production of high maltose syrups (80-90% maltose) from starch.

A thermostable, acid stable maltogenic amylase from B. stearothermophilus was used for the production of high maltose syrups containing 70-80% maltose from liquified starch and this together with pullulanase or fungal α-amylase enabled the production of 85% maltose from the same substrate (Slominska & Sturogardzka, 1986).

Amylase from B. megaterium in combination with glucoamylase was suggested for the production of high dextrose syrups from starch (Hebeda & Styrlend, 1988).

Recently, dextrins and cyclodextrins were produced from potato starch by α-amylase enzyme of B. macerans (Koto, 1989).

1.2.5 Immobilization

Biocatalysts based on immobilized cell technology has attracted wide attention in recent years (Mattiason et al., 1983; Chibata et al., 1983; D'souza, 1989). Much of the reports available on immobilized biocatalysts including enzymes, microbial, plant and animal cells and cellular organelles and their potentials in industry, medicine and analysis were reviewed extensively by D'souza (1989).

In the past years, much interest has been expressed in the use of immobilized microorganisms in the production of urocanic acid, L-citrulline, L-aspartic acid, cortisol to prednisolone, L-malic acid, L-sorbose, glycerol to dihydroxy-acetone, preparation of lactose hydrolysed milk, high fructose syrups, L-alanine, vitamin B_{12}, butanol, lactic acid, acrylamide, L-glutamate, vinegar, acetic acid, proinsulin proteases amylases, H_2, ethanol employing bacterial systems; chlorotetracycline, nikkomycin, daunorubicin,

thienamycin, proteases and cholesterol degradation using actinomycetes; sucrose to fructose and gluconic acid, NADH regeneration, lactose free milk, ethanol and alcoholic beverages, meat, citric acid, glutathione, hydrolysis of inulin employing yeasts; citric acid, cellulose, itaconic acid, alkaloid, penicillin G, Alkaline proteases, chlorinated benzoates, degradation of n-alkanes, detoxification of cynide and clarification of pectin using fungi and wastewater nutrient removal, ammonia photoproduction, ammonia production, glycollate, hydrogen, glycerol and polysaccharide production employing algae (D'souza, 1989). Both organic and inorganic supports have been used for immobilizing cells and enzymes. Some of the commonly used supports for whole cell immobilization include inorganic supports like kaoline, colloidal silica, glass particles, controlled pore glass, alumina, nickel oxides, ziconia, charcoal, hydroxyapatite iorn oxide, ceramics, sand, hornblends, magnetite, steel and bentonite, organic supports like cellulose, agarose, agar, dextran, polyacrylamide, nylon, colloidon, polystyrene, polyacrylic acid, chitin, chitosan, ca-alginate, carrageenan, polymethane, prepolymers, polyvinyl alcohol and proteinic supports like collagen, fibrin, feather protein silk, elastin, albumin, gelatin casein and hen egg white (D'souza, 1989).

Immobilization of amylases is now considered as the most suitable techniques for starch hydrolysis rather than different fermentation processes (Sen & Chakraborthy, 1987). A reduction of production cost and a more precise reaction control could be achieved by recycling immobilized amylases in industrial glucose production (Linko et al., 1975)

Alpha amylase production was studied by immobilizing Bacillus subtilis on polyacrylamid gel (Kokobu et al., 1978), Bacillus amyloliquifaciens in kappa carrageenan (Shinmyo 1982), Bacillus subtilis in alginate (Oriel, 1988), Bacillus amyloliquifaciens on ion exchange resins Groom et al., 1988) and Bacillus coagulans on alginate (Nandakumar & Chandrasekaran, 1990). Alpha amylases are known to be immobilized on cyanogen bromide activated carboxymethyl cellulose for the production of glucose and maltose (Linko et al., 1975), on Duolite DS 73141 for heating paper mill effluent (Smiley et al., 1975) on millipore filter for continuous hydrolysis of soluble starch (Okada & Urabe, 1976) on magnetic iron oxide (Kennedy, 1976; Kennedy & White, 1979) on 2-hydroxy ethyl methacrylate (Kumakura et al., 1977; Kaetsu et al., 1979) by gamma irradiation on a polymer containing methyloacrylamide and methanol (Karube et al.,

1977) on charcoal (Karube *et al.*, 1977) on polystyrene (Fisher *et al.*, 1978) on cellulose (Kucera & Kuminkova, 1980) on acrylamide (Sen & Chakraborthy, 1987), on alginate (Prabha, 1989; Nandakumar & Chandrasekaran, 1990).

1.3 OBJECTIVES OF THE PRESENT STUDY

In the present study, it was decided to use rice starch and related products like rice decanted water as a substrate for fermentation. From the review of literature it is clear that not much work has been done in India in the utilization of this substrate for the production of sugar and related products.

Main objectives of the present study included the following:

- to isolate amylolytic *Bacillus* sp. from natural environments.
- to select suitable strains that ferment rice starch efficiently.
- to identify the selected *Bacillus* sp. upto species level.

- to characterise the organism for their growth and enzyme production with respect to various physico-chemical parameters.

- to prepare, purify and characterise the amylases from the selected strains of Bacillus.

- to study the conversion of rice starch to sugars by free enzyme.

- fermentation of rice starch and rice decanted water by whole cells of selected strains of Bacillus with respect to various physicochemical parameters towards optimisation of fermentation process.

- to immobilize the whole cell and free amylases and effect conversion of rice starch.

2. MATERIALS AND METHODS

2.1 SAMPLE

Parboiled and milled grains of Oryza sativa (Family Graminae) commonly known as rice or paddy was selected as the substrate for fermentation studies.

In India, this species of rice is widely distributed and cultivated in Andhra Pradesh, Assam, Karnataka, Kerala, Punjab and Tamil Nadu. This species is a perenial grain without a rhizome. The leaves are long and narrow 30-50 cm and 1.2-2.5 cm, slightly pubescent with spiny hairs on the margin. Lemma and palea surrounding the kernel, variously coloured, golden yellow, red, purple, brown or smoky black. Grain varying in size from 5 to 14.5 mm long and 1.9 to 3.7 mm broad, the length/breadth ratio defining size and shape of the grain. Kernel most commonly white occasionally red, purple or brown.

The mature grain is botanically called caryopsis and consists of a large outer husk enclosing the kernel. The kernel is made of three parts viz., the outer layer which include pericarp (or seed coat) with the underlying aleurone

layer, the starting endosperm and the germ (or embryo) which on an average amount respectively to 6.0, 91.75 and 2.25% of the grain (Wealth of India 1966).

2.1.1 Chemical composition

The chemical composition of rice is influenced to some extent by genetic and environmental factors. Analysis of 14 types of husked rice from different parts of India gave the following range of values: moisture, 10.90-13.78%; ether extract, 0.59-2.59%; protein, 5.50-9.32%; mineral matter, 0.79-2.0%; carbohydrates, 73.35-80.81%; fibre, 0.18-0.95%, (Sen, 1917; Basu & Sarker, 1934-35; Sadasivan & Sreenivasan, 1938; Sreenivasan, 1951).

Starch is the major constituent of rice. The amylose content of the starch varies according to grain type. The longer grained and superior types contain upto 17.5% of amylose, whereas, some of the coarse types are completely devoid of it. Starch in glutinous rice is reported to consists almost entirely of amylopectin. The variation in starch composition is probably one of the factors responsible for the difference observed in the cooking quality of rices.

Freshly harvested rice contains: starch, 72.20-74.90% glucose, 1.45-2.65%; sucrose, 0.30-0.48%; and dextrin, 1.56-2.05%. No marked change is reported in the amount of carbohydrates during storage. Small amounts of fructose and galactose, maltotriose and maltotetrose identified in old rice are all formed possibly due to some starch being hydrolyzed by α-amylase during storage. Hemi-celluloses of rice are made up of arabinose and xylose (approximately 1 : 1) and small quantities of galactose, mannose and uronic acid. (Sreenivasan, 1939; Rao et al., 1952; Parihar, 1955; Bevenue & Williams, 1956).

The protein content of rice is lower than that of wheat and maize, but otherwise the three cereals do not differ materially in nutritive value. One fourth of the protein of the whole rice is contained in the bran and polish, the germ, aleurone layer and one or two layers of the cells of the endosperm adjacent to the aleurone layer are rich in protein.

The chemical composition of home-pounded and milled rice, raw as well as parboiled, is summarized in Table 1. (Sreenivasan, 1951; Sathe et al., 1951-52; Rao, 1954; Basak et al., 1961).

In its mineral content rice resembles other cereals. Most of the minerals present in rice are located in the pericarp and germ. The mineral content of milled rice is 0.5% and that of husked rice is 1.0-2.5%. Rice, particularly the polished grain, is poor in calcium and iron. Rice contain Sodium (20 mg/100 g), Potassium (100 mg/100 g), Magnesium Sulphur and Chlorine, Trace elements reported in the grain include Aluminium, Manganese, Copper, Zinc, Arsenic, Boron, Chromium, Nickel, Cobalt, Iodine, Fluorine, Selenium, Titanium, Molybdenum, Vanadium, Lead, Tin, Strontium, Barium, Rubidium and Lithium. More than 6.5% of the Iodine in the whole grain is found in the bran fraction (Pain and Banerjee, 1956).

2.2 SOURCE OF AMYLASE PRODUCING BACTERIA

2.2.1 Isolation of amylolytic bacteria from natural environments

2.2.1.1 Samples

Samples screened for the isolation of amylolytic bacteria included soil collected from different areas in and around Cochin University campus and potato, tapioca, grapes, bananas, mango, rice, wheat and barley collected from market.

Soil samples were collected using sterile spatula and transferred to sterile polythene bags. Cereals fruits and vegetables were collected in sterile polythene bags, from the market. The samples were immediately taken to the laboratory and processed for bacteriological analysis.

2.2.1.2 Preparation of media

Two different types of media were used for the isolation of amylolytic bacteria. They included (a) nutrient agar (NA) (HI-Media) added with soluble starch (E.Merck) and (b) a minimal salts--starch agar medium.

Use of nutrient agar added with starch in the isolation of amylolytic bacteria involves detection of amylase production by iodine flooding of Petri dishes grown with colonies. Iodine brings about a detrimental effect on the bacteria and hence require round about procedures of isolation of these amylolytic strains.

Therefore an attempt was made to develop a mineral salts basal medium supplemented with starch as sole carbon source with a dual objective of rapid detection and isolation of starch utilising bacteria without subjecting them to iodine staining which harm the bacteria.

After standardisation the Mineral Salts Starch Agar (MSSA) medium contains the following composition:

K_2HPO_4	-	0.1%
KH_2PO_4	-	0.1%
$MgSO_4 7H_2O$	-	0.05%
$CaCl_2$	-	0.05%
$(NH_4)_2SO_4$	-	0.1%
Potato starch	-	1%
Agar	-	2%
pH	-	7.0 ± 0.2
Distilled water	-	100 ml

The prepared medium was autoclaved and used.

2.2.1.3 Preparation of serial dilutions and plating procedures

About 10 g of soil was aseptically weighed and transferred to 90 ml of blank (Tap water) in a 250 ml conical flask. The contents were homogenised for 15 min. on a rotary shaker and allowed to settle. The supernatent was used for serial dilution preparations. Pour plate technique was employed for plating and incubation was done at room temperature (28 ± 2°C) for 3-7 days.

2.2.1.4 Isolation and maintenance of cultures

Amylase production by bacteria on Nutrient Agar (NA) added with starch was checked by iodine flooding technique (Iodine solution contained 0.3% I_2 and 3% KI in distilled water). The positive strains, which produced clear zones, were immediately transferred to nutrient agar slants. Whereas all the colonies that developed on MSSA were considered as amylolytic and transferred to NA slants.

All the isolates obtained from both NA and MSSA were purified, reconfirmed for their amylase production on NA plus starch medium and maintained on nutrient agar medium. Stock cultures were preserved under mineral oil (sterilized liquid paraffin), while working slants were kept at room temperature and subcultured once in 15 days. Their purity was checked at regular intervals of 30 days.

2.2.1.5 Identification of bacteria

The isolates were assigned to various genera based on their morphological and biochemical characters outlined in the Bergey's Manual of Systematic Bacteriology (Buchanan & Gibbons, 1974). The selected strains of Bacillus were further identified upto their species level based on the schemes suggested by Gordon (1973).

2.2.2 Reference strains

Twenty species of Bacillus (Table 2) were procured from National Collection of Industrial Microorganisms (NCL, Pune, India). These reference strains were used as standards for species identification of the isolated Bacillus strains.

2.2.3 Screening of amylolytic Bacillus sp that ferment rice starch

Although several groups of bacteria were encountered as amylase producers during isolation, Bacillus sp. was desired for the present study. Hence all the amylolytic Bacillus sp. isolated from soil were subjected to further primary and secondary screening procedures in order to select potential strains that could ferment rice effectively.

2.2.3.1 Primary screening

The primary screening of potent strains that could ferment rice, included a qualitative test described as follows. Slope cultures of all the Bacillus strains (24 hrs. old) were planted on MSSA agar substituted with rice starch instead of potato starch (SISCO), and grown for 3-5 days

at 30°C. After incubation, amylase activity of strains were detected by iodine flooding technique, and the width and size of the clear zone produced were recorded. All the strains tested were ranked in terms of maximal width of clear zone and those strains that recorded maximal width of clear zones were selected for secondary screening.

2.2.3.2 Secondary screening

Secondary screening of amylolytic Bacillus sp. that could ferment rice effectively included estimation of maximal production of growth and enzymes, total sugars, reducing sugars and maximal starch reduction.

2.2.3.2.1 Media

Mineral Salts Starch Agar medium mentioned earlier (Section 2.2.1.2) was used as a liquid medium with 1% powdered rice starch instead of potato starch. Fifty ml of MSRSB (Mineral Salts Rice Starch Broth) taken in 250 ml Erlnmeyer conical flasks, were autoclaved and used.

2.2.3.2.2 Inoculation procedures

Inoculum for secondary screening was prepared as follows:

1. Initially a loopful of 24 hrs old agar slope culture was transferred to 10 ml of NBS (Nutrient Broth added with soluble Starch) and grown for 24 hrs at room temperature (28 ± 2°C).

2. One ml of the precultured broth was then aseptically transferred into another 50 ml of the NBS media and incubated for 24 hrs in a rotary shaker (150 rpm) at room temperature (28 ± 2°C).

3. Cells were harvested by centrifugation (MB Centrifuge Model MB 20) at 5000 rpm for 20 minutes.

4. The harvested cells were made upto 10 ml volume using physiological saline (0.85% NaCl) after repeated washing with the same.

5. The prepared cell suspension was used as inoculum at 1%level (0.5 ml with $1x10^6$ cells) for further inoculation of 50 ml MSRSB.

6. All the flasks were uniformly inoculated and incubated on rotary shakers (150 rpm) for a period of 24 hrs at room temperature (28 ± 2°C).

2.2.3.2.3 Measurement of growth

The growth of bacteria in the MSRS Broth was estimated by quantifying the cell protein according to the methods suggested by Strickland (1951).

1. Ten ml of the culture broth was centrifuged at 5000 rpm for 20 minutes. The supernatent obtained after centrifugation was used for enzyme assay, estimation of starch, reducing sugar and total sugar.

2. The precipitate obtained was made upto 2 ml with distilled water.

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

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

5. After cooling at room temperature (28 ± 2°C) the samples were added with 1 ml of 2.5% $CuSO_4 5H_2O$, shaken well and centrifuged at 5000 rpm for 15 minutes.

6. After discarding the precipitate the protein extracted into the supernatent was estimated by measuring the absorbance at 555 nm in a UV visible spectrophotometer (Hitachi - Model 200).

7. A standard curve prepared with Bovine serum albumin was used for computation of the concentration of protein in the samples.

8. Cell protein was expressed as mg/ml.

2.2.3.2.4 Enzyme production

The enzyme production in MSRS Broth was assayed according to Medda and Chandra (1980).

The culture supernatent obtained after centrifugation at 5000 rpm were used as crude amylase preparations. The reaction mixture contained 1 ml of the above crude enzyme, 1 ml of phosphate buffer with pH 7 (unless otherwise stated) and 1 ml of 1% soluble starch prepared in distilled water. After incubation at 40°C for 30 min. 1 ml of the sample was withdrawn, added with 0.1 ml of I_2-KI (0.3% I_2 and 3% KI in distilled water) solution (Medda & Chandra 1980), made upto 15 ml with distilled water and the blue colour developed in the solution was measured at 650 nm.

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

2.2.3.2.5 Estimation of residual starch

The residual starch in the culture broth was determined by Iodine staining method (Medda & Chandra 1980). Same procedures mentioned under previous section (2.2.3.2.4) for the enzyme assay were followed. Residual starch in the broth is expressed as mg/ml.

2.2.3.2.6 Estimation of total sugar

Total sugar was estimated by phenol-H_2SO_4 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 H_2SO_4 and the absorbance was measured at 490 nm. The results were computed from a standard curve prepared with glucose. Total sugar is expressed as mg/ml.

2.2.3.2.7 Estimation of reducing sugar

Reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959 modified by Jones & Grainger 1983). One ml of appropriately diluted culture supernatent was added with 0.5 ml of dinitrosalicylic acid reagent, heated at 100°C

for 10 minutes in a water bath, cooled, added with 4 ml of distilled water and absorbance was 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 MSRS broth and the values are expressed as mg/ml of reducing sugar.

2.3 GROWTH STUDIES

Growth studies were carried out for the four strains of Bacillus sp., selected after secondary screening, by subjecting them to various physicochemical parameters and estimating their growth curve.

2.3.1 Effect of physico chemical factors on growth and enzyme production

Optimal conditions required for maximal growth and amylase production by the test strains were determined by subjecting them to various incubation temperatures, different levels of pH, NaCl concentrations, substrate concentrations, carbon and nitrogen sources, inoculum concentrations in the growth media and different incubation periods.

2.3.1.1 Media

Mineral Salts Rice Starch Broth with the composition mentioned earlier (Section 2.2.3.2.1 unless otherwise specified) was prepared, dispensed in 50 ml aliquots in 250 ml Erlnmeyer conical flasks, autoclaved and used for optimization studies.

2.3.1.2 Preparation of inoculum and inoculation procedures

The procedure described in the previous section (2.2.3.2.2) was followed for the preparation of inoculum and inoculation of media.

2.3.1.3 Measurement of growth

Growth was measured as cell protein following the procedures mentioned earlier (Section 2.2.3.2.3) at the end of 24 hrs of incubation.

2.3.1.4 Enzyme production

Enzyme production in the media was estimated in terms of enzyme activity as per the procedures mentioned under section (2.2.3.2.4) except for the change in the time of incubation of reaction mixture for 10 minutes instead of 30 minutes.

2.3.1.5 pH

The pH of the culture broth at the end of incubation period (24 hrs) was monitored using ELICO pH meter.

2.3.1.6 Effect of temperature

Effect of temperature on growth and enzyme production was estimated by incubating the MSRS broth inoculated with test strains at 25, 30, 35, 45 and 55°C for a total period of 24 hrs. Cell protein, enzyme production and other biochemical analysis were carried out at the end of 24 hrs of incubation as per the procedures mentioned earlier under sections 2.2.3.2.3, and 2.2.3.2.4.

2.3.1.7 Effect of pH

The effect of pH on growth and enzyme production was analysed by subjecting the organisms to various pH levels (pH ranging from 4 to 11) in MSRS broth for 24 hrs, incubated at room temperature (28 ± 2°C) in a rotary shaker at 150 rpm. Biochemical estimations were carried out after 24 hrs of growth as per the procedures described under sections 2.2.3.2.3, and 2.2.3.2.4.

2.3.1.8 Effect of NaCl concentration

The effect of NaCl concentration on growth and enzyme production of the four species of Bacillus were carried out by subjecting them to NaCl concentrations of 0, 0.5, 1, 3, 5 and 7% added to MSRS broth with pH 7 ± 0.2° at room temperature (28 ± 2°C). After 24 hrs of incubation in a rotary shaker (150 rpm), growth, enzyme production were analyzed according to the procedures described under sections 2.2.3.2.3, and 2.2.3.2.4.

2.3.1.9 Effect of substrate concentration

The effect of substrate concentration on the growth and enzyme production of all the four strains of Bacillus were checked by growing them in MSRS broth supplemented with different rice starch concentrations (0.5, 1, 2, 3, 4 and 5%). After 24 hrs of incubation at room temperature (28 ± 2°C) in a rotary shaker (150 rpm), growth, enzyme production were estimated as per the procedures mentioned under sections 2.2.3.2.3, and 2.2.3.2.4.

2.3.1.10 Effect of incubation time

The effect of incubation time on growth and enzyme production of the four strains of Bacillus were carried out

in a series of 50 ml MSRS broth distribution in 250 ml Erlnmeyer conical flasks at their optimal growth conditions for a period of 48 hrs. At periodical internals of 6 hrs, growth, and enzyme production were estimated according to the procedures described under sections 2.2.3.2.3, and 2.2.3.2.4.

2.3.1.11 Effect of inoculum concentration

Effect of inoculam size (1 to 7%) on growth and enzyme production were carried out in MSRS broth at their optimal growth conditions. After 24 hrs of incubation, growth and enzyme production in the media were estimated according to the procedures mentioned under sections 2.2.3.2.3, and 2.2.3.2.4.

2.3.1.12 Effect of carbon sources

The effect of carbon sources on growth and enzyme production was tested according to Shinke et al., (1977). Carbon sources viz., Glucose, Maltose, Lactose, Dextrin, Soluble starch and Glycerol were used instead of rice starch. Mineral media (composition mentioned in section 2.2.1.2) with 1% of carbon sources and 0.5% peptone as a nitrogen

source adjusted to a pH 7 ± 0.2 were used. After 24 hrs of incubation at room temperature (28 ± 2°C) in a rotary shaker (150 rpm), growth, pH and enzyme production were estimated as mentioned earlier (See sections 2.2.3.2.3, 2.2.3.2.4 and 2.3.1.5).

2.3.1.13 Effect of nitrogen sources

Effect of nitrogen sources on growth and enzyme production were estimated using both organic (peptone, beef extract, tryptone and yeast extract) inorganic nitrogen sources $(NH_4)_2SO_4$, potassium nitrate and sodium nitrate) at 1% concentrations in the mineral media along with 1% soluble starch as carbon source. After 24 hrs of incubation at room temperature in a rotary shaker (150 rpm), growth, pH and enzyme production were analyzed as mentioned earlier (See sections 2.2.3.2.3, 2.2.3.2.4 and 2.3.1.5).

2.3.2 Growth curve

Growth curve studies for all the four strains of Bacillus sp., were carried out in nutrient broth (HI-Media).

The prepared medium was dispensed in 10 ml aliquots in test tubes, autoclaved and inoculated with 0.1 ml ($1x10^2$ cells)

of the inoculum prepared earlier (See section 2.2.3.2.2) and incubated at room temperature (28 ± 2°C). Samples were drawn at regular intervals and growth was determined by measuring the turbidity in the tubes at 660 nm in a UV visible spectrophotometer besides enumeration of TVC. From the results obtained growth curve was made.

2.4 ENZYME STUDIES

2.4.1 Isolation of enzyme

2.4.1.1 Media

The Enzyme Production Medium (EPM) was designed based on the data obtained for optimal requirements of bacteria for maximal growth and enzyme production in MSRS broth. The final composition of the EPM after standardisation is as follows:

K_2HPO_4	-	0.1%
KH_2PO_4	-	0.1%
$MgSO_4 7H_2O$	-	0.05%
$CaCl_2$	-	0.05%
$NaNO_3$	-	0.1%
Peptone	-	1%
Soluble starch	-	1%
pH	-	7 ± 0.2
Distilled water	-	100 ml

Prepared medium was autoclaved and used.

2.4.1.2 Preparation of inoculum

Inoculum for EPM medium was prepared as follows:

(a) A loopful of 18 hrs old slope culture was transferred aseptically to 10 ml of EP medium and incubated for 18 hrs at room temperature (28 ± 2°C).

(b) One ml of the grown culture was then transferred to 50 ml of EP medium and incubated on a rotary shaker (150 rpm) at room temperature (28 ± 2°C) for a period of 18 hrs.

(c) Cells were harvested by centrifugation at 5000 rpm for 20 min., washed with physiological saline and suspended in 50 ml of the same saline.

(d) The prepared cell suspension was adjusted to 1.0 OD ($1x10^6$ cells) and used as inoculum.

2.4.1.3 Enzyme production in the medium

Hundred ml of EP medium, taken in 1000 ml conical flask, was inoculated with 10 ml of prepared inoculum aseptically and incubated on a rotary shaker at room temperature (28 ± 2°C) for 18 hrs. Later the culture broth was centrifuged at 5000 rpm for 20 min. and the cell free extract was used for further studies.

2.4.1.4 Enzyme assays

2.4.1.4.1 Buffers used for enzyme assays

Different buffers with various pH ranges were used for the assay of amylolytic enzyme. They included acetate buffer (0.2 M) with a pH ranging from 4 to 6; phosphate buffer (0.2 M) of pH 7-8 and Glycine-NaOH buffer (0.2 M) of pH 9-11.

2.4.1.4.2 Determination of enzyme activity

Enzyme activity was measured according to the methods mentioned below:

(a) Iodine method (Medda & Chandra 1980)

1. One ml of the cells free extract was incubated with 1 ml of 1% soluble starch in 1 ml of appropriate buffer at 40°C for 10 min. (unless otherwise stated).

2. The reaction was arrested by the addition of 0.1 ml of IN HCl.

3. One ml of the above sample was added with 0.1 ml of I_2-KI reagent (Medda & Chandra 1980).

4. The volume was made upto 15 ml with distilled water.

5. The blue colour developed was then measured at 650 nm in a UV visible spectrophotometer (Hitachi Model 200).

6. One unit of amylase activity, expressed as dextrinizing activity, is defined as the amount of enzyme required to bring about the hydrolysis of 0.1 mg of starch at their optimal pH and T°C within 10 min. of incubation.

(b) Dinitrosalicylic acid method

Dinitrosalicylic acid reagent was used to measure the reducing sugar released during the hydrolysis of starch and is a measure of saccharolytic activity of the enzyme.

1. The composition of the reaction mixture was same as described in the previous paragraph.

2. The reaction was carried out at 40°C for 10 min. (unless otherwise stated).

3. After incubation the mixture was rapidly cooled in an ice bath (0°C).

4. One ml of the cooled sample was mixed with 0.5 ml of DNS reagent and heated in a boiling water bath (100°C) for 10 min.

5. Four ml of distilled water was added to the sample and the optical density of the colour developed was measured at 500 nm in an UV visible spectrophotometer (Jones & Grainger 1983).

6. One unit of enzyme activity, expressed as saccharolytic activity, was defined as the amount of enzyme that released 0.1 mg of reducing sugar as glucose at their appropriate pH and temperature for 10 min. of incubation (unless otherwise stated).

2.4.2 Purification of enzymes

The crude enzyme preparation (cells free extract) was partially purified by $(NH_4)_2SO_4$ (Sisco-enzyme grade) fractionation followed by dialysis using appropriate buffers. The specific activity was determined by measuring enzyme protein using Foline Ciocalteau reagent (Lowry et al., 1951).

2.4.2.1 $(NH_4)_2SO_4$ fractionation

The crude enzyme fraction (cells free extract) was subjected to ammonium sulphate precipitation from 30 to 80% saturation by increasing slowly the $(NH_4)_2SO_4$ concentration

along with continuous stirring using a magnetic stirrer at 4°C in an ice bath. The precipitate obtained at each saturation was removed by centrifugation and dissolved in 10 ml of phosphate buffer with pH 8.0 (0.2 M) or glycine-NaOH buffer of pH 9 (0.2 M).

2.4.2.2 Dialysis

The precipitate obtained after $(NH_4)_2SO_4$ fractionation was dialysed extensively at 4°C for 24 hrs using phosphate buffer, pH 8 (0.2 M) or glycine-NaOH buffer, pH 9 (0.2 M). The dialysed enzyme was redissolved in the same buffers and used for further studies.

2.4.3 Characterisation of amylase

The partially purified enzyme was characterized according to Medda and Chandra (1980). The enzyme were initially tested for their dextrinizing and saccharifying activity on soluble starch and for the effect of various physicochemical parameters viz., pH, T°C, substrate concentration and heavy metal ions on the activity. The stability of the enzyme at different pH and temperatures were also studied.

2.4.3.1 Dextrinizing and saccharifying properties of the amylase

The dextrinizing and saccharifying activities of the partially purified amylase on soluble starch with reference to time was carried out according to Medda and Chandra (1980).

The dextrinizing and saccharolytic activity were analyzed by using a reaction mixture containing 1 ml of 1% soluble starch, 1 ml of partially purified enzyme and 1 ml of the appropriate buffer. The reaction mixture was dispensed in 3 ml aliquots in a series of test tubes and incubated at 40°C for a total period of 60 min. At the end of every 10 min., samples were removed and the reaction was arrested by cooling on ice at 0°C or by adding 0.1 ml HCl. One ml of the sample was tested for dextrinizing property by iodine method (Section 2.4.1.4.2). While the other 1 ml was added with 0.5 ml DNS reagent for checking saccharolytic activity (See section 2.4.1.4.2). Tests were done in triplicates for each analysis.

2.4.3.2 Effect of pH on activity and stability of the enzyme

Effect of pH on activity of the enzyme was studied as per the following procedure described by Morgan & Priest (1980). One ml of the partially purified enzyme was incubated with 1 ml of 1% soluble starch at different levels of pH ranging from pH 4 to 11 using acetate buffer (pH 4-6), phosphate buffer (pH 7-8) and glycine-NaOH buffer (pH 9-11). The reaction was conducted at 40°C for 10 min. and later arrested by the addition of 0.1 ml of IN HCl. One ml of the sample was withdrawn and the enzyme was assayed as per the procedures mentioned earlier under section (2.4.1.4.2).

Stability of the enzyme at various pH levels was tested (Morgan & Priest 1980) by incubating 1 ml of the partially purified enzyme in 1 ml of the buffer of appropriate pH in the range of pH 4-11 for 30 min. at 40°C. After 30 min. of incubation 1 ml of the sample was withdrawn and adjusted to its optimum pH (pH 7,8,9) with 1 ml of appropriate buffer (phosphate buffer pH 7,8 or glycine-NaOH buffer of pH 9). Residual activity of the test sample was checked by incubating it at 40°C residual activity was measured by comparing the activities at the optimum pH and the test pH.

2.4.3.3 Effect of temperature on activity and stability of the enzyme

The effect of temperature on activity of enzyme was tested (Morgan & Priest 1980) at various temperatures (40-90°C). The reaction mixture contained 1 ml of partially purified enzyme, 1 ml of buffer (optimum pH), and 1 ml of 1% soluble starch. After incubation for 10 min. the reaction was arrested by adding 0.1 ml of IN HCl and cooled to 0°C in an ice bath. One ml of the sample was withdrawn and added with 0.1 ml of iodine solution and the enzyme activity was measured according to the assay procedures described earlier (Section 2.4.1.4.2).

The effect of temperature on stability of the enzyme was measured (Morgan & Priest 1980) by subjecting 1 ml of partially purified enzyme to different temperatures ranging from 40-90°C for 30 min. After incubation the enzyme was rapidly cooled and then it was incubated along with 1 ml of 1% soluble starch and 1 ml of appropriate buffer (optimum pH) at their optimum temperature for 10 min. The residual activity was measured by comparing their activity obtained at their optimum temperature and the test temperature.

2.4.3.4 Effect of substrate concentration on the activity of the enzyme

The effect of substrate concentration on the activity of the partially purified enzyme was checked by subjected the enzyme to different concentrations of substrate (soluble starch) dissolved in distilled water ranging from 0.5 to 5% at their optimum pH and temperature. One ml of the substrate solution was incubated with 1 ml of the partially purified enzyme and 1 ml of buffer (optimum pH) solution at their optimum temperature for 10 min. After arresting the reaction by the addition of 0.1 ml of IN HCl 1 ml of the sample was added with 0.1 ml of iodine solution (See section 2.4.1.4.2) and then the activity was compared with appropriate blank solution.

2.5 CONVERSION OF RICE AND RELATED PRODUCTS BY WHOLE CELLS

2.5.1 Media

Fermentation studies were carried out in two types of media namely Mineral Salts Rice Starch Broth (MSRSB) and the cooked rice decanted water, prepared as described below:

2.5.1.1 Pretreatment of media

1. Dried parboiled rice was initially ground and seived (100 nm mesh size) to remove the large grains. Then the powdered small grains were again dried in an over at 80°C to remove the moisture and stored in a cool dry place in a plastic container.

2. Rice decanted water was prepared by boiling about 200 gm of rice grains in 1000 ml distilled water at 121°C for 1 hour. The decanted supernatent obtained after boiling was appropriately diluted with distilled water for adjusting the starch concentration to 1% by measuring the absorbance of the blue colour developed by iodine reagent.

2.5.1.2 Preparation of media for fermentation

Mineral Salts Rice Starch Broth (MSRSB) was prepared by supplementing with 1% powdered parboiled rice as described earlier (See section 2.2.3.2.1). The pH of the broth was pH 7.0 ± 0.2 after autoclaving.

The Rice Decanted Water (RDW) was appropriately diluted with distilled water, adjusted to a pH 7.0 ± 0.2, autoclaved and used for fermentation studies.

2.5.2 Inoculum preparation

The inoculum preparation for fermentation studies was carried out following the same procedures mentioned in section (2.4.1.2).

2.5.3 Inoculation procedure

Prepared inoculum ($1.2x10^6$ cells/ml) was directly added to the prepared medium under aseptic conditions. Fifteen ml of the inoculum was added to 1.5 litre medium to give a final concentration of 1% of cells in the medium.

2.5.4 Fermentation conditions

A working volume of 1.5 litre MSRSB/Rice Decanted Water (RDW), was used for fermentation. The fermentation was carried out in a Eyela mini fermentor (Model M-100, sterilizable at 121°C for 20 min. with the media) at pH 7.0 ± 0.2 at 30°C (unless otherwise stated) under continuous aeration (flow rate of 1 vvm. unless otherwise stated) at an agitation speed of 150 rpm for a total period of 24 hours.

2.5.5 Sampling procedure

Samples from the fermentor were withdrawn, in 10 ml aliquots at 4 hrs intervals, through the sampling pipe for further biochemical analysis during fermentation.

2.5.6 Product analysis

The residual starch, cell protein (growth), reducing sugar, total sugar, enzyme activity and pH were measured according to the procedures described earlier under sections 2.2.3.2.3 to 2.2.3.2.7, 2.3.1.5 and 2.4.1.4.2.

Dextrose Equivalent (DE) was calculated from the data obtained for reducing sugar and total sugar using the following formula: (Hyun & Zeikus, 1985).

$$DE = \frac{\text{Reducing sugar (mg/ml)}}{\text{Total sugar (mg/ml)}} \times 100$$

2.5.7 Effect of pH on fermentation of rice starch

The effect of pH on the fermentation of rice starch was carried out in the fermentor at pH 7.0 and 9.0 (pH selected based on the results of growth studies described in the

previous section 2.3.1). The media pH was adjusted to desired levels with IN NaOH and IN HCl. The fermentation was carried out for 24 hrs at 30°C at an aeration rate of 1 vvm with an agitation speed of 150 rpm. At 4 hrs intervals, samples were withdrawn and analyzed for all biochemical factors as mentioned earlier (See sections 2.2.3.2.3 to 2.2.3.2.7, 2.3.1.5 and 2.4.1.4.2).

2.5.8 Effect of temperature on fermentation of rice

The effect of temperature on fermentation of rice was carried out in the fermentor at 30 and 35°C (temperature selected based on the results of growth studies described in the previous section (2.3.1)). Fermentation was carried out at pH 7 with an aeration rate of 1 vvm and with an agitation speed of 150 rpm for 24 hrs. At 4 hrs intervals samples were withdrawn and analyzed for all biochemical factors as mentioned earlier (See sections 2.2.3.2.3 to 2.2.3.2.7, 2.3.1.5 and 2.4.1.4.2).

2.5.9 Effect of aeration on fermentation

The aeration effect on fermentation of rice starch was carried out at two aeration rates 1 vvm and 2 vvm

(also by intermittent arresting of aeration) using an air flow meter by supplying external air via aerator after filtration. The fermentation was carried out for 24 hrs at 30°C at pH 7.0. Samples were withdrawn at 4 hrs intervals and all the biochemical analysis were carried out as per the procedures described in earlier sections (2.2.3.2.3 to 2.2.3.2.7, 2.3.1.5 and 2.4.1.4.2).

2.6 CONVERSION OF RICE STARCH BY FREE ENZYME

2.6.1 Production of alpha amylase

2.6.1.1 Enzyme production media

The composition of the enzyme production media, described in section (2.4.1.1) was used for the production of amylases.

2.6.1.2 Enzyme production procedures

The physico-chemical conditions for the production of amylase by the Bacillus sp., were carried out following the procedures described in section (2.4.1.3).

2.6.1.3 Separation and purification of enzyme

The extracellular amylases produced by the organisms were recovered by centrifugation and removal

of cells, followed by $(NH_4)_2SO_4$ fractionation and extensive dialysis. Details are given in section (2.4.2).

2.6.2 Liquifaction and saccharification of rice starch by alpha amylase

The partially purified alpha amylase was used for the free enzyme liquifaction and saccharification of rice starch at different temperatures and at various incubation periods (Brookes & Griffin 1989).

2.6.2.1 Effect of temperatures on liquifaction and saccharification of rice starch with respect to incubation time

Five gm of powdered rice starch was gelatinized by heating at 100°C for, 30 min. in 50 ml of distilled water in a 100 ml beaker. The cold gelatinized substrate was adjusted to the pH 7,8 or 9 by the addition of 5 ml of appropriate buffer (phosphate buffer, pH 7,8 or glycine-NaOH buffer, pH 9). To this 5 ml of partially purified amylase was added, sealed with aluminium foil and then incubated at different temperatures ranging from 50°C to 90°C in a water bath shaker. After 30 min. and 60 min. of incubation, samples were withdrawn in 5 ml aliquots, and the reaction was arrested by

the addition of 1 ml of IN HCl. The contents were immediately centrifuged at 5000 rpm at 4°C for 10 min. and the supernatent was analyzed for residual starch, reducing sugar and total sugar according to the procedures described in sections 2.2.3.2.5 to 2.2.3.2.7.

The liquifaction was observed visually and by checking the percentage conversion of rice starch into sugars while saccharification was monitored by calculating the amount of reducing sugar produced in the reaction.

2.7 CONVERSION OF RICE STARCH BY IMMOBILIZED CELLS AND ENZYMES

2.7.1 Immobilization of cells and enzymes

Immobilization of whole cells of bacteria and enzymes were carried out employing entrapment techniques using calcium alginate (Chibata et al., 1974).

2.7.1.1 Preparation of whole cells and enzymes

The whole cells for immobilization were prepared as follows:

1. Eighteen hour old slope cultures were first grown in 50 ml of enzyme production media (composition of medium

presented in section 2.4.1.1) at room temperature (28 ± 2°C) for 18 hrs in a rotary shaker (150 rpm).

2. Ten ml of the culture broth obtained was then subcultured in 100 ml of EPM in a 500 ml flask.

3. After 18 hrs of incubation the cells were harvested by centrifugation (at 5000 rpm, at 4°C) and washed three times with physiological saline and suspended finally in 50 ml of the same saline.

4. This cell suspension (50 ml containing $1x10^8$ cells/ml) was then transferred to 1000 ml of EPM in a 2.5 litre Eyela mini fermentor and the cells were grown at 30-35°C (Varied for each strain studied) for 18 hrs.

5. The cells were later harvested by centrifugation at 5000 rpm at 4°C, washed repeatedly and suspended in physiological saline.

6. The prepared cell suspension was used for immobilization.

7. The cell free extract obtained during the preparation of cells was partially purified by $(NH_4)_2SO_4$ fractionation and extensive dialysis (details outlined in section 2.4.2). The prepared amylase was used for immobilization studies.

2.7.1.2 Immobilization of whole cells

1. Hundred ml of sodium alginate solution (4% w/v) was prepared by slow addition of the dry powder to the water while being stirred. The stirring was continued for a further period of one hour and warmed at 60°C to ensure complete dissolution. The solution was then left to stand for about an hour to allow the air bubbles to escape.

2. Under sterile conditions 20 ml of the prepared cell slurry was mixed with sodium aliginate solution gently together.

3. This sodium alginate-cell slurry mixture was then extruded dropwise through 10 ml syringe from a height of about 10 cms into an excess of 0.2 M $CaCl_2$ solution.

4. Beads of calcium alginate entrapped cells (mean diameter of 3 mm) were maintained in the $CaCl_2$ solution to be hardened for 30 minutes.

5. After washing with physiological saline, three times, the beads were used for further studies.

2.7.1.3 Immobilization of enzymes

Amylases prepared from Bacillus coagulans (ACMN-1), Bacillus polymyxa (ACMN-25), Bacillus cereus (ACMN-33) and Bacillus coagulans (ACMN-42) were immobilized on aliginate beads separately and used for rice starch conversion studies.

1. The sodium alginate solution prepared as mentioned in the previous section (2.7.1.2.1) was mixed thoroughly with 5 ml of 5% Gluteraldehyde (Ferak) solution prepared in phosphate buffer pH 7.

2. Twenty ml of the partially purified amylase from Bacillus sp with an activity of 30-40 u/ml was added separately to the sodium-alginate-gluteraldehyde mixture and mixed thoroughly.

3. The final mixtures were then extruded dropwise through a 10 ml syringe from a height of about 10 cm into an excess of $CaCl_2$ (0.2 M) solution (1 litre).

4. The beads (mean diameter 3 mm) were allowed to harden by leaving them as such in $CaCl_2$ solution for 30 minutes.

5. The beads prepared using bacterial amylases were later washed with their respective optimum pH buffer (phosphate buffer pH 7-8 or glycine-NaOH buffer pH 9) and suspended in the same buffer.

The prepared beads were then stored under refrigeration for further studies.

2.7.1.4 Activation of immobilized viable cell (IVC) beads

About 30 gm (wet wt) of the prepared IVC beads were suspended in 100 ml of enzyme production medium in 1 litre conical flask and incubated at room temperature (28 ± 2°C) for 24 hrs. The activated beads were then removed and washed with fresh sterile saline solution and used for further studies.

2.7.1.5 Assay of amylase activity of immobilized cells

The amylase activity of the activated immobilized cells were tested according to Sen and Chakravarthy (1987). Hundred mg (wet wt) of activated beads were incubated with 1.5 ml of 1% soluble starch and 1.5 ml of their respective optimum pH buffer (phosphate buffer, pH 7-8 (0.2 M) or glycine-NaOH buffer, pH 9 (0.2 M)) for enzyme activity and incubated for 10 min. at 40-50°C (respective of the organism). The reaction was arrested by the addition of 0.1 ml of IN HCL and the decanted supernatent was tested for amylase activity as per the procedure detailed in section 2.4.1.4.2.

2.7.1.6 Measurement of growth of cells

The cell growth inside the immobilized beads were estimated as cell protein according to Jones et al., (1984) with some modification.

About 500 mg (wet wt) of immobilized cells beads were suspended in 3 ml of phosphate buffer of pH 8. The beads got dissolved in at this pH after 2 hrs of incubation. The liberated cells were extracted with 1 ml of 3N NaOH. After removing the precipitated alginate by centrifugation, the protein dissolved in supernatent was quantified by Folin Ciocalteau method (Lowry et al., 1951). Absorbance was read at 750 nm in a UV visible spectrophotometer (Hitachi Model 200).

2.7.2 Rice starch conversion by immobilized cells

2.7.2.1 Effect of pH on rice starch conversion by immobilized cells

The effect of pH on the conversion of rice starch by immobilized cells was studied in MSRSB medium adjusted to various pH levels ranging from pH 4 to 11 according to Shinmyo et al., (1982). Fifty ml of MSRS broth was prepared

in a series of 250 ml Erlnmeyer flask and adjusted to pH levels from pH 4 to 11 using IN HCl or IN NaOH and sterilized by autoclaving. One gm (wet wt) of activated immobilized cells were added in each flask and incubated at room temperature in a rotary shaker (28 ± 2°C) under mild shaking (50 rpm) for 24 hrs. After incubation the contents were centrifuged (5000 rpm) for 20 minutes and the clear supernatents obtained were used for analysis of starch, total sugar, reducing sugar, growth and enzyme activity according to the methods described in section 2.2.3.2.4 to 2.2.3.2.7 2.7.1.5 and 2.7.1.6.

2.7.2.2 Effect of temperature on rice starch conversion by immobilized cells

Effect of temperature on rice starch conversion by immobilized cells was studied by incubating 1 gm (wet wt) of immobilized cells at different temperatures (30-50°C) (Shimnyo et al., 1982) in 50 ml of MSRS broth for 24 hrs in a rotary shaker (50 rpm). After 24 hrs of incubation the residual starch, reducing sugar, total sugar and enzyme activity were estimated (See section 2.2.3.2.4 to 2.2.3.2.7 and 2.7.1.5 and 2.7.1.6.

2.7.2.3 Effect of retention time on rice starch conversion by immobilized cells

The optimum retention time required for maximum conversion of rice starch by immobilized cells were determined by the following procedure.

Thirty gm (wet wt) of activated immobilized cell beads were packed in a column (12x4.7 cm) washed with physiological saline and filled with MSRS broth and incubated at 30°C for 24 hrs. The void volume of the column was 60 ml, which was calculated according to Sonal Vora (1989). At periodical intervals of 6 hrs, 5 ml of sample were withdrawn and analyzed for residual starch reducing sugar, total sugar and enzyme activity after centrifuging the sample at 5000 rpm (Section 2.2.3.2.4 to 2.2.3.2.7, 2.7.1.5 and 2.7.1.6.)

2.7.2.4 Reusability of the immobilized cells

Reusability of the IVC packed column was studied in batch process for the rice starch conversion for a four cycles. Each cycle had a retention time of 24 hrs, (which was found to be the optimum retention time). After the conversion fo rice starch, the eluted samples were analyzed

residual starch, reducing sugar, total sugar, enzyme activity and growth as outlined earlier (Sections 2.2.3.2.4 to 2.2.3.2.7, 2.7.1.5 and 2.7.1.6).

2.7.3 Conversion of rice starch by immobilized enzymes

2.7.3.1 Activity of immobilized enzymes

The activity of the immobilized amylase enzymes was initially checked according to the procedure described in section 2.7.1.5.

2.7.3.2 Effect of pH on rice starch conversion by immobilized enzymes

The effect of pH on the rice starch conversion by immobilized enzymes as studied according to Sen and Chakrabarty (1987) by incubating 100 mg (wet wt) of immobilized beads with 1.5 ml of 1% rice starch and 1.5 ml of buffers of varying pH ranging from 4 to 11 for 10 minutes at their respective optimum temperature (40-50°C). After 10 minutes, the reaction was arrested by the addition of 0.1 ml of IN HCl. Then the reaction mixture was decanted immediately centrifuged at 4°C at 5000 rpm for 10 minutes to

remove the soiled particles. The clear supernatent was then used for the estimation of residual starch, reducing sugar, total sugar (See section 2.2.3.2.5 to 2.2.3.2.7).

2.7.3.3 Effect of temperature on conversion of rice starch by immobilized enzyme

The effect of temperature as rice starch conversion by immobilized amylase was studied according to (Sen and Chakrabarty (1987) by incubating 100 mg of immobilized beads with 1.5 ml of 1% rice starch and 1.5 ml of 1% rice starch and 1.5 ml of their respective optimum pH buffer (phosphate buffer pH 7 or glycine-NaOH buffer pH 9 at different temperatures, ranging from 40 to 70°C for 10 minutes. After 10 minutes, the reaction mixture was cooled on an ice bath and the decanted supernatent was centrifuged at 5000 rpm for 10 minutes at 4°C o remove the solid particles and the clear supernatent was used for estimating residual starch, reducing sugar and total sugar (See section 2.2.3.2.5 to 2.2.3.2.7).

2.8 STATISTICAL ANALYSIS

2.8.1 Variables

The variables used for statistical analysis included incubation time, pH, temperature, residual starch, reducing sugar, total sugar, DE, enzyme activity and growth.

2.8.2 Correlation coefficient analysis

To test whether the numerical differences of the above said variables were significant or not a Pearson correlation coefficient 'r' was calculated using the formula

$$r = \frac{n \lesssim xy - (\lesssim X)(\lesssim Y)}{\sqrt{[n \lesssim X^2 - (\lesssim x)^2]\,[n \lesssim Y^2 - (\lesssim y)^2]}}$$

Calculations were done using a micro computer (HCL) and the final outputs are presented in the form of tables.

3. RESULTS

3.1 Screening of amyloytic *Bacillus*

Amylolytic *Bacillus* sp. (62 in number) isolated from various samples, as a part of a sponsored research project entitled "Isolation and characterization of amylolytic bacteria for their possible use in Biotechnology", and maintained in the laboratory were subjected to primary screening procedures for the further selection of best strains to be used in the present study.

3.1.1 Primary screening and selection

The primary screening of the *Bacillus* sp. included a qualitative test of detecting the ability to utilize rice starch as sole carbon source. All the 62 strains of *Bacillus* were subjected to this test and the size of the clear zones produced around their colonies in the mineral medium substituted with rice starch as carbon source was measured. In general the clear zones varied from 5 mm to 45 mm in width (radius). From this lot 20 strains whose clear zones were well above 15 mm (radius) were selected for secondary screening.

3.1.2 Secondary screening

Secondary screening of the suitable strains included quantitative determination of growth, enzyme production and rice starch conversion in a mineral salts broth containing rice as a carbon source. Results obtained for the various analysis of cell protein (growth), enzyme activity, total sugar, reducing sugar, residual rice starch and percentage of rice starch conversion for all the 20 strains are presented in Table 3.

Growth of bacteria estimated in terms of cell protein (mg/ml) varied from 0.31 to 0.73 mg/ml for the 20 strains. Amylase activities in general ranged from 3 u/ml to 23 u/ml for many strains excepting the few strains which did not even produced a detectable level of amylase. While total sugar varied from 0.22 to 0.61 mg/ml, the reducing sugar was in the range of 0.09 to 0.631 mg/ml. Residual starch and percentage of starch conversion varied from 0.92 to 9.7 mg/ml and from 3% to 89% respectively. Data obtained for the various parameters analysed were individually ranked from 1 to 20, considering the values obtained for each strain, in the order of decreasing trend. After assigning the respective

ranks for each strain, with reference to each parameter, average of ranks were computed and considered for selection of best strains. Accordingly four strains which ranked 1 to 4 on the average basis were selected for further study. The four strains were Bacillus sp. ACMN 1, Bacillus sp. ACMN 25, Bacillus sp. ACMN 33 and Bacillus sp. ACMN 42. All these strains were isolated from soil samples.

3.2 IDENTIFICATION OF THE SELECTED STRAINS

The four strains of Bacillus sp. selected after secondary screening were identified to their various species after studying their morphological, physiological and biochemical properties (Fig.1 and Table 4). Two strains were identified as species of Bacillus coagulans ACMN 1 and ACMN 42. While the other two were Bacillus polymyxa ACMN 25 and Bacillus cereus ACMN 33.

3.3 GROWTH STUDIES

3.3.1 Effect of pH

The effect of pH on growth and enzyme production of the selected strains of Bacillus were carried out by subjecting them to various levels of pH (pH 4-11) in a mineral salts

rice starch broth. The results are expressed in terms of cell protein (growth estimated as cell protein) and enzyme activity (u/ml)(Fig.2).

In general all the strains grew well and produced significant levels of amylase at pH 7 to 10 (Fig.2) while they could not grow well and produce amylase at pH 4-5 except B. cereus ACMN 33 which showed a moderate amount of growth at these pH levels (0.101 to 0.112 mg/ml cell protein).

At pH 6, except B. coagulans ACMN 1 all the other strains recorded only a meagre amount of amylase production (4-7 u/ml).

Among the two B. coagulans sp. while the strain B.coagulans ACMN 1 could grow and produce amylase to a maximum level at pH 9 (0.57 mg/ml cell protein and 35 u/ml enzyme activity), the other strain of B. coagulans ACMN 42 could do the same only at pH 8 (0.57 mg/ml cell protein and 37 u/ml enzyme activity). However, both the strains recorded significant level of growth (0.36-0.57 mg/ml for B. coagulans ACMN 1 & 0.16-0.57 mg/ml for B.coagulans ACMN 42) and enzyme production (23-35 u/ml for B. coagulans ACMN 1 & 17-37 u/ml for B. coagulans ACMN 42 at pH levels of 7-11.

B. polymyxa ACMN 25 preferred a pH of 8 and 9 for maximal growth (0.51 mg/ml cell protein) and enzyme production (39 u/ml) respectively. However, they demonstrated significant levels of growth and enzyme production at other pH levels 6-10 (0.21-0.45 mg/ml cell protein and 7-39 u/ml enzyme activity).

Whereas, unlike other strains, B. cereus preferred pH 7.0 for their maximal growth (0.791 mg/ml cell protein) and amylase production (27 u/ml) while recording significant levels of growth and enzyme production at other pH levels of pH 8, 9 and 10 (0.201 to 0.581 mg/ml cell protein and 17-25 u/ml amylase activity).

3.3.2 Effect of temperature

Effect of temperature on growth and enzyme production was tested at different temperatures viz., 25, 30, 35, 45 and 55°C. Results are presented in the Fig.3. Suggest that all the four strains preferred an optimal temperature of 35°C for their maximal growth (0.62-0.81 mg/ml cell protein) and enzyme production (32-39 u/ml). However, they could record significant level of growth and enzyme production at 30°C

also. In general all the four strains could grow and produce enzymes at significant level, at other temperatures also ranging from 25-45°C (0.302-0.810 mg/ml cell protein and 9-39 u/ml enzyme activity).

Incubation at 55°C did not favour growth and amylase production. Of all the strains, B. polymyxa ACMN 25 produced higher amount of amylase (39 u/ml) than others (B. coagulans ACMN 1, 33 u/ml; B. cereus ACMN 33, 32 u/ml and B. coagulans ACMN 42, 42 u/ml).

3.3.3 Effect of NaCl concentration

The effect of NaCl concentration on growth and enzyme production of all the strains were tested at various levels of NaCl ranging from 0 to 7% in the growth medium (MSRS broth).

Data presented in the Fig.4 indicate that an increase in the concentration of NaCl above 0.5% resulted in a decrease in the amount of growth and enzyme production in a linear fashion. Specifically concentrations above 3% of NaCl did not favour appreciable amounts of growth and enzyme

production by all the strains. Both of the B. coagulans strains ACMN 1 and ACMN 42 grew well and produced appreciable level of enzymes at 0% NaCl (0.42-0.53 mg/ml cell protein and 27 u/ml enzyme activity respectively). While B. polymyxa ACMN 25 and B. cereus ACMN 33 required 0.5% NaCl for their maximal growth and amylase production (0.41 & 0.491 mg/ml cell protein and 29 & 31 u/ml enzyme activity respectively).

3.3.4 Effect of substrate concentration

Effect of substrate concentration on the growth and amylase production of Bacillus strains was tested at substrate concentration ranging from 0.5 to 5% and the results are presented in Fig.5. Although significant levels of growth and enzyme production was recorded at concentrations ranging from 0.5 to 4% for all the strains, maximal levels of growth and enzyme production occurred at 1% concentration for all the strains (0.43 to 0.601 mg/ml cell protein and 20-34 u/ml enzyme activity). Increase in the concentration of substrate from 1 to 4% did not influence the rate of growth and enzyme production by the strains. In general the amylase activity obtained for the various strains varied from 8 to 14 u/ml for B. coagulans ACMN 1, 20-34 u/ml for

B. coagulans ACMN 42, 16-30 u/ml for B. polymyxa ACMN 25 and 18-33 u/ml for B. cereus ACMN 33. Five per cent concentration inhibited growth and amylase production of all strains.

3.3.5 Effect of incubation period

Effect of incubation period on growth and enzyme production by all the four strains was tested in the medium at their optimal conditions of growth and enzyme production for a total period of 48 hours.

Results presented in Fig.6 indicate that, in general, all the strains recorded a period of 24 hrs for maximal growth and amylase production though significant levels of growth and enzyme production could be obtained from 12 hrs onwards. Further incubation after 24 hrs to 48 hrs however did not influence the rate of enzyme production of the strains.

While B. coagulans ACMN 1 and B. polymyxa ACMN 25 could produce maximal levels of amylase at 18 hrs (36 u/ml and 40 u/ml respectively), B. cereus ACMN 33 and B. coagulans ACMN 42 required 24 hrs, (29 & 32 u/ml respectively).

Relatively B. polymyxa ACMN 25 recorded higher levels of growth (2.31 mg/ml cell protein) followed by B. coagulans ACMN 1 (1.47 mg/ml), B. cereus ACMN 33 (1.00 mg/ml) at B. coagulans ACMN 42 (1.531 mg/ml).

All the strains interestingly recorded a declining trend of pH in a linear fashion in their medium during incubation for 48 hrs. The decline in the pH ranged from pH 9.0-6.5; 9.0-6.38; 7.0-4.95; 8.0-4.98 for the four species B. coagulans ACMN 1, B. polymyxa ACMN 25, B. cereus ACMN 33 and B. coagulans ACMN 42 respectively.

3.3.6 Effect of inoculum concentration

The effect of inoculum concentration on growth and amylase production of the four strains was tested by inoculating the medium with four different concentrations of 1, 3, 5 and 7% and the results are presented in Fig.7.

Both the strains of Bacillus coagulans could produce good growth and enzyme production at all the concentrations of inoculum tested. B. polymyxa ACMN 25 showed maximal enzyme production and growth at concentrations ranging from 3 to 7%. Whereas B. cereus ACMN 33 recorded

maximal enzyme production at 1-5% inoculum concentration with a maximum at 3%. In general, it could be noted that inoculum concentration of 1% is sufficient for obtaining significant levels of growth and enzyme for all the strains although 1% for both B. coagulans and 3% for B. polymyxa ACMN 25 and B. cereus shall be their optimal concentration.

3.3.7 Effect of carbon sources

The effect of carbon sources on growth and enzyme production was tested by incorporating various carbon sources (1%) in the media. Results presented in Table 5 indicate that all the carbon sources favoured significant levels of growth and enzyme production by all the strains.

Glucose was preferred for amylase production by B. coagulans ACMN 42 (42 u/ml) followed by B. cereus ACMN 33 (38 u/ml), B. polymyxa ACMN 25 (19 u/ml) and B. coagulans ACMN 1 (11 u/ml). However, B. cereus ACMN 33 recorded more growth (1.93 mg/ml cell protein) when compared to others (0.91-1.38 mg/ml cell protein) with glucose as substrate.

Maltose, similar to that of glucose, influenced maximal enzyme production by B. coagulans ACMN 42 (58 u/ml)

followed by B. cereus ACMN 33 (53 u/ml). B. coagulans ACMN 1 (14 u/ml) and Bacillus polymyxa ACMN 25 (19 u/ml) produced lesser amounts of amylase when compared to others using maltose as carbon source.

B. coagulans ACMN 42 followed by B. polymyxa ACMN 25 could utilize dextrin for maximal enzyme production in an appreciable level of 62 u/ml and 46 u/ml respectively.

Soluble starch also favoured maximal enzyme production by B. coagulans ACMN 42 followed by B. cereus ACMN 33, B. polymyxa ACMN 25 and B. coagulans ACMN 1 (62, 49, 48 and 25 u/ml amylase activity respectively for each strains).

While lactose favoured maximal enzyme production for B. coagulans ACMN 42 (53 u/ml), B. cereus ACMN 33 (51 u/ml) and B. polymyxa ACMN 25 (38 u/ml), it did not induce higher levels of enzyme production by B. coagulans ACMN 1 (13 u/ml).

Similar to other substrates gylcerol also favoured maximal enzyme production by B. coagulans ACMN 42 (50 u/ml)

B. cereus ACMN 33 (47 u/ml), B. polymyxa ACMN 25 (42 u/ml) and B. coagulans ACMN 1 (15 u/ml).

An overall assessment of the data suggest that only B. coagulans ACMN 42 could utilize all the carbon sources tested efficiently for maximal enzyme production followed by B. cereus ACMN 33.

All the four strains could significantly reduce the pH (ranging from pH 4.54 to 6.58) of the medium when they utilized all the carbon sources tested.

Among the carbon sources tested soluble starch followed by dextrin and glycerol could effect maximal enzyme production by B. coagulans ACMN 1 and B. polymyxa ACMN 25 whereas maltose followed by lactose, starch and glycerol could influence maximal enzyme production by B. cereus ACMN 33.

In case of B. coagulans ACMN 42, both starch and dextrin followed by maltose, lactose, glucose and glycerol could influence maximal amylase activity. In general, soluble starch and related sugars could effect more amylase production than other carbon sources.

3.3.8 Effect of nitrogen sources

Effect of nitrogen sources on the growth and enzyme production was tested by incorporating various nitrogen sources in the growth media and the results are shown in Table 6.

Among the nitrogen sources tested, peptone, beef extract and tryptone alone showed some inducing effect in maximal enzyme production (7 to 44 u/ml) by the strains tested. Whereas yeast extract, $NaNO_3$, KNO_3 and $(NH_4)_2SO_4$ could not effect even a meagre amount of enzyme production by the strains.

Amylase production varied from 27 to 44 u/ml and growth from 1.1 to 1.51 mg/ml for the four strains with reference to peptone as a nitrogen source. Among the four strains, while maximal growth was shown by B. polymyxa ACMN 25 (2.25 mg/ml), maximum enzyme production (44 u/ml) was recorded by B. coagulans ACMN 42.

B. cereus ACMN 33 and B. polymyxa ACMN 25 were favoured by beef extract for maximal growth (1.58 mg/ml and 1.90 mg/ml cell protein) respectively and enzyme production

(22 and 21 u/ml respectively). Whereas the other two strains recorded only moderate amount of growth and enzyme production using this source.

Although yeast extract induced moderate levels of growth by all strains only B. coagulans ACMN 1, could record 10 u/ml enzyme production. While amylase production was not detected for other strains.

Tryptone favoured maximal growth for B. coagulans ACMN 1 (1.93 mg/ml cell protein) and B. polymyxa ACMN 25 (1.87 mg/ml cell protein) while inducing maximal enzyme production by B. cereus ACMN 33 (21 u/ml) at B. coagulans ACMN 42 (19 u/ml).

While $NaNO_3$, KNO_3 and $(NH_4)_2SO_4$ did not encourage fair growth of all the four organisms and enzyme production, B. coagulans ACMN 1 showed some response to these three inorganic nitrogen sources in their amylase production by recording 4, 5 and 3 u/ml respectively for the inorganic nitrogen sources tested. B. cereus ACMN 33 also showed minimum levels of enzyme production (3 u/ml) in response to KNO_3 and $(NH_4)_2SO_4$.

In general, peptone followed by beef extract and tryptone favoured maximal growth and enzyme production by all the four strains when compared to other nitrogen sources tested. Of all the four strains B. cereus ACMN 33 could produce maximal level of growth and enzyme utilizing the three major nitrogen sources.

3.3.9 Growth curve

Growth curves obtained for all the four strains of Bacillus sp. are presented in Figures 8-11. Uniformly all the four strains exhibited a similar pattern of growth curve with marginal differences. All of them spent only 4 hrs in logarithmic phase. After 4 hrs logarithmic phase was observed for all the strains in a similar fashion as it is evidenced from the figures. The logarithmic phase was continued till 12 hrs for all the strains except B. polymyxa which remained in the late logarithmic phase till 18 hrs. Stationary phase for all the strains was found to prolong even after 24 hrs. However, after 30 hrs, all the strains entered the decline phase.

The generation time for the four strains was recorded as 57.75, 37.8, 48.0 and 39.0 minutes respectively

for B. coagulans ACMN 1, B. coagulans ACMN 42, B. polymyxa ACMN 25 and B. cereus ACMN 33.

3.4 PURIFICATION AND RECOVERY OF AMYLASES OF BACILLUS STRAINS

Amylases prepared from the Bacillus strains were partially purified and recovered after $(NH_4)_2SO_4$ precipitation and dialysis. Results obtained for the purification steps with reference to total activity, specific activity and yields are presented in Table 7.

Specific activity of the amylases was observed to increase at each step of purification and after dialysis, a specific activity of 52.7, 62.5, and 60.9 at 41.2 u/mg of protein for B. coagulans ACMN 1, B. polymyxa ACMN 25 B. cereus ACMN 33 and B. coagulans ACMN 42 were recorded respectively. B. polymyxa ACMN 25 and B. cereus ACMN 33 could record higher specific activities than the two B. coagulans strains. Among the two B. coagulans strains, B. coagulans ACMN 42 showed relatively lesser specific activity. Of the four strains, B. cereus ACMN 33 could record 71.4% yield after dialysis followed by B. coagulans ACMN 42 (62.2%); B. coagulans ACMN 1 (54.5%) and B. polymyxa ACMN 25 (45.9%).

3.5 IDENTIFICATION OF AMYLASES

The types of amylase produced by the four strains were identified based on the dextrinizing and saccharifying activities of the enzymes using starch as substrate (Fig.12). All the four strains could record higher dextrinizing activity when compared to the saccharolytic activity. The dextrinizing activity varied from 30 to 65 u/ml for the four strains while the saccharolytic activity varied from 4 to 12 u/ml at the end of 10 minutes of reaction. These results indicate that the enzymes are α-amylases.

3.6 EFFECT OF pH, TEMPERATURE AND SUBSTRATE CONCENTRATION ON THE ACTIVITY AND STABILITY OF ENZYME

3.6.1 Effect of pH

The effect of pH on the activity and stability of the partially purified amylase prepared from the strains were tested by subjecting them to various pH levels ranging from 4 to 11. Data presented in Fig.13 indicate that enzymes of all the four strains were active and stable at pH 9 except B. cereus ACMN 33 which preferred pH 7.

In general, enzymes of all the four strains were able to show some activity at pH levels of 5-10. The results

indicate further that the α-amylase were alkaline amylases, since they could record significant levels of activity and stability at alkaline pH levels than at acidic pH levels. Amylases of B. coagulans ACMN 1 (88 u/ml) and ACMN 42 (47 u/ml) and B. polymyxa ACMN 25 (95 u/ml) were more active and stable at pH 9 than those of B. cereus ACMN 33 (47 u/ml) which was stable at pH 7.

3.6.2 Effect of temperature on activity and stability of amylases

The effect of temperature on the activity and stability of the amylases was tested by subjecting them to various temperatures ranging from 40 to 90°C.

From the results (Fig.14) it is seen that B. polymyxa ACMN 25 followed by B. coagulans ACMN 1 produce thermostable α-amylases. The enzymes of these strains recorded significant levels of activity and stability at 80°C and moderate amounts of activity at 90°C. However, the maximal activity and stability of the enzymes of both strains were recorded only at 50°C. Amylase of B. polymyxa showed a better thermostability than that of B. coagulans ACMN 1.

Whereas, B. cereus ACMN 33 and B. coagulans ACMN 42 amylases recorded a declining trend of activity and stability along with an increase in the incubation temperature from 40 to 80°C. While amylase of B. cereus ACMN 33 lost stability and activity at 80°C, enzymes of B. coagulans ACMN 42 lost their ability only at 90°C.

3.6.3 Effect of substrate concentration on activity of amylases

The effect of substrate concentration on the activity of amylases of the four strains were tested by subjecting the partially purified amylase preparation to various levels of substrate concentrations ranging from 0.5% to 5% and the results are presented in Fig.15.

Amylase of B. coagulans ACMN 1 showed maximal activity at 1% concentration and recorded a steady state of activity with further raise in the substrate concentration. All the other strains such as B. polymyxa ACMN 25, B. cereus ACMN 33 and B. coagulans ACMN 42 recorded maximal activity only at 3% concentration and then a steady state of activity at concentration above 3%.

3.6.4 Effect of heavy metal on the activity of amylases

The effect of heavy metal ions on the activity of amylases of the four strains of Bacillus were tested on various heavy metals like Ag, Hg, Cu, Ca, Mn and Fe at 100 ppm level in the reaction mixture and the results are shown in Table 8.

Among the heavy metals tested while calcium did not inhibit the activity of the amylase, Iron was found to inhibit the activity of enzymes of all the strains (100% inhibition) except B. cereus ACMN 3 which lost 54.3% activity.

Hg followed by Ag brought about significant levels of inhibition of the enzymes only to Iron. Whereas Cu and Mn did not inhibit enzyme activity at appreciable level.

Amylases of B. coagulans ACMN 1 was completely inhibited by Fe. Among the other metals, while Hg could effect 43.3% loss of activity, Mn and Hg did not inhibit the amylase activity significantly. Cu and Ca did not inhibit the activity of this enzyme.

Amylase of B. polymyxa ACMN 25 also demonstrated a similar pattern of inhibition, that was shown by B. coagulans ACMN 1. Of all the metals only Fe effected a total inhibition of enzyme action when compared to the levels of inhibition by Hg, Mn and Ag. These enzymes were also not inhibited by Cu and Ca as those of other strains.

Amylases of B. cereus ACMN 33 showed different pattern of response to the heavy metals when compared with the other three strains. Hg followed by Ag, Fe, Cu and Mn effected loss of activity ranging from 67.3% to 45.5% whereas Ca did not inhibit enzyme action.

Enzymes of B. coagulans ACMN 42 was totally inhibited by Fe and not inhibited by Mn and Ca. While Hg could effect 64.8% loss of activity, Ag and Cu could not bring about any significant loss of activity.

3.7 CONVERSION OF RICE STARCH BY FREE CELL OF BACILLUS sp

Fermentation of rice starch in Mineral Salts Rice Starch Broth (MSRSB) and in Cooked Rice Decanted Water (CRDW) was carried out employing B. coagulans ACMN 1 and ACMN 42

strains, B. polymyxa ACMN 25 and B. cereus ACMN 33 at different pH (7 and 9), temperature (30-35°C), aeration rate (1 vvm to 2 vvm) and at constant agitation speed of 150 rpm in an Eyela fermentor (2.5 liter capacity). The fermented broths were periodically analyzed at 4 hrs interval for a total period of 24 hrs for various biochemical parameters which included residual rice starch, reducing sugar, total sugar, DE (Dextrose Equivalents), growth (cell protein) and enzyme production (enzyme activity).

3.7.1 Fermentation of rice starch with B. coagulans ACMN 1

(a) Fermentation at pH 7; 30°C; 1 vvm of aeration

Results presented in the Fig.16 indicate that rapid reduction of rice starch was effected by the bacteria along with increase in incubation period. More than 85% of the initial starch (10 mg/ml) content was converted by 12 hrs itself (1.21 mg/ml of residual starch). The data obtained for all the parameters such as reducing sugar (3.82 mg/ml), total sugar (41.5 mg/ml), DE (9.2) cell protein (5.83 mg/ml), enzyme production (23 u/ml) and pH (6.59) evidences significant levels of fermentation of rice starch by the organism by 12 hrs itself. Further incubation till 24 hrs did not

enhance the rate of fermentation which was observed till 12 hrs. The levels of DE (9.9) and enzyme production (26 u/ml) observed at 16 hrs suggest that rice starch has supported significant level of α-amylase production besides sugar production. The pH of the medium indicated a probable acid production in the medium by the organisms during fermentation.

(b) Fermentation at pH 9; 30°C and 1 vvm of aeration

Results presented in Fig.17 suggest that B. coagulans ACMN 1 could bring about conversion of rice starch 90% (1.0 mg/ml residual starch) after 16 hrs of incubation with an initial pH of 9. Maximum levels of DE (6.0) and enzyme production (32 u/ml) were observed only after 20 hrs of incubation. A reduction of initial pH 9 to pH 7.5 by 16 hrs suggest the probable production of an acid which neutralized the alkaline conditions in the media. In general, the level of reducing sugar and DE observed at pH 9 was lesser than that observed at pH 7.

(c) Fermentation at pH 7; 35°C and 1 vvm of aeration

B. coagulans ACMN 1 did not show any significant difference in the pattern of starch reduction (Fig.18), when

the incubation temperature was alone changed to 35°C while maintaining all other conditions constant. Although about 80% of the starch reduction (2 mg/ml) residual starch) was observed at 12 hrs the level of DE (2.4) was not high. However, significant level of enzyme production (28 u/ml) was observed at 12 hrs.

In general, significant levels of reduction in starch (94.8%) and enzyme production (33 u/ml) were observed at 16 hrs. Further incubation after 16 hrs did not make any impact on the rate of fermentation.

The reduction in the pH of the medium after fermentation was insignificant at this temperature.

(d) Fermentation at pH 7;30°C and aeration rate of 2 vvm

Data presented in Fig.19 clearly evidences the effect of enhanced aeration on the fermentation of rice starch by B. coagulans ACMN 1. More than 86% of starch reduction (1.4 mg/ml residual starch) along with a DE of 10.98 and an enzyme production of 13 u/ml could be recorded at 4 hrs of incubation when rate of aeration was raised from 1 vvm to 2 vvm. Further incubation till 24 hrs brought about

only a marginal increase in the levels of the parameters. The maximal level of enzyme production (37 u/ml) was observed at 16 hrs and changes in the pH were insignificant. A maximum of 97% rice starch conversion was recorded at the said fermentation conditions.

(e) Fermentation at pH 7; 30°C; aeration rate of 2 vvm and with intermittent arresting of aeration after 12 hrs

At the above said conditions of fermentation 87% of rice starch reduction, with DE of 8.3 and 13 u/ml of enzyme production were observed at 4 hrs (Fig.20). At 12 hrs the percentage of conversion was 92.7% and the levels of DE, reducing sugar and enzyme production were 8.97, 5.2 mg/ml and 13 u/ml respectively. Whereas when the aeration was arrested at this hour and incubation continued till 24 hrs rapid changes in the level of reducing sugar (3.11 mg/ml), DE (6.01) enzyme production (27 u/ml) and pH (4.95) were observed.

These observations indicate rapid acid production in the medium in concordant with utilization of reducing sugar produced in the medium by the cells.

(f) Fermentation of cooked rice decanted water at pH 7; 30°C and aeration rate of 1 vvm

Results presented in Fig.21 indicate that only after 12 hrs of incubation significant levels of starch reduction (88%) along with reducing sugar, (5.21 mg/ml), DE (8.0) and maximal enzyme production could be achieved with rice decanted water, as substrate. Further incubation till 24 hrs effected only a decline in the levels of reducing sugar, DE and enzyme production.

3.7.2 Fermentation of rice starch with B. coagulans ACMN 42

(a) Fermentation at pH 7; 30°C and aeration rate of 1 vvm

B. coagulans ACMN 42 could bring about rapid reduction of rice starch (86.9%) by 12 hrs (Fig.22). Further incubation till 24 hrs contributed only a marginal increase in the percentage of reduction. While the maximal DE (5.2) was obtained at 16 hrs, maximal enzyme production was noted at 12 hrs itself. However, both of the parameters DE and enzyme production showed a decline in the later periods of incubation. pH did not show a significant reduction from the initial level.

(b) Fermentation at pH 9; 30°C and aeration rate of 1 vvm

When the pH of the fermentation broth was raised to pH 9, rate of starch conversion was accelerated (Fig.23). About 87% of reduction in starch with a DE of 7.9 and 18 u/ml of enzyme production was recorded by 8 hrs of incubation itself. Further incubation till 24 hrs did not result in a total conversion of starch. DE did not increase after 8 hrs instead recorded a decline. Whereas a maximal enzyme production (33-34 u/ml) was recorded between 12 and 16 hrs. Further incubation resulted in a decline in the enzyme production (29 u/ml at 24 hrs). pH was observed to be reduced significantly from 9.0 to 7.8 at 24 hrs.

(c) Fermentation at pH 7; 35°C and aeration rate of 1 vvm

When the incubation temperature was raised to 35°C, B. coagulans ACMN 42 activity on rice starch was also enhanced significantly since about 80% of starch reduction along with a maximal DE of 7.8 and 13 u/ml of enzyme production were observed in just about 4 hrs of incubation (Fig.24). As in other cases, further incubation did not bring about a total reduction in the starch. Similarly DE also showed a marginal decline in its levels on further incubation.

Whereas, maximal level of enzyme activities (39-37 u/ml) observed between 12-18 hrs of incubation. Changes in the pH levels were not significant.

(d) Fermentation at pH 7; 30°C and aeration rate of 2 vvm

When the aeration rate was enhanced to 2 vvm from 1 vvm, a rapid conversion of starch was effected by this strain within a very short period of 4 hrs (Fig.25). Although more than 85% of starch reduction with a DE of 7.45 was effected by 4 hrs, significant level of DE (12.64) and enzyme production (23 u/ml) could be observed only at 8 hrs. Further increase in the incubation period showed only a marginal increase in the levels of DE and the percentage of conversion. However, high levels of enzyme production could be recorded from 12 hrs onwards at these fermentation conditions. pH did not show any significant change.

(e) Fermentation at pH 7; 30°C; aeration rate of 2 vvm and with an intermittant arresting of aeration after 12 hrs

Results presented in the Fig.26 indicate that arresting of aeration did bring a decrease in the DE and enzyme production besides production of acid in the medium.

However, the level of decline in the DE from 14.5 to 11 from 62.38 to 23 u/ml in enzyme production and pH from 6.97 to 4.98 indicate that the effect of aeration is only gradual when compared with that of B. coagulans ACMN 1. The results also indicate that there was no immediate effect, in a significant level, on the rate of fermentation since even at 16 hrs, there was 90% starch reduction along with 14.49 DE, 35 u/ml of enzyme production and pH 6.3.

(f) Fermentation with cooked rice decanted water at pH 7; 30°C and aeration rate of 1 vvm

B. coagulans ACMN 42 also brought about a rapid reduction of starch (90%) along with significant levels of DE (7.6) and enzyme production (27 u/ml) by 8 hrs of incubation (Fig.27). Although there was no significant increase in the percentage of conversion, DE and reducing sugar later, the enzyme production was maximum (46 u/ml) at 16 hrs. The DE varied between 8.52-8.42 from 12 hrs onwards till 24 hrs in the medium.

3.7.3 Fermentation of rice starch by B. polymyxa ACMN 25

(a) Fermentation at pH 7; 30°C and aeration rate of 1 vvm

B. polymyxa could bring about significant level of reduction in the starch (82%) along with a DE of 5.4 and 12 u/ml of enzyme activity by 8 hrs of incubation (Fig.28). Although further increase in the percentage of rice starch conversion was very gradual till 24 hrs., the increase in the DE and enzyme production was remarkable. While the DE was maximal (8.2) at 12 hrs enzyme production (36 u/ml) was maximum at 16 hrs. Both the parameters, DE and enzyme production, gradually declined during the later periods of incubation.

(b) Fermentation at pH 9; 30°C and aeration rate of 1 vvm

When the pH of the medium was raised to 9.0, B. polymyxa required 12 hrs to bring about a significant level of starch reduction (90.7%) and to provide a maximal level of enzyme production (37 u/ml) along with DE of 4.8 (Fig.29). Although percentage of starch conversion increased gradually after 12 hrs, there was not much increase in the DE. Enzyme production also declined gradually during the periods of incubation. The initial pH of 9 was brought to 7.4 at the end of incubation due to acid production in the medium.

(c) Fermentation at pH 7; 35°C and aeration rate of 1 vvm

Data presented in Fig.30 indicate that B. polymyxa could bring about a rapid conversion of rice starch (90%) by 4 hrs when the temperature was elevated to 35°C. Further increase in the incubation period did effect only a marginal reduction in the level of starch. DE level varied between 6.4 (8 hrs) to 6.8 (12 hrs). While maximal enzyme production was observed at 12 (39 u/ml) 16 hrs (41 u/ml) of incubation, there was no significant change in pH.

(d) Fermentation at pH 7; 30°C and aeration rate of 2 vvm

When the rate of aeration was increased to 2 vvm B. polymyxa showed a rapid reduction in the starch by 4 hrs itself (1.9 mg/ml) along with significant levels of DE (17.9) and enzyme production (17 u/ml)(Fig.31). Further incubation after 4 hrs brought about only a gradual reduction in the level of starch. However, 99% (0.1 mg/ml residual starch) of starch conversion was observed unlike that of other strains. Further, unlike other strains, this strain could record a maximal level of DE of 18.79 at 8 hrs which however declined to 17.71 by 24 hrs. Maximal enzyme production was observed at 12 hrs (37 u/ml) which declined marginally on further incubation.

(e) Fermentation at pH 7; 30°C and aeration rate of 2 vvm (aeration arrested intermittantly after 12 hrs)

From the data presented in Fig.32 it is clear that significant level of starch was reduced by 12 hrs (0.313 mg residual starch) along with a DE of 17.89 and a maximal enzyme activity of 36 u/ml. Arresting of aeration at 12 hrs brought about significant levels of reduction in the enzyme production (21 u/ml) and pH (4.95). DE level did not show any significant change after 12 hrs since the values ranged between 17.46-18.53 during the later period of incubation.

(f) Fermentation of cooked rice decanted water at pH 7; 30°C and aeration rate of 1 vvm

As it was observed for rice starch, B. polymyxa could bring about rapid reduction of starch (91.7%) and maximal enzyme production (33 u/ml) by 8 hrs (Fig.33). Although further increase in the incubation period effected a decrease in the starch level, enzyme production was not much affected. DE levels varied between 6.87-9.10 during the later half of incubation while the enzyme activity reduced gradually from 37 to 30 u/ml. Changes in the level of pH were not significant.

3.7.4 Fermentation of rice starch by B. cereus ACMN 33

(a) Fermentation at pH 7; 30° and aeration rate of 1 vvm

Significant levels of reduction of rice starch, (1.81 mg/ml residual) DE (4.6) and enzyme production (11 u/ml) was obtained with B. cereus within 8 hrs (Fig.34) as it was observed for other strains. The patterns of reduction in the rice starch and changes in the DE and enzyme activity during later periods of incubation after 8 hrs were similar to that observed for other strains. While a maximal DE of 6.6 was observed at 16 hrs, maximal enzyme production (32 u/ml) was recorded at 12 hrs. The DE recorded for the B. cereus was not appreciable when compared to other strains.

(b) Fermentation at pH 9; 30°C and aeration rate of 1 vvm

Elevation of media pH to 9.0 did not bring about any marked changes in the pattern of rice starch reduction by this strain (Fig.35). Although most of the starch was reduced by 12 hrs (0.53 mg/ml residual starch), the DE (6.3) and enzyme activities were not relatively very high when compared to that observed for pH 7. Further incubation, after 12 hrs, in fact brought about a decline in the DE and enzyme activity as it was observed for other strains. Initial pH was reduced to neutral pH during fermentation.

(c) Fermentation at pH 7; 35°C and aeration rate of 1 vvm

Fermentation at 35°C did not bring about any marked changes in the rate of reduction of starch and enzyme production by the organisms (Fig.36) when compared to that of 30°C. Only after 12 hrs of incubation rapid level of starch reduction (0.68 mg/ml) along with a DE of 7.5 and 28 u/ml of enzyme production were observed. As it was noted in other experiments, further incubation till 24 hrs did not influence very much the level of DE and enzyme production and both the parameters recorded very marginal change from the levels observed at 12 hrs.

(d) Fermentation at pH 7; 30°C and aeration rate of 2 vvm

Data presented in the Fig.37 indicate that, unlike all the other three strains studied, B. cereus is not influenced by the increase in the rate of aeration in terms of rate of conversion. Rapid conversion (1.21 mg/ml residual starch) along with significant levels of DE (16.62) and enzyme production (23 u/ml) were observed only after 12 hrs. Further increase in incubation period brought about only a marginal increase in the level of starch reduction and enzyme

production, while effecting a gradual decline in DE. In this experiment the only interesting observation made was that of DE which was almost double in its level, in general, when compared to that recorded at 1 vvm of aeration rate.

(e) Fermentation at pH 7; 30°C and aeration rate of 2 vvm (intermittent arresting of aeration after 12 hrs)

Data presented in the Fig.38 indicate that rapid conversion of starch (0.93 mg/ml residual starch) along with significant levels of DE (15.69) and enzyme production (25 u/ml) was effected within 12 hrs of incubation and aeration. Even after discontinuing of aeration after 12 hrs a maximum level of DE (16.06) and enzyme production (30 u/ml) were recorded at 16 hrs indicating a negative influence of aeration on these two parameters. pH of the medium recorded a significant reduction from 7.0 to 4.98.

(f) Fermentation of cooked rice decanted water at pH 7; 30°C and aeration rate of 1 vvm

B. cereus, very much like other strains, could bring about rapid reduction of starch in the rice decanted water by 8 hrs (0.93 mg/ml residual starch) along with a

DE of 8.12 and 29 u/ml of enzyme production Fig.39. Interestingly this particular strain could yield an increased level of DE (10.12) in the fermentation broth by 20 hrs. The enzyme production was maximum (37 u/ml) during the period 12-16 hrs which recorded a marginal decrease after 16 hrs. Changes in the pH were not significant.

3.8 CONVERSION OF RICE STARCH BY FREE ENZYMES

Liquifaction of rice starch by α-amylase produced by the four strains of Bacillus sp. at different temperatures was performed by incubating the prepared enzyme with 10% rice starch powder for a total period of 60 min. After incubation the contents were analyzed for residual starch, reducing sugar and total sugar and DE. The results are presented in Table 9.

Amylase of B. coagulans ACMN 1 could bring about maximum levels of liquifaction at 60°C followed by 50°C. However, 50% conversion was observed at 70°C also. DE was more at 50°C and found to be decreased along with increase of temperatures above 50°C.

Amylase of B. coagulans ACMN 42 also recorded very high percentage of conversion (90%) at 60°C followed by 70°C (74%) and 50°C (70%). However, significant levels of reduction could also be observed at 80°C. DE (11.4) was recorded to be more at 50°C which decreased along with an increase in temperature.

Amylase of B. polymyxa also recorded maximal levels of conversion of starch (96.8%) at 60°C followed by 50°C, 70°C and 80°C. Interestingly this strain could bring about 30% conversion at 90% compared to the activity of the enzymes of other strains at this temperature. DE was found to be very high at 60°C (12.47) followed by 70°C (8-9) and 50°C (6.31).

Amylase of B. cereus ACMN 33 also recorded maximal percentage of conversion of 90% at 60°C followed by 50°C. They could also record significant levels of conversion at temperatures above 60°C. DE was found to be more at 50°C (14.9) which decreased along with increase in temperature.

Of all the four strains while amylase of B. polymyxa ACMN 25 demonstrated maximal conversion of rice starch, maximal DE was recorded by B. cereus ACMN 33 than other strains.

3.9 RICE STARCH CONVERSION BY IMMOBILIZED WHOLE CELLS OF *BACILLUS* sp

Rice starch conversion by *Bacillus* whole cells entrapped on ca-alginate beads was carried out at various pH levels and temperatures. They were also characterised for their retention time and reusability. The media after conversion were analyzed for residual rice starch, reducing sugar, dextrose equivalent and enzyme production.

3.9.1 Effect of pH on rice starch conversion by immobilized whole cells of *Bacillus* sp

Results presented in Fig.40 indicate that all the four strains could bring about significant levels of reduction in the starch content besides producing amylase, along with DE in the pH range 7-9.

B. coagulans ACMN 1 recorded a maximal rice starch reduction at pH 8 with maximal DE (8.2) and amylase activity (23 u/ml). Similar levels of DE and enzyme production was also observed for this strain at pH 9 whereas, *B. coagulans* ACMN 42 brought about only a moderate level of reduction in the starch at pH 9 (residual starch 4 mg/ml). Maximal levels of enzyme activity varying between 17-18 u/ml was observed at pH 7-9, while a maximal DE (7.5) was noted at pH 7.

B. polymyxa also recorded significant levels of reduction in the starch (residual starch 0.52-0.9 mg/ml), DE (5.83-7.30) and enzyme production (20-23 u/ml) at pH levels of 7-9.

B. cereus recorded a maximal reduction of starch (1.5 mg/ml residual starch) and maximal levels of DE (6.15) and emzyme production (17 u/ml) at pH 7. However, at pH 8 also it could record significant levels of DE and enzyme production when compared with other pH levles.

In general, B. polymyxa and B. coagulans ACMN 1 produced more enzymes and more DE at neutral pH 7-8 when compared to other strains.

3.9.2 Effect of temperature on rice starch conversion by immobilized whole cells of Bacillus sp.

The effect of temperature on the rice starch conversion by immobilized whole cells of Bacillus sp. were carried out and the results are presented in Fig.41.

All the strains could bring about significant levels of reduction in the starch at all the three

temperatures except B. polymyxa and B. coagulans ACMN 42 which did not reduce starch at 50°C. B. coagulans ACMN 1 and B. cereus recorded 50% conversion at 50°C. All the strains invariably recorded maximal level of DE at 30°C which decreased gradually on further raise in incubation temperature. While enzyme production was relatively significant at 30-40°C for all the strains, only B.cereus could produce 6 u/ml of amylase at 50°C compared to others which did not show any enzyme production at this temperature.

3.9.3 Effect of retention time on rice starch conversion by immobilized whole cells of Bacillus sp.

The results presented in Fig.42 increase in the retention time towards 24 hrs of retention yielded significant levels of reduction in the rice starch along with maximal levels of enzyme production and dextrose equivalent for all the strains.

Of the four strains, B. coagulans ACMN 42 (28-29 u/ml) followed by B. polymyxa (18-23 u/ml), B. cereus (17-23 u/ml) and B. coagulans ACMN 1 (11-18 u/ml) produced maximal levels of enzyme by 12-24 hrs of incubation.

Similarly B. coagulans ACMN 42 recorded maximal levels of DE (10.8) when compared to B. polymyxa (DE 5.8), B. cereus (3.7 DE) and 2.8 B. coagulans ACMN 1.

In general B. coagulans ACMN 42 was found to be the best strain to effect rice starch conversion by immobilization since it recorded maximal levels of rice starch reduction (0.73 mg/ml residual starch) and enzyme production (29 u/ml).

3.9.4 Reusability of immobilized Bacillus sp.

Immobilized cells of Bacillus sp. were checked for their reusability by conducting repeated conversion of rice starch using the same beads for a period of four cycles (each cycle induced 24 hrs of incubation). Data presented in the Fig.43 indicate that efficiency of the immobilized beads to reduce rice starch, and produce enzyme and reducing sugar decreased after each cycle in a gradual fashion. However, all the strains retained their ability to produce sugars in a significant levels even after 4 cycles.

3.10 CONVERSION OF RICE STARCH BY IMMOBILIZED ENZYME

3.10.1 Effect of pH

Conversion of rice starch by immobilized enzyme was carried out at different pH and temperatures and the results are presented in Fig.44.

Results obtained clearly evidences the fact that the amylases isolated from all the four strain preferred alkaline pH even after immobilization for their maximal starch reduction. Significant levels of rice starch reduction and DE were recorded at pH 8-9.

3.10.2 Effect of temperature

Immobilized enzymes showed a negative response to increase in incubation temperature by recording an increased levels of residual starch and a decreased levels of reducing sugar and DE (Fig.45).

All the enzymes preferred 40°C for effecting maximal reduction in the rice starch than higher temperature.

3.11 STATISTICAL ANALYSIS

3.11.1 Conversion of rice starch by free cells

Pearson correlation coefficient was analysed for various parameters estimated during fermentation studies. In general, all the factors analyzed showed significant levels of relations, either positive or negative between each other (Tables 10-39).

Data obtained for the statistical analyses of the fermentation studies with the B. coagulans is presented in Tables 10-15. Incubation time maintained a significant positive correlation with other factors such as residual starch, reducing sugar, DE, growth and enzyme activity, in most cases. Whereas, residual starch and pH had a significant negative correlation with all other factors. In general, DE and enzyme activity accorded significant levels of positive correlation with other parameters except for few occasions.

B. coagulans ACMN 42 also showed a significant correlation between the parameters analyzed during fermentation (Tables 16-21). A highly significant negative

correlation was observed between incubation time and residual starch, and between residual starch and reducing sugar, DE, growth and enzyme production. A highly significant positive correlation between growth and enzyme production and DE, was also noted in most cases.

The different parameters analyzed during fermentation by B. polymyxa ACMN 25 also recorded significant correlations (Tables 22-27). Insignificant negative correlations are recorded between the incubation time and residual starch in most of the cases for this species. However, highly significant negative correlations was recorded between residual starch and reducing sugar, total sugar, DE and enzyme activity. Relatively, the levels of correlations, positive or negative, were significant for the studies conducted at pH 9 and temperature 35°C (Table 24, 25). However, significant positive correlations were observed between growth and enzyme production and DE and enzyme production with B. polymyxa.

B. cereus also highlighted a significant correlation between the parameters during fermentation (Tables 28-33). A significant negative correlation was observed

between time and starch reduction in on several occasions. Similarly, highly significant negative correlations were recorded between residual starch and other parameters. However, significant positive correlations between growth and enzyme production, DE and enzyme activity were also noted.

3.11.2 Conversion of rice starch by immobilized cells and enzymes

In general, a highly significant correlation was recorded between the parameters analyzed (Table 34). For all the strains pH and residual starch showed insignificant negative correlation between them. Highly significant negative correlations were recorded between residual starch and reducing sugar. However, significant positive correlations were recorded between most of the parameters, for all the four strains.

The statistical data on the effect of temperature on rice starch conversion by immobilized whole cells of Bacillus sp. recorded significant correlations only between few parameters (Table 35). A significant negative correlation was recorded between temperature and total sugar whereas a significant positive correlation was recorded between

reducing sugar and DE which was prominant only in the case of B. coagulans ACMN 1. A significant positive correlation, was also recorded between reducing sugar and enzyme activity with B. cereus ACMN 33.

However, the correlations between the parameters analyzed were not significant level for the other two strains.

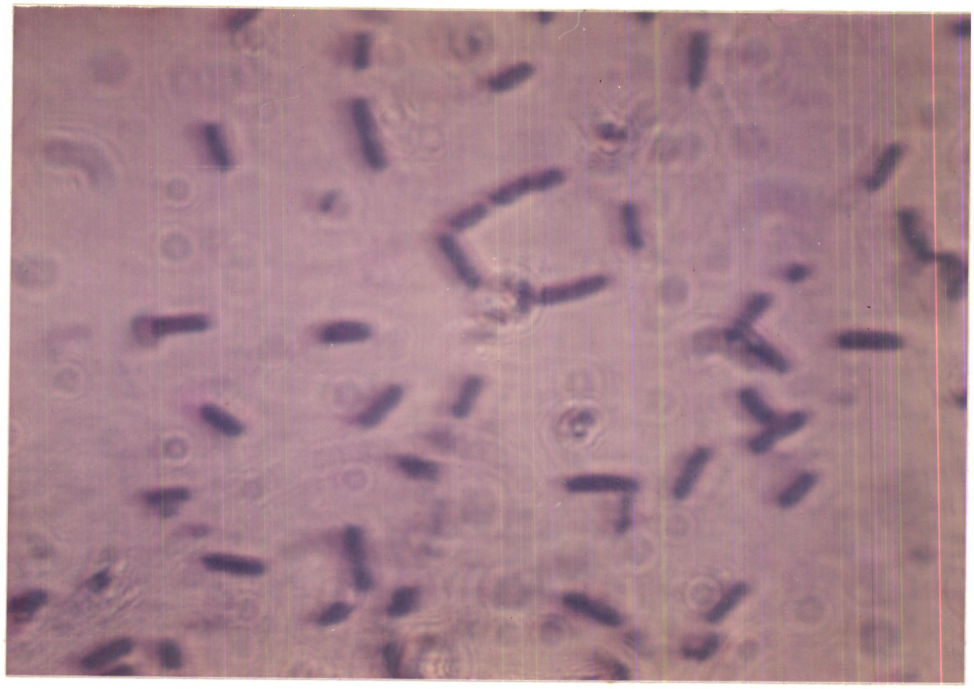
The correlations between the parameters analyzed with respect to effect of incubation time on rice starch conversion by immobilized cells was recovered at significant levels in most cases (Table 36). Significant negative correlations between time and residual starch and a significant positive correlation between time and enzyme activity were recorded for all the four species of Bacillus.

In general, correlation coefficients obtained for the various parameters analyzed in the reusability studies with immobilized cell beads for rice starch conversion were not significant levels (Table 37). However, a significant negative correlations was noted between the number of cycles and reducing sugar for all the strains except in B. cereus

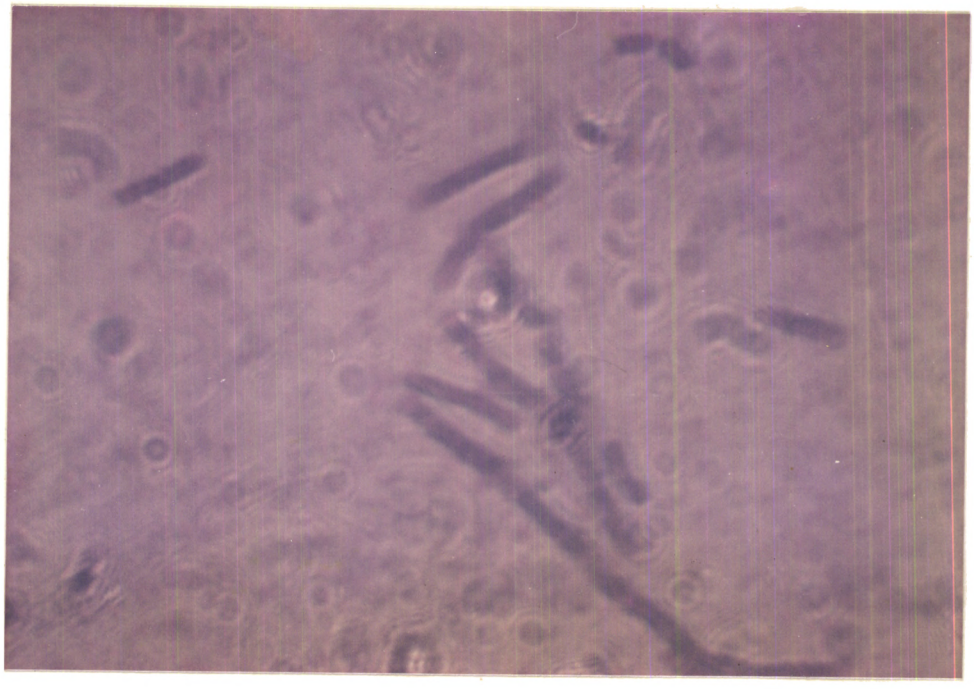
ACMN 33. Interestingly B. coagulans ACMN 1 and B. cereus ACMN 33 could recorded a significant positive correlation between enzyme activity and DE when compared to other two strains of Bacillus.

In general, significant correlation coefficients for all the parameters were recorded (Table 38). pH did not exhibit correlations with other parameters at significant level. However, significant negative correlations were recorded between residual starch and reducing sugar, total sugar and DE for all the strains. Similarly reducing sugar exhibited significant positive correlation with total sugar and DE.

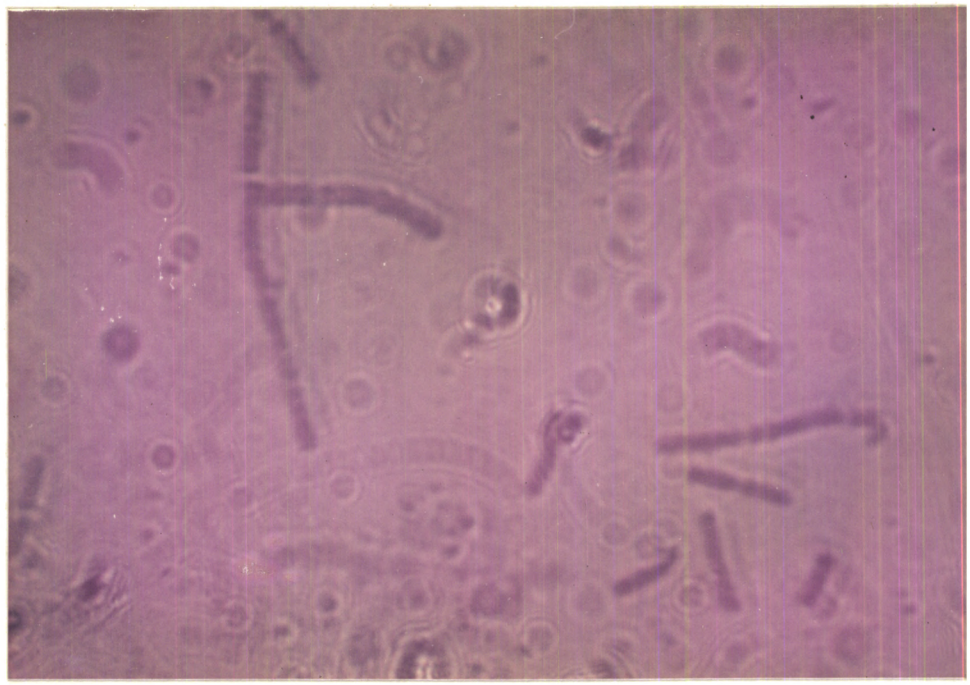
In general, the correlation coefficient obtained between the parameters were at significant levels (Table 39). However, a positive correlation between temperature and residual starch was recorded for all the strains except for B. polymyxa ACMN 25.



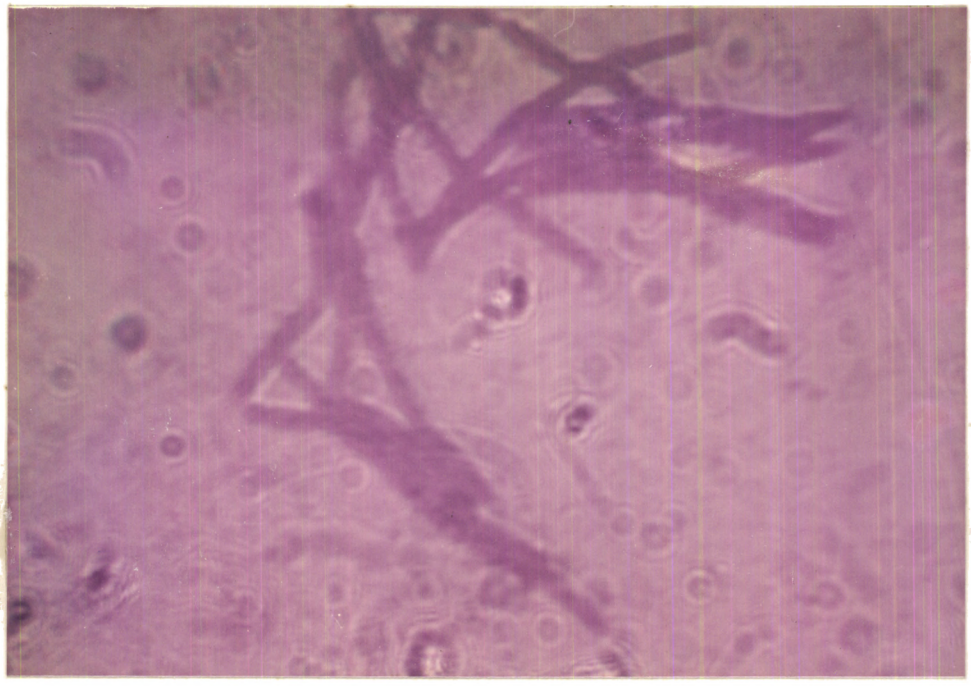
Microphotograph of B.coagulans ACMN 1



Microphotograph of B.coagulans ACMN 42



Microphotograph of B.polymyxa ACMN 25



Microphotograph of B.cereus ACMN 33

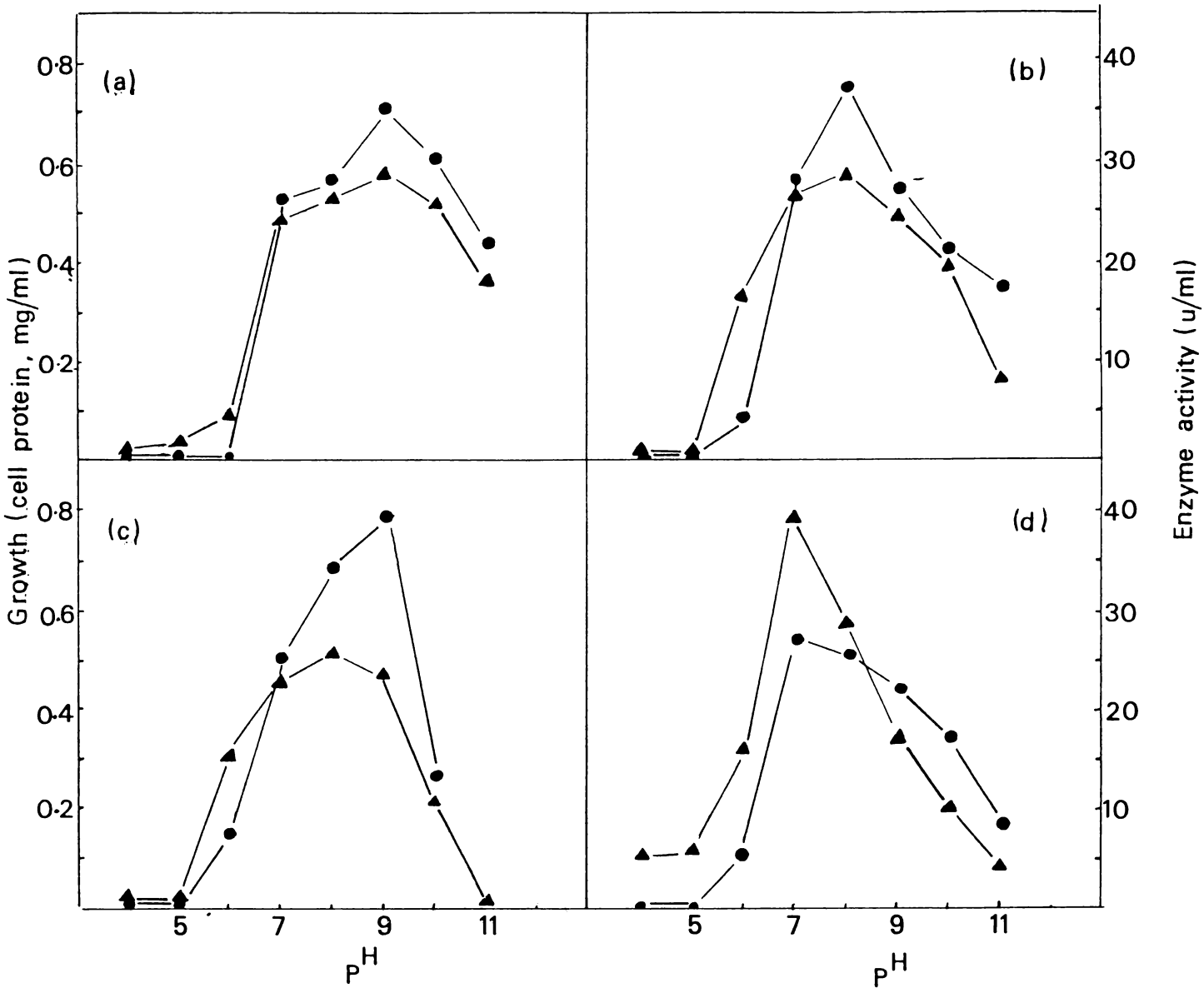


Fig.2 : Effect of pH on growth and enzyme production of Bacillus sp.

(a) B. coagulans ACMN 1
(b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25
(d) B. cereus ACMN 33

-▲- Growth
-●- Enzyme activity

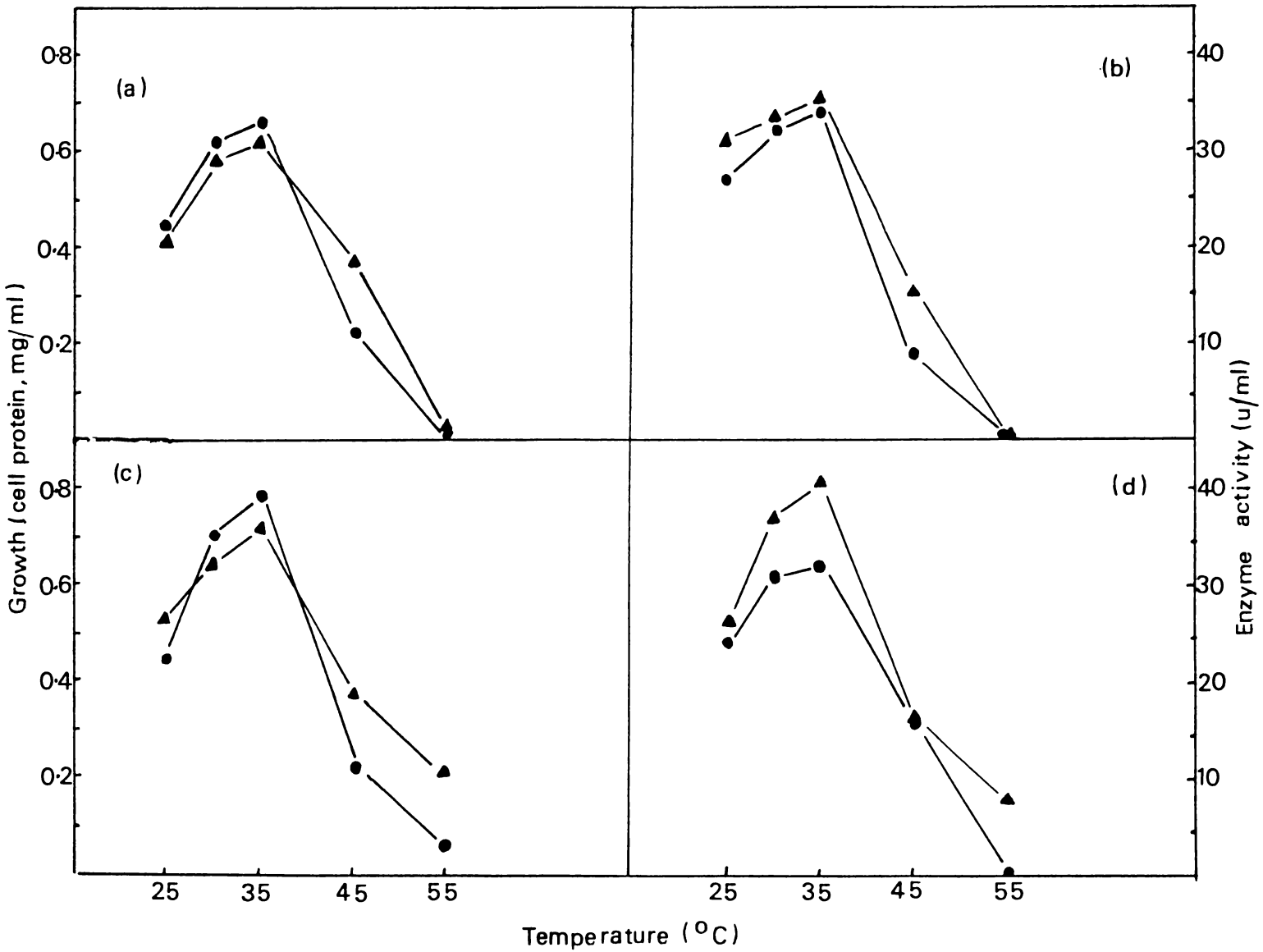


Fig.3 : Effect of temperature on growth and enzyme production of Bacillus sp.

(a) B. coagulans ACMN 1
(b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25
(d) B. cereus ACMN 33
-▲- Growth
—●— Enzyme activity

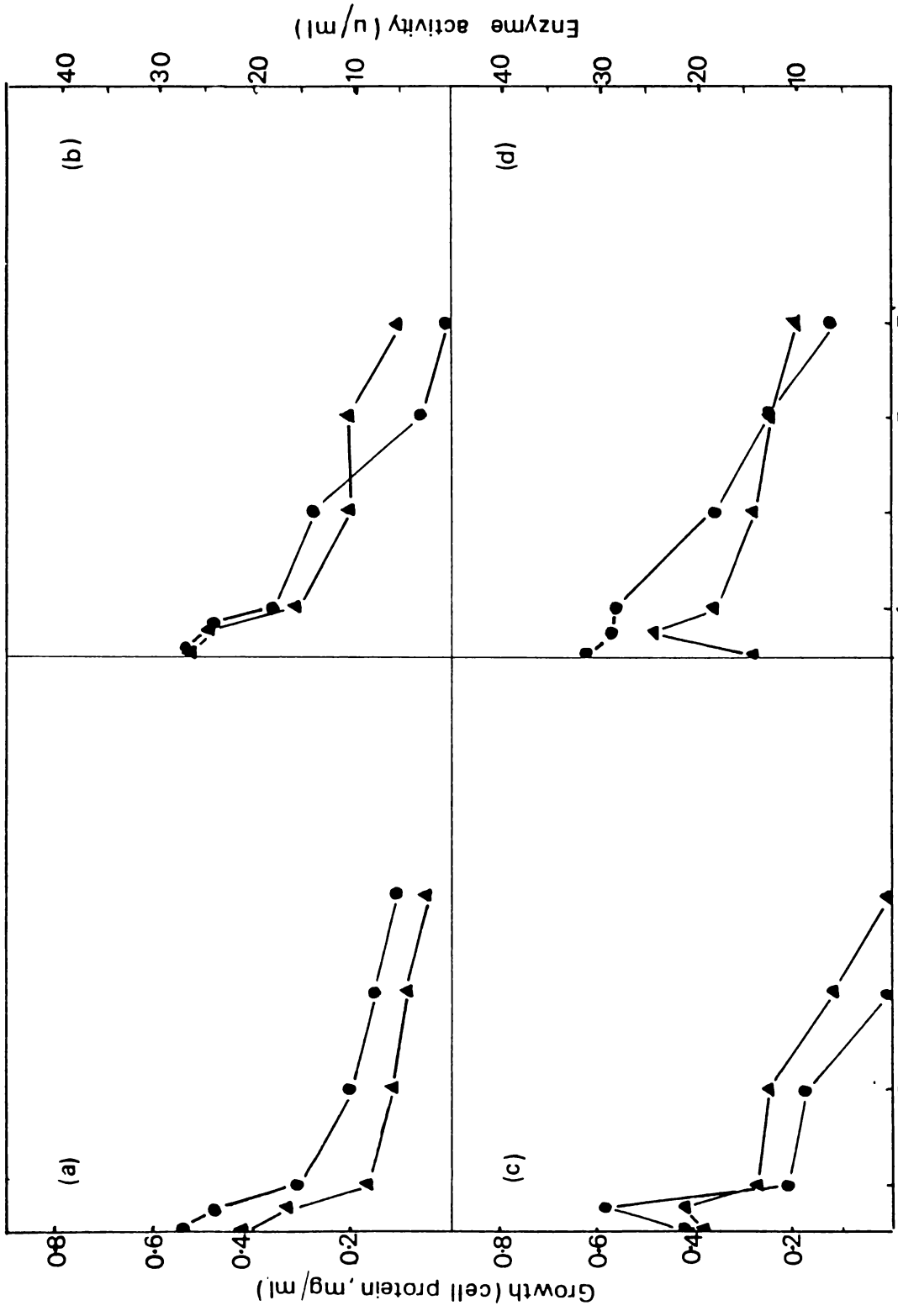


Fig.4 : Effect of NaCl concentration on growth and enzyme production of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33
-▲- Growth -●- Enzyme activity

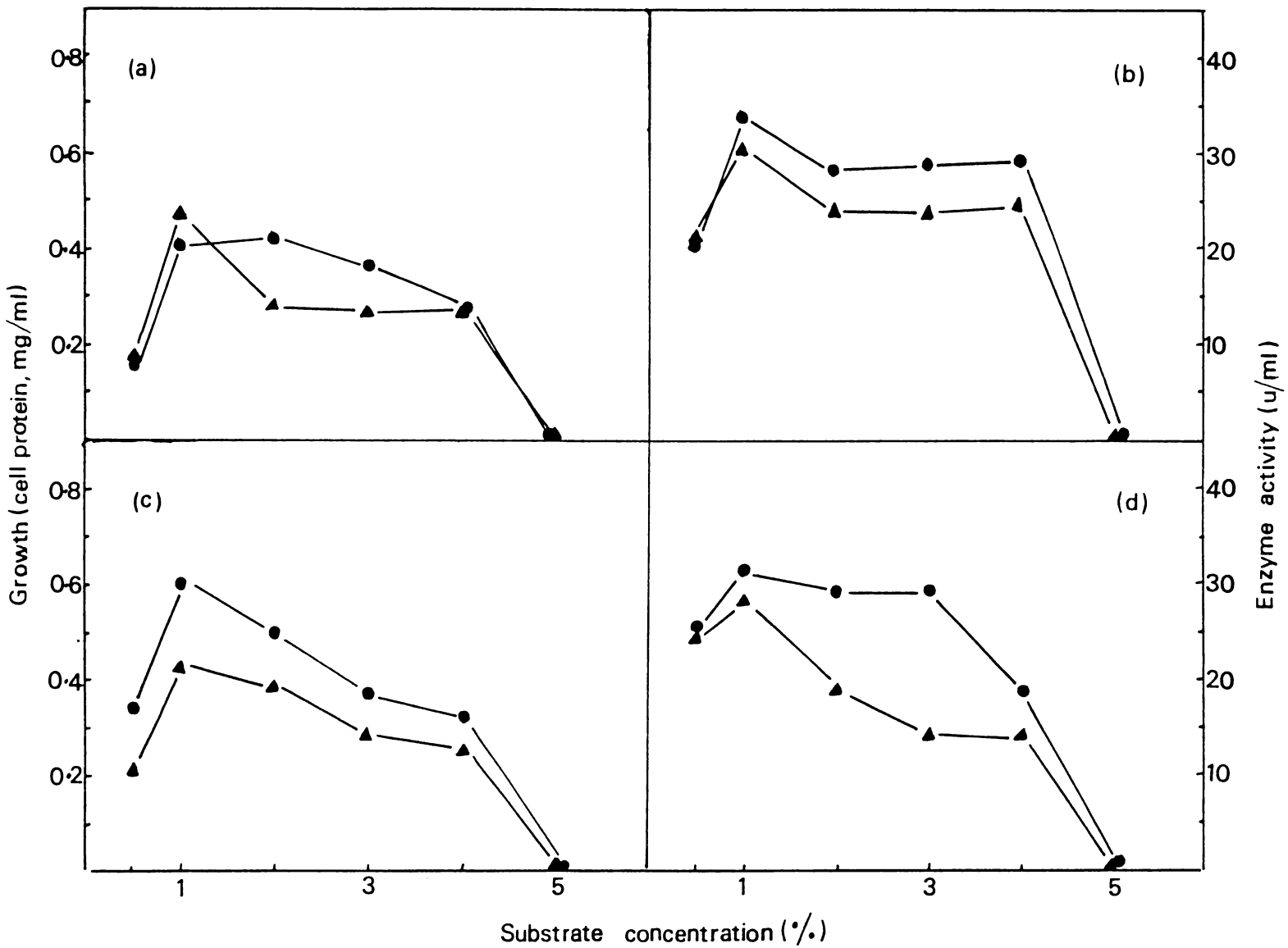


Fig. 5 : Effect of substrate concentration on growth and enzyme production of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42

(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

-▲- Growth -●- Enzyme activity

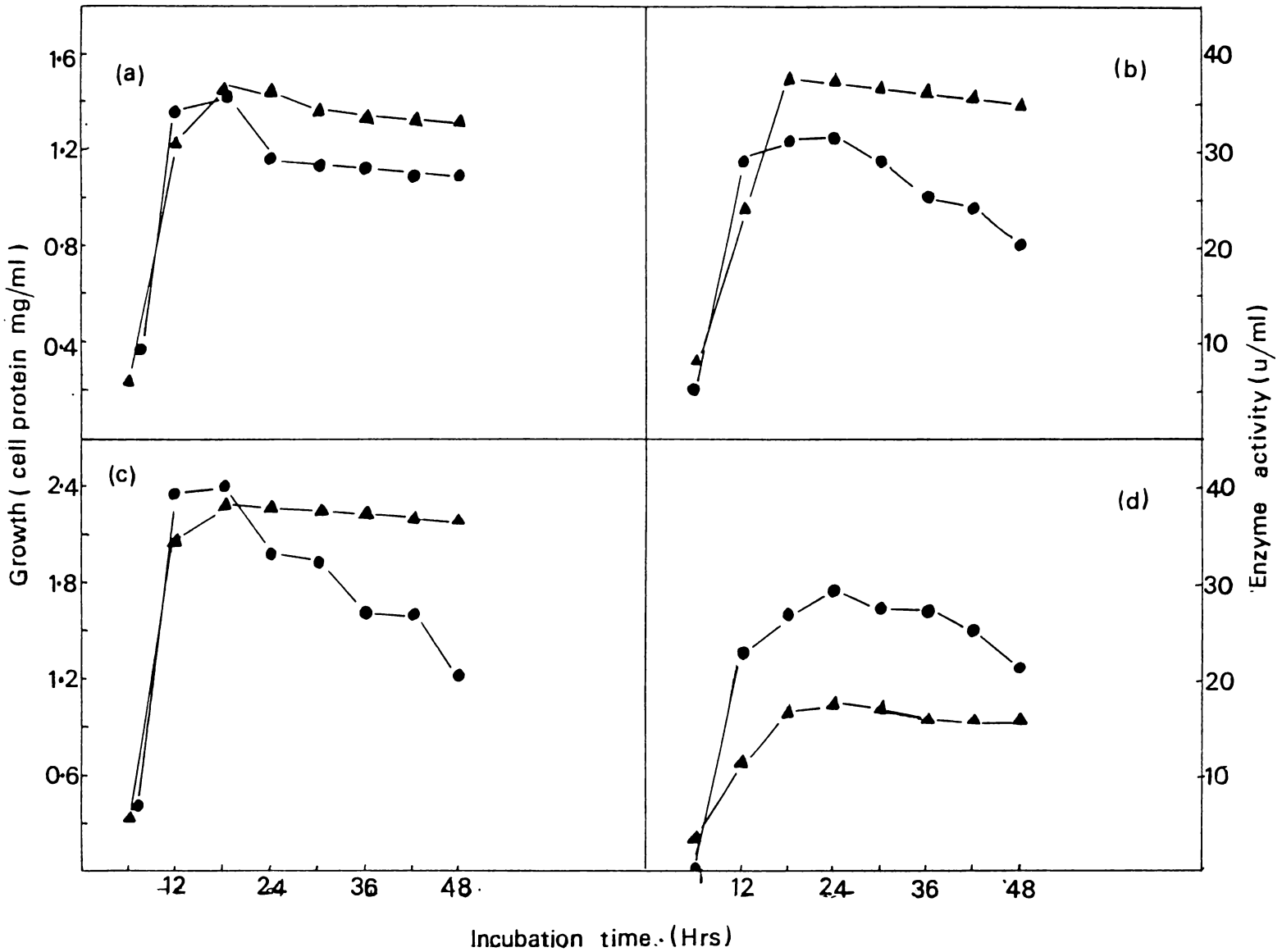


Fig.6 : Effect of incubation period on growth and enzyme production of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42

(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

-▲- Growth -●- Enzyme activity

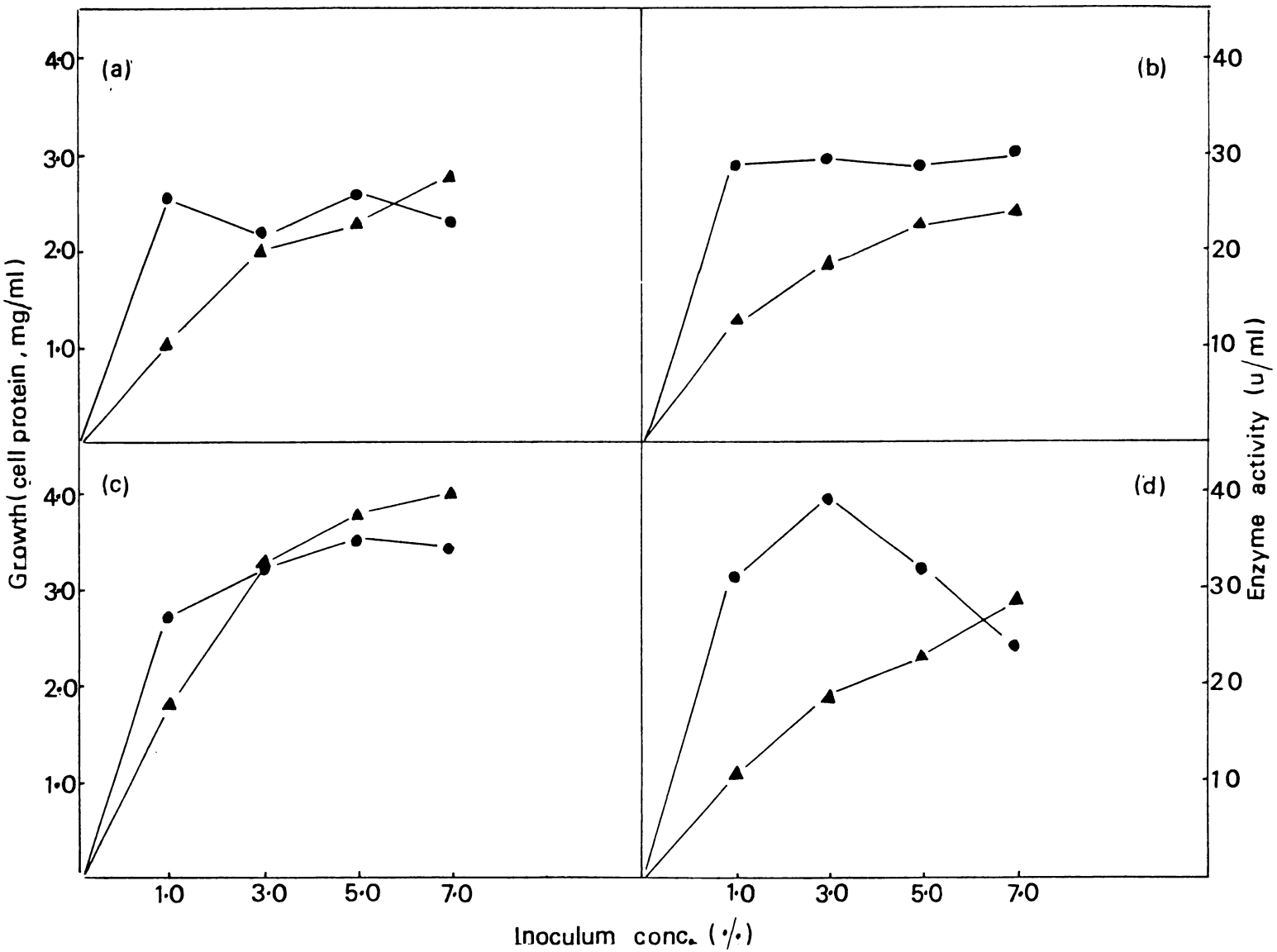


Fig.7 : Effect of inoculum concentration on growth and enzyme production of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42

(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

–▲– Growth –●– Enzyme activity

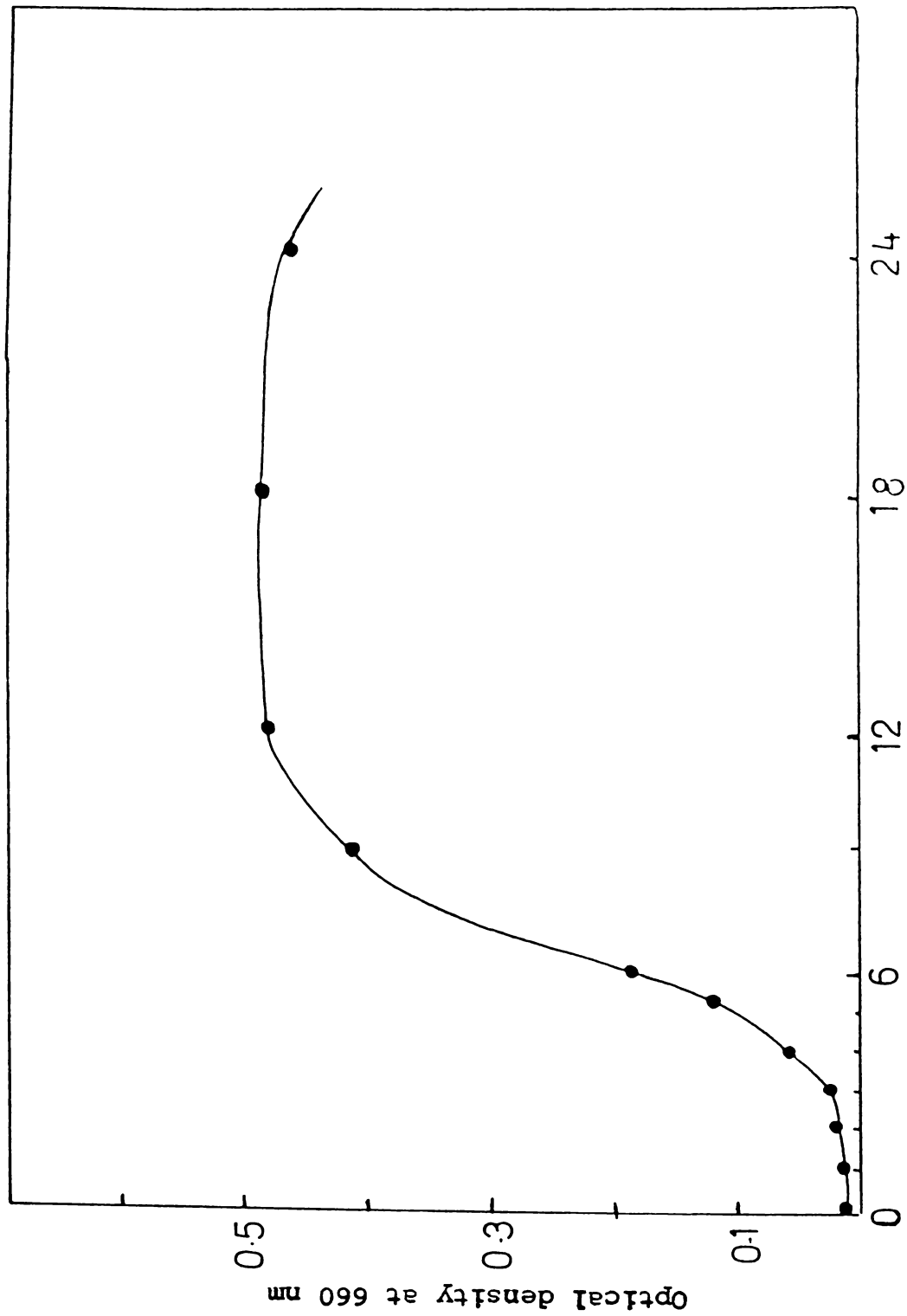


Fig.8 : Growth curve of B. coagulans ACMN 1.

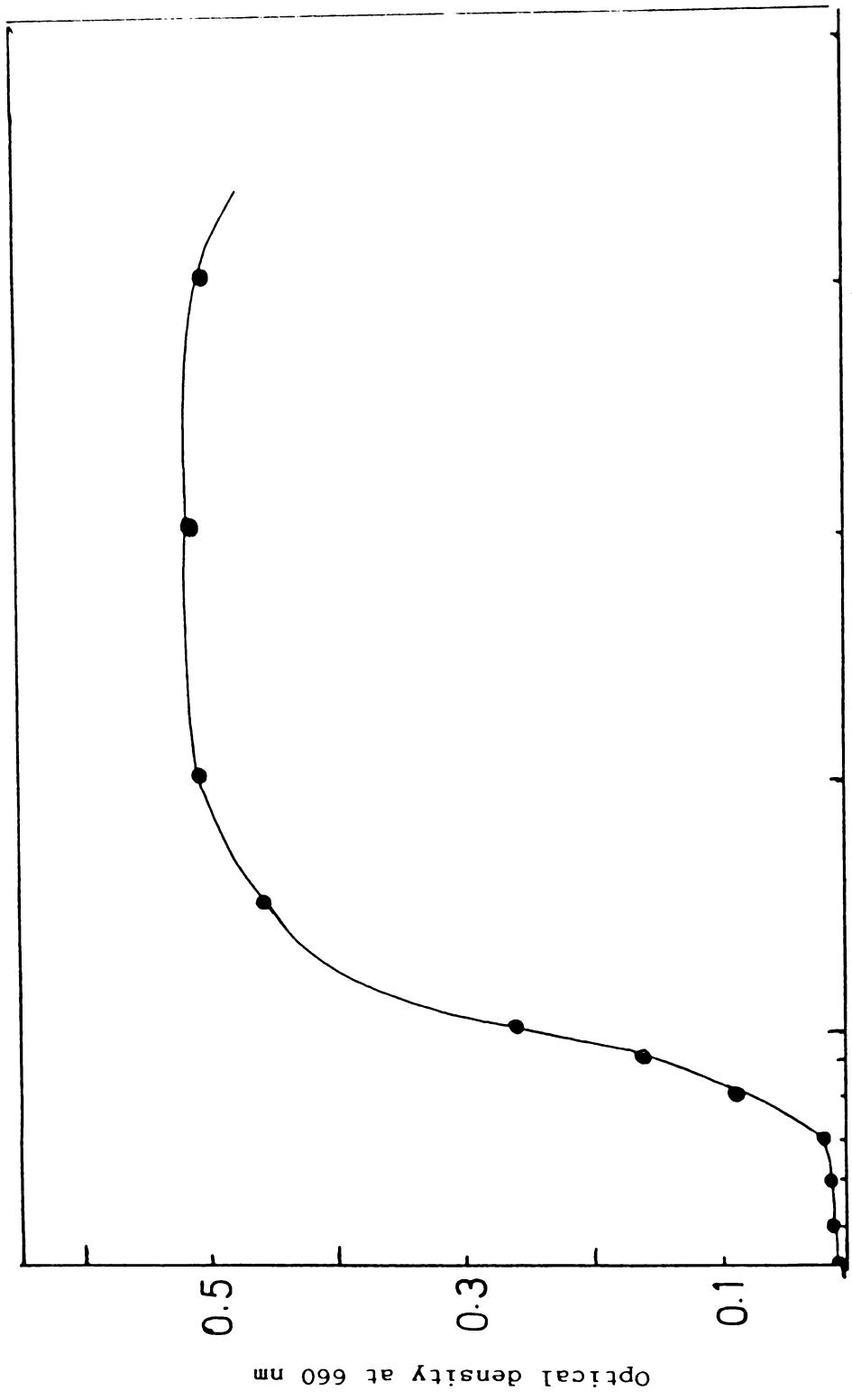


Fig.9 : Growth curve of B. coagulans ACMN 42.

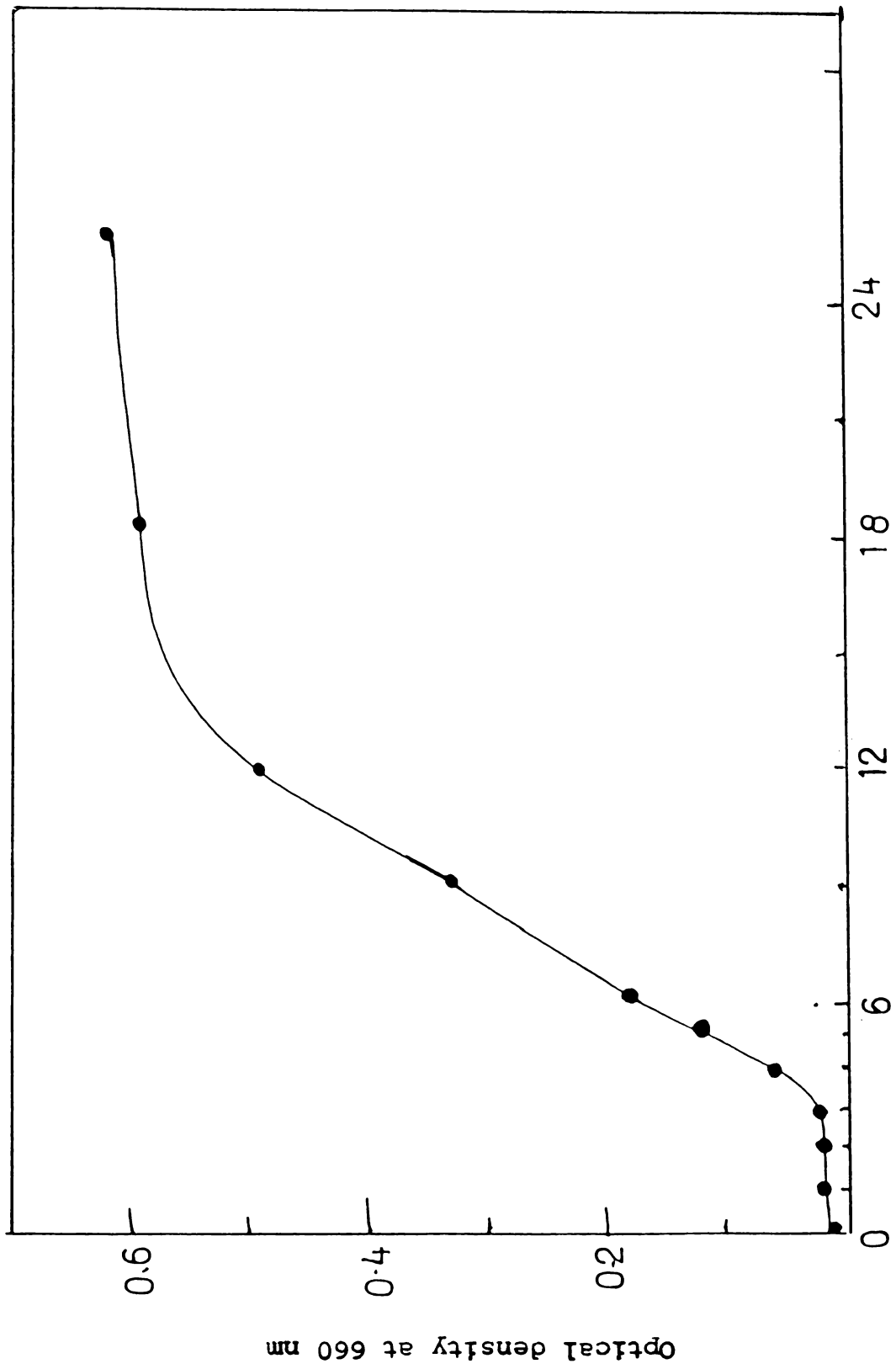


Fig.10 : Growth curve of B. polymyxa ACMN 25.

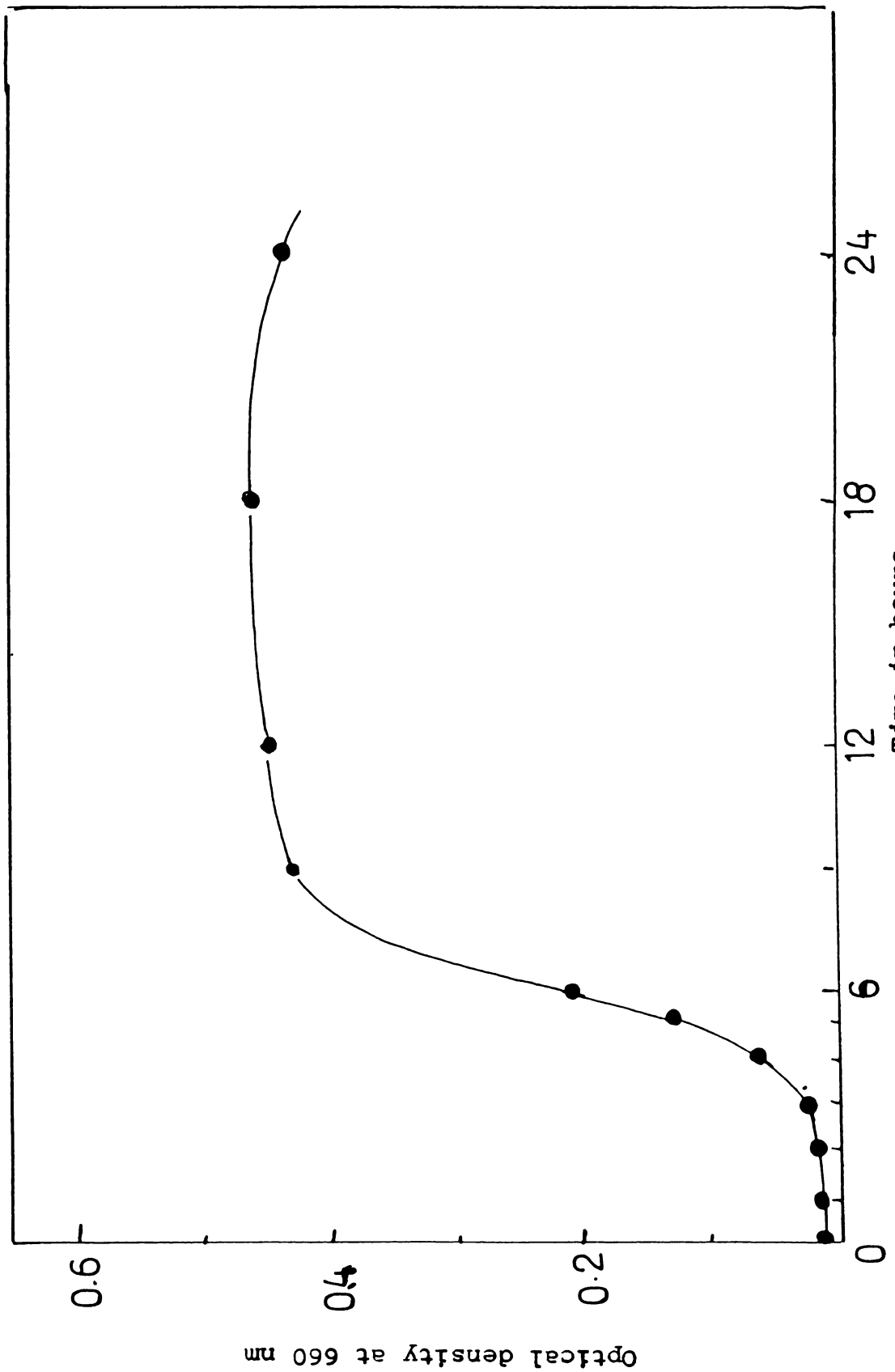


Fig.11 : Growth curve of B. cereus ACMN 33.

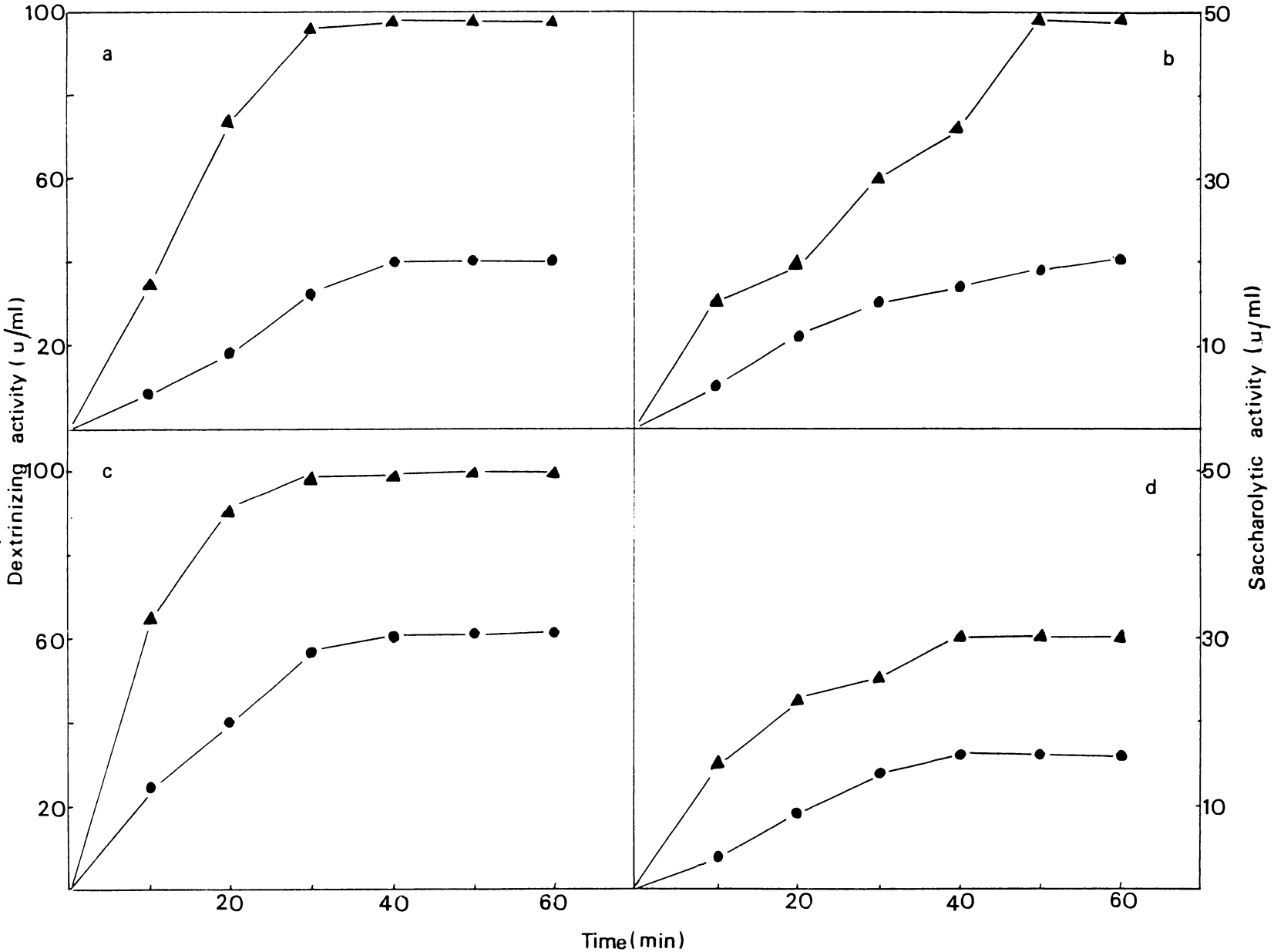


Fig.12 : Dextrinizing and saccharolytic activity of amylase from Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42

(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

—▲— Dextrinizing activity —●— Saccharolytic activity

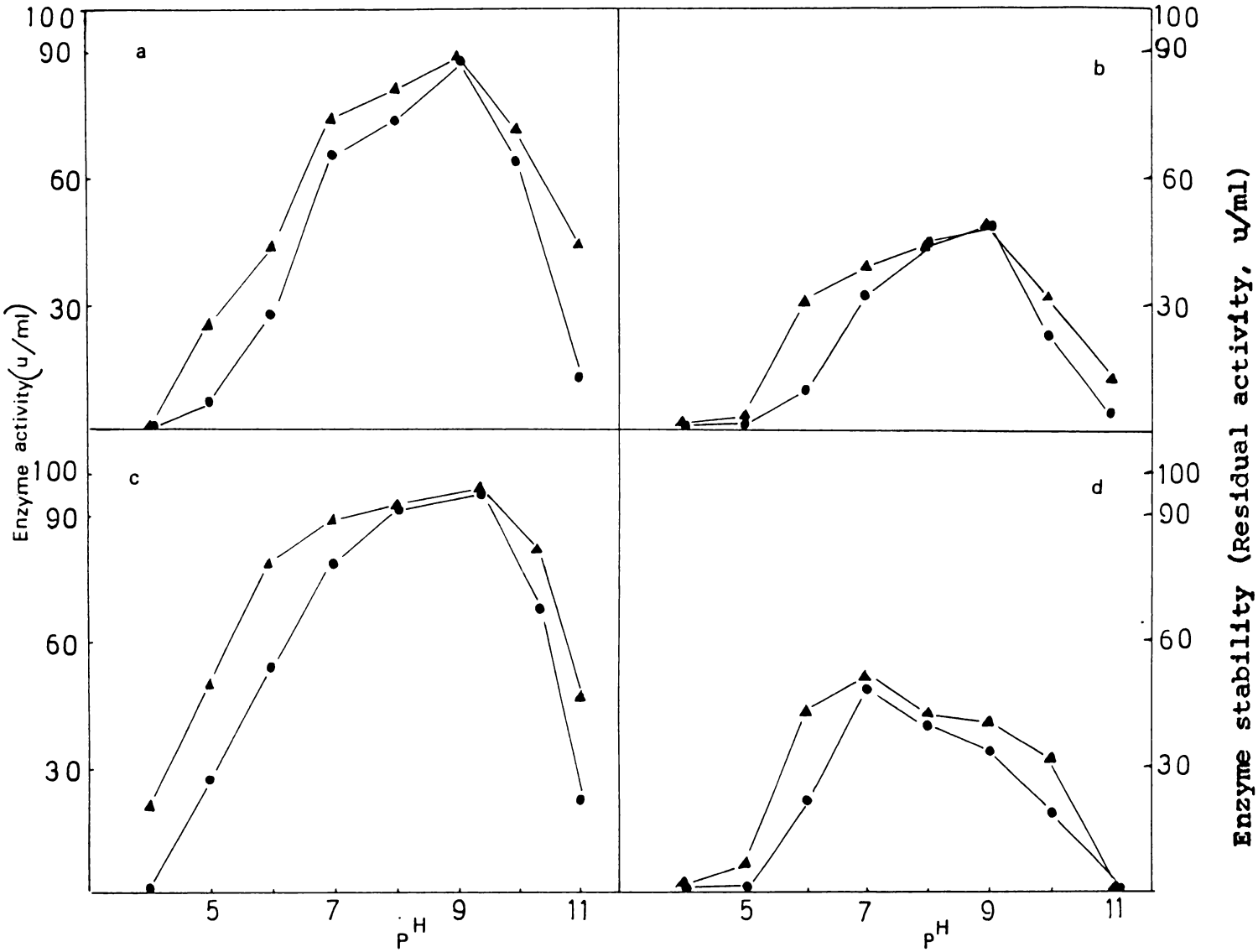


Fig.13 : Effect of pH on the activity and stability of amylase from Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42

(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

-▲- Enzyme activity -●- Enzyme stability

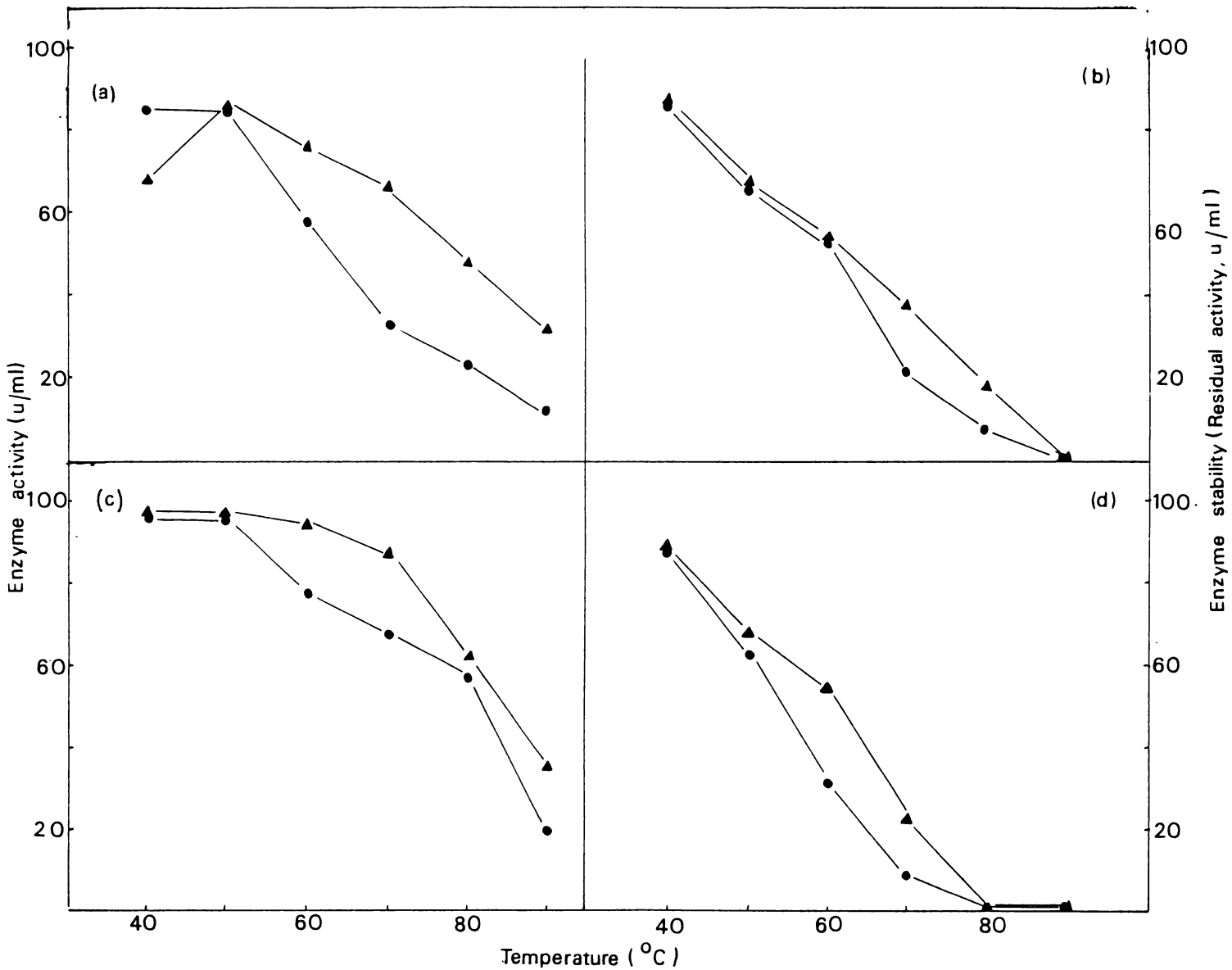


Fig.14 : Effect of temperature on the activity and stability of amylase from Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42

(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

—▲— Enzyme activity —●— Enzyme stability

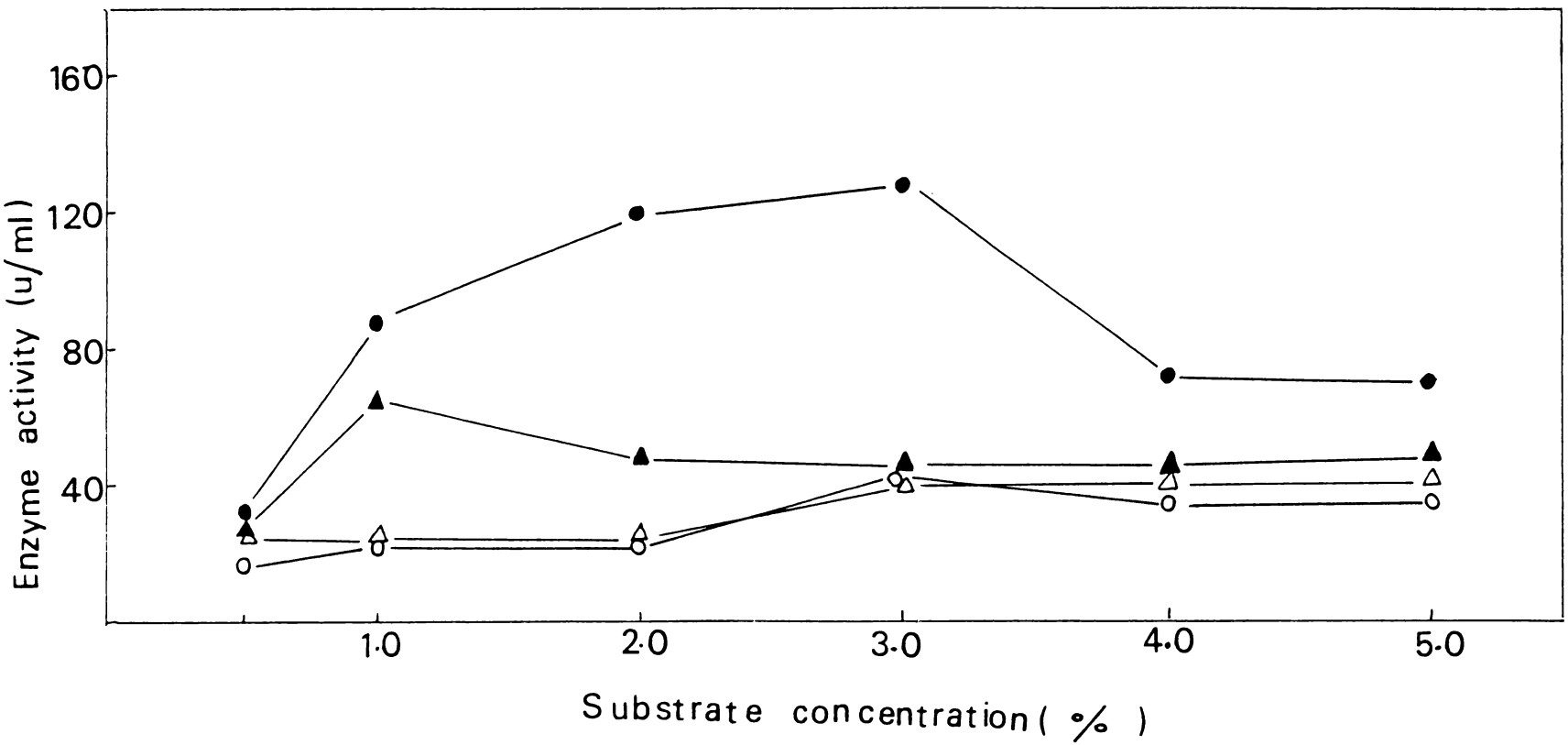


Fig.15 : Effect of substrate concentration on the activity of amylase from Bacillus sp.

B. coagulans ACMN 1 –▲– B. coagulans ACMN 42 –△–

B. polymyxa ACMN 25 –●– B. cereus ACMN 33 –o–

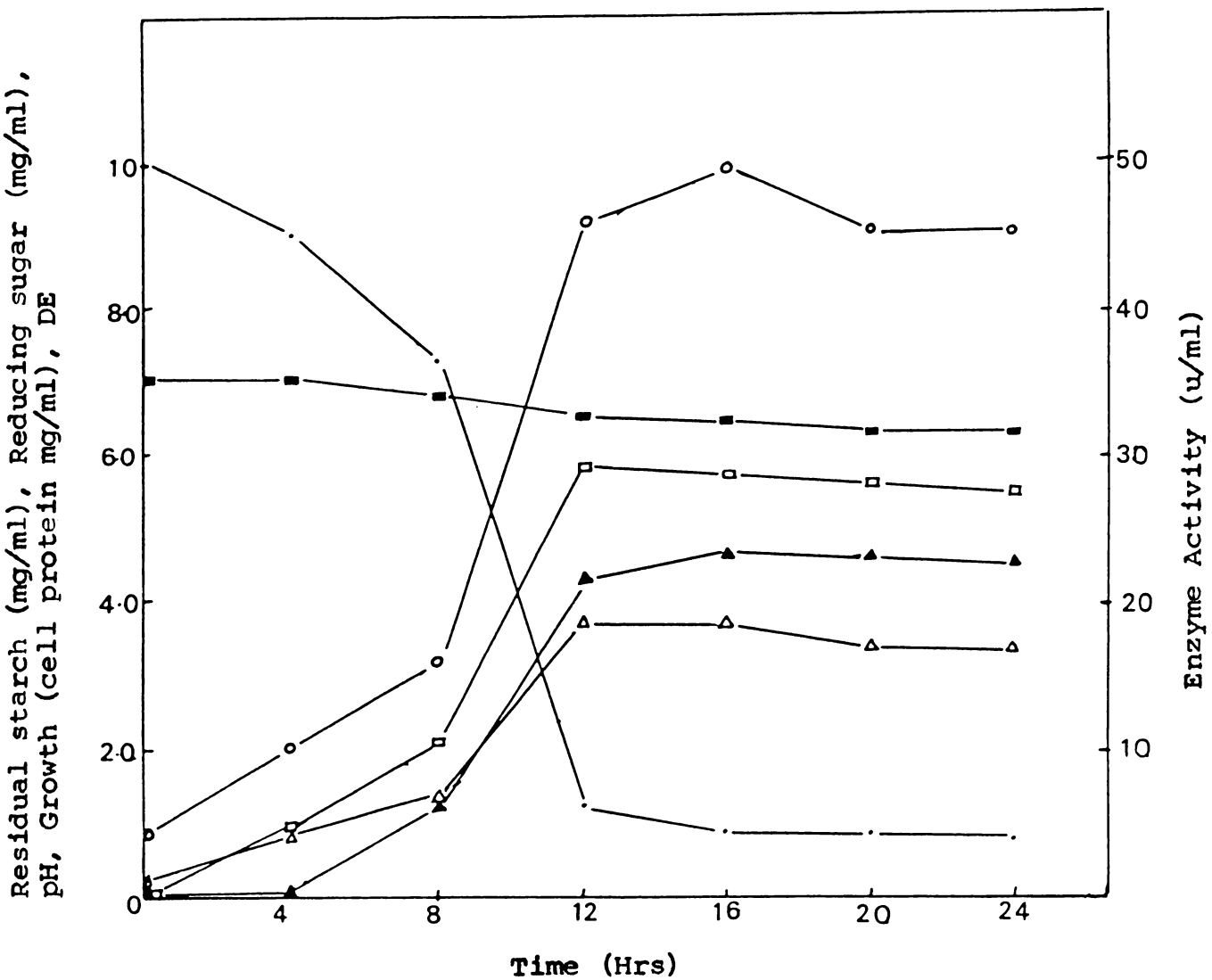


Fig.16 : Fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 30°C and at an aeration rate of 1 vvm.

·—· Residual Starch ; □—□ Growth ; ▲—▲ Enzyme Activity

△—△ Reducing Sugar ; ○—○ DE ; ■—■ pH

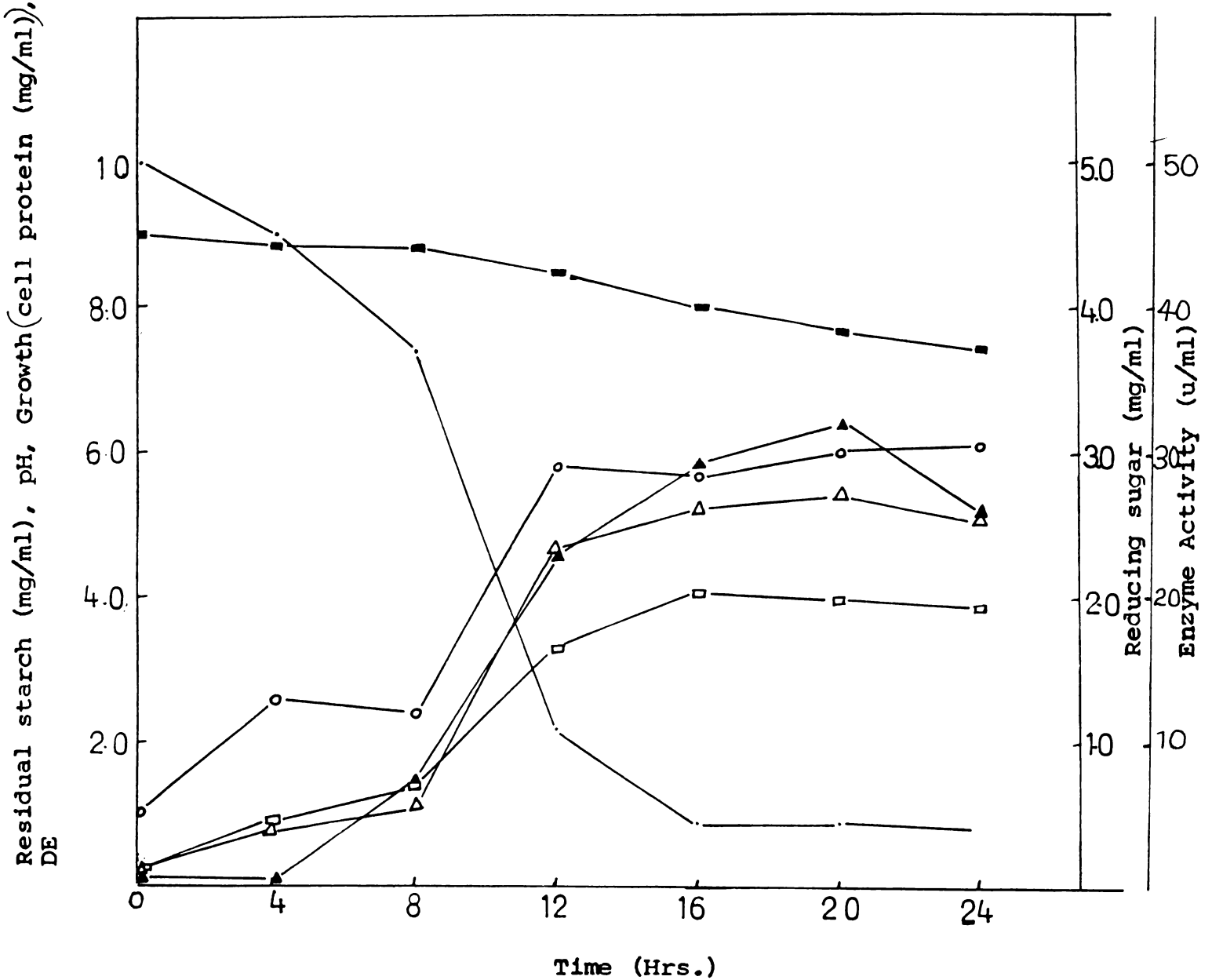


Fig.17 : Fermentation of rice starch by B. coagulans ACMN 1 at pH 9; 30°C and at an aeration rate of 1 vvm.

—•— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE : —■— pH

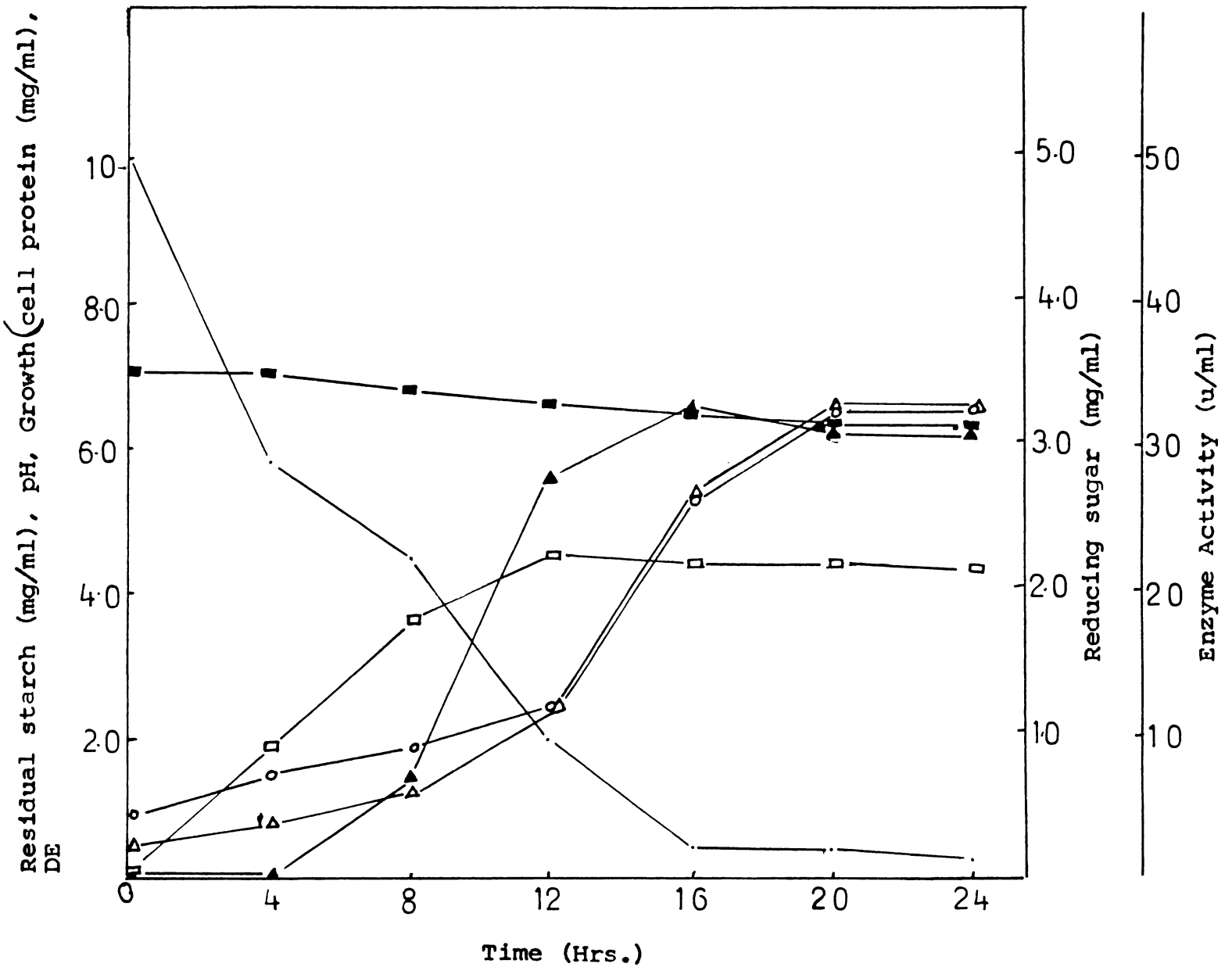


Fig.18 : Fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 35°C and at an aeration rate of 1 vvm.

—•— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

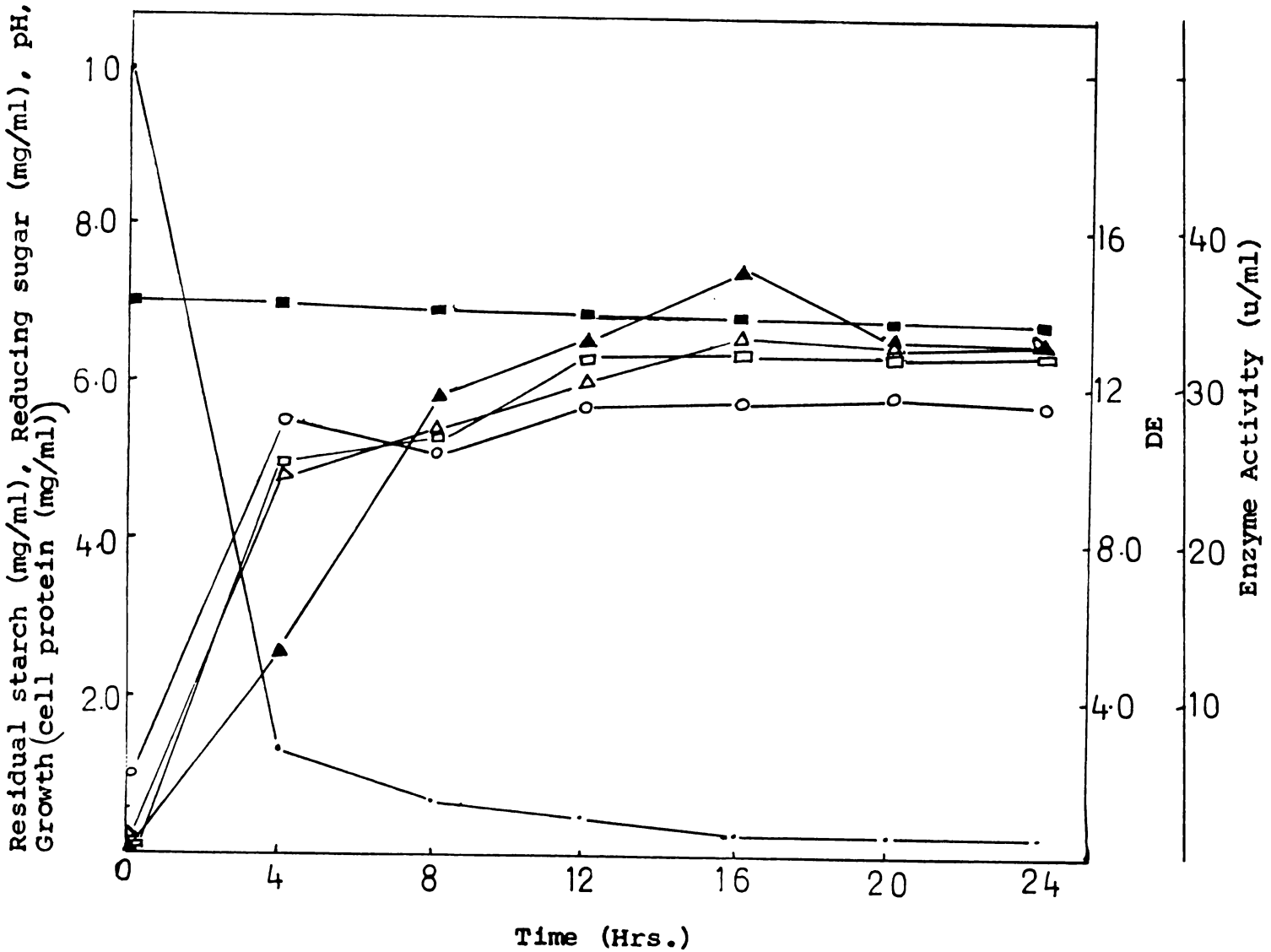


Fig.19 : Fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 30°C and at an aeration rate of 2 vvm for 24 hrs.

—•— Residual Starch ; —□— Growth : —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE : —■— pH

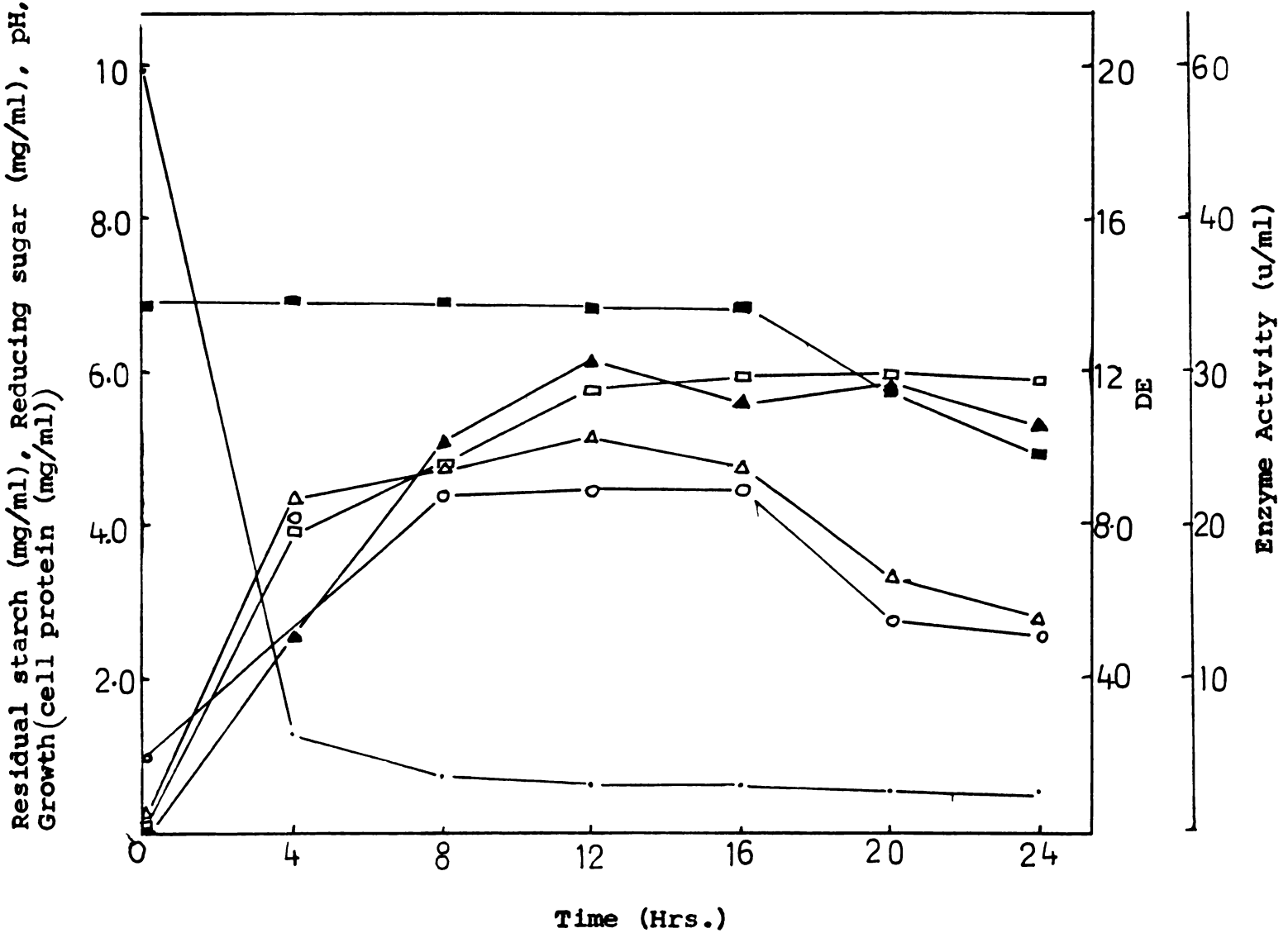


Fig.20 : Fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 30°C and at an aeration rate of 2 vvm for 12 hrs.

-·-Residual Starch ; -□- Growth : -▲- Enzyme Activity
-Δ- Reducing Sugar ; -o- DE : -■- pH

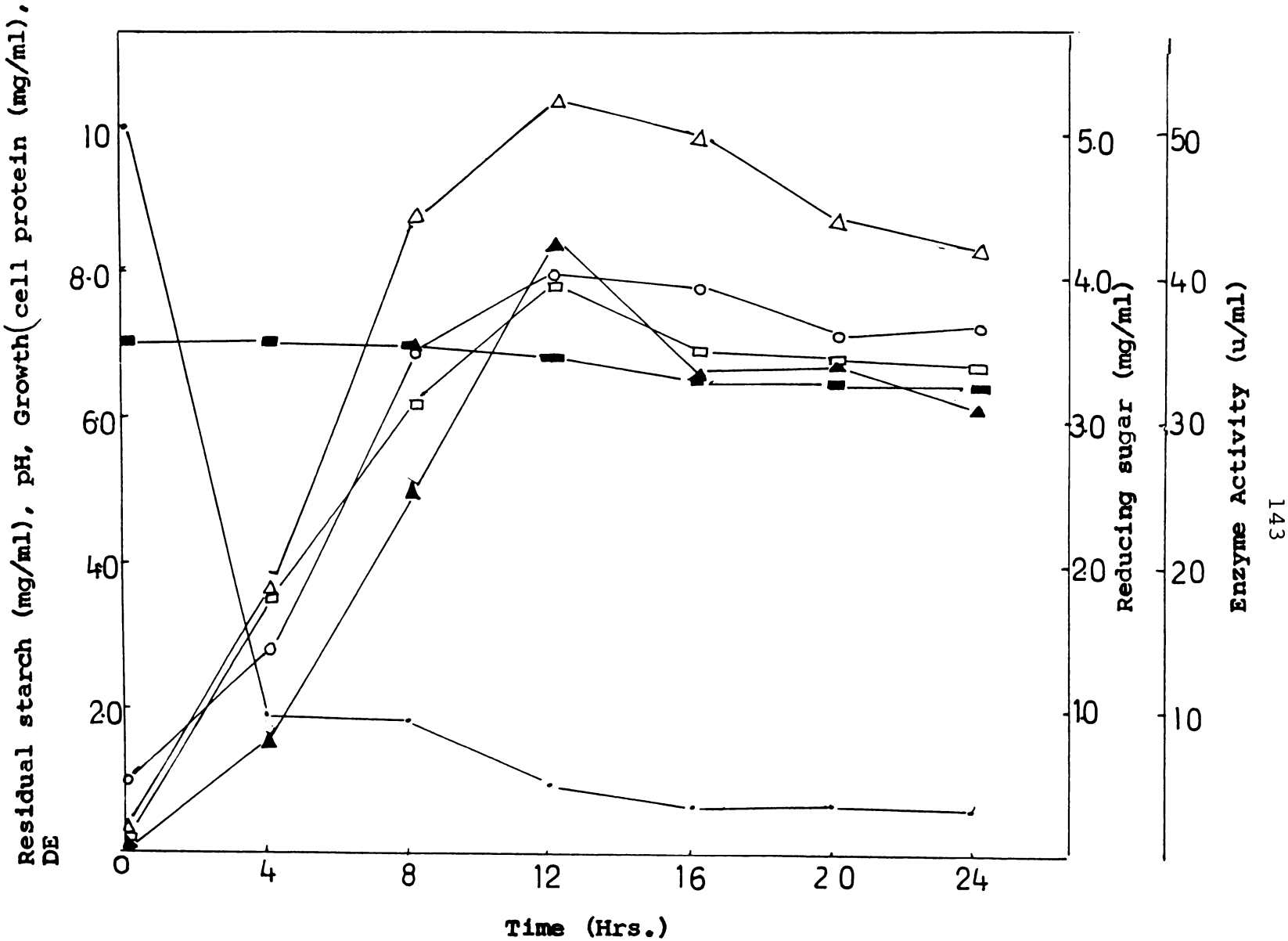


Fig.21 : Fermentation of rice decanted water by B. coagulans ACMN 1 at pH 7; 30°C and at an aeration rate of 1 vvm.

—•— Residual Starch ; —□— Growth : —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE : —■— pH

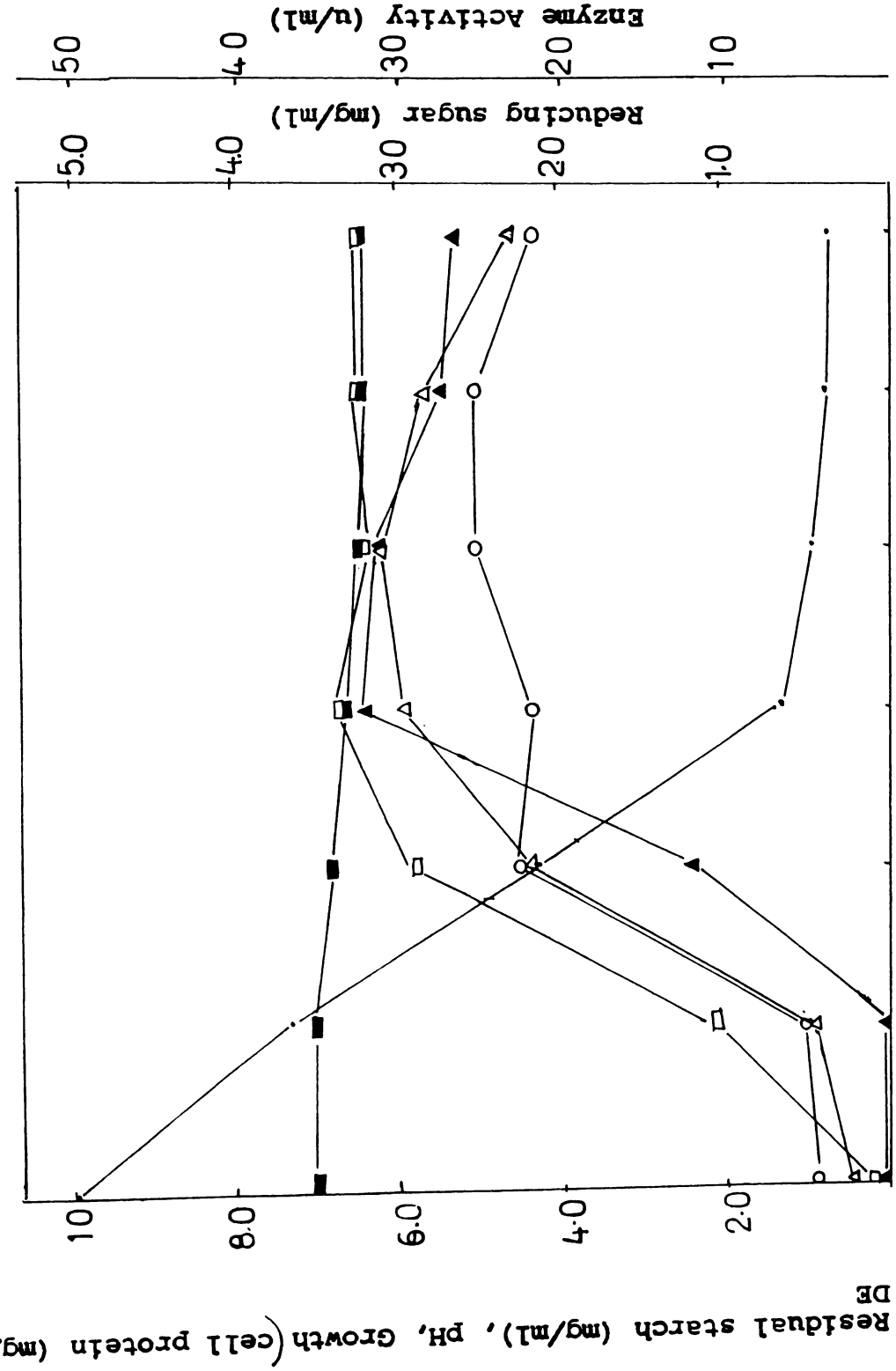


Fig.22 : Fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and at an aeration rate of 1 vvm.

-•- Residual Starch ; -□- Growth ; -▲- Enzyme Activity
-△- Reducing Sugar ; -●- DE ; -■- pH

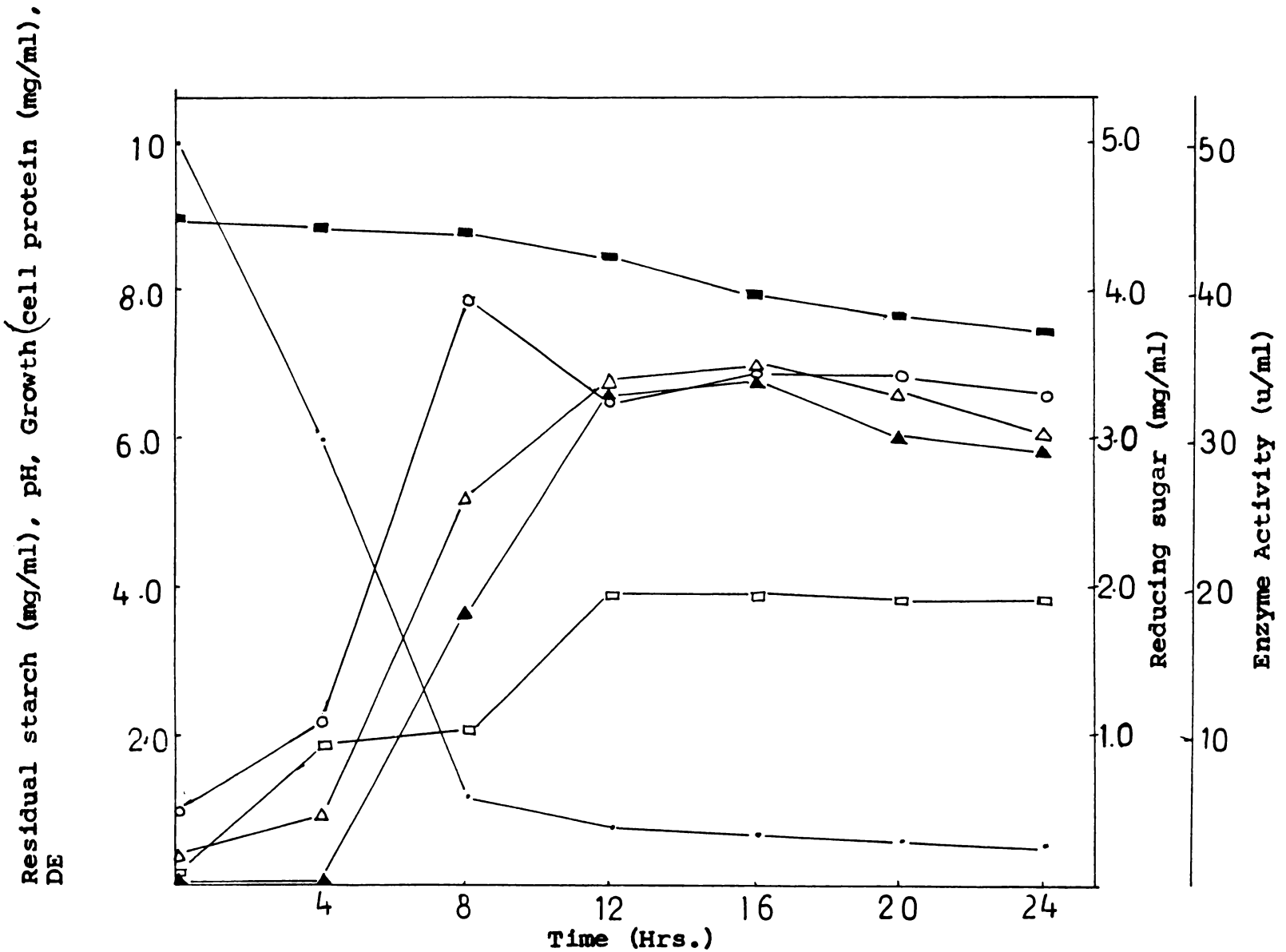


Fig.23 : Fermentation of rice starch by B. coagulans ACMN 42 at pH 9; 30°C and at an aeration rate of 1 vvm.

—•— Residual Starch ; —□— Growth : —▲— Enzyme Activity
– △ – Reducing Sugar ; —o— DE : —■— pH

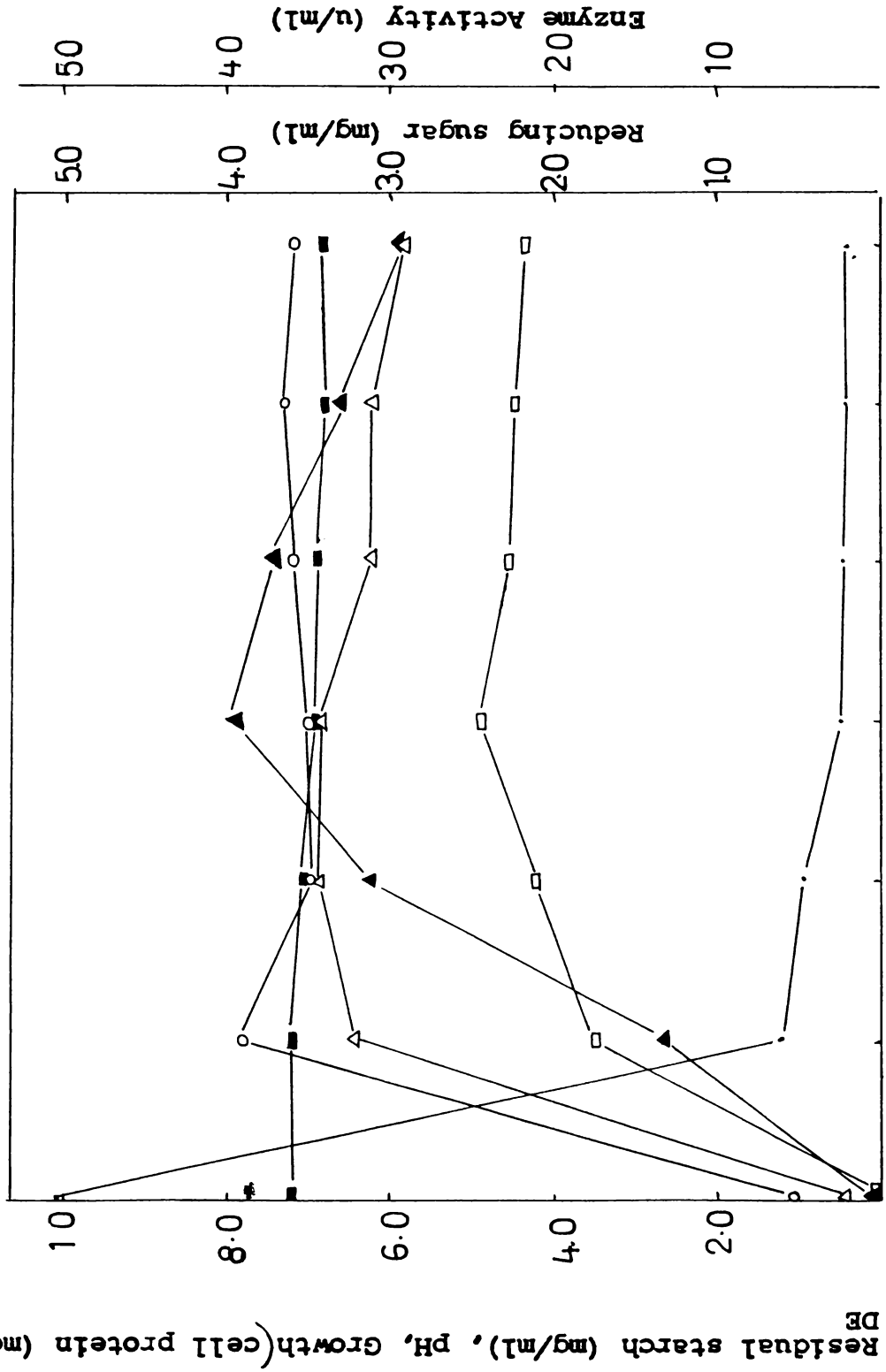


Fig.24 : Fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 35°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

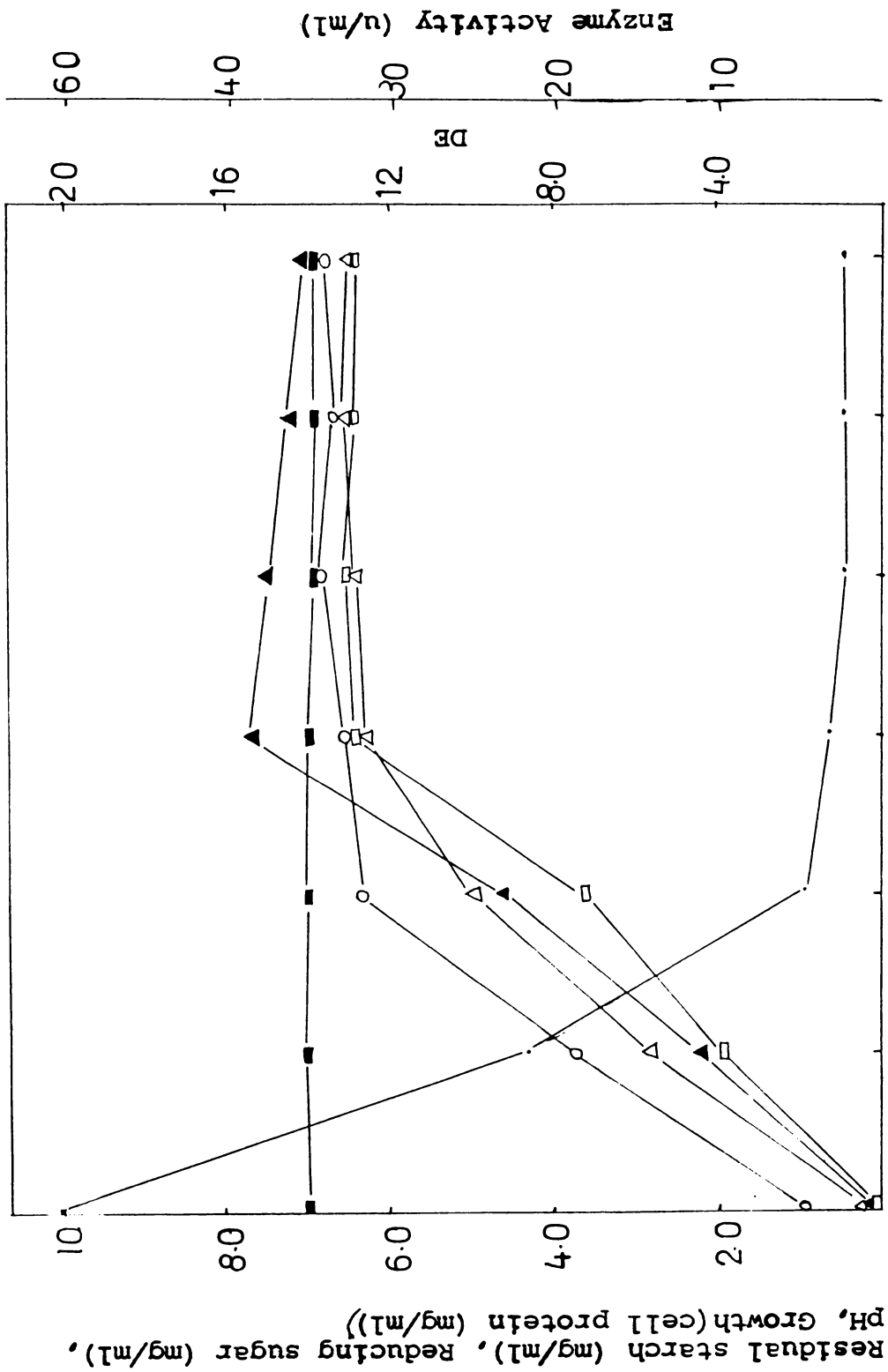


Fig.25 : Fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and at an aeration rate of 2 vvm for 24 hrs.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —o— DE ; —■— pH

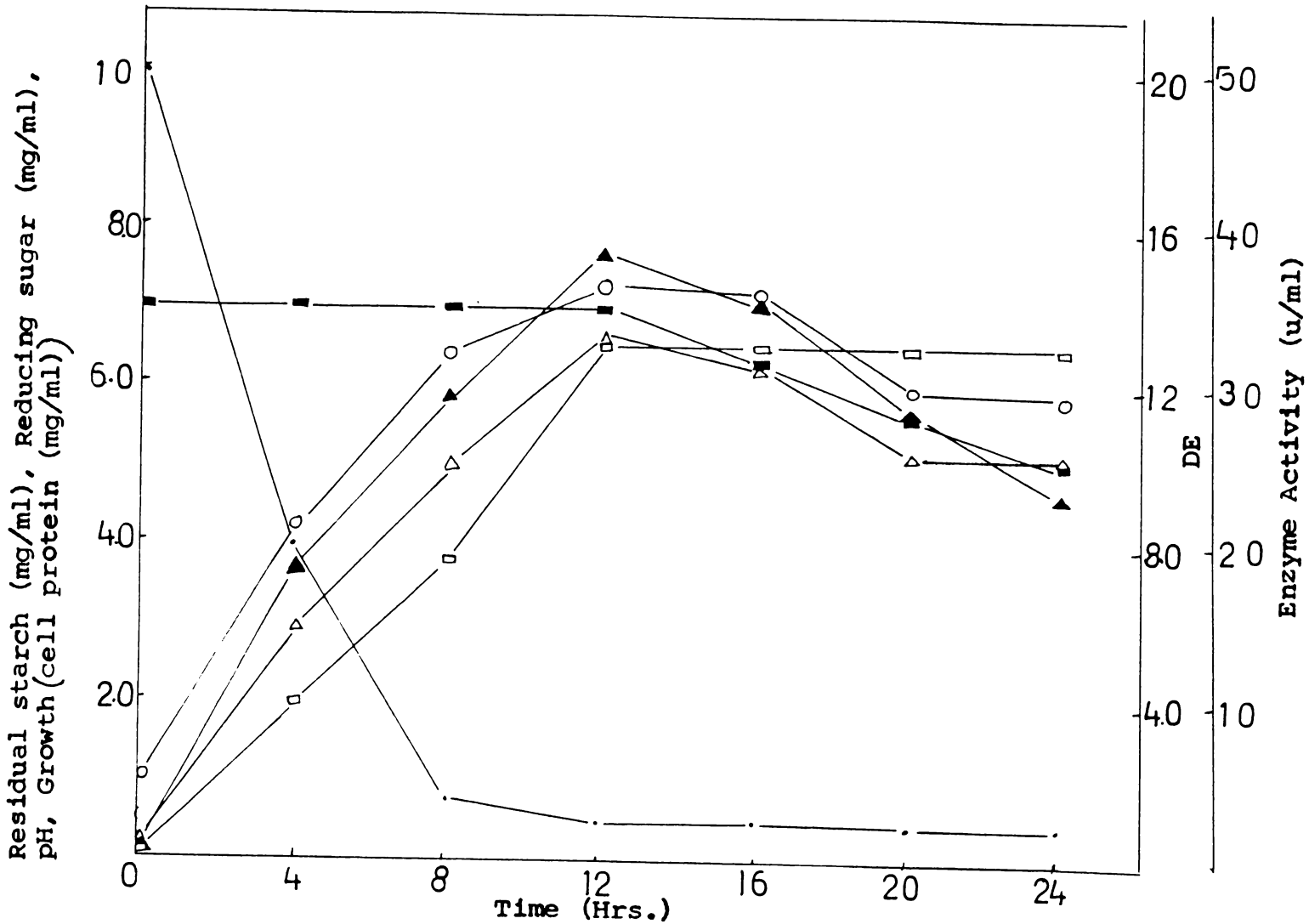


Fig.26 : Fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and at an aeration rate of 2 vvm for 12 hrs.

—•— Residual Starch ;— □ — Growth ; — ▲ — Enzyme Activity
-△- Reducing Sugar ; — ○ — DE ; — ■ — pH

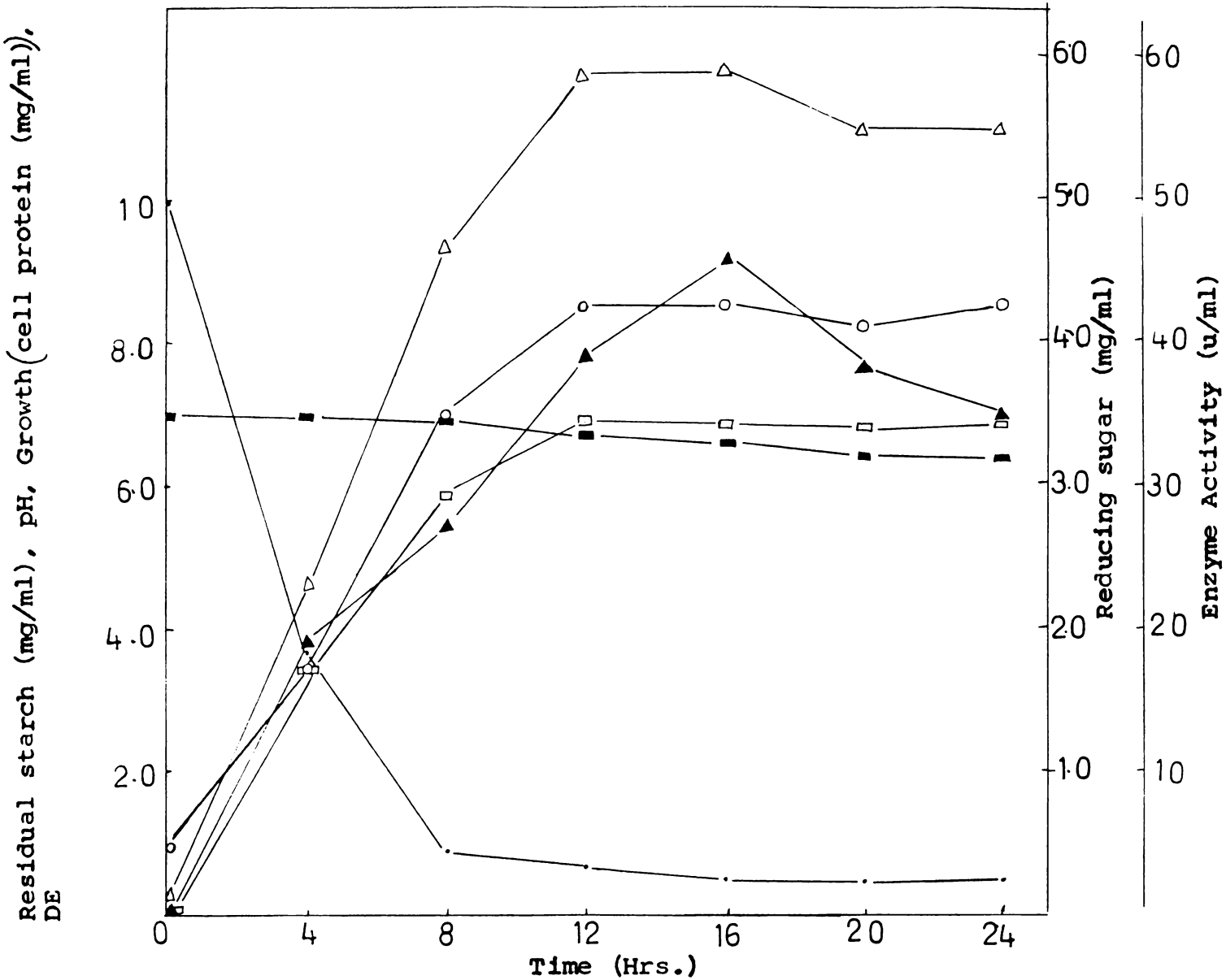


Fig.27 : Fermentation of rice decanted water by B. coagulans ACMN 42 at pH 7; 30°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

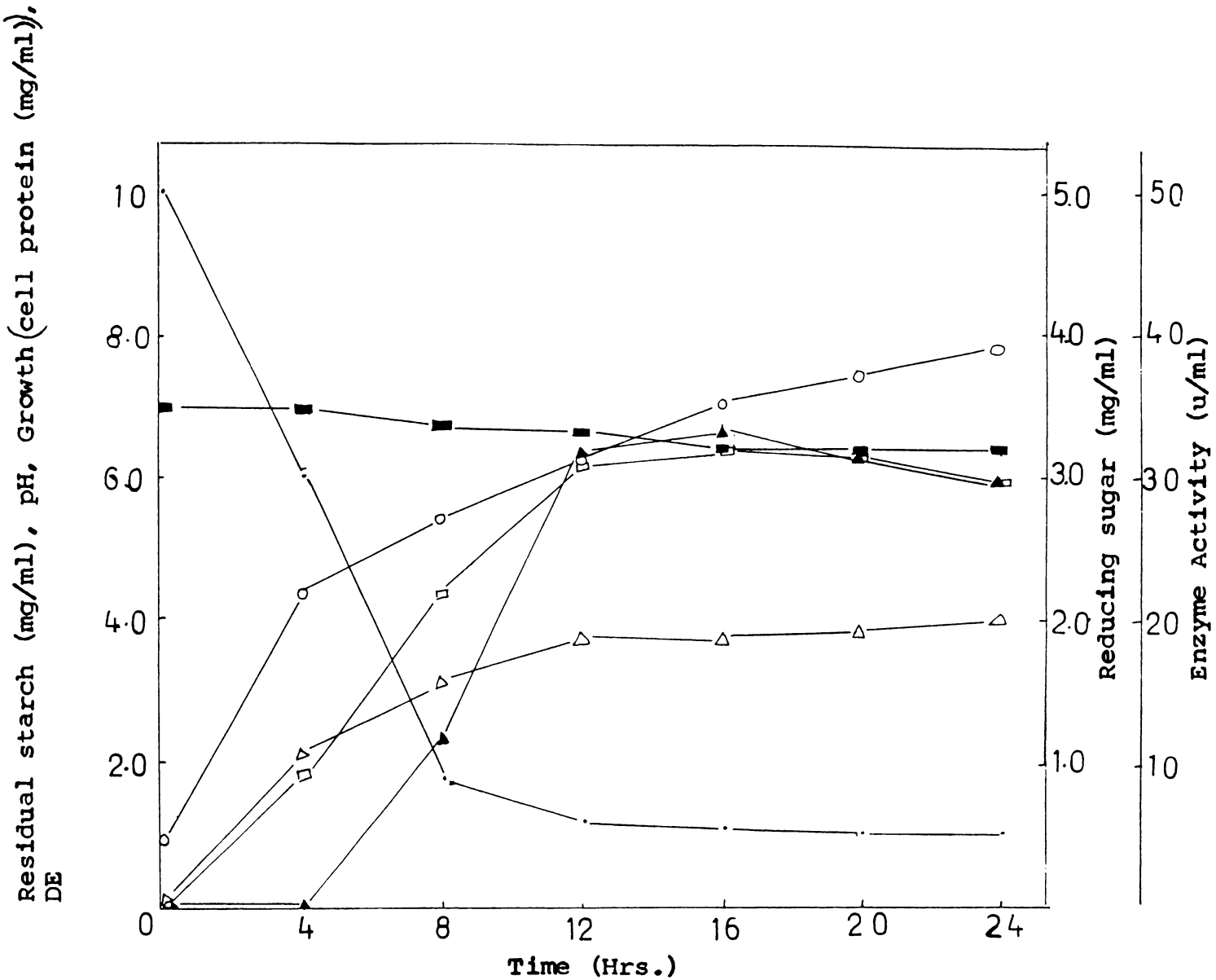


Fig.28 : Fermentation of rice starch by B polymyxa ACMN 25 at pH 7; 30°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —○— DE ; —■— pH

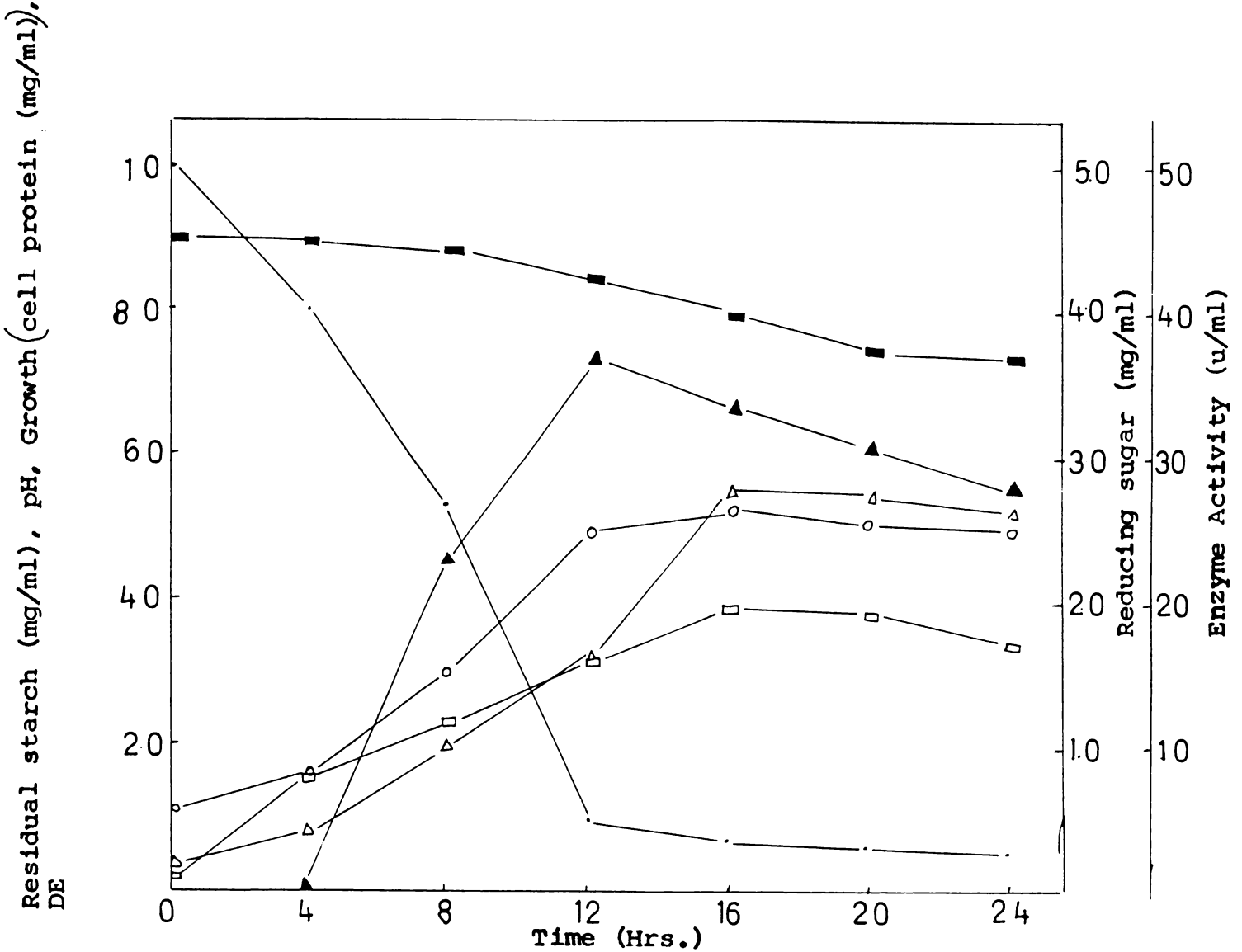


Fig.29 : Fermentation of rice starch by B polymyxa ACMN 25 at pH 9; 30°C and at an aeration rate of 1 vvm.

—•— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

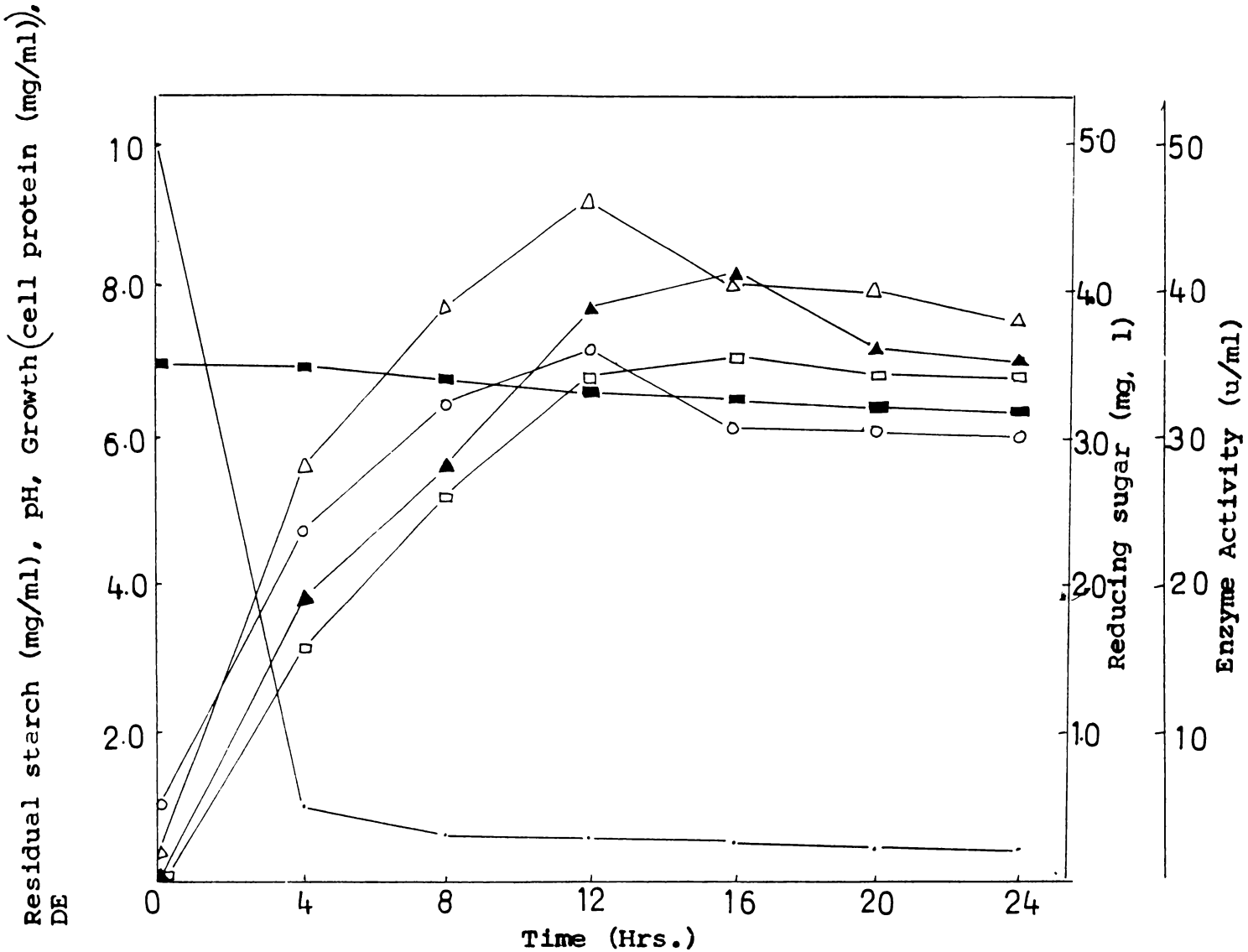


Fig.30 : Fermentation of rice starch by B polymyxa ACMN 25 at pH 7; 35°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —○— DE ; —■— pH

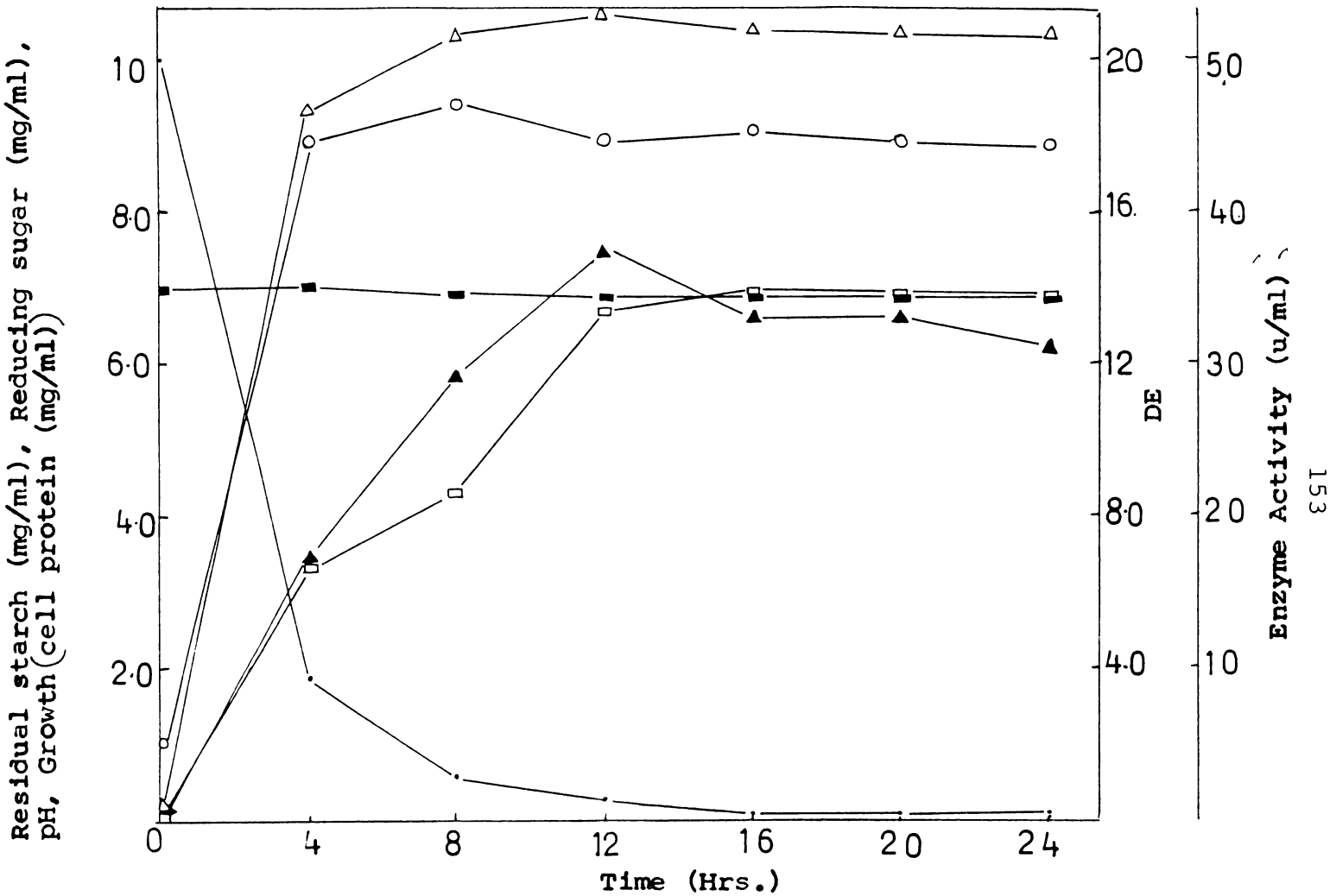


Fig.31 : Fermentation of rice starch by B polymyxa ACMN 25 at pH 7; 30°C and at an aeration rate of 2 vvm for 24 hrs.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

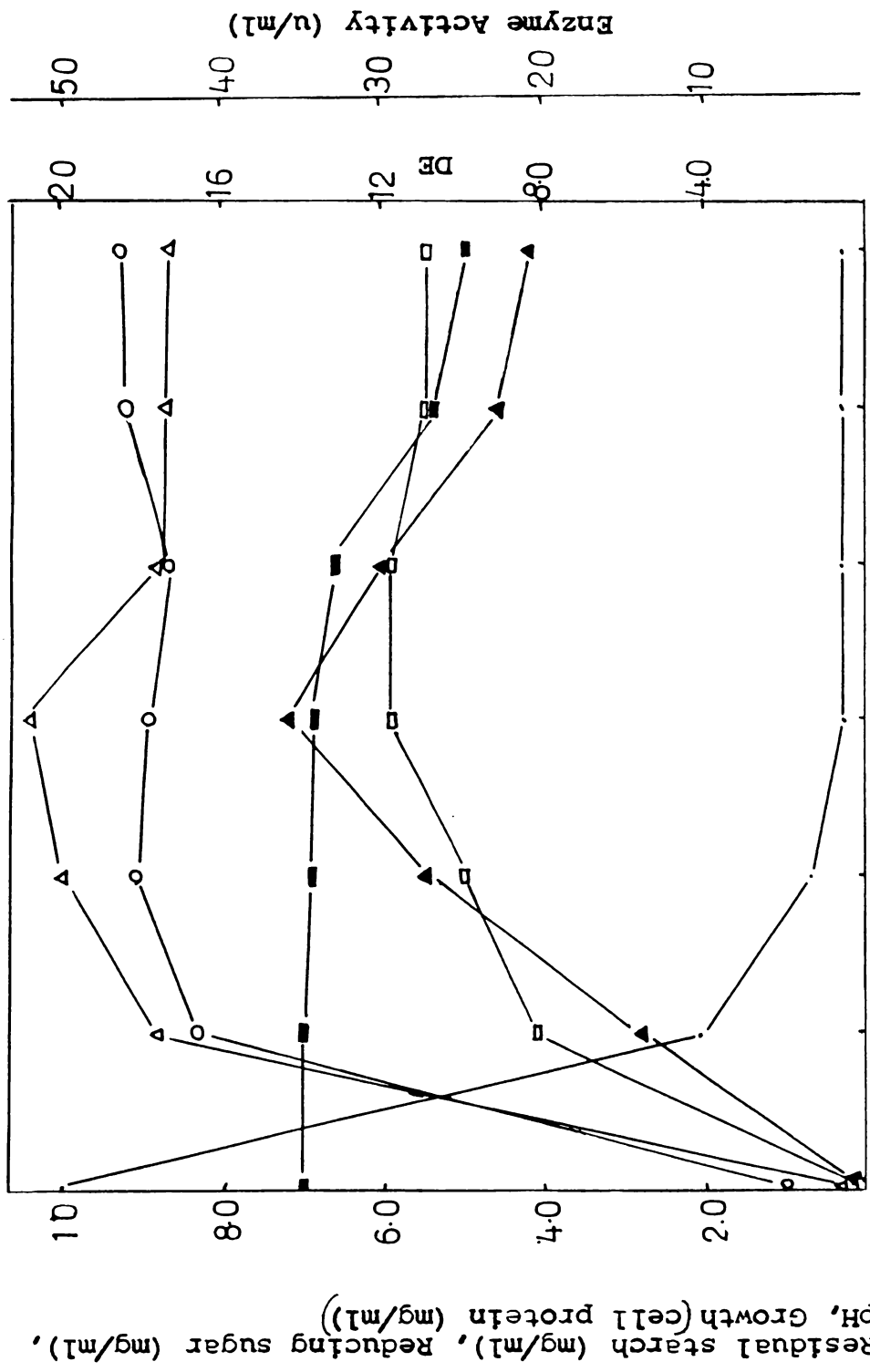


Fig.32 : Fermentation of rice starch by B polymyxa ACMN 25 at pH 7; 30°C and at an aeration rate of 2 vvm for 12 hrs.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —○— DE ; —■— pH

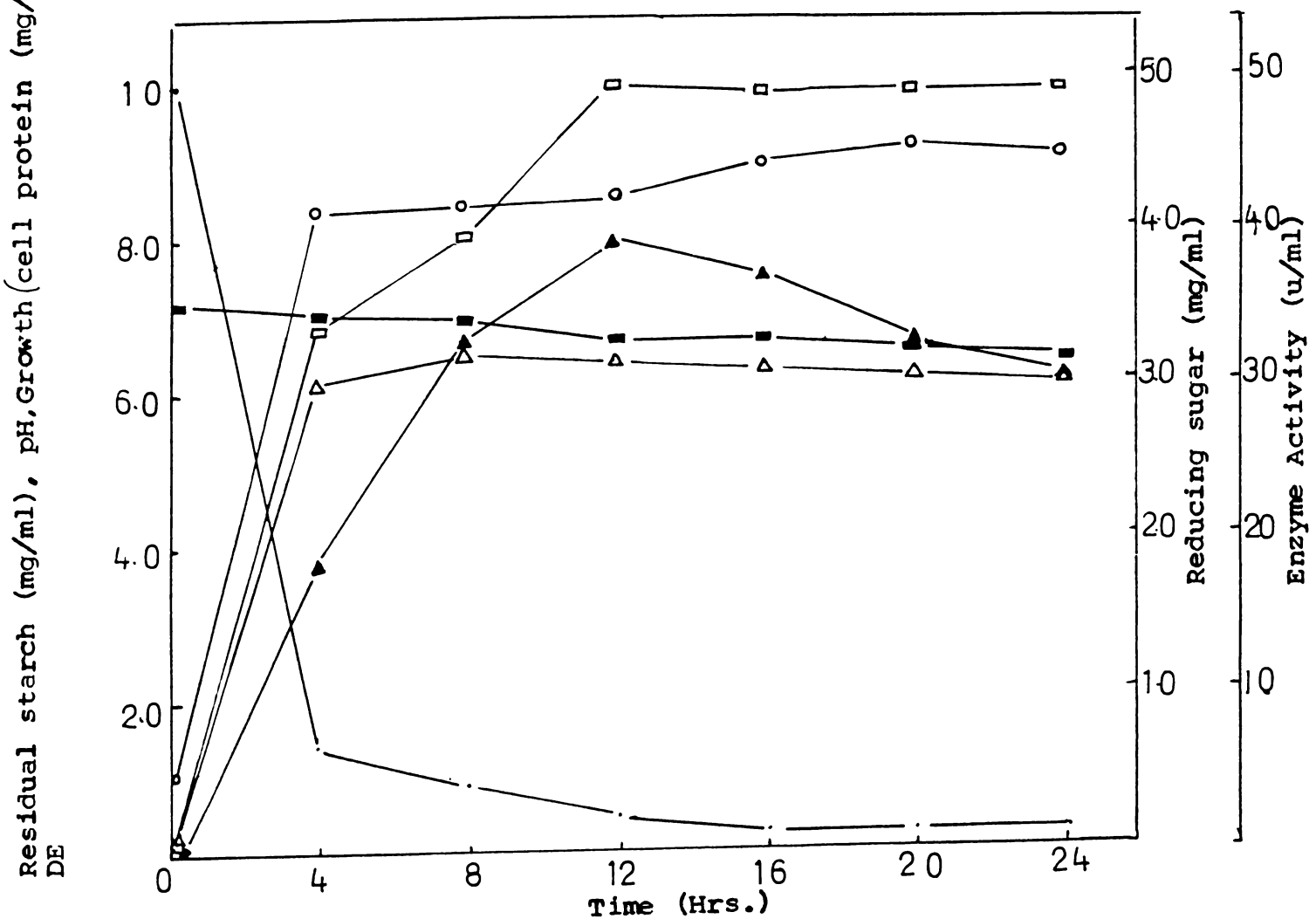


Fig.33 : Fermentation of rice decanted water by B polymyxa ACMN 25 at pH 7; 30°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

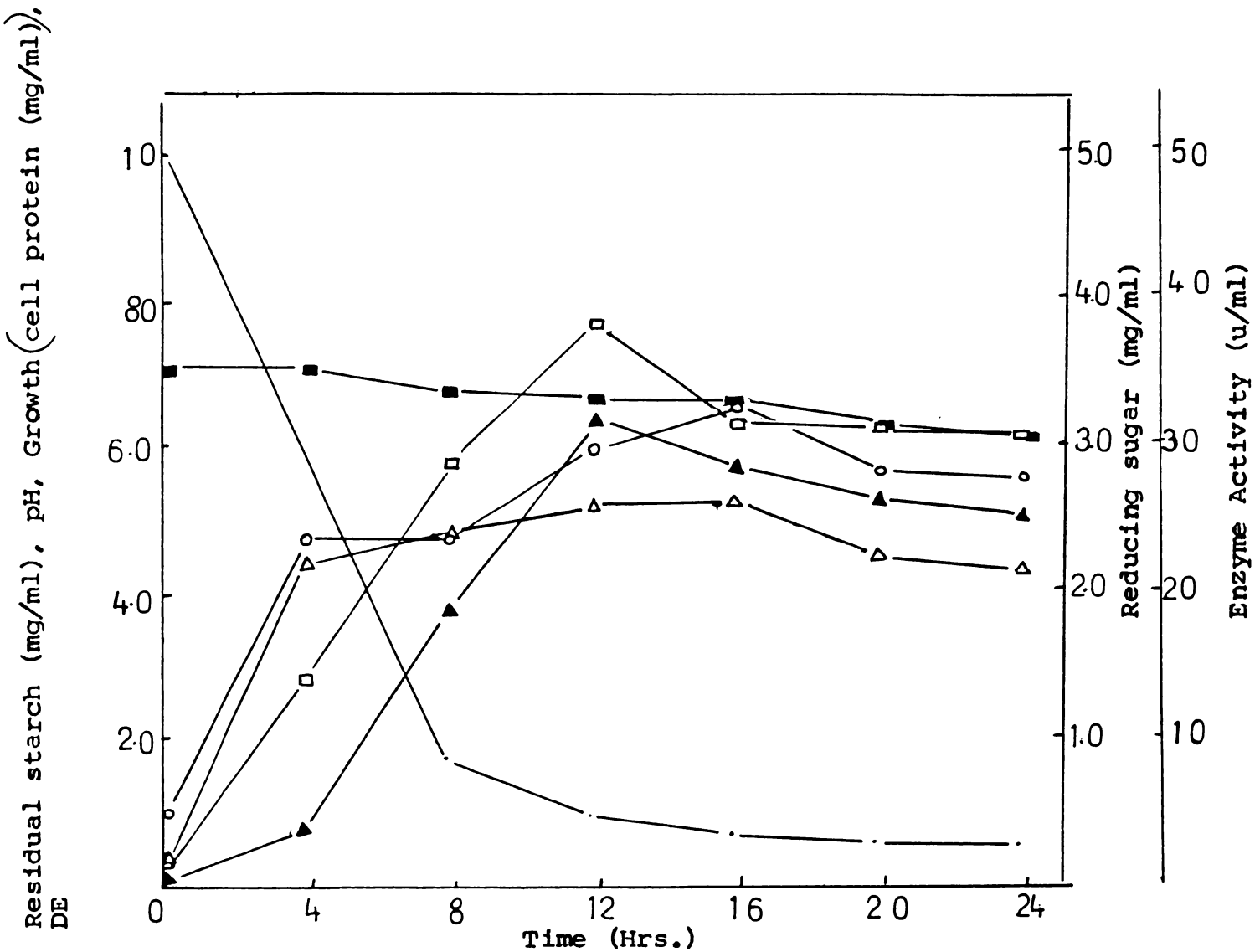


Fig.34 : Fermentation of rice starch by B cereus ACMN 33 at pH 7; 30°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing sugar ; —○— DE ; —■— pH

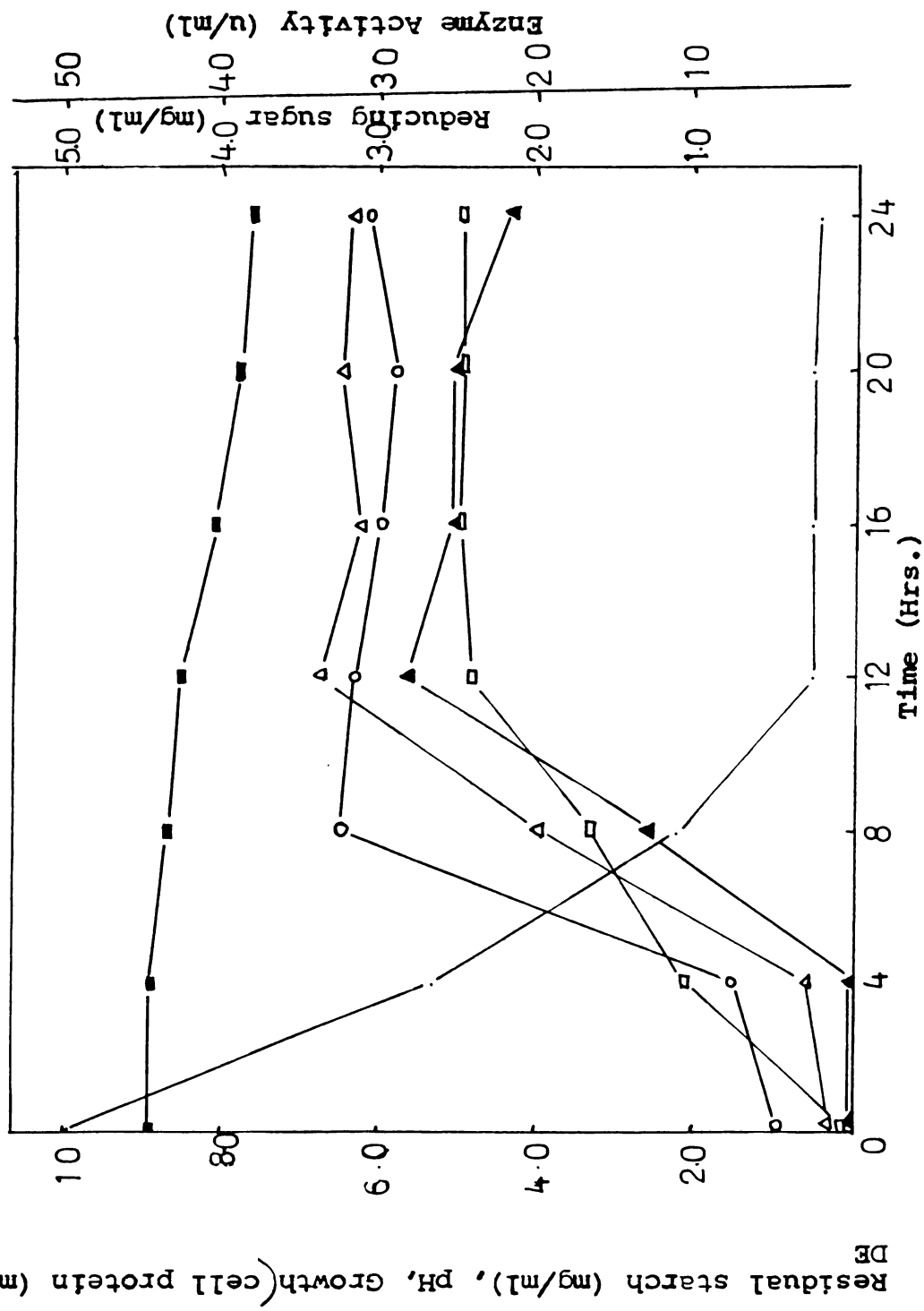


Fig.35 : Fermentation of rice starch by B cereus ACMN 33 at pH 9; 30°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —○— DE ; —■— pH

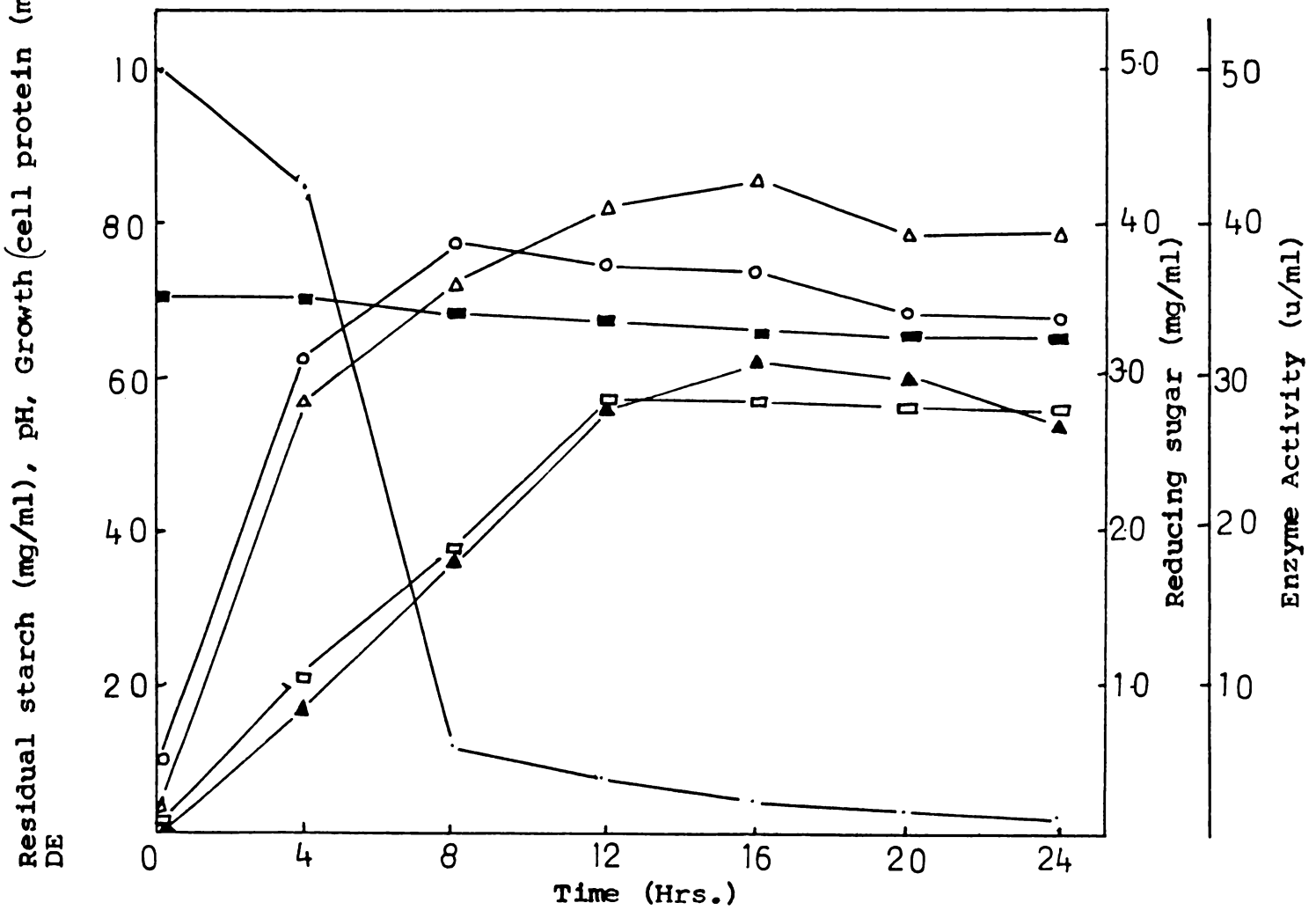


Fig.36 : Fermentation of rice starch by B. cereus ACMN 33 at pH 7; 35°C and at an aeration rate of 1 vvm.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

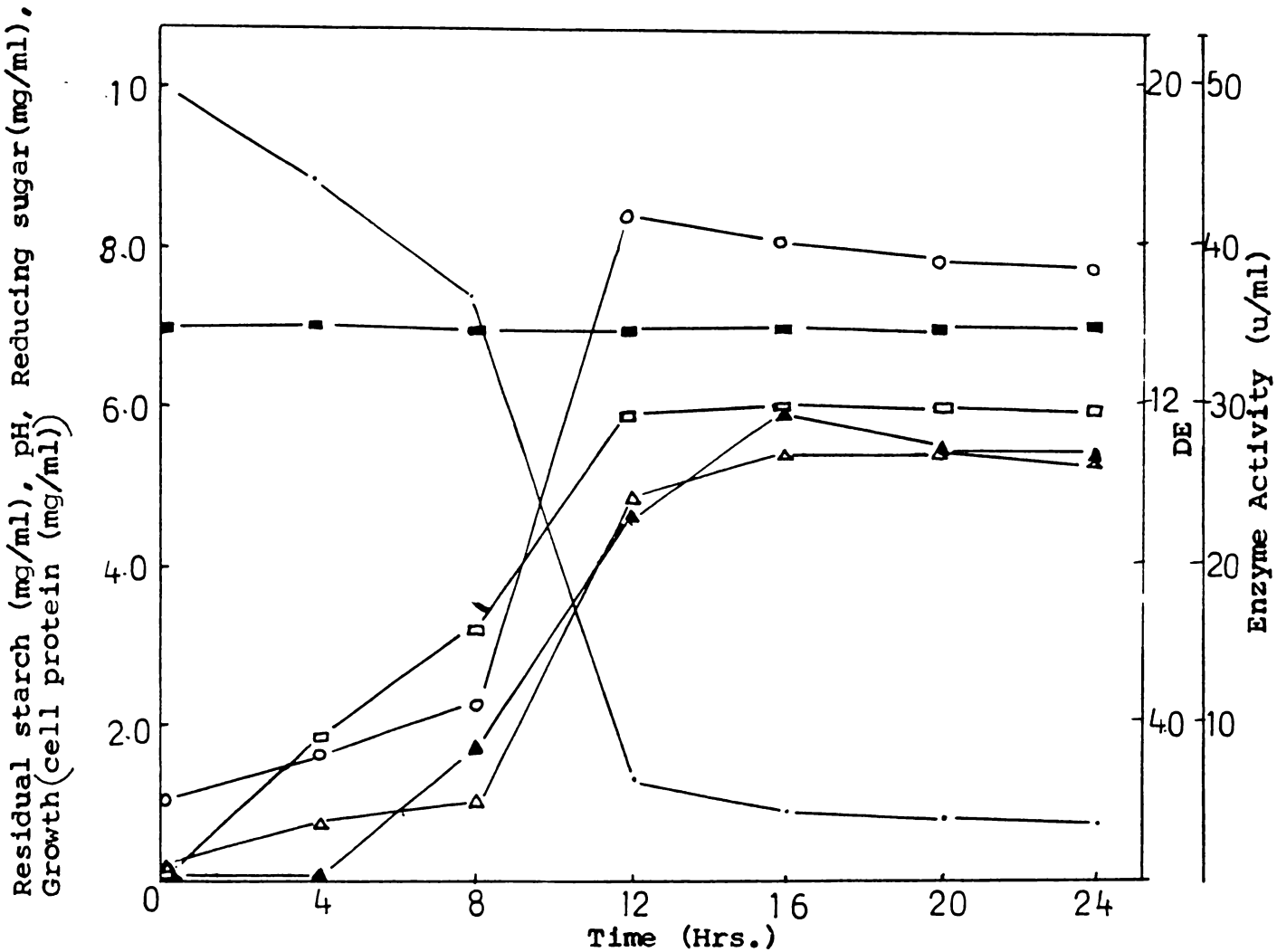


Fig.37 : Fermentation of rice starch by B. cereus ACMN 33 at pH 7; 30°C and at an aeration rate of 2 vvm for 24 hrs.

—•— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —○—DE ; —●— pH

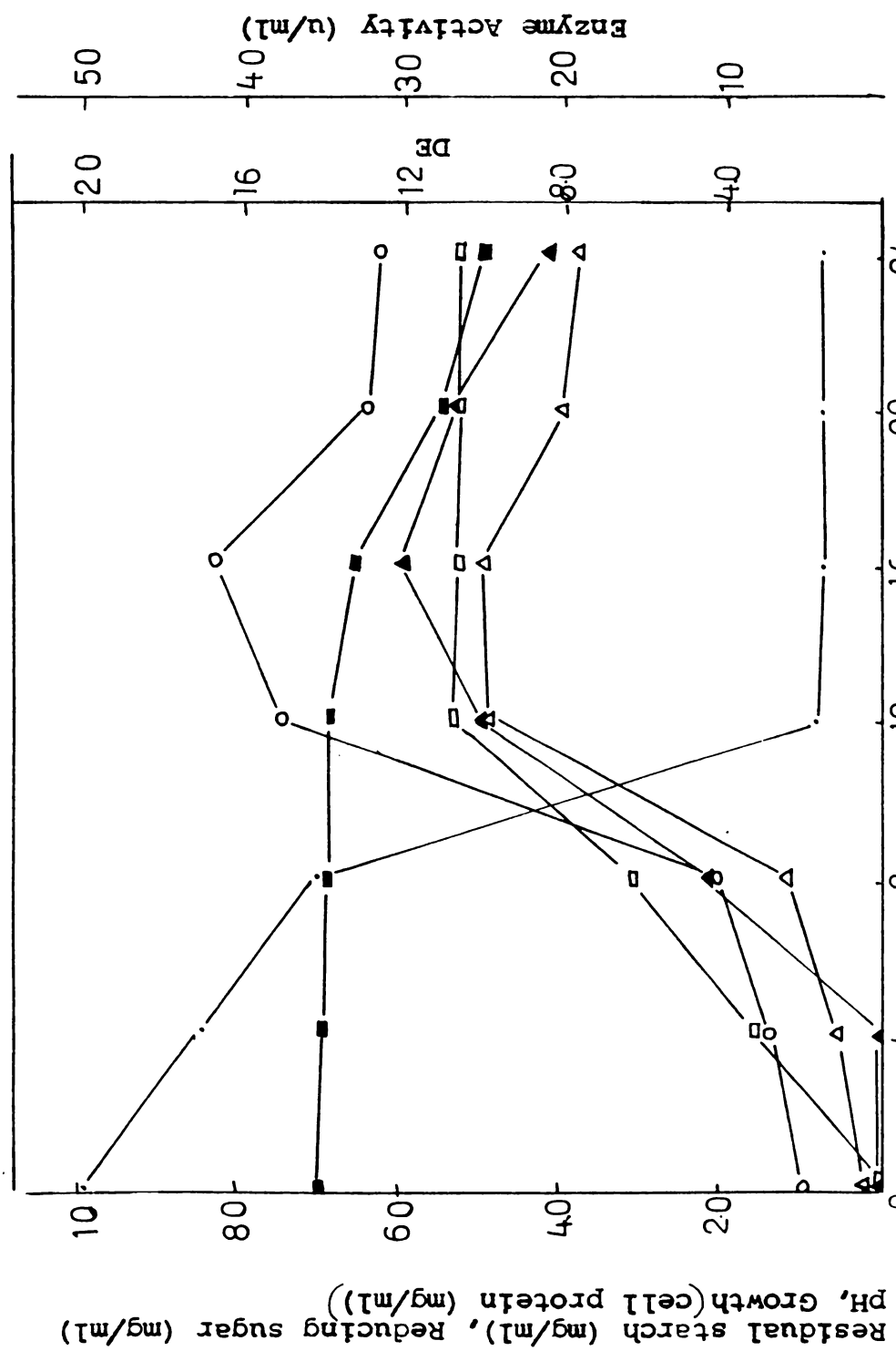


Fig.38 : Fermentation of rice starch by B. cereus ACMN 33 at pH 7; 30°C and at an aeration rate of 2 vvm for 12 hrs.

—·— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—△— Reducing Sugar ; —○— DE ; —■— pH

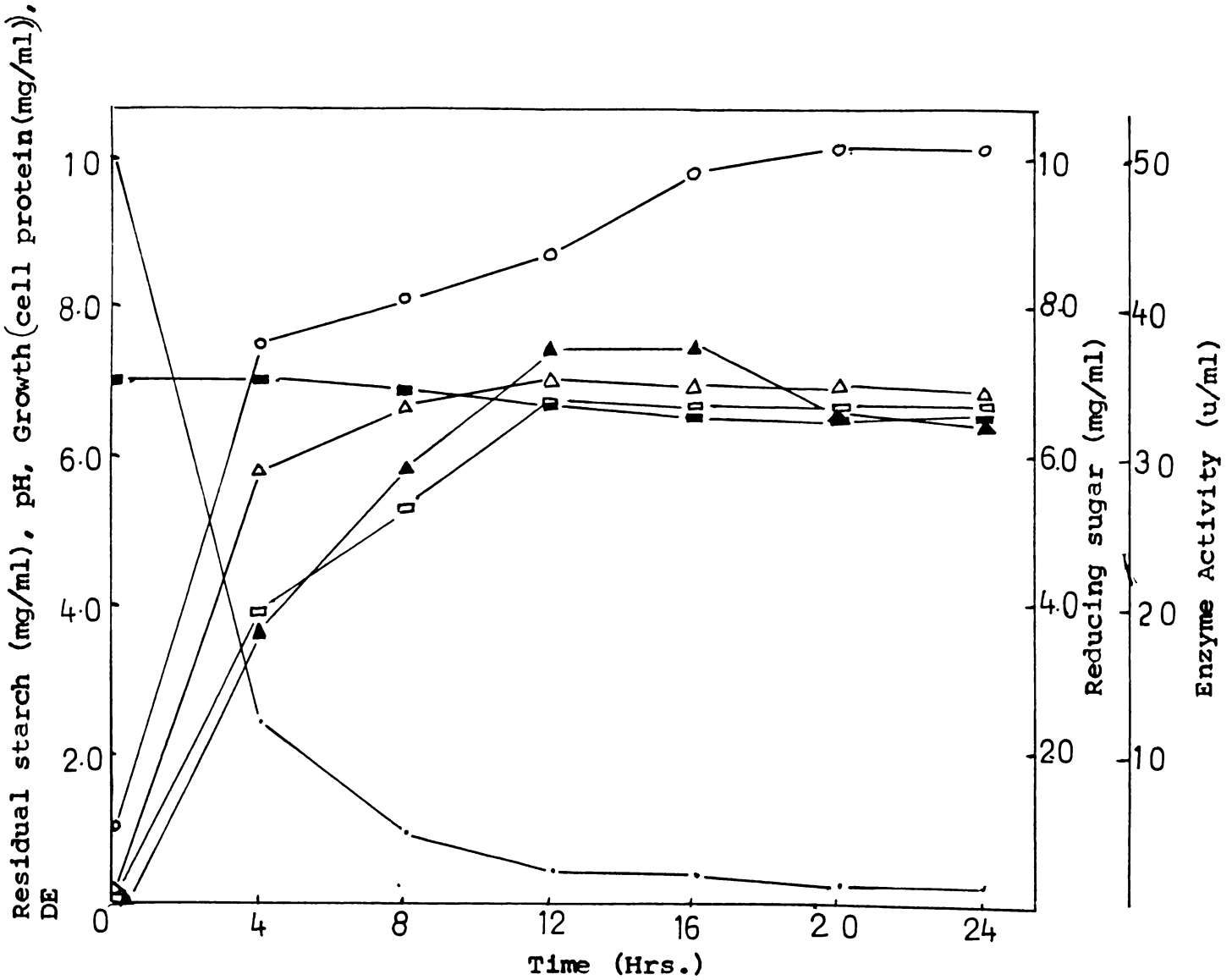


Fig.39 : Fermentation of rice decanted water by B. cereus ACMN 33 at pH 7; 30°C and at an aeration rate of 1 vvm.

—•— Residual Starch ; —□— Growth ; —▲— Enzyme Activity
—Δ— Reducing Sugar ; —o— DE ; —▬— pH

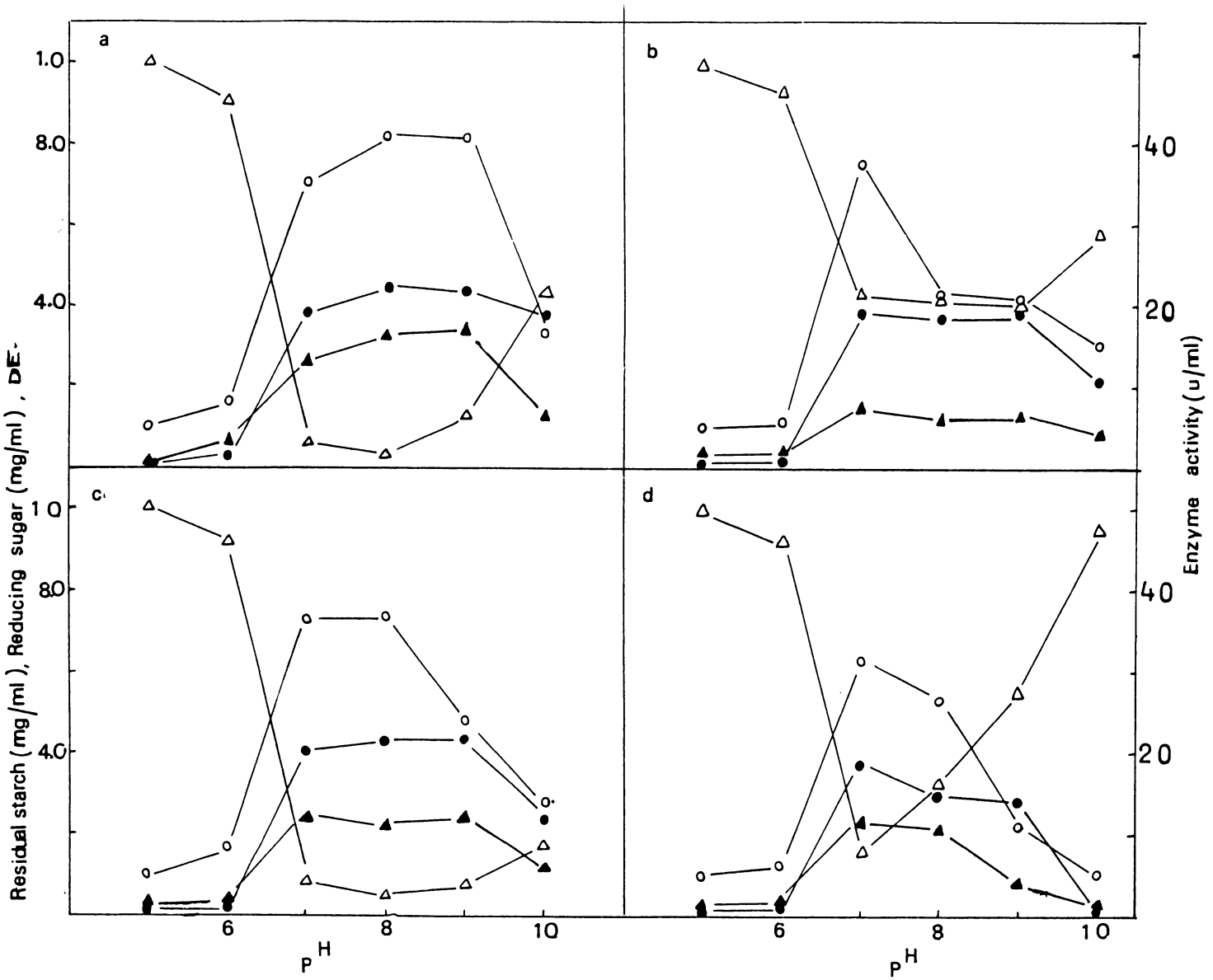


Fig.40 : Effect of pH on rice starch conversion by immobilized whole cells of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

-△- Residual Starch ; -▲- Reducing Sugar ;
-○- DE ; -●- Enzyme Activity

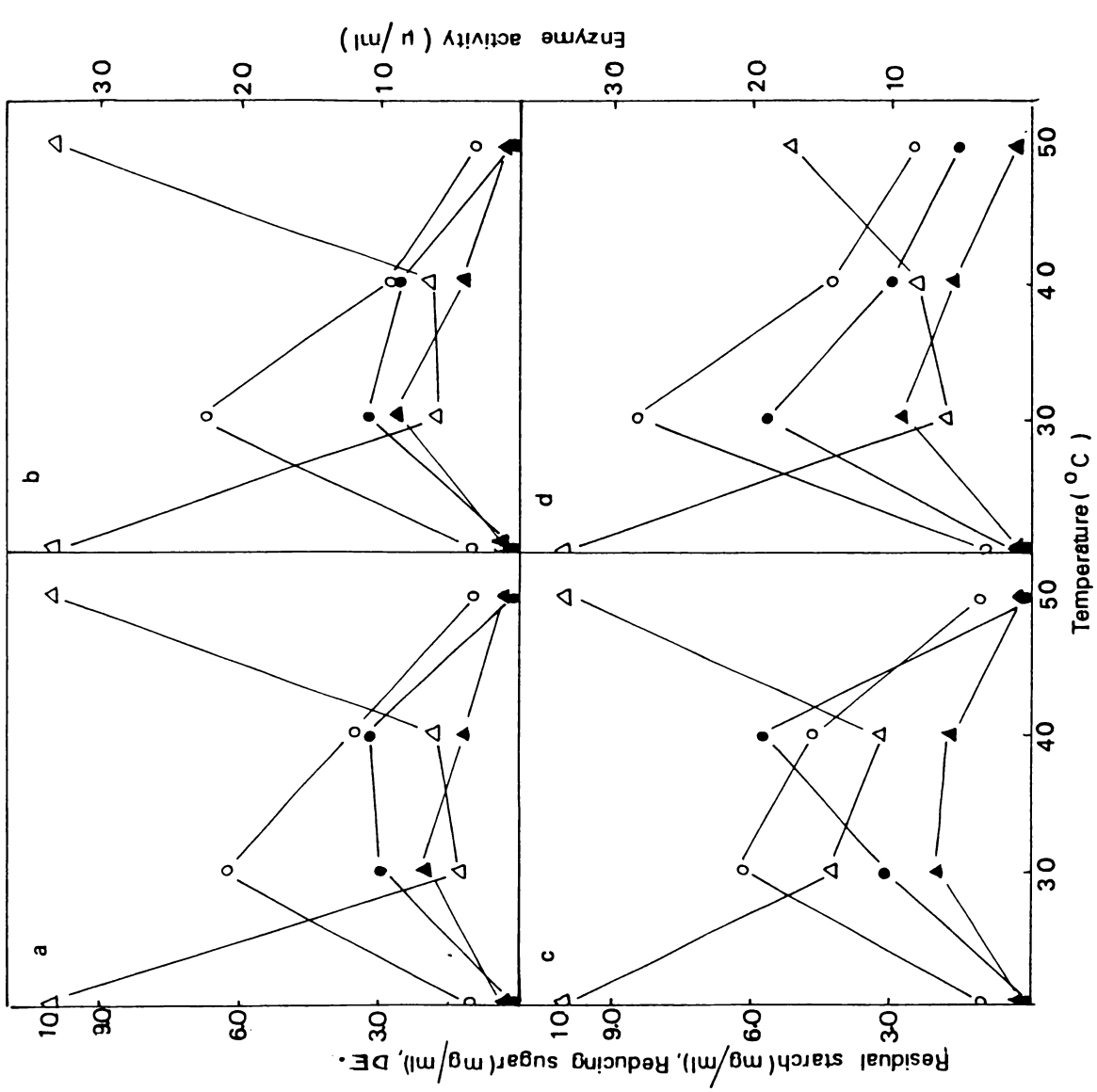


Fig.41 : Effect of temperature of rice starch conversion by immobilized whole cells of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

-△- Residual Starch ; -▲- Reducing Sugar ;
-○- DE ; —●— Enzyme Activity

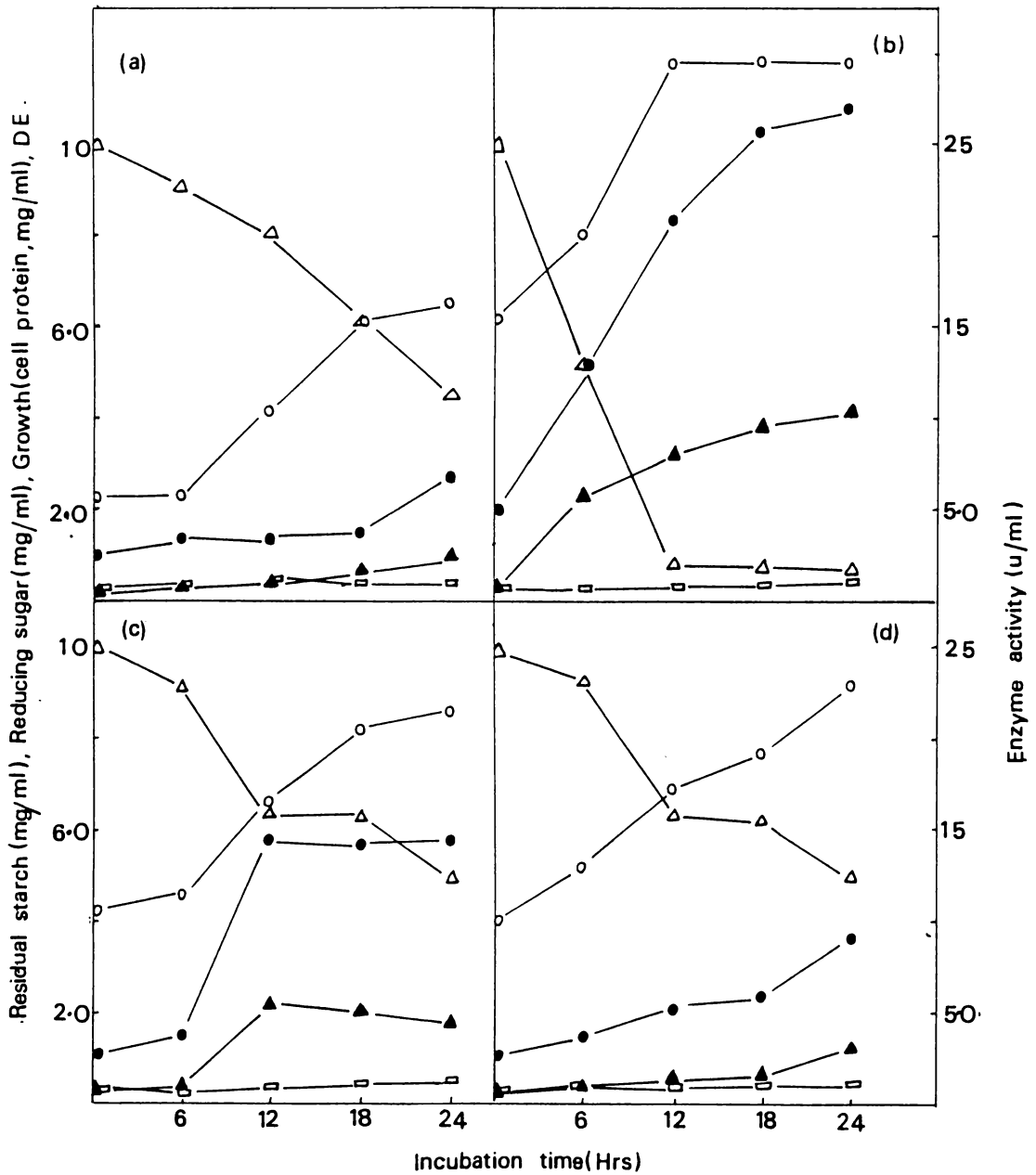


Fig.42 : Effect of incubation time on rice starch conversion by immobilized whole cell of Bacillus sp.

(a) B. coagulans ACMN 1
(b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25
(d) B. cereus ACMN 33

-△- Residual Starch ; -▲- Reducing Sugar ; -□- Growth
-O- DE ; -●- Enzyme Activity

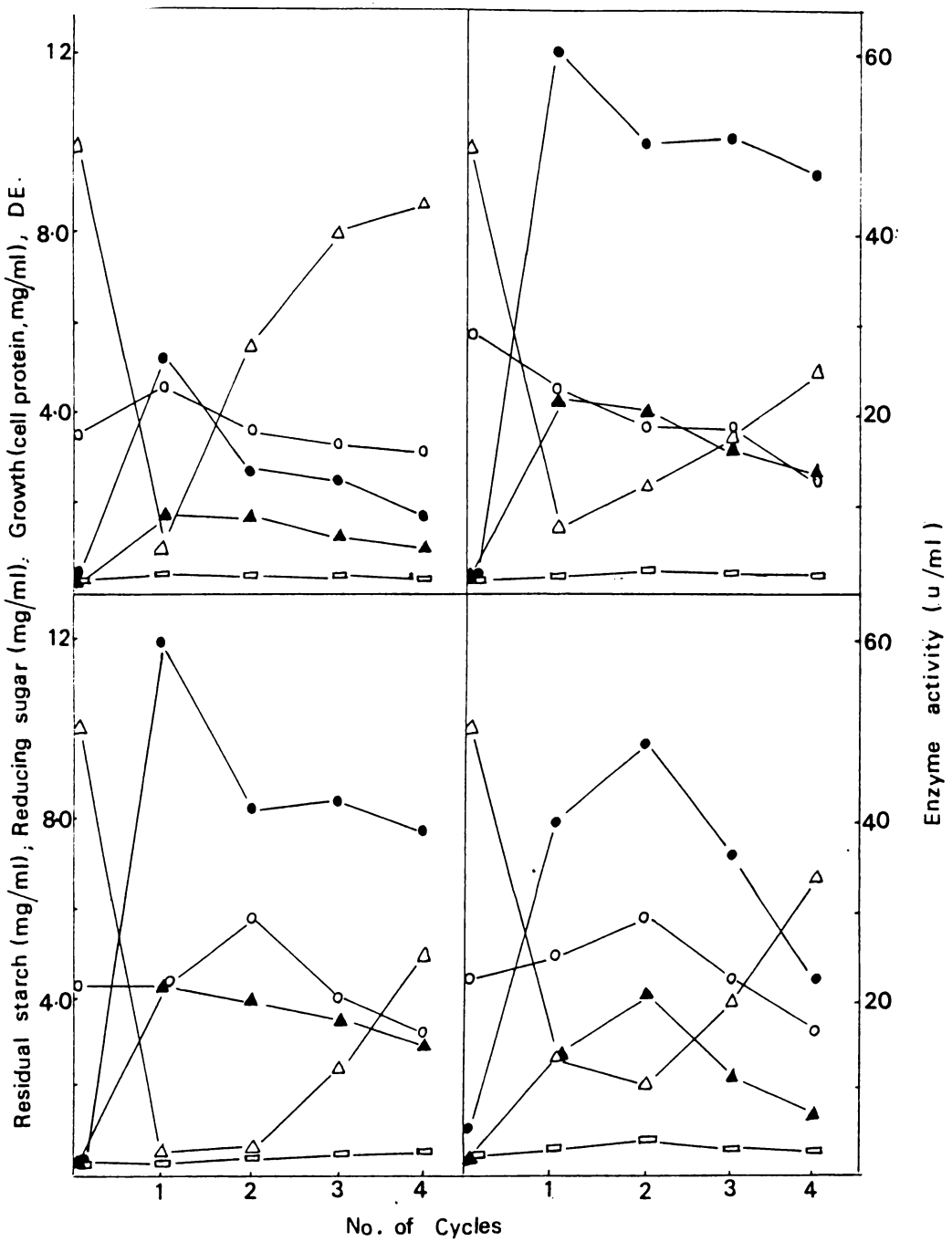


Fig.43 : Reusability of immobilized whole cells of Bacillus sp.
(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33
-△- Residual Starch ; -▲- Reducing Sugar ; -▭- Growth ; -o- DE
-●- Enzyme Activity

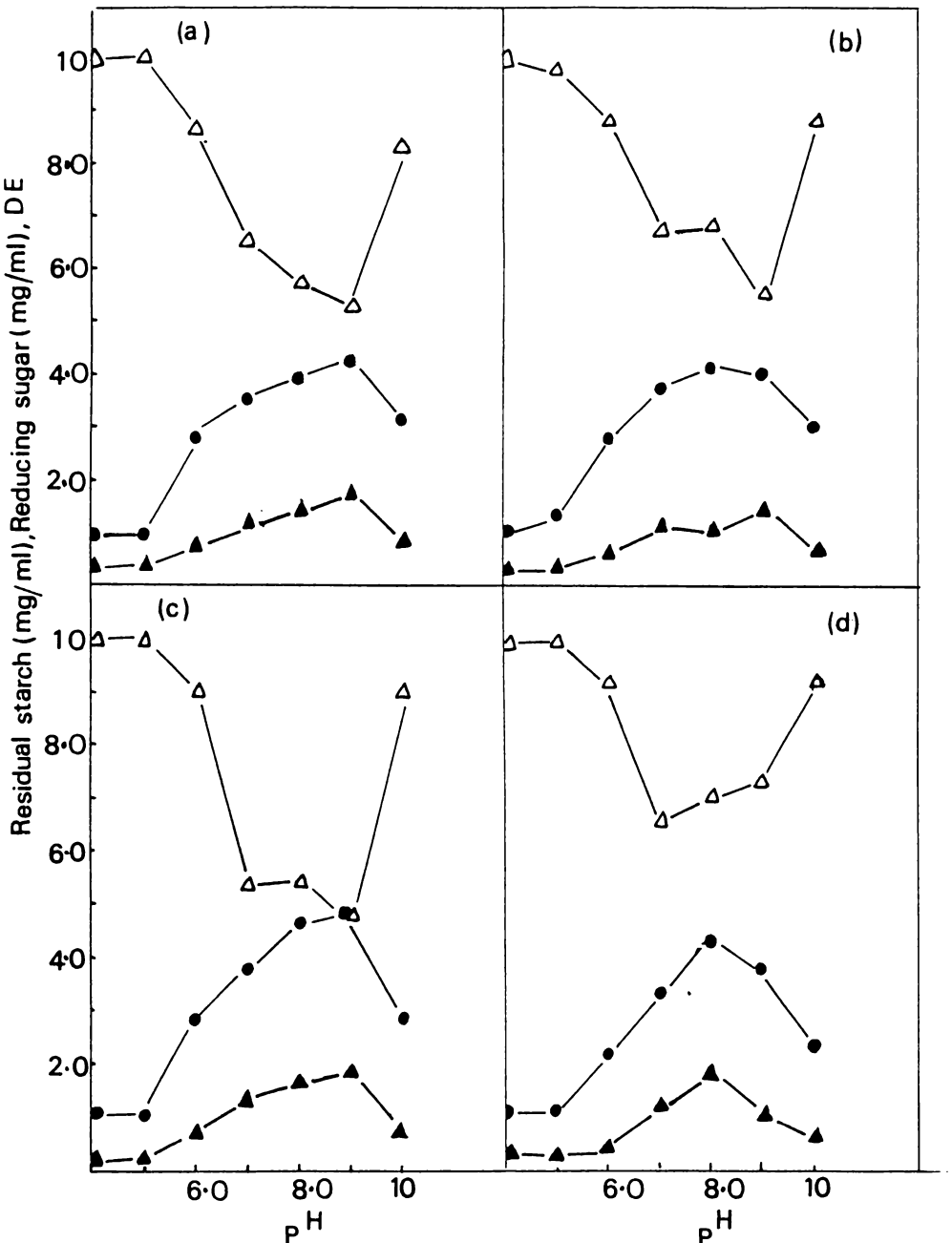


Fig.44 : Effect of pH on the conversion rice starch by immobilized amylase of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

—Δ— Residual Starch ; —▲— Reducing Sugar ; —●— DE

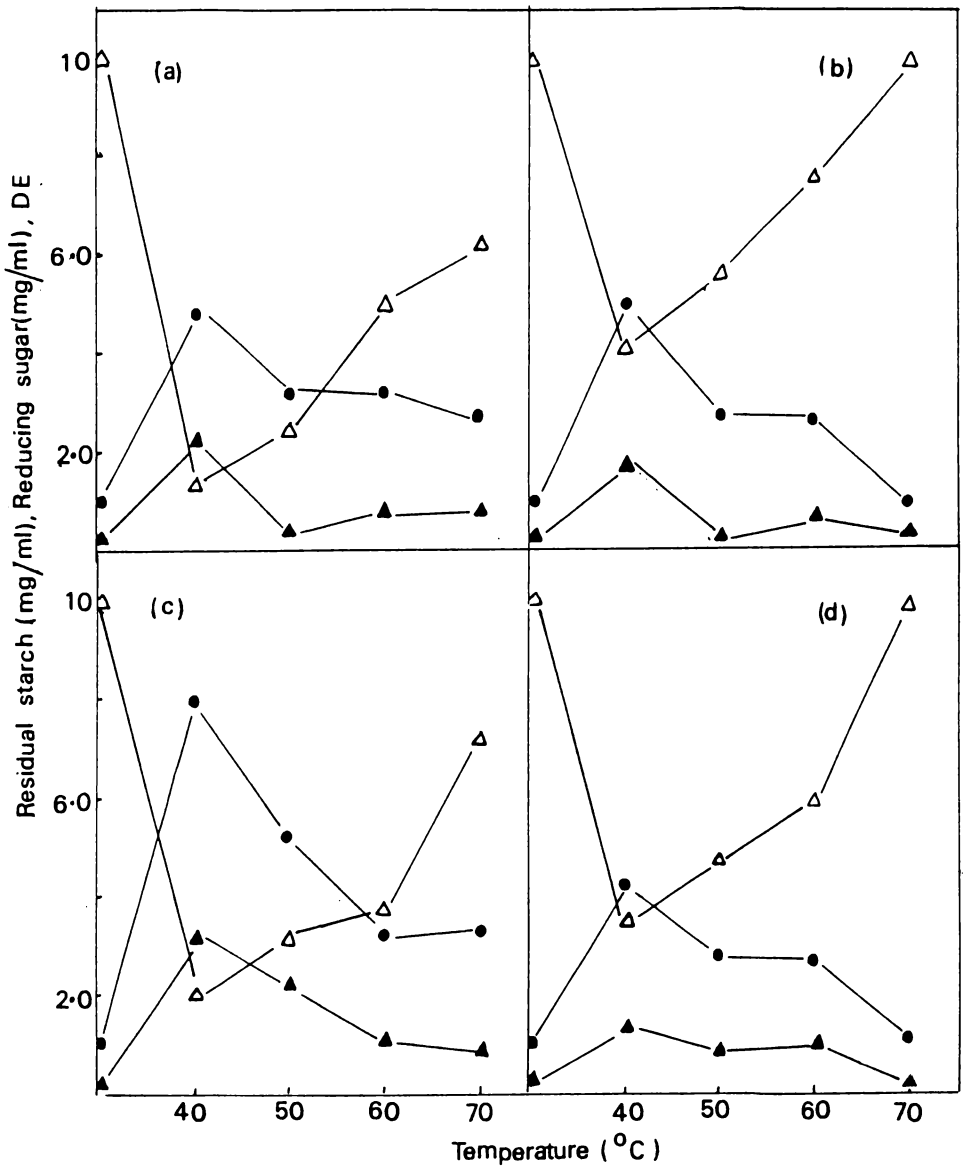


Fig.45: Effect of temperature on the conversion of rice starch by immobilized amylase of Bacillus sp.

(a) B. coagulans ACMN 1 (b) B. coagulans ACMN 42
(c) B. polymyxa ACMN 25 (d) B. cereus ACMN 33

-Δ- Residual Starch ; -▲- Reducing Sugar ; -●- DE

Table 1 : Chemical composition of different kinds of rice

	Raw rice			Parboiled rice		
	Husked	Homepounded	Undermilled	Milled	Homepounded	Milled
Moisture %	9.7	9.6	9.5	9.7	12.6	13.3
Protein %	7.7	7.3	7.2	6.9	8.5	6.4
Fat %	1.8	1.2	0.95	0.54	0.6	0.4
Carbohydrates %	78.1	80.1	80.95	82.06	77.4	79.1
Crude fibre %	1.1	0.7	0.5	0.2	--	--
Mineral matter %	1.6	1.1	0.9	0.6	0.9	0.8
Calcium mg/100 g	15.9	13.0	13.0	10.0	10.0	10.0
Phospherous mg/100 g	368.0	182.0	146.0	87.0	280.0	150.0
Iron mg/100 g	4.0	2.8	2.5	2.2	2.8	2.2
Thiamine mg/100 g	360.0	210.0	190.0	105.0	270.0	210.0
Nicotinic acid mg/100 g	3.5	2.5	2.2	1.0	4.0	3.8

Source: The Wealth of India--1966, Raw Materials, Vol.7, p.165.

Table 2 : Reference strains* used in the present study

Sl.No.	Culture No.	Identity
(1)	(2)	(3)
1	NCIM 2464	Bacillus laevolacticus
2	NCIM 2456	Bacillus aminoveran
3	NCIM 2042	Bacillus licheniformis
4	NCIM 2019	Bacillus mesentericus
5	NCIM 2465	Bacillus laterosporus
6	NCIM 2018	Bacillus lentus
7	NCIM 2189	Bacillus pumilus
8	NCIM 2234	Bacillus aporrheus
9	NCIM 2479	Bacillus macquarieusis
10	NCIM 2461	Bacillus cereus
11	NCIM 2463	Bacillus freudenrechi
12	NCIM 2131	Bacillus macerans
13	NCIM 2216	Bacillus brevis
14	NCIM 2264	Bacillus firmus
15	NCIM 2107	Bacillus circulaus
16	NCIM 2477	Bacillus pasteurii
17	NCIM 2237	Bacillus anuviuolistery

(Contd...)

(1)	(2)	(3)
18	NCIM 2032	Bacillus megaterium
19	NCIM 2034	Bacillus coagulans
20	NCIM 2542	Bacillus stearothermophilus
21	NCIM 2538	Bacillus polymyxa
22	NCIM 2478	Bacillus sphericus
23	NCIM 2010	Bacillus subtilis
24	NCIM 2476	Bacillus pantothenticus
25	NCIM 2354	Bacillus zoopfti

* Source: National Collection of Industrial Microorganisms, N.C.L (CSIR), Pune 411008.

Table 3 : Selection of *Bacillus* strains for fermentation studies.

Sl. No.	Culture No.	Clear zone width (mm)	Growth (cell protein) (mg/ml)	Enzyme activity (u/ml)	Total sugar (mg/ml)	Reducing sugar (mg/ml)	Residual rice starch (mg/ml)	Starch conversion %	Average of ranks
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1.	1	32	0.71	19	0.58	0.468	1.11	89	18.2
2.	3	41	0.52	ND	0.35	0.221	6.20	38	10.2
3.	5	38	0.56	11	0.48	0.280	5.71	43	14.4
4.	9	29	0.35	5	0.37	0.239	6.41	36	9.2
5.	17	25	0.47	ND	0.27	0.181	6.92	31	7.8
6.	18	29	0.48	7	0.46	0.271	6.31	37	12.0
7.	20	19	0.55	16	0.56	0.442	1.93	81	16.4
8.	24	24	0.52	3	0.31	0.141	7.20	28	8.2
9.	25	39	0.59	21	0.59	0.511	0.92	92	18.8
10.	26	28	0.47	ND	0.31	0.162	7.31	27	7.4

(Contd...)

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
11.	29	17	0.55	7	0.32	0.201	6.53	35	10.2
12.	32	45	0.45	6	0.45	0.271	6.14	39	11.6
13.	33	31	0.73	23	0.61	0.631	1.30	87	19.6
14.	34	22	0.55	16	0.55	0.412	1.81	82	15.8
15.	38	19	0.52	6	0.42	0.331	5.83	42	13.0
16.	40	24	0.39	ND	0.31	0.121	7.32	27	6.4
17.	42	28	0.51	23	0.52	0.539	2.10	79	16.6
18.	44	19	0.41	9	0.45	0.312	5.74	43	12.6
19.	47	30	0.31	ND	0.38	0.251	8.33	17	8.0
20.	54	32	0.39	ND	0.22	0.090	9.72	3	5.4

Table 4 : Characteristic properties of *Bacillus* sp. selected for fermentation studies.

Properties studied	*B. coagulans* ACMN 1	*B. coagulans* ACMN 42	*B. polymyxa* ACMN 25	*B. cereus* ACMN 33
(1)	(2)	(3)	(4)	(5)
Source	Soil	Soil	Soil	Soil
Coloney morphology				
Shape	Irregular	Irregular	Irregular	Irregular
Colour	Off white	Off white	Off white	Off white
Surface	Smooth flat	Smooth flat	Irregularised	Smooth flat
Consistency	Opaque	Opaque	Opaque	Opaque
Cell morphology	long thick rods	long thick rods	long thick rods	long thick rods
Gram reaction	+	+	+	+
Mortility	+	+	+	+
Spore	+	+	+	+

(Contd....)

(1)	(2)	(3)	(4)	(5)
Shape	Elliptical	Elliptical	Elliptical	Elliptical
Dominant position	Central	Central	Sub-terminal	Central
Swelling	+	+	-	-
Biochemical characters				
Catalase	+	+	+	+
Fermentation of glucose	Fermentative	Fermentative	Fermentative	Fermentative
Arabinose	-	-	-	-
Xylose	-	-	+	-
Mannitol	-	-	-	-
Hydrolysis of urea	+	+	+	+
Starch	+	+	+	+
Cellulose	+	+	+	+

(Contd...)

(1)	(2)	(3)	(4)	(5)
Casein	+	+	+	+
Gelatin	+	+	+	+
Lipid	-	-	-	+
Utilization of citrate	+	+	-	+
NO_3 - NO_2	-	-	+	+
Voges-Proskauer test	-	-	+	+
Voges-Proskauer pH	4-5	4-5	4-5	4-5
Ammonia Production	-	-	+	+
Indole Production	-	-	-	-
Growth at 5% NaCl	+	+	+	+
Growth at 7% NaCl	-	-	-	+

Table 5 : Effect of carbon sources on growth and enzyme production of Bacillus sp.

Organisms	Parameters analysed	Carbon sources					
		Glucose	Maltose	Dextrin	Soluble starch	Lactose	Glycerol
B. coagulans ACMN 1	Cell protein (mg/ml)	0.91	0.75	0.83	0.97	0.90	0.51
	Enzyme activity (u/ml)	11.00	14.00	22.00	25.00	13.00	15.00
	pH	4.91	5.12	5.28	5.11	6.58	6.21
B. coagualns ACMN 42	Cell protein (mg/ml)	1.38	1.53	3.19	2.31	1.49	1.01
	Enzyme activity (u/ml)	53.00	58.00	62.00	62.00	53.00	50.00
	pH	4.64	4.73	4.85	4.82	4.53	5.29
B. polymyxa ACMN 25	Cell protein (mg/ml)	1.10	1.25	2.00	2.25	0.91	1.25
	Enzyme activity (u/ml)	19.00	19.00	46.00	48.00	38.00	42.00
	pH	4.94	5.04	5.03	5.80	6.25	5.02
B. cereus ACMN 33	Cell protein (mg/ml)	1.93	1.81	2.53	2.58	1.58	2.19
	Enzyme activity (u/ml)	38.00	53.00	38.00	49.00	51.00	47.00
	pH	5.00	5.13	5.12	5.11	6.51	6.38

Table 6 : Effect of nitrogen sources on growth and enzyme production of Bacillus sp.

Organisms	Parameters analysed	Nitrogen sources						
		Peptone	Beef extract	Yeast extract	Tryptone	$NaNO_3$	KNO_3	$(NH_4)_2SO_4$
B. coagulans ACMN 1	Cell protein (mg/ml)	1.01	0.92	0.78	1.93	0.21	0.35	0.31
	Enzyme activity (u/ml)	27.00	11.00	10.00	13.00	4.00	5.00	3.00
	pH	5.09	5.74	6.16	6.03	6.05	6.83	6.09
B. coagulans ACMN 42	Cell protein (mg/ml)	1.51	1.19	1.31	1.32	0.21	0.32	0.11
	Enzyme activity (u/ml)	44.00	11.00	Nil	19.00	Nil	Nil	Nil
	pH	5.16	5.15	5.13	5.18	6.81	6.85	6.80
B. polymyxa ACMN 25	Cell protein (mg/ml)	2.25	1.90	2.12	1.87	0.24	0.29	0.25
	Enzyme activity (u/ml)	35.00	21.00	Nil	7.00	Nil	Nil	Nil
	pH	4.85	5.05	5.00	5.42	6.82	6.83	6.85
B. cereus ACMN 33	Cell protein (mg/ml)	2.11	1.58	1.01	1.29	0.11	0.44	0.42
	Enzyme activity (u/ml)	38.00	22.00	Nil	21.00	Nil	3.00	3.00
	pH	5.28	5.19	5.18	5.21	6.82	6.57	6.58

Table 7 : Purification of overall recovery of amylases from Bacillus sp

Organisms	Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity u/mg of protein	Yield (%)
B. coagulans ACMN 1	Crude liquor	100	8800	5200	1.70	100.00
	$(NH_4)_2SO4$ fractionation	50	7500	217	34.60	85.20
	Dialysed solution	20	4800	91.0	52.70	54.50
B. coagulans ACMN 42	Crude liquor	100	4500	3930	1.10	100.00
	$(NH_4)_2SO4$ fractionation	50	4000	143	27.90	88.90
	Dialysed solution	20	2800	68	41.20	62.20
B. polymyxa ACMN 25	Crude liquor	100	9800	5890	1.60	100.00
	$(NH_4)_2SO4$ fractionation	50	8000	198	40.40	81.60
	Dialysed solution	20	4500	72	62.50	45.90
B. cereus ACMN 33	Crude liquor	100	7000	6312	1.10	100.00
	$(NH_4)_2SO4$ fractionation	50	6000	253	23.70	85.70
	Dialysed solution	20	5000	82	60.90	71.40

Table 8 : Effect of heavy metals on the activity of amylase of *Bacillus* sp.

Organisms	Parameters analysed	Heavy metals					
		Copper	Iron	Manganese	Silver	Mercury	Calcium
B. coagulans ACMN 1	Residual enzyme activity (u/ml)	88.0	Nil	82.0	82.0	50.0	88.0
	Loss of activity (%)	0.0	100.0	6.70	6.70	43.3	0.0
B. coagualns ACMN 42	Residual enzyme activity (u/ml)	70.0	Nil	71.0	45.0	25.0	71.0
	Loss of activity (%)	1.5	100.0	0.0	36.7	64.8	0.0
B. polymyxa ACMN 25	Residual enzyme activity (u/ml)	97.0	Nil	88.0	95.0	80.0	97.0
	Loss of activity (%)	0.0	100.0	9.3	2.1	17.5	0.0
B. cereus ACMN 33	Residual enzyme activity (u/ml)	26.0	25.0	30.0	20.0	18.0	55.0
	Loss of activity (%)	52.8	54.3	45.5	63.7	67.3	0.0

Table 9 : Liquifaction of rice starch (10%) by alpha amylases of *Bacillus* sp. at different temperatures.

Organisms	Temperature (°C)	Time in minutes	Percentage of starch conversion	Reducing sugar (mg/ml)	Dextrose equivalents (DE)
(1)	(2)	(3)	(4)	(5)	(6)
	50	30	55.0	7.10	8.80
		60	75.0	33.0	11.70
	60	30	70.0	6.20	3.41
		60	75.0	25.2	9.20
B. coagulans ACMN 1	70	30	28.0	4.93	3.76
		60	50.0	23.8	9.15
	80	30	26.0	2.20	1.22
		60	35.0	2.81	1.24
	90	30	0.00	0.93	1.01
		60	0.00	0.93	1.01

(Contd....)

(1)	(2)	(3)	(4)	(5)	(6)
	50	30	55.3	7.21	1.95
		60	70.3	49.20	11.40
	60	30	79.1	7.23	4.00
		60	90.1	13.20	7.33
B. coagulans ACMN 42	70	30	55.3	4.01	1.21
		60	74.2	13.20	3.60
	80	30	62.2	2.41	1.60
		60	63.4	2.61	1.80
	90	30	0.00	1.01	0.98
		60	0.00	1.01	0.98
	50	30	73.0	21.20	4.93
		60	88.0	33.20	6.31
	60	30	94.9	21.20	9.61
		60	96.8	47.40	12.40
B. polymyxa ACMN 25	70	30	67.0	7.21	2.61
		60	75.0	29.20	8.91
	80	30	73.0	2.40	1.32
		60	74.0	2.61	1.43
	90	30	7.0	2.03	1.05
		60	30.0	2.03	1.00

(Contd...)

(1)	(2)	(3)	(4)	(5)	(6)
	50	30	50.2	10.20	2.64
		60	50.2	49.20	14.90
	60	30	52.0	10.30	3.71
		60	70.0	33.30	9.81
B. cereus ACMN 33	70	30	10.0	3.21	1.14
		60	30.0	29.20	5.81
	80	30	3.0	2.41	4.01
		60	3.0	5.41	1.52
	90	30	0.0	1.00	0.91
		60	0.0	1.01	0.91

Table 10 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9324*	0.9110*	0.5669	0.8900*	0.8644*	0.8982*	-0.9543*
Residual starch		-0.9877*	-0.7798*	-0.9975*	-0.9935*	-0.0995	0.9261*
Reducing sugar			-0.8005*	0.9901*	0.9972*	0.9895*	-0.8665*
Total sugar				0.7328	0.7736*	0.7370	-0.5065
DE					0.9938*	0.9945*	-0.9074*
Growth						0.9986*	-0.8935*
Enzyme activity							-0.9178*

* Significant at 5% level.

Table 11 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 1 at pH 9; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9316*	-0.9253*	0.9303*	0.9115*	0.9295*	0.9086*	0.6106
Residual starch		-0.9953*	-0.9883*	-0.9830*	-0.9978*	-0.9885*	-0.3810
Reducing sugar			0.9753*	0.9805*	0.9960*	0.9886*	0.3837
Total sugar				0.9593*	0.9904*	0.9580*	0.3850
DE					0.9786*	0.9580*	0.3850
Growth						0.9891*	0.3654
Enzyme activity							1.0000*

* Significant at 5% level.

Table 12 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 35°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9293*	0.9569*	0.9062*	0.9510*	0.8515*	0.9046*	-0.8703*
Residual starch		-0.8616*	-0.9845*	-0.8275*	-0.9631*	-0.9246*	0.9096*
Reducing sugar			0.8521*	0.9956*	0.7302	0.8896*	-0.7716
Total sugar				0.8081*	0.9461*	0.9673*	-0.9108
DE					0.6894	0.8486*	-0.7379
Growth						0.8657*	-0.9468*
Enzyme activity							0.2919

* Significant at 5% level.

Table 13 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 30°C and aeration at the rate of 2 vvm for 24 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.0724	-0.4600	0.7012	0.2736	-0.4296	0.8867*	-0.0294
Residual starch		-0.2089	-0.5005	0.8733*	-0.2451	-0.4834	-0.3553
Reducing sugar			-0.6726	-0.2988	0.9976*	-0.2168	0.0927
Total sugar				-0.2035	-0.6336	0.7724*	0.1644
DE					-0.3143	-0.0721	-0.3320
Growth						-0.1719	0.1405
Enzyme activity							-0.9032*

* Significant at 5% level.

Table 14 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 1 at pH 7; 30°C and aeration at the rate of 2 vvm for 12 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.6570	0.2853	0.4532	0.3033	0.8150*	0.7821*	-0.8382*
Residual starch		-0.8742*	-0.9411*	-0.8910*	-0.9551*	-0.8857*	0.3249
Reducing sugar			0.9653*	0.9961*	0.7827*	0.7575*	0.1480
Total sugar				0.9578*	0.8754*	0.8451*	-0.0699
DE					0.7926*	0.7534	0.1240
Growth						0.9632*	-0.4486
Enzyme activity							0.1243

* Significant at 5% level.

Table 15 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice decanted water by B. coagulans ACMN 1 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9152*	0.7342	0.4956	0.7936*	0.8177*	0.7732*	-0.9824*
Residual starch		-0.8536*	-0.5855	-0.8832*	-0.9105*	-0.9203*	0.9321*
Reducing sugar			0.7726*	0.9929*	0.9862*	0.9708*	-0.7580*
Total sugar				0.7161	0.7886*	0.7266	-0.4293
DE					0.9867*	0.9687*	-0.8206*
Growth						0.9820*	-0.8227*
Enzyme activity							-0.3899

* Significant at 5% level.

Table 16 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9108*	0.7716*	0.7040	0.7880*	0.8403*	0.8260*	-0.9572*
Residual starch		-0.9484*	-0.9243*	-0.9231*	-0.9684*	-0.9548*	0.9217*
Reducing sugar			0.9298*	0.9762*	0.9643*	0.9547*	-0.8477*
Total sugar				0.8425*	0.9120*	0.9417*	-0.7499
DE					0.9638*	0.8905*	-0.8746*
Growth						0.9179*	-0.9108*
Enzyme activity							0.7659*

* Significant at 5% level.

Table 17 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 42 at pH 9; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.8238*	0.8160*	0.8184*	0.7246	0.8895*	0.8390*	-0.9718*
Residual starch		-0.9497*	-0.8933*	-0.9535*	-0.8783*	-0.9058*	0.7613*
Reducing sugar			0.9680*	0.9318*	0.9137*	0.9860*	-0.7564*
Total sugar				0.8164*	0.9708*	0.9898*	-0.7782*
DE					0.7446	0.8636*	-0.6384
Growth						0.9487*	-0.8751*
Enzyme activity							-0.8561*

* Significant at 5% level.

Table 18 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 35°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.6697	0.5049	0.4724	0.5794	0.7231	0.7201	-0.9553*
Residual starch		-0.9765*	-0.9441*	-0.9826*	-0.9664*	-0.8416*	0.6532
Reducing sugar			0.9832*	0.9714*	0.9343*	0.8021*	-0.5191
Total sugar				0.9142*	0.9350*	0.8492*	-0.5368
DE					0.9073*	0.7344	-0.5264
Growth						0.9470*	-0.7620*
Enzyme activity							-0.7923*

* Significant at 5% level.

Table 19 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and aeration at the rate of 2 vvm for 24 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.7909*	0.8692*	0.8455*	0.8090*	0.9048*	0.9019*	-0.9461*
Residual starch		-0.9741*	-0.9503*	-0.9971*	-0.8685*	-0.9357*	0.6979
Reducing sugar			0.9724*	0.9827*	0.9575*	0.9895*	-0.8134*
Total sugar				0.9453*	0.9279*	0.9562*	-0.7828*
DE					0.8911*	0.9518*	-0.7269
Growth						0.9839*	-0.8996*
Enzyme activity							-0.8223*

* Significant at 5% level.

Table 20 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and aeration at the rate of 2 vvm for 12 hrs (intermittant arresting of aeration after 12 hrs).

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.7722*	0.7025	0.8019*	0.6593	0.9002*	0.5765	-0.8955*
Residual starch		-0.9550*	-0.9779*	-0.9697*	-0.8598*	-0.9226*	0.4515
Reducing sugar			0.9710*	0.9898*	0.8986*	0.9838*	-0.3164
Total sugar				0.9620*	0.9202*	0.9348*	-0.4700
DE					0.8418*	0.9843*	-0.2739
Growth						0.8232*	-0.8267
Enzyme activity							-0.8813*

* Significant at 5% level.

Table 21 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. coagulans ACMN 42 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.7694*	0.8253*	0.5769	0.8570*	0.8398*	0.8037*	-0.9792*
Residual starch		-0.9555*	-0.9378*	-0.9401*	-0.9661*	-0.9312*	0.6902
Reducing sugar			0.8203*	0.9962*	0.9975*	0.9730*	-0.7869*
Total sugar				0.7801*	0.8324*	0.8413*	-0.4714
DE					0.9956*	0.9575*	-0.8232*
Growth						0.9631*	-0.7967*
Enzyme activity							-0.1586

* Significant at 5% level.

Table 22 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. polymyxa ACMN 25 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.6745	-0.2301	0.4424	0.8055*	0.8053*	0.8281*	0.4629
Residual starch		-0.1312	-0.4277	-0.7660*	-0.7154	-0.6221	-0.9581*
Reducing sugar			-0.8143*	-0.4838	-0.5639	-0.4665	0.3451
Total sugar				0.8347*	0.8636*	0.6977	0.2472
DE					0.9854*	0.9057*	0.5729
Growth						0.9301*	0.5021
Enzyme activity							-0.9542*

* Significant at 5% level.

Table 23 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. polymyxa ACMN 25 at pH 9; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9137*	0.9266*	0.9219*	0.9094*	0.9200*	0.7473	-0.9583*
Residual starch		-0.9341*	-0.8927*	-0.9971*	-0.9570*	-0.9366*	0.8465*
Reducing sugar			0.9915*	0.9500*	0.9481*	0.8294*	-0.9505*
Total sugar				0.9142*	0.9273*	0.7639	-0.9505*
DE					0.9570*	0.9382*	-0.8517*
Growth						0.8125*	-0.8873*
Enzyme activity							-0.3872

* Significant at 5% level.

Table 24 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. polymyxa ACMN 25 at pH 7; 35°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.6442	0.7166	0.6623	0.7065	0.8676*	0.8268*	-0.9588*
Residual starch		-0.9395	-0.9883*	-0.9504*	-0.8299*	-0.8045*	0.4769
Reducing sugar			0.9650*	0.9957*	0.9387*	0.9328*	-0.5830
Total sugar				0.9627*	0.8746*	0.8592*	-0.4986
DE					0.9198*	0.9090*	-0.5729
Growth						0.9951*	-0.7446
Enzyme activity							0.1589

* Significant at 5% level.

Table 25 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. polymyxa ACMN 25 at pH 7; 30°C and aeration at the rate of 2 vvm for 24 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.7168	0.6555	0.7456	0.5887	0.8992*	0.7673*	-0.9730*
Residual starch		-0.9961*	-0.9908*	-0.9817*	-0.8691*	-0.9397*	0.6781
Reducing sugar			0.9810*	0.9934*	0.8283*	0.9199*	-0.6125
Total sugar				0.9560*	0.9159*	0.9632*	-0.7224
DE					0.7634*	0.8710*	-0.5304
Growth						0.9469*	-0.9175*
Enzyme activity							-0.6985

* Significant at 5% level.

Table 26 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. polymyxa ACMN 25 at pH 7; 30°C and aeration at the rate of 2 vvm for 12 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.7153	0.5329	0.2090	0.6664	0.7781*	-0.5497	-0.8826*
Residual starch		-0.9677*	-0.8042*	-0.9924*	-0.9888*	-0.8690*	0.4076
Reducing sugar			0.9206*	0.9774*	0.9339*	0.8701*	-0.2114
Total sugar				0.8198*	0.7613*	0.8341*	0.1518
DE					0.9647*	0.8218*	-0.4563
Growth						0.8951*	-0.4563
Enzyme activity							-0.4406

* Significant at 5% level.

Table 27 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice dacanted water by B. polymyxa ACMN 25 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.7342	0.5867	0.4487	0.6407	0.8087*	0.6956	-0.9686*
Residual starch		-0.9777*	-0.9314*	-0.9478*	-0.9823*	-0.9398*	0.6296
Reducing sugar			0.9851*	0.9605*	0.9279*	0.8893*	-0.4638
Total sugar				0.9322*	0.8580*	0.8427*	-0.3202
DE					0.9032*	0.8026*	-0.5303
Growth						0.9568*	-0.7377
Enzyme activity							0.6842

* Significant at 5% level.

Table 28 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. cereus ACMN 33 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.8222*	0.5898	0.2782	0.7461	0.8300*	0.8327*	-0.9649*
Residual starch		-0.9265*	-0.7138	-0.9451*	-0.9796*	-0.8306*	0.8048*
Reducing sugar			0.8569*	9.9504*	0.8576*	0.6547	-0.5322
Total sugar				0.6644	0.6159	0.2391	-0.2640
DE					0.8937*	0.8081	-0.6698
Growth						0.8963*	-0.8453*
Enzyme activity							-0.8355*

* Significant at 5% level.

Table 29 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. cereus ACMN 33 at pH 9; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.8475*	0.8581*	0.8735*	0.7432	0.8951*	0.7267	-0.9838*
Residual starch		-0.9162*	-0.8777*	-0.9194*	-0.9685*	-0.9030*	0.8667*
Reducing sugar			0.9779*	0.9154*	0.9748*	0.9555*	-0.9062*
Total sugar				0.8170*	0.9676*	0.8830*	-0.9357*
DE					0.8917*	0.9783*	-0.7508
Growth						0.9155*	-0.9342*
Enzyme activity							-0.9647*

* Significant at 5% level.

Table 30 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. cereus ACMN 33 at pH 7; 35°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.8336*	0.7632*	0.8279*	0.6179	0.8533*	0.8784*	-0.9522*
Residual starch		-0.9057*	-0.8791*	-0.8260*	-0.9835*	-0.9547*	0.6795
Reducing sugar			0.9828*	0.9678*	0.9411*	0.9161*	-0.5741
Total sugar				0.9198*	0.9362*	0.9345*	-0.6774
DE					0.8482*	0.7932*	-0.3952
Growth						0.9873*	-0.7053
Enzyme activity							0.6454

* Significant at 5% level.

Table 31 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. cereus ACMN 33 at pH 7; 30°C and aeration at the rate of 2 vvm for 24 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9164*	0.9037*	0.9727*	0.8689*	0.9029*	0.9076*	-0.9644*
Residual starch		-0.9952*	-0.9630*	-0.9935*	-0.9905*	-0.9889*	0.9401*
Reducing sugar			0.9602*	0.9920*	0.9739*	0.9859*	-0.9157*
Total sugar				0.9292*	0.9484*	0.9684*	-0.9542*
DE					0.9819*	0.9762*	-0.9012*
Growth						0.9835*	-0.9503*
Enzyme activity							

* Significant at 5% level.

Table 32 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice starch by B. cereus ACMN 33 at pH 7; 30°C and aeration at the rate of 2 vvm for 12 hrs.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.9064*	0.7884*	0.7563*	0.8072*	0.8952*	0.8297*	-0.0753
Residual starch		-0.9722*	-0.8721*	-0.9739*	-0.9888*	-0.9634*	-0.1155
Reducing sugar			0.8490*	0.9972*	0.9609*	0.9665*	0.2134
Total sugar				0.8338*	0.9175*	0.8607*	-0.2065
DE					0.9599*	0.9691*	0.2115
Growth						0.9736*	-0.0176
Enzyme activity							-0.9440*

* Significant at 5% level.

Table 33 : Pearson correlation coefficients obtained for the various parameters analysed during the fermentation of rice decanted water by B. cereus ACMN 33 at pH 7; 30°C and aeration rate of 1 vvm.

	Residual starch	Reducing sugar	Total sugar	DE	Growth	Enzyme activity	pH
Time	-0.6869	0.6328	0.3209	0.7765*	0.8101*	0.7078	-0.9320*
Residual starch		0.9971*	-0.8926*	-0.9845*	-0.9399*	-0.9531*	0.6716
Reducing sugar			0.9235*	0.9710*	0.9157*	0.9392*	-0.6165
Total sugar				0.8139*	0.7166	0.7852*	-0.2954
DE					0.9511*	0.9400*	-0.7525
Growth						0.9855*	-0.8579*
Enzyme activity							0.5880

* Significant at 5% level.

Table 34 : Pearsons correlation coefficients of the various parameters analysed during the studies on the effect of pH on rice starch conversion by immobilized cells of Bacillus sp.

		Residual starch	Reducing sugar	Total sugar	DE	Enzyme activity
B. coagulans ACMN 1	pH	-0.6363	0.5601	0.8641*	0.5333	0.7763
	Residual starch		-0.9496*	-0.8920*	-0.9625*	-0.9708*
	Reducing sugar			0.8891*	0.9971*	0.9238*
	Total sugar				0.8717*	0.9410*
	DE					0.9242*
B. coagulans ACMN 42	pH	-0.7306	0.6333	0.1863	0.3432	0.6662
	Residual starch		-0.9757*	0.4430	-0.8347*	-0.9932*
	Reducing sugar			-0.6018	0.9289*	0.9861*
	Total sugar				-0.8427*	-0.5192
	DE					0.8705*

(Contd....)

		Residual starch	Reducing sugar	Total sugar	DE	Enzyme activity
	pH	-0.7976	0.5600	0.7961	0.4038	-0.7003
	Residual starch		-0.9281*	-0.4522	-0.8628*	-0.9763*
B. polymyxa ACMN 25	Reducing sugar			0.2406	0.9637*	0.9729*
	Total sugar				-0.0095	0.3503
	DE					0.9207*
	pH	-0.1318	0.0707	0.3622	0.0561	0.2529
	Residual starch		-0.9877*	-0.9469*	-0.9863*	-0.9329*
B. cereus ACMN 33	Reducing sugar			0.9087*	0.9997*	0.8840*
	Total sugar				0.9004*	0.9811*
	DE					0.8766*

* Significant at 5% level.

Table 35 : Pearsons correlation coefficients of the various parameters analysed during the studies on the effect of temperature on rice starch conversion by immobilized cells of Bacillus sp.

		Residual starch	Reducing sugar	Total sugar	DE	Enzyme activity
	Temperature	-0.2401	-0.9995*	-1.0000*	-0.9994*	-0.8219
	Residual starch		0.2105	0.2401	0.2083	0.7502
B. coagulans ACMN 1	Reducing sugar			0.9995*	0.9999*	0.8043
	Total sugar				0.9994*	0.8219
	DE					0.8029
	Temperature	0.8744	-0.9849	-0.8170	-0.9768	-0.9762
	Residual starch		-0.7773	-0.9941*	-0.7504	-0.9588
B. coagulans ACMN 42	Reducing sugar			0.7049	0.9991*	0.9239
	Total sugar				0.6748	0.9226
	DE					0.9072

(Contd...)

		Residual starch	Reducing sugar	Total sugar	DE
	Temperature	0.9477	-0.9521*	-0.8898	-0.9226
	Residual starch		-0.8051	-0.8448	-0.7560
B. polymyxa ACMN 25	Reducing sugar			0.8518	0.9964*
	Total sugar				0.7716
	Temperature	0.9507*	-0.9237	-0.6984	-0.9490
	Residual starch		-0.9665*	-0.8843	-0.9636*
B. cereus ACMN 33	Reducing sugar			0.8688	0.9958*
	Total sugar				0.8238

* Significant at 5% level.

Table 36 : Pearsons correlation coefficients of the various parameters analysed during the studies on the effect of incubation time on rice starch conversion by immobilized cells of Bacillus sp.

		Residual starch	Reducing sugar	Total sugar	DE	Enzyme activity
B. coagulans ACMN 1	Time	-0.9896*	0.9549*	0.8637	0.9111	0.9668*
	Residual starch		-0.9854*	-0.8935*	-0.7376	-0.9824*
	Reducing sugar			0.8770	0.6375	0.9625*
	Total sugar				0.4817	0.9608*
	DE					0.6649
B. coagulans ACMN 42	Time	-0.8823*	0.9476*	0.6362	0.8077	0.9185*
	Residual starch		-0.9731*	-0.8273	-0.9814*	-0.9782*
	Reducing sugar			0.8255*	0.9471*	0.9559*
	Total sugar				0.9132*	0.6963
	DE					0.9206*

(Contd...)

		Residual starch	Reducing sugar	Total sugar	DE	Enzyme activity
B. polymyxa ACMN 25	Time	-0.9826*	0.9748*	0.9116*	0.9864*	0.9961*
	Residual starch		-0.9467*	-0.8248	-0.9882*	-0.9799*
	Reducing sugar			0.8832*	0.9280*	0.9760*
	Total sugar				0.8766*	0.8975*
	DE					0.9810*
B. cereus ACMN 33	Time	-0.9530*	0.8377	0.6735	0.9243*	0.9884*
	Residual starch		-0.9511*	-0.8408	-0.9236*	-0.9764*
	Reducing sugar			0.9661*	0.9190*	0.9029*
	Total sugar				0.8317	0.7668
	DE					0.9620*

* Significant at 5% level.

Table 37 : Pearsons correlation coefficients of the various parameters analysed during the studies on the reusability of the immobilized cells of Bacillus sp for rice starch conversion.

		Residual starch	Reducing sugar	Growth	Enzyme activity	DE
B. coagulans ACMN 1	Cycles	0.9454	-0.9698*	-0.8865	-0.8539	-0.9614*
	Residual starch		-0.8382	-0.9889*	-0.9716*	-0.9923*
	Reducing sugar			0.7483	0.7023	0.8674
	Growth				0.9895*	0.9701*
	Enzyme activity					0.9630*
B. coagulans ACMN 42	Cycles	0.9944*	-0.9898*	-0.3342	-0.9486	-0.7928
	Residual starch		-0.9833*	-0.4294	-0.9547*	-0.7966
	Reducing sugar			0.4093	0.9028	0.7055
	Growth				0.3216	0.1483
	Enzyme activity					0.9402

(Contd...)

		Residual starch	Reducing sugar	Growth	Enzyme activity	DE
B. polymyxa ACMN 25	Cycles	0.9350	-0.9843*	0.3392	-0.7071	-0.8222
	Residual starch		-0.9822*	-0.0117	-0.8858	-0.5777
	Reducing sugar			-0.1679	0.8148	0.7104
	Growth				0.3964	-0.8073
	Enzyme activity					0.1825
B. cereus ACMN 33	Cycles	0.8966	0.7617	-0.6677	-0.7745	-0.7623
	Residual starch		-0.8898	-0.8068	-0.9660*	-0.9644*
	Reducing sugar			0.9722*	0.9692*	0.9629*
	Growth				0.8947	0.8805
	Enzyme activity					0.9993*

* Significant at 5% level.

Table 38 : Pearsons correlation coefficients of the various parameters analysed during the studies on the effect of pH on rice starch conversion by immobilized enzymes of Bacillus sp.

		Residual starch	Reducing sugar	Total sugar	DE
B. coagulans ACMN 1	pH	-0.5625	0.6817	0.6234	0.6925
	Residual starch		-0.9666*	-0.8775*	-0.9347*
	Reducing sugar			0.9438*	0.9272*
	Total sugar				0.7537
B. coagulans ACMN 42	pH	-0.4609	0.5641	0.4398	0.6316
	Residual starch		0.9791*	0.8977*	-0.8976*
	Reducing sugar			0.9376*	0.9029*
	Total sugar				0.6998

(Contd...)

		Residual starch	Reducing sugar	Total sugar	CE
B. polymyxa ACMN 25	pH	-0.3998	0.5753	0.5179	0.6132
	Residual starch		-0.9756*	-0.9406*	-0.9038*
	Reducing sugar			0.9272*	0.9656*
	Total sugar				0.8096*
B. cereus ACMN 33	pH	-0.3113	0.3363	0.2080	0.5314
	Residual starch		-0.8316*	-0.7730	-0.9107*
	Reducing sugar			0.9740*	0.9196*
	Total sugar				0.8178*

* Significant at 5% level.

Table 39 : Pearsons correlation coefficient analysis of the various parameters analysed during the studies on the effect of temperature on rice starch conversion by immobilized enzymes of Bacillus sp.

		Residual starch	Reducing sugar	Total sugar	DE
B. coagulans ACMN 1	Temperature	0.9873*	-0.4913	-0.6980	-0.8257
	Residual starch		-0.3891	-0.7970	-0.7654
	Reducing sugar			-0.2088	0.8892
	Total sugar				0.2579
B. coagulans ACMN 42	Temperature	0.9960*	-0.6850	-0.8421	-0.9519*
	Residual starch		-0.6538	-0.8798	-0.9434
	Reducing sugar			0.6133	0.8664
	Total sugar				0.8620

(Contd...)

		Residual starch	Reducing sugar	Total sugar	DE	Enzyme activity
	Temperature	0.7822	-0.9364	-0.4271	-0.9728	-0.6061
	Residual starch		-0.9511	-0.8973	-0.9051	-0.9696
B. polymyxa ACMN 25	Reducing sugar			0.7172	0.9921	0.8467
	Total sugar				0.6246	0.9780
	DE					0.7736
	Temperature	0.9371	-0.9834	0.9332	-0.9701	-0.9819
	Residual starch		-0.8585	0.7493	-0.8244	-0.8543*
B. cereus ACMN 33	Reducing sugar			-0.9828	0.9980*	0.9999*
	Total sugar				-0.9925	-0.9843
	DE					0.9984*

* Significant at 5% level.

4. DISCUSSION

4.1 *BACILLUS* sp. USED IN THE STUDY

Members of *Bacillus* sp., namely, *B. amyloliquifaciens*, *B. polymyxa*, *B. stearothermophilus*, *B. licheniformis*, *B. subtilis*, *B. coagulans* are extensively employed in the commercial production of amylases, proteases and other enzymes and some useful products like antibiotics fine chemicals etc., owing to their characteristic properties and efficiencies. On most occasions these strains were obtained from soil.

In the present study also, *Bacillus* strains obtained from soil were more efficient in bringing about rapid conversion of rice starch, maximal enzyme production and sugar production, compared to those obtained from other terrestrial sources. This observation made in the present study indicate that soil is a rich source of enormous potent microorganisms that are capable of performing useful conversions of organic substances. The present results are also comparable with earlier observations in isolating potent *Bacillus* sp. strains from soil. The selected strains identified as *B. coagulans*, *B. polymyxa*, *B. cereus*, in the present

study, are already known to be used by earlier investigations for the production of amylases (Medda & Chandra, 1980). However, in the earlier studies, Bacillus sp. were not used for rice starch conversion either as free cells or under immobilized state.

Although reference strains of Bacillus sp. obtained from NCIM, India were used for identification purposes. No attempt was made to use them in the fermentation studies as it was desired to try only wild strains.

4.2 GROWTH STUDIES

Bacteria grow well and metabolize various substrates at certain sets of environmental conditions. In nature, different strains of the same species usually differ from each other in their requirements of carbon and nitrogen sources, optimum temperature, pH and other factors to grow well and produce maximal amounts of enzymes and useful metabolites.

Bacillus sp. is no exception to this phenomenon. Thus several earlier investigations have observed variation in their optimal conditions for growth and enzyme production.

4.2.1 Effect of pH

B. stearothermophilus produced maximal α-amylase at pH 6.7 and preferred pH 5-8.5 for optimal enzyme activities and growth (Welker & Campbell, 1963). Horikoshi (1971) reported that Bacillus sp. required alkaline pH 7.5-11 for its growth and synthesis of alkaline α-amylase. Bindu (1989) also reported that Bacillus sp. produced optimal growth and thermostable α-amylase in the range of pH 7-9. Bacillus licheniformis TCRDC B 13, although grew well at levels of pH 3-11, recorded maximal growth at pH 10-11 and produced more α-amylase at pH 6-9 (Bajpai & Bajpai, 1989). Bacillus BQ 10, a β-amylase producer, was observed to prefer pH 7 for maximal growth and enzyme production (Shinke et al., 1975).

In the present study, while B. cereus preferred pH 7 for their maximal growth and amylase production, B. polymyxa preferred pH 8. Among the four B. coagulans, while ACMN 1 preferred pH 9 for maximal growth and enzyyme production, ACMN 42 required pH 8 for the same. However, all the four strains could show significant levels of α-amylase production in the pH range 7-10. It was also observed that B. cereus could produce significant levels of growth and amylase at pH 4-5 when compared to other strains. These

observations are in agreement with the results observed with Bacillus sp. mentioned above. However, since reports on similar studies on the same organisms were not available a direct comparison could not be made.

The results, further indicate that these strains of Bacillus sp. generally preferred alkaline pH levels for their growth and enzyme production as it was observed by earlier workers.

In few reports, it is mentioned that since Bacillus sp. could isolated from foods having acidic pH. They may be able to tolerate or perhaps prefer acidic pH for their growth (Buonocore, 1972; Gordon, 1973; Sandhu & Soni, 1988). In the present study acidic pH levels, pH 4-5 have been observed to promote growth and enzyme production by B. cereus ACMN 33. This observation on B. cereus suggest their preference to acidid pH ranges.

4.2.2 Effect of temperature

Medda and Chandra (1980) observed that B. licheniformis CUMC 305 could grow and produce maximal α-amylase at 55°C while B. coagulans CUMC 512 could do the same at 60°C.

Whereas, Bajpai and Bajpai (1989) reported that B. licheniformis TCRDC B 13 grew and produced α-amylase to a maximal level at 35-40°C. Shinke et al., (1975) reported maximal β-amylase production by Bacillus BQ 10 at temperatures 30-50°C while uniform growth occurred at 25-35°C.

In the present study it was observed that all the four strains belonging to the species of B. coagulans, B. polymyxa and B. cereus preferred 35°C for their maximal growth and enzyme production, although they could produce significant levels of enzymes at 25-45°C. The present results indicate the mesophilic nature of the organisms and are comparable with the reports of Bajpai and Bajpai (1989) for B. licheniformis TCRDC B 13.

4.2.3 Effect of NaCl

Very few reports are available on the effect of NaCl on the growth and enzyme production of a Bacillus sp. Horikoshi (1971) reported that an alkalophilic Bacillus sp. A-40-2 did not show enhancement of growth and enzyme production after addition of NaCl in the medium. Whereas Medda and Chandra (1980) reported that B. licheniformis CUMC 305 could grow in the presence of 7% NaCl. In the present study,

NaCl concentrations, 0.5-3%, favoured growth and enzyme production by all the strains to an appreciable level. In general increase in the NaCl concentration effected concordant decrease in the enzyme production in a linear fashion. However, maximal enzyme production was observed only at 0% NaCl for both the B. coagulans, while B. polymyxa and B. cereus produced maximal levels of enzyme production at 0.5%. The results suggest that B. polymyxa and B. cereus isolated in the present study had a tolerance to meagre levels of NaCl when compared to B. coagulans. The results show some agreement with the reports of Horikoshi (1971). It may be suggested that all these four strains do not require NaCl to produce optimal levels of enzyme.

4.2.4 Effect of substrate concentration

B. licheniformis TCRDC B 13 was recorded to produce more amylase at 1% of corn starch than at higher concentration and an increase of concentration of starch led to decrease in the levels of enzyme production (Bajpai & Bajpai, 1989). B. coagulans produced maximal α-amylase at 2% rice starch (Babu & Satyanarayana, 1990). In the present study the B. coagulans ACMN 1 and ACMN 42 strains as well as

B. polymyxa and B. cereus produced significant levels of α-amylase at 1% concentration of rice starch. Further increase in the concentrations of substrate did not influence the rate of growth and enzyme production. These results indicate that all the organisms actively produced α-amylase with meagre amount of (1%) rice starch, which could induce maximal enzyme production. On comparison of the results observed with B. coagulans of the present study with that of Babu and Satyanarayana's (1990) report, it is inferred that the strains used in the present study are very much different from that strain of the same species isolated from another environment. Hence it may be suggested that environment influence the basic nature of bacteria. Since no similar reports on B. polymyxa and B. cereus are available no comparisons could be made.

4.2.5 Effect of incubation time

Medda and Chandra (1980) reported that B. coagulans CUMC 512 and B. licheniformis CUMC 305 produced maximal levels of α-amylase after 24 hrs of incubation. B. licheniformis TCRDC B 13 recorded maximal α-amylase production at 40-70 hrs of incubation, while the maximum growth was

obtained at 20-30 hrs of incubation (Bajpai & Bajpai, 1989). An interesting observation of maximal α-amylase production at 18 hrs of incubation by B. subtilis was reported by Pazlarova (1984). Babu and Satyanarayana (1990) in contrast to Medda and Chandra's (1980) report observed that B. coagulans produced maximal enzymes only after 48 hrs of incubation. β-amylase production by a Bacillus sp. BQ 10 was recorded at 48-72 hrs of incubation (Shinke et al., 1975).

In the present study all the four strains produced maximal growth and enzymes by 18-24 hrs although they could record significant levels of enzyme production by 12 hrs itself. It was noted that further incubation after 24 hrs till 48 hrs did not enhance enzyme production. B. coagulans ACMN 1 and B. polymyxa produced significant levels of enzymes at 18 hrs while B. cereus and B. coagulans ACMN 42 record maximal enzymes at 24 hrs. The present results are quite comparable with the reports made for B. subtilis (Pazlarova, 1984).

4.2.6 Effect of inoculum concentration

Bindu (1989) reported that Bacillus sp required inoculum concentration of 5-20% for maximal α-amylase

production. However, in the present study inoculum concentration of 1-3% yielded maximal amylase production in the media. It was also observed that further increase in the inoculum concentrations did not enhance the enzyme production by all the four species of Bacillus.

4.2.7 Effect of carbon sources

The nutritional requirements especially the carbon and nitrogen sources play an important role on the growth and metabolism of microorganisms present in various environments. Carbon sources generally either induce or repress growth and amylase synthesis. B. stearothermophilus was found to grow and synthesize higher amounts of α-amylase in the presence of soluble starch and maltose than on glucose and glycerol which showed a repressing effect (Welker & Campbell, 1963). B. subtilis although grew well and produced enhanced levels of α-amylase in the presence of glucose, the levels of enhancement was comparatively lesser than those observed for starch and maltose sources. Starch supported a rapid growth and α-amylase synthesis by B. amyloliquifaciens followed by glycerol and glucose (Coleman & Grant, 1966). Ingle and Boyer (1975) also observed that the α-amylase production by B. amyloliquifaciens was repressed in the presence of glucose.

B. licheniformis TCRDC B 13 although recorded higher growth rate in the presence of glucose, their α-amylase production was more only with starch, followed by dextrin, maltose and lactose (Bajpai & Bajpai, 1989). Yoshigi (1985) noted that B. cereus NY 14 secreted higher amounts of α-amylase in the presence of starch, dextrin and glycogen while being inhibited in the presence of glucose. A mutant of the above mentioned strain was found to synthesize α-amylase even in the presence of 5% glucose (Yoshigi & Kammura, 1988).

However, in the present study all the four strains of Bacillus sp. was found to grow and secrete significant amount of α-amylase in presence of all the carbon sources tested. In general, all the four species of Bacillus preferred starch, dextrin, lactose and maltose for higher amylase synthesis and comparatively minimal production in the presence of glucose and glycerol. These observations are similar to that recorded for B. licheniformis TCRDC B 13 (Bajpai & Bajpai, 1989). Among the four strain, B. coagulans ACMN 1, ACMN 42 and B. polymyxa ACMN 25 recorded higher enzyme production in the presence of soluble starch followed by dextrins and glycerol. Whereas, maltose followed by lactose, starch and glycerol could effect maximal α-amylase

production by B. cereus ACMN 33 significant levels of growth and enzyme production by B. cereus in the present study is in contrast with the observations made for the wild strains of B. cereus studied by Yoshigi (1985). However, the present results are in agreement with results obtained for the mutant strain of B. cereus studied by Yoshigi and Kammura (1988).

4.2.8 Effect of nitrogen sources

The secretion of α-amylase was also known to be influenced by the nitrogen sources present in the medium. Peptone and tryptone were found to induce the growth and α-amylase synthesis of Bacillus sp. (Goyal, 1978). Bajpai and Bajpai (1989) observed that growth and α-amylase production by B. licheniformis TCRDC B 13 was induced by peptone followed by meat extract, beef extract and yeast extract. Whereas, Alam and Weigand (1988) observed that yeast extract at 5% level induced maximal growth and synthesis of amylase by B. amyloliquifaciens at controlled pH. In the present study of the organic nitrogen sources, peptone favoured higher growth and maximal enzyme production as it was observed in B. licheniformis TCRDC B 13 (Bajpai & Bajpai, 1989). However, significant levels of growth and enzyme

production was also enhanced by other nitrogen sources like beef extract and tryptone. Eventhough yeast extract could effect appreciable levels of growth, it generally inhibited enzyme production by most of the strains. The inorganic nitrogen sources like $NaNO_3$, KNO_3 and $(NH_4)_2SO_4$ were not favoured, for both growth and amylase production, by all the four species of Bacillus as it has observed with B. cereus BQ 10 by Shinke (1977).

4.2.9 Growth curve

Growth curves obtained for the four strains B. coagulans ACMN 1, B. coagulans ACMN 42, B. polymyxa ACMN 25 and B. cereus ACMN 33 indicated that all the organisms are fast growing in nature since they required only 3 hrs to pass the lag phase and enter the logarithmic phase. All the strains uniformly spent only about 6-12 hrs in the logarithmic phase before entering the stationary phase. Differences in them was noted only in terms of their generation time. Among the four, B. coagulans ACMN 42 and B. cereus recorded short generation times than B. polymyxa ACMN 25 and B. coagulans ACMN 1. This observation confirmed

results obtained in other studies where rapid growth and rice starch conversion by all these strains within 12 hrs of incubation were noted. No comparisons could be drawn with other organisms as no similar reports are available.

4.3 ENZYME STUDIES

4.3.1 Purification and recovery of amylases

Thermostable alkaline α-amylases were obtained from B. licheniformis CUMC 305 and B. coagulans CUMC 512 on partial purification by acetone fractionation followed by dialysis and recorded 52.17% of yield and 204 u/mg of protein as specific activity for B. licheniformis and 49.3% yield and 151.2 specific activity for B. coagulans (Medda & Chandra, 1980). Morgan and Priest (1980) obtained 66% yield of α-amylase after purification by dialysis and ion exchange chromatography from B. licheniformis NCIB 6346. In the present study amylases obtained from species of B. coagulans ACMN 1, ACMN 42 and B. cereus and B. polymyxa ACMN 25 were purified by $(NH_4)_2SO_4$ fractionation followed by dialysis. In general, an overall recovery of 41.00-71.14% yield was recovered by the four species with specific activities ranging from

41.20-62.50 for all the strains. The yield and specific activity was quite comparable with that of B. coagulans CUMC 512 reported by Medda and Chandra (1980).

4.3.2 Identification of amylases

Moseley and Keay (1970) distinguished B. subtilis amylase as α-type by measuring the iodine staining property and reducing sugar determination using soluble starch as substrate. Medda and Chandra (1980) identified the amylase of B. licheniformis CUMC 305 and B. coagulans CUMC 512 as α-amylase by detecting the dextrinizing and saccharifying activity of the partially purified α-amylase in starch. They observed an increase in the dextrinizing activity within a short period of time and a slow and gradual increase in the saccharolytic activity and identified the enzyme as α-amylase. Enzymes from B. licheniformis NCIB 6346 was identified as α-amylase by determining the reduction in iodine staining and the release of reducing sugar from starch (Morgan & Priest, 1981). In the present study, partially purified α-amylase from all the four strains were able to hydrolyze starch within 10 min. and a complete hydrolysis within 30-40 min. with an increase in dextrinizing activity.

Whereas, only a slow and gradual increase in reducing sugar of saccharolytic activity was observed with reference to time. The present results are identical with the findings of Medda and Chandra (1980).

4.3.3 Characterization of α-amylase

A facultative thermophilic B. coagulans strain which was able to produce a thermostable enzyme with an optimal activity at 70°C was reported by campbell (1955). Amylases from B. amyloliquifaciens recorded an optimum activity at 65°C (Welker & Campbell, 1963). Saito (1972), reported a thermostable α-amylase from B. licheniformis active at 76°C an in a wide range of pH 7-9. A thermostable α-amylase from B. licheniformis NCIB 6346 which was found to be active and stable between pH 7-9 and 70-90°C (Morgan & Priest, 1980). According to Medda and Chandra (1980), B. licheniformis CUMC 305 and B. coagulans CUMC 512 showed thermostability at 90 and 85°C, and active at a wide range of pH with a pH optima of pH 9.5 and pH 7.5-8.5 respectively for the strains. Bajpai and Bajpai (1989) reported a B. licheniformis sp which recorded an optimum activity at 90°C and at a wide range of pH 5.5-10.

In the present study the partially purified enzymes from all the strains recorded activity and stability at wide ranges of pH and temperature similar to that reported by the early workers. Strains of B. coagulans ACMN 1 and ACMN 42 and B. polymyxa ACMN 25 recorded activity and stability at a wide range of pH 5-10 with an optimum at pH 9. However, B. cereus showed an optimum of pH 7. Although a temperature of 50°C was recorded as optimum for the maximum activity and stability for B. coagulans ACMN 1 and B. polymyxa ACMN 25. They were able to show reasonable activity and stability upto 80°C. However, B. cereus ACMN 33 and B. coagulans ACMN 42 showed optimum activity and stability at 40°C and were active upto 70°C.

Yankov et al., (1986) studied the effect of substrate concentration on the rate of enzyme reaction on soluble starch with a thermostable α-amylase of B. licheniformis MB 10 and found that 300 g/l (30%) substrate concentration was the optimal concentration for the maximal enzyme activity and concentration above 300 g/l inhibited the α-amylase activity at 100°C. However, in the present study it was found that a 3% substrate concentration was favourable for the maximal rate of enzyme activity for B. polymyxa

ACMN 25, B. cereus ACMN 33 and B. coagulans ACMN 42 except for B. coagulans ACMN 1 which required 1% substrate concentration as the optimum . At substrate concentrations above 3%, a decrease or no further increase in the activity was observed.

Moseley and Keay (1970) reported that α-amylase from B. subtilis NRRC B 3411 showed higher activity and stability in the presence of calcium ions. Horikoshi (1971) also reported that calcium retains 100% of original activity and found that addition of urea inhibited α-amylase from Bacillus sp No. A 40-2. Hayashi (1988) examined the effect of heavy metal ions such as Hg^{2+}, Zn^{2+}, CO^{2+}, Ni^{2+}, Pb^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Ag^{2+} on an alkaline maltohexose forming amylase from Bacillus sp. H. 167 and observed that Hg^{2+}, Zn^{2+} completely inhibited the activity while Ca^{2+}, $^{2+}$ and Pb^{2+} reduced the activity to 40-60%. Sen and Chakraborthy (1987) reported the inhibitory effect of Fe^{2+}, Zn^{2+} on immobilized α-amylase from Lactobacillus cellobiosus. In the present study it was found that calcium did not inhibit the activity of α-amylase of all the four strains while Fe inhibited the activity (100%) of the enzymes from

all the strains except B. cereus where 54.3% loss of activity was observed. Hg followed by Ag brought about significant levels of inhibition as reported by earlier investigatiors.

4.4 CONVERSION OF RICE STARCH AND RICE DECANTED WATER BY FREE CELLS OF BACILLUS sp.

During the studies on rice starch conversion by B. coagulans ACMN 1 and ACMN 42, B. polymyxa ACMN 25, B. cereus ACMN 33, at various conditions, involving pH 7 and 9, 30 and 35°C, 1 and 2 vvm of aeration and 150 rpm agitation observed, in general, that irrespective of the combinations of the above said parameters, at all sets of conditions, all the four strains uniformly brought about a rapid conversion at highly significant levels within a period of 4-12 hrs of incubation and produced reducing sugar, total sugar, DE, cell protein and enzyme at similar levels with marginal differences in their values. In fact rice decanted water favoured production of significant levels of DE and enzymes by all the four strains than powdered rice starch.

In the case of B. coagulans ACMN 1, fermentation at pH 7, 30°C along with a rate of aeration of 2 vvm influenced rapid and maximal accumulation of sugar and

enzymes in the broth within a short duration, when compared to other conditions. Similarly enhanced rate of aeration at 2 vvm enhanced the yield of DE for this strain. B. coagulans ACMN 42, has influenced by a rise in the incubation temperature and rate of aeration. When these parameters here rised, they effected an increase in the amount and rate of production in the reducing sugar, total sugar, DE, cell protein and enzyme activity. Similarly arresting of aeration midway during fermentation also changed the course of fermentation in that, there was a rapid fall in the values from the levels obtained during aeration. Results on pH for this organisms did not indicate an acid production in the media unlike others. This species required 16 hrs of incubation and 2 vvm of aeration to produce a maximal DE while under normal conditions, it could produce maximal enzyme.

B. polymyxa ACMN 25 also showed some response to changes in the fermentation conditions. Rise in temperature from 30° to 35°C enhanced the level of production of reducing sugar, total sugar, DE, cell protein and enzyme activity. However, the increase was only marginal. Whereas, a rise in pH effected a decrease in the levels of all variables except enzyme activity which was comparatively maximum at pH 9 than

at pH 7 and 30°C. Rate of aeration, when increased, also effected rapid conversion and enhanced production of sugars, DE and enzyme production at short periods of incubation. Arresting of aeration after 12 hrs not only resulted in the decrease in the enzyme production but also indicated a rapid acid production by the organisms. However, DE levels were not affected by arresting of aeration. Unlike other three strains, this species yielded maximal DE within 4 hrs.

B. cereus ACMN 33, similar to B. coagulans ACMN 1, showed poor response to changes in fermentation conditions. However, it responded to enhanced aeration than to rise in pH and temperature, especially with reference to DE levels than for any other parameters. Enhanced aeration could infact double the level of DE in the medium when compared to all other conditions. This species also recorded acid production in the medium.

Ko (1972) reported that during fermentation of a non-glutaneous rice in solid state fermentation by Chlamedo-mucor oryza and yeast Endomycopsis sp. for 5 days at 30°C. There was a rapid increase in the reducing sugar production at 10-30 hrs of incubation and a decrease in the pH of the

media to pH 4. They also recorded a maximal reducing sugar level at pH 4. According to them, reducing sugar increased along with decrease in pH in the medium.

Purushothaman (1977) reported a continuous increase in the reducing sugar in the idli batter during fermentation upto 12 hrs and followed by a decrease in the later period. Sugimoto et al., (1986) reported an increase in total sugar, reducing sugar and ethanol during the fermentation on non-glutaneous rice starch by simultaneous liquifaction and saccharifaction by amylase of Aspergillus oryza and baker's yeast for sake brewing. However, they observed the maximal sugar accumulation after 85 hrs of fermentation. Sandhu and Soni (1988) isolated B. amyloliquifaciens, B. polymyxa as the major groups of bacteria during the fermentation of the batter of dosai besides reporting an increase in the amylase activity and reducing sugar along with a decrease in media pH to acidic levels during fermentation. Soni and Sandhu (1990) reported that B. amyloliquifaciens, B. polymyxa and B. cereus reduced starch to maltose and glucose along with a reduction in pH from 6.0 to 4.3 during the fermentation of idli.

According to Gordon (1973) B. coagulans are aciduric and produce acidity in a media (pH 4-5) containing

utilizable carbohydrates and also take part in the spoilage of acidic food.

Results observed in the present study for all organisms with regard to increase in the reducing sugar, total sugar, enzyme activity along with decrease in pH during fermentation at all sets of fermentation conditions are in agreement with the reports mentioned above. The observation made with rise in temperature and its minimal effect on the sugar production indicate the fact that the organisms are measophilic in nature and are active in the range of 30-30°C and hence not influenced in a significant level. Since all the organisms were found to be active and could grow and produce optimal levels of enzymes in the pH range 7-9, the organisms were not influenced much in their activity when the pH of fermentation medium was raised to 9. These observations further suggest that the organisms might be alkalophilic in nature.

Pirt (1959) suggested that aeration increased butanediol productivity by increasing cell concentration. According to Vollbrecht (1982) the oxygen supply rate is also important. The ratio of oxygen demand to oxygen supply can control the proportions of various metabolites produced.

In the present study it was observed that increase in aeration rate from 1 vvm to 2 vvm although did not bring about a great increase in cell protein, could effect rapid reduction of starch and release of sugars and enzyme by the organism. The present results are in support to the above held view of Vollbrecht (1982).

The reduction in the pH towards acidic level from initial level from initial pH observed for organisms during fermentations may be accounted with the possible production of organic acids from the available reducing sugars in the medium by the organism. However, in the present study no attempt was made to study the type of acid produced during fermentation.

The present results are in agreement with the views of Papoutsakis and Meyer (1985) according to whom majority of carbon is directed to the formation of carbondioxide, butanediol, acetate and lactate which led to reduction in the pH of the medium.

An overall assessment of the impact of arresting of aeration after 12 hrs at 2 vvm on the strains indicated that

except B. polymyxa all the other strains opted an alternative pathway to metabolize the available sugars. Probably an anaerobic fermentation to produce acids in the medium that led to reduction of pH to acidic level.

Rice decanted water after cooking, without further addition of any nutrients, favoured significant levels of conversion of rice starch, production of reducing sugars accumulation of total sugars, higher levels of DE and enhanced enzyme synthesis by the four strains studied, when compared with their performance in the mineral salts rice starch medium prepared with powdered rice starch. These observations very strongly advocate the superiority of cooked rice decanted water over natural rice powder, as a fermentation medium for the production of sugar as well as amylase. No similar studies are reported in the literature for a comparison. Hence based on the results it is inferred that the probable reason for this supremacy of cooked rice decanted water as a supportive medium is due to repeated gelatization of rice on cooking followed by autoclaving which would have led to the liberation of minerals, proteins and vitamins from the grains besides making the gelatized starch more vulnerable for attack by the bacteria. Under such conditions it is

quite natural for a bacteria to eat with less strain and do more activity with comfort than to struggle and spend more time to complete the job on a grain.

4.5 CONVERSION OF RICE STARCH BY FREE ENZYMES

Hansen (1981) reported liquifaction of 5% rice flour and further separation and production of high protein rice flour using thermostable α-amylase of Aspergillus niger at 100°C and liquifaction of 20% of rice flour by a thermostable α-amylase from B. subtilis at 96°C for 90 min and a conversion of 82% of the total rice starch into maltodextrins which were further used for the production of high fructose syrups (Chen & Chang 1984). Sasaki (1986) observed that, commercial preparation of α-amylase from Aspergillus niger was able to hydrolyse rice starch seven times greater than that of corn starch. Brooks and Griffin (1987) used a commercial preparation of α-amylase (Termamyl 120 L) obtained from B. subtilis and liquified 10% w/v of rice flour at 60-90°C for 15 min. Eventhough the maximal liquifaction was recorded at 80-90°C, reducing sugar was recorded high only at 70°C. Brooks and Griffin (1989) also reported the liquifaction of 30% w/v of rice flour and their conversion to maltodextrins using a commercial preparation of B. subtilis α-amylase (Termamyl 120 L).

In the present study, the ∝-amylases isolated from all the four strains of Bacillus brought about significant levels of liquifaction, reducing sugar and DE at 50-60°C. However, B. polymyxa could hydrolyze starch even at 90°C. The results are very different from those mentioned above.

4.6 IMMOBILIZATION STUDIES

4.6.1 Rice starch conversion by immobilized whole cells of Bacillus sp

Kokubu et al (1978) reported that free and poly-aerylamide gel entrapped cells of B. subtilis recorded maximal ∝-amylase activity at similar levels for some period of incubation. Later the immobilized cells recorded an increase in activity compared to the free cells. Shinmyo et al., (1982) also reported that B. amyloliquifaciens immobilized synthesized ∝-amylase at maximal level after 32 hrs of incubation while growth was maximal at 8 hrs. Sen and Chakraborthy (1987) immobilized whole cells and partially purified ∝-amylase of Lactobacillus cellobiosus and observed that the optimum pH for their activity shifted to pH 7.6 from pH 7.3 on immobilization.

However, in the present study when the whole cells of the four strains of Bacillus sp. were immobilized on

ca-alginate gel, they could bring about efficient conversion of rice starch and enzyme production and DE at pH 7-9, similar to that of free cells. In general, B. polymyxa and B. coagulans ACMN 1 produced more enzyme and DE at pH 7-8 when compared to other two strains. Unlike that observed by Sen and Chakraborthy (1987), in the present study, immobilization has not been found to influence the optimal requirements of the Bacillus sp. for rice starch conversion, when compared to free cells. However, immobilized cells recorded a marginal reduction in the yield of DE and enzymes.

Temperature also did not influence the optimal temperature requirement of all the Bacillus sp. for rice starch conversion when compared to free cells. Although there was some reduction in the yield of DE and insignificant. The present results are different from the report made for Lactobacillus cellobiosus (Sen & Chakraborthy, 1987).

Incubation time influenced significantly the rate of rice starch conversion and enzyme production and DE could be obtained only after 24 hrs when compared to that of free cells, although from 12 hrs onwards they could effect reduction of starch and synthesize enzyme. This shows that the

cells in the immobilized conditions are not induced to synthesize more enzyme unlike that of free cells which come into contact with substracts easily unlike immobilized cells.

4.6.2 Conversion of rice starch by immobilized enzymes

Linko (1975) used B. subtilis α-amylase, immobilized on cyanogen bromide activated carboxymethyl cellulose, for the conversion of wheat starch into simple sugars and compared with that of free enzyme. He observed a maximum of 20.8 mg/ml reducing sugar from 4% wheat starch slurry after 60 min at temperatures above 72°C and the immobilized enzyme was less active than free enzymes. Sen and Chakraborthy (1987) observed that immobilized α-amylase obtained from Lactobacillus cellobiosus was active at pH 7.6 than at pH 7.3 which was optimum for free enzyme. Further they recorded activity of immobilized enzyme at wide range of pH and temperature. β-amylase and pullulanase immobilized on a aerylic polymer by cross linking efficiently converted soluble starch into maltose in the 60% maximal conversion (Martensson, 1974).

In the present study, immobilization process did influence the enzyme in their optimal requirements of pH and

temperature and they recorded similar levels of DE and enzyme production when compared with free enzymes. The present results are in agreement with the reports of Linko, (1975).

4.7 STATISTICAL ANALYSIS

On hydrolysis starch is reduced to the levels of dextrin, maltose and glucose in a gradual fashion. It is natural that if residual starch in the fermentation broth decrease in level reducing and total sugar should increase in the environment provided, they are left over by the growing organism. However, production of these sugars and consequent DE, growth and enzyme production are dependent on each other and the activity of the organism. In the present study in order to understand whether these parameters follow any specific type of relation among them correlation between incubation time, residual starch, reducing sugar, total sugar, DE, growth and enzyme production were drawn by a series of correlation coefficient matrices (Pearson's Correlation). The data showed that there existed a significant correlation between these variables. Incubation time had a significant negative correlation with residual starch while recording a significant positive correlation with other parameters. Similarly all parameters, while maintaining

significant positive correlations among them maintained a significant negative correlation with residual starch. Statistical data very strongly support the results obtained for rice starch conversion by free cells and immobilized cells.

4.8 CONCLUDING REMARKS

Based on the results obtained in the present investigation, the following conclusions are drawn. Rice starch is a potential raw material for fermentation production of amylases and sugars. Cooked rice decanted water, which is normally discarded after cooking rice for food, is a nutrient rich fermentation medium for rapid production of reducing sugar, sugar syrups and amylases. This raw material can be thought of in commissioning small scale industries based on starch for production of alcohol, vinegar and sugar syrups. Bacillus sp. is an ideal candidate for fermentation production of α-amylase. B. coagulans and B. polymyxa are potential sources of α-amylase production using rice starch in commercial scale. However, scaling up studies are required to get substantial support for this purpose.

5. SUMMARY

5.1 Amylolytic *Bacillus* sp. isolated from various samples were initially subjected to a primary and secondary screening and later selected four potent strains of rice fermenting *Bacillus* sp. which belonged to soil.

5.2 The isolated strains were identified as *B. coagulans* ACMN 1, *B. coagulans* ACMN 42, *B. polymyxa* ACMN 25, and *B. cereus* ACMN 33.

5.3 All the four strains were initially characterized for their optimal requirements of pH, temperature, NaCl, substrate concentration incubation time and inoculum concentration and carbon and nitrogen sources for maximal growth and enzyme production.

5.4 All the four strains could grow and produce enzyme at a wide range of pH (6-10) especially at alkaline range of pH. An optimum pH 9 was recorded for *B. coagulans* ACMN 1 and in *B. polymyxa* ACMN 25 and pH 7 and 8 for *B. cereus* ACMN 33 and *B. coagulans* ACMN 42 respectively.

5.5 All the strains showed maximal growth and enzyme production between 30-45°C.

5.6 All the strains preferred NaCl concentration up to 0-5% for maximal growth and enzyme production. While both B. coagulans strains opted for absence of NaCl, the other two strains B. polymyxa and B. cereus required 0.5% NaCl for maximal growth and enzyme production.

5.7 All the four strains could record maximal levels of growth and enzyme production at 1% substrate concentration.

5.8 An incubation period of 12-24 hrs was found sufficient for maximal growth and enzyme production by all the four strains.

5.9 Although inoculum concentrations 1-3% promoted the four strains to grow to a maximum, even 1% inoculum level led to maximal growth and enzyme production by all the four strains.

5.10 All the carbon sources tested were found to favour both growth and enzyme production for all the four strains.

Starch, maltose and dextrin encouraged higher enzyme production when compared to glucose, lactose and glycerol.

5.11 Among the nitrogen sources tested organic nitrogen sources favoured more growth and enzyme production than inorganic nitrogen sources. Peptone favoured maximal amylase production for all the strains. Yeast extract did not enhances amylase production.

5.12 All the four strains were found to grow fast and spend about 3-4 hrs in logarithmic phase and 6-12 hrs in logarithmic phase before entering stationary phase. The growth curve patterns for the strains did show much differences among the strains. Generation time for the strains were 57.75, 37.8, 48.0 and 39.0 minutes for B. coagulans ACMN 1, B. coagualns ACMN 42, B. polymyxa ACMN 42 and B. cereus respectively.

5.13 The amylolytic enzymes from the four species of Bacillus were partially purified by $(NH_4)_2SO_4$ fractionation followed by dialysis. An overall recovery of 54.5, 62.2, 45.9, 71.4% and a specific activities of 52.7, 41.2, 62.5 and 60.9 were obtained for B. coagulans ACMN 1, B. coagulans ACMN 42, B. polymyxa ACMN 25 and B. cereus ACMN 33 respectively.

5.14 The partially purified amylase recorded higher dextrinizing activity and a meagre level of saccharolytic activity within 10 min. They were identified as α-amylase.

5.15 The partially purified amylase from all the four strains recorded activities and stability at wide range of pH and temperature. They preferred alkaline range of pH for maximal activity and stability. An optimum pH of 9 was recorded for all the four strains except for B. cereus which preferred pH 7 for maximal activity and stability. Amylase from B. coagulans ACMN 1 and B. polymyxa ACMN 25 showed an optimum temperature of 50°C while the other two strains preferred 40°C for maximal activity and stability. Interestingly B. coagulans ACMN 1 and B. polymyxa ACMN 26 recorded activity and stability even at 80-90°C.

5.16 Substrate concentration of 1 and 3% were found to be preferred for maximal activity of the enzymes while enzymes of B. coagulans ACMN 1 recorded maximal activity at 1% substrate concentration, the enzymes of all the other strains preferred 3%.

5.17 Heavy metals like Fe, Hg, Ag, Cu, Mn were found to inhibit activity of the α-amylase especially Fe which effected 100% inhibition.

5.18 Fermentation in the case of B. coagulans ACMN 1, at pH 7, 30°C along with a rate of aeration of 2 vvm influenced rapid and maximal accumulation of sugar and enzymes in the broth within a short duration, when compared to other conditions. Similarly enhanced rate of aeration at 2 vvm enhanced the yield of DE for this strains. Fermentation of rice decanted water also found to enhance enzyme production, reducing sugar accumulation with significant reduction of starch.

5.19 This species, B. coagulans ACMN 42 unlike that of B. coagulans ACMN 1 was influenced by a rise in the incubation temperature and rate of aeration. Hence when these parameters were rised, they effected an increase in the amount and rate of production in the reducing sugar, total sugar, DE, cell protein and enzyme activity. Similarly arresting of aeration midway during fermentation also changed the course of fermentation in that, there was a rapid fall in the values from the levels achieved during aeration. This organism did not indicate acid production in the media unlike other species. This species required 16 hr incubation and 2 vvm aeration to produce a maximal DE while under normal conditions, it could produce maximal enzyme. However, rice decanted water could

enhance enzyme production, DE and enhance the growth. Like B. coagulans ACMN 1, this species was also recorded significant level of enzyme production, reducing sugar and DE during the fermentation on this substrate.

5.20 B. polymyxa ACMN 25 also showed some response to changes in the fermentation conditions. Rise in the temperature from 30° to 35°C enhanced the level of production of reducing sugar, total sugar, cell protein, enzyme activity and DE. However, the increase was only marginal. When rise in pH effected a decrease in the levels of all variables except enzyme activity which was comparatively more at pH 9 than at pH 7 at 30°C. Rate of aeration when increased, also effective rapid conversion and enhanced production of sugars, DE and enzymes at short period of incubation. Arresting of aeration after 12 hrs not only resulted in the decrease in the enzyme production but also indicates a rapid acid production by the organisms. Fermentation of rice decanted was also found to favour significant level of enzyme production, growth, reducing sugar, accumulation and DE.

5.21 B. cereus ACMN 33, similar to B. coagulans ACMN 1 showed poor response to change in fermentation conditions.

However, it responded to enhanced aeration than the rise in pH and temperature. Enhanced aeration could in fact double the level of DE in the medium when compared to all other conditions. Like other strains, rice decanted water found to favour significant level of amylase production, reducing sugar, growth, and DE during fermentation by this species of Bacillus also.

5.22 α-amylase obtained from all the four strains brought about a significant level of hydrolysis of starch and production of reducing sugar and DE at 50-60°C. Interestingly enzymes of B. polymyxa ACMN 25 could bring about the rice starch hydrolysis even at 90°C.

5.23 Whole cells of all the strains on ca-alginate beads showed no marked differences in the rate of conversion, reducing sugar production, DE and enzyme activity when compared to that of free cells, at different pH and temperatures. However, the cells, upon immobilization required higher incubation time than that of free cells for maximal conversion and enzyme production.

5.24 The enzymes in the immobilized conditions were less active than that of free enzyme. However, they were not influenced by the immobilization process, in terms of their optimal conditions for activity.

REFERENCES

1. Aiba, S., Kitai, K., Imanaka, T. (1983) Appl.Environ. Microbiol., 46: 1059.

2. Alam, S., Juan, H., William, W. (1989) Biotech.Bioengg., 33: 780.

3. Ana, A.L., Steren, R.T. (1978) J.Food Science, 43: 1012.

4. Anderson, E., Aun-C.J., Barbel, H.H. (1985) Enzyme Microbiol.Technol., 7(7): 333.

5. Anon. (1984) Bulletin 248B (Bagsuaerd Novo Industri A/S).

6. Armbruster, F.C., Jacaway, W.A. (1970) US Patent 3459, 496.

7. Babu, K.R., Satyanarayana, T. (1990) Proceedings on National Symposium on Current Trends in Biotechnology, Cochin.

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

9. Basak. (1961) Indian J.Agric.Sci., 31: 113.

10. Basu, S. (1934-35) Indian J.Med.Res., 22: 745.

11. Batra, L.R., Mill, P.D. (1976) Dev.Ind.Microbiol., 17: 117.

12. Batra, L.R. (1981) Advances in Biotechnology, Moo-Young, M., Robinson, C.W. (eds), Pergamon Press, Toronto, p.54.

13. Bergaman, W.F., Jun, I.A., Susumu, H. (1988) Appl.Microbiol.Biotechnol., 27: 443.

14. Bevenue, W. (1956) J.Agric.Fd.Chem., 4: 1014.

15. Bindu, C.K. (1989) M.Sc.Project, Cochin University of Science and Technology.

16. Boyer, E.W., Ingle, M.B. (1972) J.Bacteriol., 110(3): 992.

17. Brooks, J.R., Griffin, V.K. (1987) J.Food Science, 52(3): 712.

18. Brooks, J.R., Griffin, V.K. (1989) J.Food Science, 54(1): 190.

19. Buchanan, R.E., Gibbons, N.C. (1974) Bergey's Manual of Determinative Bacteriology, 8th edn., Williams and Wilkins Co., Baltimore.

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

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

22. Campbell, L.L. (1955) Arch.Biochem.Biophys., 54: 154.

23. Chen, W.P., Yung-Chi, Chang (1984) J.Sci.Food Agric. 35: 1128.

24. Chiang, J.P., Alter, J.E., Sternberg-E.M. (1979) Die. Starke, 31: 86.

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

26. Chibata, I., Wingard, L.L. (1983) In Immobilized Microbial Cells, Applied Biochemistry and Bioengineering, Vol.4, Wingard, L.L., Katechalski E.K. and Goldstein, L. (eds), Academic Press, New York.

27. Chua, J.W., Nobuyuki, F., Yuzuru, W., Toshiomi, Y., Hisaharu, T. (1984) J.Ferment.Technol., 62(2): 123.

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

29. Coleman, G., Grant, M.A., (1966) Nature, 211: 306.

30. Corfield, V.A., Reid, S.J., Bodmer, J., Thomson, J.A. (1984) Gene., 30: 17.

31. Cronk, T.C., Steinkarun, K.H., Hakler, L.R., Mattick, L. (1977) Appl.Environ.Microbiol., 33: 1067.

32. De nault, U. (1963) Cereal Chemistry, 40(6): 618.

33. De Queiroz, M.S., Rodrigues, E.G.C., Da Silva, G.H., Coutinho, P.H.A., Scofield, R.L., Leucos, A.L. (1982) Biol.Tech PETROBAS, 25(3): 177.

34. Desikacher, H.S.R., Radhakrishnamurthy, R., Rama Rao, G., Kadkoi, S.B., Sreenivasan, N., Subramanyan, V. (1960) J.Sci.Ind.Res., 19: 168.

35. D'Souza, S.F. (1989) Indian J.Microbiol., 29(2): 83.

36. Dondero, L.M., Montgomery, M.W., Mc Gill, L.A., Law, D.K. (1978) J.Food Science, 43: 1698.

37. Dubois, M., Gills, K.A., Hamilton, T.K., Robers, P.A., Smith, F. (1956) Analyst Chem., 28: 350.

38. Elegado, B., Yusaku, F., Yojiro, K. (1986) Annual Reports of ICME, Osaka University, 9: 143.

39. FAO Nutr.Stud. (1954) 1: 15.

40. Fischer, J., Ulbrich, R., Schellenberg, A. (1978) Acta. Biol.Med.Ger., 37: 1413.

41. Fugita, E., Sugimoto, Y., Tanaka, N., Yoshida, T., Taguchi, H. (1983) Hakko Kogaku, 61: 331.

42. Fugita, E., Sugimoto, Y., Tanaka, N., Yoshida, T., Taguchi, H. (1984) Hakko Kogaku, 62: 63.

43. Gordon, R.E., Haynes, W.C., Pang, C.H.N. (1973) The Genus Bacillus, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.

44. Goyal, S.G., Khandeparker, V.G. (1978) Ind.J.Microbiol., 13: 73.

45. Groom, C.A., Daugulis, A.J., White, B.N. (1988) Appl. Microbiol.Biotechnol., 28: 8.

46. Hansen, L.P., Hosek, R., Callan, M., Jones, F.T. (1981) Food Technol., November: 38.

47. Harden, J.D. (1972) Eng., December: 59.

48. Harden, J.D. (1973) Eng., January: 65.

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

50. Hayashi, T., Zeruhiko, A., Koki, H. (1988) Agric.Biol. Chem. 52(2): 443.

51. Heady, R.E., Armbruster, F.C. (1970) US Patent, 3565-765.

52. Hebeda, R.E., Styrlund, C.R., Teague, W.M. (1988) Starch/ Starke, 40(1): 112.

53. Heisler, E.G., Treadway, R.H., Osborne, M.F., Clennan,M.L. (1952) Am.Pot.J. 29: 37.

54. Hiroshi, M., Yogiro, K., Seinosuke, U. (1982) J.Ferment. Technol., 60(6): 599.

55. Howling, D. (1973) J.Appl.Chem.Biotechnol., 23: 164.

56. Hupkes, J.V., Van Telburg, R. (1976) Die Starke, 28: 356.

57. Hyun, H.H., Zeikus, J.G. (1985) Appl.Environ.Microbiol., 5: 1174.

58. Jaleel, S.A., Srikanta, S., Ghildyal, N.P., Lonsane, B.K. (1988) Starch/Starke, 40(2): 45.

59. Jones, K.L., Grainger, J.M. (1983) Eur.J.Appl. Microbiol. Biotechnol., 18: 181.

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

61. Joyet, P., Guerineau, M., Heslot, H. (1984) FEMS Microbiol. Lett., 21: 353.

62. Kaetsu, I., Kumakura, M., Yoshida, M. (1979) Biotechnol. Bioengg., 21: 847.

63. Kalevi, V., Alexander, M.K. (1987) Biotechnol.Bioengg., 30: 917.

64. Karube, I., Hirano, K.I., Suzuki, S. (1977a) J.Solid. Phase.Biochem., 2: 241.

65. Karube, I., Suzuki, S., Kusano, T., Sato, I. (1977b) J.Solid Phase Biochem., 2: 273.

66. Kawazu, T., Nakanishi, Y., Vozumi, N., Sasaki, T., Yamagata, H., Tsukagoshi, N., Udaka, S. (1987) J.Bacteriol. 169: 1564.

67. Kennedy, J.F. (1976) J.Chem.Soc.Perkin Trans., 1: 329.

68. Kennedy, J.F., White, C.A. (1979) Starke, 31: 375.

69. Kingma, W.G.Ir. (1969) Process Biochemistry, April, 19.

70. Ko, S.D. (1972) Appl.Microbiol. 23: 973.

71. Kochhar, S., Raniyi, D.D. (1990) Biochenol.Lett., 12(5): 393.

72. Kokubu, T., Isao, K., Shuichi, S. (1978) Appl.Microbiol. Biotechnol., 5: 233.

73. Kondo, H. (1984) Sake Kopdansha International Ltd.

74. Koto, Y., Tunko, N., Katsuhiko, M. (1989) J.Fermentn. Bioengg., 68(1): 14.

75. Kuccra, J., Kuminkova, M. (1980) Collect.Czceh.Comm., 45: 298.

76. Kumakura, M., Yoshida, M., Asano, M., Kaetsu, I. (1977) J.Solid Phase Biochem. 2: 279.

77. Kunhi, A.A.M., Ghildyal, N.P., Lonsane, B.K., Ahmed,S.V., Natarajan, C.P. (1981) Starch/Starke, 33: 275.

78. Lakshmi, I. (1978) M.Sc.Project, Biochemistry Department, M.S.Univ. of Baroda.

79. Langois, D.P., Dale, J.K. (1940) US Patent, 2: 201.

80. Leach, H.W., Hebeda, R.E., Holik, D.J. (1975) US Patent, 3: 922, 197.

81. Lin, L., Kang, J. (1988) Shipin Kexue (Beijing), 5(9): 104.

82. Linko, Y., Saarinen, P., Linko, M. (1975) Biotech.Bioengg. 17: 153.

83. Lonsane, B.K. (1990) In Proceedings on National Symposium on Current Trends in Biotechnology, Cochin.

84. Lowry, O.H., Roseburough, N.S., Farr, A.L., Randall, R.Y. (1951) J.Biol.Chem., 193: 265.

85. Madsen, G.B., Norman, B.E., Slott, S. (1973) Die Starke, 25(9): 304.

86. Magee, R.J., Kosarie, N. (1987) Adv.Appl.Microbiol., 32: 89.

87. Marshall, J.J. (1974) FEBS Lett., 46: 265.

88. Martensson, R. (1974) Biotech.Bioengg., 16: 567.

89. Mattiason, B. (ed.) (1983) Immobilized Cells and Organelles, CRC Press, Boca, Raton, Vols.1 and 2.

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

91. Miller, G.L. (1959) Analytical Chem., 31(3): 426.

92. Miyoshi, T., Gyozo, F. (1973) Fermentn.Technol., 51(5): 305.

93. Morgan, F.J., Priest, F.G. (1981) J.Appl.Bacteriol., 50(1): 107.

94. Moseley, H.M., Leonard, K. (1970) Biotech.Bioengg. 12(2): 251.

95. Muller, H. (1978) US Patent, 4, 069, 103.

96. Munk, L., Rexen, F., Haastrup, L. (1988) Starch/Starke, 40(3): 81.

97. Nakumura, I.M., Sawada, H., Abad, E.J., Chaitiumvong,S., Yoshida, T., Taguchi, S.H., Suuahegul, N. (1978) Annual Reports of ICME, Osaka University, Japan, 1: 31.

98. Nandakumar, M.P., Chandrasekaran, M.C. (1990) In Proceedings of National Symposium Cum Workshop on Immobilized Cells and Enzymes, Trivandrum.

99. Ngaba, P.R., Lee, J.S. (1979) J.Food Science, 44: 1570.

100. Oesterguard,J., Knudson, S.L. (1976) Die Starke, 28: 350.

101. Ogasahara, K., Imanishi, A., Isemura, T. (1970) J.Biochem., 65: 65.

102. Okada, H., Urabe, I. (1976) Am.Chem.Abstr., 85: 16336.

103. Oriel, P. (1988) Enzyme.Microbiol.Technol., 10: 518.

104. Ortlepp, S.A., Ollington, J.F., Mc Connel, D.J. (1983) Gene., 23: 267.

105. Pain, Banerjee. (1956) Indian J.Med.Res. 44: 749.

106. Palsmer, J.J. (1975) Process Biochemistry, December: 19.

107. Palva, I. (1982) Gene., 19: 81.

108. Papoutsakis, E.T., Meyer, C.L. (1985) Biotechnol.Bioengg., 27: 50.

109. Parihar (1955) Nature, London, 175: 42.

110. Park, Y.K., Papini, R.S. (1970) Rev.Brasil.Technol., 1: 13.

111. Pazlarova, J., Baig, M.A., Votruba, J. (1984) Appl.Microbiol.Biotechnol., 20: 331.

112. Pfueller, S.L., Elliott, W.H. (1969) J.Biol.Chem., 48: 244.

113. Pinches, A., Louw, M.E., Watson, T.G. (1985) Biotech.Lett., 7(9): 621.

114. Pirt, S.J., Callow, D.S. (1959) Selected Scientific Papers from the Instituto Superiore di Sanita, 2: 292.

115. Prabha, K.C. (1989) M.Sc.Project, Cochin University of Science and Technology, Cochin.

116. Prema, P., Ramakrishna, S.V., Madhusudhana Rao, J. (1986) Biotech.Lett., 8(1): 449.

117. Purushothaman, D., Dhanapal, N., Rangaswami, G. (1977) Symposium on Indigenous Fermented Foods, Bangkok, Thailand.

118. Rajalakshmi, R., Vanjana, K. (1967) Brit.J.Nutr. 21: 467.

119. Ramakrishna, C.V. (1979) Baroda J.Nutr., 6: 1.

120. Ramesh, M.V., Lonsane, B.K. (1989) Biotech.Lett., 11(1): 49.

121. Ramesh, M.V., Lonsane, B.K. (1989) Process Biochemistry, October: 176.

122. Rao (1952) Proc.Indian Acad.Sci., 36B: 70.

123. Reade, E.A., Gregory, F.K. (1975) Appl.Microbiol., 30: 897.

124. Rexen, F., Munk, L. (1984) Report Prepared for the Commission of the European Communities, DG XII.

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

126. Rose, A.H. (1980) Microbial Enzymes and Bioconversions in Economic Microbiology, Vol.5, Academic Press, New York.

127. Sadasivan, Sreenivasan (1938) Indian J.Agric.Sci., 8: 307.

128. Saha, C.B., Gregory, J.Z. (1987) Process Biochemistry, 6: 78.

129. Saha, C.B., Gregory, J.Z. (1989) Biotech.Bioengg., 34: 299.

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

131. Saito, N., Yamamoto, K. (1975) J.Bacteriol., 121: 848.

132. Sandhu, D.K., Waraich, M.K. (1984) Kawaka, 12: 73.

133. Sandhu, D.K., Soni, S.K. (1988) Biotech.Lett., 10: 277.

134. Sasaki, H., Kurosawa, K., Takao, S. (1986) Agric.Biol. Chem., 50: 1661.

135. Sathe (1951-52) Sci. and Cult., 17: 134.

136. Sen (1917) Bull.Agric.Res.Inst. Pusa, 70: 39.

137. Sen, S., Chakraborthy, S.L. (1987) Enzyme Microbiol. Technol., 9: 112.

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

139. Shakunthala, M.M., Shadakshara Swamy (1987) In Food--Facts and Principles, Wiley Eastern Ltd., New Delhi, p.262.

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

141. Shinke, R., Yuji, K., Hiroshi, N. (1975) J.Fermentn. Technol., 53(10): 693.

142. Shinke, R., Yugi, K., Kenji, A., Hiroshi, N. (1977) J.Fermentn.Technol., 55(12): 103.

143. Shinmyo, A., Hiroyuki, K., Hirosuki, O. (1982) Eur.J. Appl.Microbiol.Biotech., 14: 7.

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

145. Sinclair, P.M. (1965) Chem.Engg., 30: 90.

146. Slominska, L., Starogardzka, G. (1986) Starch/Starke, 38(6): 205.

147. Smiley, K.L., Hofreiter, B.T., Boundy, J.A., Rogovin,S.P. (1975) Proceedings of the International Symposium on Biodegradation 1001 Applied Science.

148. Soni, S.K., Sandhu, D.K. (1990) Indian J.Microbiol., 30(2): 135.

149. Sreenivasan, J. (1939) Indian J.Agric.Sci., 9: 208.

150. Sreenivasan, J. (1951) J.Sci.Industr.Res., 10A: 438.

151. Srikanta, S., Jaleel, S.A., Sreekantiah, K.R. (1987) Starch/Starke, 39: 132.

152. Strickland, L.H. (1951) J.Gen.Microbiol., 5: 698.

153. Sugimoto, Y., Tanaka, N., Furukawa, A., Watanabe, K., Yoshida, T., Taguchi, H. (1984) (Abstr.), Annual Meeting of Soc.Fermentn.Technol. Japan, p.46.

154. Sugimoto, Y., Tanaka, N., Furukawa, A., Watanabe, K., Yoshida, T., Taguchi, H. (1986) J.Fermentn. Technol., 64: 187.

155. Takasaki, Y. (1976) Agr.Biol.Chem., 40(8): 1515.

156. Takasaki, Y., Takahara, Y. (1976) US Patent, 3992, 261.

157. Tanaka, N., Sugimoto, Y., Suzuki, Y., Furukawa, A., Watanabe, K., Yoshida, T., Taguchi, H. (1984) Hakkokogaku, 62: 391.

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

159. Thammarutwasi, P., Yusaku, F., Seinosuke, U. (1978) Annual Reports of ICNE, Osaka University, Japan, 1: 346.

160. Tsuchiya, K., Akihisa, S., Koore, S., Mitosuo, O., Yoshiharu, M. (1975) J.Fermentn.Technol., 53(4): 199.

161. Ueda, S., Celia, T.Z., Domingos, A.M., Young, K.P. (1981) Biotech.Bioengg., 23: 291.

162. Uzumi, N., Kyoko, S., Takuji, S., Shiro, T., Hideo,Y., Nirihiro, T., Shigezo, U. (1989) J.Bacteriol., 171(1): 375.

163. Vance, R.V., Rock, A.O., Carr, P.W. (1972) US Patent 3, 654, 081.

164. Venkatasubhaiah, P., Dwarkanath, C.T., Sreenivasa Murthy, V. (1984) J.Fd.Sci.Technol., 21: 59.

165. Venkatasubramanian, K. (1978) In Enzyme, The Interface between Technology and Economics, (ed.), J.P.Dansky and B.Wolnak, Marcel Dekker, New York, p.35.

166. Vollbrecht, D. (1982) Eur.J.Appl.Microbiol.Biotechnol., 15: 111.

167. Welker, N.E., Campbell, L.L. (1963) J.Bacteriol., 86(4): 681.

168. Welker, N.E., Campbell, L.L. (1963) J.Bacteriol., 86(4): 687.

169. Wang, H.L., Herseltine, C.W. (1970) J.Agric.Food Chem., 18: 572.

170. Wayman, M., Parekh, S.R., Parekh, R.S.,Trass,Grandolfi,E. (1988) Starch/Starke, 40: 418.

171. Wealth of India, Raw Materials (1966) CSIR Publication, New Delhi, p.110.

172. Wiseman, A. (1985) Handbook of Enzyme Biotechnology, John Wiley & Sons, Australia.

173. Yankov, D., Dobreva, E., Besehkov, V., Emannilova, E. (1986) Enzyme Microbiol.Technol., 8(11): 665.

174. Yamoto, C., Adachi, T., Nakajima, Y., Hidaka, H., Yoshida, T., Sugawa, F. (1976) US Patent, 3998, 696.

175. Yang, S.S. (1988) Biotech.Bioengg., 32: 886.

176. Yoo, J.Y., Theodore, W.C., Juan, H., Randolph, T.H. (1988) Biotech.Bioengg., 31: 357.

177. Yoon, M., Yoo, Y., Cadman, T. (1989) Biotechn.Lett., 11(1): 57.

178. Yoshigi, N., Chikano, T., Kamimura, M. (1985) Agric. Biol.Chem., 49: 2379.

179. Yoshigi, N., Kamimura, M. (1988) Agric.Biol.Chem., 52(9): 2365.

180. Yoneda, Y. (1980) Appl.Environ.Microbiol., 39: 274.