STUDIES ON AMYLOLYTIC BACTERIA IN COCHIN BACKWATERS

THESIS

SUBMITTED TO

THE COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MARINE MICROBIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

BY

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To
My Parents

CERTIFICATE

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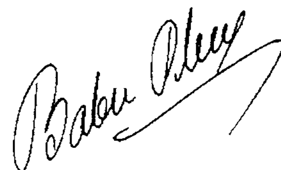
This is to certify that this thesis entitled 'Studies on amylolytic bacteria in Cochin backwaters' is an authentic record of the research carried out by **Smt. A.V. SARAMMA,** under my supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements of the Ph.D. Degree of the Cochin University of Science and Technology and no part thereof has been presented before for any other degree in any University.



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DECLARATION

I hereby declare that this thesis entitled 'STUDIES ON AMYLOLYTIC BACTERIA IN COCHIN BACKWATERS' has not previously formed the basis for the award of any degree, diploma or associateship in any University.

Cochin - 682 016,
December 1992.

A.V. SARAMMA

ACKNOWLEDGEMENTS

The material presented in this thesis is the report of a study undertaken by me during 1987-1992. In this connection, I have received immense help from various esteemed persons and institutions.

I owe a deep debt of gratitude to Dr. Babu Philip, Reader in Marine Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, for his expert guidance and supervision at various stages of the study.

I am grateful to Dr. P. Lakshmanaperumal Samy, Reader in Microbiology, Bharathiar University for suggesting the problem and for the useful discussions.

I would like to thank Dr. N.R. Menon, Director, School of Marine Sciences for all the facilities and support given to me in this regard. The constant encouragement and support given by Dr. Y.L. Dora and the late Dr. P.N.K. Nambisan, former Directors of the School of Marine Sciences are gratefully acknowledged here.

I am grateful to Dr. A. Mohandas, Head of the School of Environmental Studies and to Dr. M. Shahul Hameed, Head of the Department of Industrial Fisheries CUSAT for providing me with

laboratory facilities. I am also grateful to the Director, Central Institute of Fisheries Technology, Cochin for giving me library facilities.

I am obliged to Dr. D. Chandramohan, Scientist, National Institute of Oceanography, Goa and to the late Dr. George Philip, Professor of Biochemistry, School of Marine Sciences for their valuable academic advice.

I greatly appreciate and acknowledge the thought-provoking ideas given to me by Dr. I.D. Konikkara, Reader in Microbiology, University of Calicut.

I would like to extend my sincere thanks to Dr. M. Chandrasekharan, Head of the Centre for Biotechnology, Cochin University of Science and Technology, for the useful discussions.

I wish to thank Dr. Rosamma Philip, Lecturer in Microbiology, for isolation and preliminary identification of the cultures. I would like to thank Dr. Bright Singh, Lecturer in Microbiology, for the useful suggestions and for taking the photomicrographs.

I acknowledge the timely help rendered to me by Dr. K.J. Joseph, Dr. C.K. Radhakrishnan, Dr. N. Chandramohan, Dr. Sophy John, Dr. V.J. Kuttyamma, Dr. Saleena Mathew,

Dr. Suguna Yesodharan, Dr. Ammini Joseph, Dr. Rajathy and all other colleagues.

Let me thank Shri. P.J. George, Shri. T.K. Maqbool and Shri. R. Padmasenan, Research Scholars, School of Marine Sciences for the various help I received from them. I am also thankful to the library and office staff of the School of Marine Sciences, for their help and co-operation.

I would like to acknowledge the services rendered by Radiant Copy Centre, Ernakulam for typing and binding of the thesis.

Finally, I place on record my gratitude to my husband, son, brothers, sisters and all my teachers and well-wishers for their moral support.

A.V. SARAMMA

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Chapter I

1. INTRODUCTION

1.1. PREFACE

Bacteria are well-known for their ability to elaborate extracellular enzymes. Most organic matter in nature consists of molecules that cannot directly enter cells, because of their polymeric structure, high molecular weights and large size. The hydrolysis of such polymers is a rate limiting step in the utilization of organic matter in the environment. Before they can be incorporated into the microbial cells, polymeric materials must undergo step-wise degradation by a variety of enzymes. Extracellular enzymes - enzymes released from the microorganisms into the environment as free dissolved enzymes - play a very important role in the degradation of these complex organic molecules in the environments. Low-molecular-weight compounds - the products of their enzymatic degradation - can be taken up by microbial cells to meet their energy requirements and to build up biomass.

Amylases are a very important group of extracellular enzymes secreted by microorganisms. They are involved in the degradation of starch in the environment. Amylases are commercially very important enzymes. They are used for making starch syrups, adhesives, sizings, paper coating and in brewing, baking, textiles,

pharmaceuticals, animal feed, digestive aids and detergents. They are also used for treatment of sewages. In these industries, amylases have gradually been replacing acid as a hydrolytic agent.

The commercially significant amylolytic enzymes are the following :

a) Amyloglucosidase (Glucoamylases; α -1, 4 – Glucan glucanohydrolase, E.C. 3.2.1.3.)

These enzymes hydrolyse α -1, 4 and α -1, 6 linkages and produce glucose as the sole end-product from starch and related polymers. They are exoenzymes and remove glucose units from the non-reducing end of the substrate.

b) β-Amylases (α-1,4 Glucan maltohydrolase, E.C. 3.2.1.2)

They are also exo-acting enzymes and hydrolyse α -1, 4 bonds from the non-reducing ends of the substrate. They cannot bypass α-1,6 linkages in amylopectin and glycogen. They produce maltose from amylase and maltose and β -limit dextrin from amylopectin and glycogen.

c) α -Amylases (α -1,4 Glucan-4 Glucanohydrolase, E.C. 3.2.1.1)

These are endo-acting enzymes and hyrolyse α -1,4 bonds and

bypass α-1,6 linkages in amylopectin and glycogen. The end products include maltose, glucose, oligosaccharide mixtures and α-limit dextrin from amylopectin.

Even though several terrestrial bacteria are known to produce amylase, only very few amylolytic halophilic bacteria are reported. Detailed studies have not been made on the amylolytic bacteria from Indian waters. Hence, this work is undertaken.

The important objectives of the study are :

1. to find out the distribution of amylolytic bacteria in water, sediment, fishes, clams and prawns from Cochin backwaters;
2. to select and identify some of the most potent amylolytic bacteria;
3. to study their cultural conditions for maximum growth and amylase production;
4. to study the mechanisms involved in the regulation of amylase production such as induction and repression;
5. to identify the amylase secreted by selected strains, and to study the kinetics of the enzymes;
6. to find out the ability of the amylase to degrade raw-starch from different sources; and

7. to determine the effect of various ions and organic compounds on amylase action.

The thesis consists of six chapters. The introduction covers preface and review of literature. The second chapter deals with screening and selection of amylolytic bacteria. The third chapter explains the effect of various conditions on growth and amylase production. The fourth chapter deals with the regulation of amylase synthesis. The fifth chapter describes the characters of amylases and the last chapter gives the summary of the work, followed by the list of references.

1.2. REVIEW OF LITERATURE

1.2.1. AMYLOLYTIC BACTERIA

There are several reports on the production of amylases by bacteria. Occurrence of amylases as extracellular enzymes has been demonstrated in bacteria like *Bacillus cereus* var. *mycoides*[1], *Bacillus megaterium*[2,3], *Bacillus subtilis* IMD. 198[4] *Pseudomonas* sp. BQ6[5], *Streptomyces* sp.[6], *Clostridium thermosulfurogenes*[7], *Bacillus acidocaldarius*[8], *Bacillus amyloliquefaciens*[9], *Bacillus caldolyticus*[10], *Bacillus coagulans*[11], *Bacillus licheniformis*[12,13], *Bacillus stearothermophilus*[14], *Bacillus subtilis* R. 623[15], *Bacterioides amylophilus*[16], *Bacillus* HOP.40[17], *Clostridium*

acetobutylicum[18], Clostridium butyricum[19], Clostridium sp.[20] Streptomyces aureofaciens[21], Thermoactinomyces vulgaris strain 42[22], Thermoactinomyces viridis[23], Paseudomonas saccharophila[24] Streptomyces albus G.[25], genetically engineered Escherichia coli[26-29] Thermoanaerobacter finnii, Thermobacterioides acetoethylicus, Thermoanaerobacter ethanolicus and Clostridium thermosaccharolyticum[30], Thermomonospora curvata[31,32], Bacillus circulans[33], Lactobacillus[34], Thermophilic Actinomycetes[35], Bacillus coagulans ACMN 1 and ACMN 42, Bacillus polymyxa ACMN 25 and Bacillus cereus ACMN 33[36], Streptococcus bovis JBI, Rumibacter amylophilus H18, Butyrivibrio fibrosolvens and Bacterioides ruminicola[37]. Olukayode et al[38] observed extracellular amylase production by cassava-fermenting bacteria, Bacillus subtilis, Bacillus licheniformis and Bacillus cereus. Production of heat-stable amylase complex from Dictyoglomus thermophilum was reported by Yasuhiko et al[47].

The halophilic bacteria known to produce amylase include Vibrio gazogenes[39], Micrococcus halobius[40], Halobacterium vallismortis[41], Acinetobacter sp[42], Vibrio parahaemolyticus[43], Micrococcus varians sub sp. halobius[44] and Alteromonas rubra[45]. Lindgren and Refai[46] have reported amylolytic lactic acid bacteria in fish ensilage.

1.2.2. CULTURAL CHARACTERS

Pau-Tsung[48] reported optimum ∝-amylase production by Bacillus subtilis 220 in a medium containing defatted soya bean powder, ammonium sulphate, tapioca, dipotassium hydrogen phosphate, magnesium sulphate and calcium chloride. Gandhi and Kjaergaard[49] studied the effect of CO_2 on the formation of ∝-amylase by Bacillus subtilis and found that highest activity was obtained using 6% CO_2 (V/V). Gasparyan et al[50] observed highest rate of ∝-amylase production by a new strain of Bacillus subtilis in a medium containing maltose and peptone as carbon and nitrogen sources respectively. Tryasogolova et al[51] reported maximum amylase production by Bacillus subtilis 83 in a fermentation medium containing corn extract, corn meal, ammonium phosphate, urea and calcium carbonate. Fogarty and Bourke[4] observed highest levels of amylase production by a strain of B. subtilis (IMB 198) in a salt medium containing soya bean meal and starch. The effect of iron salts on the production of Bacillus subtilis β-glucanase and ∝-amylase was studied by Fumio et al[52].

Emannilova et al[53] studied the influence of aeration and agitation on the thermostable ∝-amylase and proteinase from Bacillus licheniformis 44 MB-82. Chandra et al[12] studied the cultural and nutritional requirements of Bacillus licheniformis CUMC 305 and found the optimum pH, temperature and incubation period as 6.5, 48°C and 15 to 20 hours respectively. They observed

an increased enzyme production by using vitamins and aminoacids. Ammonium phosphate and peptone were the ideal phosphate and nitrogen sources and galactose was the best carbohydrate carbon source for enzyme production. Krishnan and Chandra[54] studied the effect of oil seed cakes on ∝-amylase production by Bacillus licheniformis CUMC-305. Yankov et al[55] studied the optimum conditions for amylase production by B. licheniformis. Priest and Thirunavakkarasu[56] found that ∝-amylase produced by B. licheniformis was invariably extracellular.

Young et al[57] studied the kinetics of ∝-amylase synthesis from Bacillus amyloliquefaciens. Zhang et al[58] observed increased production of ∝-amylase by Bacillus amyloliquefaciens in the presence of glycine.

Takaya et al[59] determined the cultural conditions for amylase production by alkalophilic Bacillus H-167 isolated from soil and found initial medium pH 9.4; temperature 37°C and 50 to 60 hours cultivation period as optimum. Ramesh and Lonesane[17] observed that the ratio of buffer to wheat bran, incubation temperature and initial pH influence ∝-amylase production by Bacillus megaterium under solid state fermentation. Gurlev[60] found that the optimum culture medium for amylase production by strains of B. subtilis and B. amyloliquefaciens included sucrose as C source, ammonium nitrate as nitrogen source and calcium, magnesium and zinc salts.

Naohira et al[61] reported optimum culture condition for amylase production by Bacillus cereus NY-14 as pH 8.0 to 8.5, temperature 30 to 33°C and a medium containing soluble starch, peptone and NaCl. Ghosh and Chandra[62] studied the nutritional requirements and cultural characteristics of Bacillus apiarius CBML-152 for the production of thermostable α-amylase. They observed optimum incubation periods for α-amylase production in still and shake cultures as 32 to 38 hours and 20 to 25 hours respectively.

Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, Na^{+} and K^{+} ions were important for α-amylase production. Ammonium phosphate was best utilised as inorganic nitrogen and phosphate sources. Aminoacids and vitamins increased α-amylase production[62]. Srivastava and Baruah[63] studied the culture condition for production of thermostable amylase by Bacillus stearothermophilus. Esther et al[64] studied growth and extracellular enzyme production by strains of Bacillus sp. isolated from fermenting African locust bean iru.

Hostinova et al[65] studied production of extracellular and cell bound amylases from Streptomyces aureofaciens and found that cell bound amylase showed maximum production in the logarithmic phase of growth whereas, extracellular amylase was maximum in the stationary phase of growth. Dong Hewi et al[66] determined the optimum culture conditions for amylase production by Streptomyces H.A. 40 isolated from soil and found that peptone and starch

were the favourable carbon and nitrogen sources. Enzyme production was promoted by the addition of Ca^{2+} and Na^{+} but inhibited by Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+} and Ag^{+}. Optimal initial pH and temperature for enzyme formation were pH 7.0 and 45°C respectively. Abd-el-Malek et al[67] observed maximum amylase production by streptomycetes from Egyptian soils, in starch-nitrate medium within 48 hours at pH 7 to 8 with an optimum pH of 7.5. Sinha and Chandra[68] isolated amylase-producing streptomycetes from soil. Optimum cultural conditions for amylase production were temperature: 30°C, period of incubation: 4 days and a medium containing ammonium acetate as a source of nitrogen and starch as carbon source.

Avendano and Cornejo[69] studied the effect of carbon sources on amylase production and found that starch at 3% concentration was the best carbon source for enzyme production. Effect of carbon source on production of thermostable α-amylase, pullulanase and α-glucosidase by Clostridium thermohydrosulfuricum was studied by Hannes[70] who found that starch, pullulan, dextrin or maltose in the medium supported amylase production. Hanchul et al[18] studied the production of extracellular α-amylase by Clostridium acetobutylicum ATCC 824. They found optimum temperature for secretion of amylase as 45°C, optimal initial pH 6.5 and starch concentration 2.5%.

Therion *et al*[71] studied the effect of pH on growth rates of rumen amylolytic and lactilytic bacteria and found pH optima for growth between pH 6.1 and 6.6.

Onishi[72] observed a novel halophilic α-amylase produced by *Micrococcus halobius* in a medium containing 0.2 to 2% starch, 1 to 3 M NaCl or KCl at 30 to 37°C and pH 6.8 under aerobic conditions. Kobayashi *et al*[44] found that amylase production by *Micrococcus varians halophilus* ATCC 21971 was highest in the medium containing 2 M NaCl, with maltose as inducer. Amylase production was also supported by 1.5 to 3.0 M NaBr or 2 to 4M $NaNO_3$.

Uptron and Fogarty[23] studied the production of thermostable amylase and protease from *Thermomonospora viridis* in a medium containing 1.5% corn starch and 0.5% mycological peptone with an initial pH of 7.0. Best yields of amylase were obtained after incubation for 48 hours. Qadeer *et al*[73] observed that wheat bran was an ideal substrate for amylase synthesis by a locally isolated culture of *Bacillus subtilis*. Zherebtsov and Korneeva[74] studied the amylase accumulation dynamics in *Clostridium acetobutylicum* and found that maximum amylase production occurs in starch medium at 36°C for 24 hours incubation and pH: 4.5 to 4.6.

1.2.3. REGULATION OF AMYLASE SYNTHESIS

Several reports are available on studies on regulation of amylase production in microorganisms. Bodisko and Yurkevich[75] observed self regulation of α-amylase formation by Bacillus subtilis. Fumio et al[76] found that glucose and its metabolites act as repressors of amyloglucosidase but not of α-amylase synthesis. Wayne and Glenn[77] studied the effect of decoyinine on the regulation of α-amylase synthesis in Bacillus subtilis. Wambutt et al[78] studied the formation of extracellular α-amylase by Bacillus subtilis in relation to guanosine polyphosphate and found a positive involvement of it in the regulation of the expression of the α-amylase gene.

Rothstein et al[13] found that α-amylase production by Bacillus licheniformis depends on the presence or absence of a catabolite-repressing carbon source in the growth medium. Thirunavakkarasu and Priest[79] studied regulation of amylase synthesis in B. licheniformis NCIB 6346 and observed that amylase synthesis in that organism was constitutive and not dependent on exogenous α-glucan for induction and was subject to catabolite repression.

Sata et al[80,81] studied regulation of amylase synthesis in Bacillus circulans F2 and observed induction of amylase by cross linked starches and repression by glucose.

Srivastava and Mathur[82] observed induction of amylase synthesis by starch in growing cells of Bacillus stearothermophilus. Glucose and maltose were found to repress the enzyme synthesis. Khovrychev et al[83] noted a decrease in amylase production when glucose at a concentration of 0.25 to 1.0 g/l was added to continuous cultures of Bacillus stearothermophilus. Srivastava et al[84] observed that glucose had a repressive effect on amylase synthesis by thermophilic Bacillus sp. but the presence of ammonium sulphate and glutamic acid eliminated this repression. Young et al[57] found that ∝-amylase synthesis by B. amyloliquefaciens was not inducible but was subjected to catabolite repression.

Hannes[70] observed partial repression of amylase production by glucose and complete repression by fructose in Clostridium thermohydrosulfuricum. Hyun and Zelkus[85] studied the regulation of ∝-amylase production in Clostridium thermosulfurogenes and observed that ∝-amylase was expressed at high levels only when the organism was grown on maltose or other carbohydrates containing maltose units.

Iuchi and Tanaka[43] observed that production of extracellular amylase and protease in V. parahaemolyticus was repressed by various carbohydrates present in the medium. Shigenobu and Shuji[86] also observed catabolite repression of extracellular amylase

synthesis in *V. parahaemolyticus*. Ratcliffe *et al*[39] observed that amylase secretion by the marine bacterium *Vibrio gazogenes* was inducible by starch or maltose and was repressed by glucose.

Gavrilovic *et al*[45] observed induction of amylase synthesis by starch or maltose and repression by glucose in *Alteromonas rubra*.

Regulation of amylase synthesis has been reported in various fungi also. Catabolite repression has been observed in *Endomycopsis fibuligera*[87], *Filobasidium capsuligenum*[88], *Saccharomyces fibuligera*, *Schwanniomyces castellii* and *Schwanniomyces alluvius*[89] and *Aspergillus* sp.[90].

Induction of amylase production by starch has been reported in *Aspergillus oryzae*[91] and *Aspergillus niger*[92].

Sadhukan *et al*[93] studied induction and regulation of α-amylase synthesis in a cellulolytic thermophilic fungus *Myceliophthora thermophila* D14 (ATCC 48104) and found that extracellular α-amylase synthesis in that organism was inducible by starch and was subjected to catabolite repression by glucose.

1.2.4. PROPERTIES OF BACTERIAL AMYLASES

Amylases from several bacterial sources have been purified

and characterised by many workers. Amylases from different strains of Bacillus subtilis have been purified by Bakhmatova et al[94], Mantsala and Zalkin[95], and Takasaki[96,97]. Bacillus licheniformis α-amylase has been purified and characterised by Krishnan and Chandra[98], Morgan and Priest[99], Dobreva et al[100] and Galabova and velcheva[101].

Srivastava[102,103] and Srivastava et al[104,105] purified and characterised amylases from a thermophilic Bacillus stearothermophilus. Patrick et al[106] and William et al[107] purified amylase elaborated by Bacillus polymyxa. Amylases from Bacillus amyloliquefaciens have been purified by Granum[108] and Suzuki et al[109]. Naohire et al[110] purified and determined the properties of an amylase from Bacillus cereus NY.14. Thomas et al[111] characterised an extracellular β-amylase from Bacillus megaterium. Mutsuo[112] purified a Bacillus acidocaldarius α-amylase that was highly stable to heat under acidic conditions.

Hannes[113] characterised α-amylase of Clostridium thermohydrosulfuricum. Wako et al[114] purified and studied some properties of a maltotriose producing amylase from Streptomyces griseus NA 468. Hostinova et al[115] purified Streptomyces aureofaciens α-amylase and studied its action on starch. Mizokami et al[116] crystallised and studied the properties of raw-starch hydrolysing enzyme produced by Streptococcus bovis. Goldberg and Edward[117] purified and characterised an extracellular

amylase from thermophilic Streptomyces thermoviolacens sub sp. apingens.

Onishi and Hidaka[42] purified and determined the properties of amylase produced by a moderately halophilic Acinetobacter sp. Onishi and Sonoda[40] purified and studied some properties of an extracellular amylase from another moderate halophile Micrococcus halobius.

Yeong Soo et al[118] partially purified and characterised amylases from Herpetosiphon geysericola. Von Tigerstorm and Stelmaschunk[119] purified and partially characterised an amylase from Lysobacter brunescens. Amylase from Lactobacillus cellobiosus has been purified and characterised by Sensirbir and Chakrabarty[120]. Abramov et al[22] purified and studied the properties of ∝-amylase from Thermoactinomyces vulgaris strain 42. Obi and Odibo[121] partially purified and studied the characters of a highly thermostable ∝-amylase from a Thermoactinomyces sp. Amylase of the thermophilic actinomycete Thermomonospora vulgaris has been partially purified and characterised by Allam et al[122].

Toshio et al[123] purified and studied some properties of raw-starch binding amylase of Clostridium butyricum T-7 isolated from mesophilic methane sludge.

Stability of technically pure ∝-amylase of Bacillus subtilis was studied by Daniela and Jozef [124]. Thermostability of amylase produced by thermophilic bacterium Bacillus sp. was studied by Emayavaramban and Ramabadran [125]. Galabova and Velcheva [126,127] studied the thermostability of Bacillus licheniformis M.B.80 ∝-amylase in presence of urea and monovalent cations. Mechanisms of irreversible thermal inactivation of Bacillus ∝-amylase was studied by Susan and Alexander [128]. Karen et al [129] studied the characters of thermostable ∝-amylase having a low requirement for calcium ions derived from a Bacillus sp.. Dua and Kochhar [130,131] studied the substrate binding nodes of Bacillus amyloliquefaciens ∝-amylase. Kochhar and Dua [132] identified an active site centre tryptophan residue in liquefying ∝-amylase from Bacillus amyloliquefaciens.

Action pattern and substrate specificity of ∝-amylase K, a novel amylase from a strain of Bacillus subtilis was studied by Kennedy et al [133]. Balsis et al [134] studied the catalytic properties of ∝-amylase of morphological variants of Bacillus subtilis R.623. Marie et al [135] studied the catalytic properties of Bacillus megaterium amylase. Activity and action pattern of Bacillus licheniformis ∝-amylase in aqueous ethanol was studied by Blakeney and Stone [136]. Kinetics of catalytic action and thermodenaturation of ∝-amylase from B. licheniformis MB.80 was determined by Galabova et al [137].

1.2.5. RAW-STARCH DEGRADATION

Hajime and Yoshihara[138] observed raw-starch digesting amylase from *Bacillus circulans* F2, capable of digesting potato starch. Paje *et al*[139] detected amylase activity on local strains of *Streptococcus bovis* on raw cassava starch. Avendano and Cornejo[69] detected raw-starch hydrolysing ∝-amylase produced by *Clostridium* 2021. Toshio *et al*[140] observed raw-starch hydrolysing amylase produced by *Clostridium butyricum*. Lerluck *et al*[141] studied the characters of raw-starch digesting amylase from non-sulphur purple photosynthetic bacterium. Masataka *et al*[142] studied the action pattern of β-amylase from *Bacillus* strains on raw-starch. Degradation of starch granules by ∝-amylase of *Streptomyces praecox* N.A 273 was studied by Takaya *et al*[143]. Shamala and Sreekantiah[144] studied the degradation of starches by a crude enzyme preparation and utilisation of the hydrolysate for lactic fermentation.

Chapter 2

2. SCREENING AND SELECTION OF AMYLOLYTIC BACTERIA FROM COCHIN BACKWATERS

Bacteria are distributed widely in the aquatic environment. They play a central role in a number of processes in the system. They are important components of the food chain, and are required for nutrient turnover. They are also responsible for biodegradation of organic materials present in the aquatic system. Biodegradation is brought about by the secretion of extracellular enzymes. Amylolytic bacteria are involved in the production of amylase and play a very important role in the degradation of starch and related polymers.

Bacteria from water, sediment, fishes (Etroplus suratensis and Liza parsia), clams (Sunetta scripta and Meretrix casta) and prawns (Penaeus indicus and Metapenaeus dobsoni) from Cochin backwaters were isolated and preserved in our laboratory[145]. They were tested for their ability to produce amylase and to find out the distribution of amylolytic bacteria in the aquatic environment. Three of the most potent strains were selected for further studies and were identified.

2.1. MATERIALS AND METHODS

2.1.1. COLLECTION AND IDENTIFICATION OF CULTURES

Methods used for collection and identification up to generic

level were described by Philip[145].

2.1.2. SCREENING FOR AMYLASE PRODUCTION

Basal nutrient agar plates containing 0.3% (W/V) soluble starch were spot-inoculated with bacterial cultures and incubated for 2 days. The plates were flooded with Lugol's iodine solution to detect the zones of clearance which are indicative of a positive result[146,147]. The area of the zone of clearance in each plate was measured to find out the extent of amylase production.

2.1.3. IDENTIFICATION OF THE SELECTED STRAINS

Identification of the selected strains up to species level was done by employing morphological, biochemical and physiological tests[148].

In all the methods described below, the media contained 1.5% NaCl unless otherwise stated.

2.1.3.1. Incubation

The usual temperature of incubation was 28 $\pm$ 2°C.

2.1.3.2. Gram reaction and morphology

Cultures were Gram-stained and examined microscopically after 18-24 hours of incubation in nutrient agar.

2.1.3.3. Motility

Motility was determined by microscopic examination of a hanging drop preparation after 24 hours of incubation in nutrient broth.

2.1.3.4. Swarming

Nutrient agar plates were prepared and allowed to set but not to dry. Plates were spot-inoculated with the cultures and incubated for 24 to 48 hours. Plates were examined daily for up to 2 days for spreading over the entire surface[239].

2.1.3.5. Pigmentation

Pigmentation was observed by growing the cultures in nutrient agar plates.

2.1.3.6. Oxidase test

The filter paper method described by Kovacs[149] using tetramethyl-p-phenylene diamine was used.

2.1.3.7. O/129-sensitivity

Sensitivity to the vibriostatic agent O/129 (2,4-diamine - 6,7, diisopropyl pteridine) was tested at 150 µg/ml and 10 µg/ml concentration as described by Lee et al[150].

2.1.3.8. Dissimilation of glucose

Fermentation of glucose was tested by using the medium of Hugh and Leifson[151].

2.1.3.9. Salt-tolerance

Tryptone water (1% Tryptone) containing 0,3,6,8 and 10% of NaCl was used. The media were distributed in 3 ml amounts in test tubes and inoculated with the cultures. The tubes were incubated for 24 to 48 hours and the growth recorded.

2.1.3.10. Growth at 42°C

Tryptone broth with 2% NaCl was inoculated and growth recorded after 24 hours of incubation at 42°C.

2.1.3.11. Nitrate reduction

Nitrate broth (Nutrient broth + 0.1% KNO_3) was inoculated with the cultures, incubated for 24 hours and tested for the presence of nitrite, using reagent A and B of Crosby[152].

2.1.3.12. Voges - Proskauer test (VP test)

Glucose phosphate broth was prepared and distributed in 3 ml volumes in test-tubes. Inoculated with the cultures and incubated for 48 hours. The presence of acetyl methyl carbinol was detected using α-naphthol and KOH reagents.

2.1.3.13. Arginine dihydrolase

The method of Thornley[153] was used for the detection of arginine dihydrolase.

2.1.3.14. Lysine and Ornithine decarboxylase

They were detected by the method of Moller[154].

2.1.3.15. Growth on compounds as the sole source of carbon

The basal medium of Mac Leod[155] was used. The carbon sources were used at a final concentration of 0.2%. The plates were incubated at 28± 2°C and examined for growth every day for a total of 6 days. Utilisation of the following compounds – sucrose, cellobiose, D-gluconate, γ-aminobutyrate and putrescine was tested.

2.1.3.16. Production of gelatinase

Gelatin hydrolysis was detected using the methods of Cowan[146].

2.1.3.17. Production of lipase

Lipolytic activity was tested in tributyrin agar medium. Plates were spot-inoculated and incubated for 2 days and observed for clear zone around the inoculum.

2.2. RESULTS

2.2.1. SAMPLE-WISE DISTRIBUTION

Results of the screening tests are shown in Table 1 and Fig.1. Of the 858 isolates tested, 455 were amylolytic (53%). The percentage of positive isolates present in various samples were: Metapenaeus dobsoni-90.22%, Etroplus suratensis-56.3%, Penaeus indicus-55.96%, Liza parsia-48.9%, Sunetta scripta-47.3%, water-42.99%, sediment-36.93% and Meretrix casta-32.6%.

Table 1. Sample-wise distribution of amylolytic bacteria

Sample	No. of isolates tested	No. of positive isolates	Percentage of positive isolates
Metapenaeus dobsoni	133	120	90.22
Penaeus indicus	109	61	55.96
Etroplus suratensis	119	67	56.30
Liza parsia	92	45	48.90
Sunetta scripta	95	45	47.36
Meretrix casta	92	30	32.60
Water	107	46	42.99
Sediment	111	41	36.93
Total	858	455	53.03

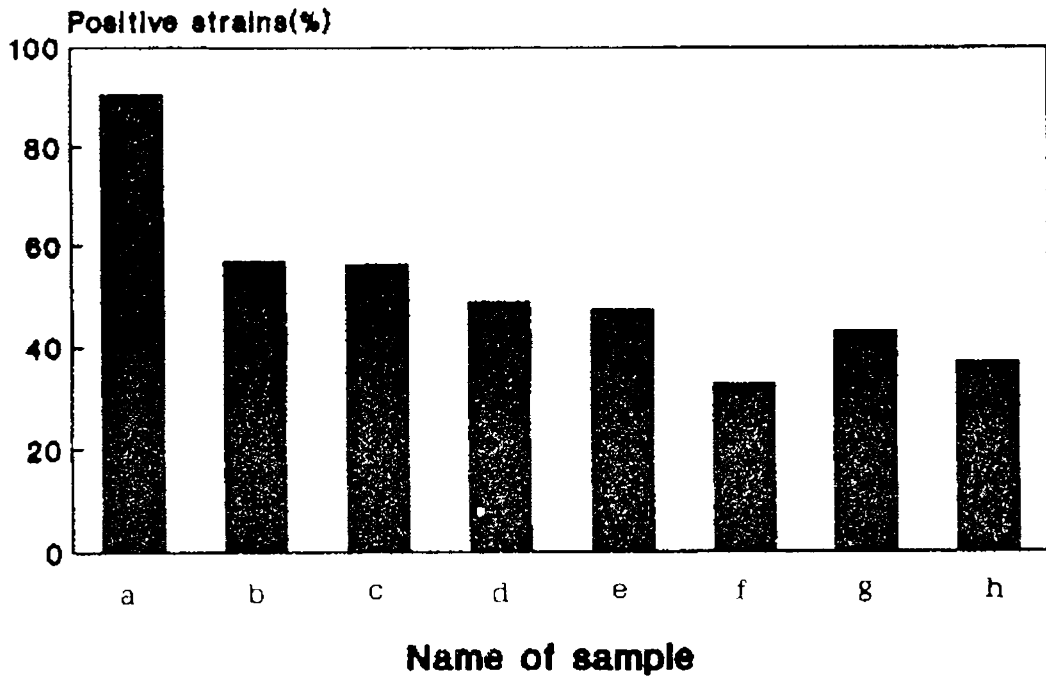


Fig. 1. Sample-wise distribution of amylolytic bacteria. (a) *M. dobsoni* (b) *P. indicus* (c) *E. suratensis* (d) *L. parsia* (e) *S. scripta* (f) *M. casta* (g) Water (h) Sediment.

2.2.2. REGION-WISE DISTRIBUTION

Region-wise distribution of amylolytic strains in different marine animals are presented in Table 2 and Fig. 2. In *M. dobsoni*, the distribution pattern was gill > surface > gut. In *P. indicus*, the pattern was gill > gut > surface. In *E. suratensis*, the gut region, harboured more amylolytic population than gill or surface. The distribution pattern was gut > surface > gill. In *L. parsia*, 35% of bacteria in the surface were found to be amylolytic whereas, in the gut, 33% and in gill, 24% were amylolytic. In clams also, the gut region harboured more amylolytic population than mantle or gill. The distribution pattern in *S. scripta* was gut > mantle > gill whereas, in *M. casta*, it was gut > gill > mantle.

2.2.3. GENERA-WISE DISTRIBUTION

Genera-wise distribution of amylolytic bacteria are shown in Table 3 and Fig.3. The percentage of amylolytic population was maximum in the genus *Vibrio* and minimum in the genus *Pseudomonas*. Occurrence of positive forms were in the order: *Vibrio* > Enterobacteriaceae > *Staphylococcus* > *Bacillus* > *Flavobacterium* > *Acinetobacter* > *Micrococcus* > *Corynebacterium* > *Moraxella* > *Pseudomonas*.

Amylolytic bacteria constitute a higher percentage of Gram-negative bacteria (64.29%) than Gram-positive bacteria (21.33%)

Table 2. Region-wise distribution of amylolytic bacteria in marine animals

Sample	Region	No. of isolates tested	No. of isolates positive	Percentage of positive isolates
M. dobsoni	Surface	45	41	91.10
	Gill	51	47	92.15
	Gut	37	32	86.48
P. indicus	Surface	32	15	46.87
	Gill	41	22	53.65
	Gut	36	24	66.66
E. suratensis	Surface	38	14	36.84
	Gill	37	25	67.56
	Gut	44	28	63.63
L. parsia	Surface	35	16	45.70
	Gill	24	16	66.66
	Gut	33	13	39.39
S. scripta	Mantle	37	14	37.83
	Gill	16	3	18.75
	Gut	42	28	66.66
M. casta	Mantle	18	4	22.22
	Gill	34	10	29.40
	Gut	40	16	40.00

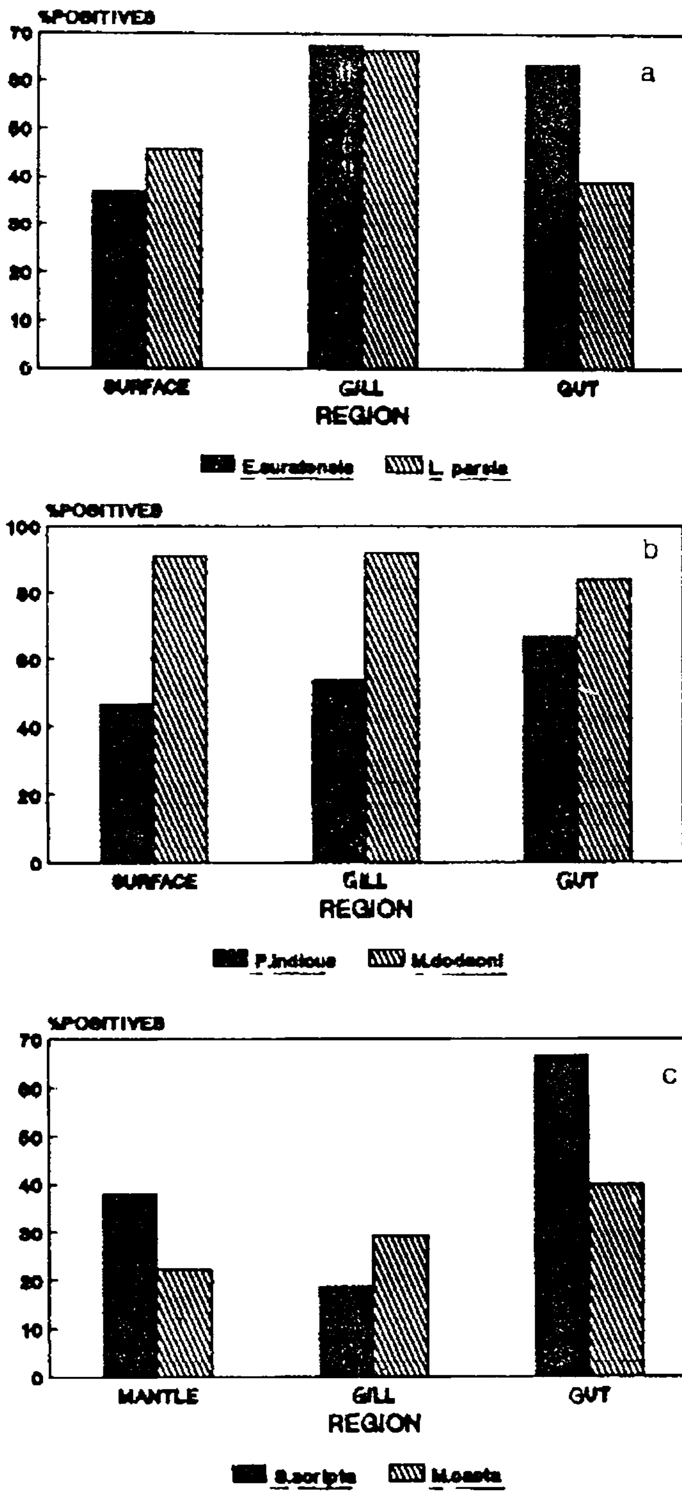


Fig. 2 a-c. Region-wise distribution of amylolytic bacteria. (a) Fishes (b) Prawns (c) Clams.

Table 3. Genera-wise distribution of amylolytic bacteria

Genera/Family	No. of isolates tested	No. of isolates positive	Percentage of positive isolates
Vibrio	397	338	85.12
Enterobacteriaceae	58	42	72.41
Staphylococcus	9	6	66.66
Bacillus	35	12	34.28
Flavobacterium	17	5	29.41
Acinetobacter	48	10	20.83
Micrococcus	137	24	17.51
Corynebacterium	44	6	13.63
Moraxella	15	2	13.33
Pseudomonas	98	10	10.20
Total	858	455	53.03

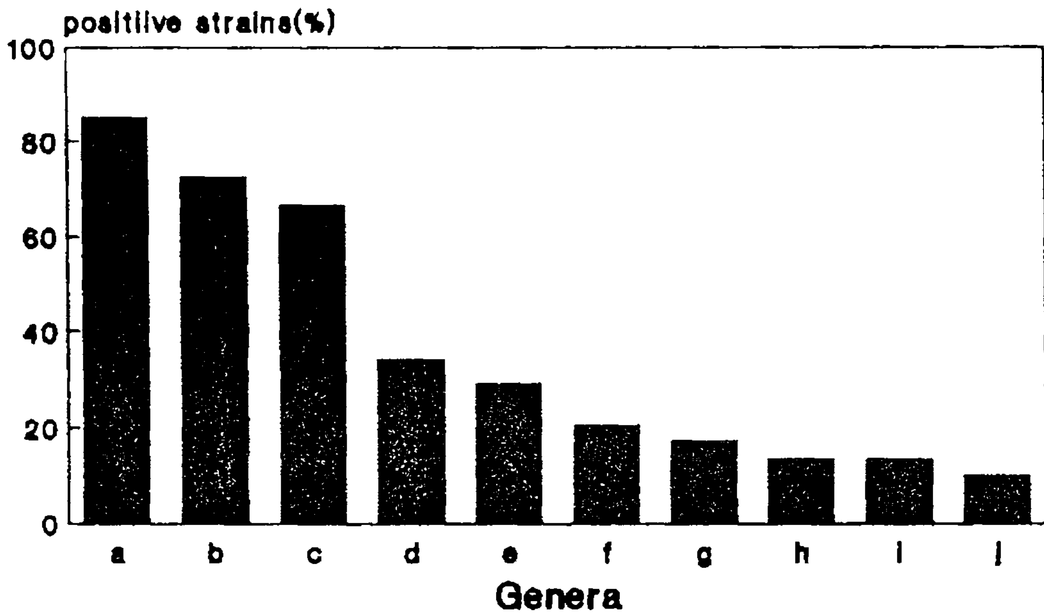


Fig. 3. Genera-wise distribution of amylolytic bacteria. (a) *Vibrio* (b) Enterobacteriaceae (c) *Staphylococcus* (d) *Bacillus* (e) *Flavobacterium* (f) *Acinetobacter* (g) *Micrococcus* (h) *Corynebacterium* (i) *Moraxella* (j) *Pseudomonas*.

2.2.4. IDENTIFICATION OF VIBRIO ISOLATES

The results of the various morphological, biochemical and physiological tests are presented in Table 4. Their characters were compared with those of standard strains according to Bergey's Manual of Systematic Bacteriology [148]. They were identified as *Vibrio alginolyticus* (MB1), *Vibrio parahaemolyticus* (MB2) and *Vibrio* sp. (MB3).

2.3. DISCUSSION

Bacteria capable of elaborating hydrolytic enzymes are distributed widely in the aquatic system. The important factors which determine the activity of various groups of bacteria in an environment are the availability of organic matter susceptible to degradation and the source of organic matter [156]. Cochin backwater is rich in sewage [157] and it is natural to expect high occurrence of bacteria, producing hydrolytic enzymes. Naturally, the animals living in such area will also harbour higher load of hydrolytic enzyme producers. These enzymes produced by the bacteria are involved in the degradation of various organic substances in the water and purify the system.

In the present study, 53% of bacteria isolated from different sources from Cochin backwaters had the ability to produce amylase.

Table 4. Characters used in the identification of *Vibrio* spp.

Sl. No.	Character	Isolate No. 1	Isolate No. 2	Isolate No. 3
1.	Gram's reaction	Negative	Negative	Negative
2.	Morphology	Rod	Rod	Rod
3.	Motility	Motile	Motile	Motile
4.	Pigmentation	-	-	Yellow-brown diffusible pigment
5.	Swarming on solid media	+	-	-
6.	Cytochrome oxidase	+	+	+
7.	Nitrate reduction	+	+	+
8.	O/129 Sensitivity			
	10 µg	-	-	+
	150 µg	+	+	+
9.	Thornley's arginine dihydrolase	-	-	-
10.	Lysine decarboxylase	+	+	+
11.	Ornithine decarboxylase	+	+	+
12.	Growth at 42°C	+	+	+
13.	Growth at % NaCl			
	0% NaCl	-	-	-
	3% NaCl	+	+	+
	6% NaCl	+	+	+
	8% NaCl	+	+	+
	10% NaCl	+	-	-

14.	VP test	+	-	-
15.	Gas from glucose	-	-	-
16.	Enzyme production			
	Amylase	+	+	+
	Gelatinase	+	+	+
	Lipase	+	+	+
17.	Utilization of compounds as sole source of carbon			
	γ-Aminobutyrate	-	-	-
	Cellobiose	-	-	+
	D. Gluconate	+	+	+
	Putrescine	-	+	-
	Sucrose	+	-	-

Maximum number of amylolytic population were present in *M. dobsoni* followed by *E. suratensis*. The percentage of amylolytic strains was lowest in *M. casta*.

Amylolytic population in the water constituted 46%. This observation is almost in agreement with the observation made by Kjelleberg and Hakansson[158]. They have reported that carbohydrate decomposers in the bulk water represented 49%. Philip[145] found 59.75% of caseinolytic bacteria isolated from water as amylolytic.

Amylolytic strains were found to be less in the sediment sample. This may be due to the non-availability of readily degradable carbohydrates in the sediment.

Occurrence of amylolytic strains in different marine animals showed great variation. Among the clams, *S. scripta* harboured more amylolytic population than *M. casta*. In the case of fishes, more amylolytic strains were present in *E. suratensis* than in *L. parsia*. Among prawns, more number of amylolytic bacteria were seen in *M. dobsoni* than in *P. indicus*. Of all the animals tested, amylolytic population were maximum in *M. dobsoni*. In her study, Mary[159] found that only very little starch hydrolysers were present in the gut microflora of fresh mullet. Fathima *et al*[160] found higher percentage of amylolytic strains in mackeral than in mullet (*Liza dissumeri*).

Distribution of amylolytic bacteria in various regions of animals like body surface, gill and gut also showed considerable variation. In clams, the gut region was found to harbour more amylolytic bacteria than gill or mantle but in fishes, gill region showed the presence of more amylolytic bacteria. In the case of prawns, amylolytic strains were maximum in the gut region of P. indicus but in M. dobsoni, maximum number were seen in gill region.

In general, the gut region of animals showed the occurrence of a higher percentage of amylolytic strains. The hydrolytic enzymes produced by these strains may be involved in breaking down of complex carbohydrates present in the food and help in their digestion.

Genera-wise studies have shown that maximum number of amylolytic strains were present in the genus Vibrio, followed by members of Enterobacteriaceae. Lowest number of amylolytic strains were present in the genus Pseudomonas. In the aquatic system, Gram-negative bacteria are more prominent and active. In the present study also, Gram-negative forms are found to be capable of producing amylase in higher percentages than Gram-positive forms. Only 21.33% of Gram-positive forms were amylolytic whereas, 64.29% of Gram-negative bacteria studied were amylolytic.

Vibrios are primarily aquatic inhabitants found in the sea and freshwater and in association with aquatic animals. The production of extracellular enzymes is common among marine members of this genus[161]. The observation of a very high percentage of vibrios in the present study is consistent with the observation made by Philip[145]. She found that 75.86% of caseinolytic vibrios were capable of producing amylase.

All the three isolates selected for further studies, based on their ability to elaborate amylase in large quantities, belonged to the genus Vibrio. The genus Vibrio contains a number of species of marine origin[162]. They bear a close resemblance to the terrestrial enterobacteria in a number of physiological and metabolic properties[161].

The marine vibrios are facultatively anaerobic, Gram-negative rods. Some are heterotrophic, others are pathogenic to humans and marine animals. They inhabit oceans, estuaries, bays and lagoons. They are known for their ability to utilize a wide variety of organic compounds as sources of energy and play a vital role in biodegradation[190].

The extracellular hydrolases produced by most vibrios include amylase, gelatinase, lipase, chitinase, alginase and deoxyribonuclease. Only very few reports are available on

detailed studies on enzyme secretion by Vibrio species and their characters. Reid et al[163,164] have studied peptone induction and rifampicin-insensitive collagenase production by Vibrio alginolyticus. Tanaka et al[165] studied the regulation of amylase synthesis by V. parahaemolyticus. Induction and repression of an extracellular proteinase in V. parahaemolyticus was studied by Tanaka and Iuchi [166]. Driesbech and Merkel[167] studied induction of collagenase production in Vibrio B-30. Cellulolytic and chitinolytic activities of V. harveyi, V. fischeri, V. parahaemolyticus and V. anguillarum were studied by Venugopalan et al[168].

Identification of the selected Vibrio strains was done based on their biochemical and physiological characters. The cells were Gram-negative rods, motile, incapable of forming endospores, chemoorganotrophs, facultative anaerobes, oxidase-positive, capable of utilising D-glucose as a sole or principal source of carbon and energy, require sodium chloride or a seawater base for optimum growth and were sensitive to the vibriostatic agent O/129 pteridine compound.

Two of the isolates were identified up to species level as V. alginolyticus (MB1) and V. parahaemolyticus (MB2). The important characters used for identification of V. alginolyticus (MB1) were their ability to swarm on solid media, production of

acetoin, growth at 40°C and utilization of sucrose. They were negative for arginine dihydrolase and utilisation of cellobiose, β-hydroxybutyrate and γ-aminobutyrate.

The characters showed by V. parahaemolyticus (MB2) were ability to grow at 40°C, utilization of putrescine and gluconate, negative for arginine dihydrolase and acetoin, swarming on complex solid media and utilization of sucrose, cellobiose, β-hydroxybutyrate and γ-aminobutyrate.

The third isolate could not be identified up to species level. They were found to produce a brownish-yellow, diffusible pigment when grown on solid nutrient agar medium. Such a character was not described for any of the Vibrio species in the Bergey's Manual of Systematic Bacteriology[148]. So it may be considered as a new species of Vibrio.

It may be concluded that amylolytic bacteria are distributed widely in Cochin backwaters and they play a very important role in biodegradation. The most potent strains of amylolytic bacteria in the backwaters belong to the genus Vibrio.

Chapter 3

3. EFFECT OF CULTURAL CONDITIONS ON GROWTH AND AMYLASE PRODUCTION

The rate of growth and metabolism of bacteria depend very much on the composition of the medium and the prevalent environmental conditions. Different bacterial strains differ in their nutritional requirements and cultural conditions for maximum growth and enzyme production.

To culture microorganisms, it is necessary to establish a suitable environment, one in which a particular microbe can survive and reproduce. For each type of microorganisms, there are minimal nutritional requirements, tolerance limits for a variety of environmental factors and optimal conditions for growth and enzyme production. By understanding the specific requirements of a microbial species, it is possible to establish conditions *in vitro* to support the optimal growth and enzyme production of that organism.

The effect of various factors like temperature of incubation, pH, NaCl concentration, carbon source, nitrogen source, period of incubation etc. on growth and amylase production by the three *Vibrio* species were studied to establish suitable environment for amylase production by these bacteria.

3.1. MATERIALS AND METHODS

3.1.1. ORGANISMS

Vibrio alginolyticus (MB1), *V. parahaemolyticus* (MB2) and *Vibrio* sp. (MB3) isolated from Cochin backwaters and preserved in our laboratory were used for the study. The cultures were maintained in nutrient agar slants.

3.1.2. GROWTH MEDIUM

Nutrient broth containing 0.2% starch and a mineral medium containing artificial seawater base (ASW) of Mac Leod[155] and 0.5% starch or maltose as carbon source were used.

Medium Composition

a. Nutrient broth

Peptone	:	5 gm
Beef extract	:	3 gm
NaCl	:	15 gm
Soluble starch	:	2 gm
pH	:	7.2
Water	:	1000 ml

b. Mineral medium

Tris (hydroxymethyl) amino methane (adjusted to pH 7.5 with HCl)	:	50 mM (6.1 gm/litre)
NH_4Cl	:	19 mM (1.0 g/litre)
$K_2HPO_4.3H_2O$	:	0.33 mM (75 mg/litre)
$FeSO_4.7H_2O$	:	0.1 mM (28 mg/litre)
Maltose/Starch	:	5 gm/litre
Yeast extract	:	100 mg/litre
Half strength ASW	:	1000 ml

Composition of ASW [155]

NaCl	:	400 mM (23.4 g/litre)
$MgSO_4.7H_2O$	:	100 mM (24.6 g/litre)
KCl	:	20 mM (1.5 g/litre)
$CaCl_2.2H_2O$	:	20 mM (2.9 g/litre)

Salts were dissolved separately and combined.

3.1.3. INOCULUM PREPARATION

The cultures were inoculated on nutrient agar slopes and incubated for 24 hours. The cells were harvested and washed twice in sterile saline and cells separated by centrifugation. The pellets were resuspended in sterile saline and their optical density (O.D.) was adjusted to 1. One ml of this cell suspension was used to inoculate 49 ml of the sterile broth (initial O.D. 0.02) taken in 100 ml Erlenmeyer flasks.

3.1.4. MEASUREMENT OF GROWTH

Growth was determined by measuring the O.D. of the culture at 700 nm using a Hitachi 200 model U.V-visible Spectrophotometer.

3.1.5. COLLECTION OF ENZYME

The inoculated flasks were incubated for 24 hours at 30°C and the cells separated from the culture fluid by centrifugation. The supernatant was collected and retained for assay of amylase activity.

3.1.6. ASSAY OF AMYLASE

Amylase was assayed by either of the following 2 methods:

1. Dextrinogenic amylase activity was assayed by using soluble starch as a substrate by the modified method of Fuwa[169]. One ml of 1% soluble starch in 0.02 M sodium phosphate buffer (pH 7.0) was mixed with 1 ml of enzyme solution. Incubated at 35°C for 15 minutes and 0.2 ml portion was added to 5 ml of 0.167 mM I_2-KI solution. The O.D. at 700 nm was measured in a spectrophotometer. Soluble starch was used as the standard.

Dextrinizing unit of amylase activity

One dextrinizing unit of amylase activity is defined as the

amount of enzyme required to hydrolyse 1 mg of soluble starch in 15 minutes under the assay conditions.

2. Saccharolytic amylase activity was determined by measuring the formation of reducing sugar by the method of Bernfeld[170]. One ml of properly diluted enzyme was incubated for 15 minutes with 1 ml of 1% starch in 0.02 M phosphate buffer. The reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent. The tube containing this mixture was heated for 5 minutes in a boiling water bath and cooled in running tap water. After the addition of 20 ml of distilled water, the O.D. of the solution was determined at 540 nm. The amount of reducing sugar produced was read from the standard curve prepared with known concentrations of maltose.

Saccharolytic amylase activity unit

One unit of saccharolytic amylase activity is defined as the amount of enzyme required to liberate 1 mg of maltose in 15 minutes.

3.1.7. EFFECT OF PHYSICO-CHEMICAL FACTORS ON GROWTH AND AMYLASE PRODUCTION

3.1.7.1. Effect of pH

The effect of pH on growth and enzyme production was analysed by inoculating the organisms in nutrient broth having varying pH (pH ranging from 5 to 10). Cultures were incubated

at room temperature (28±2°C) for 24 hours and growth and enzyme activity were measured.

3.1.7.2. Effect of NaCl concentration

Effect of NaCl concentration on growth and enzyme production was studied by inoculating the cultures in nutrient media having varying NaCl concentrations (0-15%). Growth and enzyme activity were determined after 24 hours of incubation at room temperature (28±2°C).

3.1.7.3. Effect of various ions

The effect of various ions on growth and amylase production was studied by adding separately 0.25 M concentration of the salts, calcium chloride, sodium chloride, magnesium chloride, potassium chloride, sodium nitrate, sodium fluoride, sodium bicarbonate, sodium sulphate and sodium phosphate as the only source of minerals to nutrient broth and inoculating the media with the cultures. Growth and enzyme activity were detected after 24 hours of incubation at room temperature (28 ±2°C).

3.1.7.4. Effect of temperature

Effect of temperature on growth and enzyme production was determined by inoculating the nutrient agar media with bacterial cultures and incubating them at different temperatures (5°C to 60°C) for 24 hours. Growth and enzyme activities were determined.

3.1.7.5. Effect of starch concentration

Effect of starch concentration on growth and enzyme production was studied by inoculating the cultures in both nutrient broth and mineral medium having varying concentrations of starch (0.1% to 3%). Growth and enzyme activities were measured after 24 hours of incubation at room temperature.

3.1.7.6. Effect of carbon source

Effect of source of carbon on growth and enzyme production was determined by inoculating the cultures in mineral medium containing 0.5% of either glucose, lactose, maltose, mannitol, dextrin or starch as carbon sources. Incubated for 24 hours and growth and enzyme activities were determined.

3.1.7.7. Effect of nitrogen source

Effect of nitrogen source on growth and enzyme production was determined by inoculating the cultures in mineral medium containing starch as carbon source and 0.5% of either ammonium chloride, peptone or yeast extract as nitrogen sources. Growth and enzyme activities were determined after 24 hours of incubation at room temperature.

3.1.7.8. Effect of native starches

Preparation of native starches[171]

Cassava, plantain and potato were first peeled and reduced

to pulps using a hand grater. Subsequently, they were homogenized in a wet grinder. From each homogenate, contained in a bag of fine, white cloth, starch was leached into a beaker by churning with excess water. The crude starch suspension in the vat was allowed to settle down overnight, after which, the sediment was separated from the supernatant by decantation, and dried at 50°C for 48 hours. The resultant flakes were ground to fine powder and used as native starches.

Ability of the isolates to grow and produce amylase using raw-starches was tested by inoculating the cultures in mineral medium containing 0.5% of starch from either cassava, potato, plantain or soluble starch as carbon sources. Incubated for 24 hours at room temperature and growth and enzyme activity were determined.

3.1.8. EFFECT OF PERIOD OF INCUBATION

3.1.8.1. Still culture

The incubation period required for maximal growth and enzyme production was determined by inoculating the cultures in mineral medium containing 0.5% maltose as carbon source. Growth and enzyme production were monitored at different intervals up to a period of 36 hours.

3.1.8.2. Shaker culture

The cultures were inoculated in mineral medium containing maltose as carbon source and incubated on a shaker at room temperature (140 rev/minute). Growth and enzyme activity were measured at different intervals up to 12 hours.

3.1.9. PHASE OF AMYLASE PRODUCTION

To determine the phase of enzyme production, cultures were inoculated in starch medium, incubated on the shaker and growth and enzyme production were determined at different intervals.

3.1.10. DETERMINATION OF INTRACELLULAR AMYLASE ACTIVITY

The cultures were grown in 500 ml of medium containing 0.5% maltose as carbon source, to the late-logarithmic phase of growth and were harvested by centrifugation at 10000 g for 20 minutes at 0°C. The cells were washed twice with 0.1 M sodium phosphate buffer and resuspended in phosphate buffer at pH 7. The cells were disrupted by grinding with glass powder and cell debris were removed by centrifugation. The supernatant fraction was used for enzyme assays.

3.2. RESULTS

3.2.1. EFFECT OF pH ON GROWTH AND AMYLASE PRODUCTION

The effect of pH on growth and enzyme production of the three Vibrio species selected, was studied by the assaying growth and enzyme production in nutrient broth having varying pH (pH 5 to 10). Results are presented in Table 5 and Fig. 4.

All the strains showed maximum growth and enzyme production at pH 7. Low pH (below 5) and high pH (above 9) were found to be unfavourable for growth and enzyme production. Acidic pH was found to be more harmful than alkaline pH.

V. alginolyticus (MB1) showed a sharp decline in growth and enzyme production below and above pH 7. V. parahaemolyticus (MB2) exhibited a broad pH range for growth from pH 6 to 9; but, enzyme production was maximum at pH 7 and 8. Vibrio sp. (MB3) also preferred to grow and produce amylase at pH 7 and there was considerable decrease in growth and enzyme production above and below pH 7.

3.2.2. EFFECT OF NaCl CONCENTRATION ON GROWTH AND AMYLASE PRODUCTION

The effect of NaCl concentration on growth and enzyme

Table 5. Effect of pH on growth and amylase production

pH	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
5	0.09	0.1	0.05	0.08	0.09	0
6	0.673	0.34	0.68	0.23	0.66	0.17
7	0.712	0.68	0.7	0.4	0.8	0.6
8	0.507	0.58	0.67	0.4	0.476	0.4
9	0.434	0.5	0.65	0.34	0.43	0.39
10	0.31	0.4	0.41	0.2	0.3	0.1

* mg starch hydrolysed per 15 minutes

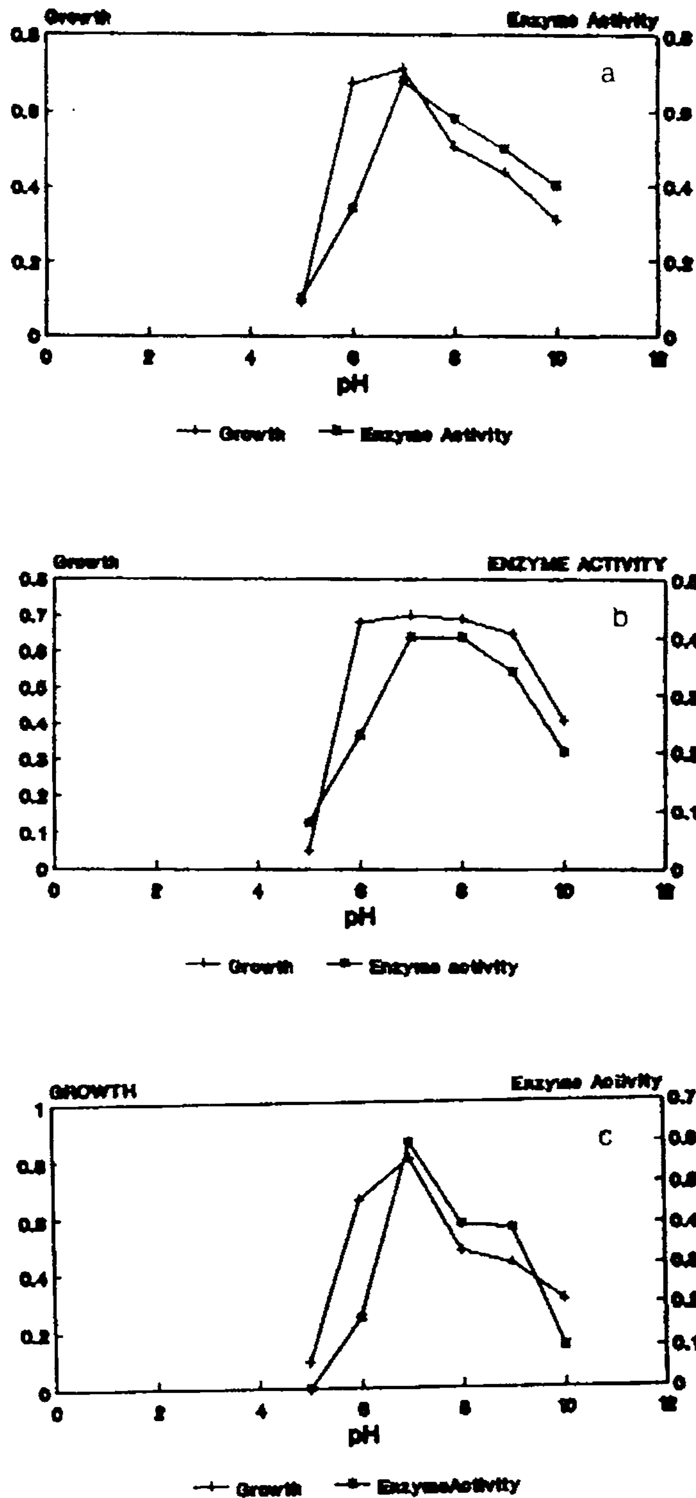


Fig. 4 a-c. Effect of pH on growth and amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

production was studied by measuring growth and enzyme activity of these bacteria in nutrient broth having different levels of NaCl concentration (0-15%).

Results (Table 6 and Fig. 5) showed that all the three strains require NaCl for optimum growth and enzyme production. Higher levels (above 10%) were found to be inhibitory to growth and amylase production.

V. alginolyticus (MB1) could grow optimally when the NaCl concentration of the medium was in the range 3% to 10%. Amylase production was maximum in the medium containing 1.5% NaCl.

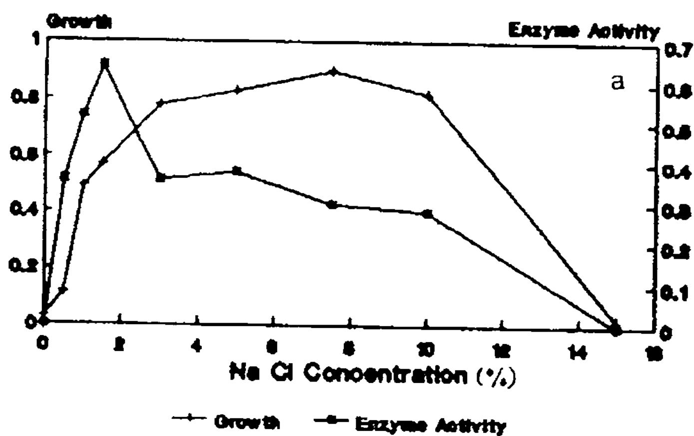
V. parahaemolyticus (MB2) exhibited maximum growth when the NaCl concentration in the medium was in the range 1.5% to 5%. Amylase production was maximum at 1% NaCl concentration. There was no growth when the NaCl concentration in the medium was 10% or more. Enzyme production stopped when the salt concentration in the medium was above 4%.

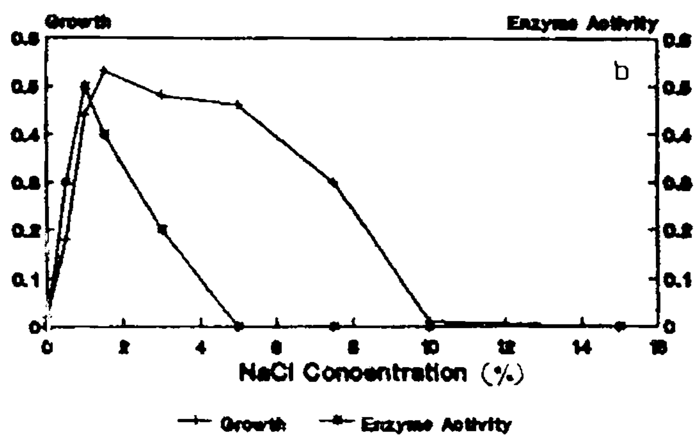
The optimum NaCl required for maximal growth of Vibrio sp. (MB3) was found to be 3%. It could not grow above 8% NaCl concentration. Enzyme production was maximum at 1% NaCl concentration. There was no enzyme production when the salt concentration in the medium was 5% or above.

Table 6. Effect of NaCl on growth and amylase production

NaCl Concentration %	*V. alginolyticus*(MB1)		*V. parahaemolyticus*(MB2)		*Vibrio* sp.(MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity * (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
0	0.02	0	0.02	0	0.02	0
0.5	0.117	0.36	0.18	0.3	0.166	0.2
1	0.49	0.52	0.44	0.5	0.35	0.98
1.5	0.57	0.64	0.53	0.4	0.48	0.6
3	0.78	0.36	0.48	0.2	0.61	0.4
5	0.83	0.38	0.46	0	0.49	0
7.5	0.9	0.3	0.3	0	0.3	0
10	0.82	0.28	0.01	0	0.01	0
15	0.02	0	0	0	0	0

* mg starch hydrolysed per 15 minutes





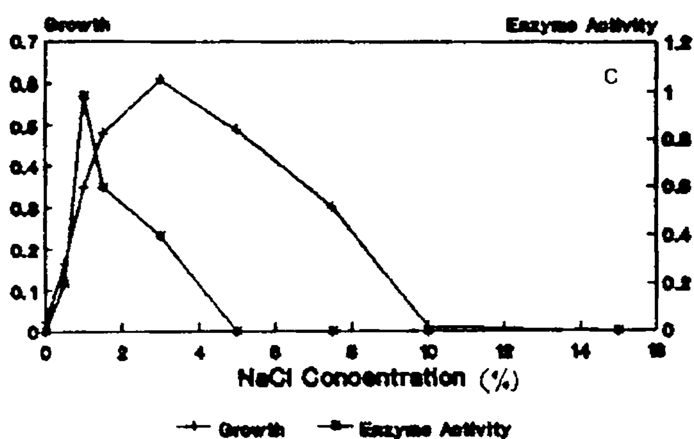


Fig. 5 a-c. Effect of NaCl on growth and amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

3.2.3. EFFECT OF VARIOUS IONS ON GROWTH AND AMYLASE PRODUCTION

Results are shown in Table 7 and Fig. 6. All the isolates showed maximum growth and enzyme production in the presence of phosphate. Calcium and fluoride at 0.25 M concentration were found to be inhibitory to the growth of these bacteria.

The pattern of growth exhibited by V. alginolyticus (MB1) in the presence of cations was $Mg^{++} > \substack{Na^{+}\\K^{+}} > Ca^{++}$ and anions was $HPO_4^{--} > SO_4^{--} > Cl^{-} > NO_3^{-} > HCO_3^{-} > F^{-}$. The pattern of enzyme production in the presence of cations was $K^{+} > Mg^{++} > Na^{+} > Ca^{++}$ and anions was $HPO_4^{--} > NO_3^{-} > Cl^{-} > SO_4^{--} > \substack{HCO_3^{-}\\F^{-}}$.

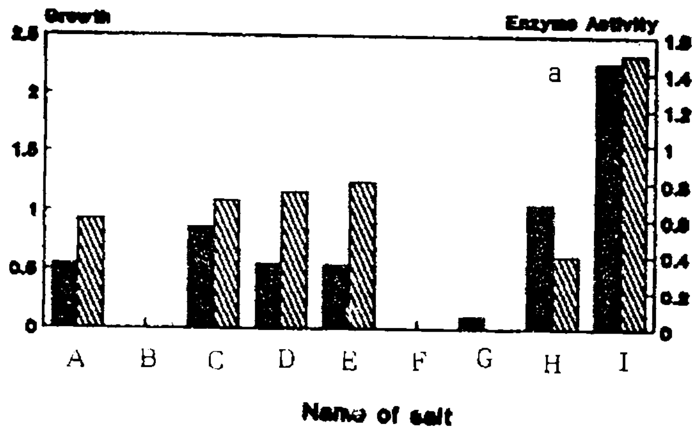
The pattern of growth shown by V. parahaemolyticus(MB2) in the presence of cations was $Na^{+} > K^{+} > Mg^{++} > Ca^{++}$ and anions was $HPO_4^{--} > Cl^{-} > SO_4^{--} > HCO_3^{-} > NO_3^{-} > F^{-}$. The pattern of enzyme production in the presence of cations was $Na^{+} > Mg^{++} > K^{+} > Ca^{++}$ and anions was $HPO_4^{--} > Cl^{-} > SO_4^{--} > HCO_3^{-} > NO_3^{-} > F^{-}$.

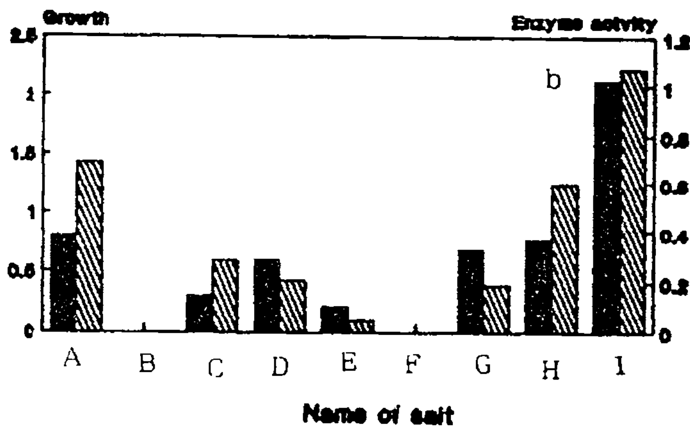
The growth pattern shown by Vibrio sp. (MB3) in the presence of cations was $Na^{+} > K^{+} > Mg^{++} > Ca^{++}$ and anions was $HPO_4^{--} > Cl^{-} > HCO_3^{-} > NO_3^{-} > SO_4^{--} > F^{-}$. The enzyme production pattern in the presence of cations was $Mg^{++} > Na^{+} > K^{+} > Ca^{++}$ and anions was $HPO_4^{--} > SO_4^{--} > NO_3^{-} > Cl^{-} > HCO_3^{-} > F^{-}$.

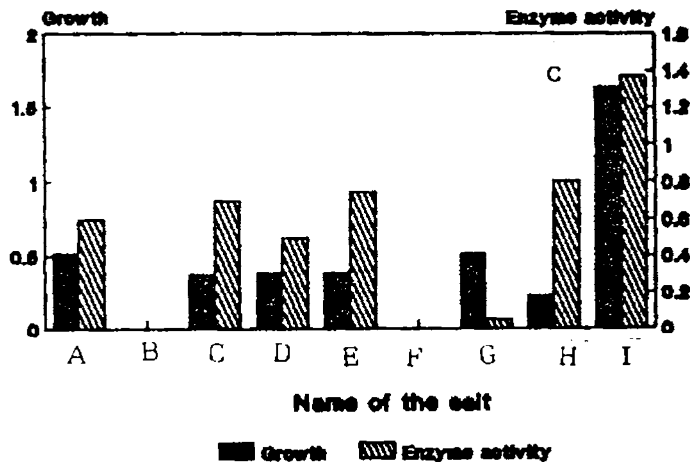
Table 7. Effect of various ions on growth and amylase production

Salt (0.25 M)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
NaCl	0.56	0.6	0.8	0.685	0.52	0.6
$CaCl_2$	0	0	0	0	0	0
$MgCl_2$	0.87	0.7	0.3	0.285	0.37	0.7
KCl	0.56	0.75	0.6	0.205	0.38	0.5
$NaNO_3$	0.54	0.8	0.21	0.05	0.38	0.75
NaF	0	0	0	0	0	0
$NaHCO_3$	0.104	0	0.686	0.19	0.51	0.05
Na_2SO_4	1.067	0.4	0.765	0.59	0.22	0.8
Na_2HPO_4	2.27	1.5	2.13	1.075	1.63	1.37

* mg starch hydrolysed per 15 minutes







(A) NaCl (B) $CaCl_2$ (C) $MgCl_2$ (D) KCl (E) $NaNO_3$ (F) NaF (G) $NaHCO_3$

(H) Na_2SO_4 (I) Na_2HPO_4

Fig. 6 a-c. Effect of various ions on growth and amylase production by *Vibrio* species. (a) *V. alginolyticus* (MB1) (b) *V. parahaemolyticus* (MB2) (c) *Vibrio* sp. (MB3).

3.2.4. EFFECT OF TEMPERATURE ON GROWTH AND AMYLASE PRODUCTION

Effect of temperature on growth and enzyme production was studied by incubating the cultures at different temperatures (5°C to 60°C). All the three species showed optimum growth at 35°C. There was no growth and enzyme production below 5°C and above 45°C. Results are presented in Table 8 and Fig. 7.

V. alginolyticus (MB1) showed maximum amylase production at 30°C. Above and below this temperature, there was considerable reduction in enzyme production. Although there was some growth at 45°C, enzyme production was negligible.

V. parahaemolyticus (MB2) and Vibrio sp. (MB3) showed maximal enzyme production at 35°C. V. parahaemolyticus (MB2) showed very feeble growth and no enzyme production at 45°C whereas, Vibrio sp. (MB3) showed some growth and enzyme production even at 45°C.

3.2.5. EFFECT OF STARCH CONCENTRATION ON GROWTH AND AMYLASE PRODUCTION

Effect of starch concentration on growth and enzyme production was studied by varying the starch concentration of the medium from 0.1% to 3% in both nutrient broth and mineral medium.

Table 8. Effect of temperature on growth and amylase production

Incubation Temperature °C	*V. alginolyticus*(MB1)		*V. parahaemolyticus*(MB2)		*Vibrio* sp.(MB3)	
	Gorwth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
5	0.003	0	0.015	0	0.018	0
10	0.111	0.1	0.096	0.1	0.05	0.08
15	0.297	0.23	0.23	0.28	0.205	0.36
20	0.347	0.3	0.321	0.36	0.353	0.4
25	0.56	0.47	0.48	0.4	0.52	0.44
30	0.759	0.62	0.896	0.48	0.702	0.6
35	1.158	0.55	1.257	0.6	1.110	0.7
40	0.824	0.2	0.663	0.2	0.473	0.5
45	0.362	0.015	0.096	0	0.313	0.2
50	0.05	0	0.03	0	0.21	0
55	0.002	0	0.02	0	0.134	0
60	0	0	0	0	0.02	0

* mg starch hydrolysed per 15 minutes

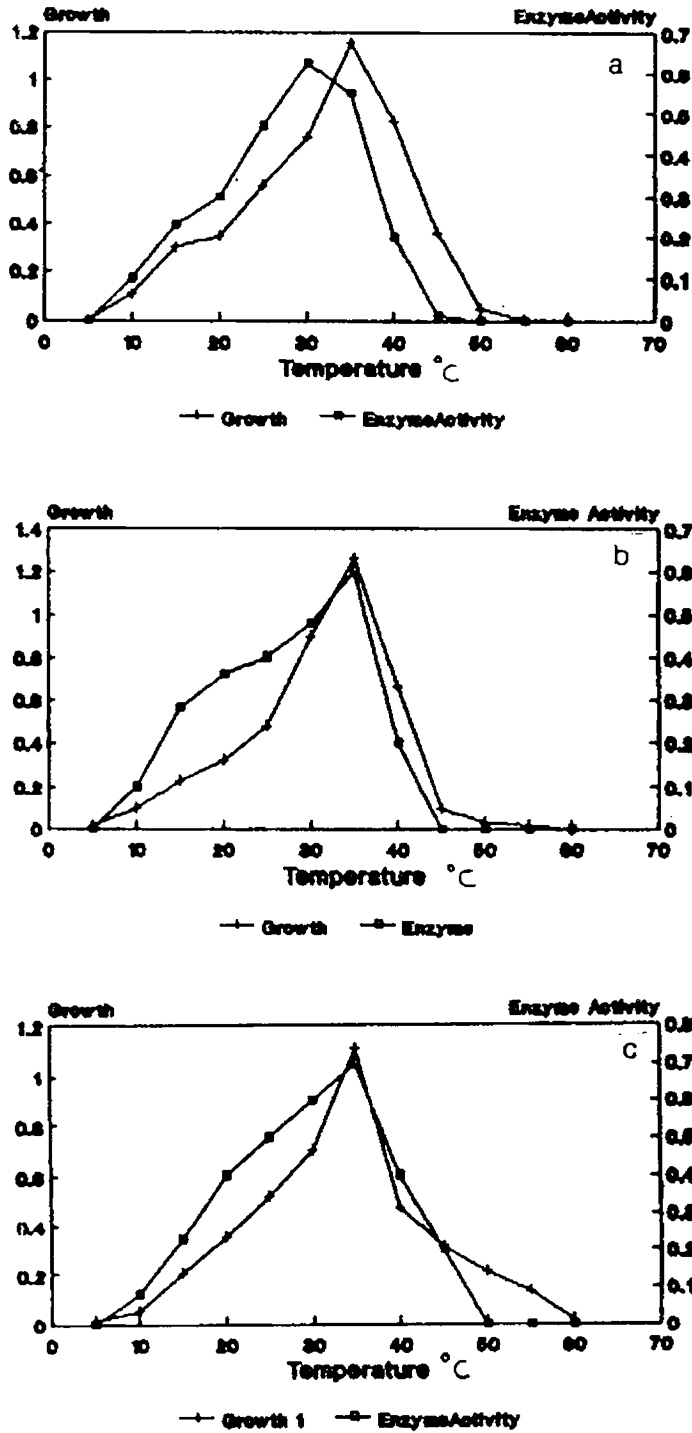


Fig. 7 a-c. Effect of temperature on growth and amylase production by Vibrio species. (a). V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

Results are shown in Table 9 and 10 and Fig. 8 and 9. In nutrient broth, the optimum starch concentration required for growth and enzyme production showed much variation among different strains, whereas, in mineral medium, the variation was less.

V. alginolyticus (MB1) showed an optimum starch concentration of 1% for growth and 0.3% for amylase production in nutrient broth. It could grow well in the medium containing 0.8% to 3% starch. Enzyme production declined considerably as the concentration of starch in the medium was increased. Even then, it could retain 20% of the maximum enzyme activity at 3% starch concentration.

In the mineral medium, V. alginolyticus (MB1) expressed maximum growth at 1.5% starch concentration and maximum enzyme production at 0.5% starch.

V. parahaemolyticus (MB2) showed maximal growth and enzyme production at a starch concentration of 0.2%. There was around 50% reduction in growth when the starch concentration in the medium was more than 0.5%. Enzyme production was completely inhibited when the starch concentration was above 1%.

In the mineral medium, V. parahaemolyticus (MB2) showed maximal growth and enzyme production when the starch concentration was in the range 0.4% to 0.6%.

Table 9. Effect of starch concentration on growth and amylase production in nutrient broth

Starch Concentration (%)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
0	0.6	0.4	0.872	0.2	0.93	0.4
0.1	0.8	0.8	1.061	0.46	1.14	0.8
0.2	1	0.83	1.765	0.47	1.605	0.4
0.3	1.2	0.94	1.321	0.28	0.830	0.22
0.4	1.4	0.8	1.041	0.28	0.665	0.28
0.5	1.62	0.8	0.933	0.28	0.673	0.28
0.6	1.73	0.6	0.891	0.28	0.629	0.24
0.7	1.62	0.55	0.84	0.2	0.622	0.22
0.8	1.83	0.5	0.83	0.15	0.63	0.2
0.9	1.92	0.5	0.84	0.1	0.64	0.2
1	1.98	0.45	0.847	0	0.668	0.1
1.5	1.77	0.4	0.84	0	0.649	0.1
2	1.84	0.4	0.83	0	0.677	0
2.5	1.6	0.3	0.865	0	0.628	0
3	1.55	0.2	0.851	0	0.687	0

* mg starch hydrolysed per 15 minutes

43 b

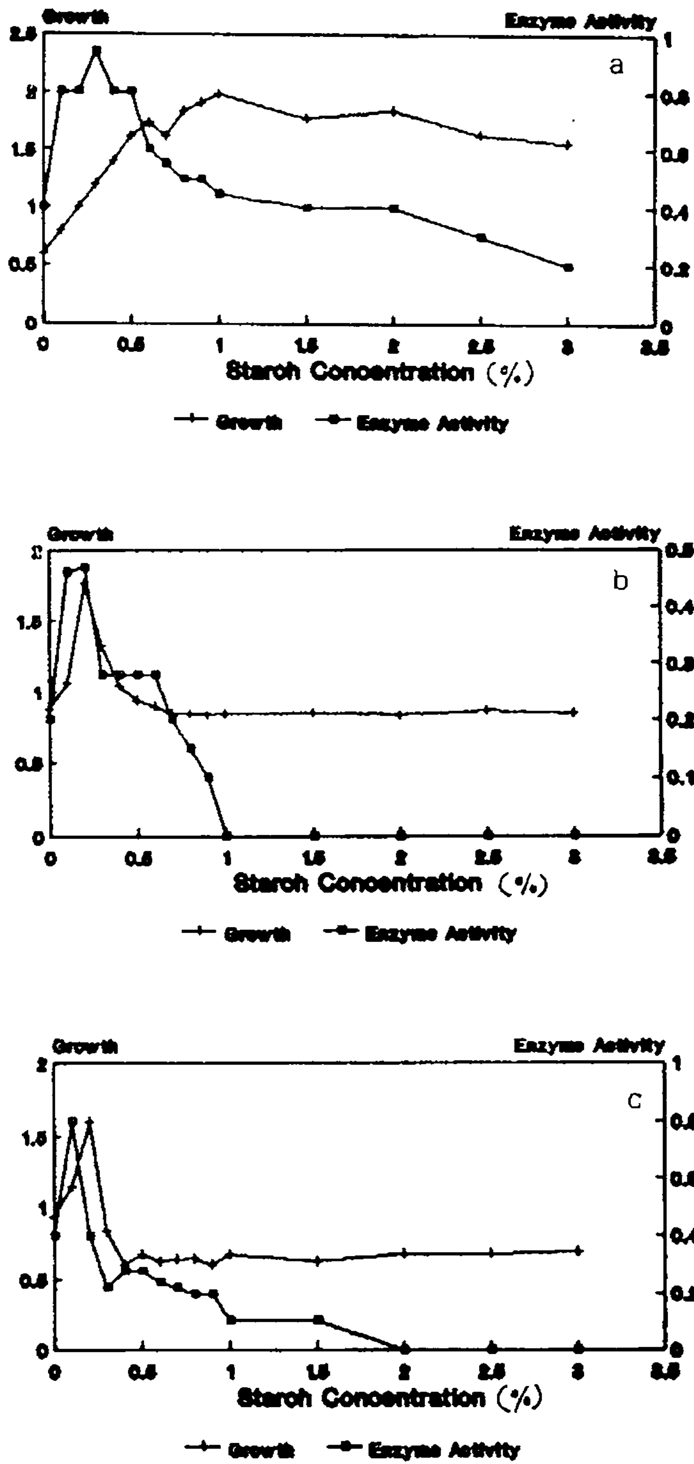
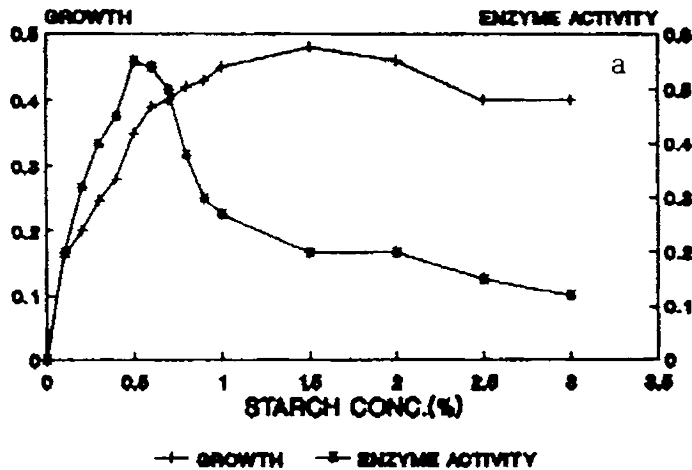


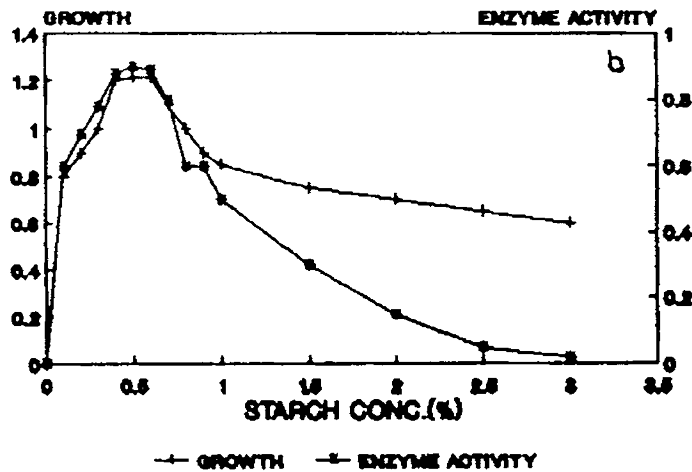
Fig. 8 a-c. Effect of starch concentration on growth and amylase production by *Vibrio* species in nutrient broth. (a) *V. alginolyticus* (MB1) (b) *V. parahaemolyticus* (MB2) (c) *Vibrio* sp. (MB3).

Table 10. Effect of starch concentration on growth and amylase production in mineral medium

Starch concentration (%)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
0	0	0	0	0	0	0
0.1	0.16	0.2	0.8	0.6	0.5	0.45
0.2	0.2	0.3	0.9	0.7	0.61	0.6
0.3	0.24	0.4	1	0.78	0.65	0.8
0.4	0.28	0.45	1.2	0.88	0.7	0.82
0.5	0.3	0.55	1.214	0.9	0.72	0.8
0.6	0.33	0.5	1.23	0.89	0.74	0.75
0.7	0.4	0.48	1.1	0.8	0.75	0.7
0.8	0.42	0.4	0.9	0.6	0.7	0.6
0.9	0.43	0.39	0.8	0.6	0.65	0.5
1	0.45	0.38	0.75	0.4	0.6	0.4
1.5	0.48	0.3	0.7	0.2	0.55	0.34
2	0.46	0.27	0.65	0.1	0.54	0.15
2.5	0.4	0.2	0.6	0.05	0.5	0.1
3	0.4	0.2	0.5	0.02	0.4	0.05

* mg starch hydrolysed per 15 minutes





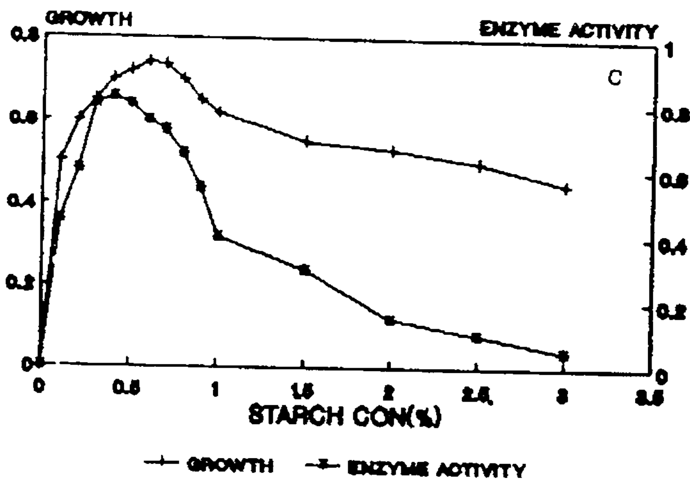


Fig. 9 a-c. Effect of starch concentration on growth and amylase production by *Vibrio* species in mineral medium. (a) *V. alginolyticus* (MB1) (b) *V. parahaemolyticus* (MB2) (c) *Vibrio* sp. (MB3).

In the case of Vibrio sp. (MB3), maximum growth occurred at a starch concentration of 0.2% whereas, enzyme production was maximum at 0.1% starch concentration in nutrient broth. When the starch concentration was above 0.5% in the medium, there was 50% reduction in growth. Enzyme production was completely inhibited when the starch concentration in the medium was above 2%. In the mineral medium, Vibrio sp. (MB3) expressed maximum growth when the starch concentration was in the range 0.4% to 0.8% and maximum enzyme production in the range 0.3% to 0.5%. Above these values, there was considerable reduction in growth and enzyme production.

In general, all the three species showed good growth and enzyme production in mineral media containing 0.5% starch as carbon source.

Growth and enzyme production of the three species were compared in nutrient broth and mineral medium. It was found that in all the three cases, growth was maximum in complex medium (nutrient broth containing starch at their optimal concentration) than in mineral medium. But, enzyme production was maximum in mineral medium containing 0.5% starch.

3.2.6. EFFECT OF CARBON SOURCE ON GROWTH AND AMYLASE PRODUCTION

To find out the suitable source of carbon for growth and

enzyme production, bacteria were grown in mineral media containing 0.5% of either glucose, lactose, maltose, mannitol, dextrin or starch as carbon sources and growth and enzyme prooduction were monitored. Results are given in Table 11 and Fig. 10.

Glucose was the C source of choice for growth of *V. alginolyticus* (MB1) and *Vibrio* sp. (MB3), whereas, *V. parahaemolyticus* (MB2) showed maximum growth in starch medium. There was no enzyme production in glucose media in all the three cases. Enzyme production was found to be maximal in maltose medium and there was also good growth in that medium.

The pattern of growth and enzyme production expressed by the different strains in various media were:

V. alginolyticus (MB1): growth: glucose > lactose > maltose > starch> mannitol > dextrin.

Enzyme production : maltose > starch > mannitol > lactose > dextrin> glucose.

V. parahaemolyticus (MB2): growth: starch > maltose > glucose> lactose > mannitol > dextrin.

Enzyme production : maltose > starch > mannitol > lactose > dextrin> glucose.

Table 11. Effect of carbon source on growth and amylase production

Source of carbon (0.5%)	*V. alginolyticus*(MB1)		*V. parahaemolyticus*(MB2)		*Vibrio* sp.(MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D.) at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
Glucose	0.63	0	1.047	0	0.787	0
Lactose	0.4	0.12	1.01	0.53	0.642	0.443
Maltose	0.34	0.6	1.129	0.98	0.684	0.94
Mannitol	0.26	0.1	0.9	0.3	0.508	0.16
Starch	0.3	0.48	1.21	0.96	0.75	0.856
Dextrin	0.2	0.1	0.05	0.05	0.04	0.02

* mg starch hydrolysed per 15 minutes

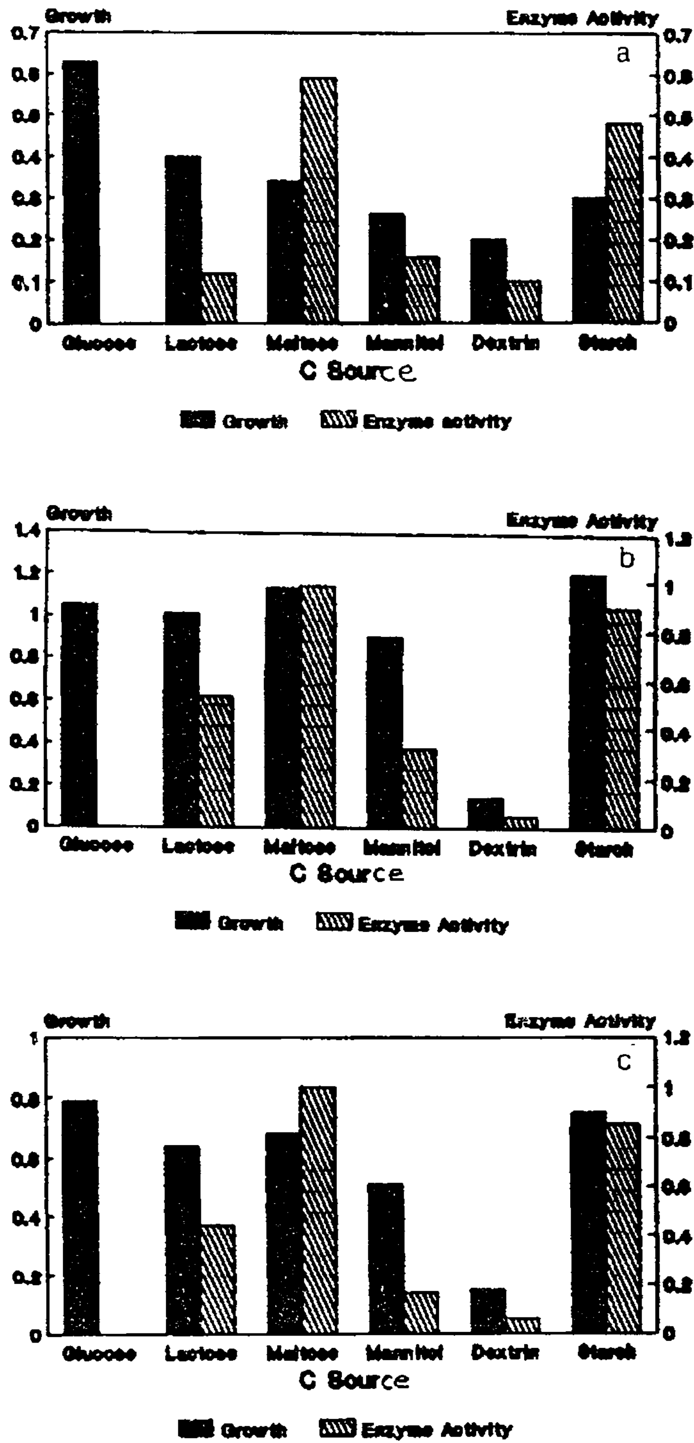


Fig. 10 a-c. Effect of carbon source on growth and amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

Vibrio sp. (MB3): growth: > glucose > starch > maltose > lactose > mannitol > dextrin.

Enzyme production : maltose > starch > lactose > mannitol > dextrin > glucose.

3.2.7. EFFECT OF NITROGEN SOURCE ON GROWTH AND AMYLASE PRODUCTION

The effect of ammonium chloride, peptone and yeast extract as source of nitrogen on growth and enzyme production was tested. Results are shown in Table 12 and Fig. 11.

V. alginolyticus (MB1) preferred to grow and produce amylase maximally in the medium containing yeast extract as the source of nitrogen. V. parahaemolyticus (MB2) and Vibrio sp. (MB3) produced enzyme maximally in the medium containing NH_4Cl as nitrogen source. All the three strains showed good growth in peptone and yeast extract media, but peptone was found to be a poor source of nitrogen for enzyme production. V. alginolyticus (MB1) showed very little growth when the source of nitrogen was ammonium chloride.

The pattern of growth expressed in different media were: V. alginolyticus (MB1): peptone > yeast extract > NH_4Cl, V. parahaemolyticus (MB2): yeast extract > NH_4Cl > peptone and Vibrio sp. (MB3): yeast extract > peptone > NH_4Cl. The pattern of

Table 12. Effect of nitrogen source on growth and amylase production.

Source of nitrogen (0.5%)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D.) at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
Peptone	0.42	0.3	1.02	0.3	0.782	0.32
Yeast extract	0.41	0.6	1.23	0.5	0.802	0.37
NH_4Cl	0.2	0.1	1.1	0.9	0.684	0.92

* mg starch hydrolysed per 15 minutes

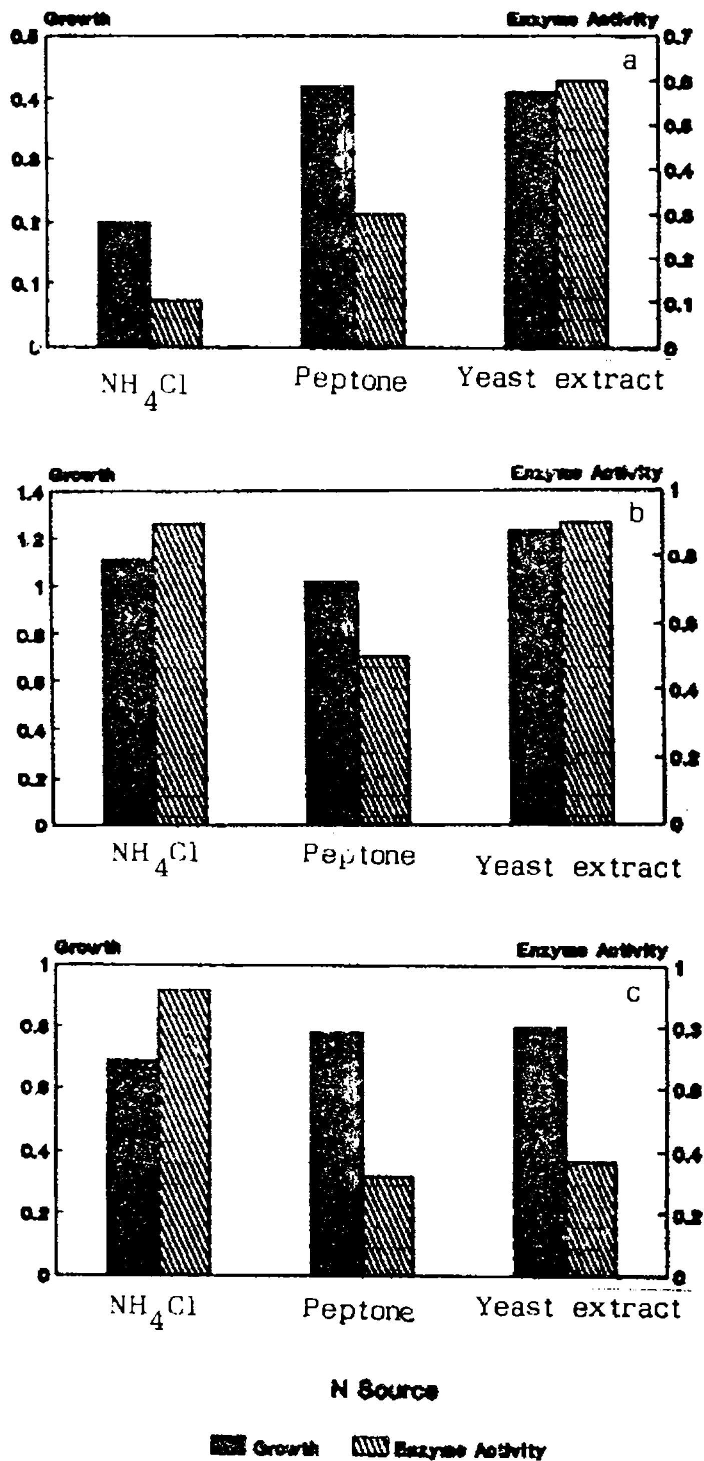


Fig. 11 a-c. Effect of nitrogen source on growth and amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

enzyme production was: V. alginolyticus (MB1): yeast extract > peptone > NH_4Cl; V. parahaemolyticus (MB2): NH_4Cl > yeast extract > peptone; Vibrio sp. (MB3): NH_4Cl > yeast extract > peptone.

3.2.8. EFFECT OF NATIVE STARCHES ON GROWTH AND AMYLASE PRODUCTION

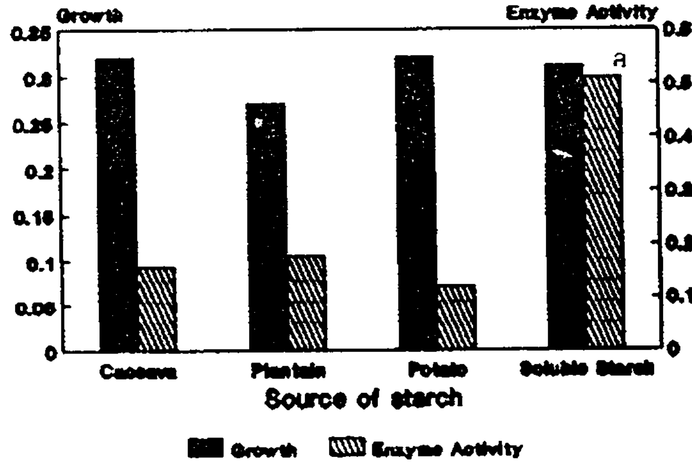
Ability of the isolates to grow and produce amylase in raw-starches was tested using either cassava, plantain or potato starches along with soluble starch. Results are presented in Table 13 and Fig. 12.

V. alginolyticus (MB1) could show almost uniform growth in all the four media. Enzyme production was maximal in the medium containing soluble starch. The growth pattern was: Potato starch > cassava starch > soluble starch > plantain starch and amylase production was: soluble starch > plantain starch > cassava starch > potato starch. V. parahaemolyticus (MB2) and Vibrio sp. (MB3) showed maximum growth and enzyme production in soluble starch medium. The pattern of growth shown by V. parahaemolyticus (MB2) was: soluble starch > potato starch > cassava starch > plantain starch and Vibrio sp. (MB3) was: soluble starch > plantain starch > potato starch > cassava starch. The pattern of enzyme production by V. parahaemolyticus (MB2) was: soluble starch > cassava starch > plantain starch > potato starch and Vibrio sp. (MB3) was: soluble starch > potato starch > cassava starch > plantain starch.

Table 13. Effect of native starches on growth and amylase production

Source of starch (0.5%)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D.) at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
Soluble starch	0.31	0.51	1.032	0.93	0.75	0.856
Cassava "	0.32	0.16	0.31	0.46	0.38	0.204
Plantain "	0.27	0.18	0.24	0.32	0.5	0.14
Potato "	0.33	0.12	0.32	0.22	0.44	0.32

* mg Reducing sugar formed per 15 minutes



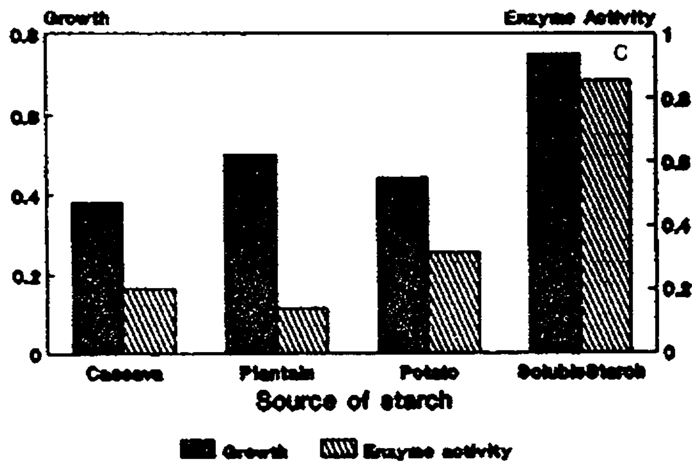


Fig. 12 a-c. Effect of native starches on growth and amylase production by *Vibrio* species. (a) *V. alginolyticus* (MB1) (b) *V. parahaemolyticus* (MB2) (c) *Vibrio* sp. (MB3).

3.2.9. EFFECT OF PERIOD OF INCUBATION ON GROWTH AND AMYLASE PRODUCTION

The effect of period of incubation on growth and amylase production was studied in both still culture and shaker culture. Results are presented in Table 14 and 15 and Fig. 13 and 14.

3.2.9.1. Still culture

In still culture, an incubation period of 24 hours was found to be necessary for maximal growth and enzyme production. Vibrio alginolyticus (MB1) began enzyme production after 12 hours of incubation and enzyme production continued for up to 30 hours. In V. parahaemolyticus (MB2) and Vibrio sp. (MB3), enzyme production started at an earlier stage (after 6 hours) and reached maximal level after 24 hours. Enzyme produced was not very stable in the medium and the activity decreased considerably by further incubation.

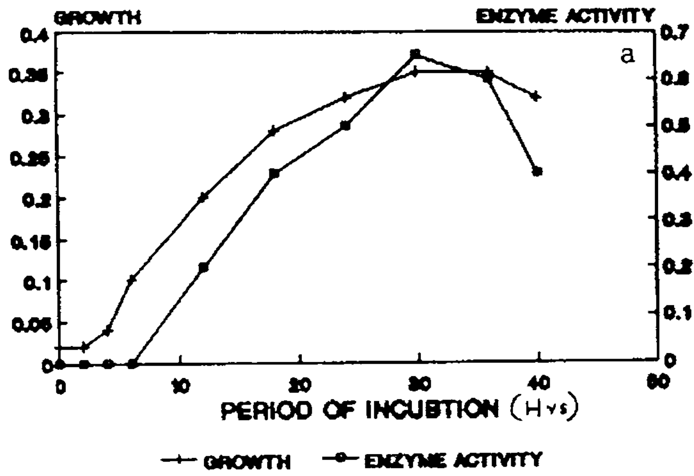
3.2.9.2. Shaker culture

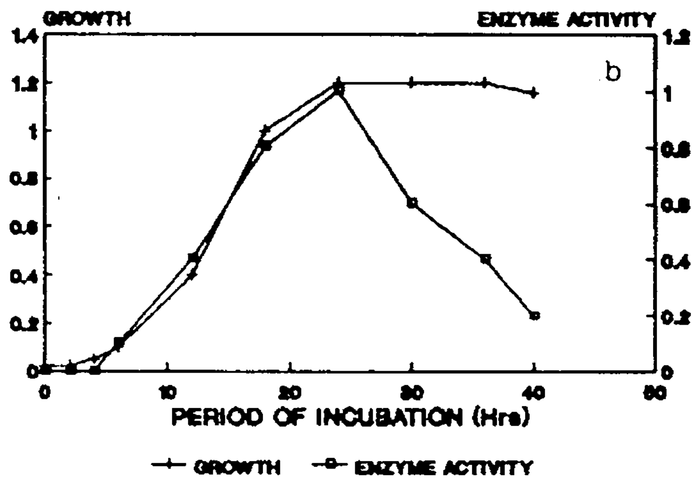
To find out the effect of shaking on incubation period, cultures were inoculated in maltose medium and incubated on shaker (140 rev/minute). Growth and enzyme production were monitored at different intervals. Shaking of the cultures resulted in a considerable reduction in the period of incubation required for growth and enzyme production. V. alginolyticus (MB1) required

Table 14. Effect of period of incubation on growth and amylase production in still cultures (Maltose medium)

Period of incubation (hours)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
0	0.02	0	0.02	0	0.02	0
2	0.02	0	0.02	0	0.02	0
4	0.05	0	0.05	0	0.035	0
6	0.1	0	0.1	0.1	0.095	0.15
12	0.2	0.2	0.4	0.4	0.35	0.4
18	0.31	0.4	1	0.8	0.65	0.6
24	0.35	0.51	1.2	1	0.7	0.8
30	0.35	0.65	1.2	0.6	0.7	0.4
36	0.34	0.6	1.2	0.4	0.7	0.2
40	0.3	0.4	1.1	0.2	0.67	0.1

* mg starch hydrolysed per 15 minutes





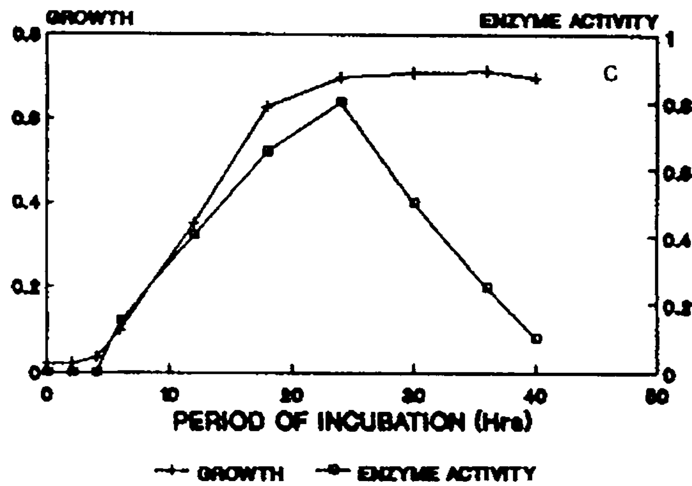
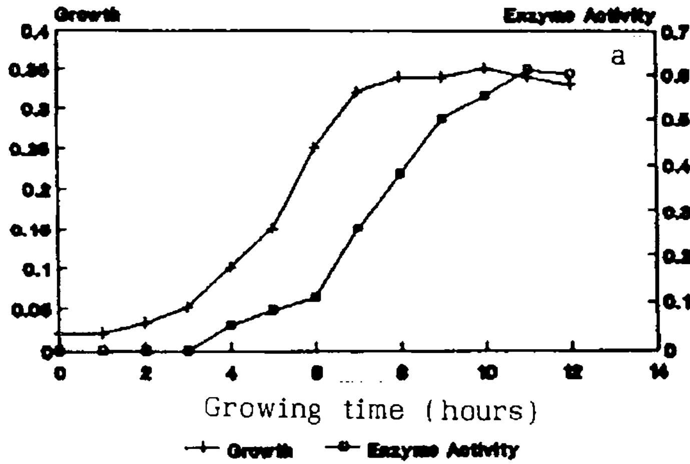


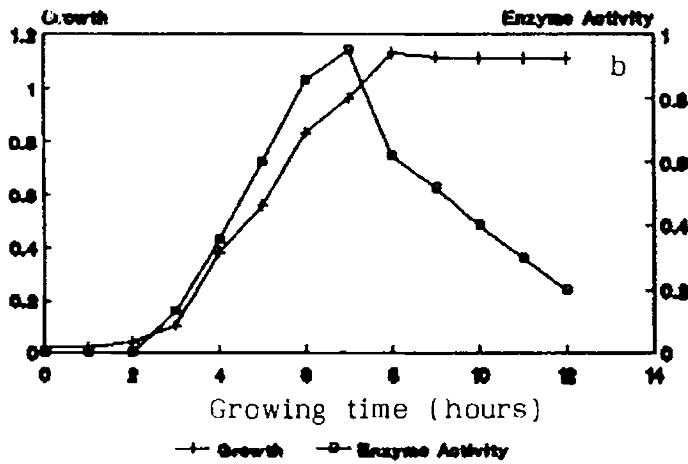
Fig. 13 a-c. Effect of period of incubation on growth and amylase production by *Vibrio* species in still culture. (a) *V. alginolyticus* (MB1) (b) *V. parahaemolyticus* (MB2) (c) *Vibrio* sp. (MB3).

Table 15. **Effect of period of incubation on growth and amylase production in shaker cultures (Maltose medium).**

Period of incubation (hours)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
0	0.02	0	0.02	0	0.02	0
1	0.02	0	0.02	0	0.02	0
2	0.025	0	0.04	0	0.04	0
3	0.05	0	0.1	0.12	0.18	0
4	0.1	0.05	0.38	0.35	0.379	0.13
5	0.15	0.08	0.556	0.6	0.581	0.52
6	0.25	0.11	0.833	0.86	0.677	0.69
7	0.32	0.26	0.964	0.95	0.757	0.85
8	0.34	0.35	1.129	0.8	0.75	0.83
9	0.34	0.5	1.129	0.6	0.749	0.6
10	0.35	0.55	1.114	0.5	0.745	0.4
11	0.34	0.61	1.112	0.4	0.73	0.2
12	0.33	0.6	1.11	0.2	0.721	0.1

* mg starch hydrolysed per 15 minutes





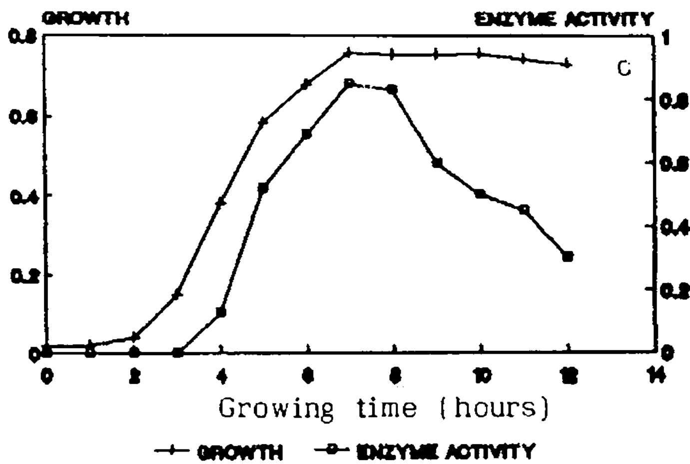


Fig. 14 a-c. Effect of period of incubation on growth and amylase production by Vibrio species in shaker culture. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

10 hours of incubation for maximal growth and enzyme production whereas, V. parahaemolyticus (MB2) and Vibrio sp. (MB3) required only seven hours of incubation for maximal growth and enzyme production.

3.2.10. GROWTH PHASE OF AMYLASE PRODUCTION

To determine the growth phase at which enzyme production occurs, cells were inoculated in starch medium and incubated on a shaker. Growth and enzyme production were monitored at short intervals. Results are shown in Table 16 and Fig. 15.

In general, these organisms spent about 2 hours in the lag phase, 4 to 6 hours in logarithmic phase and then entered the stationary phase. In all the strains, enzyme production started at about 4 hours of incubation, when the cells were in their early logarithmic phase of growth, and amylase production continued till they entered the stationary phase. In Vibrio parahaemolyticus (MB2) and Vibrio sp. (MB3), enzyme secretion discontinued when the cells were in the stationary phase of growth whereas, in V. alginolyticus (MB1), enzyme secretion continued in the early stationary phase also.

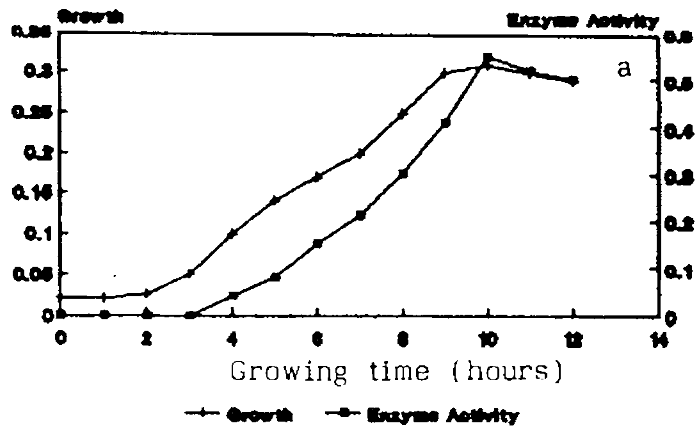
3.2.11. LOCATION OF AMYLASES

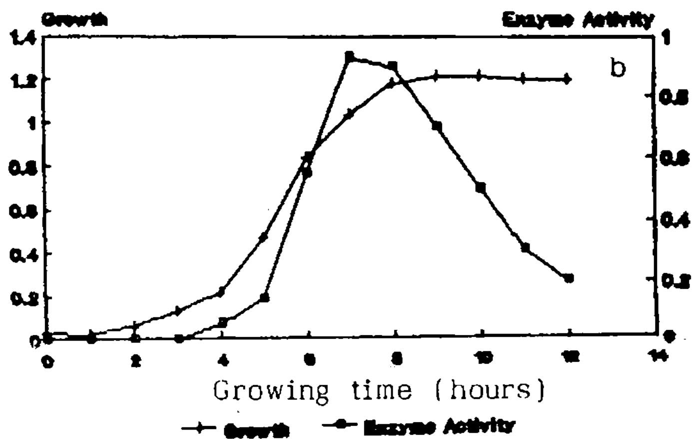
To find out whether the amylase activity was truely

Table 16. **Phase of amylase production (starch medium)**

Period of incubation (hours)	*V. alginolyticus* (MB1)		*V. parahaemolyticus* (MB2)		*Vibrio* sp. (MB3)	
	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)	Growth (O.D. at 700nm)	Enzyme activity* (Units/ml)
0	0.02	0	0.02	0	0.02	0
1	0.02	0	0.025	0	0.021	0
2	0.025	0	0.06	0	0.08	0
3	0.05	0	0.134	0	0.187	0
4	0.11	0.04	0.220	0.05	0.379	0.03
5	0.15	0.08	0.471	0.137	0.581	0.2
6	0.18	0.15	0.836	0.55	0.677	0.6
7	0.215	0.21	1.032	0.93	0.757	0.85
8	0.25	0.3	1.18	0.9	0.75	0.83
9	0.3	0.4	1.213	0.7	0.749	0.6
10	0.31	0.51	1.214	0.5	0.745	0.4
11	0.3	0.55	1.2	0.3	0.74	0.3
12	0.29	0.5	1.2	0.2	0.74	0.24

* mg starch hydrolysed per 15 minutes





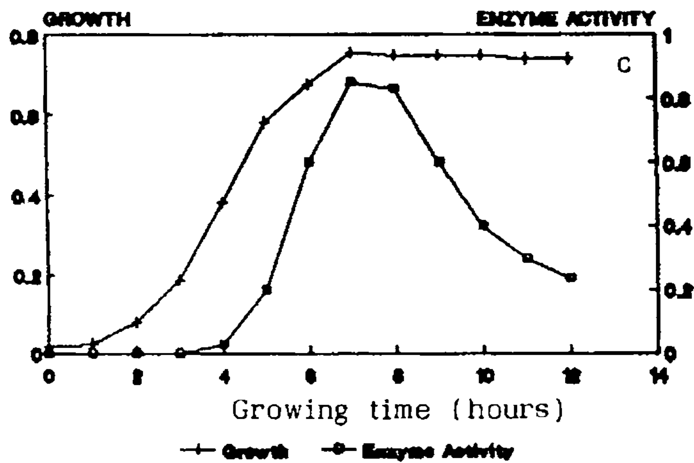


Fig. 15 a-c. Growth phase of amylase production by Vibrio species in starch medium. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

extracellular, or released by cell lysis, the activity of the enzyme in the cell-free culture supernatant and in the extract of whole cells was determined. Results are presented in Table 17.

Only very little amylase activity was detected in the cell extracts. The intracellular amylase activity detected in *V. alginolyticus* (MB1) was 4.5%, in *Vibrio parahaemolyticus* (MB2) 1.42% and in *Vibrio* sp. (MB3) 1.1% of the total activity. Results showed that amylase activity is mostly extracellular.

3.3. DISCUSSION

The growth of aquatic microorganisms is influenced by a number of physical and chemical factors. They influence the size, composition, morphology and physiology of the individual bacterium. Thus, the incubation temperatures, pH values and salt concentration above or below the optimum may lead to considerable changes in metabolism and reproduction. The synthesis of enzymes and the ability to break down substances may be either promoted or inhibited by these factors[172].

3.3.1. EFFECT OF pH ON GROWTH AND AMYLASE PRODUCTION

pH is a very important factor affecting growth and metabolism of bacteria. They show wide variation in their optimum pH for growth and amylase production. Most bacteria can grow only within

Table 17. Location of amylases: Total intracellular enzyme activity and extracellular enzyme activity

Name of the isolate	Total enzyme activity* (Units/500 ml)	Extracellular enzyme activity*	Intracellular enzyme activity*	Percentage of intracellular activity
V. alginolyticus (MB1)	314.5	300	14.5	4.5
V. parahaemolyticus (MB2)	458.5	452	6.5	1.42
Vibrio sp. (MB3)	439.84	435	4.84	1.1

* mg starch hydrolysed per 15 minutes

the pH range 4 to 9[173]. More pronounced deviations from the optimum pH cause physiological changes[172].

B. stearothermophilus produced α-amylase maximally at pH 6.7[174]. Bacillus sp. producing alkaline α-amylase required alkaline pH (pH 7.5-11) for growth and amylase production[175]. Bacillus subtilis produced α-amylase in the pH range 6.5 to 8.0[176, 48]. B. cereus produced amylase at pH 8.0 to 8.5[109]. Bacillus licheniformis synthesised α-amylase maximally at pH ranging from 6 to 9[56,12,177]. Takaya et al[59] reported that Bacillus H 167, isolated from soil, produced amylase maximally when the initial pH of the medium was 9.4. Bacillus sp. isolated from fermenting African locust bean had their optimum pH for growth between 7.0 and 9.0[64]. Nandakumar[36] reported that Bacillus strains produced amylase at significant levels in the pH range 7 to 10.

Different species of Clostridium also showed variation in their optimum pH for growth and enzyme production. Clostridium acetobutylicum[18] and Clostridium thermohydrosulfuricum[178] produced amylase maximally at pH 6.5 whereas, a new clostridial isolate produced amylase maximally at pH 5.9[179].

Micrococcus halobius, a halophilic bacterium produced amylase maximally at pH 6.8[72].

In general, most microorganisms prefer pH 7 for their maximal growth and activity[180]. The bacteria used in the present study also preferred pH 7 for growth and amylase production. V. alginolyticus (MB1) and Vibrio sp. (MB3) showed a sharp decline in enzyme production below or above this pH value, whereas, V. parahaemolyticus (MB2) could produce amylase at significant levels in the pH range 7 to 9. Acidic pH was found to be more harmful for growth and amylase production than alkaline pH.

3.3.2. EFFECT OF NaCl ON GROWTH AND AMYLASE PRODUCTION

Growth of all species of Vibrio is stimulated by Na^+. The minimal concentration required for optimal growth ranges from 5 to 15 mM for V. cholerae and V. metschinikovii to 600 to 700 mM, for V. costicola[162,181,182].

Only very few reports are available on the effect of sodium chloride on growth and amylase production by bacteria. Micrococcus halobius, a moderate halophile, produced amylase maximally when cultivated in media containing 1-3M NaCl[183,184]. Micrococcus varians sub sp. halophilus produced amylase maximally in media containing 2 M NaCl[44]. Acinetobacter 204.1, produced amylase in media containing 1 to 2 M NaCl[42]. Alteromonas rubra[45] and Vibrio gazogenes[39] produced amylase in high quantities in artificial seawater medium containing 600 mM NaCl.

In the present study, NaCl was found necessary for growth and enzyme production. The optimal salt concentration required by the different species showed great variation. V. alginolyticus (MB1) could grow maximally in the NaCl range 3% to 10%, whereas, enzyme production was maximal at 1.5% NaCl concentration. For V. parahaemolyticus (MB2), NaCl optima for growth was 1.5% and for enzyme production 1% . Vibrio sp. (MB3) exhibited salt optima for growth and enzyme production at 3% and 1% respectively.

In general, at higher concentration of NaCl, (up to 8%) all the three species showed significant growth whereas enzyme production was found to be inhibited by higher NaCl levels in the medium. Lower amounts of NaCl were essential for enzyme production and growth and enzyme production were completely absent in the media without salt.

3.3.3. EFFECT OF VARIOUS IONS ON GROWTH AND AMYLASE PRODUCTION

Growth and enzyme production of halophilic bacteria depend upon the presence of various ions in the media. They are unable to grow in the absence of ions in the media. The results, obtained in the present study, have shown that this ionic requirement is not very specific. Except calcium chloride and sodium fluoride, all other salts used in the experiment were able

to support growth and amylase production by these bacteria. It may be concluded that the important function of these ions is to maintain the osmotic balance between the cells and their environment. Similar observations were also made by other workers. Onishi[72] found that a halophilic bacterium, Micrococcus halobius, could grow and produce amylase in starch medium containing either 1 to 3 M NaCl or KCl. Kobayashi et al[44] also observed that amylase production by Micrococcus varians halophilus ATCC 21971 was supported by 1.5 to 3.0 M NaBr or 2 to 4 M $NaNO_3$.

3.3.4. EFFECT OF TEMPERATURE ON GROWTH AND AMYLASE PRODUCTION

All bacteria exhibit a characteristic minimum growth temperature, optimum growth temperature and maximum growth temperature[185]. Optimum temperature required for α-amylase production by different bacteria shows wide variation. Different strains of the same species also show considerable variation in their temperature optima for amylase production.

Temperatures used for α-amylase production by different strains of Bacillus subtilis were 37°C and 40°C[176], 30°C[186], 37°C[187] and 35°C[48]. The optimal temperatures employed for the production of amylase by Bacillus licheniformis were 37°C[188], 35-40°C[177], 55°C[189] and 45°C[12]. Bacillus coagulans CUMC 512

produced amylase maximally at 50°C[189]. Bacillus stearothermophilus also produced amylase maximally at 50°C[68]. The temperature optima for amylase production by other Bacillus strains were 30°C for Bacillus cereus NY-14[110], 37°C for Bacillus sp. H.167[59] and 35°C for Bacillus coagulans, B. polymyxa and B. cereus[36].

Clostridium acetobutyricum produced amylase maximally at 45°C[18] whereas, Clostridium sp. strain EMI produced amylase maximally at 55°C[20]. The halophilic bacterium Micrococcus halobius preferred a growth temperature of 30 to 37°C for maximal enzyme production[40].

From previous studies, it is evident that, except a few, majority of bacteria prefer to grow and produce amylase below 40°C. The observation made in the present study also agrees with this general pattern. All the strains showed maximal growth and enzyme production in the temperature range 30 to 35°C. Vibrio alginolyticus (MB1) and Vibrio sp.(MB3) showed some growth at 45°C. There was no growth below 5°C. This observation shows that, though they are aquatic forms, they are mesophiles. Their growth in the natural environment may be suboptimal.

Species of Vibrio vary with respect to temperature at which growth will occur. All grow at 20°C and most at 30°C, some grow at 4°C and 45°C and none grows at 50°C[190]. This general

observation was found to be true in the case of the Vibrio species used in the present study also.

3.3.5. EFFECT OF STARCH CONCENTRATION ON GROWTH AND AMYLASE PRODUCTION

Starch is used widely as a source of carbon for amylase production. The concentration of starch required for maximal amylase production shows wide variation among different bacterial strains.

Bacillus stearothermophilus produced amylase maximally at 0.3% starch concentration. Above this level, amylase production decreased[63]. B. cereus NY-14 produced amylase maximally at 0.5% soluble starch[109]. Bacillus licheniformis TCRDC B13 was reported to produce amylase maximally at 1% starch[177]. Bacillus coagulans showed maximal amylase production at 2% starch[191]. Nandakumar[36] reported α-amylase production by Bacillus strains at 1% starch. Clostridium acetobutyricum required 2.5% starch for maximal amylase production[18]. Clostridium 2021 produced amylase maximally at 3% starch[69] whereas, a new Clostridium isolate[179] required only 1% starch for maximal amylase production. Optimum starch concentration for Streptomyces rimosus was 1.5%[68] and for Streptomyces HA 40, it was 1% starch[62]. Micrococcus halobius ATCC 21729 produced amylase maximally in a medium containing 0.2 to 2% starch[44].

In the present study, the three strains showed marked variation in optimal starch concentration for growth and enzyme production in nutrient medium. The concentration optima for growth and enzyme production also showed great differences. Vibrio alginolyticus (MB1) and Vibrio sp. (MB3) required higher concentration of starch for optimal growth and lesser concentration for maximal amylase production. Vibrio alginolyticus (MB1) required 1% starch for optimal growth in nutrient broth and 0.3% for enzyme production. Vibrio sp.(MB3) showed maximum growth at 0.2% starch and enzyme production at 0.1% starch. But Vibrio parahaemolyticus (MB2) showed optimal growth and enzyme production at the same starch concentration (0.2%). In all the three cases, higher concentration of starch was found to be inhibitory to enzyme production.

In the mineral media, starch concentration required for maximal growth and enzyme production was higher compared to nutrient broth. This may be due to the fact that in the mineral media, starch is the only source of carbon whereas, in nutrient broth, other ingredients like beef extract and peptone can also serve as sources of carbon. In mineral medium, V. alginolyticus (MB1) showed maximum growth at 1.5% starch and enzyme production at 0.5%. V. parahaemolyticus (MB2) showed optimum growth and enzyme production in the range 0.4% to 0.6% starch. Vibrio sp.(MB3) showed maximum growth at 0.4% to 0.8% starch

concentration and maximal enzyme production in the concentration range 0.3% to 0.5%. In general, it was found that at 0.5% starch concentration in mineral media, all the strains showed good growth and enzyme production.

Growth and enzyme production were compared in complex medium and mineral medium. All the strains showed maximal growth in complex medium whereas, amylase production was maximal in synthetic medium. In complex medium, since other sources of carbon are available, they can grow even without producing amylase. Whereas, in mineral medium where the only source of carbon is starch, the organisms have to produce amylase for growth and their metabolism. Ustyuzhania et al[192] also observed higher production of amylase by Aspergillus oryzae in a synthetic medium than in complex medium.. Alteromonas rubra[45] and Vibrio gazogenes[39] also produced amylase in high quantities in mineral medium containing starch or maltose as carbon source. Whereas, Bacillus stearothermophilus was found to produce amylase in higher quantities in complex media than in semi-synthetic or synthetic media[63].

3.3.6. EFFECT OF SOURCE OF CARBON ON GROWTH AND AMYLASE PRODUCTION

The composition and concentration of media greatly affect the growth and production of extracellular amylase in bacteria.

Starch is considered to be an inducer for amylase production but there are reports that starch may not be required for amylase production[193].

Starch was found to be a good C source for amylase production by Bacillus stearothermophilus[68], Bacillus subtilis[38,4] Bacillus licheniformis[38], Bacillus cereus[109,38], Bacillus megaterium[111], Clostridium acetobutylicum, Clostridium 2021[69], Clostridium thermosulfuricum[70], Clostridium butyricum[19], Streptomyces rimosus[68] and Streptomyces sp. H.A.40[62].

Starch or maltose served as a good source of carbon for amylase production by Pseudomonas saccharophila[24], Vibrio gazogenes[39] and Alteromonas rubra[45].

Arabinose, maltose, maltotriose or xylose served as carbon source for amylase production by Bacillus licheniformis[56]. Starch dextrin, lactose and maltose served as carbon sources for amylase production by different strains of Bacillus[36]. Starch, pullulan, maltotriose and maltose served as good C source for amylase production by a new Clostridium isolate[20]. Maltose served as a carbon source for amylase production by a new strain of Bacillus subtilis[50]. Galactose served as the carbon source for production of amylase by Bacillus licheniformis[12]. Bacillus subtilis and Bacillus brevis 47-5 provided large amounts of amylase in medium supplemented with glucose as carbon source[194].

In the present study, Vibrio alginolyticus (MB1) and Vibrio sp.(MB3) showed maximal growth in glucose media whereas, Vibrio parahaemolyticus (MB2) expressed maximum growth in starch medium.

Maltose was found to be the best carbon source for amylase production by all the three strains. Starch also supported enzyme production in high quantities. But, glucose at 0.5% concentration was found to inhibit amylase production completely. So the amylases produced by these bacteria may be considered as subjected to catabolite repression.

Among the different types of starches tested, soluble starch was found to be the best C source for growth and amylase production by all the strains. Vibrio alginolyticus showed almost uniform growth in all the starches tested but amylase production was maximal in soluble starch.

3.3.7. EFFECT OF SOURCE OF NITROGEN ON GROWTH AND AMYLASE PRODUCTION

The source of nitrogen also influences growth and amylase secretion by bacteria. Clostridium acetobutylicum secreted amylase in high quantities in the presence of fish powder[18]. Soy flour was found to be a good nitrogen source for amylase production by Bacillus megaterium, sensu stricto (NCIB 7581)[111]. Peptone was

a good source of nitrogen for amylase production by Bacillus cereus NY-14 [110], Bacillus sp.[195], Bacillus licheniformis[12], Bacillus licheniformis TCRDC B13[177], Bacillus subtilis[50] and Streptomyces sp. H.A.40[62]. Yeast extract was found to be a good nitrogen source for amylase production by Bacillus amyloliquifaciens[57].

In the present study, of the three nitrogen sources tested, yeast extract was the preferred nitrogen source for Vibrio alginolyticus (MB1) whereas, V. parahaemolyticus (MB2) and Vibrio sp.(MB3) produced amylase maximally in the presence of ammonium chloride.

3.3.8. EFFECT OF PERIOD OF INCUBATION ON GROWTH AND AMYLASE PRODUCTION

The incubation period required for maximal growth and enzyme production depends on the bacterial species and the type of culture. The optimum incubation period for production of α-amylase by Bacillus licheniformis CUMC.305 was 15 to 20 hours[12]. Alkalophilic Bacillus sp. H 167 required 50 to 60 hours of incubation period[59]. Bacillus subtilis required 72 hours of incubation period[186]. Clostridium acetobutylicum ATCC 824 required 15 hours of cultivation period for maximal amylase production[18] whereas, Clostridium butyricum produced amylase maximally in 18 hours[19].

Optimum incubation periods for α-amylase production by Bacillus licheniformis in still and shake cultures were 32 to 38 hours and 20 to 25 hours respectively[62]. In the present study also, the incubation period showed variation in still and shake cultures and a shorter incubation was sufficient for maximal amylase production in shake cultures. V. parahaemolyticus (MB2) and Vibrio sp.(MB3) required only 7 hours of incubation under shake cultures for maximal amylase production whereas, under still culture, 24 hours of incubation was essential. Vibrio alginolyticus (MB1) required 10 hours of incubation under shake culture whereas, 36 hours were essential under still cultures. But Bacillus stearothermophilus[63] did not show any difference in incubation period due to shaking and in case of Aspergillus oryzae, stationary cultures gave better yields of amylase than shake cultures[196].

3.3.9. GROWTH PHASE OF AMYLASE PRODUCTION

In many bacteria, amylase secretion takes place at the exponential or logarithmic phase of growth. This was found to be true in the case of Bacillus stearothermophilus[14], Bacillus licheniformis[56,13], Clostridium isolate[20], Pseudomonas saccharophila[24], Vibrio gazogenes[39] and Alteromonas rubra[45]. In the present study also, Vibrio parahaemolyticus (MB2) and Vibrio sp.(MB3) secreted enzyme in the logarithmic phase of growth and further increase in incubation period resulted in the loss of

enzyme activity. This may be due to the production of proteolytic enzymes towards the early stationary phase of growth.

In some bacteria, enzyme production takes place in the stationary phase of growth or can continue in the stationary phase. Streptomyces aureofaciens showed maximal production of cell-bound amylase in the logarithmic phase whereas, extracellular amylase was produced maximally in the stationary phase of growth[61]. Bacillus megaterium sensu stricto (NCIB 7581) produced β-amylase through out the exponential phase and during the early stationary phase[111]. The yeast Saccharomycopsis fibuligera ST2 produced amylase during the stationary phase of growth. Vibrio alginolyticus (MB1) employed in the present study started enzyme production in the logarithmic phase and the production continued in the early stationary phase of growth also.

3.3.10. LOCATION OF AMYLASES

The amylases produced by bacteria may be either cell-bound or extracellular. Amylolytic enzyme produced by a new Clostridium isolate was largely cell-bound during the growth of the organism with 0.5% starch but an increase in the starch concentration in the medium resulted in excretion of amylase into the culture broth[20]. Clostridium thermosulfuricum also exhibited similar pattern of enzyme liberation[70].

Bacillus licheniformis NCIB 6346 α-amylase was invariably extracellular and could not be detected in the cytoplasm or cell surface[56]. Similar observations were made in the case of *Vibrio gazogenes*[39] and *Alteromonas rubra*[45]. The bacteria employed in the present study also produced mostly extracellular amylase. Only less than 5% of the total activity was found to be associated with cell. So, the amylases produced by *Vibrio alginolyticus* (MB1), *V. parahaemolyticus* (MB2) and *Vibrio* sp.(MB3) may be considered as extracellular.

Chapter 4

4. REGULATION OF AMYLASE SYNTHESIS

The synthesis of extracellular enzymes by bacteria is regulated by environmental stimuli, in a similar fashion to the regulation of catabolic cytoplasmic enzymes. Thus, extracellular enzymes may be either inducible or constitutive[197,198].

Synthesis of extracellular enzymes may be inducible, partially constitutive or totally constitutive depending on the microorganism and the enzyme involved. Since the substrates for extracellular enzymes are often too large to enter the cell, it is, generally, assumed that the inducing molecule is a product of the enzyme's activity rather than the substrate itself. It is thought that a small level of constitutive extracellular enzymes degrades the high-molecular-weight substrates and that the low-molecular-weight product induces further enzyme synthesis on entering the cell. It was found that digalacturonic acid residue which is a product of the cleavage of pectic acid by pectic acid lyase, acts as an inducer of pectic acid lyase[199]. Similarly, maltooligosaccharides induce α-amylase synthesis in some strains of _Bacillus licheniformis_[200] and _Bacillus stearothermophilus_[174].

Experiments were carried out to study whether any induction or repression in amylase synthesis occurs in halophilic bacteria.

4.1. MATERIALS AND METHODS

4.1.1. EFFECT OF VARIOUS CARBOHYDRATES ON AMYLASE PRODUCTION

Effect of maltose, starch, glucose, lactose, mannitol and dextrin on amylase induction was studied by inoculating the cultures in mineral medium containing 0.5% each of these carbohydrates individually as sole source of carbon. Enzyme activity was estimated after 24 hours.

4.1.2. EFFECT OF GLUCOSE ON AMYLASE PRODUCTION

Effect of glucose on enzyme production was studied by adding different concentrations of glucose to cultures growing and secreting amylase in maltose medium. The enzyme activity was assayed at short intervals.

4.1.3. EFFECT OF CHLORAMPHENICOL ON AMYLASE SECRETION

Effect of chloramphenicol on amylase secretion was studied by adding chloramphenicol (5 µg/ml) to cells actively secreting amylase in maltose medium. Enzyme activity was determined at short intervals.

4.1.4. EFFECT OF ACTINOMYCIN D ON AMYLASE SECRETION

Effect of actinomycin D on amylase production was studied by adding actinomycin D (70 μg/ml) to cultures actively secreting amylase in maltose medium. Enzyme secretion was followed by assaying enzyme activity at short intervals.

4.2. RESULTS

4.2.1. EFFECT OF VARIOUS CARBOHYDRATES ON AMYLASE PRODUCTION

All the 3 strains showed amylase secretion at high levels in both starch as well as maltose medium (Table 18 and Fig. 16). In all the strains, amylase secretion was found to be more in maltose medium than in starch medium. There was no enzyme production in glucose medium and very little amylase production in dextrin and mannitol media.

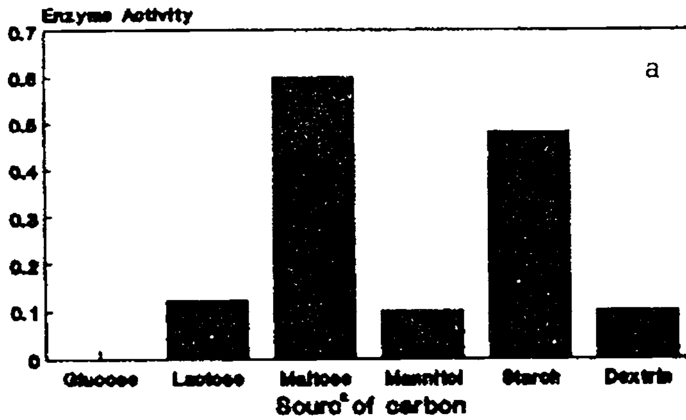
4.2.2. EFFECT OF GLUCOSE ON AMYLASE PRODUCTION

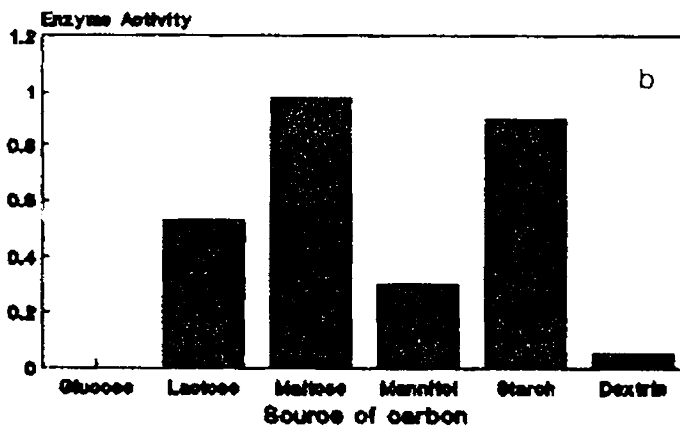
As it was found that glucose at 0.5% level completely inhibited enzyme production, the effect of lower concentration of glucose was tested (Table 19 and Fig. 17). It was found that in all the three strains at 0.5 mg/ml of glucose, enzyme production

Table 18. Effect of various carbohydrates on amylase production

Carbon source (0.5%)	Enzyme activity* (Units/ml)		
	V. alginolyticus (MB1)	*V. parahaemolyticus* (MB2)	*Vibrio* sp. (MB3)
Glucose	0	0	0
Lactose	0.12	0.53	0.443
Maltose	0.6	0.98	0.94
Mannitol	0.1	0.3	0.16
Starch	0.48	0.9	0.856
Dextrin	0.1	0.05	0.04

* mg starch hydrolysed per 15 minutes





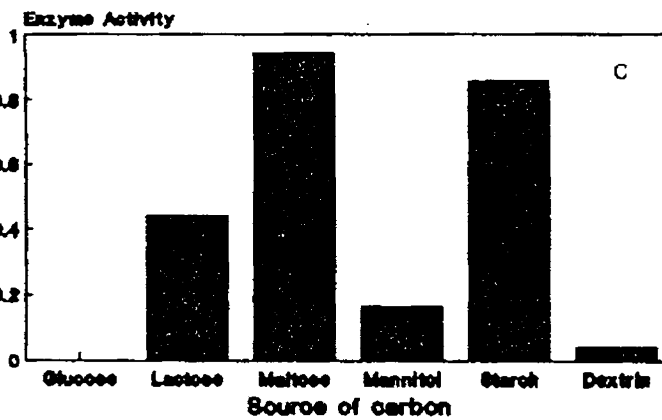


Fig. 16 a-c. Effect of various carbohydrates on amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp (MB3).

Table 19a. Effect of addition of different concentrations of glucose to <u>Vibrio</u> <u>alginolyticus</u> (MB1) cultures secreting amylase in maltose medium.

Period of incubation (hours)	Enzyme activity* (Units/ml)		
	Concentration of glucose 0 mg/ml	Concentration of glucose 0.5 mg/ml	Concentration of glucose 2.5 mg/ml
0	0	0	0
1	0	0	0
2	0	0	0
3	0	0	0
4	0.05	0.05	0.05
5	0.08	0.08	0.08
6	0.11	0.11	0.11
7	0.26	0.12	0.12
8	0.4	0.27	0.121
9	0.5	0.4	0.122
10	0.55	0.5	0.12
11	0.61	0.55	0.11
12	0.6	0.6	0.122

* mg starch hydrolysed per 15 minutes

Table 19b. Effect of addition of different concentrations of glucose to Vibrio parahaemolyticus (MB2) cultures secreting amylase in maltose medium.

Period of incubation (hours)	Enzyme activity* (Units/ml)		
	Concentration of glucose 0 mg/ml	Concentration of glucose 0.5 mg/ml	Concentration of glucose 2.5 mg/ml
0	0	0	0
1	0	0	0
2	0	0	0
3	0.12	0.12	0.12
4	0.35	0.35	0.35
5	0.6	0.36	0.35
6	0.86	0.6	0.346
7	0.95	0.8	0.34
8	0.8	0.85	0.35
9	0.6	0.6	0.34
10	0.5	0.55	0.35

* mg starch hydrolysed per 15 minutes

Table 19c. Effect of addition of different concentrations of glucose to *Vibrio* sp. (MB3) cultures secreting amylase in maltose medium.

Period of incubation (hours)	Enzyme activity* (Units/ml)		
	Concentration of glucose 0 mg/ml	Concentration of glucose 0.5 mg/ml	Concentration of glucose 2.5 mg/ml
0	0	0	0
1	0	0	0
2	0	0	0
3	0	0	0
4	0.13	0.13	0.13
5	0.5	0.13	0.13
6	0.69	0.35	0.2
7	0.85	0.55	0.35
8	0.83	0.8	0.47
9	0.6	0.75	0.5
10	0.4	0.6	0.5

* mg starch hydrolysed per 15 minutes

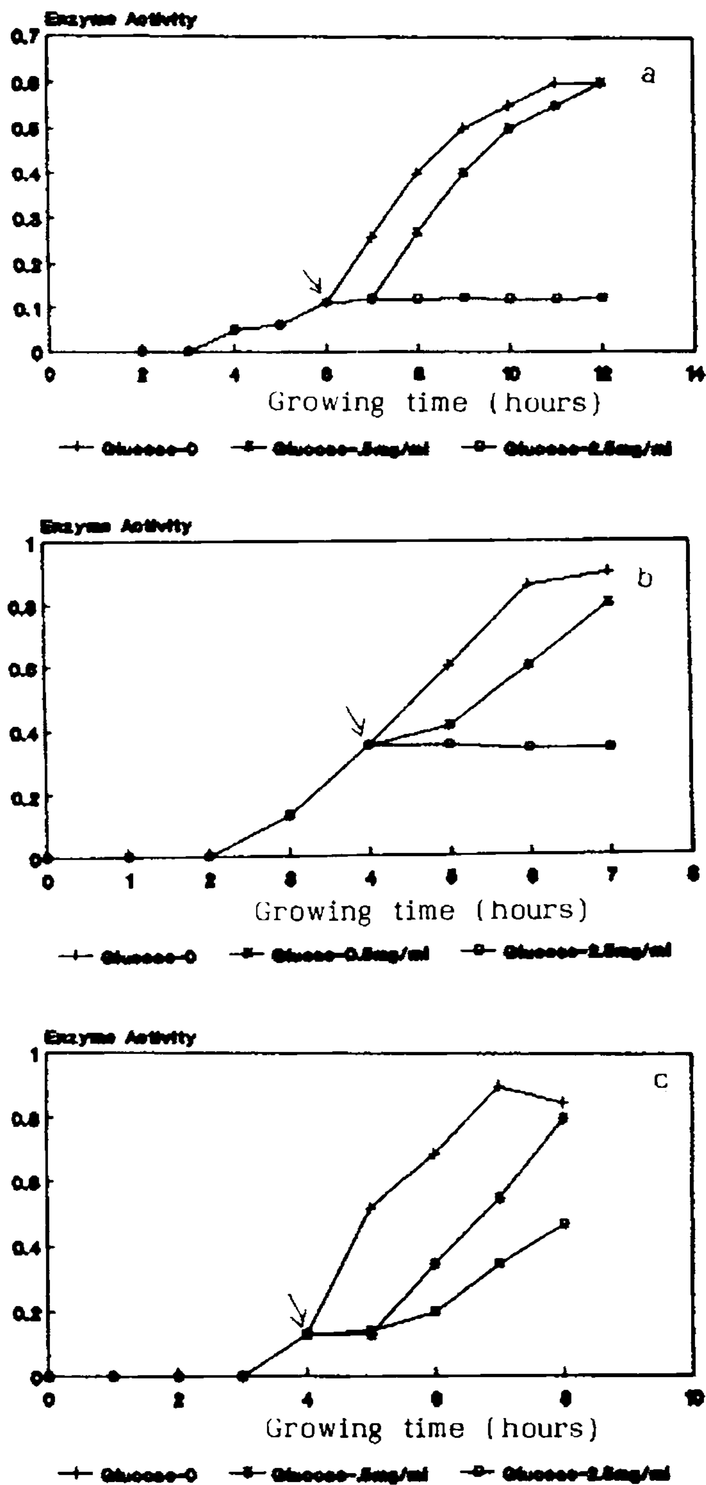


Fig. 17 a–c. Effect of adding different concentrations of glucose (at the time indicated by the arrow) on amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

was delayed for a period of one hour and then resumed. But, at higher levels, (2.5 mg/ml) amylase production was completely inhibited in V. alginolyticus (MB1) and V. parahaemolyticus (MB2). There was considerable reduction in amylase production by Vibrio sp.(MB3) at this concentration of glucose.

4.2.3. EFFECT OF CHLORAMPHENICOL ON AMYLASE SECRETION

When chloramphenicol was added to cells actively secreting amylase, there was an immediate and complete inhibition of amylase secretion in all the three cases (Table 20 and Fig. 18).

4.2.4. EFFECT OF ACTINOMYCIN-D ON AMYLASE SECRETION

When actinomycin D was added to cell suspension, amylase secretion continued for a short period, followed by inhibition. It was found that inhibition did not occur until about one hour after the addition of the antibiotic. All the strains showed similar pattern of inhibition by actinomycin D (Table 21 and Fig. 19).

4.3. DISCUSSION

4.3.1. INDUCTION OF AMYLASE SYNTHESIS

Maltose, one of the products of α-amylase action, was found

Table 20a. Effect of chloramphenicol on amylase secretion by *V. alginolyticus* (MB1).

Period of incubation (hours)	Enzyme activity* (Units/ml)	
	Without chloramphenicol	With chloramphenicol
0	0	0
1	0	0
2	0	0
3	0	0
4	0.05	0.05
5	0.08	0.079
6	0.15	0.15
7	0.26	0.15
8	0.36	0.149
9	0.5	0.145
10	0.6	0.14

* mg starch hydrolysed per 15 minutes

Table 20b. Effect of chloramphenicol on amylase secretion by *V. parahaemolyticus* (MB2).

Period of incubation (hours)	Enzyme activity* (Units/ml)	
	Without chloramphenicol	With chloramphenicol
0	0	0
1	0	0
2	0	0
3	0.12	0.12
4	0.35	0.35
5	0.6	0.35
6	0.86	0.34
7	0.95	0.35

* mg starch hydrolysed per 15 minutes

Table 20c. Effect of chloramphenicol on amylase secretion by *Vibrio* sp. (MB3).

Period of incubation (hours)	Enzyme activity* (Units/ml)	
	Without chloramphenicol	With chloramphenicol
0	0	0
1	0	0
2	0	0
3	0	0
4	0.13	0.133
5	0.52	0.134
6	0.69	0.134
7	0.85	0.136

* mg starch hydrolysed per 15 minutes

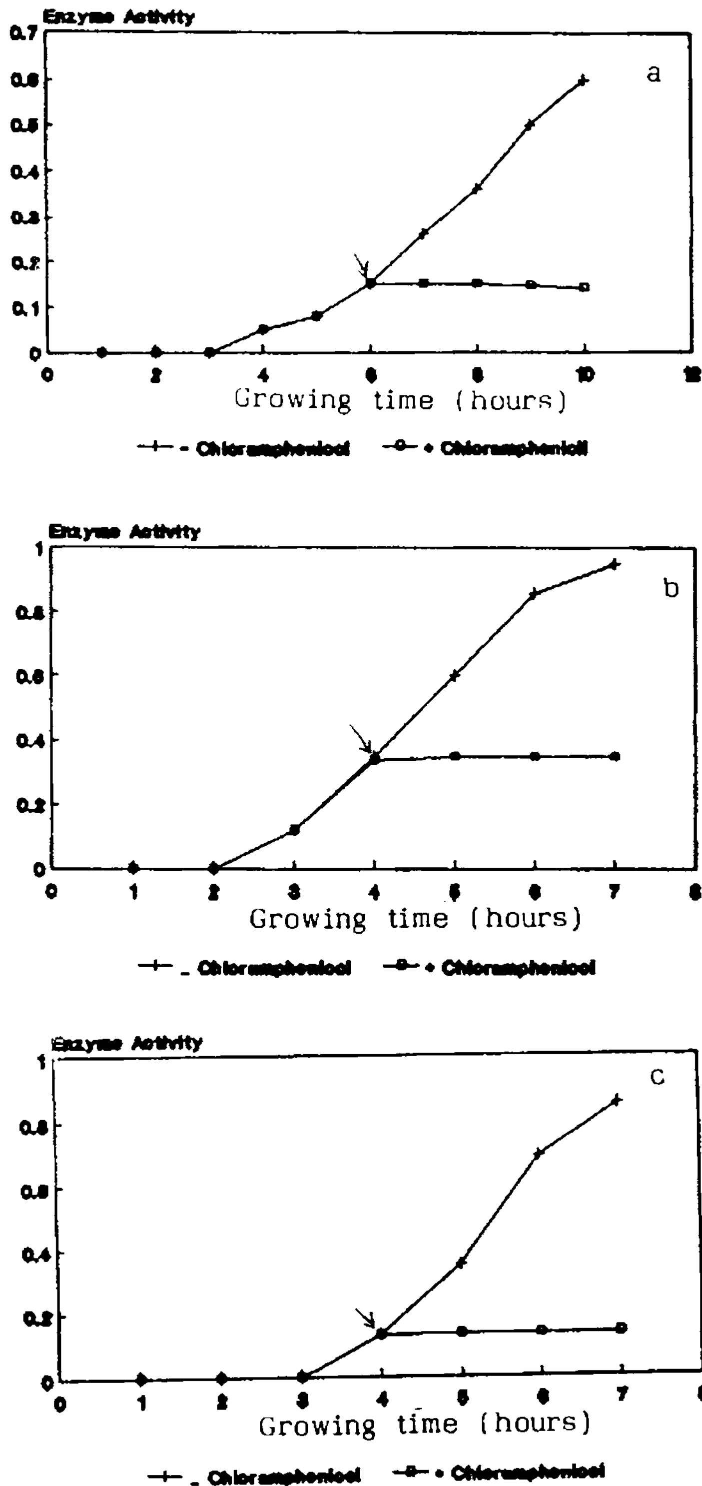


Fig. 18 a-c. Effect of adding chloramphenicol (at the time indicated by the arrow) on amylase production by Vibrio species. (a) V. alginolyticus (MB1) (b) V. parahaemolyticus (MB2) (c) Vibrio sp. (MB3).

Table 21a. Effect of actinomycin D on amylase secretion by *Vibrio alginolyticus* (MB1).

Period of incubation (hours)	Enzyme activity* (Units/ml)	
	Without actinomycin D	With actinomycin D
0	0	0
1	0	0
2	0	0
3	0	0
4	0.05	0.045
5	0.08	0.082
6	0.15	0.14
7	0.27	0.2
8	0.38	0.24
9	0.5	0.25
10	0.62	0.255

* mg starch hydrolysed per 15 minutes

Table 21b. Effect of actinomycin D on amylase secretion by *Vibrio parahaemolyticus* (MB2).

Period of incubation (hours)	Enzyme activity* (Units/ml)	
	Without actinomycin D	With actinomycin D
0	0	0
1	0	0
2	0	0
3	0.12	0.125
4	0.35	0.359
5	0.6	0.45
6	0.86	0.455
7	0.9	0.45

* mg starch hydrolysed per 15 minutes

Table 21c. Effect of actinomycin D on amylase secretion by *Vibrio* sp. (MB3).

Period of incubation (hours)	Enzyme activity* (Units/ml)	
	Without actinomycin D	With actinomycin D
0	0	0
1	0	0
2	0	0
3	0	0
4	0.13	0.132
5	0.52	0.25
6	0.69	0.256
7	0.85	0.254

* mg starch hydrolysed per 15 minutes

68 h

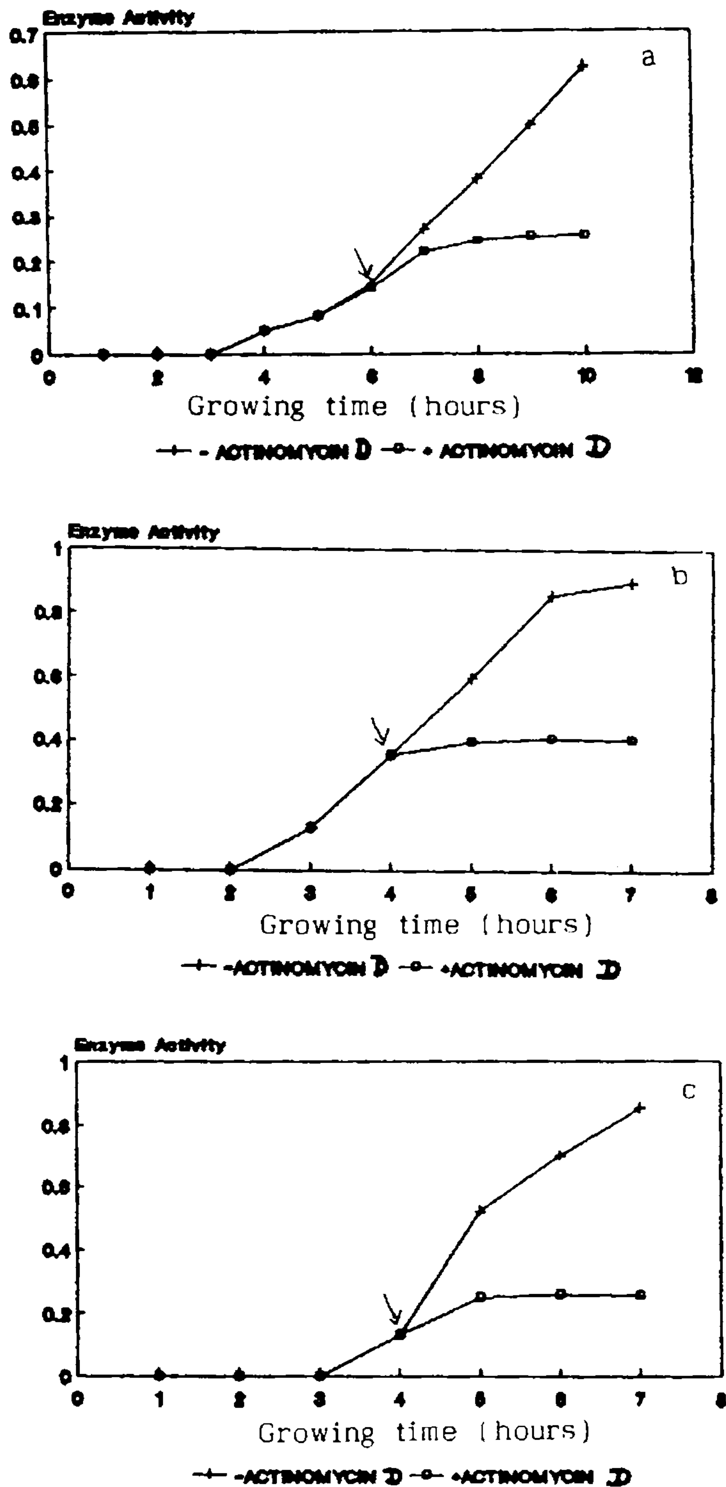


Fig. 19 a-c. Effect of adding actinomycin D (at the time indicated by the arrow) on amylase production by _Vibrio_ species. (a) _V. alginolyticus_ (MB1) (b) _V. parahaemolyticus_ (MB2) (c) _Vibrio_ sp. (MB3).

to be an inducer of amylase production in Micrococcus varians halophilus ATCC 21971[44]; Vibrio gazogenes[39], Alteromonas rubra[45], Aspergillus niger[92], Bacillus circulans[81], Vibrio parahaemolyticus[86], Streptomyces rimosus[68] and Aspergillus oryzae[201].

Synthesis of α-amylase by Streptomyces olivaceus was induced in media containing maltose or starch[202]. Higher yields of amylase were known to be produced in media containing complex starch materials like ground corn rather than in artificially defined media[203, 204, 205], because these carbon compounds do not exert catabolite repression and it would appear that they permit maximal induction of α-amylase.

Starch, pullulan, maltotriose and maltose induced the synthesis of α-amylase and pullulanase by a new Clostridium strain[20]. Maltotriose induced amylase synthesis in V. parahaemolyticus[86]. Clostridium thermosulfurogenes expressed β-amylase at high levels when maltose or other carbohydrates containing maltose units were present in the medium[7]. Amylase from Bacillus circulans F2 was induced by cross-linked starches[80]. Starch, maltose and β-cyclodextrin induced amylase production by the yeast Filobasidium capsuligenum[88]. Starch induced amylase synthesis in Bacillus stearothermophilus[82], Streptomyces rimosus[68] and Aspergillus oryzae[91]. In the present study also, the amylases of all the three strains were found to be inducible. Maltose and

starch were found to induce higher amylase production in these species. Of these, maltose was a better inducer than starch because higher amylase activity was found in the medium containing maltose. In contrast, the amylase produced by *Bacillus licheniformis* NCIB 6346 was found to be constitutive and not dependent on exogenous α-glucans for induction[79]. Similarly, the amylase of *Bacillus subtilis*[206, 207] was also produced constitutively. The enzymes produced by the present strains may be considered partially constitutive, since, small quantities of enzymes were secreted into the medium, even in the absence of starch or maltose.

In general, compounds containing α-1,4 glucosidic linkages are effective inducers of amylases. In contrast to this general observation, Tomomura *et al*[208] reported that isomaltose and pentose were most effective inducers of α-amylase in *Aspergillus oryzae*. Suzuki and Tanabe[209] observed that the highest levels of α-amylase were produced when high concentration of disaccharides, identified as isomaltose and maltose were present in the medium.

4.3.2. CATABOLITE REPRESSION

Repression of constitutive and inducible enzyme synthesis in the presence of readily utilizable carbon source is known as catabolite repression. It explains many aspects of extracellular

enzyme synthesis[210] and contributes to the regulation of these enzymes. In E. coli, it was found that the rate of synthesis of the enzyme β-galactosidase in constitutive mutants or after induction was inversely proportional to the growth rate of the organism. It was higher in cells when the growth rate was low, with lactose or succinate as carbon sources, and low in cells growing rapidly with glucose or galactose[211]. Meers[212] reported that catabolite repression and growth rate were the main factors controlling α-amylase synthesis in Bacillus licheniformis. α-Amylase synthesis in Bacillus subtilis is related to the nature of the carbon source, being highest in media containing lactate or glutamate but lowest with glucose which gave the fastest growth rate[207]. In both Bacillus stearothermophilus[174] and Bacillus subtilis[213], the rate of synthesis of α-amylase is inversely related to the rate of growth of the organism, when starch, glycerol or glucose are used as carbon sources. Continuous culture studies on Bacillus subtilis[214] showed that α-amylase was catabolite - repressed. The amylase production by Bacillus licheniformis depended on the presence or absence of a catabolite-repressing carbon source in the growth medium[13]. Thirunavakkarasu and Priest[79] have reported that amylase production by Bacillus licheniformis was subjected to catabolite repression. If either glucose or mannitol was present in the medium, amylase synthesis could not be detected until the stationary phase by which time the carbon source was exhausted.

Saito and Yamamoto [200] also observed that B. licheniformis α-amylase is sensitive to catabolite repression.

Glucose inhibited amylase production in Aspergillus oryzae [215,216], Endomycopsis fibuligera [87], Schwanniomyces castellii and Endomycopsis fibuligera [217], Streptomyces olivaceus [202], Bacillus amyloliquefaciens [218], Bacillus licheniformis [56] and Bacillus licheniformis CMB 88 [55].

Glucose repressed α-amylase synthesis in Bacillus subtilis [219], Bacillus subtilis and Bacillus licheniformis [212], Bacillus stearothermophilus [83], Thermophilic Bacillus sp. [84], Clostridium thermosulfurogenes [7], Vibrio gazogenes [39], Alteromonas rubra [45], Saccharomycopsis fibuligera [220], Saccharomycopsis fibuligera, Schwanniomyces castellii and Schwanniomyces alluvium [89].

Glucose and 2-deoxy D-glucose repressed amylase secretion in the yeast Filobasidium capsuligenum [88]. Glucose, maltose and sucrose repressed amylase synthesis in B. stearothermophilus [82,63]. In presence of glucose, sucrose, lactose or isomaltose, no enzyme secretion was noticed in Bacillus circulans [81].

Iuchi and Tanaka [43] observed repression of extracellular amylase and protease production in Vibrio parahaemolyticus by various carbohydrates. Monosaccharides like mannose, glucose,

galactose or gluconate and also pyruvate repressed amylase synthesis in Vibrio parahaemolyticus[86,43].

In the present study also, higher concentration (2.5 mg/ml) of glucose in the culture medium inhibited amylase synthesis in all the strains. At lower concentration (0.5 mg/ml) there was a transient repression of amylase synthesis which continued at the same rate after one hour. This may be due to the fact that once glucose concentration comes to a low level as a result of its metabolism, amylase synthesis is derepressed. So, the amylases produced by V. alginolyticus (MB1), V. parahaemolyticus (MB2) and Vibrio sp. (MB3) may be considered as catabolite-repressible. Similar observations were made by Ratcliffe et al[39] in amylase production by V. gazogenes and by Gavrilovic et al[45] in the case of Alteromonas rubra.

4.3.4. EFFECT OF CHLORAMPHENICOL ON AMYLASE SECRETION

The antibiotic, chloramphenicol, inhibits protein synthesis in prokaryotic organisms. At lower concentrations, it does not affect RNA synthesis. Chloramphenicol was added to culture medium when the cells were actively engaged in amylase synthesis to find out whether the synthesis is a de novo process. It was found that, in all the strains, there was a rapid and complete inhibition of amylase production when chloramphenicol was added at a concentration of 5 µg/ml. This confirms that the secretion

of amylase, in these strains was the result of de novo synthesis. This, together with the finding that there was only very little active intracellular amylase, supports the hypothesis that the enzyme is synthesised on the cytoplasmic membrane [221]. Similar effect of chloramphenicol was observed in the case of amylase secretion by Alteromonas rubra [45] and V. gazogenes [39]. Chloramphenicol did not affect the activity of the enzyme. It abolishes the inductive effect of maltose in a maltose medium.

4.3.5. EFFECT OF ACTINOMYCIN D

Actinomycin D is an antibiotic, inhibiting RNA synthesis. Its effect on amylase production was studied by adding 70 µg/ml to growth medium when the cells have started amylase secretion. All the strains showed a transient continuation of amylase secretion followed by inhibition, indicating the presence of a pool of mRNA specific for amylases. The exact cause of this transient continuation of amylase secretion by actinomycin D is unknown, but, it is reminiscent of the findings of Coleman and Elliott[222] who observed a stimulation of the synthesis of extracellular ribonuclease by a Bacillus sp. after addition of actinomycin D to cells actively secreting the enzyme. However, the stimulation they observed lasted for several hours.

Two hypotheses have been proposed which are consistent with the present data. Both et al [223] postulated that the mRNA

pool for extracellular enzymes in Bacillus amyloliquefaciens resulted from a positive imbalance of transcription over translation. An alternate hypothesis was proposed by O' Connor et al [224] who suggested that there were 2 forms of mRNA for the extracellular protease produced by Bacillus amyloliquefaciens, a short-lived form, immediately available for translation and a relatively stable, non-translatable form. They suggested that in the presence of inhibitors of transcription, the stable form was converted into the translatable species which would account for the continued production of the enzyme in the absence of transcription. The existence of mRNA pools specific for extracellular protease was first reported by Both et al [223]. Similar observations were also made with protease secretion by Vibrio alginolyticus[164], amylase and protease secretion by Vibrio gazogenes[39] and amylase production by Alteromonas rubra[45].

Chapter 5

5. CHARACTERS OF AMYLASES

Amylases obtained from different sources differ widely in their properties. There are variations in the type of amylase produced, their tolerance to acid or alkali, temperature stability, K_m and V_{max} values, effect of ions on enzyme action etc. Experiments were carried out to study the properties of amylases from the halophilic bacteria.

5.1. MATERIALS AND METHODS

5.1.1. PARTIAL PURIFICATION OF THE AMYLASES

Partial purification of amylase was done by ammonium sulphate precipitation followed by dialysis.

To the chilled culture fluid, solid ammonium sulphate was added with gentle stirring to 30% saturation; kept in the refrigerator for two hours and subjected to centrifugation. After centrifugation, the precipitate was discarded. More ammonium sulphate was added to the supernatant to 70% saturation. The precipitate was collected and dissolved in 0.01 M sodium phosphate buffer (pH 7) and dialysed overnight against the same buffer at 4°C. The dialysed enzyme was used for further studies.

5.1.2. DETERMINATION OF PROTEIN

Protein was determined by the method of Lowry et al[225], with bovine serum albumin as standard.

5.1.3. POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

Polyacrylamide disc gel electrophoresis was carried out following the method of Davis[241].

5.1.4. IDENTIFICATION OF THE ENZYMES

To determine whether the amylase was of the α or β type, incubation of the reaction - mixture was made at 30°C in a final volume of 15 ml: 14 ml starch solution containing 10 mg starch/ml prepared in 0.01 M phosphate buffer at the optimal pH of 7 and 1 ml partially purified enzyme. Samples were removed at 5 minutes intervals up to 80 minutes and the decrease in blue colour of reaction of iodine with starch and the amount of reducing sugar formed were measured. Decrease in the intensity of the iodine stain of starch was plotted against the conversion of starch into reducing sugar (measured as maltose).

The action-pattern of enzymes from the cultures were compared with salivary α-amylase (assayed in 0.01 M sodium

phosphate buffer, pH 7) and sweet potato β-amylase (assayed in 0.01 M acetate buffer, pH 4.8).

5.1.5. EFFECT OF pH ON AMYLASE ACTIVITY AND STABILITY

Effect of pH on amylase activity was determined as per the procedure described by Morgan and Priest[99]. 0.1 ml of the partially purified enzyme solution was incubated in 1 ml starch solution and 1 ml of different buffers (0.02 M) showing varying pH (accetate buffer: pH 4 to 6, phosphate buffer: pH 7 to 8 and glycine - NaOH buffer: pH 9 to 10). The reaction-mixture was incubated for 15 minutes. Enzyme activity was assayed colorimetrically by measuring the intensity of the blue colour of the starch-iodine complex. Stability of the enzyme at various pH values were tested by incubating 1 ml of the enzyme solution in 1 ml of different buffers in the pH range 3 to 10 for 2 hours at room temperature. After incubation, the pH was adjusted to 7 and residual enzyme activity assayed.

5.1.6. EFFECT OF TEMPERATURE ON ENZYME ACTIVITY AND STABILITY

Effect of temperature on enzyme activity was measured by incubating the reaction-mixture at different temperatures (10°C to 60°C) for 15 minutes and assaying the activity by the starch - iodine method.

Effect of temperature on stability was determined by incubating the enzyme solution in phosphate buffer at different temperatures (20°C to 60°C) for 2 hours. Later, the residual activity was measured at 35°C by starch-iodine method.

5.1.7. EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF THE ENZYMES

Effect of substrate concentration on amylase activity was tested by varying the starch concentration in the reaction-mixture from 0.2 mg to 1.2 mg/ml. 0.1 ml enzyme solution was added and incubated for 5 minutes. Enzyme activity was determined by measuring the reducing sugar formed, by the dinitrosalicylic acid method [170]. The same experiment was repeated using native starches isolated from cassava, potato and plantain. LineWeaver-Burk plot was constructed by plotting the inverse of initial velocity against the inverse of substrate concentration. From the plot, the K_m and V_{max} for different substrates were calculated.

5.1.8. EFFECT OF CATIONS, ANIONS AND ORGANIC COMPOUNDS ON AMYLASE ACTION

Effect of various ions and organic compounds on enzyme activity was determined by adding varying concentrations of different salts and organic compounds to the reaction-mixture.

The pH was adjusted to 7. Incubated for 15 minutes and determined the enzyme activity either by the starch-iodine method or by estimating the reducing sugar.

5.2. RESULTS

5.2.1. PARTIAL PURIFICATION OF AMYLASES

Amylases from the culture fluids of the strains were partially purified by ammonium sulphate precipitation followed by dialysis. Results are presented in Table 22.

Amylase from *Vibrio alginolyticus* (MB1) could be purified 3.7 fold with 57% yield and specific activity 63.33 units/mg protein. Amylase from *V. parahaemolyticus* (MB2) was purified 3.6 fold with 68% yield and specific activity 87.71 units/mg protein. *Vibrio* sp. (MB3) amylase was purified 4.2 fold with 64% total yield, and specific activity 96 units/mg protein. The extracellular amylase of each species was found to be homogeneous by polyacrylamide gel electrophoresis.

5.2.2. IDENTIFICATION OF AMYLASES

The amylases produced by all the three strains showed typical pattern of α-amylases and were identified as α-amylase (Table 23 and Fig. 20).

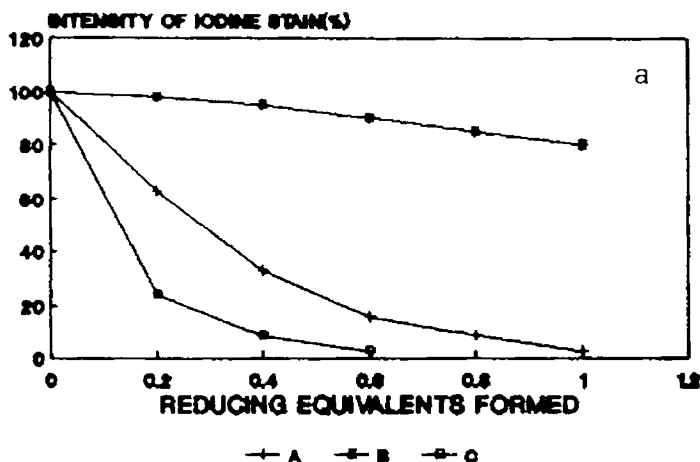
Table 22. Partial purification of amylases from the *Vibrio* isolates

Name of the isolate	Purification step	Volume (ml)	Total activity* units	Total protein (mg)	Specific activity (Units/mg protein)	Yield (%)	Purification (fold)
V. alginolyticus (MB1)	Culture fluid	500	300	17.7	16.94	100	1
	Ammonium sulphate precipitation and dialysis	20	171	2.7	63.33	57	3.7
V. parahaemolyticus (MB2)	Culture fluid	500	452	18.72	24.14	100	1
	Ammonium sulphate precipitation and dialysis	20	307	3.5	87.71	68	3.6
Vibrio sp. (MB3)	Culture fluid	500	435	19.4	22.42	100	1
	Ammonium sulphate precipitation and dialysis	20	278	2.9	96	64	4.2

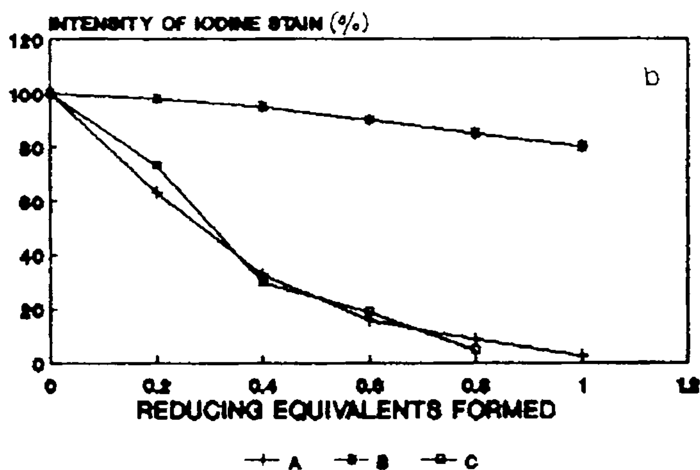
* mg starch hydrolysed per 15 minutes

Table 23. Identification of amylases

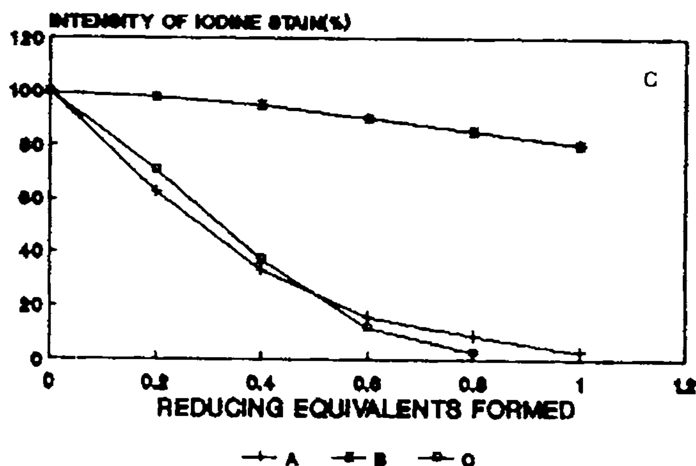
Reducing sugar formed (mg)	Intensity of starch-iodine stain (%)				
	Salivary α-amylase	Sweet potato β-amylase	*V. alginolyticus* (MB1) amylase	*V. parahaemolyticus* (MB2) amylase	*Vibrio* sp. (MB3) amylase
0	100	100	100	100	100
0.2	63	98	58	73	71
0.4	33	95	24	30	37
0.6	16	90	8.74	19	12
0.8	9	85	3	5	3
1	3	80	-	-	-



(A) Salivary α-amylase (B) Sweet potato β-amylase (C) V. alginolyticus (MB1) amylase.



(A) Salivary α-amylase (B) Sweet potato β-amylase (C) V. parrahaemolyticus (MB2) amylase.



(A) Salivary α-amylase (B) Sweet potato β-amylase (C) Vibrio sp. (MB3) amylase.

Fig. 20 a-c. Identification of amylase produced by Vibrio species. (a) V. alginolyticus (MB1) amylase (b) V. parahaemolyticus (MB2) amylase (c) Vibrio sp. (MB3) amylase.

5.2.3. EFFECT OF pH ON AMYLASE ACTIVITY AND STABILITY

Effect of pH on enzyme activity was measured by assaying the activity in different buffers (pH 4 to 10). Results are given in Table 24 and Fig. 21. Amylases from all the three isolates showed optimum activity at pH 7. In general, they could show considerable activity in the pH range 6 to 8. There was no activity at pH 4. Amylase from V. alginolyticus (MB1) retained 50% activity at pH 10, V. parahaemolyticus (MB2) amylase retained only very little activity at pH 10 and Vibrio sp. (MB3) amylase retained about 50% activity at pH 10. Acidic pH was found to be more harmful to the enzyme activity than alkaline pH.

The effect of pH on the stability of the enzymes was tested by incubating the enzyme for two hours in buffers having different values of pH (pH 3 to 10) and then assaying the activity at pH 7 (Table 25 and Fig. 22).

Amylases from all the three strains were considerably stable from pH 6 to 8. V. alginolyticus (MB1) amylase showed maximum stability at pH 7 and retained around 90% activity at pH 6 and 8. It was completely inactivated at pH 3 and retained 11% activity at pH 4 and 25%, at pH 10. V. parahaemolyticus (MB2) amylase showed maximum stability from pH 6 to 8. It retained only 18% activity at pH 4 and 45% activity at pH 10. There was no

Table 24. Effect of pH on amylase activity

pH	Activity* of amylase from (Units/ml):		
	V. alginolyticus (MB1)	*V. parahaemolyticus* (MB2)	*Vibrio* sp. (MB3)
3	0	0	0
4	0	0.68	1.14
5	2.8	6.8	6.54
6	7.9	14.5	12.26
7	8.5	15.35	13.89
8	7.79	14.49	12.75
9	6.37	6.8	9.81
10	4.24	0.68	6.54

* mg starch hydrolysed per 15 minutes

Table 25. Effect of pH on stability of amylases

pH	Residual activity (%) after 2 hours of incubation		
	V. alginolyticus (MB1)	*V. parahaemolyticus* (MB2)	*Vibrio* sp. (MB3)
3	0	0	20
4	11	18	56
5	33	60	82
6	93	100	90
7	100	100	100
8	90	100	100
9	75	60	100
10	25	45	70

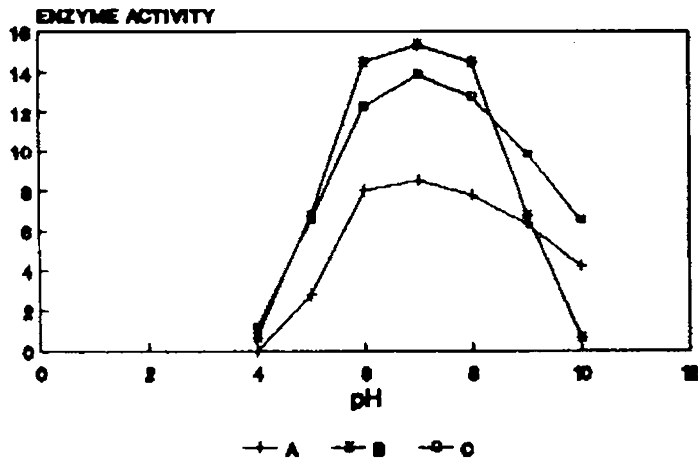


Fig. 21. Effect of pH on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

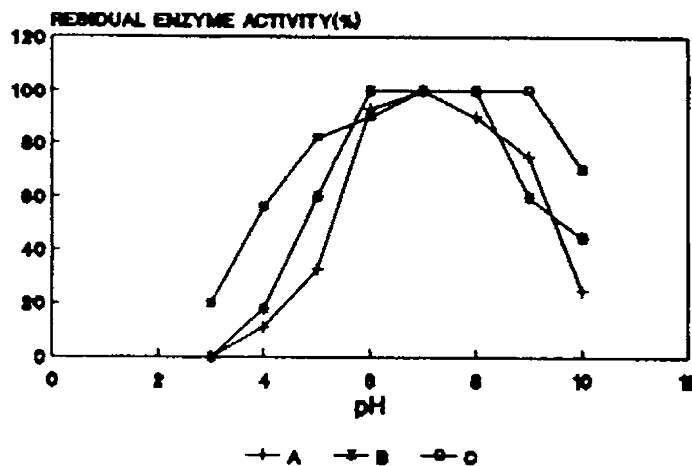


Fig. 22. Effect of pH on stability of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

activity at pH 3. Vibrio sp.(MB3) amylase showed 100% stability from pH 7 to 9. It retained 90% stability at pH 6 and 70% stability at pH 10. When compared to the amylases of the other two strains, amylalse from Vibrio sp. (MB3) was more stable from pH 5 to 10.

5.2.4. EFFECT OF TEMPERATURE ON AMYLASE ACTIVITY AND STABILITY

Effect of temperature on enzyme activity was determined by incubating the reaction-mixture at different temperatures (10°C to 60°C). Results are shown in Table 26 and Fig. 23. V. alginolyticus (MB1) and V. parahaemolyticus (MB2) showed maximum activity between 35°C to 40°C whereas, Vibrio sp.(MB3) showed maximum activity between 30°C to 35°C. There was only 25 to 45% activity at 60°C.

Effect of temperature on stability of the enzymes was assessed by incubating them at different temperatures (20°C to 60°C) for two hours and then assaying their residual activity at 35°C (Table 27 and Fig. 24).

Amylases from all the strains showed 100% stability up to 40°C. Their stability decreased gradually as the temperature was raised. V. alginolyticus (MB1) amylase showed 30% stability at 60°C, 75% at 50°C and 90% at 45°C. V. parahaemolyticus (MB2)

Table 26. Effect of temperature on amylase activity

Temperature (°C)	Activity* of amylase from (Units/ml):		
	V. alginolyticus (MB1)	*V. parahaemolyticus* (MB2)	*Vibrio* sp. (MB3)
10	2.8	3.75	2.4
15	4.24	5.96	4.04
20	5.94	8.69	7.27
25	6.94	10.92	10.99
30	7.5	12.45	13.9
35	8.5	15.35	13.9
40	8.5	15.35	12.9
45	7.65	10.23	11
50	6.37	7.67	9.6
55	4.24	5.11	8.08
60	2.8	3.41	6.5

* mg starch hydrolysed per 15 minutes

Table 27. Effect of temperature on stability of amylases

Temperature (°C)	Residual activity (%) after 2 hours of incubation		
	V. alginolyticus (MB1)	*V. parahaemolyticus* (MB2)	*Vibrio* sp. (MB3)
20	100	100	100
25	100	100	100
30	100	100	100
35	100	100	100
40	100	100	100
45	90	66	93
50	75	50	87
55	50	43	60
60	30	22	50

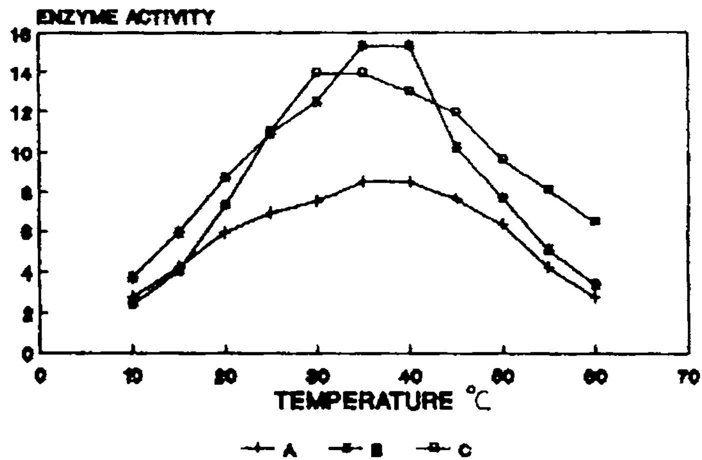


Fig. 23. Effect of temperature on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase and (C) Vibrio sp. (MB3) amylase.

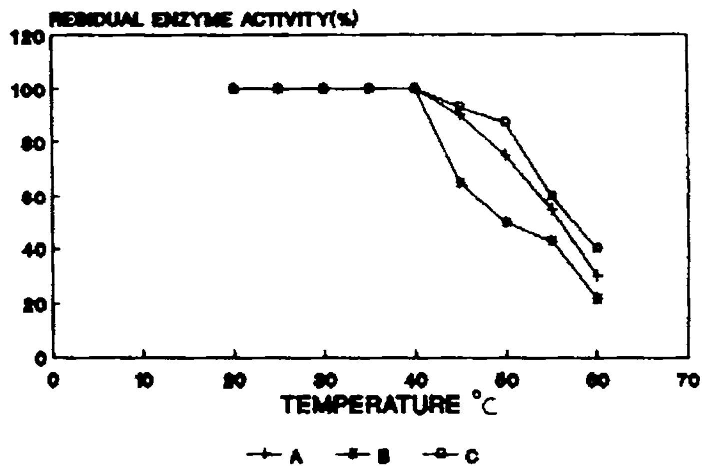


Fig. 24. Effect of temperature on stability of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

amylase showed only 22% stability at 60°C, 50% at 50°C and 66% at 45°C. Amylase from Vibrio sp.(MB3) was more stable. It exhibited 50% stability at 60°C, 87% at 50°C and 93% stability at 45°C.

5.2.5. EFFECT OF SUBSTRATE CONCENTRATION ON AMYLASE ACTIVITY

The effect of substrate concentration on enzyme activity was tested using starches from cassava, potato and plantain and soluble starch. Amylases from the three strains were found to hydrolyse all the substrates tested at varying rates (Table 28 and Fig. 25).

Amylase from V. alginolyticus (MB1) showed maximum activity when the substrate concentration was 0.8 mg/ml, in the case of soluble starch and starch from plantain and cassava. A starch concentration of 1 mg/ml was required for maximal activity in the case of potato starch. The K_m and V_{max} values for the various substrates were calculated from the LineWeaver-Burk plot (Fig.26). The K_m values were: soluble starch - 0.82 mg/ml, cassava starch - 0.65 mg/ml, plantain starch - 0.82 mg/ml and potato starch - 0.59 mg/ml. The V_{max} values were: soluble starch - 0.36 mg/min/ml, cassava starch - 0.19 mg/min/ml, plantain starch - 0.22 mg/min/ml and potato starch - 0.138 mg/min/ml.

V. parahaemolyticus (MB2) amylase also required 0.8 mg/ml starch concentration for maximal activity. The K_m values for

Table 28a. Effect of substrate concentration on velocity of amylase from *Vibrio alginolyticus* (MB1)

Substrate concentration (mg/ml)		Initial velocity (mg reducing sugar formed/minute)							
		Soluble starch		Cassava starch		Plantain starch		Potato starch	
S	$\frac{1}{S}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$
0.2	5	0.07	14.28	0.05	20	0.04	23.2	0.033	30
0.4	2.5	0.117	8.55	0.07	14.28	0.067	14.9	0.055	18
0.6	1.66	0.129	7.75	0.1	10	0.095	10.5	0.072	13.3
0.8	1.25	0.187	5.31	0.122	8.2	0.1	10	0.095	10.8
1	1	0.187	5.3	0.123	8.1	0.1	10	0.122	8.19
1.2	0.83	0.187	5.3	0.123	8.1	0.1	10	0.122	8.19

Table 28b. Effect of substrate concentration on velocity of amylase from *Vibrio parahaemolyticus* (MB2)

Substrate concentration (mg/ml)		Initial velocity (mg reducing sugar formed/minute)							
		Soluble starch		Cassava starch		Plantain starch		Potato starch	
S	$\frac{1}{S}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$
0.2	5	0.102	9.8	0.1	10	0.038	26.3	0.05	20
0.4	2.5	0.18	5.5	0.154	6.49	0.061	16.39	0.09	11
0.6	1.66	0.22	4.54	0.196	5.10	0.13	7.69	0.13	7.6
0.8	1.25	0.26	3.84	0.205	4.87	0.144	6.9	0.169	5.9
1	1	0.26	3.84	0.218	4.58	0.144	6.9	0.168	5.9
1.2	0.83	0.26	3.84	0.219	4.58	0.144	6.9	0.168	5.9

Table 28c. Effect of substrate concentration on velocity of amylase from *Vibrio* sp. (MB3)

Substrate concentration (mg/ml)		Initial velocity (mg reducing sugar formed/minute)							
		Soluble starch		Cassava starch		Plantain starch		Potato starch	
S	$\frac{1}{S}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$
0.2	5	0.068	14.70	0.092	10.86	0.05	18.89	0.1	10
0.4	2.5	0.09	10.5	0.1	10	0.08	12.5	0.145	6.9
0.6	1.66	0.1	10	0.12	8.3	0.09	11.1	0.169	5.91
0.8	1.25	0.12	8.55	0.14	7.14	0.097	10.3	0.186	5.37
1	1	0.14	7.14	0.14	7.14	0.1	10	0.211	4.73
1.2	0.83	0.14	7.14	0.14	7.14	0.1	10	0.211	4.73

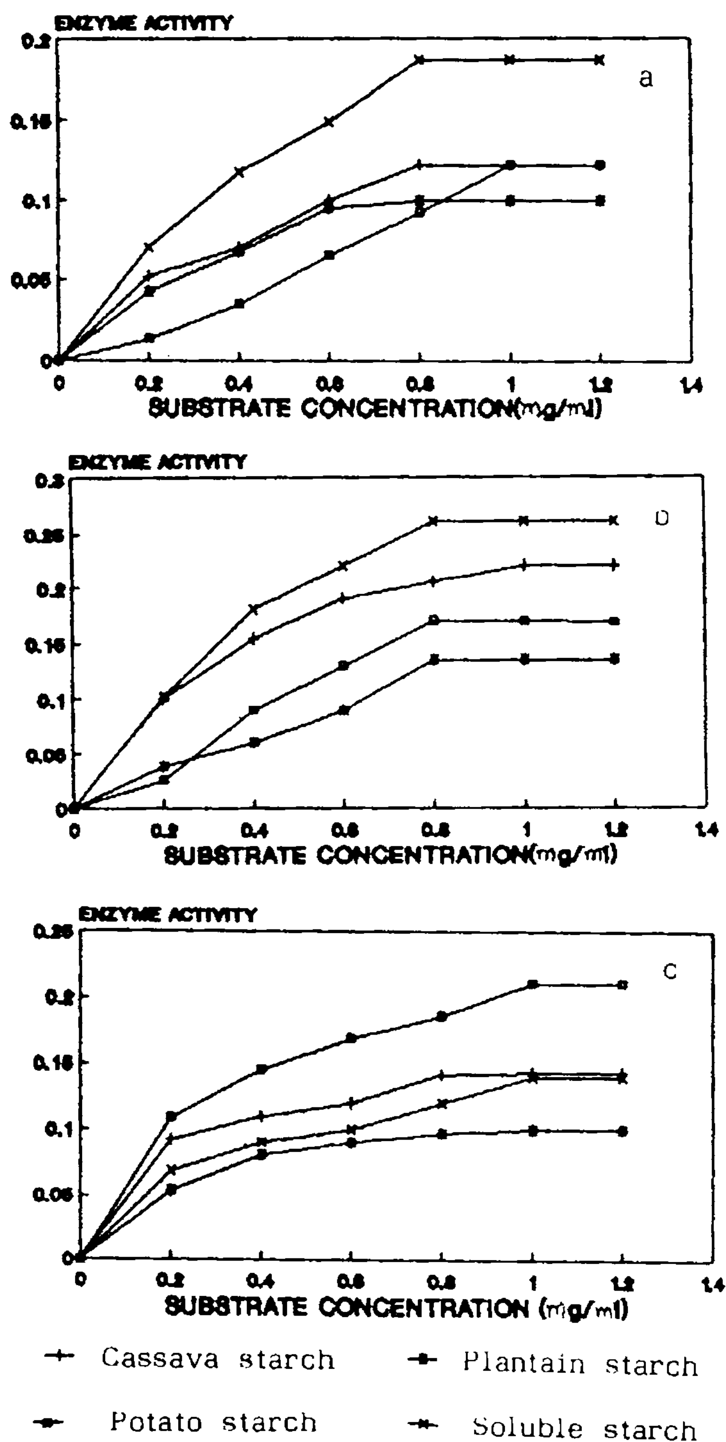


Fig. 25 a-c. Effect of substrate concentration on activity of amylase produced by *Vibrio* species. (A) *V. alginolyticus* (MB1) amylase (B) *V. parahaemolyticus* (MB2) amylase (C) *Vibrio* sp. (MB3) amylase.

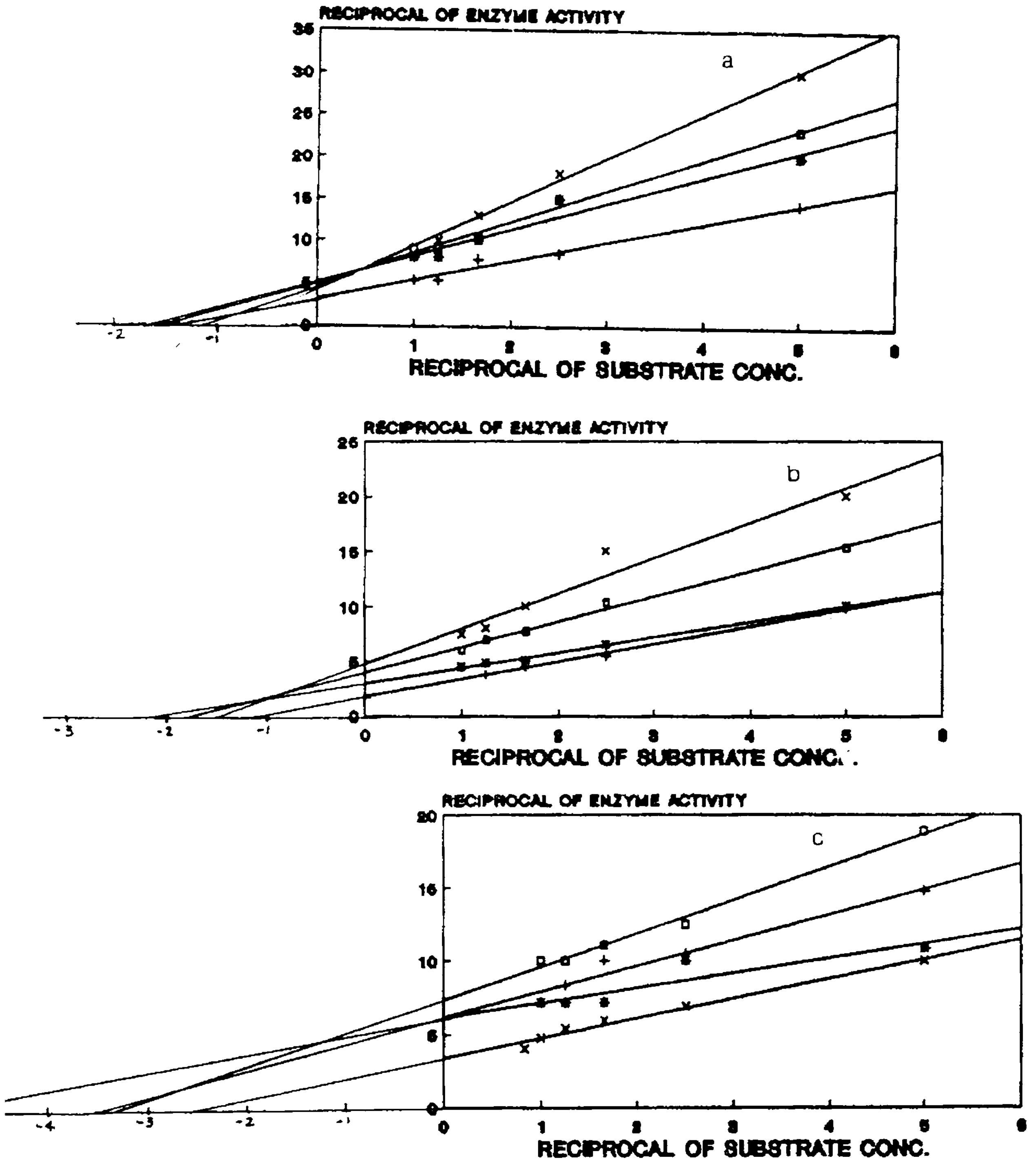
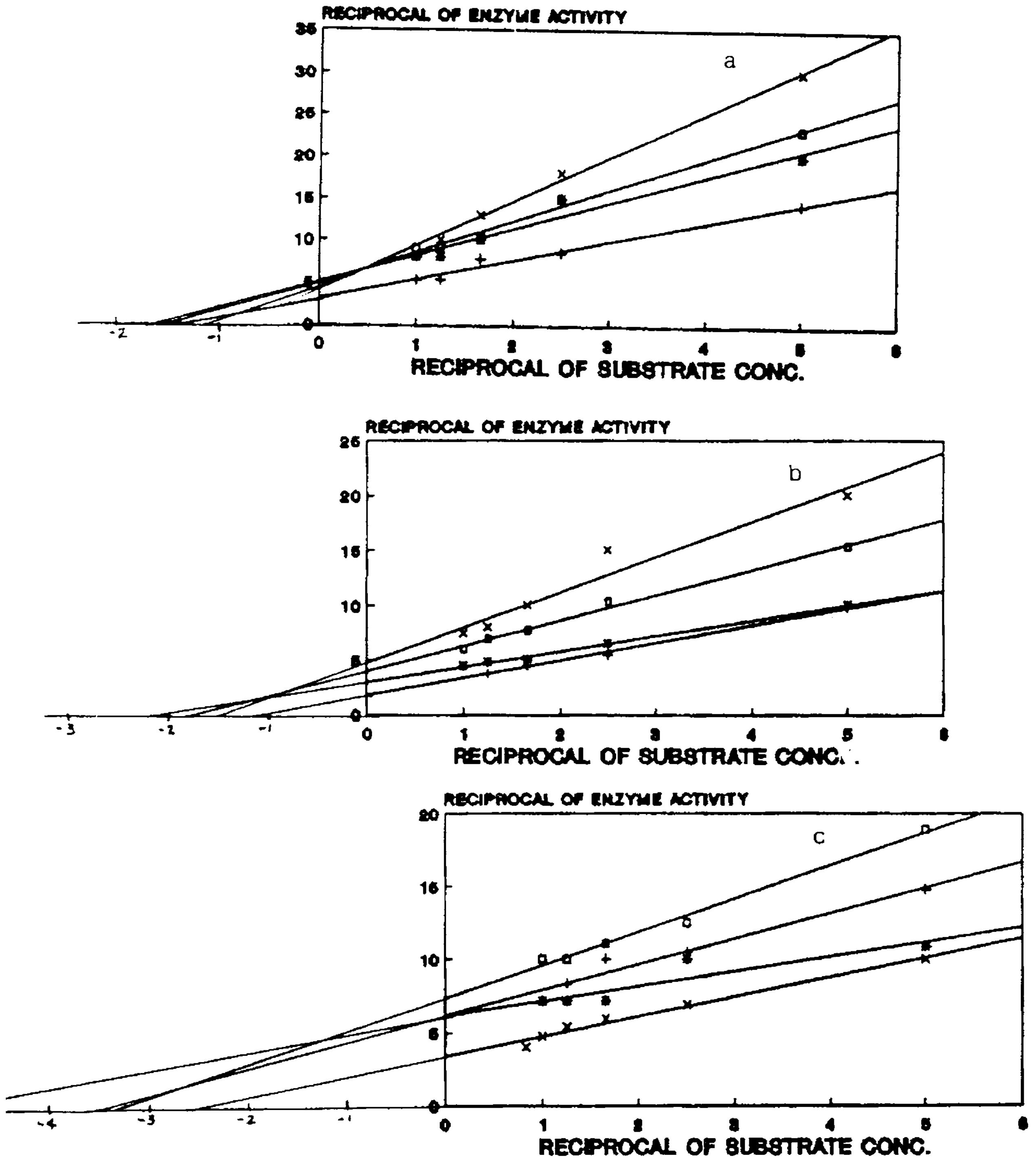


Fig. 26 a-c. Line Weaver-Burk Plot. (a) *V. alginolyticus* (MB1) amylase (b) *V. parahaemolyticus* (MB2) amylase (c) *Vibrio* sp. (MB3) amylase.



Soluble starch — Cassava starch

Plantain starch — Potato starch

Fig. 26 a-c. Line Weaver-Burk Plot. (a) V. alginolyticus (MB1) amylase (b) V. parahaemolyticus (MB2) amylase (c) Vibrio sp. (MB3) amylase.

various substrates were: soluble starch - 0.9 mg/ml, cassava starch - 0.48 mg/ml, plantain starch - 0.57 mg/ml and potato starch - 0.68 mg/ml. The V_{max} values were: soluble starch - 0.55 mg/min/ml, cassava starch - 0.34 mg/min/ml, plantain starch - 0.22 mg/min/ml and potato starch - 0.2 mg/min/ml.

Amylase from Vibrio sp.(MB3) showed maximum activity when the starch concentration in the medium was 1 mg/ml. The K_m values for the various starches were: soluble starch - 0.288 mg/ml, cassava starch - 0.22 mg/ml, plantain starch - 0.35 mg/ml and potato starch - 0.33 mg/ml. The V_{max} values were: soluble starch - 0.162 mg/min/ml, cassava starch - 0.198 mg/min/ml, platain starch - 0.147 mg/min/ml and potato starch - 0.283 mg/min/ml.

5.2.6. EFFECT OF VARIOUS IONS ON AMYLALSE ACTIVITY

5.2.6.1. Effect of sodium (Na^+) and chloride (Cl^-) ions

Effect of sodium chloride on enzyme activity was determined by adding different concentrations of NaCl (from 5 mM to 40 mM) to the assay medium containing soluble starch and dialysed enzyme solution (dialysed against water). Results are given in Table 29 and Fig. 27. NaCl was found to have no significant effect on enzyme activity. In all the cases, the enzyme activity remained, more or less, the same at different concentration of NaCl.

Table 29. Effect of various ions on amylase action

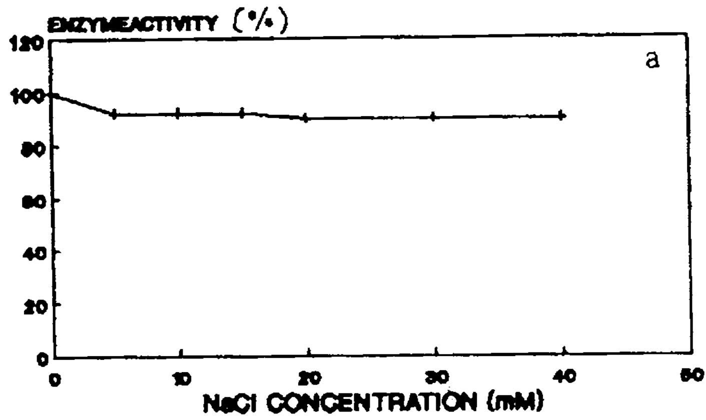
Name of the salt	Concentration (mM)	Residual activity (%) of amylasse from:		
		V. alginolyticus(MB1)	*V. parahaemolyticus*(MB2)	*Vibrio* sp.(MB3)
NaCl	0	100	100	100
	5	92	95	98
	10	92	95	95
	15	92	95	95
	20	90	94	95
	30	90	94	93
	40	90	94	91
$CaCl_2$	0	100	100	100
	5	85	96	90
	10	78	92	90
	15	70	89	85
$CoCl_2$	0	100	100	100
	5	70	64	49
	10	65	60	41
	20	51	57	35

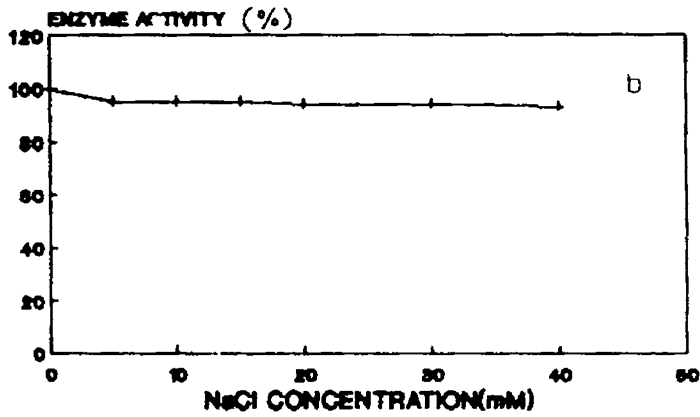
$MnCl_2$	0	100	100	100
	5	77	72	75
	10	74	72	66
	20	67	64	62
$MgCl_2$	0	100	100	100
	5	102.6	105	67.3
	10	107.5	92	60.6
	20	91.2	70	56.3
KCl	0	100	100	100
	5	102	130	100
	10	105	132	100
	20	106	135	101
$FeCl_3$	0	100	100	100
	5	17	0	30
	10	13	0	25
	20	10	0	23

(Contd...)

$HgCl_2$	0	100	100	100
	0.5	20	15	29
	1	0	0	0
	5	0	0	0
$CuCl_2$	0	100	100	100
	0.5	66	58	60
	1	42	30	35
	5	0	0	0
$ZnCl_2$	0	100	100	100
	0.5	80	70	84
	1	45	32	52
	5	0	0	0
Na_2SO_4	0	100	100	100
	5	106	120	83
	10	104	110	74
	20	101	100	68

Na_2CO_3	0	100	100	100
	5	75.6	83	56
	10	20.3	0	29
	20	18.7	0	8
$NaNO_3$	0	100	100	100
	5	99	130	83.7
	10	100	120	83
	15	101	110	83
NaI	0	100	100	100
	5	103	103	90.6
	10	100	103	90.4
	20	100	100	84.9





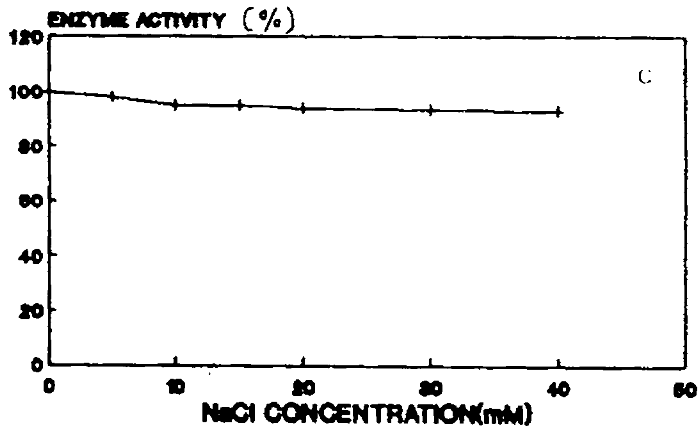


Fig. 27 a-c. Effect of NaCl on activity of amylase produced by *Vibrio* species. (a) *V. alginolyticus* (MB1) amylase (b) *V. parahaemolyticus* (MB2) amylase (c) *Vibrio* sp. (MB3) amylase.

5.2.6.2. Effect of calcium (Ca^{++})

Calcium chloride was also found to have no significant effect on amylase activity. Amylase produced by V. alginolyticus (MB1) showed a decrease in activity at higher concentrations of calcium chloride (Table 29 and Fig. 28).

5.2.6.3. Effect of cobalt (Co^{++})

Cobalt chloride was found to reduce amylase activity. There was 50 to 65% reduction in activities of the amylases in the presence of cobalt chloride at 20 mM level (Table 29 and Fig. 29).

5.2.6.4. Effect of manganese (Mn^{++})

Manganese chloride was found to reduce amylase activity. There was 30 to 40% reduction in activity in the presence of manganese chloride at 20 mM level (Table 29 and Fig. 30).

5.2.6.5. Effect of magnesium (Mg^{++})

The effect of magnesium chloride showed marked variation among species (Table 29 and Fig 31). Amylase from V. alginolyticus (MB1) showed 7.5% increase in activity at 10 mM magnesium chloride level, but further increase in concentrations resulted in decrease in enzyme activity. V. parahaemolyticus (MB2) amylase

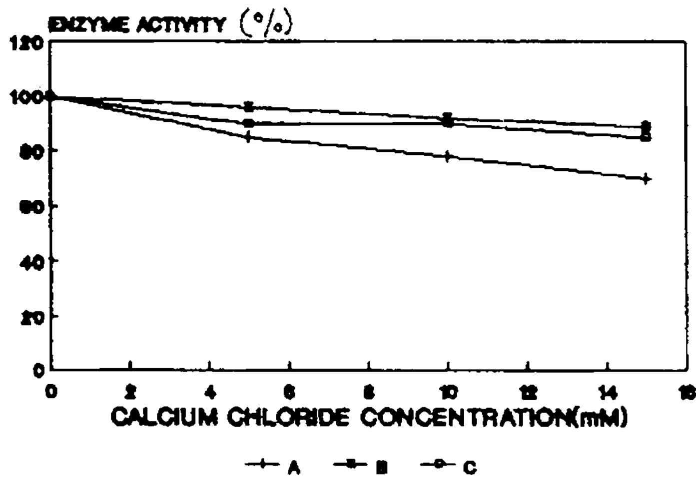


Fig. 28. Effect of calcium chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

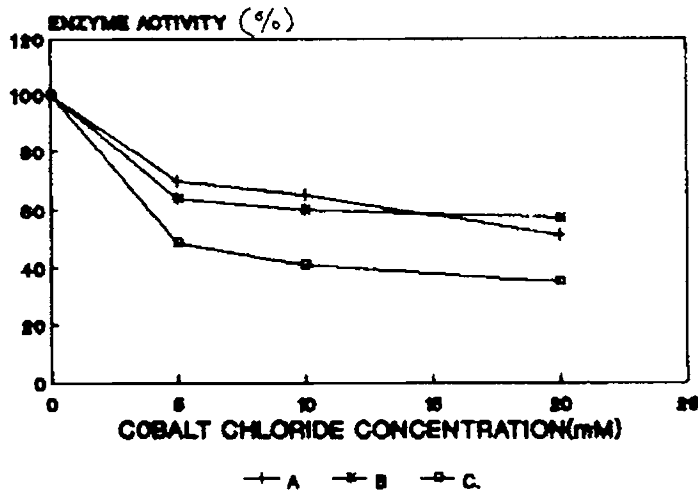


Fig. 29. Effect of cobalt chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

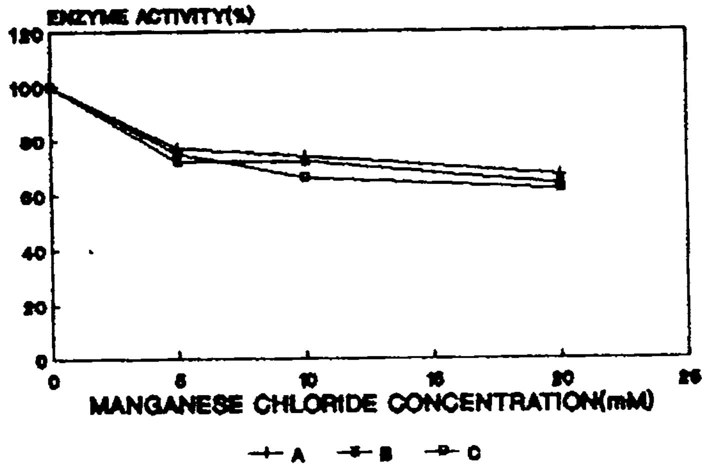


Fig. 30. Effect of manganese chloride on activity of amylase from Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

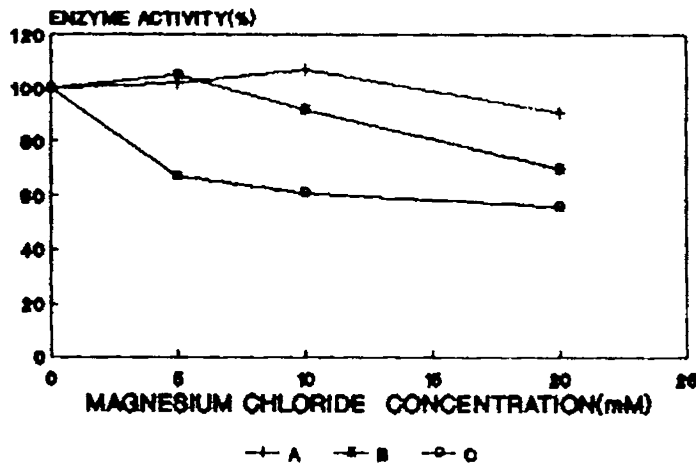


Fig. 31. Effect of magnesium chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

also showed an increase in activity at 5 mM level, and there was 30% decrease in activity at 20 mM magnesium chloride level. Amylase produced by Vibrio sp.(MB3) did not show any increase in the activity in presence of magnesium chloride. There was 35% reduction in activity at 5 mM level, and 45% reduction in activity at 20 mM magnesium chloride.

5.2.6.6. Effect of potassium (K^{+})

Potassium chloride was found to enhance enzyme action to some extent. The effect shown by amylases of different species showed variation (Table 29 and Fig. 32). The activity of amylase from V. alginolyticus (MB1) was enhanced by 2 to 6% by potassium whereas, amylase from V. parahaemolyticus (MB2) showed 30 to 35% increase in activity due to potassium. The activity of amylalse from Vibrio sp.(MB3) remained unaffected in presence of potassium ions.

5.2.6.7. Effect of ferric ion (Fe^{3+})

Ferric chloride was found to be inhibitory to the activity of amylases produced by these strains (Table 29 and Fig. 33). At 5 mM concentration amylase from V. parahaemolyticus (MB2) lost its activity completely whereas, that of V. alginolyticus (MB1) retained about 18% activity and amylase from Vibrio sp.(MB3)

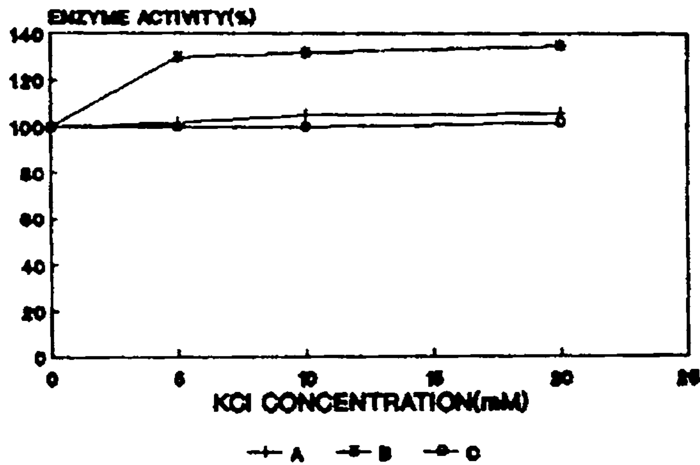


Fig. 32. Effect of potassium chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

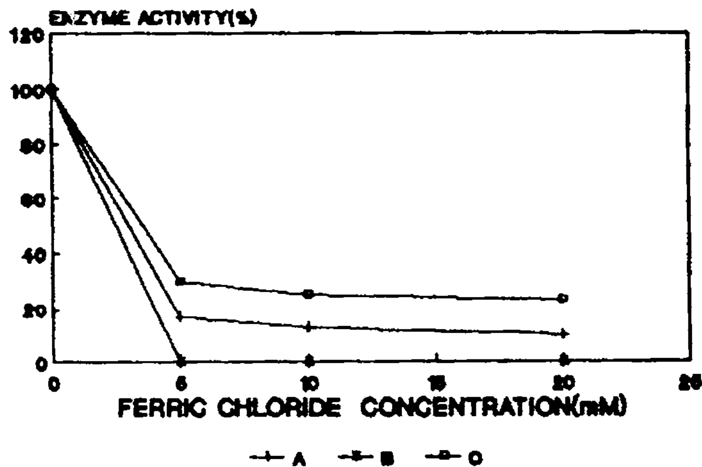


Fig. 33. Effect of ferric chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

retained 30% activity. Further increase in ferric chloride concentration did not decrease the enzyme activity considerably. At 20 mM concentration, Vibrio sp.(MB3) amylase retained 23% of its activity whereas, amylase from V. alginolyticus (MB1) retained only 10% of its activity.

5.2.6.8. Effect of mercuric ion (Hg^{++})

Amylase activities were completely inhibited in presence of 1 mM mercuric chloride (Table 29 and Fig. 34). At 0.5 mM level, amylase from V. alginolyticus (MB1) showed 80% reduction in activity and that of V. parahaemolyticus (MB2) showed 85% decrease in activity and that of Vibrio sp.(MB3) showed 71% decrease in activity.

5.2.6.9. Effect of copper (Cu^{++})

Cupric chloride was also found to be inhibitory to amylase action (Table 29 and Fig. 35). Cupric chloride, at 0.5 mM concentration, reduced amylase activity by 35 to 45%. At 1 mM concentration, amylase from V. alginolyticus (MB1) showed 60% reduction in activity, that from V. parahaemolyticus (MB2) 70% reduction and that from Vibrio sp. (MB3) 65% reduction in activity. At 5 mM level, there was 100% loss in amylase activities.

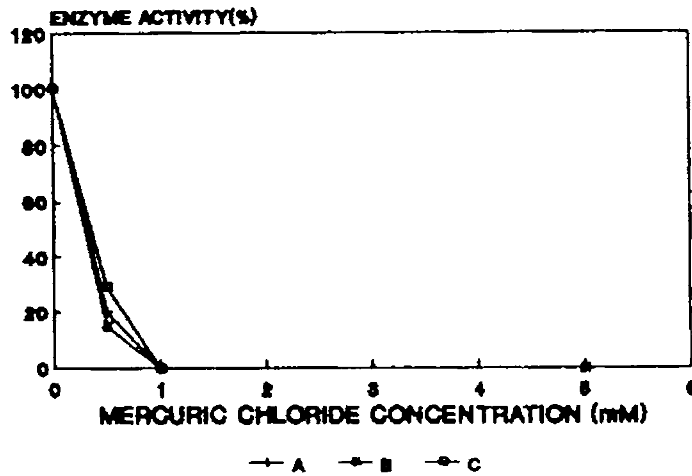


Fig. 34. Effect of mercuric chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

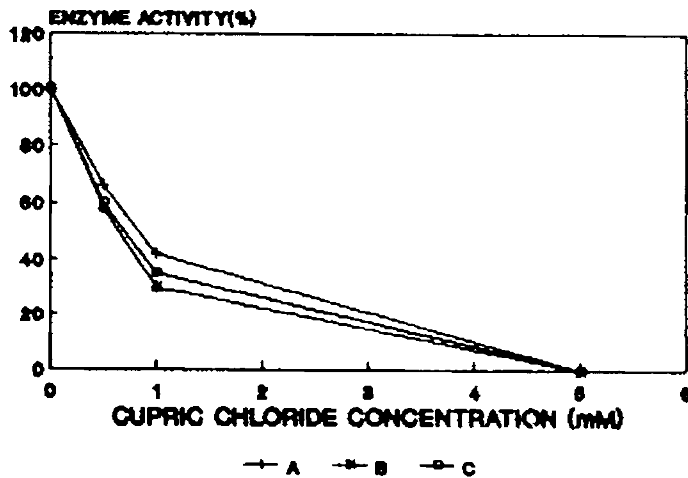


Fig. 35. Effect of cupric chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

5.2.6.10. Effect of Zinc (Zn^{++})

Amylase activities were completely inhibited in presence of 5 mM zinc chloride (Table 29 and Fig. 36). At 0.5 mM concentration, there was 20% loss of activity in the case of amylase from V. alginolyticus (MB1), 30% in the case of V. parahaemolyticus (MB2) and 18% in Vibrio sp.(MB3). At 1 mM level, V. alginolyticus (MB1) amylase showed 55% reduction in activity, that from V. parahaemolyticus (MB2) 65% and Vibrio sp.(MB3) 50% reduction in activity.

5.2.6.11. Effect of sulphate

Effect of sulphate on the amylases varied considerably (Table 29 and Fig. 37). Amylase from V. alginolyticus (MB1) was not affected much by the presence of sulphate. V. parahaemolyticus (MB2) amylase showed an increase in activity (about 20%) at 5 mM sodium sulphate concentration. Further increase in sulphate concentration reduced amylase action. Amylase from Vibrio sp.(MB3) showed a decrease in activity in presence of sulphate. There was about 20% reduction at 5 mM concentration, 25% at 10 mM and 30% at 20 mM concentration.

5.2.6.12. Effect of carbonate

Carbonate reduced amylase activity to a large extent (Table 29 and Fig. 38). Amylase from V. parahaemolyticus (MB2) was

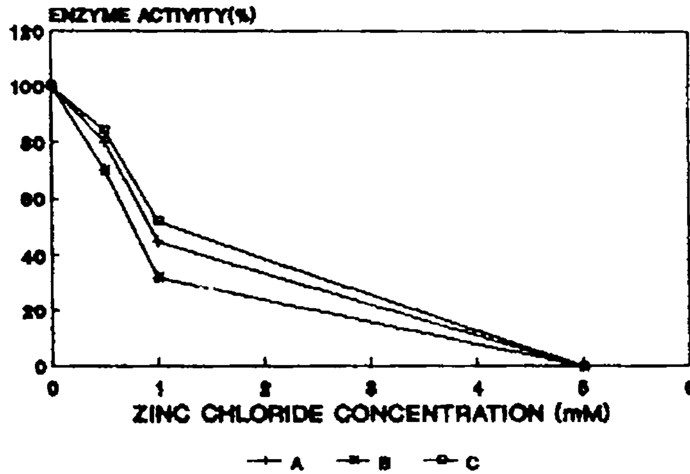


Fig. 36. Effect of zinc chloride on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

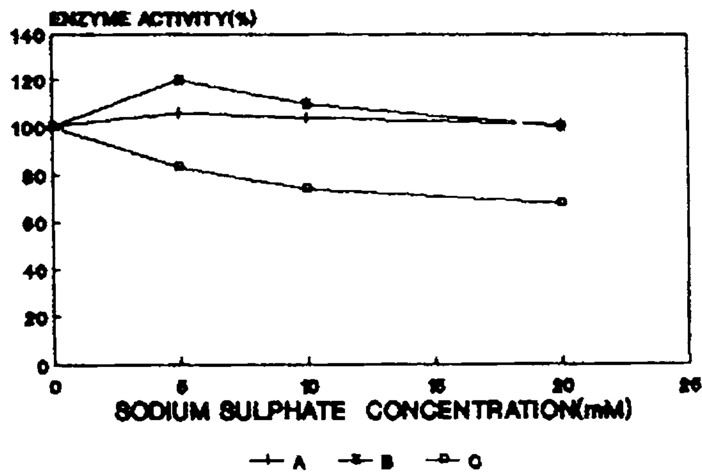


Fig. 37. Effect of sodium sulphate on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

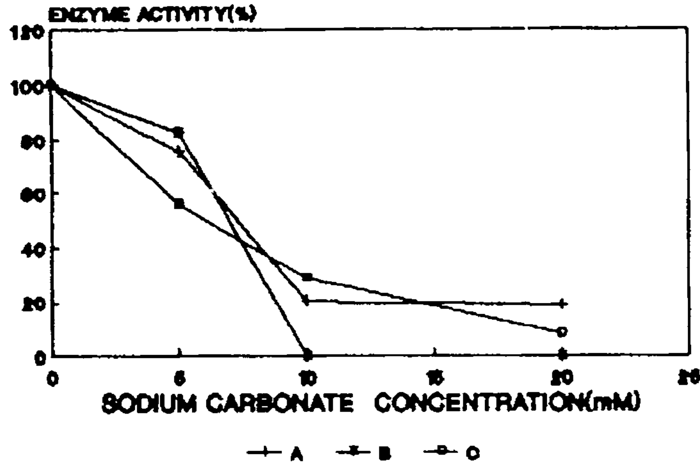


Fig. 38. Effect of sodium carbonate on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

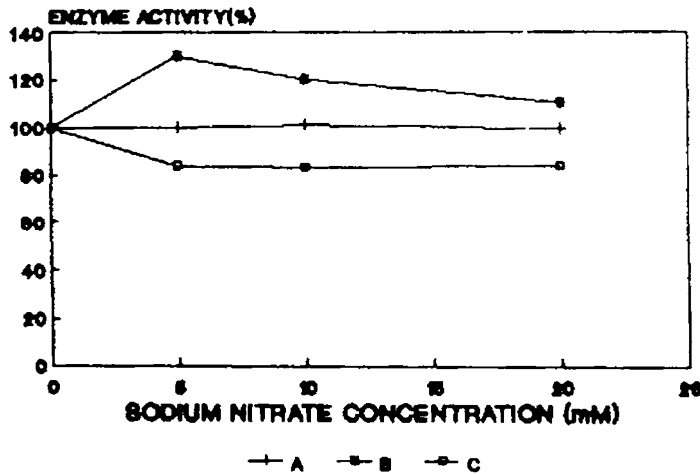


Fig. 39. Effect of sodium nitrate on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

completely inhibited at 10 mM concentration of sodium carbonate. Amylase from V. alginolyticus (MB1) showed 80% decrease in activity at 10 mM level. Vibrio sp.(MB3) showed 70% reduction at 10 mM concentration. There was 80 to 90% loss of activity of amylase from V. alginolyticus (MB1) and Vibrio sp.(MB3) at 20 mM level.

5.2.6.13. Effect of nitrate

Nitrate was found to have no significant effect on the activity of amylase from V. alginolyticus (MB1). At lower concentrations, it stimulated amylase activity of V. parahaemolyticus (MB2). Amylase from Vibrio sp. (MB3) showed slight reduction in activity in presence of nitrate (Table 29 and Fig. 39).

5.2.6.14. Effect of iodide

The activities of amylases from V. alginolyticus (MB1) and V. parahaemolyticus (MB2) remained unaffected in the presence of iodide whereas, amylase from Vibrio sp. (MB3) showed slight reduction in activity in presence of iodide (Table 29 and Fig. 40).

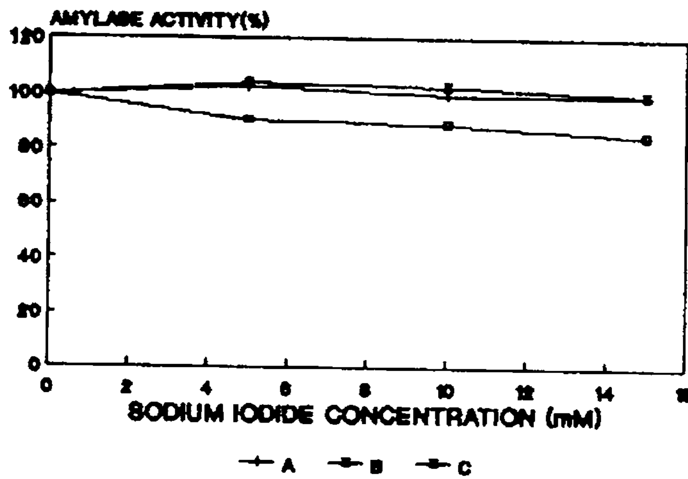


Fig. 40. Effect of sodium iodide on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

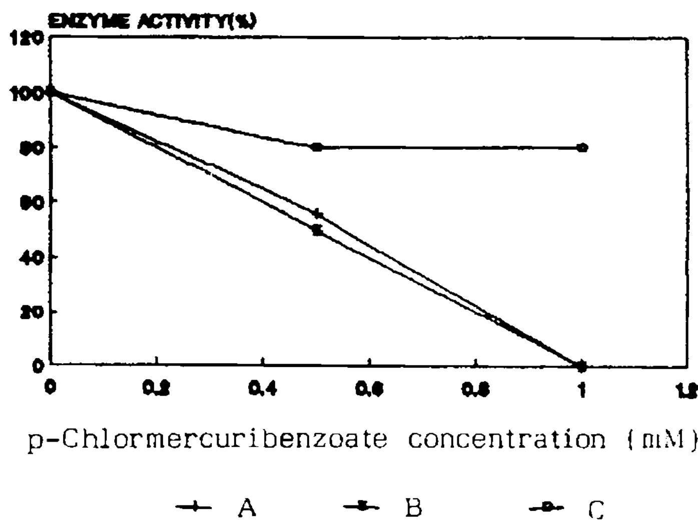


Fig. 41. Effect of p-chloromercuribenzoate on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

5.2.7. EFFECT OF ORGANIC COMPOUNDS ON AMYLASE ACTION

5.2.7.1. Effect of p-hydroxymercuribenzoate on amylase action

Amylases from V. parahaemolyticus (MB2) and V. alginolyticus (MB1) were completely inhibited at 1 mM concentration of p-hydroxymercuribenzoate whereas, amylase from Vibrio sp.(MB3) showed only very little loss of activity (20%) at this concentration (Table 30 and Fig. 41).

5.2.7.2. Effect of ethylene diamine tetra-acetic acid (EDTA) on amylase action

At high concentrations, (10 mM) EDTA inhibited amylase activities completely (Table 30 and Fig. 42). At 5 mM level, there was about 65% to 85% reduction in amylase activity.

5.2.7.3. Effect of reduced glutathione, β-mercaptoethanol and thiourea on amylase action

Results are given in Table 30 and Figs. 43,44 and 45. Amylases from all the three strains retained almost full activity in presence of all these compounds.

Table 30. Effect of organic compounds on amylase action

Name of the compound	Concentration (mM)	Residual activity (%) of amylase from:		
		V. alginolyticus (MB1)	*V. parahaemolyticus* (MB2)	*Vibrio* sp. (MB3)
p-Chloromercuribenzoate	0	100	100	100
	0.5	56	50	80
	1	0	0	80
	5	0	0	79
EDTA	0	100	100	100
	0.5	50	50	87.4
	1	40	45	66
	5	22	40	52
	10	0	0	0
Reduced glutathione	0	100	100	100
	0.5	98	96	102
	1	99	94	104
	5	90	94	108

(Contd...)

β-Mercaptoethonol	0	100	100	100
	0.5	102	101	96
	1	100	99	97
	5	98	99	96
Thiourea	0	100	100	100
	0.5	99	119	104
	1	96	100	102
	5	100	100	98

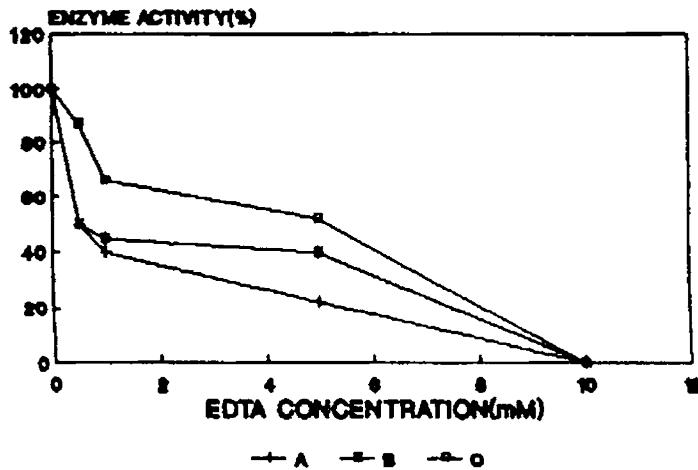


Fig. 42. Effect of EDTA on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

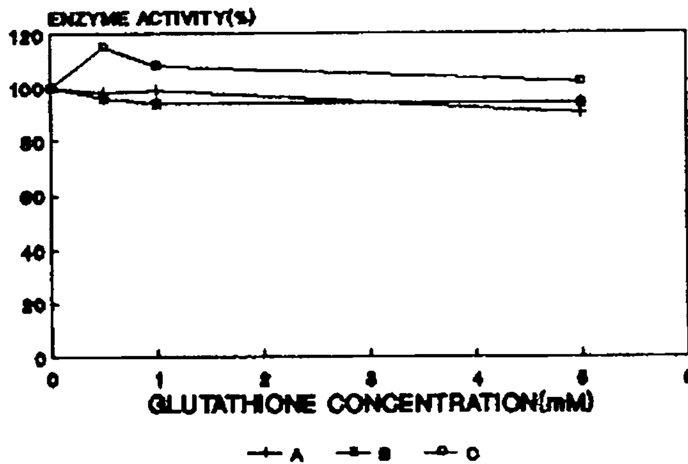


Fig. 43. Effect of glutathione on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

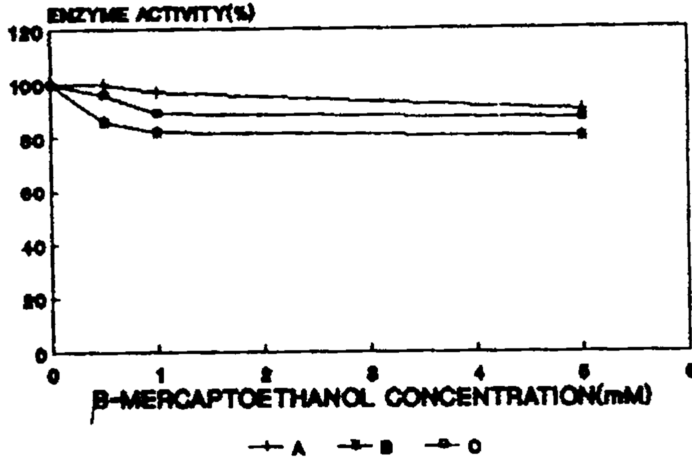


Fig. 44. Effect of β-mercaptoethanol on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. parahaemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

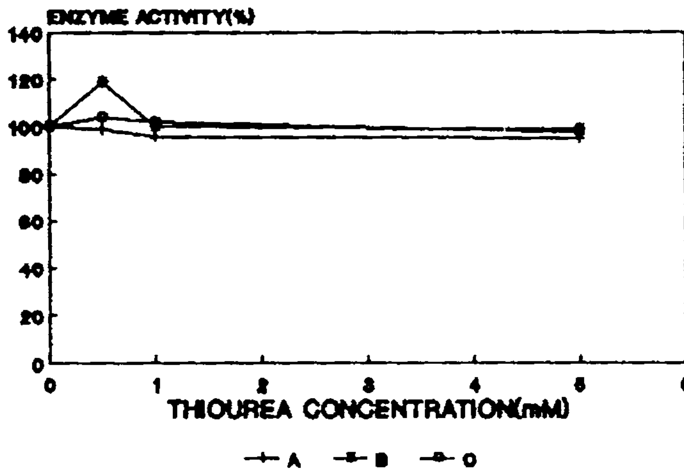


Fig. 45. Effect of thiourea on activity of amylase produced by Vibrio species. (A) V. alginolyticus (MB1) amylase (B) V. paraemolyticus (MB2) amylase (C) Vibrio sp. (MB3) amylase.

5.3. DISCUSSION

5.3.1. PARTIAL PURIFICATION OF AMYLASES

Partial purification of amylases from the Vibrio strains resulted in 57% to 68% yield. Their specific activity varied from 63.3. units/mg protein to 96 units/mg protein. The results obtained are comparable to other amylases. Medda and Chandra [189] partially purified amylase from Bacillus licheniformis CUMC 305 and Bacillus coagulans CUMC 512 by acetone fractionation followed by dialysis, and recorded 52.12% yield for B. licheniformis with specific activity 204 units/mg protein and 49.3% yield and specific activity 151.2 units/mg protein for Bacillus coagulans. Morgan and Priest [99] obtained 66% yield of amylase from B. licheniformis after partial purification. Nandakumar[36] recorded 41.00 to 71.14% yield and specific activity 41.24 to 62.5 units/mg protein for various Bacillus strains.

5.3.2. IDENTITY OF THE ENZYMES

The mode of action of amylases from the three strains shows that they are ∝-amylases. Preliminary studies showed that the enzymes gave a clear zone of hydrolysis on starch-agar plates following the iodine test. This phenomenon is accepted as suggestive of ∝-amylase[6]. The relationship between the release

of reducing power and iodine-staining value as compared with ideal pattern of exo and endo-splitting enzymes further confirms the conclusion. Starch hydrolysis by exo-attacking enzymes is accompanied by a large release of reducing power, with relatively little effect on the iodine-staining capacity of the substrate. Conversely, endo-attacking enzymes rapidly reduce the iodine-staining capacity but, release little reducing power[99].

The amylases produced by halophilic bacteria are reported to be mainly of the ∝-type. Baumann and Baumann[161] have reported ∝-amylase production by Beneckea species.The extracellular amylase produced by Alteromonas rubra was of ∝-type[45]. Similarly, Vibrio gazogenes also produced ∝-amylase[39].

5.3.3. EFFECT OF pH ON AMYLASE ACTIVITY AND STABILITY

Enzyme activities are markedly influenced by the hydrogen ion concentration of the solution. Some enzymes act best in acid solution; others require alkaline solution and still others need a neutral environment. For every enzyme, there exists a maximum, an optimum and a minimum pH[226]. An enzyme can tolerate slight changes in pH without being destroyed. If the solution is made strongly acidic or alkaline, the enzyme undergoes an irreversible loss of activity[226].

Amylases produced by several bacteria have pH optima in

the range 6 to 8. They include amylases from Bacillus polymyxa[106] Bacillus stearothermophilus[103], Bacillus cereus[110], Bacillus licheniformis MB 80[99,100], Bacillus subtilis[96], Bacillus megaterium[17] Acinetobacter sp.[42], Micrococcus halobius[40], Vibrio gazogenes[39] Alteromonas rubra[45], Streptomyces thermoviolaceus sub sp. apingens[117], Lysobacter brunescens[119], Thermomonospora vulgaris[122] and Thermoactinomyces sp. No.15[121].

In the present study also, the optimal pH for the activity of the amylases was found to be pH 7. The optimum pH activity profile is in consonance with the characteristic single pH peak shown by most amylases[227]. However, the pH optima shown by some other bacterial strains show variation from this pH range. The amylase produced by Bacillus acidocaldarius showed pH optimum as 3.5[112]. The optimum pH for the activity of the amylase produced by Bacillus licheniformis CUMC 305 was pH 9[98]. Amylase from Bacillus subtilis showed pH optima in the range 4.5 to 7.2[124]. The pH optima for amylases from different Clostridium strains were: C. butyricum pH 5[19,140] and C. thermohydrosulfuricum pH 5.6[70].

α-Amylases are usually stable in the pH range 5.5 to 8.0[240]. Amylases produced by different bacterial strains show considerable variation in pH stability also. Bacillus cereus amylase showed stability in the pH range 5.5 to 12[110]. Bacillus sp. No.P. 206

amylase was stable in the pH range 6 to 10[175]. Bacillus licheniformis NCIB 6346 amylase was stable in the pH range 7 to 9[99].

In the present study, the amylases showed 100% stability in the pH range 6 to 8. Vibrio sp.(MB3) amylase was highly stable in the alkaline range. It showed 100% stablity at pH 9 and 70% stability at pH 10. In general, acidic pH was found to be more harmful than alkaline pH to bacterial amylases. However, Mutsuo[112] reported that amylase from Bacillus acidocaldarius was stable at pH 2.

5.3.4. EFFECT OF TEMPERATURE ON AMYLASE ACTIVITY AND STABILITY

The velocity of an enzyme catalysed reaction is accelerated by an increase in temperature. This continues until a maximum is reached, after which, the velocity decreases, and finally results in the destruction of the enzyme. Each enzyme has its characteristic optimum, maximum and minimum temperatures and an enzyme displays its greatest activity at the optimum temperature[226].

The optimum temperature required for activity and stability of amylase activity of different bacteria differs widely. 37°C was found to be optimal for the amylase activities of Clostridium

butyricum[140] and Bacillus polymyxa[106]. Bacillus subtilis amylase showed maximum activity at 45°C[124]. Amylases produced by Bacillus cereus[110], Bacillus subtilis[96], Lactobacillus cellobiosus[120], Acinetobacter sp.[42], Micrococcus halobius[40], Clostridium butyricum[19] and Streptomyces thermoviolaceus[117] showed their optimum activity in the temperature range 50 to 55°C. A temperature of 50°C was recorded as optimum for the maximum activity and stability of amylases from B. coagulans ACMN 1 and B. polymyxa ACMN 25 and 40°C for B. cereus ACMN 33 and B. coagulans ACMN 42[36]. Higher temperature was required for maximal activity of amylases of some bacterial strains. B. amyloliquefaciens recorded optimum activity at 65°C[174]. Optimum temperature of 60 to 70°C was required for the maximal activities of amylases from Bacillus acidocaldarius[112], B. megaterium[17], Clostridium sp.[20] and Thermoactinomyces[121]. Amylases produced by Clostridium thermohydrosulfuricum[70] showed optimum temperature for activity in the range 85°C to 90°C. Bacillus licheniformis NCIB 6346 amylase was active and stable in the temperature range 70 to 90°C[99]. Amylases produced by Dictyoglomus thermophilum[47], Bacillus licheniformis MB 80[100] and Bacillus licheniformis CUMC 305[54] showed optimum activity at 90°C.

The amylases used in the present study were found to be not very thermostable, and considerable loss of enzyme activity was noticed when the temperature was raised above 50°C. V. alginolyticus (MB1) and V. parahaemolyticus (MB2) amylases

showed maximum activity and stability in the temperature range 35 to 40°C. Vibrio sp.(MB3) showed maximum activity and stability at 30 to 35°C. The temperature activity pattern suggests the mesophilic nature of these organisms.

5.3.5. ABILITY OF AMYLASES TO DEGRADE RAW-STARCHES AND EFFECT OF SUBSTRATE CONCENTRATION

The amylases produced by halophilic bacteria employed in the present study were found to be capable of degrading native starches like cassava starch, potato starch and plantain starch. This is suggestive of their role in the degradation of native starches present in the environment. Since they show the ability to degrade different types of starches, they may be employed for commercial starch saccharification.

Amylases produced by different bacterial species show variation in their K_m and V_{max} values. The K_m for amylase from Bacillus stearothermophilus was 1.05 mg soluble starch/ml [103], that from B. acidocaldarius was 1.6 mg/ml [112], that from Thermomonospora vulgaris was 1.4 mg/ml [122] and that from a new Clostridium isolate was 0.35 mg/ml [20]. The K_m for B. licheniformis amylase for different substrates were: soluble starch- 1.274 mg/ml, amylose - 1.818 mg/ml, amylopectin - 1.94 mg/ml and glycogen - 2.28 mg/ml [98]. Lysobacter brunescens showed a

K_m of 2.08 mg/ml for soluble starch[119]. In the present study also, amylases from the three strains showed considerable variation in their kinetic parameters. V. alginolyticus (MB1) showed highest K_m value for soluble starch and plantain starch and lowest value for potato starch. V. parahaemolyticus (MB2) showed highest K_m for soluble starch and lowest value for cassava starch. Vibrio sp.(MB3) showed highest K_m value for plantain starch and lowest value for cassava starch. Compared to others, Vibrio sp.(MB3) showed a lower K_m value for all the substrates. In the case of V_{max} also, there was considerable variation. B. licheniformis amylase showed V_{max} values for soluble starch, amylose, amylopectin and glycogen as 0.738 mg/min/ml, 1.08 mg/min/ml, 0.8 mg/min/ml and 0.5 mg/min/ml respectively[98]. In the present study, V. alginolyticus (MB1) and V. parahaemolyticus (MB2) showed highest V_{max} values for soluble starch and the lowest value for potato starch. Vibrio sp.(MB3) showed highest V_{max} value for cassava starch and lowest value for plantain starch.

5.3.6. EFFECT OF DIALYSIS ON AMYLASE ACTIVITY

Amylases produced by all the three strains retained 100% activity after dialysis. This observation does not agree with earlier information obtained from amylases produced by other halophilic bacteria. Amylase activities of Acinetobacter sp.[42],

Micrococcus halobius[40] and Vibrio gazogenes[39] were lost completely after dialysis. In the case of Alteromonas rubra, there was 98% loss of activity due to dialysis[45].

5.3.7. EFFECT OF VARIOUS IONS ON AMYLASE ACTIVITY

5.3.7.1. Effect of sodium^{+} and chloride^{-} ions on amylase activity

Previous studies have shown that sodium chloride is required for the activity of the amylases from marine sources[39,45]. Amylase from Acinetobacter sp. required 0.2 to 0.6 M NaCl for maximum activity[42] and that of Micrococcus halobius required 0.25 M NaCl for optimal activity[40]. In contrast, the amylases produced by the bacteria of the present study were found to be active even in the absence of NaCl. Though their activity was unaffected by the presence of NaCl, they did not show any specific requirement for NaCl.

5.3.7.2. Effect of calcium on amylase action

α-Amylases have been classified as metallo-enzymes having calcium as a cofactor[228]. They bind one mole of calcium per mole of enzyme[229]. In the presence of calcium ions, amylases are quite resistant to extremes of pH, temperature, treatment with urea or exposure to some proteases[230,231]. Once calcium containing amylases have been denatured, they are susceptible to

protein degradation[232,233]. Ca^{2+} was found to stabilize the activities of the amylases of Clostridium thermohydrosulfuricum[70] and Bacillus No P 206[175]. Calcium was found to reactivate the amylases of Vibrio gazogenes[39] and Alteromonas rubra[45]. Mosely and Keay[234] reported that α-amylase from Bacillus subtilis NRRC 3411 showed higher activity and stability in presence of calcium ions. Hsiu et al.[235] found that to reactivate B. subtilis α-amylase, whose activity was lost by chelation with EDTA or by electro dialysis, four gram atoms of calcium per mole of enzyme were required.

In the present study also, when EDTA, the metal chelating agent, was added to the reaction-mixture at 10 mM level, there was complete inhibition of enzyme activities. The lost activity could not be recovered by further addition of $CaCl_2$. Similar result was also shown by the amylase produced by Micrococcus halobius[40]. Since these calcium ions are strongly bound to the enzyme molecules, dialysis against distilled water could not remove them and they retained full activity even after dialysis. Addition of calcium to the reaction-mixture did not show significant effects on enzyme action.

5.3.7.3. Effect of other ions on enzyme activity

The activity of amylases is influenced by the presence of ions in the systems. The effects of different types of ions vary

depending upon the source of amylase. Srivastava[103] found that Fe^{3+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Ni^{2+} and Ag^{2+} were potent inhibitors of amylases produced by Bacillus stearothermophilus whereas, Zn^{2+} Mg^{2+} and Al^{3+} were mild inhibitors. Ca^{2+}, Ba^{2+}, Sr^{2+} and K^+ stimulated amylase in the order: $Ca^{2+} > Ba^{2+} > Sr^{2+} > K^+$. Amylase produced by Bacillus subtilis was strongly inhibited by Cu^{2+}, Zn^{2+}, Ni^{2+} and Fe^{2+} [97]. Obi and Odibo[121] reported activation of amylase produced by Thermoactinomyces sp. by Mn^{2+}, Ag^{2+}, and Fe^{2+} whereas, Hg^{2+} and Zn^{2+} were inhibitory. Cations such as, Na^+, Co^{2+} and Mg^{2+} showed stimulatory effect on amylase produced by Bacillus licheniformis whereas, Hg^{2+}, Cu^{2+}, Ni^{2+}, Ag^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Al^{3+} and Mn^{2+} were inhibitory[98]. Metal ions such as Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} were not required for the activity of α-amylase from Lysobacter[119]. Takaya et al[59] observed that Hg^{2+} and Zn^{2+} completely inhibited the activity of amylase from Bacillus sp. H. 16 while Ca^{2+} and Pb^{2+} reduced the activity to 40 to 60%. Sensirbir and Chakraborthy[120] reported the inhibitory effect of Fe^{2+} and Zn^{2+} on immobilized α-amylase from Lactobacillus cellobiosus. Hg^{2+}, Ag^{2+} and Fe^{2+} were found to be inhibitory to the amylase produced by various Bacillus strains[36].

In the present study also, several metal ions were found to be inhibitory to amylase action. Different metal ions showed inhibitory effect to varying degrees. Amylase from Vibrio

alginolyticus (ME1) was inhibited by metal ions in the order: Hg^{2+} > Cu^{2+} > Zn^{2+} > Fe^{3+} > Co^{2+} > Mn^{2+} > Mg^{2+}. Na^{+}, Ca^{2+} and K^{+} were not inhibitory to its action and K^{+} showed some stimulatory effect. In the case of V. parahaemolyticus (MB2) amylase, the inhibitory action was in the order: Hg^{2+} > Cu^{2+} > Zn^{2+} > Fe^{3+} > Co^{2+} > Mn^{2+} > Mg^{2+} and in Vibrio sp.(MB3) the order was: Hg^{2+} > Cu^{2+} > Zn^{2+} > Fe^{3+} > Co^{2+} > Mg^{2+} > Mn^{2+}. K^{+} was found to have some stimulatory effect on V. parahaemolyticus (MB2) amylase also.

Of the anions tested, carbonate was also found to be inhibitory to all the amylases. In the case of Vibrio sp.(MB3) amylase, sulphate, iodide and nitrate also showed some inhibitory effects. Sulphate and nitrate, at lower concentrations, showed stimulatory effect on amylase from V. parahaemolyticus (MB2). Other anions tested did not affect amylase activity significantly.

5.3.8. EFFECT OF ORGANIC COMPOUNDS ON AMYLASE ACTION

Effect of p-chloromercuribenzoic acid, thiourea, β-mercaptoethanol and reduced glutathione on amylase activities were studied to find out whether sulfhydryl group is present in the active site of the amylases. p-Chloromercuribenzoic acid inhibits enzyme activity by combining with the sulfhydryl groups of the enzyme and thiol compounds usually give protection to enzyme activity

In the present study, amylases produced by V. parahaemolyticus (MB2) and V. alginolyticus (MB1) were completely inhibited by 1 mM concentration of p-chloromercuribenzoate whereas, the activity of the amylase from Vibrio sp.(MB3) did not show much reduction in presence of p-chloromercuribenzoate. It may be inferred that the amylase from V. parahaemolyticus (MB2) and V. alginolyticus (MB1) require a functional-SH group for their activity whereas, Vibrio sp.(MB3) amylase does not require-SH group. p-Chloromercuribenzoate was found to be inhibitory to Taka-amylase[236], Bacillus stearothermophilus amylase[103] and Bacillus licheniformis CUMC 305[98]. Bacillus subtilis α-amylase has neither sulfhydryl nor disulphide group[237,238]. Thiol compounds afforded protection to amylase activities of all the three species. There was no reduction in activity in presence of any of these compounds tested. This suggests that the-SH group of the enzyme protein is responsible for the normal catalytic activities of amylases from V. alginolyticus (MB1) and V. parahaemolyticus (MB2). Similar observations were mady by Krishnan and Chandra[54] in the case of α-amylase from Bacillus licheniformis CUMC 305.

Chapter 6

6. SUMMARY

This thesis presents a detailed account of the distribution of amylolytic bacteria in water, sediment, fishes (*Etroplus suratensis* and *Liza parsia*), prawns (*Penaeus indicus* and *Metapenaeus dobsoni*) and clams (*Sunetta scripta* and *Meretrix casta*) from Cochin backwaters, genera-wise distribution of amylolytic bacteria, ability of selected strains to grow and produce amylase at various physico-chemical conditions, regulation of amylase synthesis and characters of amylases produced by these halophilic bacteria.

The important findings are:

1. Amylolytic bacteria are distributed widely in water, sediment, fishes, prawns and clams of Cochin backwaters. 53% of the total isolates tested were capable of producing amylase. Maximum number of amylolytic bacteria were present in *Metapenaeus dobsoni*.

2. In general, the gut region of aquatic animals harboured more amylolytic bacteria than the gill or surface. These bacteria may help in the digestion of starch present in their food.

3. The percentage of amylolytic population was maximum in the genus *Vibrio*. Most potent strains selected for detailed

studies also belonged to the genus *Vibrio*. They are identified as *Vibrio alginolyticus* (MB1), *Vibrio parahaemolyticus* (MB2) and *Vibrio* sp.(MB3).

4. The selected strains showed maximum growth and enzyme production at pH 7. Low pH (below 5) and high pH (above 9) were found to be unfavourable for growth and amylase production. Acidic pH was more harmful than alkaline pH.

5. Presence of ions in the medium was found to be essential for growth and amylase production. It was found that this ionic requirement is not highly specific. Sodium chloride could be replaced by potassium chloride, or magnesium chloride to some extent, without affecting growth and amylase production. The important function of these ions may be to maintain the osmotic balance between the cells and their environment.

6. The optimum temperature for growth of these strains was found to be 35°C. Except *V. alginolyticus* (MB1), others produced amylase also maximally at 35°C. *V. alginolyticus* (MB1) preferred 30°C for maximal amylase production. This suggests that though they are aquatic forms, they are mesophiles.

7. The starch concentration required for optimum growth and enzyme production showed great variation among different strains. In general, higher starch concentration was required for maximal growth and lower concentration for amylase production. Very high starch concentration was found to be inhibitory to amylase production.

8. These bacteria preferred complex medium for maximum growth whereas, enzyme production was maximum in mineral medium.

9. Glucose was the favourable carbon source for growth of V. alginolyticus (MB1) and Vibrio sp. (MB3), whereas, V. parahaemolyticus (MB2) showed maximum growth in starch medium. Enzyme production was maximal in maltose medium.

10. Ammonium chloride was the preferred nitrogen source for amylase production by V. parahaemolyticus (MB2) and Vibrio sp.(MB3) but V. alginolyticus (MB1) showed maximum amylase production in the presence of yeast extract.

11. All the isolates showed the ability to grow and produce amylase using raw-starches from cassava, plantain and potato. This property suggests their role in the degradation of native starches in the environment.

12. The incubation period required for maximal amylase production showed much variation in still culture and shaker

culture. The incubation period can be considerably brought down by employing shaker culture.

13. The enzyme production by these bacteria was found to begin at the early-logarithmic phase of growth and continued till the late-logarithmic phase. In the case of _V. alginolyticus_ (MB1), it continued in the early-stationary phase also. Further incubation resulted in loss of amylase activity mainly due to the production of toxic end-products and proteolytic enzymes.

14. The amylases produced by these strains are mostly extracellular. Only less than 5% intracellular enzyme activity could be detected.

15. Amylase production by these halophilic bacteria are found to be inducible. Presence of maltose or starch in the medium was found to induce higher production of amylase. The true inducer may be maltose since, starch is a long polymer and cannot enter the cell directly. The small level of constitutive enzyme liberated by the cells may degrade the starch, and maltose, the product of this enzymatic action, may act as the true inducer.

16. Amylases produced by these bacteria are found to be subjected to catabolite repression. Higher levels of glucose in the medium completely repressed amylase synthesis.

17. Chloramphenicol, when added to cultures actively secreting amylase, caused rapid and complete inhibition of amylase synthesis, suggesting amylase synthesis as a de novo process.

18. When actinomycin D was added to cultures actively secreting amylase, there was a transient continuation of amylase synthesis followed by inhibition. This indicates the presence of a pool of mRNA specific for amylases.

19. The amylases could be partially purified by ammonium sulphate precipitation, followed by dialysis. The yield varied from 57% to 64% and specific activity 63.3 units/mg protein to 96 units/mg protein.

20. The amylases produced by these strains are of the α-type.

21. The amylases showed optimum activity at pH 7. In general, they could show considerable activity in the pH range 6 to 8. They were also stable from pH 6 to 8.

22. Amylases from V. alginolyticus (MB1) and V. parahaemolyticus (MB2) showed maximum activity between 35°C and 40°C. Whereas, Vibrio sp.(MB3) showed maximum activity at 30°C to 35°C. Amylase from all the strains showed 100% stability up to 40°C. Their stability decreased

gradually, with increase in temperature. This suggests that these enzymes are not very thermostable.

23. The optimum substrate concentrations required for maximal activity ranged from 0.8 mg/ml to 1 mg/ml. The K_m and V_{max} values for different substrates showed variation. The K_m ranged from 0.22 mg/ml to 0.9 mg/ml. The V_{max} values were in the range 0.13 mg/min/ml to 0.55 mg/min/ml.

24. Sodium chloride was found to be not essential for the activity of these amylases.

25. Even though addition of calcium to the reaction-mixture did not increase amylase activity, EDTA treatment resulted in the loss of activity, suggesting possible requirement of metal ions for enzyme activity.

26. Several metal ions were found to be inhibitory to enzyme action. Mercury, copper, zinc and iron were very potent inhibitors and cobalt, manganese and magnesium were mild inhibitors. Sodium and calcium did not affect enzyme activity considerably, and potassium caused a slight activation.

27. Carbonate was found to be inhibitory to all the amylases. Other anions tested did not affect enzyme activity significantly.

28. p-Chloromercuribenzoate inhibited the activities of amylases produced by *V. alginolyticus* (MB1) and *V. parahaemolyticus* (MB2). Thiol compounds afforded protection to the enzyme activity, suggesting the presence of SH groups in the active site of these enzymes. Activity of amylase from *Vibrio* sp.(MB3) was not inhibited considerably in the presence of p-chloromercuribenzoate.

The investigation suggests that amylolytic bacteria are distributed widely in the aquatic environment, and they play a very important role in biodegradation. Members of *Vibrio* species may be considered as potential commercial sources of amylase as they grow rapidly in a readily available low-cost medium based on seawater and raw-starches. The problem of contamination in industries from terrestrial forms can be minimised by using such a medium.

REFERENCES

1. Takasaki, Y. 1976. Agric. Biol. Chem. **40**: 1515.
2. Higashihara, M., and Okada, S.C. 1974. Agric. Biol. Chem. **38**: 1023.
3. Yamane, T., and Tsukano, M. 1977. J. Ferment. Technol. **55**: 233.
4. Fogarty, W.M., and Bourke, E.J. 1983. J. Chem. Technol. Biotechnol. **33**: 145-154.
5. Shinke, R., Kunimi, Y., and Nishira, H. 1975. J. Ferment. Technol. **53**: 687.
6. Shinke, R., Nishira, H., and Mugibayashi, N. 1974. Agric. Biol. Chem. **38**: 665.
7. Hyun, H.H., and Zelkus, J.G. 1985a. Appl. Environ. Microbiol. **49**: 1162-1167.
8. Buonocore, V., Caporale, C., De Rosa, M., and Gambacorta, A. 1976. J. Bacteriol. **128**: 515.
9. Rutten and A.J. Dougulis. 1987. Biotechnol. Lett. **9**: 505-519.
10. Grootegoed, J.A., Lauwer, A.M., and Heinen, W. 1973. Arch. Microbiol. **90**: 223.
11. Bliesmer, B.O., and Hartman, P.A. 1973. J. Bacteriol. **113**: 526.
12. Chandra, A.K., Medda, S., and Bhadra, A.K. 1980. J. Ferment. Technol. **58**: 1-10.
13. Rothstein, D.M., Delvin, P.E., and Cata, R.L. 1982. J. Bacteriol. **168**: 839-842.

14. Davis, P.E., Cohen, D.L., and Whitaker, A. 1980. Antonie Van Leeuwenhock. **46**: 391-398.

15. Balsis, A.B., Baratova, L.A., Bakhmatova, I.A., Glemzha, A.A., and Janulaitiene, K.K. 1985. Biokhimiya. **50**: 928-935.

16. Mc Wethy, S.J. and Hartman, P.A. 1977. J. Bacteriol. **129**: 1537.

17. Ramesh, M.V., and Lonsane, B.K. 1987. Biotechnol. Lett. **9**: 501-504.

18. Hanchul, Y., and Young, L.K. 1984. Nouglim Nonjip. **24**: 113-21.

19. Jacqueline, M.C., Catherine, K., and William, F.M. 1988. Biochem. Soc. Tran. **16**: 184-185.

20. Madi, E., Antranikian G., Ohmiya K., and Gottschalk, G. 1987. Appl. Environ. Microbiol. **53**: 1661-1667.

21. Hostinova, E., Bacova, M., Poliuko, L., Gasperik, J., and Zelinka, J. 1979. Biologia. **34**: 939-946.

22. Abramov, Z.T., Tsaplina, I.A., Stepanov, V.M., and Longinova, L.G. 1986. Biokhimiya. **51**: 118-124.

23. Uptron, M.E., and Fogarty, W.M. 1977. Appl. Environ. Microbiol. **33**: 59-64.

24. Kadam, S.K., and Fogarty, W.M. 1984. Can. J. Microbiol. **30**: 950-955.

25. Andrews, G., Lasley., and John, W. 1987. Biochem. Soc. Tran. **15**: 522-23.

26. Oriel, P., and Schwacha, A. 1988. Enz. Microb. Technol. **10**: 42-48.

27. Ilari, S., Matti, K., Matte, L., Anja, K., Tuomo, G., Peter, M., and Pekka, M. 1987. Gene. **61**: 167-176.

28. Kohichi Tachibana., Koji, Y., Satori, W., Hiroshi, H., Yoshihiro, Y., Kunio, Y., Makari, Y., and Gakuzo, T. 1987. J. Gen. Microbiol. **133**: 1775-1782.

29. Shibuya, I., Limura, Y., Ishikawa, T., Ouchi, K., Matsuyama, A., Yamamoto, T., Morikawa, M., and Nishiya, T. 1986. Agric. Biol. Chem. **50**: 875-882.

30. Koch, R., Zablowski, P., and Antranikian G. 1987. Appl. Microbiol. Biotechnol. **27**: 192-198.

31. Glymph, J.L., and Stutzenberger, F.J. 1977. Appl. and Environ. Microbiol. **34**: 391-397.

32. Stutzenberger, F., and Carnall, R. 1977. Appl. Environ. Microbiol. **34**: 234.

33. Takasaki, Y. 1983. Agric. Biol. Chem. **47**: 2193-2199.

34. Champ, M., Szylit, O., Raiband, P., and Aiet Abdelkader, N. 1983. J. Appl. Bacteriol. **55**: 487-493.

35. Khandeparkar, V.G., and Dhala, S.A. 1973. Indian J. Microbiol. **13**: 73-80.

36. Nandakumar, M.P. 1991. 'Fermentation of rice and related products by Bacillus species.' Ph.D. Thesis, Cochin University of Science and Tehnology.

37. Michael, C.A. 1988. Appl. Environ. Microbiol. **54**: 772-776.

38. Olukayode, A.O., and Olusola, O.A. 1987. J. Ind. Microbiol. **2**: 123-127.

39. Ratcliffe, C.R.L., Ludmila, S.T., and R.W.O. Brien. 1982. Aust. J. Biol. Sci. **35**: 457-67.

40. Onishi, H., and Sonoda, K. 1979. Appl. Environ. Microbiol. **38**: 616-620.

41. Gonzalez, C., Gutiereez, C., and C. Ramrirez. 1978. J. Microbiol. **24**: 710-715.

42. Onishi, H., and Hidaka, O. 1978. Can. J. Microbiol. **24**: 1017-1023.

43. Iuchi, S., and Tanaka, S. 1980. Microbiol. Immunol. **24**: 803-814.

44. Kobayashi, T., Kamakura, M., Kanlayakrit, W., and Onishi, H. 1986. Microbios. **46**: 188-189.

45. Gavrilovic, L., R.W.O., Brien., and R.L. Sanders. 1982. Aust. J. Biol. Sci. **35**: 111-124.

46. Lindgren, S., and Refai, O. 1984. J. Appl. Bacteriol. **57**: 221-228.

47. Yasuhiko, K., Mahoko, M., Shoji, F., Toyoji, O., Takashi, S., and Teruhiko, B. 1988. Agric. Biol. Chem. **52**: 615-616.

48. Pau-Tsung, H. 1973. Taiwan Sugar. **20**: 189-192.

49. Gandhi, A.P., and Kjaergaard, L. 1975. Biotechnol. Bioeng. **17**: 1109-1118.

50. Gasparyan, A.V., Avakyan, Z.G., and Makarova, E.N. 1987. Biol. Zh. Arm. **40**: 275-280.

51. Tryasagolova, T.V., Kazakov, A.V., Burtseva E., Dvadtsatova, E.A., and Ustinnikov, B.A. 1986. Fermentn. Spirit. Prom-st. **5**: 36-37.

52. Fumio, H., and Yasuhiro, N. 1987. Yakagaku. **36**: 791-801.

53. Emannilova, E., Manolov, R., Tonokova, A., and Kosturkova, P. 1987. Biotechnol. Biotekh. **1**: 20-23.

54. Krishnan, T., and A.K. Chandra. 1982. Appl. Environ. Microbiol. **44**: 270-274.

55. Yankov, D., Dobreva, E., Beshkov, V., and Emannilova, E. 1986. Enz. Microb. Technol. **8**: 665-667.

56. Priest, F.G., and Thirunavukkarasu, M. 1985. J. Appl. Bacteriol. **58**: 381-390.

57. Young, Y.J., Theodore, C.W., Juan, H., and Randolph, H.T. 1988. Biotechnol. Bioeng. **31**: 357-365.

58. Zhang, Q., Tsukagoshi, Z., Miyashiro, S., and Udaka, S. 1983. Appl. Environ. Microbiol. **4**: 293-295.

59. Takaya, H., Teruhiko, A., and Koki, H. 1988. Agric. Biol. Chem. **52**: 443-448.

60. Gurlev, B. 1986. Getreide. Mehl. Brot. **40**: 204-208.

61. Naohira, Y., Takahide, C., and Minoru, K. 1985. Denpun Kagaku. **32**: 217-221.

62. Ghosh, S.B., and Chandra, A.K. 1984. Zentralbl. Mikrobiol. **139**: 293-304.

63. Srivastava, R.A.K., and Baruah, J.N. 1986. Appl. Environ. Microbiol. **52**: 179-184.

64. Esther, Y. Aderibigde., and S.A. Odunfa.1990. J. Appl. Bacteriol. **69**: 662-671.

65. Hostinova, E., Bacova, M., Polivka, L., Gasperik, J., and Zelinka, J. 1979. Biologia. **34**: 939-946.

66. Dong Hewi, Y., Tae Jong, K., and Ho Kwon, H. 1987. Sanop Misaengmul Hakhoechi. **15**: 190-195.

67. Abd-el-Malek, Y., El-Leithy, M.A., and Ibrahim, S.A. 1973. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hugg. **128**: 304-315.

68. Sinha, S., and A.L. Chandra. 1981. Zentralbl. Bakteriol. Parasitenk . Infektionskr. Hugg. **136**: 209-216.

69. Avendano, M.C., and Cornejo, I. 1987. Biotechnol. Lett. **9**: 123-124.

70. Hannes, M. 1987. J. Gen. Microbiol. **133**: 883-890.

71. Therion, J.J., Kistner, A., and Kornelius, J.H. 1982. Appl. Environ. Microbiol. **44**: 428-434.

72. Onishi, H. 1973. U.S. Patent 3,767,530.

73. Qadeer, M.A., Anjum, J.I., and Akhtar, R. 1980. Pak. J. Sci. Ind. Res. **23**: 25-29.

74. Zherebtsov, N.A., and Korneeva, O.S. 1985. Fermentn. Spirit. Prom-st. **6**: 35-38.

75. Bodisko, Q.D., and Yurkevich, V.V. 1986. Biol. Nauki. **12**: 22-25.

76. Fumio, Y. and Alfredo, V.A. 1973. Cienc. Cult. **25**: 321-323.

77. Wayne, N.L., and Glenn, C.H. 1987. J. Bacteriol. **169**: 5867-5869.

78. Wambutt, R., Riesenberg, D., Krueger, M., and Schultze, M. 1984. Z. Allg. Mikrobiol. **24**: 575-579.

79. Thirunavakkarasu, M., and Priest, F.G. 1980. FEMS Microbiol. Lett. **7**: 315-318.

80. Sata, H., Yaniguchi, H., and Murayama, Y. 1986. Agric. Biol. Chem. **50**: 2803-2809.

81. Sata, H., Taniguchi, H., and Murayama, Y. 1987. Agric. Biol. Chem. **51**: 1521-1527.

82. Srivastava, R.A.K., and Mathur, S.N. 1984. J. Appl. Bacteriol. **57**: 147-151.

83. Khovrychev, M.P., Lirova, S.A., and Rabotnova, I.L. 1987. Mikrobiologiya. **56**: 425-430.

84. Srivastava, R.A.K., Nigam, J.N., Pillai, K.R., and Barugah, J.N. 1981. Indian J. Exp. Biol. **19**: 271-276.

85. Hyun, H.H., and Zelkus, J.G. 1985 b. J. Bacteriol. **164**: 1162-1170.

86. Shigenobu, M., and Shuji, T., 1987. Nippon Saikingaku Zasshi. **42**: 837-845.

87. Stepanov, A.I., Afanaseva, V.P., Zaifseva, G.V., Mednikova, A.P., and Lupadina, I.B. 1975. Prikl. Biokhim. Mikrobiol. **11**: 682-685.

88. Rene, D.M., Hubert, V. 1987. Appl. Microbiol. Biotechnol. **26**: 258-262.

89. Ajay, P.B., Roger, K.A., Ronald, W.A., and Richard; H.C. 1987. Biotechnol. Bioeng. **30**: 363-367.

90. Reddi, C. Adinarayana., and Abouzied, M.M. 1986. Enz. Microb. Technol. **8**: 659-664.

91. Eratt. J.A., and Douglas, P.E. 1984. Can. J. Biochem. Cell. Biol. **62**: 678-680.

92. Le Van Chi., and Dement'ev, D.S. 1975. Izv. Akad. Nauk. Mold. SSR. Ser. Biol. Khim. Nauk **3**: 42-45.

93. Sadhukan, R., Roy, S.K., Raha, S.K., Subha Manna., and S.L. Chakrabarty. 1992. Indian J. Expt. Biol. **30**: 482-486.

94. Bakhmatova, I.V., Balsis, A.B., Churlis, T.K., Glemzha, A.A. and Yanulaytene, K.K. 1984. Prikl. Biokhim. Mikrobiol. **20**: 804-809.

95. Mantsala, P., and Zalkin, H. 1979. J. Biol. Chem. **254**: 8540-8547.

96. Takasaki, Y. 1985. Agric. Biol. Chem. **49**: 1091-1097.

97. Takasaki, Y. 1987. Agric. Biol. Chem. **51**: 9-16.

98. Krishnan, T., and Chandra, A.K. 1983. Appl. Environ. Microbiol. **46**: 430-432.

99. Morgan, F.J., and Priest, F.G. 1981. J. Appl. Bacteriol. **50**: 107-114.

100. Dobreva, E., Emannilova, E., Pancheva, V., Filipova, T.S., and Panchev, S. 1987. Biotechnol. Biotekh. **22**: 30-32.

101. Galabova, D., and Welcheva, P. 1986. Acta Microbiol. Bulg. **19**: 56-62.

102. Srivastava, R.A.K. 1984. Enz. Microb. Technol. **6**: 422-426.

103. Srivastava, R.A.K. 1987. Enz. Microb. Technol. **9**: 749-754.

104. Srivastava, R.A.K., Nigam, J.N., Pillai, K.R., and Baruah, J.N. 1980. Indian J. Exp. Biol. **18**: 972-976.

105. Srivastava, R.A.K., Mathur, S.N. and Baruah, J.N. 1984. Acta. Microbiol. Pol. **33**: 57-66.

106. Patrick, G.J., and William, F.M. 1973. Biochem. Soc. Trans. **1**: 1097-1100.

107. William, F.M., and Patrick, G.J. 1975. J. Appl. Chem. Biotechnol. **25**: 229-238.

108. Granum, E. 1979. J. Food Biochem. **3**: 1-12.

109. Suzuki, Y., Nagayama, T., Nakano, H. and Onishi, K. 1987. Starch/Staerke. **39**: 211-214.

110. Naohire, Y., Takahide, C., and Minoru, K. 1985b. Agric. Biol. Chem. **49**: 3369-3376.

111. Thomas, M., Priest, F.G. and Stark, J.R. 1980. J. Gen. Microbiol. **118**: 67-72.

112. Mutsuo, K. 1986. Agric. Biol. Chem. **50**: 23-31.

113. Hannes, M. 1988. Biochem. J. **250**: 813-818.

114. Wako, K., Hashimoto, S., Kubomura, S., Yokota, K., Aikawa, K., and Kanaeda, J. 1979. J. Jap. Soc. Starch Sci. **26**: 175-181.

115. Hostinova, E., Pokorna, M., and Zelinka, J. 1980. Biologia. **35**: 191-194.

116. Mizokami, K., Kozaki, M., and Kitahara, K. 1978. J. Jap. Soc. Starch Sci. **25**: 132-139.

117. Goldberg, J.D., and C. Edwards. 1990. J. Appl. Bacteriol. **69**: 712-717.

118. Yeong Soo, J., Young Ki, H., and Jung Hwn, S. 1987. Han'guk Yong Yang siklyong Hakhoechi. **16**: 128-135.

119. Von Tigerstrom, R.G., and Stelmaschunk, S. 1987. J. Gen. Microbiol. **133**: 3437-3443.

120. Sen Sirbir., and Chakraborty, S.L. 1986. J. Appl. Bacteriol. **60**: 419-423.

121. Obi, S., and Odibo, F.J.C. 1986. Can. J. Microbiol. **30**: 780-785.

122. Allam, A.M., Hussein, A.M., and Ragab, A.M. 1975. Z. Allg. Mikrobiol. **15**: 393-398.

123. Toshio, T., Eiji, I., Yuichi, S., Makoto, T., and Qi Susumu 1987a. Agric. Biol. Chem. **51**: 399-405.

124. Daniela, S., and Jozef, A. 1986. Kvasny Prum. **32**: 212-215.

125. Emayavarmban, N., and Ramabadran, R. 1980. Sci. Cult. **46**: 271.

126. Galabova, D., and Velcheva, P. 1985. Acta Microbiol. Bulg. **16**: 67-72.

127. Galabova, D., and Velcheva, P. 1986. Dokl. Bolg. Akad. Nauk. **39**: 75-78.

128. Susan, T.J., and Alexander, K.M. 1988. J. Biol. Chem. **263**: 3086-3091.

129. Karen, K.L., Stanley, M.E., Deborah, M.L., and Constance, S.B. 1986. U.S. US 4, 600, 693.

130. Dua, R.D., and Kochhar, S. 1985. Mol. Cell. Biochem. **66**: 13-20.

131. Dua, R.D., and Kochhar, S. 1986. Indian J. Biochem. Biophys. **23**: 133-139.

132. Kochhar, S., and Dua, R.D. 1985. Biochem. Biophys. Res. Commun. **126**: 966-973.

133. Kennedy, J.F., White, C.A., and Riddiford, C.L. 1979. Starke. **31**: 235-241.

134. Balsis A., Bakhmatova, I.V., Ciurlis, T., Glemza, A., Sinitsyn, A.P., and Taleborovskaya, I.K. 1986. Biokhimiya **51**: 378-386.

135. Marie, D.H., Hosti, G., and Harold, R. 1987. Starch/Staerke **39**: 436-440.

136. Blakeney, A.B., and Stone, B.A. 1985. Febs Lett. **186**: 229-232.

137. Galabova, D., Velcheva, P., and Dudev, T. 1986. Dokl. Bolg. Akad. Nauk. **39**: 97-100.

138. Hajime, T., and Yoshihara, M. 1985. Denpun Kagaku. **32**: 142-151.

139. Paje, N.F., Taymundo, A.K., Dalmacio, I.F., and Sakai, H. 1986. Philipp. J. Sci. **115**: 129-138.

140. Toshio, T., Yuichi, S., Makoto, T., and Oi Susumu. 1987b. Agric. Biol. Chem. **51**: 591-592.

141. Lerluck, B., Kazutoshi, I., Kazuo, I., Hajime, T. 1988. Enz. Microb. Technol. **10**: 173-179.

142. Masataka, H., Shinsuke, M., and Shigetaka, O. 1987. Denpun Kagaku. **34**: 106-112.

143. Takaya, T., Sugimoto, Y., Wako, K. and Fuma, H. 1979. Starke. **31**: 205-208.

144. Shamala, T.R., and Sreekantiah, K.R. 1987. Enz. Microb. Technol. **9**: 726-229.

145. Philip, R. 1987. Studies on proteolytic bacteria in fish spoilage Ph.D. Thesis, Cochin University of Science and Technology.

146. Cowan, S.T. 1974. Cowan and Steel's manual for the identification of medical bacteria. 2nd edition. University Press, Cambridge.

147. Mac Faddin, J.F. 1980. Biochemical tests for identification of medical bacteria. 2nd edition. The Williams and Wilkins Co., Baltimore.

148. Krieg, N.R., and Holt, J.G. 1984. Bergey's Manual of systematic bacteriology, Williams and Wilkins, Baltimore, London.

149. Kovacs, N. 1956. Nature. **178**: 703.

150. Lee, J.V., H.S. Hendrie., and J.M. Shewan. 1979. Identification methods for microbiologists, Society for Applied Bacteriology Technical series No.14, Academic, London.

151. Hugh, R., and Leifson, E. 1953. J. Bacteriol. **66**: 24-26.

152. Crosby, N.T. 1967. Proc. Soc. for water Treatment and Examination. **16**: 51-55.

153. Thornley, M.J. 1960. J. Appl. Bacteriol. **23**: 37-52.

154. Moller, V. 1955. Acta Pathologica et Microbiologica Scandinavica. **36**: 158-172.

155. Mac Leod, R.A. 1965. Bacteriol. R. **29**: 9-13.

156. Iturriga, R., and Hoppe, 1977. Mar. Biol. **40**: 101-108.

157. Pradeep, R. 1986. Studies on the indicators and pathogen Vibrio parahaemolyticus in Cochin Backwater. Ph.D. Thesis. Cochin University of Science and Technology, 228.

158. Kjelleberg. S., and Hakanksson, N. 1977. Marine Biology **39**: 103-109.

159. Mary, P.P. 1977. Studies on the gastro-intestinal microflora of the mullet Liza dissumeri (Valenciennes) Mugiliformes: Teleostei) Ph.D. Thesis, Annamalai University, 122.

160. Fathima, J.E., P. Lakshmanaperumalsamy., D. Chandramohan., and R. Natarajen. 1980. Bull. Dept. Mar. Sci., Univ. Cochin, **11**: 97-111.

161. Baumann, P., and Baumann, L. 1977. Annu. Rev. Microbiol. **31**: 39-61.

162. Baumann, P., Baumann, L., Bang, S.S., and Woolkalis, M.J. 1980. Curr. Microbiol. **4**: 127-132.

163. Reid, G.C., Robb, F.T., and Woods, D.R. 1978. J. Gen. Microbiol. **109**: 149-154.

164. Reid, G.C., Woods, D.R., and Robb, F.J. 1980. J. Bacteriol. **142**: 447-454.

165. Tanaka, S., Fujita, T., and Hagihara, H. 1969. Biken J. **12**: 119-124.

166. Tanaka, S., and Iuchi, S. 1971. Biken J. **14**: 81-96.

167. Driesbach, J.H., and Merkel, J.R. 1978. J. Bacteriol. **135**: 521-527.

168. Venugopalan, V.K., A. Ramesh., and B. Loganathan, 1988. In 'Marine Biodeterioration', Mary - Frances Thompson, Rachakonda Sarojini and Rachakonda Nagabhushanam (Eds), Oxford & IBH Publishing Co., Pvt. Ltd., New Delhi.

169. Fuwa, H. 1954. J. Biochem. **41**: 583-603.

170. Bernfeld, P. 1955. Amylases, α and β, Methods Enzymol. **1**: 149-158.

171. Obi, S.K.C., and F.J.C. Odibo. 1984. Appl. Environ. Microbiol. **47**: 571-575.

172. Rheinheimer, G. 1980. (Ed) Aquatic Microbiology. A wiley-International Publication. John Wiley and Sons, 235 .

173. Thimann, K.V. (Ed.) 1964. Das Leben der Bakterien. Jena, Fischer, 875 .

174. Welker, N.E., and L.L. Campbell. 1963. J. Bacteriol. **86**: 681-686.

175. Horikoshi, K., Ikeda, Y. and Tanaka, Y. 1974. United States Patent 3, 826, 715.

176. Markkanen, P.H., and Bailey, M.J. 1975. J. Appl. Chem. Biotechnol. **25**: 863.

177. Bajpai, P., and Promod, K.B. 1989. Biotech. Bioengg. **33**: 72.

178. Antranikian, G., Herzberg, C., and Gottschalk, G. 1987a. Appl. Environ. Microbiol. **53**: 1668-1673.

179. Antranikian G., Zablowski, P., Gottschalk, G. 1987b. Appl. Microbiol. Biotechnol. **27**: 75-81.

180. Jay, J.M., 1966. In 'The Physiology and Biochemistry of mussel as a food'. (Eds.) E.J. Briskey, R.G. Cassens and J.C. Trautman, University of Wisconsin Press, Madison, WI, P. 387-402.

181. Kushner, D.J. 1978. In D.J. Kushner (Ed.) 'Microbial life in extreme environments', Academic, New York.

182. Reichelt, J.L., and P. Baumann. 1974. Arch. Microbiol. **97**: 329-345.

183. Onishi, H. 1972. J. Bacteriol. **109**: 570-574.

184. Onishi, H., and M. Kamekura. 1972. Int. J. Syst. Bacteriol. **22**: 233-236.

185. Pelczar, M.J., R.D. Reid., and E.C.S. Chan. 1977 (Ed.) Microbiology, Tata Mc Graw-Hill Publishing Co., Ltd., New Delhi, 952 .

186. Abd El. Akher, M., El-Leithy, M.A., E.L. Marsafy, M.K., and Kassim, S.A. 1973. Zentralbl. Bakteriol. Parasitenk. Infektionskr Hyg. Abt. **128**: 483-490.

187. Fukomoto, J., and Negoro, H. 1966. United States patent 3, 272, 717.

188. Outtrup, H., and Aunstrup, K. 1975. Proceedings of the First International Congress, International Association of Microbiological Societies **5**: 204.

189. Medda, S., and Chandra, A.K. 1980. J. Appl. Bacteriol. **48**: 47.

190. Baumann, P. and Schubert, 1984. In 'Bergey's Manual of systematic bacteriology' Vol.1. Krieg N.R. and J.H. Holt (Eds.) Williams and Wilkins, Baltimore/London.

191. Babu, K.R., and Satyanarayana. T. 1990. Proceedings of National symposium on current trends in Biotechnology, Cochin.

192. Ustyuzhanina, S.V., Yarovenko, V.L. and Voinarskii. I.N. 1984. Prikl. Biokhim. Microbiol. **20**: 612-621.

193. Tobey, J.F., and Yousten, A.A. 1976. Developments in industrial Microbiology **18**: 499.

194. Tsukagoshi, N., Iritani, S., Sasaki, T., Takemura, T. Ihara, H., Idiota, Y., Yamagata, H., and Shigezo Udaka. 1985. J. Bacteriol, **164**: 1182-1187.

195. Goyal, S.G., and Khandeparker, V.G. 1978. Ind. J. Microbiol, **13**: 73.

196. Gudrun, V., and Meyath, J. 1973. Mitt. Versuchssta, Gaerung Sgewerbe Wien. **27**: 167-176.

197. Debabov, V.G. 1982. The Molecular Biology of the <u>Bacilli</u> **1**: 331-370. New York, London Academic Press.

198. Priest, F.G. 1983. Microbial enzymes and Biotechnology (Ed.) Fogarty W.M, 319-366, London: Applied Sciences.

199. Tsuyumu, S. 1977. Nature, **269**: 237-238.

200. Saito, N., and Yamamoto, K. 1975. J. Bacteriol. **121**: 848-856.

201. Yabuki, M., Obo, N., Hohino, K., and Fukui, S. 1977. Appl. Environ. Microbiol. **34**: 1.

202. Wojs Kowicz, J. 1977. Acta Microbiologica Polonica, **26**: 146-171.

203. Adama, P.R., and Deploey, J.J. 1976. Mycologia. **68**: 934.

204. Nyiri, L. 1971. International chemical engineering. 11: 447.

205. Kvesitadze, G.I. Kokonashvili, G.N., and Fenixova, R.V. 1974. U.S. patent 3, 826, 716.

206. Coleman, G. 1967. J.G. Microbiol. **49**: 421.

207. Sekigiuchi, J., and Okada, H., 1972. J. Fermtn. Technol. **50**: 801.

208. Tomomura, K., Suzuki, H., Nakamura, N., Kuraya, K., and Tanabe, O. 1961. Agric. Biol. Chem. **25**: 1.

209. Suzuki, H., and Tanabe, O. 1962. Reports of the Fermentation Research Institute. Japan. 57.

210. Schaeffer, P. 1969. Bacteriological Reviews. **33**: 48.

211. Moses, V., and Sharp, P.B. 1972. J. Gen. Microbiol. **71**: 81.

212. Meers, J.L. 1972. Antonie Van Leeuwenbock. **38**: 585.

213. Coleman, G., and Grant, M.A. 1966. Nature. **211**: 306.

214. Heineker, F.G., and O' Connor, R.J. 1972. J. Gen. Microbiol. **73**: 35.

215. Nikolov, T., Kibarska, T., and Nedeva, D. 1970. Compets Rendus de L' Academic des science Agricoles en Bulgarie **3**: 145.

216. Andrzejczuk - Hybel, J., and Kaczkowski, J. 1971. Bulletin de L' Academic Potonaise des Sciences XIX N. 5: 315.

217. Clementi, F., Rossi, J., Costamagna, L., and Rosi, J. 1980. Antonie Van Leeuwenhoek **46**: 399-405.

218. Ingle, M.B., and Boyer, E.W. 1976. 'Microbiology' C.D. Schelessninger (Ed.), 420. American Society for Microbiology, Washington, D.C.

219. Priest, F.G. 1975. Biochemical and Biophysical Research Communication. **63**: 606.

220. Gogoi, B.K., R.L. Bez Baruah., K.R. Pillai., and J.N. Baruah. 1987. J. Appl. Bacteriol. **63**: 373-379.

221. May, B.K., and Elliott, W.H. 1968. Biochemica et Biophysica Acta. **157**: 607-615.

222. Coleman, G., and Elliott, W.H. 1965. Biochem. J. **95**: 699-706.

223. Both, G.W., Mclnnes, J.L., Hanlon, J.E., May, B.K., and Elliott, W.H. 1972. J. Mol. Biol. **67**: 199-207.

224. O' Connor, R., Elliott, W.H., and May, B.K. 1978. J. Bacteriol. **136**: 24-34.

225. Lowry, O.H., N.J. Rosebrough., A.L. Farr., and R.J. Randall. 1951. J. Biol. Chem. **193**: 265-275.

226. Salle, A.J. (Ed.). 1979. Fundamental Principles of Bacteriology. Tata MC Grw - Hill publishing Company Ltd., New Delhi.

227. Robyt, J.F., and Whelan, W.J. 1968. The α-amylase In 'starch and its derivatives', 4th Edn. (Ed. J.A. Radley) 430-476. Chapman and Hall, London.

228. Fisher, E.H., and Stein, E.A. 1960. In 'The Enzymes' P.D. Boyer, H. Lardy., and K. Myrback, (Eds.) Vol. **4**: 313. Academic Press, New York.

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229. Thoma, J.A., Spradlin, J.G., and Dygert, S. 1971. In 'The Enzymes' (Ed.) Boyer, P.D. Vol. **5**: 115-189. London, Academic Press.

230. Stein, E.A., and Fischer, E.H. 1958. J. Biol. Chem. **232**: 867.

231. Yamamoto, T. 1955. Bull. Agr. Chem. Soc. Japan. **19**: 121.

232. Hagihara, B., Matsubara, H., and Okunuki, K. 1956. J. Biochem. Tokyo. **43**: 469.

233. Yamanaka, T., and Higashi, T. 1957. J. Biojchem. Tokyo. **44**: 637.

234. Mosely, M.H., and Keay, L. 1970. Biotech. Bioeng. **12**: 251.

235. Hsiu, J., Fisher, E.H., and Stein, E.A. 1964. Biochemistry, New York. **3**: 61.

236. Isumura, T., Takagi, T., Maeda., and K. Yutani. 1963. J. Biochem. (Tokyo) **53**: 155.

237. Akabori, S., Okada, Y., Fujiwara, S., and K. Sugai. 1956. J. Biochem. (Tokyo) **43**: 741.

238. Junge, J.M., E.A. Stein., H. Neurath., and E.H. Fisher. 1959. J.B.C. **234**: 556.

239. West, P.A., and Colwell, R.R. 1984. In 'Vibrios in the environment' Colwell, R.R., (Ed.) John Wiley and sons.

240. Fogarty, W.M., and Kelly, C.T. 1980. In 'Microbial enzymes and bioconversions' Rose, A.H. (Ed.). Academic Press, London.

241. Davis, B.J. 1964. Ann. N.Y. Acad. Sci. **121**: 404-427.