"THERMOSTABLE ALPHA AMYLASE PRODUCTION BY *BACILLUS COAGULANS*"

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By

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CERTIFICATE

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

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DECLARATION

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

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INTRODUCTION

1.1 Preface

Microbial enzymes are in great demand owing to their importance in several industries such as brewing, baking, leather, laundry detergent, dairy, starch processing and textiles besides pharmaceuticals. About 80% of the enzymes produced through fermentation and sold in the industrial scale are hydrolytic enzymes. Due to recognition of new and new applications, an intensive screening of different kinds of enzymes with novel properties, from various microorganisms, is being pursued all over the world.

Amylases hold greater significance, next to proteinases, as a commercial enzyme among the various enzymes that are used in industries. Amylases of microbial orgin are broadly classified as below:

1. Exo-acting amylases, which include amyloglucosidases, β-amylases.
2. Endo-acting amylases, including α-amylase
3. Debranching enyzmes.
4. Cyclodextrin - producing enzymes.

Among these, α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1, endo amylase) occurs widely in microorganisms. It hydrolyzes α-1,4-glucosidic bonds in amylose, amylopectin and glycogen in an endofashion, but not the α-1,6-glucosidic linkages in the branched polymers. Hydrolysis of amylose by α-amylase effects its complete conversion into maltose and maltotriose, (Walker and Whelan, 1960a). Whereas in the case of amylopectin, glucose and maltose along with a series of branched α-limit dextrins are the end products of α-amylase action.

Bacillus sp are largely known to produce α-amylase, among the different groups of microoganisms, at industrial level. They are known to produce both saccharifying and liquefying α-amylases (Fukumoto 1963; Welker and Campbell, 1967a), which are distinguishable by their mechanisms of starch degradation by the fact that the saccharifying α-amylases produce an increase in reducing power about twice that of the liquefying enzyme (Fukumoto, 1963; Pazur and Okada, 1966).

Bacterial amylases are used to produce modified starches with low DE (Dextrose Equivalent) value as well as a

range of malto-dextrins with different DE values. When bacterial amylases are used in combination with amyloglucosidase, glucose is the sole end-product, while in association with fungal amylase, maltose syrups with high values are obtained.

Production of starch hydrolysates involves firstly liquefaction of gelatinized starch and secondly saccharification of the thinned or liquefied product with the desired enzyme. The liquefaction step produces dextrin and is sometimes referred to as the dextrinizing step. Bacterial thermostable α-amylases are used for liquefaction. The reaction is generally terminated before significant hydrolysis takes place and when the average degree of polymerization is about 10-12. Saccharification is the second stage and utilizes thermolabile amylases which are not sufficiently heat stable to be used in the liquefaction step.

In enzyme catalysed processes, enzyme costs are usually a very significant process cost element. Indeed, enzyme cost is the greatest single obstacle limiting expansion of industrial enzyme technology. The cost of enzyme can be

reduced (a) by maximising enzyme yield from microbial strains by strain improvement and further fermentation optimization, (b) by developing cost effective systems for recovery and reuse of enzymes; including immobilised enzyme process, and (c) by carrying out enzyme conversions at higher temperatures using thermostable enzymes.

There are afew benefits of operating reaction at higher temperatures. (1) required enzyme can be decreased or the reaction time shortened. (2) Reaction temperatures above 60-62^{o}C markedly reduces microbial contamination of the material being processed (Brock et al., 1986). (3) Increase the solubility of substrates and other chemicals, decrease the viscosity of liquids and thereby improve their mechanical handling, and rendering them more amenable to enzymatic attack (Ward and Moo-Young, 1988, Stefen Janeck, 1993).

In its native state, starch consists of microscopic granules, each possessing a highly complex internal structure. At ambient temperatures these granules are insoluble in water, but if an aqueous starch suspension is heated above 60^{o}C the granules swell and eventually disrupt, dispersing the starch

molecules into solution. The temperature required for complete dispersion or gelatinization depends on the source of starch but 100 to 105°C is sufficient for most starches.

Normally the dry substance content of the starch suspension used for liquefaction is greater than 30% and because of this the viscosity is extremely high after gelatinization. This necessitates the use of a thinning agent, which apart from reducing viscosity also partially hydrolyses the starch so that starch precipitation or "retrogradation" is prevented during subsequent cooling.

The traditional thinning agent used in the starch industry is acid. In the so called "acid liquefaction" process the pH of a 30-40% dry solid starch slurry is adjusted to about 2.0 with a strong acid. HCl is normally used, but in the Far East it is the practice to use oxalic acid.

The starch slurry is heated to 140-150°C either by direct steam injection or indirect heating in a converter for approximately 5 minutes, after which it is flash-cooled to atmospheric pressure and neutralized. This treatment results in complete starch gelatinization and a final product which is

easy to filter. However the non-specific catalytic action of the acid can result in the formation of undesirable by products such as 5-hydroxymethyl 2-fur-furaldehyde and anhydro-glucose compounds. Moreover, the colour and ash content are high so that purification costs become significant (Greenshields and MacGillivray, 1972; Birch and Challenberger, 1973).

If a theromostable endo-amylase is used as a catalyst, the processing conditions are milder so that by-product formation is not a problem and furthermore refining costs are lower. Thus enzymatic hydrolysis of starch has been practiced on an industrial scale for many years and is gradually replacing the traditional acid catalysed processes (Underkofler et al., 1965; Barfoed, 1976). The most important application of thermostable endo amylases in the starch processing is in the liquefaction process.

Thermostable alpha amylases are known to be produced by limited groups of bacteria. *B. amyloliquefaciens* and *B.licheniformis* are the only two species which are used for industrial production of thermostable α-amylase, although other species such as *B.caldolyticus* (Heinen and Heinen, 1972,

Brootegoed et al., 1973, Emanuilova and Toda, 1984). *B.stearothermophilus* (Outtrp and Norman 1984) *Thermus* sp (Koch, et al., (1987) *C.thermosaccharolyticum* (Koch et al., 1987) *C.thermohydrosulfuricum, Dictyoglomus thermophilum* (Saiki et al., 1985). *Fervidobacterium* sp (Plant et al., 1987) *Pyrococcus* sp (Brown et al., 1990, Koch et al., 1990) *Thermoanaerobacter ethanolicus* (Koch et al., 1987) *Thermoanaerobium sp.* and *Thermobacterides sp* are reported(Priest, 1992).

In fact most industral applications of α-amylases require their use at high temperatures and this sustains the ongoing search for new enzymes having increasingly better thermostability properties. Consequently, there is a need to screen new sources for thermostable amylases.

There are basically two types of microbial processes for enzyme production:- (1) solid state fermentation and (2) submerged fermentation. The traditional method involve growing microorganisms in solid or semi solid media (the koji process). Today's industry however, is dominated by deep tank or submerged culture fermentation with its process control capabilities and reduced contamination problems. Batch

process still predominate in large scale fermentation, but fed batch and continuous operations are rapidly taking hold. Continuous processes have the advantage that microbial population can be kept in a constant environment (Wang, et al., 1979).

Recently there is a renewed interest in exploring the scope of utilising solid state fermentation process for industrial enzyme production, inspite of its use over a long time, owing to its several advantages over submerged fermentation, particularly with respect to the cost of down stream processing of enzyme and the consequent product cost. Emergence of modern biotechnologies, particularly mutation and recombinant DNA technology , do hold the key for maximising enzyme yield through amplification of genes coding for the enzymes, if studies are held towards strain improvement over and above process optimisation.

Under this circumstances, the present study was undertaken, with a view to utilise a fast growing *B.coagulans* isolated from soil, for production of thermostable and alkaline α-amylase under different fermentation processes.

1.2 REVIEW OF LITERATURE

1.2.1 Distribution of Bacterial α-Amylases

α-amylases are known to be produced among various groups of micro-organisms including bacteria, actinomycetes, yeasts and fungi. However, this review is limited to their distribution in bacteria. α-amylase is widespread among both aerobic as well as anaerobic microbes. Its production is not restricted to a specific class of bacteria and instead reported among varied species of bacteria (Table 1). The principal groups of genera that produce α-amylase include, among aerobes, species of *Acinetobacter, Aeromonas, Bacillus, Halobacterium, Pseudomonas, Thermus, Lactobacillus, Micrococcus, Streptococus* and *among anaerobes Bacteroides, Clostridium, Dictyoglomus, Fervidobacterium, Pyrococcus, Theromoanaerobium* and *Thermobacteroides.*

1.2.2. Distribution of Thermostable α-amylases

In general, α-amylases that are stable above 45°C are considered as thermostable enzymes (Ward and Moo-Young,1988). Bacteria, both aerobes and anaerobes, which

Table 1 DISTRIBUTION OF AMYLASE PRODUCING BACTERIA

ORGANISM	REFERNCE
B.megaterium	David et al., 1987
B.acidocaldarius A-2	Kanno et al., 1986
B.subtilis	Orlando et al., 1983
B.licheniformis CUME 305	Krishnan & Chandra, (1983)
Lactobacillus cellobiosus	Sen & Chakrabarty 1986
B.megaterium S-218	Stark et al., 1982
B.stereothermophilus	Outtrup and Norman 1984
*Bacillus sp.*11-15	Uchino 1982
B.thermoamyloliquifaciens KP 1071	Suzuki et al., 1987 C
B.subtilis	Takasaki 1985
Pseudomonas stutzeri	Robyt & Ackerman 1971
B.circulans	Takasaki 1983
B.licheniformis	Saito 1973
B.cereus NY-14	Toshigi et al., (1985 a, 1986)
Pseudomonas sp. (KO-8940)	Okemoto et al., 1986
Micrococcus halobius	Onishi, H. 1972
Streptococcus sp	Hobson & Macpherson 1952
Acinetobacter sp.	Onishi & Hidaka 1978
B.amyloliquefaciens	Granuim, 1979

B.caldolyticus	Grootegoed et al.,1973 Heinen and Heinen, 1972 Emanuilova and Toda, 1984
B.coagulans	Campbell et al., 1955
B.licheniformis	Morgan and Priest 1981
Halobacterium halobium	Onishi, 1972
Thermomonospora curvates	Glymph & Stutzenberger 1977
Clostridium butyricum	Whelan & Nasar 1951
Clostridium thermosulfurogenes EM 1	Madi et al., 1987
C.thermosacharolyticum	Koch et al., 1987 Hyun et al., 1985 Plant et al., 1988
Dictyoglomus thermophilus	Saila et al., 1985
Fervidobacterium sp	Plant et al., 1987
Pyrococcus woesei	Koch et al., 1990
P.furiosius	Kocj et al., 1990b Brown et al., 1990
Thermoanaerobacter ethandius	Koch et al., 1987
T.finni	Koch et al., 1977 Antranikian 1987
Thermobacteriodes actoethylicus	Koch et al., 1987

are known to produce α-amylases that have temperature optimum above 45°C are presented in Table 2. A large number of these bacteria belong to *Bacillus.sp.* Further several anaeobic moderately thermophilic bacteria which have temperature optimum of 90-100°C are also reported. The most thermoactive and thermostable enzymes, known so far, are secreted by *Pyrococcus woesei* and *P.furiosus.* Hyperthermophilic archaebacteria are capable of growth on starch and are potentially a good source of extremely thermostable enzymes. However the level of amylolytic enzymes from these microbes is low compared to aerobes and hence they are yet to be tried for industrial production.

1.2.3. Properties of α-Amylase:

1.2.3.1 Theromostability

Majority of the aerobic bacteria which are capable of growing on starch and produce α-amylase are mesophiles which grow between 30-37°C. However, α-amylases produced by them are active above 45°C. α-amylase from *B.licheniformis* is the most thermostable of all the industrial enzymes and it can

Table 2. Distribution of Thermostable a-Amylase Producing Organisms

ORGANISM	OPTIMUM TEMP(^{0}C)	REFERENCE
Acinetobacter sp.	50-55	Onishi & Hidaka, 1978
Bacillus acidocaldarius A.2	70	Kanno, 1986
B.amyloliquifaciens	50-70	Granum, 1979
B.caldolyticus	70	Heinen and Heinen, 1972 Grootegoed et al., 1973 Emanuilova & Toda, 1984
B.cereus	55	Yoshigi et al., 1983
B.circulans	50	Takasaki, 1983
B.coagulans	45-55	Campbell et al., 1955
B.licheniformis	90	Morgan and Priest 1981
B.stearothermophilus	70-80	Gallaboca, 1969, Manning & Campbell, 1961 Ptueller and Elliot, 1969
B.subtilis	55	Mitricia and Granum, 1979 Robyt and French, 1979
Halobacterium halobium	55	Onishi, 1972
Thermus sp. AmD-33	70	Nakamura et al., 1989
Lactobacillus cellobiosus	50	Sen & Chakrabarty, 1984
Micrococus halobius	50-55	Onishi 1972
Streptococcus sp	48	Hobson and Macpherson 1952
Clostridium butyricum	48	Whelan and Nasr, 1951
C.thermosulfurogenes EM 1	65-70	Madi et al., 1987

C.thermosaccharolyticum	70-75	Koch et al., 1987
C.thermohydrosulfuricum	85-90	Melasniemi 1987, Hyun et al., 1985 Plant et al., 1988
Dictyoglomus thermophilum	90	Saiki et al., 1985
Fervidobacterium sp	100	Koch et al., 1990
Pyrococcus woesei	100	Koch et al., 1990b Brown et al., 1990
Thermoanaerobaide ethanolicus	90	Koch et al., 1987
T. finni	90	Koch et al., 1977 Antranikian 1989
Thermoanoaerobium brockii	85	Colemen et al., 1987
Thermobacteriodes acetoethylicus	90	Koch et al., 1987

liquefy starch upto 110°C (Saito, 1973) and that from *B.amyloliquefaciens* was effective at 75-80°C and upto 90°C for short periods (Reichelt, 1983). *B.licheniformis* which grew at 30°C produced α-amylase active at an exceptionally high temperature of 90-100°C (Morgan and Priest 1981, Krishnan and Chandra 1983, Asther and Meunier, 1990, Ramesh and Lonsane, 1989, Takasaki et al., 1994). *B. stearothermophilus* and *B.acidocaldarus* which grew at 55 and 50°C, were optimally active at 80 and 70°C respectively (Manning et al., 1961, Gala bova, 1969, Pfueller et al, 1969, Kanno, 1986). *Lactobacillus cellobiosus* D-39 isolated from vegetable wastes produced an α-amylase maximally active at 50°C (Sen and Chakrabarty, 1986).

Enzymes from the species of *Streptomyces, Themoactinomyces* and *Thermomonospora* were optimally active between 35 and 65°C (Glymph and Stutzenberger 1977, Abramov et al., 1986 Fairbairn et al., 1986). *Thermoactinomyces sp* No.15 produced an α-amylase which on purification was maximally active at 80°C and retained 74% activity after 30 min. at 100°C (Obi and Odibo, 1984). *Pyrococcus woesei and P.furiosus* produced extremely thermoactive α-amylase which, after purification, was active between 50 and 140°C and

retained activity after autoclaving for 4 hrs at 120°C. However the temperature optimum was recorded as 100°C (Koch et al., 1990a).

1.2.3.2 pH optima

Amylases with extreme pH optima of pH 3.5 or 10.5 are produced by *B.acidocaldarius* and *B.alcalophilus* which grow optimally at extreme pH values, pH 2-4.5 and 9-10.5 respectively (Kanno, 1986, Yamamoto et al, 1972). In fact enzymes from species of *B.acidocaldarius* showed pH optima in the range of 2.0-4.5 (Table 3). α-amylases from *B.licheniformis* were reported to have optimum pH of 9.0 (Krishnan & Chandra, 1983), 5-6.5 (Umeshkumar et al., 1990), 5.5-6.0 (Wind et al., 1994), and 4.5-5.3 (Takasaki et al., 1994). Whereas α-amylases of *B.megaterium* had an optimum pH of 5.5. (David et al., 1987).

Alpha amylases of *Streptomyces*, *Thermoactinomyces* and *Thermomonospora* are optimally active at pH 5-7 (Glymph and Stutzenberger 1977, Abramov et al., 1986, Fairbairn et al., 1986).

Table 3 Properties of Bacterial α-Amylases

Organism	Optimum temp. (°C)	Optimum pH	M.W Daltons	pI	Reference
B.megaterium	.75	5.5	55000	9.5	David et.al., 1987
B.licheniformis CUMC 305	90	9.0	28000	--	Krishnan & Chandra 1983
B. subtilis	--	6-7	93000	5.0	Orlando et.al., (1983
B. acidocaldarius A-2	70	3.5	66000	--	Kanno 1986
L.cellobiosus D-39	50	7.3	22500	--	Sen & Chakrabarty 1986
B.acidocaldarius agnano 101	75	3.5	68000	--	Buonocore et.al., 1976
B.acidocaldarius 104-1A	60	4.5	---	--	Boyer et.al., 1979
B.acidocaldarius 11-1 S	70	2.0	54000	--	Uchino 1982
Pyrococcus uriosus	100	5	---	--	Koch et.al., 1990 Brown et.al., 1990
Thermoanaerobacter ethanolicus	90	5.5	---	--	Koch et al 1990
B.circulans F-2	60	6.0-6.5	93000	--	Takasaki 1982
B.amyloliqufaciens	70	5.9	49000	--	Welker & Campbell 1967 Norman 1979
B.licheniformis CUMC 305	91°	9.5	28000	--	Fogarty 1983 Krishna and Chandra 1983
B.coagulans CUMC 150	85°C	7.5-8.5	---	--	Medda & Chandra 1980
C.butyricum T-7	60°C	5.0	89000	--	Tanaka et al 1987
C. butyricum	50°	5.0	81000	--	Macarthy et al, 1988

Lacto bacillus plantharum strain A-6	65°C	5.5	50000	--	Giraud et al 1993
B.licheniformis BLM 1777	85°C	6-7	62000	--	Chiang etal 1979
B. stearo thermophilus	60-70	5.3	70000	8.5	Outtrup & Norman 1984
B.stearo thermophilus KP 1064	55	5.8	11,5000	4.4	Suzuki & Imai 1985
B.stearo thermophilus J	80	5.0	---	--	Brosnan etal. 1989
B. SP-11-15	70	2.0	54000	--	Uchino 1982
B.subtilis	50	6.0-7.0	25000	--	Takasaki 1985
Pseudomonas stutzeri	50	8.0	12,5000	--	Robyt & Ackerman 1978
P.stutzeri FI	45	8.0	55,000	5.6	Sankano etal 1982b
F_2	"	"	"	5.3	
B.Circulans	50	70	45000	--	Tanasaki 1983
B.caldolyticus	70	5.5	--	--	Heinen & Heinen 1972
					Grobfegoed et al 1973
					Emanuilova et al 1984
Thermus sp AM 33	70	5.5	--	--	Koch et al 1990
Clostridum thermosulfuricum	85-90	5.5	--	--	Melasniemi 1987
					Hyun et al 1985
					Plant et al 1987
C.thermo saccharolyticum	70-75	5.5	--	--	Koch et al 1987

All α-amylases from anaerobic bacteria are optimally active at acidic pH values (between 4.5 and 5.5) (Table - 3).

1.2.2.3. Impact of Metal ions and Sugars

Low concentrations of divalent cations such as Ca^{2+}, Mg^{2+} and Zn^{2+} have a specific role in increasing the thermostability of certain enzymes (Wisemean, 1978, Schmid, 1979, Brock, 1986). α-Amylases contain at least one calcium atom per enzyme molecule and are much resistant to temperature denaturation in the presence of calcium (Fogarty, 1983). *B.subtilis* required at least four gram-atoms of calcium per mole of enzyme for full activity. The commercial α-amylase produced by *B.amyloliquiefaciens* requires a calcium concentration of 150 ppm to maintain good enzyme stability. In the presence of high substrate concentration (30-40% starch) this enzyme is active up to 85°C (Godfrey, 1983). Whereas the thermostable α-amylase from *B.licheniformis* had a lower (50 ppm) calcium requirement for stability (Norman, 1979) at 105°C and 70 ppm calcium at 110°C (Saito, 1979). At calcium levels below 20 ppm, thermotolerance is very much pH related. Other α-amylases, from *B.stearothermophilus*, and *B. caldolyticus* (Heinen and Lauwers, 1976), Acidophilic α-amylase

from *B. acidocaldarius* (Buonocore et al, 1976) and the maltose producing α-amylase from *Thermoactinomyces vulgaris* (Shimizu et al., 1978) depend on calcium for good thermotolerance. Metal ions enhanced thermostability of α-amylase of *B.stearothermophilus* (Wind et al., 1994) and thermostability of α-amylase of *B.licheniformise* was enhanced to 100°C in the presence of Ca^{2+} (5mM)(Takasaki et al., 1994). Inhibiton of α-amylase of *B.licheniformis* MYIO by increasing concentration of calcium along with increase in temperature was also reported (Umeshkumar et al., 1990). However calcium is thought to play a role in the maintenance of the tertiary structure of the α-amylases (Priest and Sharp, 1989).

Glucose, maltose and glycerol exhibited thermostabilizing effect with α-amylase of *B.licheniformis* (Asther and Meunier 1990) while free aminoacids stimulate α-amylase synthesis in *B.amyloliquefaciens* (Fogarty and Kelly, 1980).

1.2.4. Nature of Enzyme Thermostability

In native water soluble proteins, hydrophobic amino acids tend to predominate in the interior of the molecule with

a greater number of polar, more hydrophilic, amino acids located on the external surface. All buried polar atoms are hydrogen bonded (Chotia, 1975). Non covalent force which dictate the protein secondary and teritary structures are the principal determinants of enzyme stability and disulphide bonds are not considered to contribute significantly (Ponnuswamy et al., 1982). Thermostable enzymes posses the same primary, secondary, tertiary and quaternary features as more labile enzymes (Friedman, 1976).

Factors which contribute significantly to thermostability include formation of new ion pairs (Argos et al., 1979), hydrophobicity (Heinen and Lauwers, 1976, Frommedl and Hohne,1981, Merkler et al., 1976), metal binding (Heinen and Lauwers, 1976), substrate binding (Pace and McGrath, 1980, Greenberg et al., 1985), and glycosylation (Chu et al., 1978, Harris et al., 1980). It has been estimated that a free energy of stabilization of less than 13 KJ/mole will account for the thermostability of most thermophilic proteins, indicating that subtle rather than gross structural alterations should be expected in these proteins when compared with more thermostable counterparts (Sundaram, 1986).

According to Stellwagen (1984), since internal amino acids are optimally packed, altering the size, shape or polarity of a single internal amino acid side chain would very likely destabilize the enzyme structure and that replacement of a polar groups on the surface of the enzyme should enhance the stability of the enzyme structure. In closely related mesophilic and thermophilic *Bacillus sp* there was a correlation between increasing thermostability of enzymes with an increase in hydrophobic index of the proteins, a decrease in the ratio of polar to non-polar volumes and an increase in the arginine to arginine plus lysine ratio (Merkler et al., 1981).

1.2.5 Mutation and Recombinant DNA Technology in Strain Improvement

Markkanen (1975) and, Bailey and Markkanen (1975) used ultraviolet radiation, ethylene imine and nitrosoguanidine (NTG) to obtain a mutant of *B.subtilis* producing double the yield of α-amylase obtained with the parent strain. Outtrup and Anustrup (1975) treated *B.licheniformis* with gamma rays, ultra violet radiation and NTG and recorded an increase in yield of α-amylase of 25 fold.

Tunicamycin resistant mutants of *B.subtilis* NA64 obtained after treatment of the cells with NTG yielded a five fold increase in α-amylase production. Genetic analyses indicated that resistance to tunicamycin and hyper productivity of α-amylases were induced by a single mutation (Sasaki et al., 1976). *In B.subtilis* 6160, the regulator gene amy RI was replaced by amyR2 (from *B.natto*) and a three to four fold increase in amylase production over *B.subtilis* 6160 was obtained (Yamaguchi et al., 1974). Very high producers of α-amylase were also obtianed by crossing *B.subtilis* var. *amylosacchariticus* as donor with *B.subtilis* NA20 as recipient (Yoneda et al., 1974). α-amylase production of *B.amyloiquefaciens, B.lichenifermens* and *A. niger* has been enhanced using successive mutagenesis. (Bailey and Markkanen, 1975, Ilczuk et al., 1983, Maruo and Tojo, 1985). *B.subtilis* mutated with NTG and UV irradiation secreted 5 fold more α-amylase activity than the parent strain (Shah et al., 1989).

Enzyme production can be improved by genetic recombination by both an increase in the rate of production and by transferring advantageous characters to high yielding strains. Many thermostable enzymes are produced by thermophiles or extreme thermophiles which may not be suited

to industral enzyme production processes for reasons such as the high temperature growth conditions, lower enzyme titres produced, or non acceptability of the producing strains by regulatory agencies. Recombinant DNA technology provides the opportunity for cloning of the genes for these thermostable enzymes into more suitable industrial enzyme producing organisms (Wasserman, 1984). Ultimately the major commercial potential of cloning α-amylases genes is to obtain increased enzyme yields (Workman et al., 1986). The gene coding for the thermostable α-amylase from *B.licheniformis* has been cloned into B.subtilis (Ortlepp et al., 1983). The investigation of another factor, SacQ, which is also involved in the sporulation process and which has been cloned from the industrial strain *B.licheniformis* revealed a hypersecretion phenotype based on the factor SacQ. Using the synergistic effects of different factors involved in the α-amylase production, new industrial production strains have been developed by mutagenesis and transformation. One of the industrial strains, *B.subtilis* T2 N26, contained the genetic elements, amy R, tmr A7, Pap, amyB, SacU, Clo8 and A-2 and showed a 1500 to 2000 fold icrease in the α-amylase production compared to the parental *B.subtilis* 6160 (Kallio, 1986).

Cloning of the gene for malto hexose producing amylase from an alkalophilic *Bacillus sp* #707 and expression in *B.subtilis* [pTUB 812] produced more than 70 times higher activity in the culture medium than did *Bacillus sp* #707 (Kimura et al., 1988).

1.2.6 Regulation of α-Amylase Synthesis

Extracellular enzymes are generally secreted at a basal level and if conditions are favourable, hydrolyse the substrate in the environment. The low molecular weight products are transported in to the cell and assuming other conditions are satisfied, such as absence of catabolite repression, effect induction of the enzyme, since extracellular enzymes are inducible to varying extents (Priest, 1992).

Amylases of *B.licheniformis*, *B.subtilis* and *B.amyloliquefaciens* are constitutive although the levels of synthesis vary with carbon source. This is generally due to relief of catabolite repression. Whereas amylases of *streptomyces limosus* (Faibairn et al., 1986) and *S. hygroscopicus* (Grafe et al., 1986) are induced upto 30 fold by maltose.

Amylase synthesis in *B.subtilis*, *B.licheniformis* and *B.amyloliquefaciens* is subject to repression by glucose and other rapidly metabolized carbon sources such as mannitol and glycerol (Priest, 1977). Isolation of a mutant of *B.subtilis* (Gra -10) that is catabolite derepressed for amylase synthesis (Nicholson and Chamlis, 1985;86) has shown that catabolite repression is different from, and should not be confused with the derepression of amylase synthesis that occurs towards the end of exponential growth (Priest, 1992). Amylase synthesis in *B.licheniformis* is also subject to catabolite repression. When a promotorless copy of the *B.licheniformis* amylase gene (amyL) was introduced in to *B.subtilis* it remained subject to catabolite repression irrespective of promoter used. This gene contained an operator like sequence similar to that seen in amy RI in the vicinity of the transcription initiation site which was presumably responsible for the repression (Laoide et al., 1989).

In *Bacilli*, synthesis of many inducible and constitutive enzymes is repressed during exponential growth and derepressed during stationary phase. Amylase synthesis is often controlled in this way, in *B.subtilis*, *B.licheniformis* and *B.amyloliquefaciens* (Priest, 1977). In some strains of

B.licheniformis α-amylase is synthesised throughout the growth (Priest & Thirunavukkarasu, 1985), while in others it is only synthesized at the onset of staionary phase (Laoide et al., 1989). Catabolite repression has been observed for all the common commercial enzymes, including the amylases of *B.licheniformis* (Thirunavukkarasu and Priest, 1980) and *B.amyloliquefaciens* (Ingle and Boyer, 1976).

1.2.7 Production Media

Amylase production is based on naturally occuring materials, such as corn, wheat, malt, soy extracts and corn steep liquor. Starch is widely used as the carbon source in production of α-amylase (Fogarty and Kelly, 1980). A variety of nitrogen sources have been used for α-amylase production including ammonium citrate (Allan et al., 1977) and mycological peptone (Upton and Fogarty, 1977). Fukumoto et al., (1957) described a medium for the production of α-amylase from *B.amyloliquefaciens* based on soybean cake extracts (6%), potato starch (3.5% dextrinized with bacterial amylase), $(NH_4)_2PO_4$ (1.2%), KCl and Mg $SO_4.7H_2O$ (0.02%). Studies on amylase production from *B.subtilis* indicated that when carbohydrate was used as the carbon source, phosphate had a

significant effect on enzyme production. The optimum phosphate concentation was in the region of 0.1M. When Casein and soybean extracts were used as nitrogen sources, the choice of carbon source was not critical and starch, most sugars, and organic acids were suitable. Peptones and amino acids provided a more effective source of nitrogen than ammonia (Priest and Sharp 1989). Addition of glycine in a chemically defined medium led to a 300 fold increase in α-amylase production from *B.amyloliquefaciens* ATCC 23350 (Zhang et al., 1983). Ammonium hydrogen phosphate was the prefered source of nitrogen and phosphate for α-amylase production from *B.apirarius* CBML - 152 (Ghosh and Chandra, 1984).

Enzyme production was enhaced by Mn^{2+}, Ca^{2+} and Zn^{2+} although there was little effect on cell growth. Mg^{2+}, Fe^{2+}, Fe^{3+} and Na^{+} enhanced both growth and enzyme production. In a starch based mineral salts medium amino acids including alanine, arginine, asparagine, aspartic acid, cystine, histidine, phenylalanine, lysine and valine stimulated growth and enzyme production. Biotin, pantothenic acid, pyridoxine and thiamine all enhanced growth and enzyme production (Ghosh and Chandra, 1984).

α-Amylase production from *B.licheniformis* CUMC - 305 was supported by alanine, arginine, aspartic acid, cystine isoleucine, phenylalanine and serine. The omission of aspartic acid and cystine resulted in a 50% reduction in enzyme production while $(NH_4)_2 HPO_4$ and peptone were the optimum sources of phosphate and nitrogen. Galactose, dextrin, glycogen and inulin were the optimum sources of carbon, but higher concentrations were inhibitory. Na^{2+}, Cu^{2+}, Mn^{2+}, and Fe^{3+} all stimulated enzyme production (Chandra et al., 1980). For *B.licheniformis*, oilseed cakes in a basal medium containing peptone and beef extract provided an ideal fermentation base for the production of amylase (Krishnan and Chandra, 1983).

Production of a thermostable α-amylase from *B.stearothermophilus* was examined in a defined medium. Maxiumum production was observed on starch and maltose which induced enzyme activity. Glycerol, glucose and sucrose exhibited an inverse relationship between the rate of growth and total amylase production indicating catabolite repression (Welker and Campbell, 1963a). Studies with maltodextrins, as individual constituents, showed maximum enzyme induction with maltotetraose followed by maltopentose maltohexose,

maltotriose and maltose (Welker and Campbell, 1963b). Another strain of *B.stearothermophilus* recorded maximal production of α-amylase on a complex medium based on 0.4% corn steep liquor. Starch was required to induce amylase synthesis and maximal enzyme production was observed in the late exponential and stationary phases of growth. Isoleucine, phenylalanine, cystine, and aspartic acid were essential for enzyme production. Diammonium hydrogen phosphate, ammonium sulfate and ammonium nitrate were used as nitrogen sources. Ca^{2+}, Sr^{2+}, Mg^{2+} and Ba^{2+} slightly stimulated enzyme production, whereas Ag^{2+}, Cu^{2+}, Fe^{2+}, Sb^{2+}, $As^{3+}$and Au^{3+}inhibited enzyme production and, Zn^{2+} and Mn^{2+} had no effect. Growth on glucose, galactose, fructose and sucrose suppressed enzyme production. Amylase was induced with maltotriose, maltotetrose, maltopentose and maltohexose. Maximum production was observed on starch, dextrin and glycogen and the disaccharide cellobiose (Srivastava and Baruah, 1986). In another strain of *B.stearothermophilus*, α-amylase production was induced by starch, amylopectin, maltose and malto oligosaccharides. With glucose, fructose or sucrose as the carbon source only a low basal level of α-amylase as produced (Wind et al., 1994).

1.2.8 Fermentation Process for α-Amylase Production

Laboratory and Pilot-scale fermentation studies of amylase production by a number of different species of *Bacillus* have been reported. These have mainly involved small batch and continuous fermentation studies to evaluate the control of enzyme production, optimization of media, and physical growth parameters.

Continuous culture studies of amylase and protease synthesis by *B.amyloliquefaciens* and some strains of *B.subtilis* have often, after initially inhibiting high enzyme levels on batch culture, been followed by fluctuating enzyme yields that subsequently decline during continous culture (Heinekeh and O' Counor, 1972, Fencl et al., 1972, Sikyta and Fencl, 1976). During α-Amylase production by *B.subtilis*, using a two-stage chemostat, the enzyme concentration in the second stage reached more than 75% of the maximum concentration achieved in batch fermentation (Fencl and Pazlarova,1982).

B.stearothermophilus (Davis et al., 1980), *B.licheniformis* (Emanuilova and Toda, 1984, Priest and

Thirunavakkarasu, 1985), and *B.polymyxa* (Griffin and Fogarty, 1973), in which amylase production occurs during the exponential growth, all exhibited excess levels of amylase production during continuous culture studies.

Chemostat studies of *B.licheniformis*, under nitrogen limitation using glucose, glucose with glutamate, and glucose with alanine as carbon sources moderated minimal production of α-amylase. Under carbon limitation with glucose, alanine, or glutamate as carbon source, amylase production was optimal at a dilution rate (D) of 0.05 hr^{-1} and decreased by more than 5% as D was increased to 0.2 hr^{-1}. Although low growth rate gave an increased rate of enzyme production per gram of substrate, the rate of enzyme production per unit fermentation volume was independent of dilution rate (Meers, 1972).

In batch fermentation studies using soluble starch, a range of polymers from glucose to malto hexose were detected within 10hrs of inoculation. The sugar concentration decreased to a level at which it was no longer detectable and α-amylase production was initiated. Increasing the concentration of glucose significantly diminished the rate of amylase production until the glucose was dissmilated. Glucose

produced in the fermentor, as a result of α-amylase activity, was metabolized and did not accumulate to detectable levels (Priest and Sharp, 1989).

Davis et al., (1980) studied α-amylase production from a strain of *B.Stearothermophilus* in a 1 litre fermentation vessel with air flow at 1.5 vvm in and impeller speed at 300 rpm. In batch culture the pH increased to 8.2 after 3.5 hr, yielding a maximum of 790 U of α-amylase per ml of culture.

Emanuilova and Toda, (1984) studied α-amylase production from *B.caldolyticus* during batch and continous fermentation using a 2 litre fermentor with a working volume of 1.5 liters. Growth temperature was maintained at 60°C, air flow rate of 0.5 vvm and impeller speed 500 rpm. Amylase production occured during exponential growth, when the cell doubling time was approximately 15 min. Mohandass and Chandrasekaran (1994) reported an increase in α-Amylase by *B.polymyxa*, under immobilised state, along with decrease in flow rate, during continuous production in a packed bed reactor.

Continuous culture studies of those strains in which α-amylase production occurs during exponential growth indicate that as the growth rate is increased above a maximum value the level of enzyme activity begins to decrease quite rapidly.

Solid state fermentation (SSF), a cultivation technique for micro-organisms, involves growth of micro-organism and metabolism of the culture on a moist solid substrate in the absence of free water (Lonsane et al., 1982, 1985). SSF technique is used for the commercial production of different exoenzymes such as pectinase, fungal alpha amylases, amyloglucosidases and cellulases since it offers many advantages over submerged fermentation (Lonsane & Karanth, 1990). SSF technique was used to produce amylases using fungi (Alazard & Raimbault, 1981) and bacteria. *B.licheniformis* (Ramesh and Lonsane, 1987 a,b 1989; Ramesh, 1989). Lipase (Aunstrup, 1979, Godfrey, 1983, Munoz et al., 1991) cellulase (Chakal, 1983) and L-glutaminase (Renu, 1991, Nagendra Prabhu and Chandrasekaran, 1995).

1.3 Objectives of the Present Study

The review of literature, presented in the previous section, reveals the fact that the quantum of research work carried out on thermostable α-amylase production by *Bacillus* is restricted to few reports on *B.licheniforms* *B.amyloliquefaciens* and *B.stearothermophilus*. But for the occasional report of one or two workers, there is no detailed study made on thermostable α-amylase production by *B.coagulans*. Hence in the light of the need for thermostable α-amylases in the industry the present study was undertaken using a fast growing *B.coagulans* with a view to explore the prospects of developing a bioprocess for production of thermostable α-amylase.

The specific objectives of the present study include the following .

1. To optimize the growth characteristics of *B.coagulans*
2. To study α-amylase synthesis under submerged fermentation(SmF)
3. To compare the rate of α-amylase synthesis under SmF with respect to batch vs continuous process
4. To study α-amylase synthesis under solid state fermentation

5. To study the effect of UV irradiation and NTG mutagenesis on the yield of α-amylase under SmF

6. To purify and characterize α-amylase

MATERIALS AND METHODS

2.1 Microorganism

Bacillus coagulans ACMN 1, isolated from the soil environment of Cochin University of Science and Technology Campus and, available in the culture collection of the Centre for Biotechnology was used in the present study.

In an earlier study (Nandakumar, 1991) it was observed that this strain produced α-amylase rapidly utilising native starch. Hence this strain was selected for further studies.

Culture was maintained on nutrient agar (HI media)and sub-cultured periodically at regular intervals of 15 days. Stock cultures were maintained in the same medium. Purity of the culture was checked once in a month by repeated streaking on nutrient agar plates.

2.2 Growth Studies

2.2.1 Media

Growth studies were carried out using nutrient broth (HI media) enriched with 1% soluble starch, and mineral salts starch medium (MSSM) (Nandakumar, 1991).

The composition of the mineral salts starch medium (MSSM) is as follows:

$K_2H\ PO_4$	0.1%
KH_2PO_4	0.1%
$(NH_4)_2SO_4$	0.1%
$MgSO_4.7H_2O$	0.05%
$CaCl_2$	0.05%
$NaNO_3$	0.1%
Soluble starch	1%
Distilled water	100 ml
pH	7 ± 0.2

The prepared medium was autoclaved at 121^{o}C for 15 minutes and used.

2.2.2 Preparation of Inoculum

1. A loopful of 24 hrs old agar slope culture of *B.coagulans* was first grown in 10ml of nutrient broth for 18 hrs at room temperature (28 ± 2°C).
2. One ml of the culture broth was then aseptically transferred into 50 ml of nutrient broth, and incubated in a rotary shaker at 150 rpm, for 18 hrs at room temperature.
3. Cells were harvested by centrifugation (Kubota 6700 model) at 10,000 rpm for 15 minutes.
4. The harvested cells were made upto 10ml using sterile physiological saline (0.85% NaCl), after repeated washing with the same.
5. The prepared cell suspension was used as inoculum.

2.2.3 Inoculation and Incubation

The prepared inoculum was aseptically transferred to 100 ml of the medium at 1% (v/v) level (unless otherwise stated) and incubated at room temperature (28 ± 2°C) on a rotary shaker for 24 hrs (unless otherwise specified).

2.2.4 Measurement of Growth

The growth of bacteria in the medium was determined, in terms of turbidity in the culture broth, by measuring absorbance at 620 nm in a UV-Visible spectrophotometer (Shimadzu 160 A). Growth was expressed as optical density (OD).

2.2.5 Determination of Protein

The enzyme protein was estimated following the method of Lowry et al(1951).

1. 1 ml of enzyme added with 5 ml of alkaline reagent
2. Mixed thorouhly and left for 10 minutes.
3. 0.5 ml of Folins reagent diluted with an equal volume of water was added to each tube.
4. After 40 minutes, absorbance was measured at 750 nm in a UV-Visible spectrophotometer.
5. Bovine Serum Albumin was used as standard.
6. Protein was expressed in mg/ml

2.2.6 Optimization of Growth Conditions

Various environmental parameters that influence the growth of bacteria were optimized by subjecting them to different levels of incubation temperatures, pH, substrate concentrations, additional carbon and nitrogen sources, inoculum concentrations and incubation periods.

Growth optimization studies were carried out using both nutrient broth (HI media) and in the mineral salt starch medium (MSSM).

2.2.6.1. Temperature

Optimal temperature for maximal growth was checked by growing the bacteria at various incubation temperatures (25, 30, 35, 40, 45 & 55^{o}C) for a period of 24 hrs. Growth was determined as mentioned under section 2.2.4.

2.2.6.2 pH

Optimal pH required for maximal growth was determined by subjecting the bacteria to various pH levels (pH

2-11.0), adjusted in the media using IN HCl/NaOH. After inoculation and incubation for 24 hrs at room temperature (28 ± 2°C) on a rotary shaker, growth was determined as per the procedure described under section 2.2.4.

2.2.6.3 NaCl Concentration

Optimal requirement of NaCl concentration for maximal growth was determined by subjecting the bacteria to various levels of NaCl concentrations in the media (0-10%). After incubation for 24 hrs on a rotary shaker, growth was measured as detailed under section 2.2.4.

2.2.6.4 Substrate Concentration

Optimal substrate concentration required for maximal growth was checked by growing the bacteria in media supplemented with different concentrations of starch (0.1-2%w/v). After 24 hrs of incubation at room temperature (28 ± 2°C) on a rotary shaker (150 rpm), the growth was estimated according to the procedure given under section 2.2.4.

2.2.6.5. Carbon Sources

The effect of carbon sources other than starch on growth was tested by the addition of mannose, lactose, xylose, galactose, maltose, glycerol and glucose in the media at 0.5% (w/v) level. After 24 hrs of incubation at room temperature (28 ± 2°C) on a rotary shaker, the growth was estimated as mentioned under section 2.2.4.

2.2.6.6 Nitrogen Sources

The effect of additional nitrogen sources that support maximal growth was estimated using beef extract, peptone, yeast extract, tryptone, malt extract, asparagine, aspartic acid, cysteine, proline and glutamic acid individually at 0.5% (w/v) level. After 24 hrs of incubation on a rotary shaker at room temperature (28 ± 2°C), growth was estimated as detailed under section 2.2.4.

2.2.6.7 Inoculum Concentration

Optimal inoculum concentration required for maximal growth was determined by inoculating the medium with different

levels of inoculum (1-10% v/v). After 24 hrs of incubation at room temperature (28 ± 2°C) growth was determined as mentioned under section 2.2.4.

2.2.7 Growth Curve

Growth curve studies were carried out in both nutrient broth enriched with soluble starch and mineral salt starch medium, after optimising the growth conditions.

Prepared inoculum (section 2.2.2) was added at optimal level to the media and incubated for a total period of 48 hrs. Samples were analysed for growth at regular intervals of 60 minutes as detailed under section 2.2.4 and from the values the growth curve was computed.

From the growth curve generation time of the bacteria was also calculated.

2.3 α-Amylase production by *B.coagulans* under Submerged Fermentation (SmF)- Batch process

2.3.1 Medium

α-Amylase production by *B.coagulans* under submerged condition was optimised using mineral salts starch medium (MSSM) mentioned under section 2.2.1.

2.3.2 Preparation of Inoculum

The procedure described under section 2.2.2 was followed for the preparation of inoculum.

2.3.3 Inoculation and Incubation

The autoclaved medium was inoculated with the prepared cell suspension at the optimum level, standardised for maximal growth, and incubated at room temperature (28 ± 2°C), on a rotary shaker (150 rpm) for 24 hrs unless otherwise stated.

2.3.4 Isolation of Enzyme

After incubation, the culture broth was centrifuged at 10,000 rpm for 30 minutes at 4°C, and the cell free extract was used as the crude enzyme for further studies.

2.3.5 Buffers used for Enzyme Assay

Enzyme assay was standardised using different buffers towards selection of suitable buffer that promote maximal enzyme activity. They included acetate buffer(4-6) phosphate buffer (7-8) and NaOH-glycine buffer (9-11).

2.3.6 Assay of α-Amylase

α-Amylase was assayed according to Medda & Chandra, (1980), as detailed below.

1. One ml of the cell free extract was incubated with one ml of 1% soluble starch and one ml of phosphate buffer (0.1M), pH 8, (unless otherwise stated) at 40°C for 10 minutes.
2. The reaction was arrested by the addition of 0.1ml of IN HCl.

3. One ml of the above sample was added with 0.1ml of I_2-KI reagent and the volume was made up to 15 ml with distilled water (DW).
4. The blue color developed was measured at 650 nm in a UV-Visible spectrophotometer.
5. One unit of alpha-amylase activity was defined as the amount of enzyme required to bring about the hydrolysis of 0.1mg of starch at 40°C in 10 min., at pH 8.

2.3.7 Impact of Process Parameters on α-Amylase Production by *B.coagulans* under Submerged Fermentation

2.3.7.1 Temperature

The effect of temperature on α-amylase production by *B.coagulans* was estimated by incubating the inoculated medium at various temperatures (25, 30, 35, 40, 45, 50 and 55°C) for a period of 24 hrs. After centrifugation the enzyme production was determined using the cell free extract as described under the section 2.3.6.

2.3.7.2 pH

Impact of pH on α-amylase production by the bacteria was determined by subjecting the bacteria to various pH levels adjusted in the medium using IN HCl/NaOH. After incubation for 24 hrs at room temperature (28 ± 2°C), on a rotary shaker, the culture broth was centrifuged and enzyme was assayed using the cell free extract as per the procedure described under section 2.3.6.

2.3.7.3 Substrate Concentration

Effect of substrate concentration on enzyme production by bacteria was checked by the addition of soluble starch at various concentrations (0-3%) in the medium. After 24 hrs of incubation at room temperature (28 ± 2°C) on a rotary shaker (150 rpm), the enzyme production was estimated according to the procedures given under section 2.3.6.

2.3.7.4 NaCl Concentration

The effect NaCl on enzyme production by bacteria was carried out by incorporating NaCl at various levels in the

media (0-10%). After incubation for 24 hrs, enzyme production was measured according to the procedure mentioned under section 2.3.6.

2.3.7.5 Carbon Sources

The effect of additional carbon sources, other than starch, on enzyme production by bacteria was tested by incorporating maltose, mannose, lactose, galactose, xylose, glycerol and glucose in MSSM broth, individually at 0.5% (w/v) level. After 24 hrs of incubation at room temperature (28 ± 2°C) on a rotary shaker, the enzyme production was estimated as detailed under section 2.3.6.

2.3.7.6 Nitrogen Sources

The effect of different nitrogen sources on enzyme production by bacteria was estimated by the addition of peptone, beef extract, yeast extract, tryptone, malt extract and amino acids viz, asparagine, aspartic acid, cystine, proline and glutamic acid, individually at 0.5% (w/v) level in the MSSM. After incubation for 24 hrs on a rotary shaker at

room temperature (28 ± 2°C) enzyme activity was assayed as mentioned under section 2.3.6.

2.3.7.7 Metal Ions

Effect of metal ions on enzyme production by bacteria was carried out by incorporating Cu^{2+}, Hg^{2+}, Zn^{2+}, Sn^{2+}, Cd^{2+}, Mn^{2+}, Fe^{2+} and Ag^{+} each individually at 0.1% (w/v) level in the medium. After 24 hrs of incubation at room temperature (28 ± 2°C) on a rotary shaker, enzyme production was estimated (section 2.3.6).

2.3.7.8 Inoculum Concentration

Effect of inoculum size on enzyme production by bacteria was determined in MSSM broth with various levels of prepared inoculum (1-10%). After 24 hrs of incubation at room temperature (28 ± 2°C) on a rotary shaker enzyme production was estimated as mentioned under section 2.3.6.

2.3.7.9 Incubation Period

Impact of incubation period on enzyme yield by bacteria was determined, at optimized levels of process parameters in MSSM, by analysing the enzyme yield at various time intervals of incubation up to a total period of 42 hrs. After centrifugation, the cell free extract was assayed for α-amylase as mentioned under section 2.3.6.

2.4 α-Amylase Production by *B.coagulans* under Continuous Fermentation

Continuous fermentation production of α-amylase by of *B.coagulans* was performed in 500ml of MSSM (section 2.3.1) added with 0.1% maltose in a Mini fermenter (Eyela) with a capacity of 1.5 litres, at 35°C, pH 8, aeration 1vvm and agitation rate 150 rpm, adopting the method suggested by Nakumura et.al., (1979) with slight modifications.

After precultivation for 8 hrs (which was the optimal time for the bacteria to remain under steady state in log phase) under batch process the fermentation was converted into a continuous process by supplying nutrient by means of a

peristaltic pump and the fermented liquid was withdrawn through the outlet of the fermenter. Experiments were carried at 3 flow rates (20, 35, and 50 ml/hr) at the aeration rate of 1VVM. Individual experiments were conducted for the three different flow rates of the medium, in triplicate. Careful attention was paid to guarantee a constant feed rate in each run and 5-6 samples were withdrawn to confirm whether a steady state was attained at each flow rate before sampling was done for enzyme assay. At steady state samples were withdrawn, centrifuged and supernatent was used for enzyme assay as mentioned under section (2.3.6). Cell density was measured in terms of turbidity (OD at 620nm)

2.5 α-Amylase Production by *B.coagulans* under Solid State Fermentation (SSF)

α-Amylase production under SSF was carried out following the methods suggested by Ramesh and Lonsane (1987). However necessary changes were made in the medium composition and methodology after optimization of process parameters.

2.5.1 Preparation of Solid Substrate

Commercially available wheat bran (particle size < 2400 μ), unless otherwise stated, was used as solid support for production of α-amylase.

2.5.2 Preparation of Solid Substrate Medium

10 gm of wheat bran, taken in a 250ml Erlenmeyer flask, was thoroughly mixed with mineral salts medium (composition given under section 2.3.1 added with 1% maltose) to impart 60% moisture content (unless otherwise stated). The contents were autoclaved for 60 minutes and used after cooling down to room temperature (28 $\pm$ 2°C).

2.5.3 Preparation of Inoculum

Inoculum was prepared as per the procedures mentioned under section 2.2.2.

2.5.4 Inoculation and Incubation

The prepared inoculum was adjusted to a concentration of 2mg dry cell equivalent in one ml of cell suspension and added to the sterilised moist wheat bran medium in the flask giving a final inoculum to bran ratio of 1:10 (v/w)(unless otherwise stated). The inoculated bran medium was mixed thoroughly and the flasks were incubated in a slanting position at 35°C in an incubator with 60-70% relative humidity,for 48 hrs (unless otherwise stated).

2.5.5 Extraction of Enzyme

After SSF, alpha amylase from the bacterial wheat bran was extracted following the procedures recommended by Ramesh and Lonsane, (1987) using phosphate buffer (0.1M, pH 7) at a bran buffer ratio of 1:10 and a contact time of 30 minutes. Enzyme was extracted using dampened of cheese cloth. The process was repeated twice, extracts pooled and centrifuged at 10,000 rpm in a refrigerated centrifuge at 4°C, 15 minutes. The supernatent was used as crude enzyme for alpha amylase assay.

2.5.6 Enzyme Assay

Alpha amylase was assayed as mentioned under section 2.3.6. Enzyme activity is expressed as U/gram dry substrate (U/gds).

2.5.7 Influence of Process Parameters on Enzyme Production by *B. coagulans* during SSF

Effect of process parameters viz., particle size, moisture content, temperature, pH, substrate concentration, carbon sources, nitrogen sources, inoculum concentration, and incubation period on enzyme production by bacteria during SSF were studied as detailed below. Preparation of solid substrate medium, inoculation and incubation, extraction and assay of enzyme were carried out as given under section 2.5.2 to 2.5.5 respectively.

2.5.7.1 Particle Size

Commercial wheat bran contained particles of different sizes. Hence, using standard sieves (ASTM) of

different mesh size the wheat bran particles were graded to various sizes ranging from (425 μ to 1400μ) and their impact on enzyme production by bacteria was studied under SSF.

2.5.7.2 Initial Moisture Content

The effect of initial moisture content of the medium on enzyme production by bacteria was determined by preparing the wheat bran medium with varying initial moisture content (30-70%).

2.5.7.3 Temperature

Effect of temperature on enzyme production by bacteria during SSF was estimated by incubating the inoculated SSF medium at different temperatures (30 to 55°C).

2.5.7.4 pH

Effect of initial pH on enzyme production by bacteria during SSF was estimated by adjusting the pH of the mineral salt medium used to moisten the substrate to various levels (pH 4-11).

2.5.7.5 Carbon Sources

Effect of carbon sources on enzyme production by during SSF was determined by incorporating glucose, galactose, maltose, glycerol, mannose, sucrose, xylose and lactose individually at 1% concentration (w/w) to the wheat bran medium.

2.5.7.6 Nitrogen Sources

Effect of different nitrogen sources on enzyme production by bacteria under SSF was estimated by the addition of NH_4Cl, $(NH_4)_2SO_4$, asparagine, aspartic acid, cysteine, proline, glutamic acid, tryptone, yeast extract, beef extract and malt extract individually to the wheat bran medium at 1% (v/w) level.

2.5.7.7 Inoculum Size

Effect of inoculum size on enzyme production by bacteria during SSF was estimated by using different levels of inoculum concentration (1-10% v/w).

2.5.7.8 Incubation time

Effect of incubation time on enzyme production by bacteria during SSF was determined by incubating the inoculated wheat bran medium upto a total period of 102 hrs and estimating the enzyme production at regular intervals of 12 hrs.

2.6 Mutation Studies

Effect of mutation, uv irradiation and chemical mutagenesis on α-amylase production *B.coagulans* was studied by following the methods suggested by Kunthala Jayaraman and Jayaraman (1979).

2.6.1 Medium

Nutrient Broth (HI media) was used for cultivation of cells and subculture studies. α-Amylase production was estimated in mineral salt starch medium (section 2.3.1) added with 0.5% Starch and 0.1% maltose, with pH 8 (after optimisation studies).

2.6.2 Preparation of Cell Suspension

1. Bacterial cells from the log phase culture grown in nutrient broth was centrifuged (10,000 rpm; 15 minutes), washed in sterile physiological saline repeatedly, and suspended in 25 ml of sterile phosphate buffer.
2. The cell density in the cell suspension was adjusted to 10^{8} cell/ml with sterile buffer.

2.6.3 Mutagenesis using Ultra Violet Irradiation

1. One ml of the cell suspension was serially diluted to 10^{-1} to 10^{-8} and plated on nutrient agar. The plates were incubated overnight at room temperature ($28\pm2^{o}C$ and total viable counts were made. The data obtained was used as control.
2. 15 ml of the cell suspension was taken in sterile petridish with the lid open and was exposed to UV irradiation from the source of UV lamp at about a distance of 15 cm. Care was taken to switch on the UV lamp 15 minutes before the commencement of experiment in order to stabilize the emission.

3. 1 ml aliquot of samples, in duplicate, were drawn at regular intervals of 0, 20, 40, 60, 80, 100, 120 seconds after exposure to irradiation into sterile test tubes.
4. One set of samples were wrapped with dark paper and stored in the refrigerator overnight.
5. The other set of irradiated cells were serially diluted, 10^{-4} to 10^{-6} dilutions for the early sample upto 60 seconds, and 10^{-1} to 10^{-4} dilutions for the late samples.
6. Diluted samples were plated on nutrient agar and incubated overnight at room temperature (28 ± 2°C).
7. Total viable count in each plate was made and the survivors per ml for each sample were calculated.
8. Using a semi log graph, a graph was plotted with time of exposure to UV vs survivors. Optimal time for mutagenesis was determined based on 99% lethality.

2.6.4 Mutagenesis with NTG (N-Methyl-N'-nitro-N'-nitrosoguanidine)

1. Aquous solution of NTG was prepared using distilled water and filter sterilised. The final concentration was 1mg/1ml.
2. This NTG solution was dispensed in duplicate in ependoroff tubes with concentrations varying from 10μg - 100μg. One ml of the prepared cell suspension, in nutrient broth was added to the NTG solution in ependoroff tubes, and incubated for 30 minutes at 30°C with shaking.
3. Cells were separated by centrifugation at 10,000 rpm for 20 minutes, washed with sterilized phosphate buffer repeatedly, and resuspended in 50 ml of the same buffer.
4. 0.1ml of the cell suspension was transferred to nutrient broth, and rest of the solution was stored in refrigerator.
5. Inoculated nutrient broth was incubated overnight on a rotary shaker at 30°C.
6. Cells were harvested by centrifugation at 10,000 rpm after 15 minutes, washed with sterile phosphate buffer repeatedly, and used for α-amylase production studies.

2.6.5 α-Amylase Production by UV-irradiated and NTG Treated cells

Cells, obtained after UV irradiation and NTG treatment, were checked for α-amylase production using mineral salt starch medium (mentioned under section 2.3.1) added with 0.5% starch and 0.1% maltose at pH 8. Preparation of inoculum, inoculation, incubation, and enzyme assay were carried out as mentioned under section 2.3.2, 2.3.3 and 2.3.6 respectively.

2.7 PURIFICATION OF THE ENZYME

α-Amylase was purified by ammonium sulphate fractionation followed by dialysis, ion exchange chromatography and electrophoresis.

2.7.1 $(NH_4)_{2.}SO_4$ fractionation

(a) Finely powdered $(NH_4)_2SO_4$ (enzyme grade, SRL) was added slowly and increasing the concentration upto 40% saturation, along with continuous stirring using a magnetic stirrer, at 4^oC in an ice bath.

(b) The precipitate obtained was removed by centrifugation at 10,000 rpm, 20 min. at 4^oC.

(c) Then supernatant was added with $(NH_4)_2.SO_4$ to increase the concentration slowly upto 80% saturation. The precipitate obtained for each concentration (50, 60, 70 and 80 %)was removed by centrifugation at 10,000 rpm for 20 min. at 4^oC

(d) The precipitate was resuspended in minimum volume of 0.1M phosphate buffer of pH-8.

(e) Enzyme activity and protein content of each fraction was determined.

2.7.2 Dialysis

(a) The fractions obtained by $(NH_4)_2.SO_4$ precipitation were dialyzed overnight against the same buffer with occasional changes in the buffer at 4^oC.

(b) The protein content and enzyme activityof the dialyzate were determined.

2.7.3 Chromatography upon DEAE Cellulose

The dialyzate was concentrated by lyophilization in a lyophilizer (Yamoto, Neocool) and subjected to further

purification by anion exchange chromatography upon DEAE Cellulose.

(1).DEAE Cellulose was packed in a column (5 x 30 cm) and kept at $4^{o}C$.

(2).The packed column was equilibrated with phosphate buffer of pH-8.5 (0.1 M).

(3).The dialyzate was adjusted to pH 8.5 with 0.1 M NaOH and added on to the top of the column.

(4).The column was washed with phosphate buffer of pH 8.5 (0.1 M)

(5).The enzyme was eluted using phosphate buffer(0.1 M) of pH 8 containing different concentrationsofNaCl (0-1M) in a sequential manner along with increasing concentration of NaCl in buffer. Flow rate was adjusted to 3 ml / 5 min and the fractions were collected using a Redifrac fraction collector (Pharmacia).

(6).Each fraction was analyzed for enzyme activity and protein (section 2.2.5 and 2.3.6).

(7).The fractions with higher specific activities were pooled and dialyzed against dilute sodium phosphate buffer of pH 8 (0.1 M).

(8).The dialyzed fraction were concentrated by lyophilization in a lyophilizer (Yamato Neocool) Neocool)

2.7.4. Electrophoresis

The purified fractions were further subjected to electrophoretic studies. Polyacrylamide gel electrophoresis (PAGE) was performed under denaturing and nondenaturing conditions. The methodology of Laemmili (1970) was followed.

2.7.4.1 Preparation of gel

Slab gel electrophoresis was done using the modified Studier apparatus using the notched glassplate system. Thin perspex spacers of uniform thickness of 1.5 mm were used to get a gel of 1.5 mm thickness.

Two gel layers, a stacking gel polymerised on top of the resolving gel, were prepared using 2 buffer systems. Sample wells were prepared in the stacking gel using a perspex comb (10 wells) of 1.5 mm thickness.

2.7.4.1.1 Native PAGE

Stacking gel composition is as given below:

Acrylamide-bisacrylamide (30:0.8 g/100 ml DW)	2.5 ml
Stacking gel buffer stock	5.0 ml
1.5% Ammonnium persulphate	1.0 ml
Distilled water	11.5 ml
TEMED	0.015 ml

Resolving gel of 7.5% and 10% were tried.

Composition of 5% gel

Acrylamide-bisacrylamide	5.0 ml
Resolving gel buffer stock	3.75 ml
1.5% ammonium persulphate	1.5 ml
Distilled water	19.75 ml
TEMED	0.015 ml

Composition of 7.5% gel

Acrylamide-bisacrylamide	7.5 ml
Resolving gel buffer stock	3.75 ml
1.5% Ammonium persulphate	1.5 ml
Distilled water	17.25 ml
TEMED	0.015 ml

2.7.4.1.2 SDS-PAGE (denaturing)

Stacking gel composition

Acrylamide-bisacrylamide	2.5 ml
Stacking gel buffer stock	5.0 ml
10% SDS	0.2 ml
1.5% Ammonium persulphate	1.0 ml
Distilled water	11.3 ml
TEMED	0.015 ml

Resolving gel 7.5% and 10% were tried.

Composition of 7.5% gel

Acrylamide-bisacrylamide	7.5 ml
Resolving gel buffer stock	3.75 ml
10% SDS	3.0 ml
1.5% Ammonium persulphate	1.5 ml
Distilled Water	16.95 ml
TEMED	0.015 ml

Composition of 10% gel

Acrylamide-bis-acrylamide	10.0 ml
Resolving gel buffer stock	3.75 ml
10% SDS	0.3 ml
1.5% Ammonium persulphate	1.5 ml
Distilled Water	14.45 ml
TEMED	0.015 ml

2.7.4.2. Preparation of buffer

2.7.4.2.1 Stacking gel buffer stock

Tris-HCl (pH 6.8):

6 g of Tris was dissolved in 40 ml of distilled water and titrated to a pH of 6.8 with 1M HCl (approx. 48 ml). Distilled water was added to 100 ml final volume.

2.7.4.2.2 Resolving gel buffer stock

Tris-HCl (pH 8.8):

36.3 g of Tris and 48 ml 1 M HCl were mixed and made up to final volume of 100 mlof 100 ml

2.7.4.2.3 Reservoir buffer

2.7.4.2.3.1 Native PAGE

Tris-glycine (pH - 8.3) :

3 g of Tris and 14.4 g of glycine were dissolved in DW and made to 1 l with distilled water.

2.7.4.2.3.2 SDS PAGE

Tris-glycine-SDS (pH 8.3) :

3 g of Tris, 14.4 g of glycine and 1 g of SDS were dissolved in and made to 1 l with water.

2.7.4.3 Sample Preparation

2.7.4.3.1 Native PAGE

Sample was prepared as given below at 4^{o} C.

Protein sample solution	100 μl
Bromophenol blue	40 μl
100% Sucrose	20 μl

2.7.4.3.2 SDS PAGE

Sample was prepared as given below:

Protein sample solution	100 μl
Bromophenol blue	40 μl
10% SDS	40 μl
100% Sucrose	20 μl
β-mercaptoethanol	10 μl

Mixed well and heated in a boiling water bath for 3 min.. After heating, the sample was cooled to room temperature. Insoluble materials if present were removed by centrifugation.

2.7.4.4 Sample loading

Slab gel was attached to the electrophoretic apparatus before sample loading. Reservoir buffer was poured into the reservoirs. Any air bubble trapped in the bottom of the gel was removed by using a Pasteur pipette with a bent tip. Leak was also checked. 100 μl of sample was carefully loaded on to the gel surface using a micropipette.

2.7.4.5 Running of the electrophoresis

2.7.4.5.1 Native PAGE

Native PAGE was performed in a cold room (10^{o} C) to minimise the heating due to electrophoresis. A constant voltage of 50 V was applied until the sample entered the resolving gel. Then it was electrophoresed at a constant voltage of 75 V for 10 hrs.

2.7.4.5.2 SDS PAGE

SDS-PAGE was performed at room temperature. A constant voltage of 50 V was applied until the sample entered the resolving gel. Then it was electrophoresed for 7 hrs at a constant voltage of 75 V.

2.7.4.6 Detection of bands

2.7.4.6.1 Protein staining

After electrophoresis the gel was put in Coomassie brilliant blue solution (0.1% in water:methanol:glacial acetic acid (5:5:2))in a gel staining tray. Gel was stained fully for about 6hrs at room temperature (28 +/- 2^{o} C).

2.7.4.6.2 Destaining

After staining, the excess stain was removed by putting the gel in destaining solution (30% methanol, 10% acetic acid). The destaining solution was renewed regularly as stain leaches out of the gel. The stained bands were viewed using an illuminator (Hofman). The destained gel was preserved in sealed plastic bags containing 7% acetic acid.

2.7.4.6.3 Detection of amylase

After electrophoresis, the gel was incubated for 1hr at 30^{o}C in 0.1 molar phosphate buffer, pH8 containing 1% soluble starch. After two washes with distilled water, light lanes(representing starch hydrolisis area of amylase activity) were detected by immersing the gel in lougols solution (Giraud et al,1993)

Table 4- Characteristic Properties of Bacillus coagulans

Properties Studied	
Source	Soil
Colony morphology	
Shape	Irregular
Colour	Off white
Surface	Smooth flat
Consistency	Opaque
Cell Morphology	Long thick rods
Gram reaction	+
Motility	+
Spore	+
Shape	Elliptical
Dominant Position	Central
Swelling	+
Biochemical Characters	
Catalase	+
Fermentation of Glucose	Fermentative
Arabinose	—
Xylose	—
Mannitol	—

Hydrolyses of	
Urea	+
Starch	+
Cellulose	+
Casein	+
Gelatin	+
Lipid	—
Utilisation of citrate	+
NO_3 — NO_2	—
Voges — Proskauer test	—
Voges — Proskauer pH	4 - 5
NH_3 Production	—
Production	+
Growth at 5% NaCl	+
Growth at 7% NaCl	—

Table 5 Composition of Wheat Bran

Constituents	%
Moisture	8.80
Total nitrogen	2.33
Fat	4.10
Fibres	10.80
Pentosan	25.10
Ash	6.38
Total sugar	5.40
P_2O_5	3.15
Carbohydrate content	
Starch	14.1
Sugar	7.60
Cellulose	35.20
Hemi cellulose	43.10

Source: CFTRI, Mysore.

RESULTS

3.1 Growth Studies:

3.1.1 Optimization of Growth Conditions

3.1.1.1 Temperature

Effect of temperature on growth was tested in nutrient broth containing 1 % starch at different temperatures viz., 25 to 55°C. Results presented on Fig.1 suggest that *B.coagulans* preferred an optimal temperature of 35°C for maximal growth although it could record significant levels of growth at 30°C. Higher temperature of 55°C did not favour growth.

3.1.1.2 pH

Effect of pH on growth of *B.coagulans* was determined by subjecting them to various levels of pH (4-11) in the nutrient broth enriched with 1 % starch. From the results presented in Fig.2 it is inferred that the bacteria could achieve significant levels of growth over a wide range of pH 7-11, with an optimum at pH 9. The organism could not grow well at acidic pH (4-6) conditions.

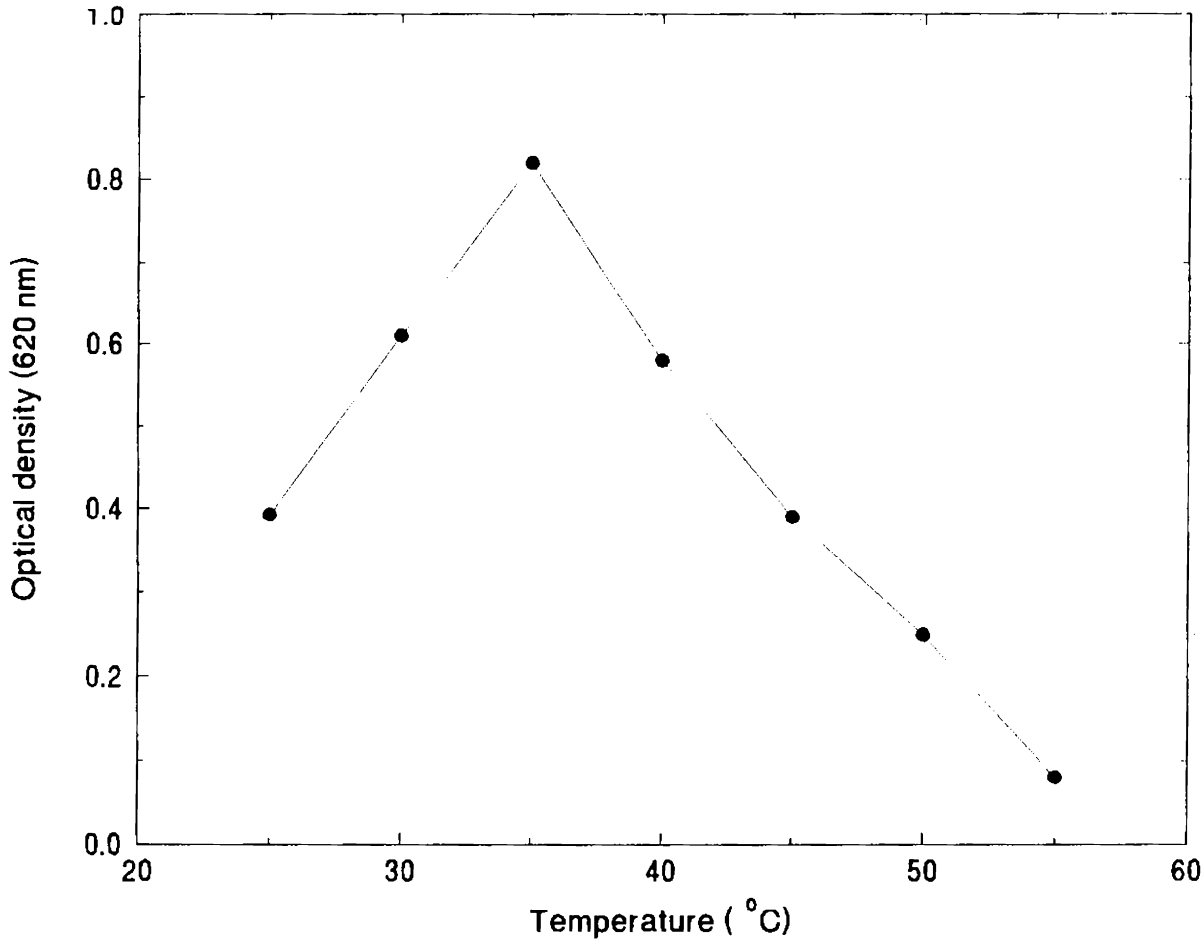


Figure 1. Effect of temperature on growth of B.coagulans.

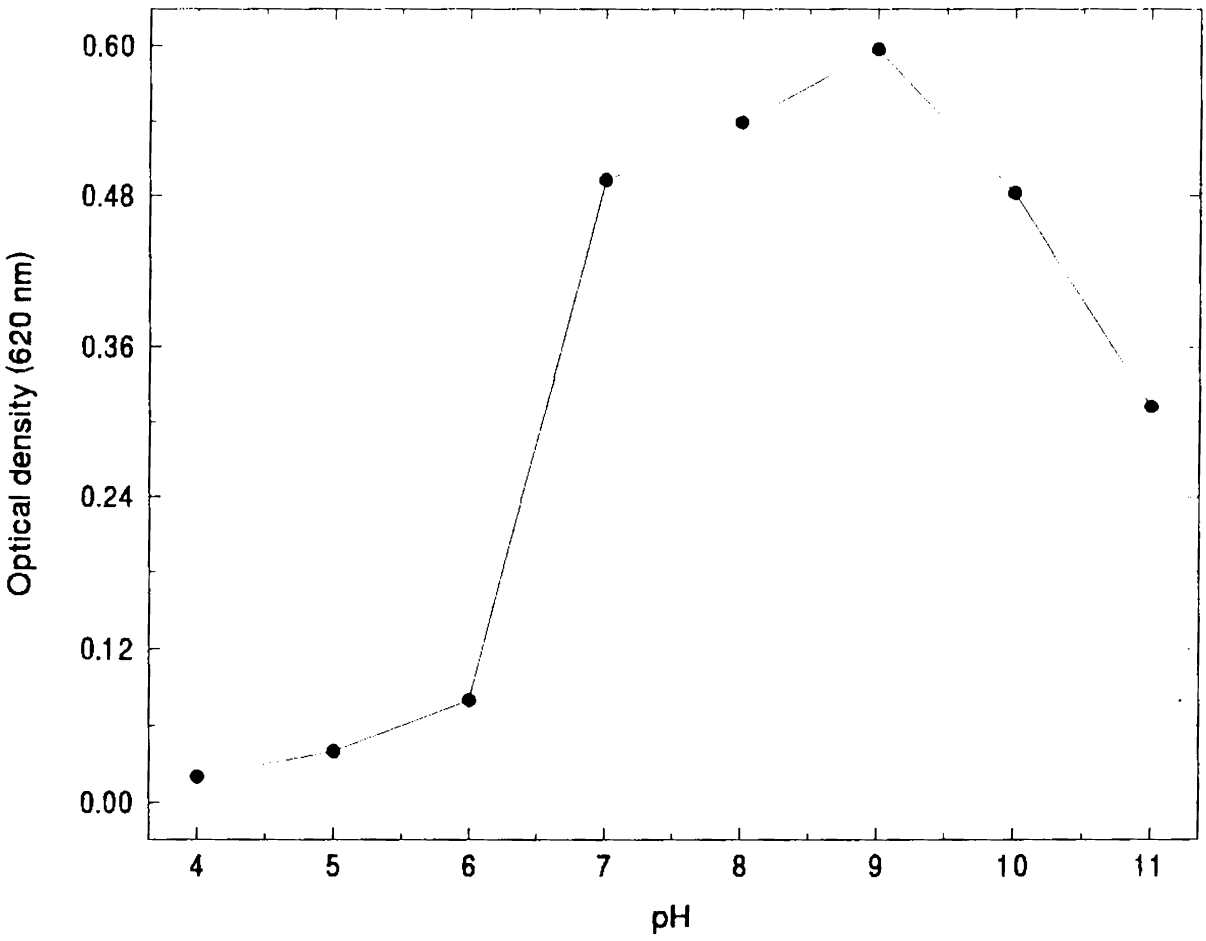


Figure 2. Effect of pH on growth of B.coagulans.

3.1.1.3 Substrate Concentration

Effect of substrate (starch) concentration on growth was tested at concentrations ranging from 0.5 to 5% in the nutrient broth and the results are presented in Fig.3. Significant levels of growth was recorded at concentration ranging from 0.5 to 4%,while maximal level of growth occurred at 1% substrate concentration. Increase in the concentration of substrate from 1 to 4% did not influence the growth rate while 5 % inhibited the growth.

3.1.1.4 Carbon sources

The effect of carbon sources on growth was tested by incorporating different carbon sources in the nutrient broth at 1% level. Data presented on Fig.4 indicated that all carbon sources favoured significant level of growth. It was noticed that the pH of the medium was reduced when the carbon sources were utilised.

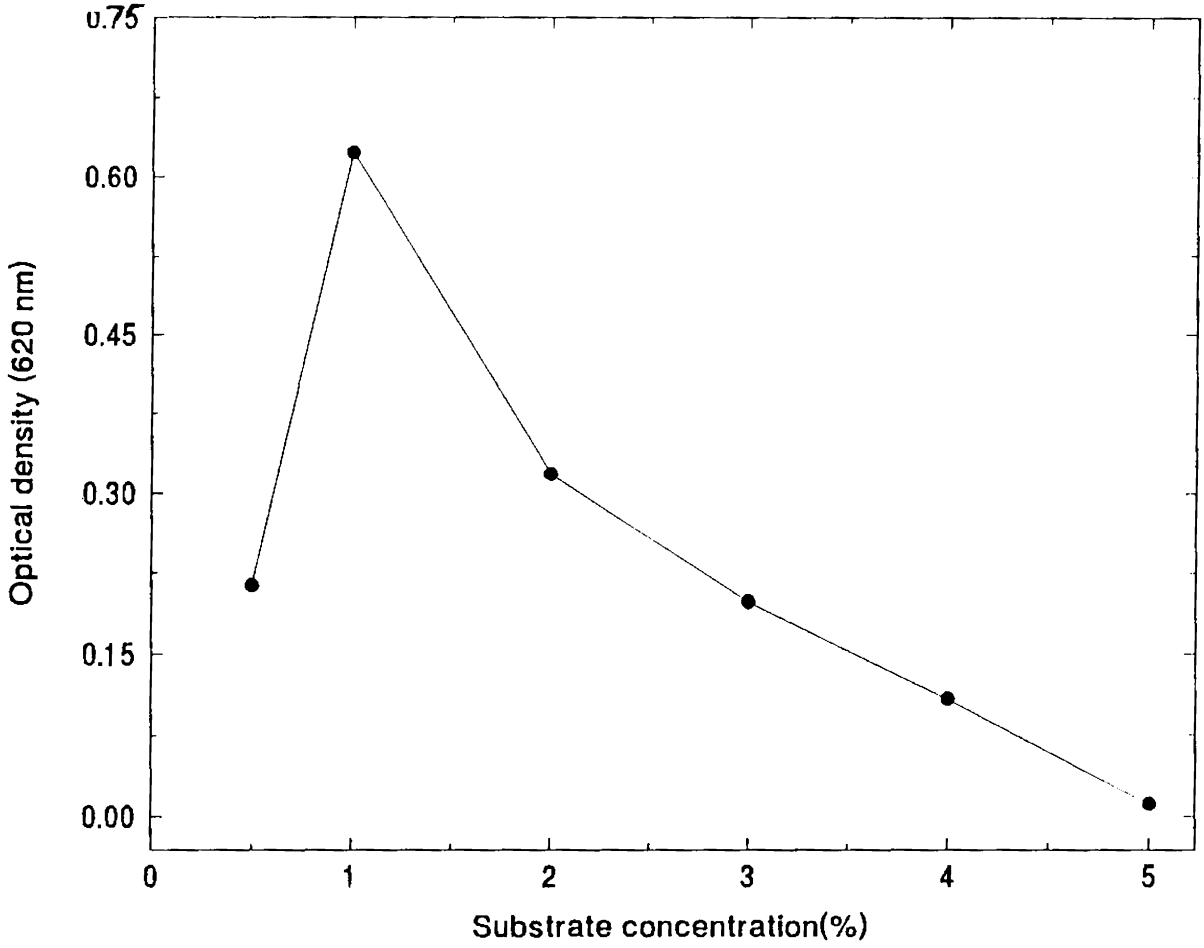


Figure 3. Effect of substrate (starch) concentration (% w/w) on growth of B.coagulans.

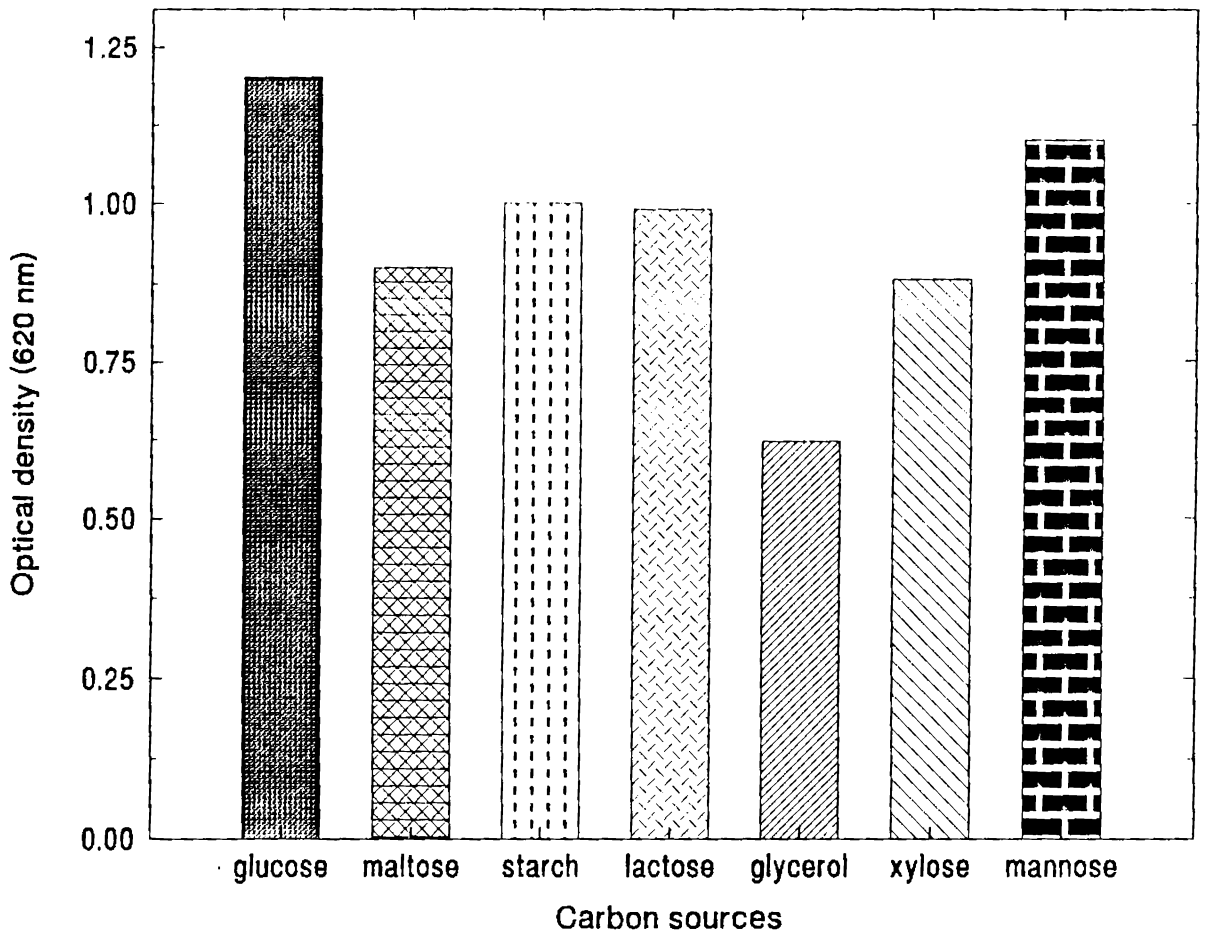


Figure 4. Effect of carbon sources on growth of B.coagulans.

3.1.1.5 Nitrogen Sources

The effect of nitrogen sources on growth was determined by incorporating various nitrogen sources in nutrient broth and the results are shown in Fig.5. Of the different nitrogen sources tested tryptone supported maximum growth. Peptone, beef extract and yeast extract could support significant levels of growth, while inorganic nitrogen sources, did not encourage fair growth.

3.1.1.6 Sodium Chloride

Impact of NaCl concentrations on growth was tested at various levels of NaCl (Fig.6). Maximal levels of growth was observed in the absence of NaCl. Further, the growth declined progressively along with increase in NaCl concentration.

3.1.1.7 Inoculum Concentration

The effect of inoculum concentration on growth was tested by inoculating the medium with different concentration of inoculum. Results presented in Fig.7. suggested that

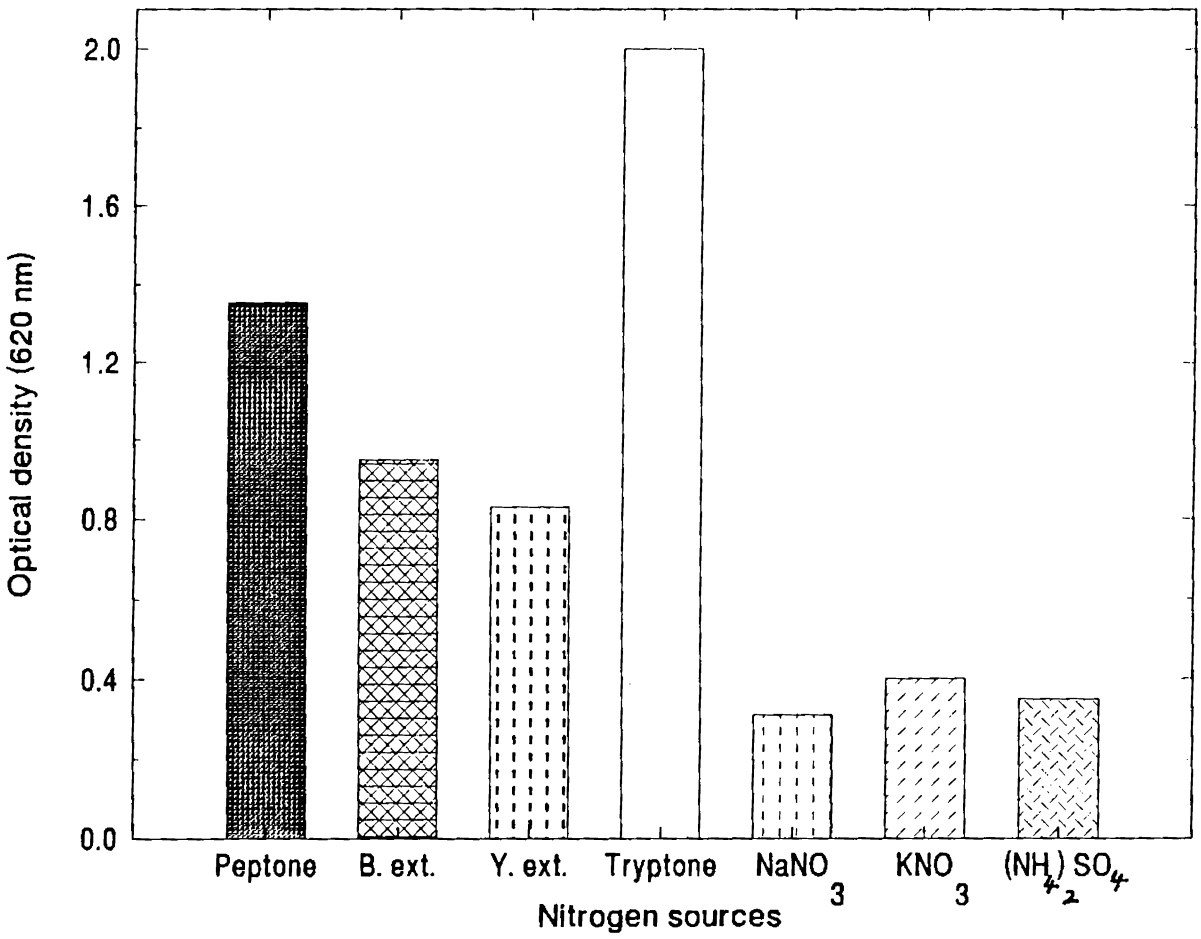


Figure 5. Effect of nitrogen sources on growth of B.coagulans.

B. ext. - Beef extract, Y. ext. - Yeast extract.

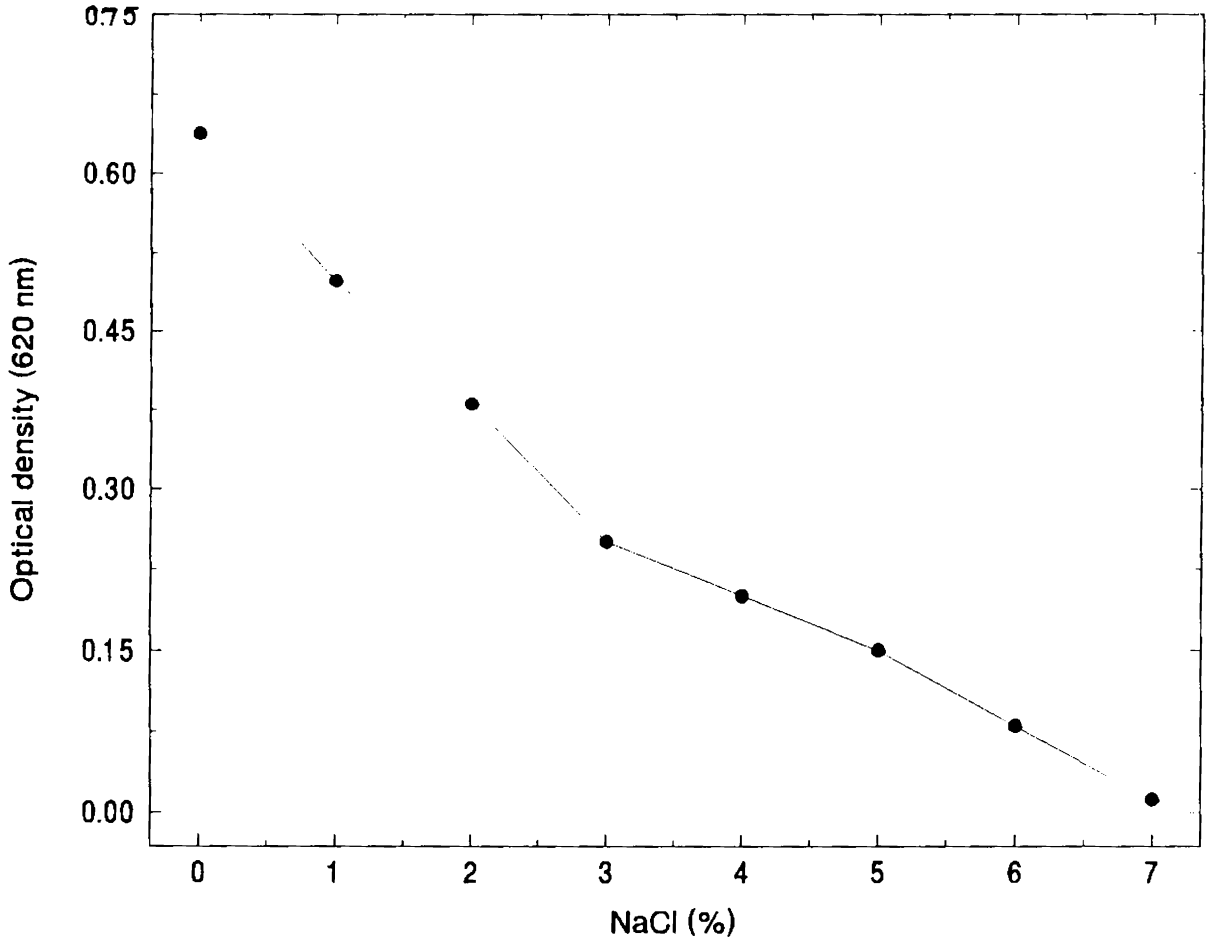


Figure 6. Effect of sodium chloride concentration (% w/w) on growth of B.coagulans.

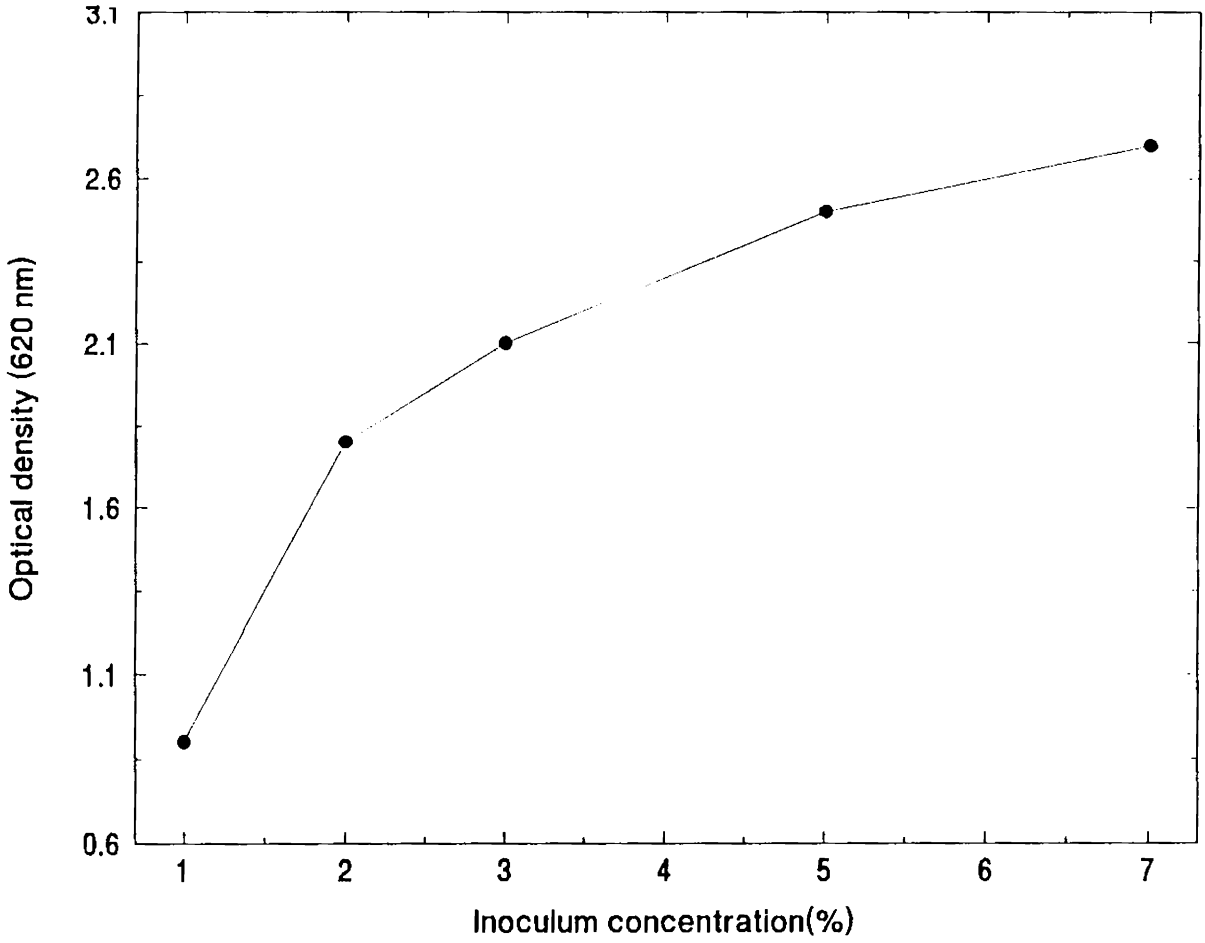


Figure 7. Effect of inoculum concentration (% v/v) on growth of B.coagulans.

significant growth could be attained at all the concentrations of inoculum tested. It was observed that, 2% inoculum is adequate for obtaining significant levels of growth.

3.1.1.8 Incubation Time

Effect of incubation period on growth was tested in at their optimal growth conditions for varying periods of incubations. Data presented in Fig.8 indicate that, maximum growth occurred at 18 hrs of incubation. Incubation after 24 hrs led to decline in growth.

3.1.2 Growth Curve and Generation Time

Growth curve obtained for *B.coagulans* is presented in Fig.8. From the results it is clearly evident that *B.coagulans* enter into the logarithmic phase after 2 hrs and continued till 12 hrs. A long stationary phase is noted, which extend upto 24 hrs. The generation time for the organism was recorded as 57.55 minutes.

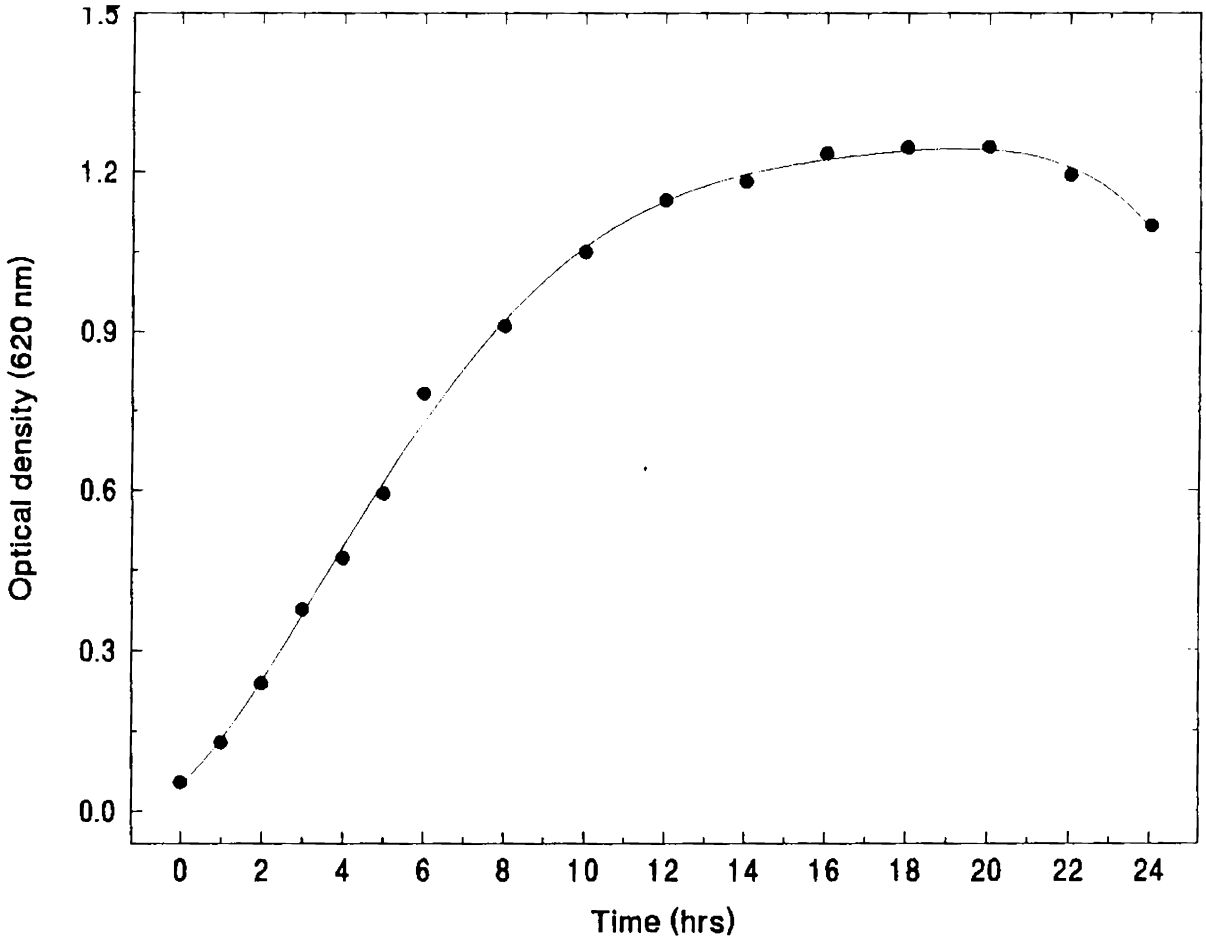


Figure 8. Growth curve of B.coagulans.

3.2 α-Amylase Production by *B.coagulans* under Submerged Fermentation (SmF)- Batch Process

Initially the process parameters that influence enzyme production during SmF under batch process were studied.

3.2.1 Effect of Temperature

Effect of temperature on α-amylase production was tested at different temperatures viz. 25 to 55°C. Results presented in Fig.9 suggest that the bacteria required an incubation temperature of 35°C for maximal production of α-amylase (108 U/ml). However, they could also record significant levels of amylase production at 30 and 40°C. Incubation at higher temperatures above 40°C showed a decline in amylase production. In general amylase production recorded at various temperatures varied between minimum 4 U/ml- maximum 108 U/ml.

3.2.2 Effect of pH

Effect of pH on α-amylase production was determined by subjecting them to various levels of pH adjusted in the

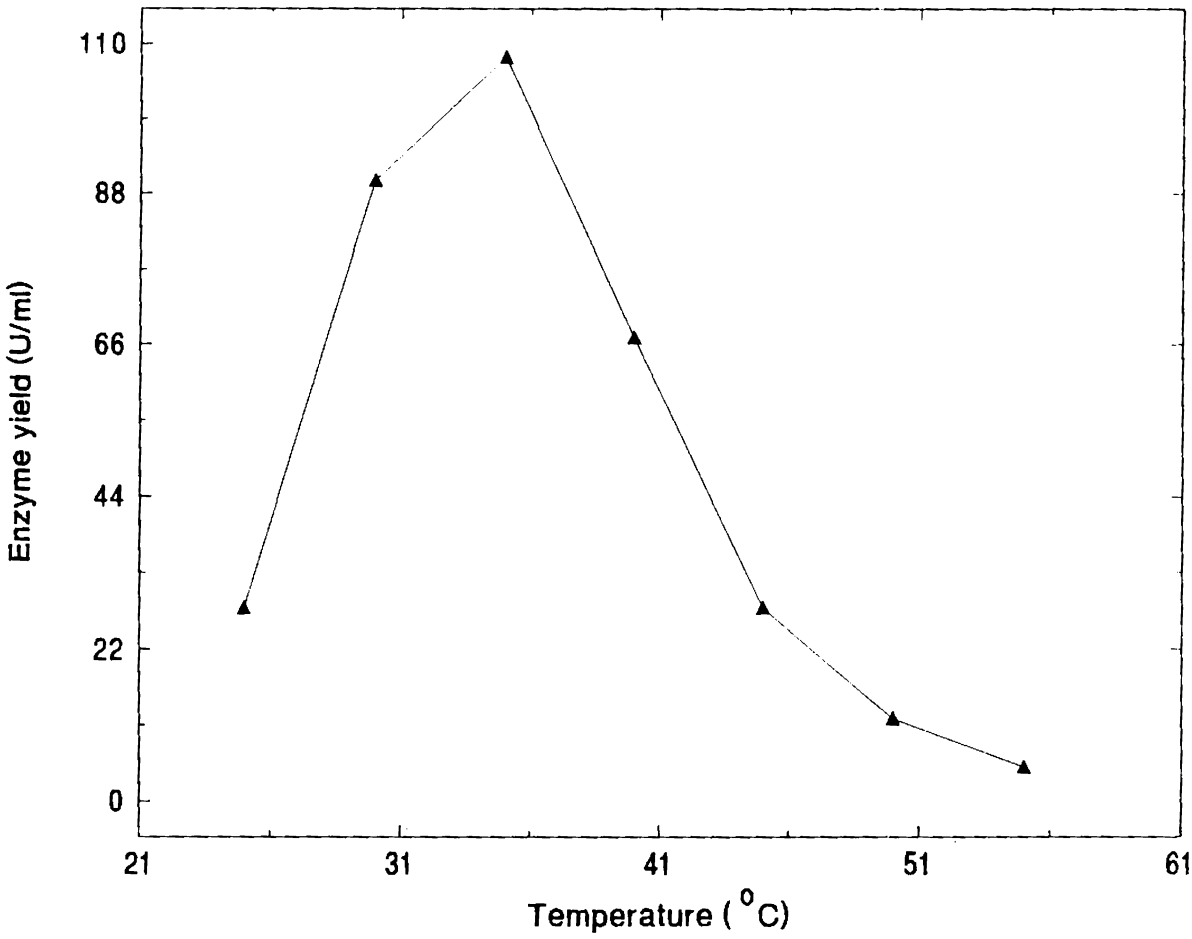


Figure 9. Effect of temperature on ∝ -Amylase production by B.coagulans during submerged fermentation.

medium. From the data depicted in Fig.10 it was inferred that *B.coagulans* could produce α-amylase over a pH 6-10 with a maximal at pH 8 (84 U/ml). Both acidic (4-5) and highly alkaline pH(11) did not support enzyme production.

3.2.3 Effect of Substrate Concentration

Effect of substrate concentration on α-amylase production was estimated by incorporating soluble starch at various concentrations from 0.1-3%. Results presented in Fig.11 indicate that significant levels of α-amylase could be produced at substrate concentrations ranging from 0.1-2%. However, a maximum of 114 U/ml was recorded at 0.5% substrate concentration. Whereas, concentrations above 2% led to a decline in the enzyme yield along with increase in concentrations.

3.2.4 Effect of Sodium Chloride

Impact of NaCl concentrations on enzyme production was tested at various levels of NaCl. Maximal levels of α-amylase was recorded in the absence of NaCl (104 U/ml)

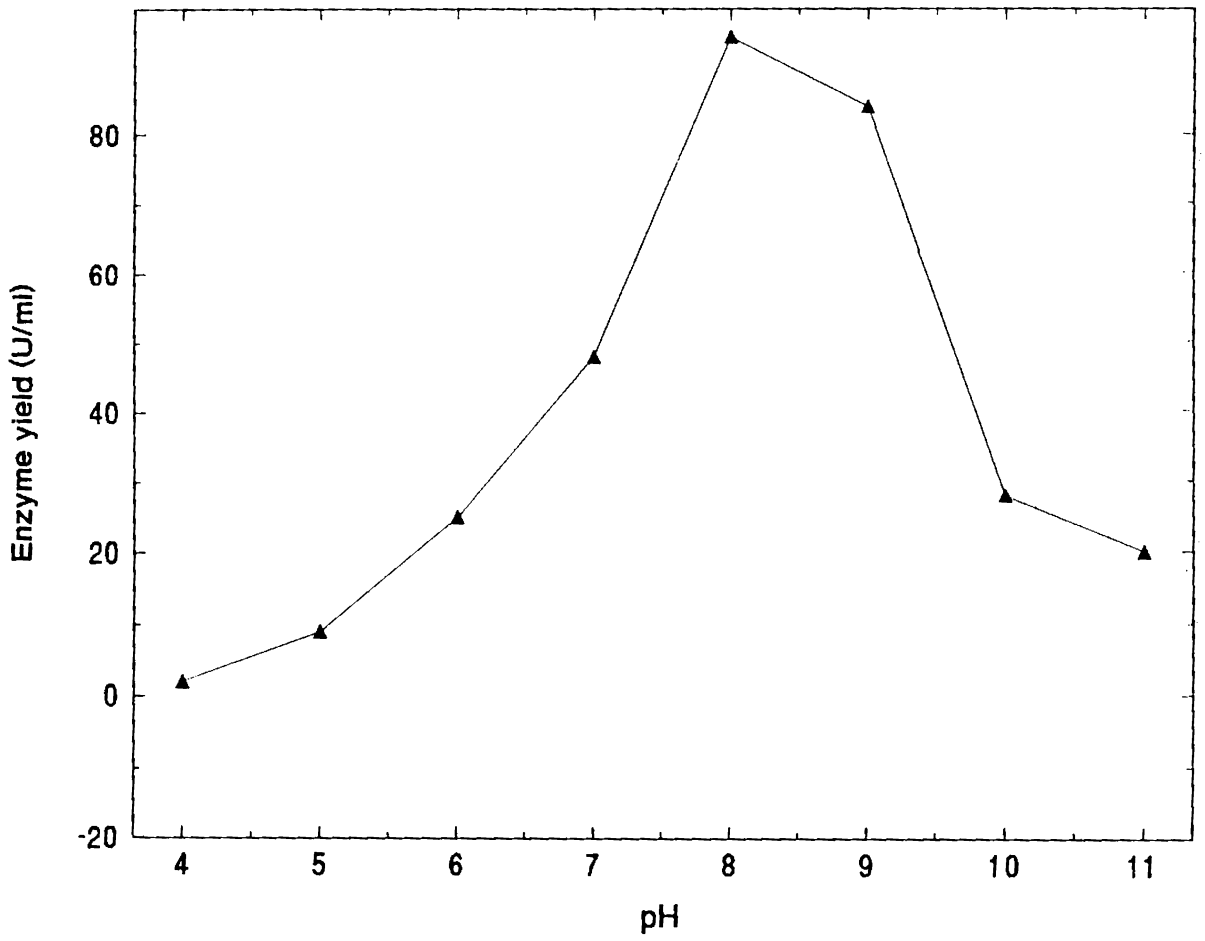


Figure 10. Effect of pH on ∝ -Amylase production by B.coagulans.

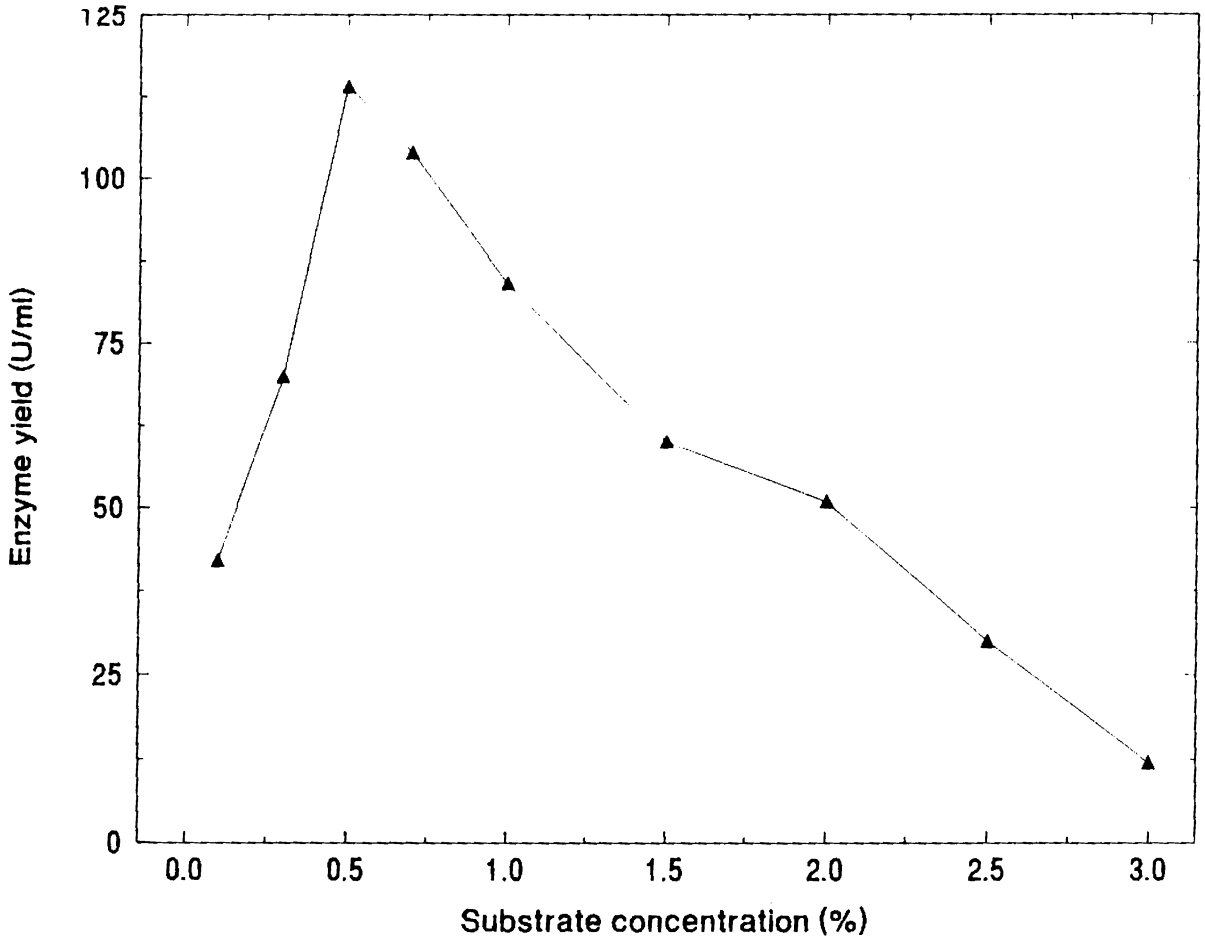


Figure 11. Effect of substrate concentration on ∝ -Amylase production by B.coagulans under submerged fermentation.

(Fig.12). Further, the enzyme yield declined progressively along with increase in NaCl concentration.

3.2.5 Effect of Carbon Sources

The effect of additional carbon sources on enzyme production was estimated by incorporating various sugars at 0.5% level in the medium. Results depicted in Fig.13 suggest that all carbon sources, tested, could promote significant levels of enzyme production. However, maximum enzyme production was enhanced by maltose (129 U/ml), followed by galactose, lactose, mannose, xylose, glycerol and glucose.

3.2.6 Effect of Nitrogen Sources

Effect of additional nitrogen sources on α-amylase production was determined by adding various nitrogen sources in the medium. From the results given in Fig.14 it was inferred that organic nitrogen sources could enhance significant levels of enzyme production compared to inorganic nitrogen sources, which, infact, led to a decrease in enzyme production. Among the organic nitrogen sources tested,cysteine and proline could promote maximal enzyme

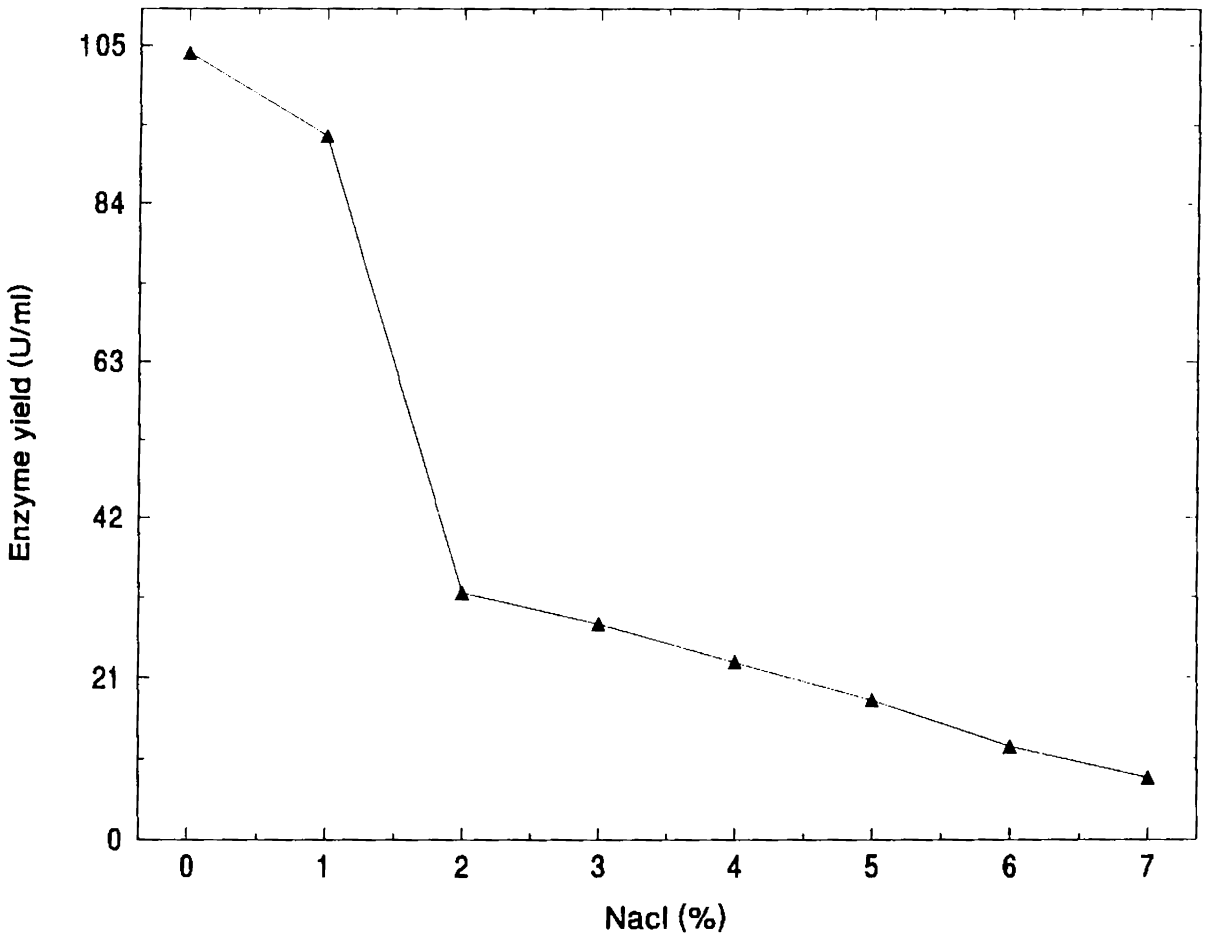


Figure 12. Effect of sodium chloride on ∝ -Amylase production by B.coagulans under submerged fermentation.

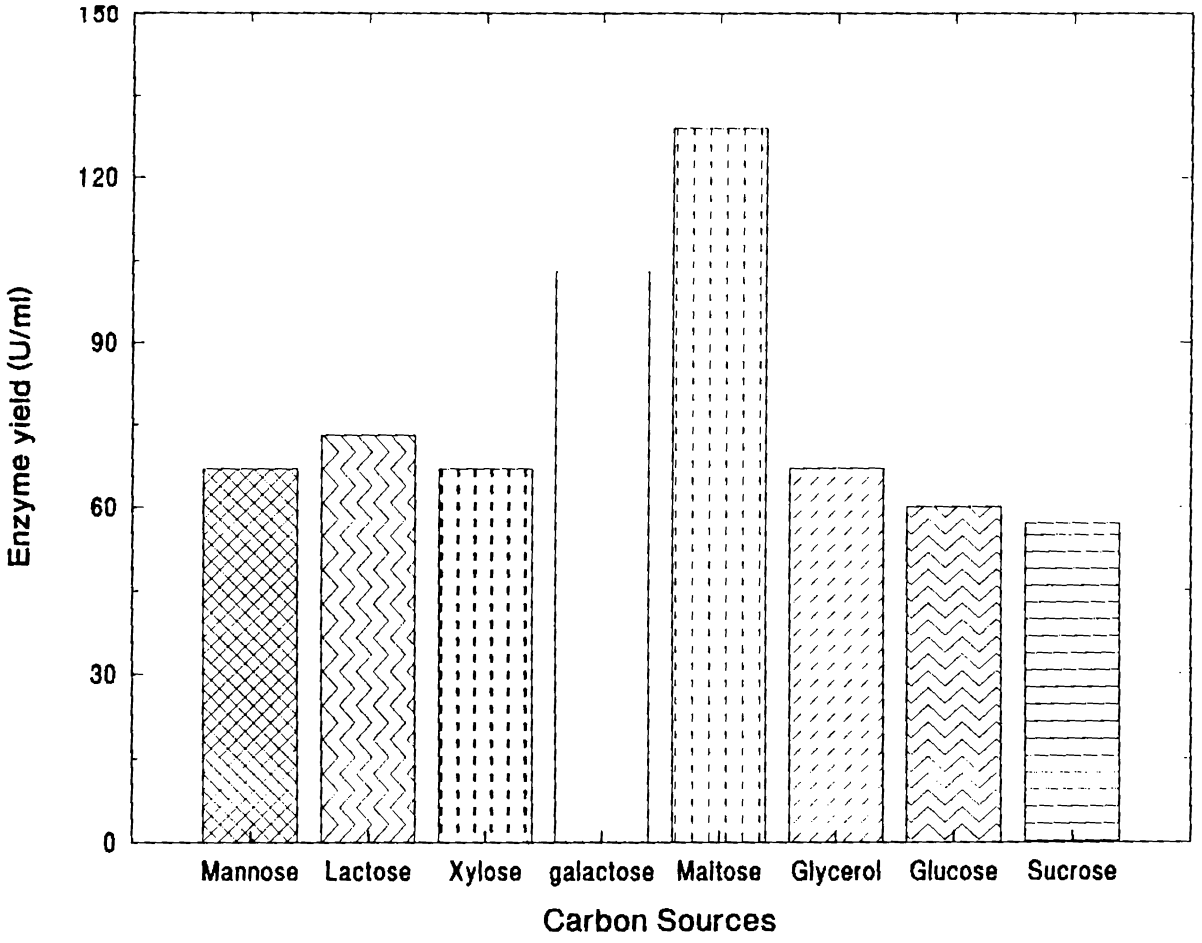


Figure 13. Effect of carbon sources on ∝ -Amylase production by B.coagulans during submerged fermentation.

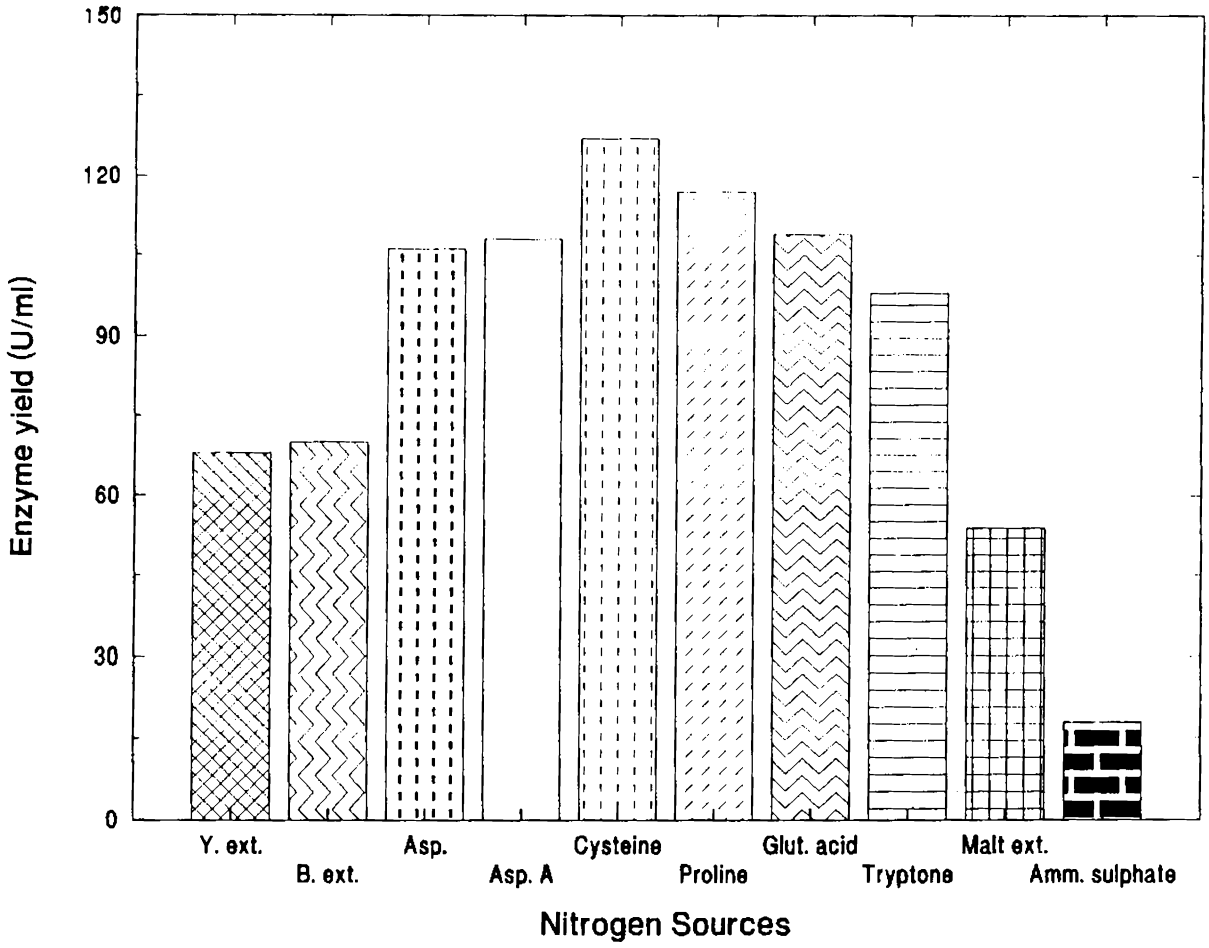


Figure 14. Effect of nitrogen sources on ∝ -Amylase production by B.coagulans. under submerged fermentation.

Y. ext - Yeast extract, B.ext. - Beef extract,
Asp. - Asparagine, Asp. A - Aspartic Acid,
Glut. acid - Glutamic Acid, Malt ext. - Malt extract

production (127 U/ml and 117 U/ml respectively). Nevertheless other amino acids could also enhance enzyme production to significant levels, compared to yeast extract, beef extract and malt extract.

3.2.7 Effect of Metal ions

Impact of metal ions on α-amylase production was determined by incorporating different metal ions in the medium. Interestingly all the metal ions tested showed a negative impact on amylase production (Fig.15). Thus the enzyme titres recorded for the various metal ions were less compared to control (130 U/ml).

3.2.8 Effect of Inoculum Concentration

The effect of inoculum concentration on α-amylase production was carried out by inoculating the medium with different concentrations of inoculum. Results presented in Fig. 16 indicate that significant levels of α-amylase could be produced at all inoculum concentrations tested, although maximum was recorded at 0.2% (125 U/ml).

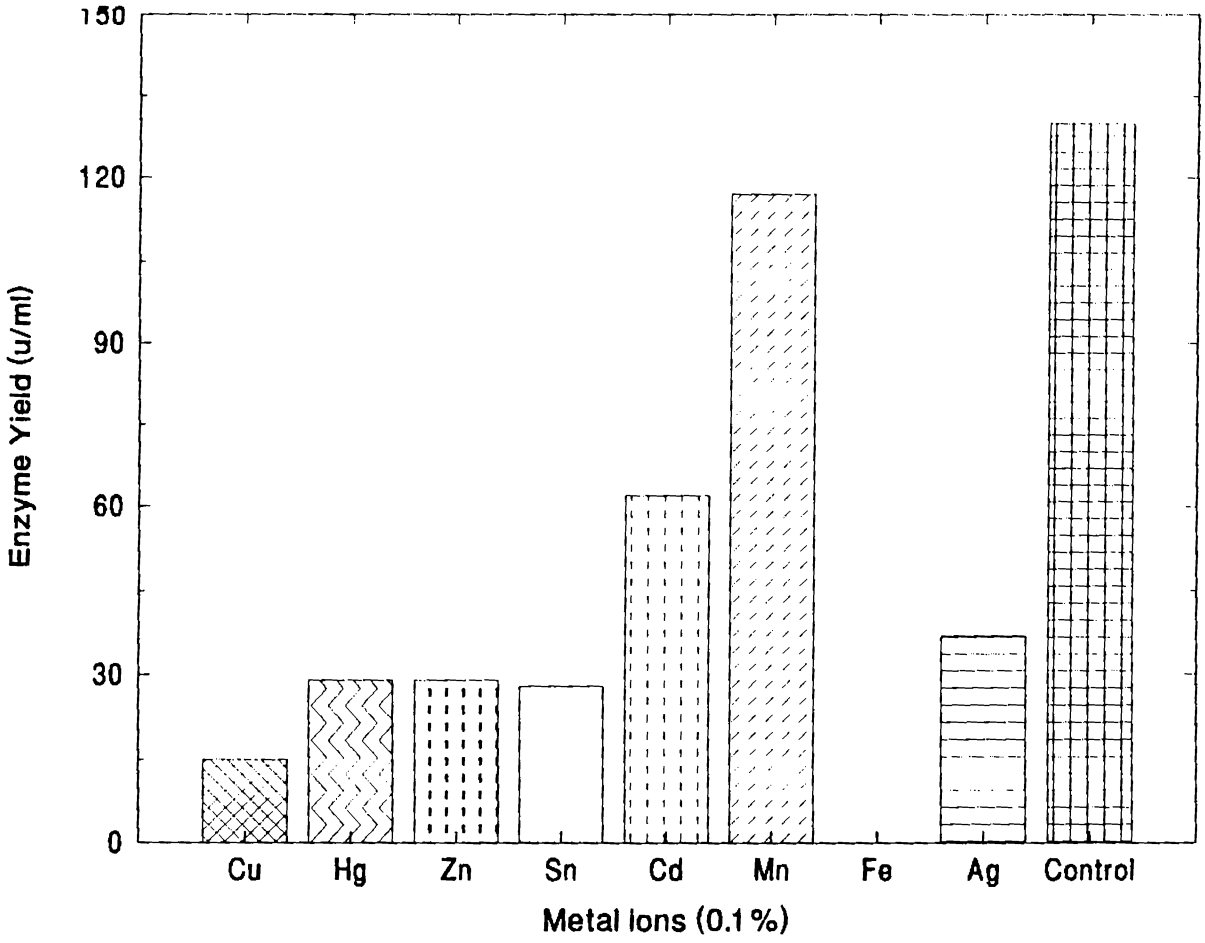


Figure 15. Effect of metal ions on ∝ -Amylase production by B.coagulans during submerged fermentation.

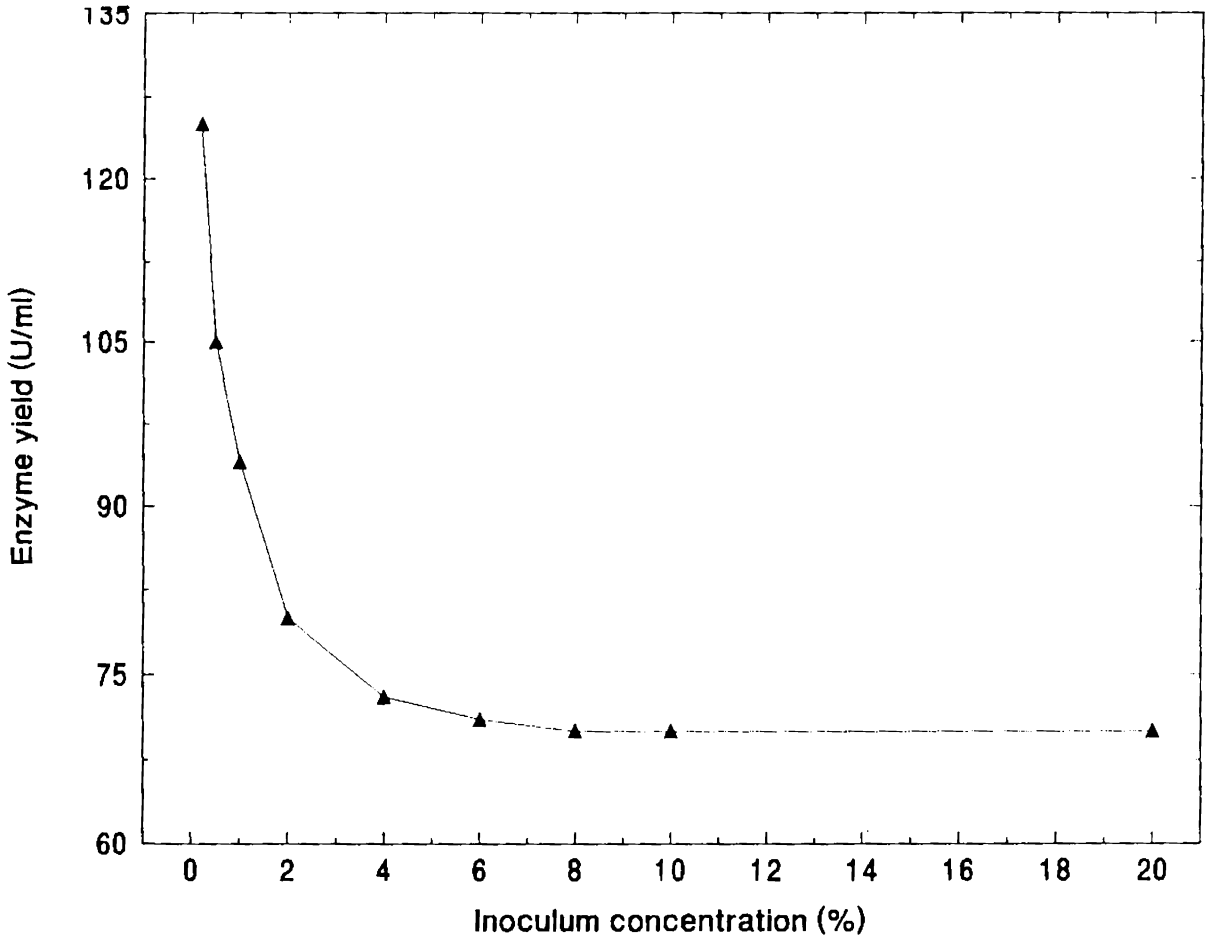


Figure 16. Effect of inoculum concentration on ∝ -Amylase production by B.coagulans during submerged fermentation.

3.2.9 Effect of Incubation Time

Optimum incubation period required for attaining maximal α-amylase production by *B.coagulans* was tested by incubating the inoculated enzyme production medium for a total period of 24 hrs. at their optimal growth conditions, after optimization studies. Results documented in Fig.17 indicate that 14 hrs of incubation is adequate for maximal enzyme production (136 U/ml). Nevertheless, significant levels of enzyme titres could be recorded, from 8 hrs of incubation (94 U/ml) on wards. However, incubation over 14 hrs led to a decline in enzyme titres. It was also observed that increase in enzyme titres followed a positive relationship with growth and the results suggest that α-amylase synthesis is enhanced during late exponential phase.

3.3 α-Amylase Production by B.coagulans under Submerged Fermentation - Continuous Process

Results obtained for the studies on the continuous production of α-amylase by *B.coagulans*, under submerged fermentation, in a stirred tank reactor at, three different flow rates are presented in Fig 18. It is evident from the

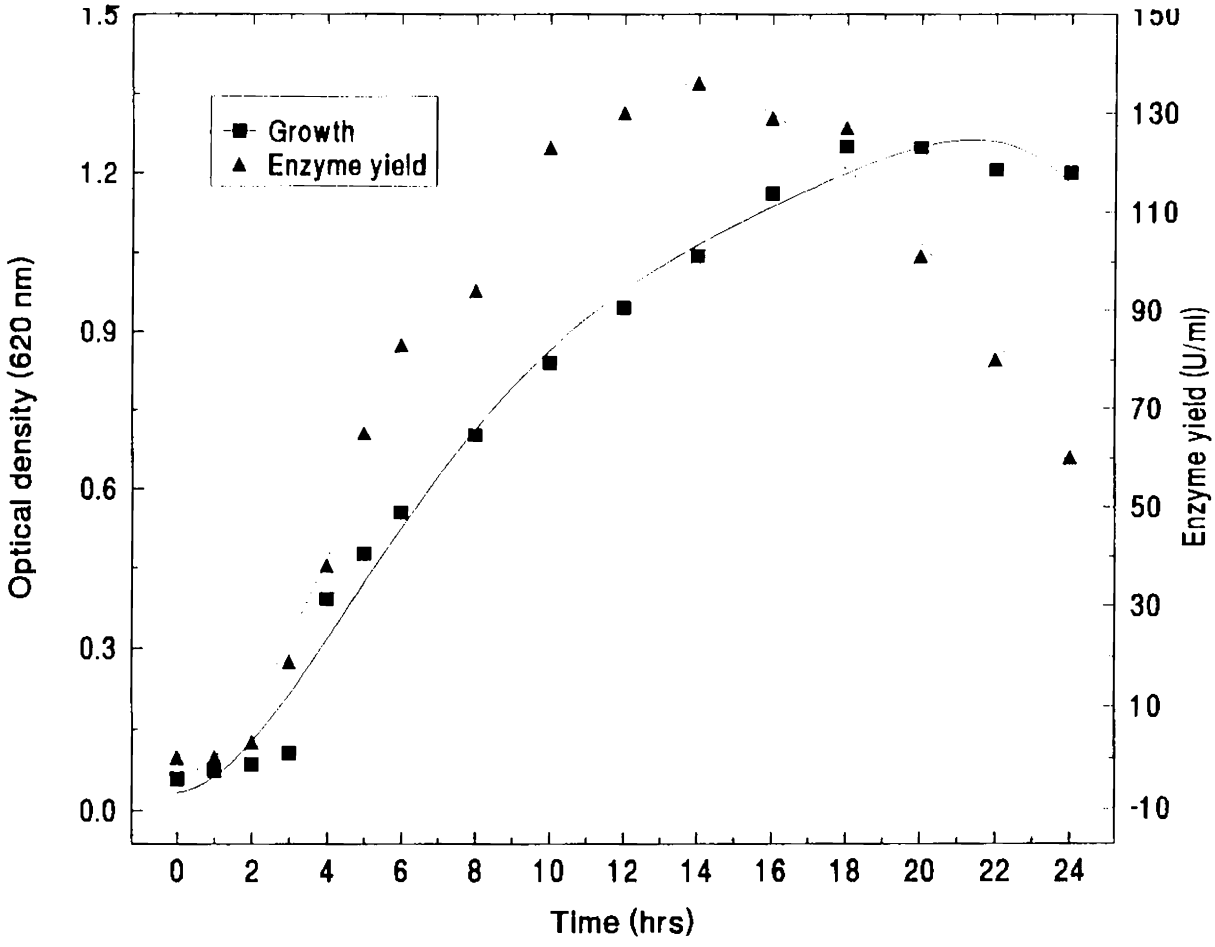


Figure 17. Effect of incubation period on ∝ -Amylase production by B.coagulans during submerged fermentation.

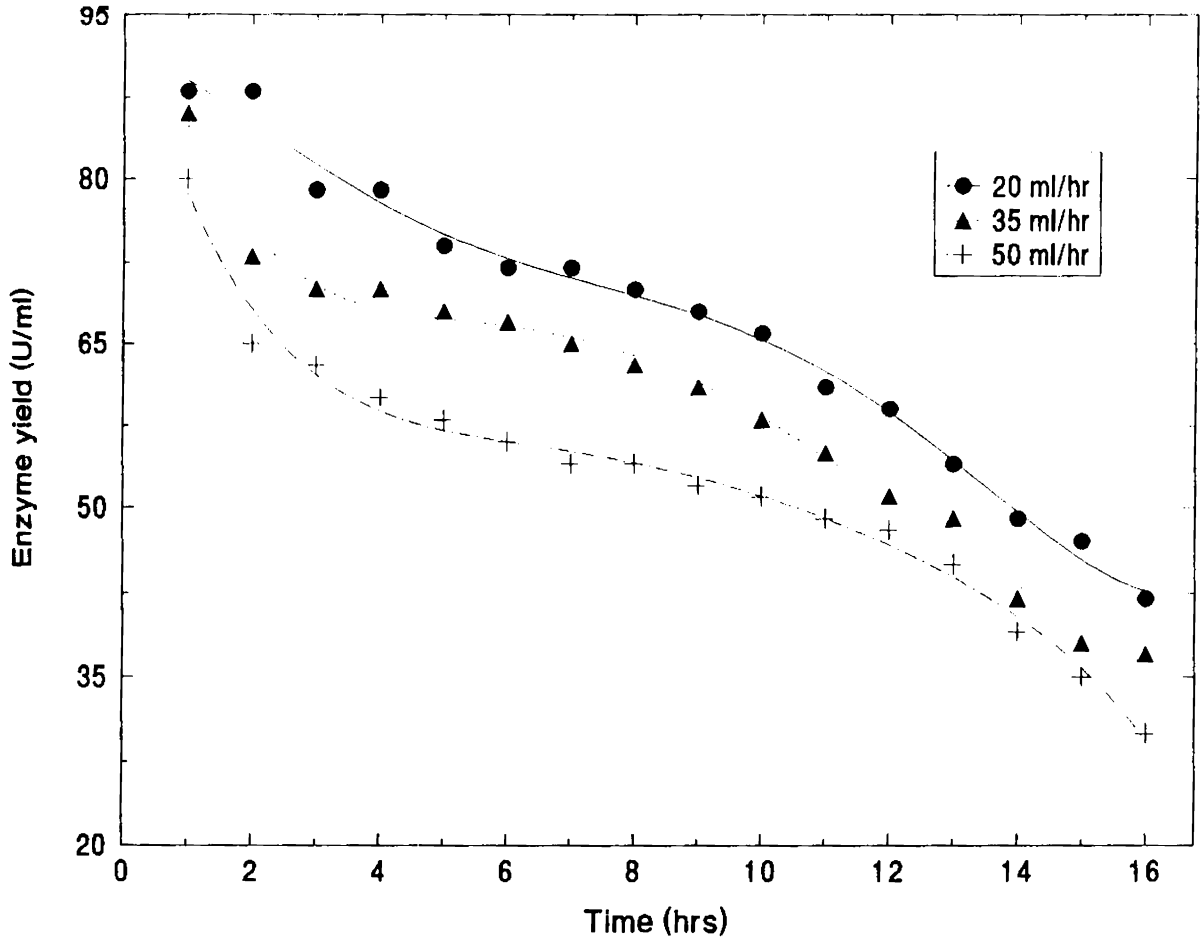


Figure 18. Continuous production of ∝ -Amylase by B.coagulans during submerged fermentation.

figure that there was a decline in enzyme yield during the course of continuous fermentation compared to batch process. However, on cumulative basis higher levels of α-amylase could be obtained compared to batch process. Increase in the flow rate of nutrient solution led to a decline in the rate of α-amylase production. Results presented in Table 6 clearly indicate that increase in dilution rates from $0.05h^{-1}$ to $0.10h^{-1}$, correspondingly led to increase in the volumetric productivity of the enzyme from 2.67 U/ml/hr to 5.25 U/ml/hr respectively, although the enzyme activities decreased from 66.75 U/ml to 52.5 U/ml along with increase in flow rates from 20ml h^{-1} to 50ml h^{-1}.

3.4 α-Amylase production by B.coagulans under SSF

The effect of operational parameters viz., particle size of wheat bran, initial moisture content, pH, incubation temperature, additional carbon and nitrogen sources, inoculum concentration and incubation time on α-amylase production during solid state fermentation was evaluated and the results obtained are presented below.

Table 6 Volumetric Productivity of α-Amylase under Continuous Fermentation

Flow rate ml/hours	Dilution rate (h^{-1})	Enzyme activity (U/ml)*	Productivity U/ml/hr
20	0.05	66.75	2.67
35	0.07	59.63	4.17
50	0.10	52.50	5.25

* Mean of three experiments

3.4.1 Effect of Particle Size of Wheat Bran

Effect of particle size of wheat bran on enzyme production during solid state fermentation was carried out by using wheat bran of different particle sizes. Results presented in the Fig.19 suggest that particle size of 600μ favoured maximal amylase production (4894 U/gds). Further, larger particles with size > 1000μ led to a decline in enzyme titres, indicating their unsuitability.

3.4.2 Effect of Moisture Content

Effect of initial moisture content on enzyme production during SSF was tested by preparing the wheat bran medium with varying initial moisture content (30-70%). Results presented in Fig.20 show that 60% initial moisture content recorded maximal enzyme titre.

3.4.3 Effect of Temperature

Effect of incubation temperature on α-amylase production during SSF was tested at different temperatures

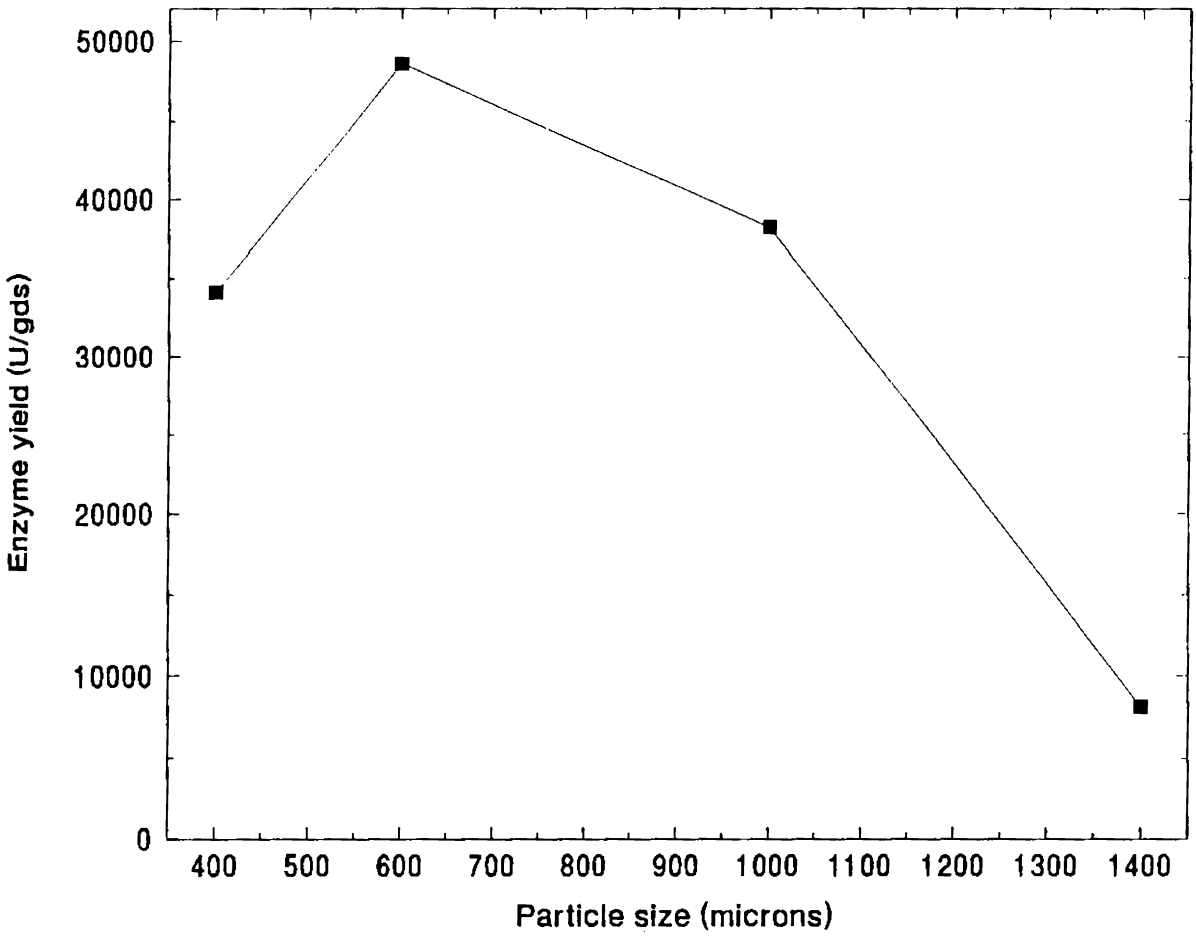


Figure 19. Effect of particle size on ∝ -Amylase production by B.coagulans under solid state fermentation.

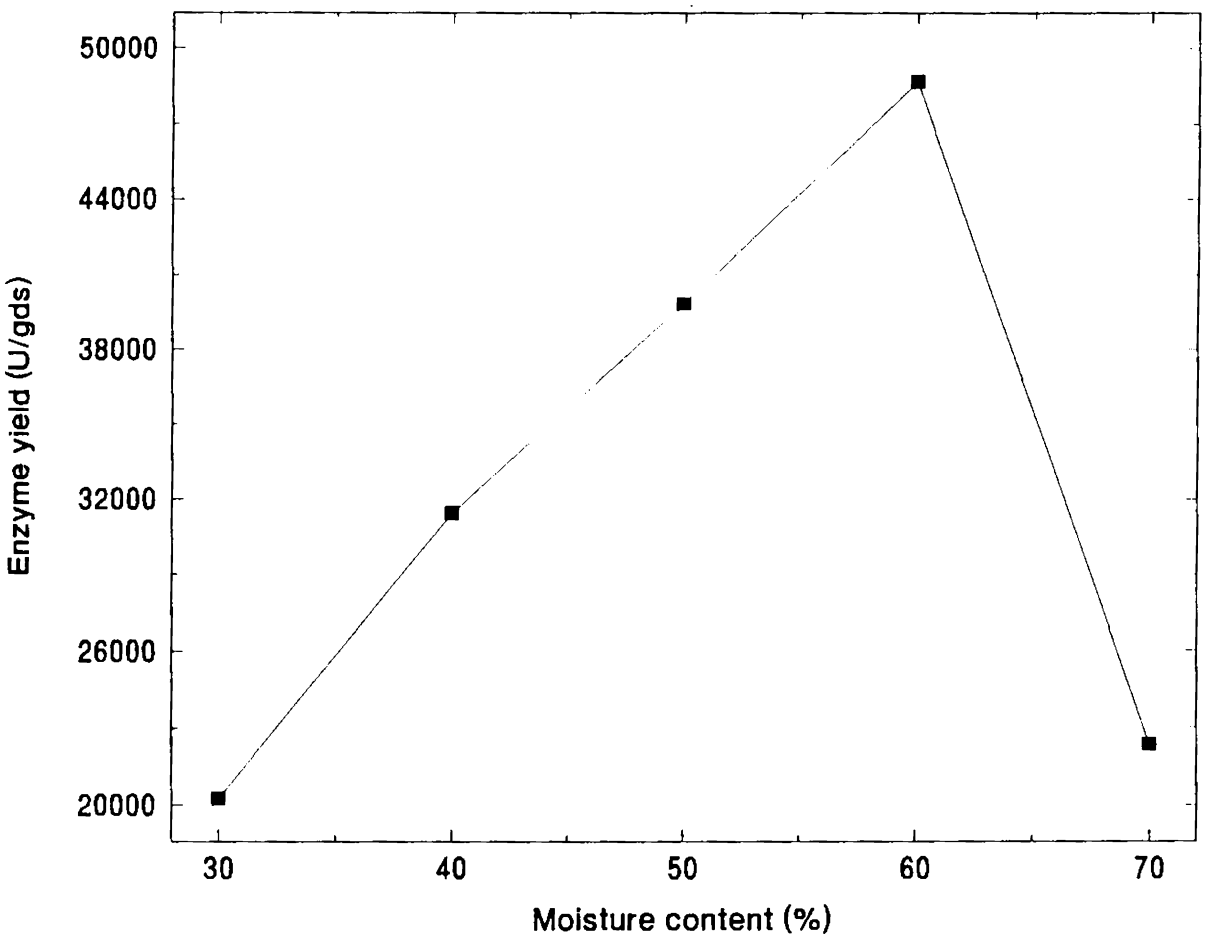


Figure 20. Effect of initial moisture content on ∝ -Amylase production by B.coagulans under solid state fermentation.

ranging from 30-55°C. Results presented in Fig. 21 indicate that significant amount of α-amylase could be produced at 30-45°C, while 35°C supported maximal enzyme yield (49878 U/gds). At temperature above 45°C the enzyme yield was decreased.

3.4.4. Effect of pH

Effect of pH on the enzyme production during SSF was tested at various levels of pH (4-10). Results presented in Fig.22 show that significant levels of α-amylase could be produced at wide range of pH from 5-10. However, maximal enzyme production was recorded at pH 6 (48525 U/gds). Alkaline pH above 10 and acidic pH below 5 did not enhance amylase production.

3.4.5 Effect of Carbon Sources

Effect of additional carbon sources on alpha amylase production during solid state fermentation was estimated by incorporating various carbon sources in the wheat bran medium. From the results presented in the Fig.23 it is inferred that

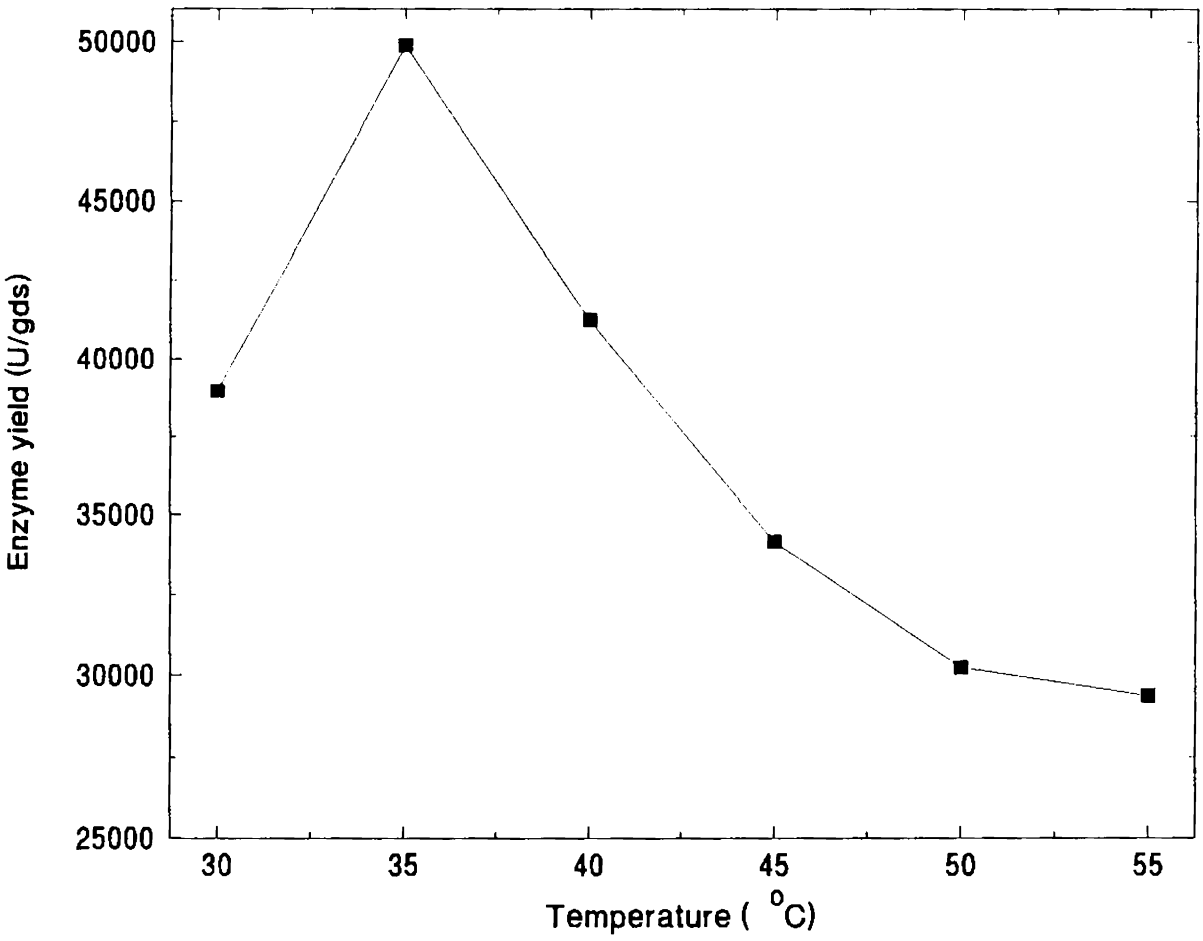


Figure 21. Effect of temperature on ∝ -Amylase production by B.coagulans under solid state fermentation.

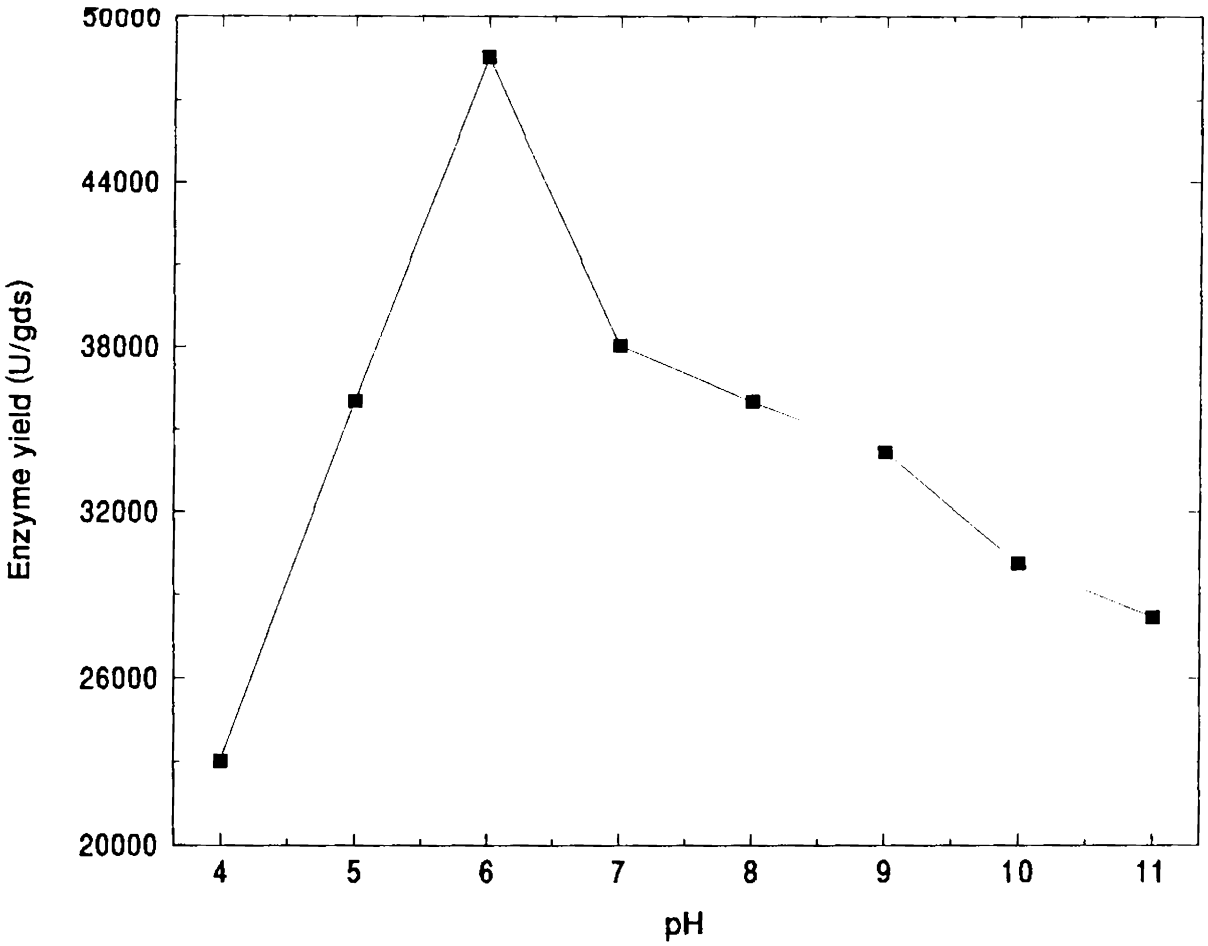


Figure 22. Effect of pH on α -Amylase production by B.coagulans under solid state fermentation.

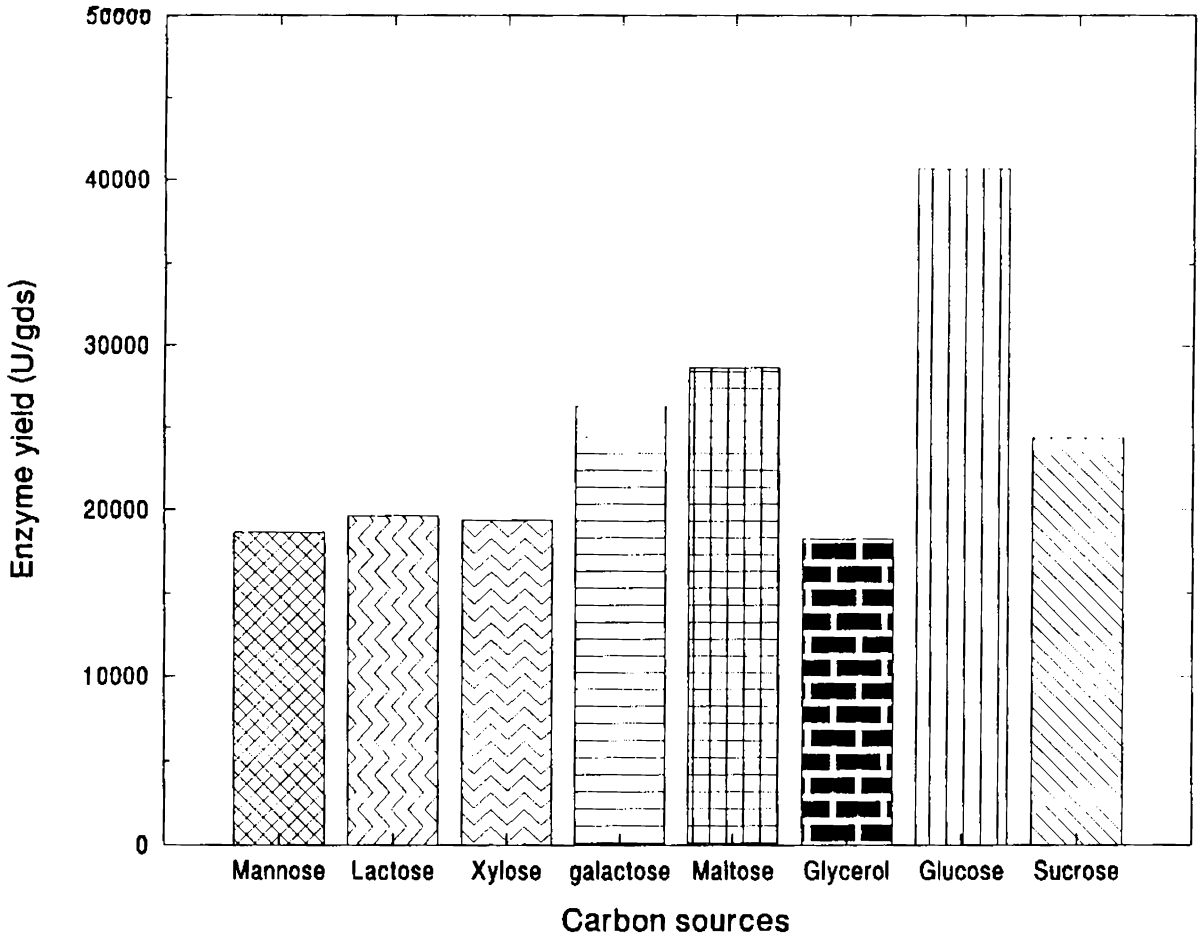


Figure 23. Effect of carbon sources on ∝ -Amylase production by B.coagulans under solid state fermentation.

only glucose, enhanced enzyme yield to an appreciable level (40634 U/gds) while others supported a slight increase in the enzyme yield.

3.4.6 Effect of Nitrogen Sources

Effect of nitrogen sources on α-amylase production during solid state fermentation was studied by the addition of various nitrogen sources at 1% w/w in the medium. Data presented on Fig.24 indicate that cysteine could enhance maximal enzyme production (49120 U/gds) followed by beef extract, glutamic acid, proline, asparagine, aspartic acid and others.

3.4.7 Effect of Inoculum Concentration

The effect of inoculum concentration on α-amylase production during SSF was carried out at different levels of inoculum. From the results presented in Fig.25, it is noted that 10% inoculum promoted maximum level of enzyme production (49690 U/gds) compared to other levels.

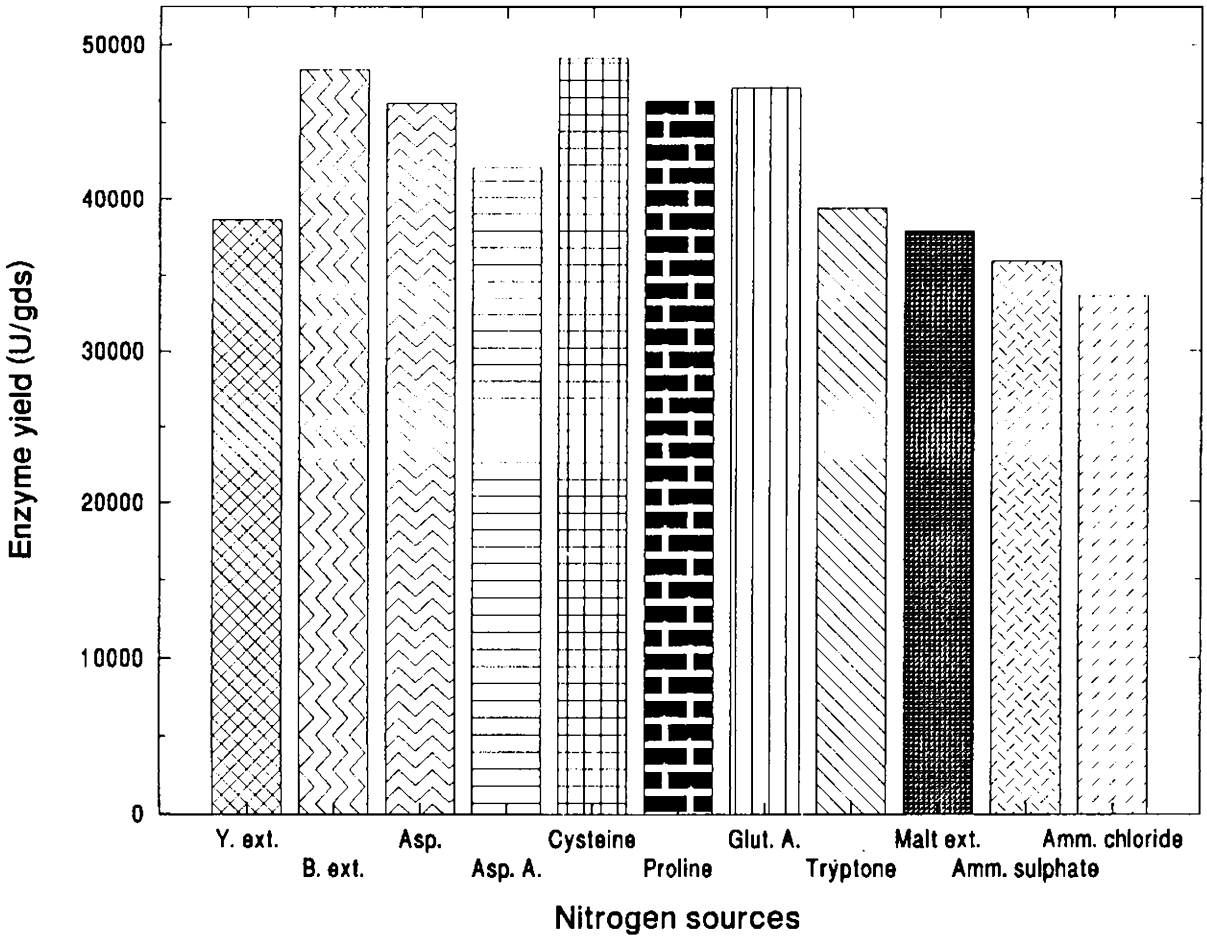


Figure 24. Effect of nitrogen sources on ∝ -Amylase production by B.coagulans under solid state fermentation.

Y.ext. - Yeast extract, B.ext. - Beef extract,
Asp. - Asparagine, Asp.A - Aspartic Acid,
Glut. acid - Glutamic Acid, Malt ext. - Malt extract

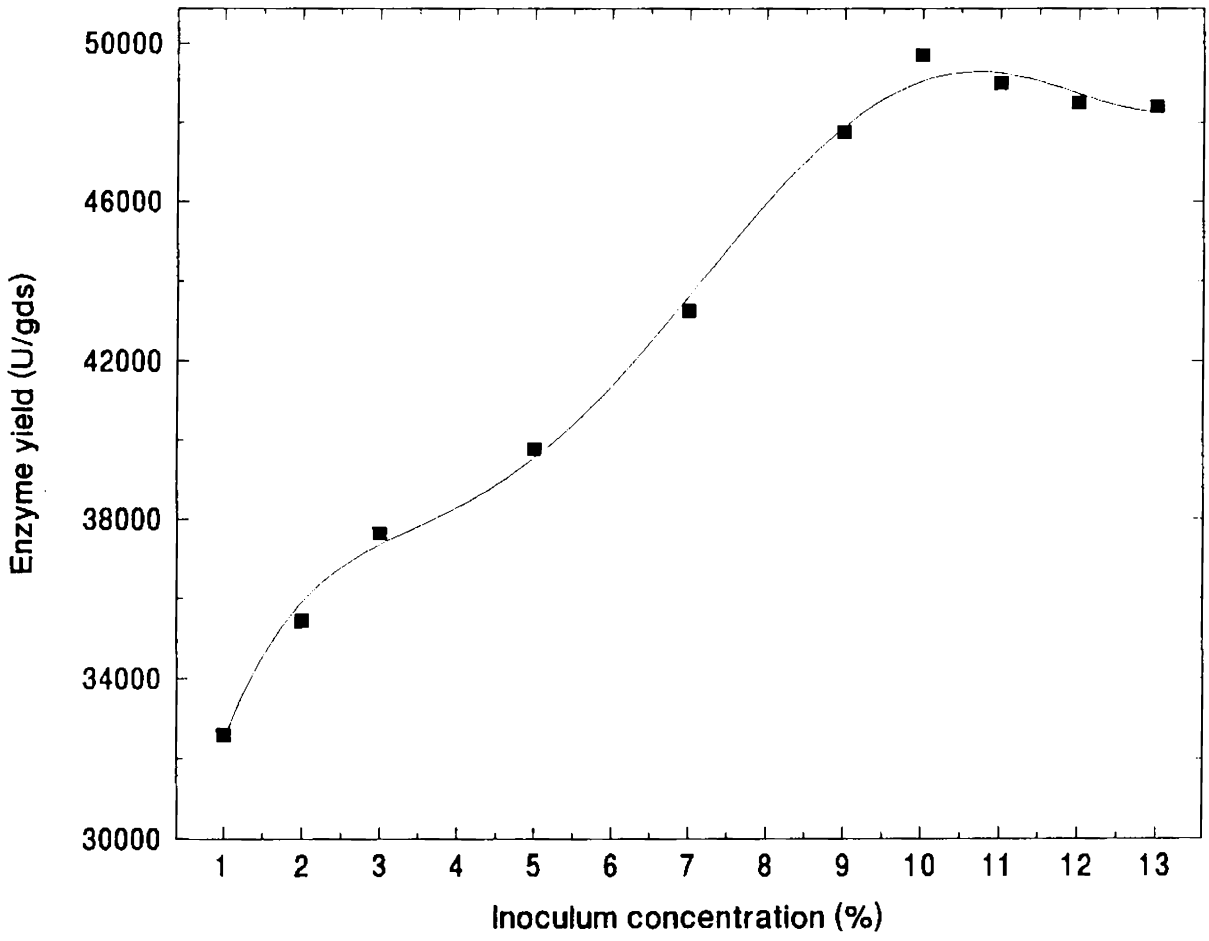


Figure 25. Effect of inoculum concentration on ∝ -Amylase production by B.coagulans under solid state fermentation.

3.4.8 Effect of Incubation Time

Effect incubation period on α-amylase production under SSF was determined by incubating the inoculated wheat bran medium for various periods, at the optimal fermentation conditions. Data presented in Fig.26 suggest that maximum enzyme yield could be obtained at 72 hrs of incubation (52989 U/gds) though appreciable amount of enzyme could also be recorded at 48 hrs of incubation. Incubation beyond 72 hrs led to decline in enzyme production.

3.5 Impact of Mutation on α-Amylase Production

Impact of mutation on α-amylase production by *B.coagulans* was studied by subjecting the bacteria to UV irradiation and chemical mutagenesis with nitrosoguanidine (NTG).

3.5.1 UV Mutagenesis

Exposure of cells to UV irradiation for a period of 120 seconds caused 99% lethality. The surviving cells were isolated, sub cultured and checked for their enzyme yield.

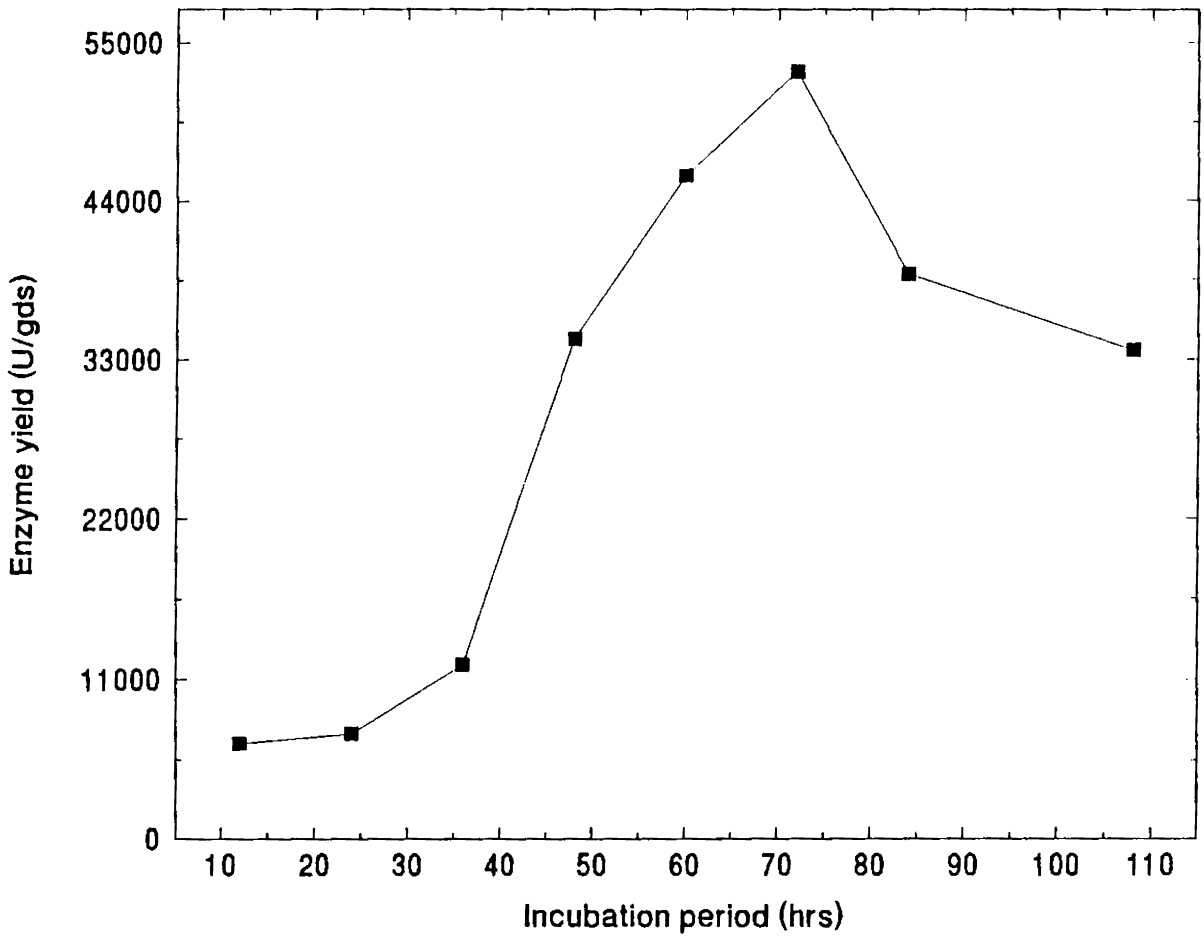


Figure 26. Effect of incubation time on ∝ -Amylase production under solid state fermentation.

The UV irradiated cells could produce 142 U/ml compared to their control cultures which produced 99 U/ml (Table 7.)Results evidence that UV mutagenesis have influenced positively a 43.4% increase in α-amylase production.

3.5.2 Mutagenesis with NTG

B.coagulans subjected to mutagenesis with NTG at various concentrations yielded mutants that produced enhanced levels of α-amylase compared to controls (Table 8). It was observed that strains mutated with 50μg of NTG could yield maximal enzyme titres (165 U/ml) compared to other concentrations.

The mutant cells obtained with 50μg NTG conc was further mutated by incubating 1 ml of 1st mutant cell suspension with NTG (50μg NTG)for different periods of contact up to 75 minutes. From results presented in Table 9, it is inferred that contact time of 60 minutes promoted favourable mutation, in the 1st mutant, towards enhanced amylase production (195 U/ml), by a double fold, when compared to other concentrations and treatment periods studied.

Table 7 α-Amylase Production by *B.coagulans* Treated with UV Irradiation.

	No of experiment	Amylase activity U/ml
A	Control	99*
B	Mutant	
	1st	145
	2nd	143
	3rd	142
	4th	140
	5th	140
	Mean	142

* Mean of Duplicate

Table 8 α-Amylase Production by B.coagulans ACMN 1 Treated with NTG at Different Concentrations

Conc. of NTG (μg/ml)	Yield* (U/ml)
10	90
20	110
30	145
40	145
50	165
60	150
70	115
80	105
90	105
100	90

* Mean of three Experiments

Table 9 α-Amylase production by *B.coagulans* treated with 50 mg. NTG at different contact time

Contact time (minutes)	Yield* (U/ml)
15	152
30	158
45	162
60	195
75	165

* Mean of three experiments

3.6. Purification of α - Amylase

α-amylase from *B.coagulans* was purified by ammonium sulfate precipitation followed by dialysis, chromatography and electrophoresis. The overall yields are presented in Table 10.

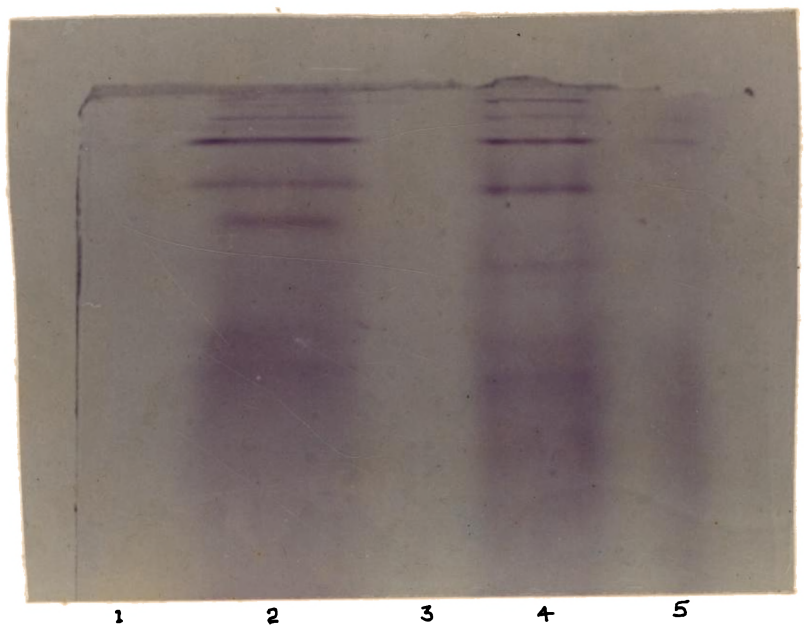
Specific activity of the amylase was observed to increase at each step of purification and after dialysis a specific activity of 65.41 U/mg protein was recorded. Specific activities recorded after chromatographic separation with DEAE cellulose column was recorded as 176.21. A single major band was obtained after PAGE. The overall yield and fold purification obtained after purification were 23 % and 51.98 respectively.

The molecular weight of the purified α-amylase was in the range of 90,000-95,000 inferred from native PAGE, using β-galactosidase (MW 116,400), Fructose-6-phosphatase (MW 85,200) and Glutamate dehydrogenase (MW 55,600) as molecular weight markers (Sigma).

Table 10 Purification of Thermostable αAmylase Produced by *B.coagulans*- Data on Overall Purification of the Enzyme

Purification step	Total enzyme units	protein mg	Specific activity U/mg protein	Purification factor (X)	Yield or recovey (%)
Initial Crude extract	9500	2794.7	3.39	1.0	100.0
Ammonimum sulphate Percipitation	7968	230.2	34.61	10.21	83.87
Dialysis	4232	64.7	65.41	19.29	44.55
DEAE- Cellulose Column chromotography	2185	12.4	176.21	51.98	23.0

PLATE - I



Native Polyacrylamide gel electrophoresis and staining of proteins obtained from various steps of purification

Lane. 2 · Crude extract

Lane. 4 · Ammonium sulphate precipitation

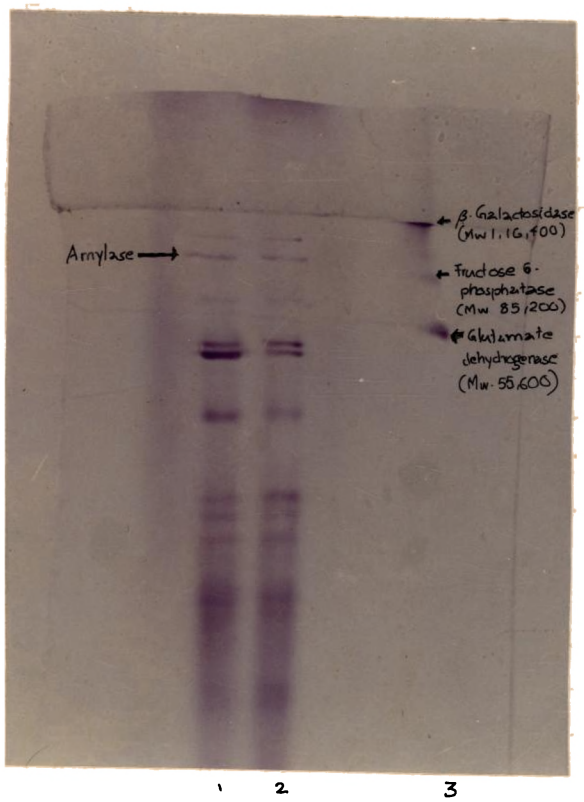
Lane. 5 · After DEAE cellulose column chromatography

PLATE - II

DETECTION OF AMYLASE

After electrophoresis, the gel was incubated with 1% soluble starch in phosphate buffer (pH - 6) Light lane (area of amylase activity) were detected by immersing the gel in Lugol's solution.

PLATE - III



Polyacrylamide gel (10%) stained with Coomassie Brilliant Blue.

Lane 1, 2. Ammonium sulphate fraction.
Lane 3 - Molecular markers
β-Galactosiclase (Mw 1,16,400)
Fructose-6-phosphatase (Mw 85,200)
Glutamate dehydrogenase (Mw 55,600)

3.7 Enzyme Characteristics:

3.7.1. Effect of Temperature on α-amylase

Effect of temperature on the activity and stability of α-amylase was tested by subjecting them to various temperatures ranging from 50 to 100°C. Results presented in Fig 27 indicate that the temperature optima for this enzyme is 70°C, while significant levels of enzyme activity could be recorded at 50-90°C. It was also noted that the enzyme is thermostable for a period of 30 minutes at 100°C and 90°C with a residual activity of 70% and 78% respectively (Fig.28)

3.7.2 Effect of pH on α-amylase

Effect of pH on the activity and stability of α-amylase was determined by subjecting them to various levels of pH (4-11). Results shown in Fig.29 clearly qualifies this enzyme as an alkaline amylase since the optimal activity and stability was recorded at pH 9.0. There was reduction in the level of residual activity at pH 8 and pH 10, and at acidic pH conditions(Fig.30).

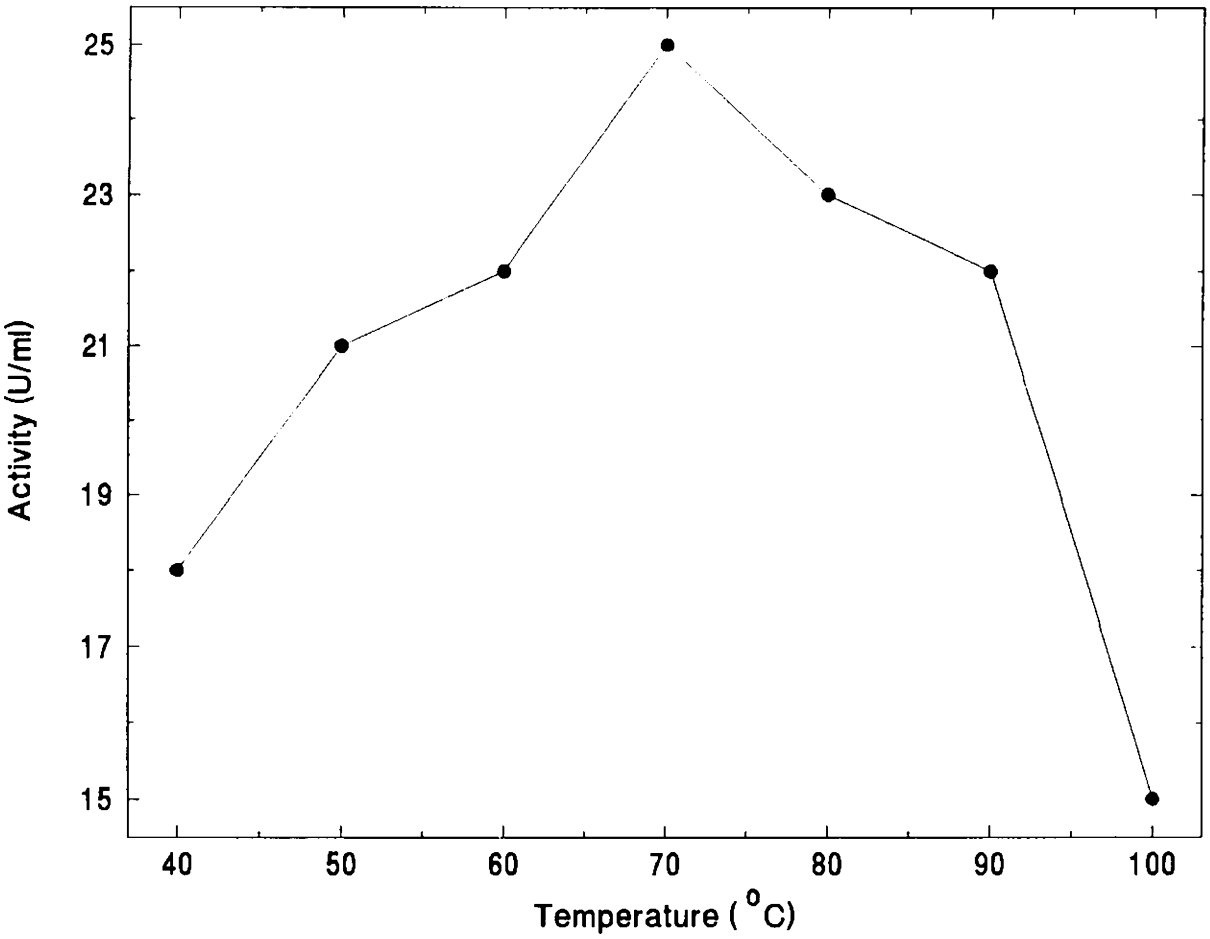


Figure 27. Effect of temperature on the activity of ∝-Amylase produced by B.coagulans.

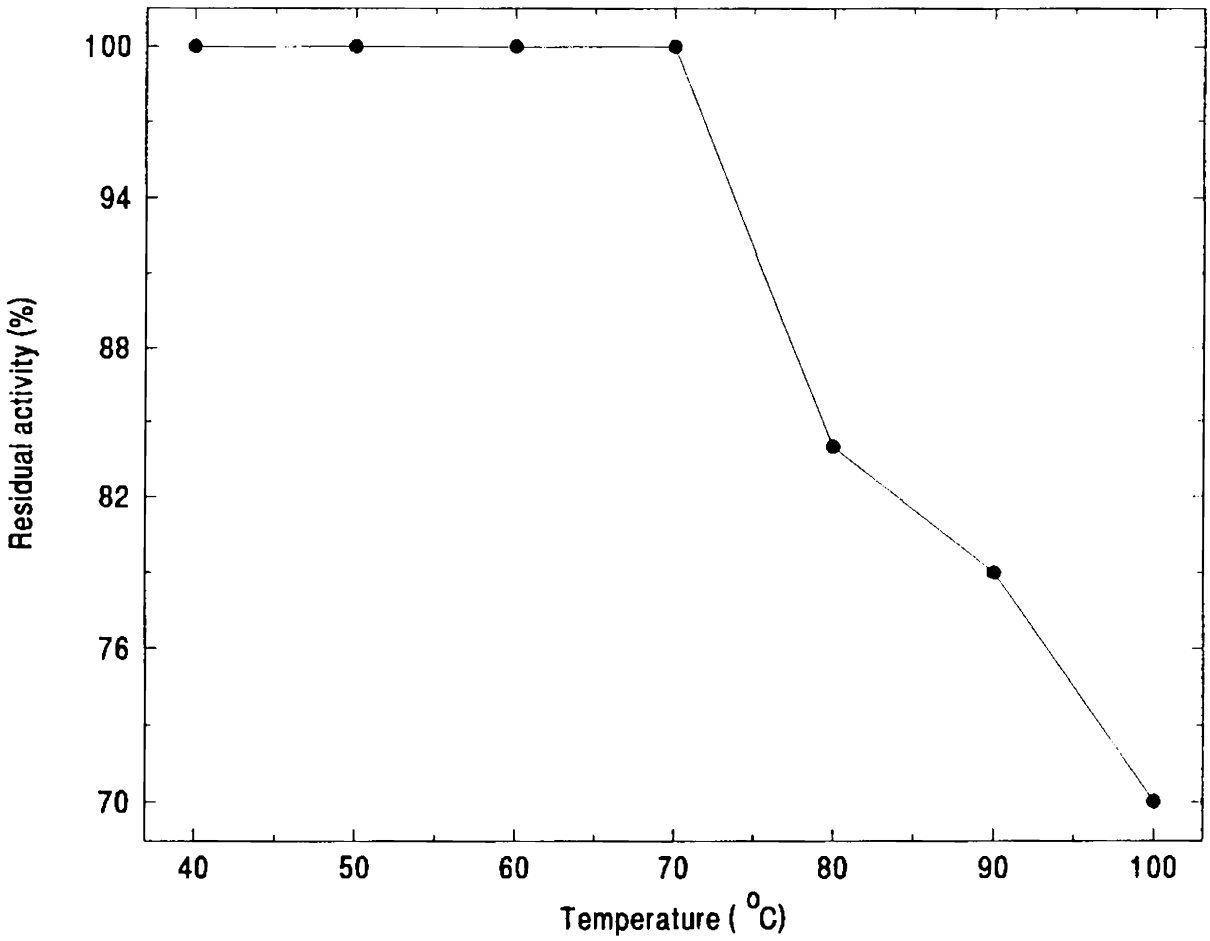


Figure 28. Effect of temperature on the stability of α - Amylase produced by B.coagulans.

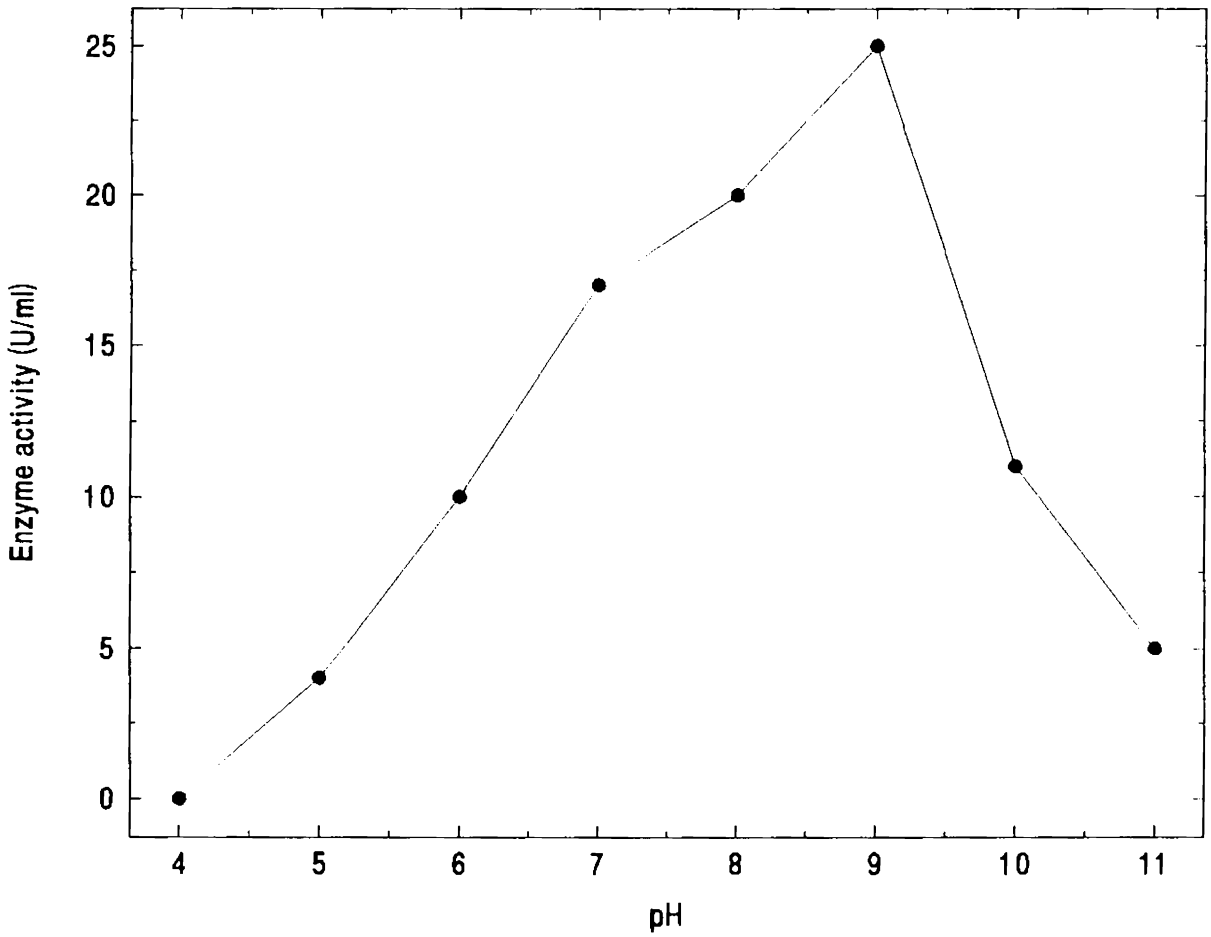


Figure 29. Effect of pH on the activity of α -Amylase produced by B.coagulans.

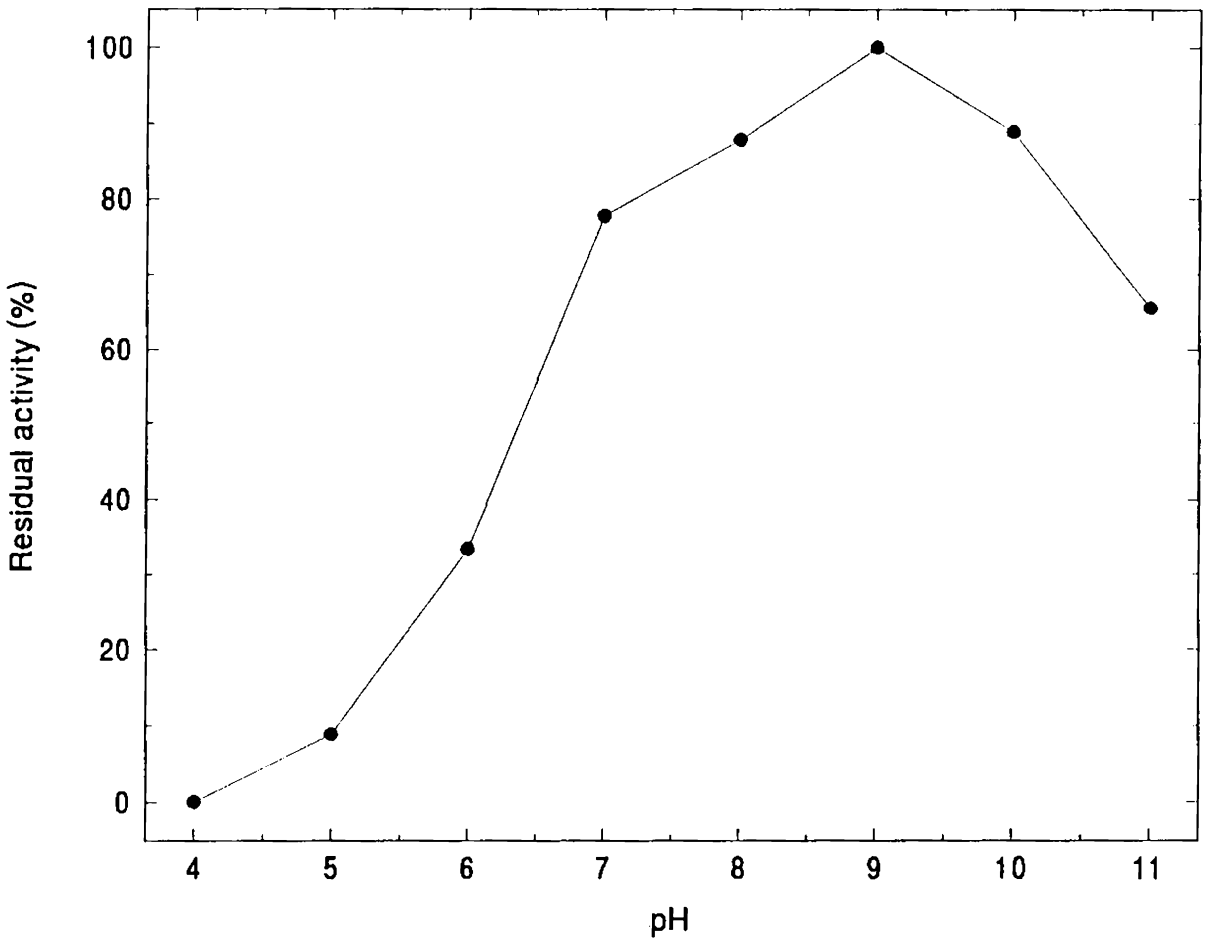


Figure 30. Effect of pH on the stability of α -Amylase produced by B.coagulans.

3.7.3 Effect of substrate concentration on α-amylase

Impact of substrate concentration on the activity and stability of α-amylase was checked at various levels of soluble starch in the reaction mixture. Data presented in Fig 31 suggest that optimal activity of this α-amylase could be recorded at 1% soluble starch and increase in concentrations of substrate led to decline in enzyme activity. The enzyme was observed to have a Km of 4.7 mg ml^{-1}. It was also found that 30% soluble starch, supported thermostability at 100°C (90% of initial activity (25 U/ml) retained) for 2 hours adding evidence to the thermostable nature of this α-amylase.

3.7.4 Effect of metal ions on α-Amylase

Data presented in Table 11 clearly evidence the fact that calcium not only provides support for retaining activity at 70°C but also promoted thermostability at 100°C unlike other metal ions. Among others tested, Cu, Sn, Mn, Co & Zn could support the enzyme to retain activities over 90%, while Fe and Cd inhibited enzyme activity. Further they did not support thermostability at 100°C unlike calcium.

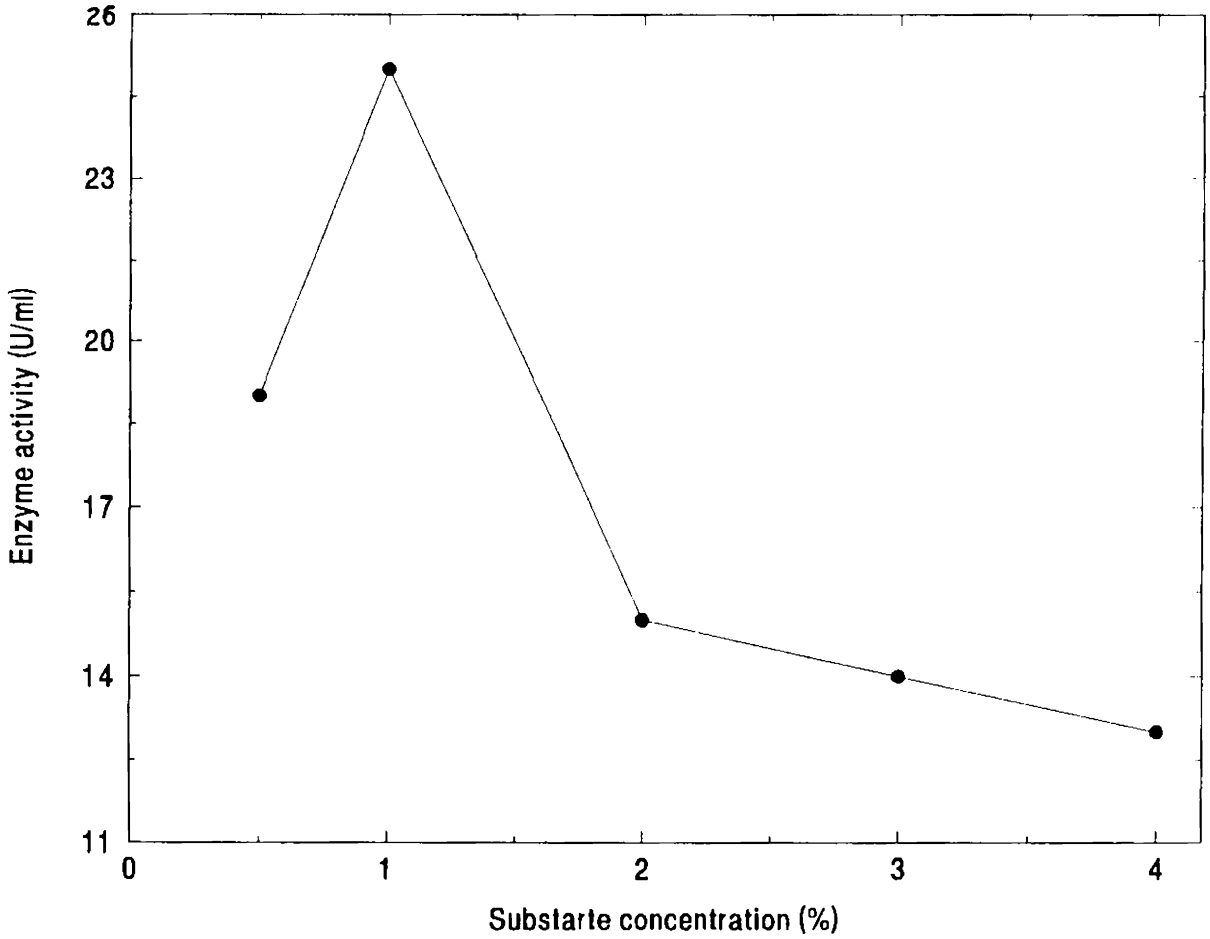


Figure 31. Effect of substrate concentration on the activity of ∝ -Amylase produced by B.coagulans.

Table 11 Effect of Metal ions on the Activity and Stability of α-Amylase Produced by *B.coagulans*

Metal ions	Relative activity at 70°C (%)	Stability at 100°C ** for 30 minutes (%)
Ca	100*	100*
Co	98	45
Sn	95	33
Cu	92	20
Mn	92	31
Zn	90	21
Ag	88	15
Hg	50	21
Cd	0	0
Fe	0	0

* 100% activity = 25 U/ml, at 70°C.

** Activity checked at 70°C after incubation with the metal ions for 30 minutes. Reation mixture was the same as mentioned under section 2.3.6.

3.7.5 Effect of sugars and other additives on α-Amylase

Results presented in Table 12 recommend the use of xylose, maltose, dextrose and polyethylene glycol as additives for retaining thermostability of α-amylase at 100°C, since there was no loss of activity in their presence for a period of incubation of 30 minutes.

Table 12 Effect of sugars and other additives on the thermostability of *B. coagulans* tested at 100^{O}C for 30 minutes

Additives	Residual Activity Checked at 70^{O}C *
Polyethylene Glycol	100.00
Xylose	100.00
Maltose	100.00
Dextrose	100.00
Glycerol	66.66
Sorbitol	20.00

* 100% activity = 25 Uml^{-1} Reaction mixture was the same described under section 2.3.6.

DISCUSSION

4.1. Growth and α-Amylase Production under Submerged Fermentation

Bacteria grow well and metabolize various substrates at certain sets of environmental conditions. In nature, different strains of the same species usually differ from each other in their requirements of carbon and nitrogen sources, optimum temperature, pH and other factors to grow and produce maximal amounts of enzymes and secondary metabolites. Thus variations in optimal environmental conditions for growth and enzyme production have been recorded by several earlier investigators for different species of *Bacillus*.

B.Stearothermophilus preferred pH 5-8.5 for optimal enzyme activities and growth, producing maximal α-amylase at pH 6.7 (Welker & Campbell, 1963). Few *Bacillus sp* required alkaline pH 7.5-11 for their growth and synthesis of alkaline α-amylase (Horikoshi, 1971) and pH 7-9 for production of thermostable α-amylase (Bindu, 1989). *B. licheniformis* TCRDC B13 required pH 10-11 for maximal growth while growing well over the pH 3-11. However, more α-amylase was recorded over a pH range 6-9 (Bajapai & Bajpai, 1989). *B.polymyxa* and *B.cereus* preferred pH 8 and PH 4-5 for optimal growth and

amylase production (Nandakumar, 1991). Whereas in the present study *B.coagulans* preferred pH 9 and 8 for maximal growth and amylase production respectively. These results indicate the ability of this organism to survive and produce α-amylase better in alkaline pH conditions similar to that observed with other strains by early workers.

B.coagulans required 35°C for optimal growth (Campbell, 1955). *B.licheniformis* CUMC 305 could grow and produce maximal α-amylase at 55°C, while *B.coagulans* preferred 60°C (Medda and Chandra, 1980). *B.licheniformis* TCRDC B 13 grew well and produced α-amylase at 35-40°C (Bajpai & Bajpai, 1989). Recently it was observed that *B.coagulans* could grow and produce α-amylase at 50°C during SSF (Sathyanarayana, 1994). *B.stearothermophilus* prefered 65°C as optimum temperature for growth while producing α-amylase to a maximum at 40°C (Wind et al., 1994). In the present study *B.coagulans* exhibited maximum growth and α-amylase production at 35°C, suggesting a mesophilic nature of the organism. However the strain could survive at 50°C which is comparable with the reports of Campbell (1955) for *B.coagulans*.

Very few reports are available on the effect of NaCl on the growth and enzyme production by *Bacillus*. Medda and Chandra (1980) reported that *B.licheniformis* CUMC 305 could grow in the presence of 7% NaCl. Horikoshi (1971) reported that an alkalophilic *Bacillus* sp A-40-2 did not show enhancement of growth and enzyme production after addition of NaCl in the medium. Nandakumar (1991) observed that *B.polymyxa* and *B.cereus* grew well and produced amylase in the presence of 0.5% NaCl. Whereas in the present study, *B.coagulans* showed maximal growth and enzyme production in the absence of NaCl.

B.licheniformis TCRDC B 13 produced amylase in the presence of 1% of corn starch and an increase in conc. of starch led to decrease in the levels of enzyme production and growth (Bajpai & Bajpai, 1989). *B.coagulans* could grow well and produce maxial α-amylase at 2% rice starch (Babu & Satyanarayana, 1990). *B.polymyxa* and *B.cereus* produced significant levels of α-amylase at 1% conc. of rice starch (Nandakumar,1991). α-Amylse production in *B.stearothermophilus* MFF4 was dependent on the starch concentrations and low starch concentrations (0.1-0.2%) were more favourable for enzyme production by the organism (Wind et al., 1994). In a similar

fashion *B.coagulans* ACMN 1, in the present study could grow to a maximum with 1% starch and produced maximal enzyme with 0.5% starch. Higher concentration of starch led to decline in enzyme yield. A possible explanation for the observed relationship between starch concentration and α-amylase synthesis is repression of enzyme synthesis at higher starch concentrations by starch-hydrolysis products such as glucose or a difference in O_2 availability due to a change in viscosity at higher starch concentrations or a combination of both (Wind et al., 1994).

Medda and Chandra (1980) reported that *B.coagulans* CUMC 512 and *B.licheniformis* CUMC 305 produced maximal levels of α-amylase after 24 hrs of incubation. *B.licheniformis* TCRDC - B 13 recorded maximal α-amylase production at 40-70 hrs of incubation while the maximal growth was obtained at 20-30 hrs of incubation (Bajpai & Bajpai, 1989). *B.subtilis* was reported to produce maximal α-amylase at 18 hrs (Pazlarova, 1984). Babu and Satyanarayana (1990) observed that *B.coagulans* produced maximal enzymes only after 48 hrs of incubation. Nandakumar (1991) reported that *B.polymyxa* and *B.cereus* produced maximal growth and enzymes by 18-24 hrs although they could record significant levels of enzyme at 12

hrs. In *B.stearothermophilus*, α-amylase production was initiated in the early exponential growth phase and became maximal in the late exponential phase. After 12hrs of growth total α-amylase production reached a maximum of 108 U/ml and declined thereafter (Wind et al., 1994). Whereas in the present study *B.coagulans* could grow to a maximum at 12-18 hrs while recording maximal enzyme production at 14 hrs. The present results are comparable with the results obtained for *B.stearothermophilus* (Wind et al., 1994).

Bindu (1989) observed that *Bacillus sp* produced maximal α-amylase at inoculum concentration of 5-20%. Nandakumar (1991) reported that 1-3% of inoculum concentration of *B.polymyxa* and *B.cereus* yielded maximal amylase production and further increase in the inoculum concentrations did not enhance the enzyme production. Whereas in the present study *B.coagulans* recorded maximal growth with 2.0% inoculum concentration while maximal enzyme could be recorded with 0.2% inoculum concentration, suggesting that this strain is very different from other *B.coagulans* species reported earlier in terms of ability to hyper produce α-amylase.

Carbon and nitrogen sources play an important role in the growth and metabolism of microorganisms present in various environments. Carbon sources generally either induce or repress growth and amylase synthesis. *B.stearothermophilus* was found to grow and synthesize higher amounts of α-amylase in the presence of soluble starch and maltose than glucose and glycerol, which showed a repressing effect (Welker & Campbell, 1963). *B.stearothermophilus* produced α-amylase on induction by starch, while glucose, galactose, fructose, and sucrose suppressed enzyme production (Srivastava and Baruah, 1986). α-Amylase production by *B.amyloliquefaciens* was enhanced by starch followed by glycerol and glucose (Coleman & Grant, 1966). *B.licheniformis* TCRDC B 13 produced higher levels of amylase with starch followed by dextrin, maltose and lactose as substrates, although higher rate of growth was recorded in the presence of glucose (Bajpai & Bajpai, 1989). *B.licheniformis* (ATCC 39326) produced α-amylase in a medium containing 10% lactose (Horwath, 1984). A mutant of *B.cereus* NY14 was found to synthesize α-amylase in the presence of 5% glucose (Yoshigigi and Kanimura, 1988). α-Amylase production in *B.stearothermophilus* was induced by starch, amylopectin, and maltose, while glucose, fructose or sucrose reduced enzyme production (Wind et al., 1994).

In the present study *B.coagulans* could grow well in the presence of all the sugars as carbon sources while maximum growth was recorded with glucose. However maximal enzyme production was observed with maltose followed by galactose, lactose, mannose, xylose, glycerol, glucose and sucrose. The present results are comparable with *B.licheniformis* TCRDC B 13 (Bajpai & Bajpai, 1989). As observed by earlier workers glucose did not support maximal amylase production while promoting growth. This indicates an immediate cessation of de-novo enzyme synthesis, probably due to glucose catabolite repression of enzyme synthesis in *Bacillus coagulans*. α-Amylase synthesis in most *Bacilli* is susceptible to catabolite repression by glucose and fructose (Priest, 1977). The results are in agreement with the general ideas on regulation of exo enzyme synthesis.

Nitrogen source present in the medium influenced the production of α-amylase significantly. Peptone and tryptone were reported to induce the growth and α-amylase synthesis of *Bacillus* sp (Goyal, 1978). *B. subtilis* produced α-amylase at significant levels with peptone and aminoacids compared to ammonia (Fukumoto et al.,1957). Glycine enhanced α-amylase to 300 fold from *B.amyloliquefaciens* (ATCC 23350) (Zhang et al.,

1983). Alanine, arginine, asparagine, aspartic acid, peptone, yeast extract, cysteine, histidine, phenylalanine, lysine and valine all stimulated growth and enzyme production by *B.apiarius* (Ghosh and Chandra, 1984). *B.licheniformis* TCRDC B 13 produced α-amylase at enhanced levels in the presence of peptone followed by meat extract, beef extract and yeast extract. In the present study *B.coagulans* recorded maximal α-amylase synthesis in the presence of cysteine followed by proline, glutamic acid, aspartic acid, asparagine while inorganic nitrogen sources did not favour enzyme production.

4.2. Continuous Production of α-Amylase under Submerged Fermentation

α-Amylase synthesis, by *B.amyloliquefaciens* and some strains of *B.subtlis*, after exhibiting initially high enzyme levels in batch culture, declined during continuous culture (Heineken and O'Connor, 1972; Fencl et al., 1972; Sikyta and Fencl, 1976). *B.stearothermophilus* (Davis et al., 1980) *B.licheniformis* (Emanuilova and Toda, 1984; Priest and Thirunavukkarasu, 1985) and *B.polymyxa* (Griffin and Fogarty,1973) in which amylase production occur during

exponential growth, all exhibit constant levels of amylase production during continuous culture studies. In continuous culture, amylase activity was low (3 U/ml) at dilution rates above 1.2 hr^{-1}. Peak amylase activity was observed at a dilution rate of 0.1- 0.25 hr^{-1}. Continuous culture studies of *Bacillus* in which α-amylase synthesis occurs during exponential growth indicate that as the growth rate is increased above a maximum value the level of enzyme activity begins to decrease quite rapidly (Emanuilova and Toda, 1984). In the present study, α-amylase production by *B.coagulans*, under continuous cultivation was initially high during the commencement of continuous cultivation, after 8 hrs, when the cells were in exponential phase. However enzyme production declined, as observed with *B.amyloliquefacies* and *B.subtilis*, referred above, during continuous culture. Whereas, in contrast to the observations of Emanuilova and Toda (1984), lower dilution rates of 0.05–0.10h^{-1} led to appreciable levels of α-amylase production in the range of 30-88 U/ml, and higher dilution of 0.10h^{-1} resulted in a significantly high volumetric productivity of enzyme at the rate of 5.251 U/ml/hr. These results suggest the suitability of *B.coagulans* as a potential strain for continuous production of α-amylase in stirred tank reactor. However, no comment could be made

about the catabolite repression and induction of α-amylase during continuous production, since those aspects were not studied.

4.3. α-Amylase Production under Solid State Fermentation

Greater interest in solid state fermentation (SSF) has been generated in recent years throughout the world (Stein Kraus, 1984) as it not only yields product with higher concentration (Arima, 1964; Gildyal et al., 1985; Kumar & Lonsane,1987; Renu, 1991) but also offers many other economic and practical advantages, mainly cheap medium, lower capital investment and lower plant operating costs (Forage & Righelato, 1974, Hesseltine, 1977, Lonsane et al., 1985).

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals and as well as growth factors. The bacteria and yeast grow by adhering to the surface of solid substrate particles like wheat bran (Lonsane & Ramesh, 1990).

As the yield of product from any fermentation process, irrespective of the process, submerged or solid

state, is governed by the environmental variables it becomes mandatory to optimize these variables in order to obtain maximal yields of the target product. In the present study an effort was made to optimize moisture content, particle size, substrate concentration, NaCl concentration, carbon sources, nitrogen sources, inoculum concentration and incubation period for maximal α-amylase production under SSF.

The critical importance of moisture content of the medium and its control during fermentation are extensively documented for SSF processes involving fungal cultures (Lonsane et al., 1985). The optimum water requirement for SSF production of α-amylase was reported as 65% for *B.licheniformis* (Ramesh & Lonsane, 1990). In the present study 60% moisture content yielded maximal α-amylase from *B.coagulans*. Similar levels of moisture content required for *B.licheniformis* and *B.coagulans* adds evidence to the fact that moisture content is a critical factor in SSF.

Wheat bran of particle size between 0.2-0.8 cm was used for the production of amylase by *B.licheniformis* which yielded high titres of α-amylase (Ramesh & Lonsane, 1989).

Whereas wheat bran particles of >600μ supported maximal α-amylase production by *B.coagulans* in the present study.

Results of the present study with reference to effect of pH on α-amylase production by *B.coagulans* under submerged fermentation as well as solid state fermentation clearly indicated the response of the organism towards pH. Thus, while the optimum pH was 8 for α-amylase production under SmF it was pH 6 under SSF. This might be due to difference in the complexity of environmental variables that vary between SSF and SmF, such as moisture content and particle size, which could have influenced the pH of the medium. The interesting difference is that an alkaline pH under SmF and slightly acidic pH under SSF were observed to be optimal for this organism for maximal α-amylase production unlike some strains reported earlier. These results also testify the tolerance of this organism to wide range of pH conditions for maximal growth and enzyme production.

In contrast to pH, the incubation temperature did not influence the rate of enzyme production by *B.coagulans*. Both under SSF and SmF 35°C was observed to be the optimum

incubation temperature for maximum enzyme production testifying the general fact that the temperature is independent of other environmental variables in its effect.

Glucose which was observed to effect catabolite repression on α-amylase production by *B.coagulans* during SmF was recorded to enhance α-amylase production under SSF. A similar observation was reported earlier for *B.licheniformis* M-27 (Ramesh and Lonsane, 1991). According to them, this might be due to the ability of the SSF technique to significantly minimize catabolite repression of α-amylase production by the culture. This ability of the SSF system has economic implication as it dominates the need to use lower substrate concentration in fermentation medium, or cost intensive fermentor operation strategies, which otherwise would be vitally essential for overcoming catabolic repression in SmF process (Ramesh and Lonsane, 1991).

Among the nitrogen sources tested, cysteine was observed to enhance α-amylase production both under SmF and SSF compared to other nitrogen sources indicating the requirement of this aminoacid by *B.coagulans*. Beef extract was also observed to enhance amylase production under SSF.

This might be probably due to the complex minerals, vitamins, and aminoacid content of beef extract which could have enhanced the enzyme production.

Earlier investigators have observed high ratios of inoculum vs bran for α-amylase production. 1:10 in the case of *B.licheniformis* (Ramesh and Lonsane,1990) and *B.coagulans* (Sathyanarayana, 1994). The present results are in agreement with the earlier observations where *B.coagulans* B 49 was observed to produce maximal α-amylase after 72 hours of incubation. In the present study also *B.coagulans* ACMN-1 recorded maximum amylase yield after 72 hours. Further it was also observed that the bacteria responded to the difference in the environment, in which it was growing, in terms of the time taken for secreting maximal levels of enzyme. Thus while it could produce maximal levels at 14 hrs under SmF, it required 72 hrs under SSF. This difference may be attributed to the free state of organism under SmF, where it can have ready access to the available nutrients through motility besides movement of particles compared to their static state due to surface adhesion on solid substrate particles under SSF.

4.4 Effect of Mutation on α-Amylase Production

Mutation of a producer strain and screening or preferably selection for a hyperproducing phenotype has proved successful for the improvements of yields of enzymes and primary and secondary metabolites (Yamane and Maruo, 1980). Several independent mutations that increased amylase synthesis in the parental strain of *B.subtilis* were obtained either by screening for increased zones of starch hydrolysis following mutagenesis or by selecting for antibiotic-resistant mutants such as tunicamycin or cycloserine-resistant strains that might be pleiotropically affected in enzyme secretion. These individual mutations were then combined in a single strain using DNA mediated transformation which finally led to a 1500 fold increase in amylase production (Yamane and Maruo, 1980). Mutation with ultraviolet radiation, ethylene imine and nitrosoguanidine (NTG) in *B.subtilis* produced a double fold yield of α-amylase obtained with the parent strain (Markkanen, 1975, Bailey and Markkanen, 1975). A 5 fold increase in α-amylase was recorded in *B.subtilis* mutated through successive treatments with NTG followed by UV irradiation (Shah et al., 1989). In the present study, mutation of *B.coagulans* with UV irradiation and NTG treatment resulted in

a double fold yield of α-amylase which is comparable to the observations of Markkanen (1975) and Bailey and Markkanen (1975) with *B.subtilis*. The present study indicated scope for further improvement in α-amylase yield through mutation.

4.5. Enzyme Studies

Partial purification by acetone fractionation followed by dialysis of thermostable alkaline α-amylase from *B.licheniformis* CUMC 305 and *B.coagulans* CUMC 512 resulted in 52.17% and 49.3% yield and 204 U/mg of protein and 151.2 U/mg of protein as specific activities, respectively (Medda & Chandra, 1980). Morgan and Priest (1981) obtained 66% yield of α-amylase after purification by dialysis and ion exchange chromatography from *B.licheniformis* NCIB 6346. Ramesh and Lonsane (1989) obtained α-amylase, produced by *B.licheniformis* under solid state fermentation, upto 7.9 fold after ammonium sulphate fractionation and 50 fold after chromatographic separation with CM-cellulose. In the present study a maximum of 23% yield of α-amylase with 51.98 fold purification and 176.21 U/mg protein specific activity could be obtained after purification. These results are comparable with that reported for *B.coagulans* earlier.

α-Amylase obtained from *B.coagulans* isolated by Campbell (1955) had optimal activity at 70°C. Medda and Chandra (1980) reported that α-amylases from *B.licheniformis* CUMC 305 and *B.coagulans* CUMC 512 were thermostable at 90°C and 85°C, and active at a wide range of pH with an optima of pH 9.5 and pH 7.5-8.5 respectively for the strains. Whereas α-amylase from *B.licheniformis* sp recorded an optimum activity at 90°C and at a wide range of pH 5.5-10.0. Nandakumar (1991) recorded that α-amylases of *B. polymyxa* and *B.cereus*, were active upto 80°C and at pH 5-10. α-amylase from *B.stearothermophilus* had temperature optima of 70°C and pH optimum in the range of 5.5-6.0 (Wind et al., 1994). In the present study the α-amylase from *B.coagulans* showed 70°C temperature optima and thermostability at 100°C and optimal activity and stability at pH 9 and stable over pH 6-11. These results are in agreement with earlier reports.

Yankov et al., (1986) studied the effect of substrate concentration on the rate of enzyme action on soluble starch with a thermostable α-amylase of *B.lichenoformis* MB10 and found that 300 g/l (30%) substrate concentration was the optimal for maximal enzyme activity and concentration above 300 g/l inhibited the α-amylase activity

at 100°C. Nandakumar (1991) recorded that a 3% substrate concentration was favourable for maximal activity of amylase from *B.polymyxa* and *B.cereus*. Whereas, *B.coagulans* recorded 1% substrate concentration as optimal with a Km of 4.9 mg/ml.

Moseley and Keay (1970) reported that α-amylase from *B.subtilis* NRRC B 3411 showed higher activity and stability in the presence of calcium ion. Horikoshi (1971) also observed that calcium retains 100% of original activity and found that addition of urea inhibited α-amylase from *Bacillus* sp No.A40-2. Hayashi (1988) studied the effect of heavy metal ions such as Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Ag^{2+} on an alkaline maltohexose forming amylases from *Bacillus* sp M.167 and observed that Mg^{2+}, Zn^{2+} completely inhibited the activity while Ca^{2+}, and Pb^{2+} reduced the activity to 40-60%. Nandakumar (1991) observed that calcium did not inhibit activity α-amylase from *B.polymyxa* and *B.cereus*. In the present study also calcium was observed to support 100% activity at optimal conditions and stability of α-amylase at 100°C. Cu^{2+}, Sn^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ag^{+} led to residual activity in the range of 88-95% indicating a partial inhibition unlike earlier reports. Another interesting observation made in the present study was that Polyethylene

glycol, Xylose, Maltose and Dextrose demonstrated insulating effect on α-amylase in retaining thermostability at 100°C similar to that recorded by Wind et al., (1994).

4.6 Concluding Remarks

Based on the results obtained in the present study, the following conclusions are drawn. *B.coagulans* ACMN 1, isolated from soil, holds potential for large scale production of themostable α-amylase employing solid state fermentation as well as under continuous cultivation in a stirred tank reactor. The bacteria is susceptible to mutation and hence by subjecting to repeated mutagenesis, using UV irradiation and chemical mutagens, the α-amylase yield could be considerably enhanced. The purified α-amylase showed thermostability at 100°C and remained stable at 100°C in the presence of some sugars. This information suggests scope for further improving the enzyme thermostability for industrial application. However, further studies are required before qualifying this enzyme for industrial use.

SUMMARY

5.1 *Bacillus coagulans* ACMN 1 isolated from soil environments of Cochin University campus and available in the culture collection of Centre for Biotechnology, was used in the present study for α-amylase production.

5.2 Initially *B. coagulans* was characterized for their optimal requirements of temperature, pH, NaCl, substrate concentration, additional carbon and nitrogen sources and inoculum concentration in nutrient broth.

5.3 *B. coagulans* recorded maximal growth at 35°C, pH 8-9, absence of NaCl, and 1% soluble starch.

5.4 Addition of glucose and mannose enhanced the growth. Tryptone and peptone promoted more growth.

5.5 *B.coagulans* was found to grow maximally at all levels of inoculum ranging from 1-7%. Results suggested that 2% will be adequate for maximal growth.

5.6 From the growth curve it was observed that the bacteria enters in to the logarithmic phase after 2 hrs and remains in the same phase till 20 hrs.hrs.

5.7 Generation time of *B.coagulans* was calculated as 57.5 minutes.

5.8 α-Amylase production by *B.coagulans* in mineral salts starch medium, under submerged fermentation was studied. Both batch and continuous fermentation studies were conducted.

5.9 Under batch process of SmF, optimum conditions for maximal α-amylase production by *B.coagulans* was recorded as 35°C, pH 8.0, absence of NaCl. and 0.5% soluble starch.

5.10 Addition of maltose and galactose in MSSM promoted maximal enzyme production under SmF.

5.11 Addition of cysteine, proline and glutamic acid enhanced maximal α-amylase production under SmF.

5.12 Optimal inoculum concentration for maximal α-amylase under SmF was recorded as 0.2%.

5.13 α-Amylase synthesis was maximum at 14 hrs of incubation (136 U/ml) although significant levels of α-amylase production could be recorded from 8 hrs onwards. It appears that α-amylase synthesis occurs during exponential phase.

5.14 Continuous production of α-amylase by *B.coagulans* under submerged fermentation at three different flow rates 20 ml/h - 50ml/h was studied.

5.15 Increase in dilution rates ($0-10h^{-1}$) enhanced volumetric productivity of α-amylase up to 5.25 U/ml/hr. While maximal enzyme/ml was recorded with reduced flow rate (20ml/hr).

5.16 α-amylase production by *B.coagulans* was studied under solid state fermentation using commercial wheat bran. Initially the bioprocess was optimized.

5.17 Commercial wheat bran with particle size > 600 μm enhanced maximal yield of α-amylase under SSF.

5.18 Initial moisture content of the wheat bran required for enhanced α-amylase production was recorded as 60%.

5.19 Optimal conditions for maximal α-amylase production under SSF was 35°C and pH 6.0.

5.20 Addition of glucose in the SSF medium enhanced α-amylase production. Similarly maltose and galactose boosted α-amylase synthesis.

5.21 Addition of cysteine/beef extract/glutamic acid/ asparagine all promoted maximal α-amylase production under SSF.

5.22 Optimal inoculum concentration required for maximal α-amylase production under SSF was 10%.

5.23 Maximal α-amylase production 52986 U/gds) under SSF was obtained after 72 hrs, at optimized conditions

5.24 *B.coagulans* cells irradiated with UV for 120 seconds resulted in improvement in enzyme yield (142 U/ml) compared to control (99 U/ml).

5.25 Chemical mutagenesis of *B.coagulans* with 50mg of NTG led to a double fold yield of α-amylase (195 U/ml) over the control (99 U/ml).

5.26 α-Amylase was purified by ammonium sulphate precipitation followed by dialysis and chromatrographic separation with DEAE - cellulose.

5.27 The final yield of purified enzyme was 23 % with 51.98 fold purification. The specific activity of purified enzyme was 176.2 U/mg protein. Molecular weight of α-amylase was approximately 93000.

5.28 The purified α-amylase was thermostable at 100°C with a temperature optima of 70°C for maximal activity. Optimal pH for maximal activity was pH 9.0.

5.29 The purified enzyme had a Km of 4.7 mg/ml and optimal substrate conc. was 1.0%.

5.30 Addition of calcium in the reaction mixture retained 100% of original activity and promoted stability at 100°C. Similarly addition of polyethylene glycol, xylose, maltose and dextrose promoted thermostability at 100°C.

REFERENCES

Abramov, A.T., Tsalpina, I.A., Stepanov, V.M., Loginova, L.G., (1986) *Biochem.* (USSR), 51, 100.

Alzard, D. and Raimbault, M., (1981) *Eu. J. Appl. Microbiol. Biotechnol.*, 12, 113.

Allen, A.M., Hussein, A.M. and Ragab, A.M., (1977) *Zentralblatt fur Bakteriologie Prasitenkunde Infektionskrankheiten and Hygine, Abteilung*, 11, 109, 143.

Antranikian, G., (1989) *Appl. Biochem. Biotechnol.*, 20, 267.

Argos, P., Rossmann, M.G., Grau, J.M., Zuber, H., Frank, G. and Tratschin, J.D., (1979) *Biochem.*, 18, 5698.

Arima, K., (1964) *In Global Impacts of Applied Microbiology*, (Ed.), Starr, M.P., John Wiley and Sons, New York, p.277.

Asther Michele and Meunie Jean-Claude, (1990) *Enzyme Microb. Technol.*, 12, 902.

Aunstrup, P., (1979) *Appl. Environ. Microbiol.*, 45, 205.

Babu, K.R. and Satyanarayana, T., (1990) *Proceedings of National Symposium on Current Trends in Biotechnology*, Cochin, India.

Bailey, M.J. and Markkanen, P.H., (1975) *J. Appl. Chem. Biotechnol.*, 25, 73.

Bajpai, P. and Bajpai, P.K., (1989) *Biotechnol. Bioeng.*, 33, 72.

Barfoed, H.C., (1976) *Cereal Food World*, 21, 588.

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

Birch, G.G. and Schallenberger, R.S., (1973) *In Molecular Structure and Function of Food Carbohydrates*, (Eds.), G.G.Birch and L.F. Green, Applied Science, London, p.20.

)yer, E,W., Ingle, M.B. and Mercer, G.D., (1979) *Starch* , 31, 36.

:ock, T.D., (1986) In *Thermophiles, General Molecular and)plied Microbiology*, (Ed.), T.D. Brock, Wiley, New York, P.1.

:ooks, J.R., Griffin, V.K. and Kattan, M.W., (1986) Cereal iemi., 63, 465.

:own, S.H., Costantio, H.R., Kelly, R.M.,(1990) *Appl, Environ. :crobiol.*, 56, 1985.

ionocore, V., Caporale, C., De Rose, M. and Gambacorta, A., 976) J. Bacteriol., 128, 515.

mpbell, L.L., (1955) *Arch. Biochem. Biophys.* 54, 154.

andra, A.K., Medda, S. and Bhadra, A.K., (1980) *J. Ferment. chnol.*, 58, 1.

ahal, D.S., (1983) Appl. Envion. *Microbiol.*, 45, 205.

otia, C., (1975) Nature, 254, 304.

u, P.K., Trimble, R.B. and Maley, F., (1978) *J. Biol. Chem.* 3, 8691.

leman, G. and Grant, M.A., (1966) *Nature*, 211, 306.

leman, R.D., Yang, S.S. and Mc Alister, M.P., (1987) *J. cteriol.*, 169, 4302.

vis, P.E., Cohen, D.L. and Whitaker, A., (1980) *Antonie Van euwenhoek*, 46, 391.

vid, M.H., Güñther, H. and Roper, H., (1987) *Starch*, 39, 436.

ancilova, E.I. and Toda, K., (1984) *Appl. Microbiol. otechnol.*, 19, 301.

Fairbairn, D.A., Priest, F.G. and Stark, J.R., (1986) *Enzyme Microbiol. Technol.*,8, 89.

Fencl, Z., Ricica, J. and Kodesova, J., (1972) *J. Appl. Chem. Biotechnol.*, 22, 405.

Fencl, Z., Pazlarova, J., (1982) *Folia Microbiol.*, 27, 340.

Fogarty, W.M. and Kelly, C.T., (1980) *In Economic Microbiology*, Vol.V, (Ed.), A.H. Rose, Academic Press, London, p.115.

Fogarty, W.M., (1983) *In Microbial Enzyme and Biotechnology.* (Ed), W.M. Fogarty, Applied Science Publishers, London, P.1.

Forage, A.J. and Righelato, R.C., (1974) *In Economic Microbiology, Vol. IV.*, (Ed.), A.H. Rose, Academic Press, London, p.289.

Friedman, S.M., (1976) (Ed.), *Biochemistry of thermophily*, Academic press, New York, 1976.

Frommel, C. and Hohne, W.E., (1981) *Biochem. Biophys. Acta*, 670, 25.

Fukumoto, J., Yamamoto, T., Tsuru, D. and Ichikawa, M., (1957) In Proceedings of the International Symposium on Enzyme Chemistry, Pergamon Press, London, p.479.

Fukumoto, J. and Okada, S., (1963) *J. Ferment. Technol.*, 41, 427.

Galabova, D., (1969) *Act. Microbiol. Bulg.*, 11, 24.

Ghildyal, N.P., Lonsane, B.K., Sreekantaiah, K.R. and Murthy, V.S., (1985) *J. Fd. Sci. Technol.*, 22, 171.

Ghosh, S.B. and Chandra, A.K., (1984) *Zentralbl. Microbiol.*, 139, 293.

Giraud. F., Gosselin, L., Marin, B., Parada, J.L. and Raimbault.M., (1993) *J. Appl. Bacteriol.*, 75, 276.

Glymph, J.L. and Stutzenberger, F.J., (1977) *Appl. Enviorn, Micriobiol.*, 34, 391.

Godfrey, R.J., (1983) (Ed.), *Industrial Enzymology,* The Application of Enzymes in Industry, The Nature Press, New York.

Goyal, S.G. and Khandeparker, V.G., (1978) *Ind. J. Microbiol.*, 13, 73.

Granum, P.E., (1979) *J. Food. Biochem.*, 3, 1.

Grafe, U., Bormann, E.J., Roth, M. and Niegenfiend, M., (1986) *Biotech. Lett.*, 8, 615.

Greenberg, M.A., Wilder, T. and Mahoney, R.R., (1985) *J. Dairy Res.*, 52, 439.

Greenshields, R.N. and Mac Gillivray, A.W., (1972) *Process Biochem.*, 7, 11.

Griffin, P.J. and Fogarty, W.M., (1973) *Biochem.* Soci. Trans., 1, 397.

Grootegoed, J.A., Lauwers, A.M. and Heinen, W., (1973) *Arch. Microbiology*, 90, 223.

Harris, J.I., Hocking, J.D., Runswick, M.J., Suzuki, K. and Walker, J.E., (1980) Eur. *J. Biochem.*, 108, 535.

Hayashi, T., Aleiba, T. and Horikoshi, K., (1988) *Appl. Microbiol. Biotech.*, 28, 281.

Heinen, U.J. and Heinen, W., (1972) *Archv. Microbiol.*, 82, 1.

Heineken, F.G. and O'Couner, R.J., (1973) *J. Gen, Microbiol.*, 73, 35.

Heinen, W. and Lauwers, A.M., (1976) In Enzymes and Proteins from Thermophilus Microorganisms, (Ed.), H. Zuber, Birkhauser Verlag, Basel, p.77.

Hesseltine, C.W., (1977) *Process Biochem.*, 12, 24.

Hobsen, P.N. and Macphersan, M., (1952) *Biochem.* J. 52, 671.

Horikoshi, K., (1971) *Agric. Biolog. Chem.* 35, 1783.

Horwath, R.O., (1984) Us Patent, No.4, 473, 645.

Hyun, H.H. and Zeikeus, J.G., (1985) *Appl, Environ. Microbiol.*, 49, 1168.

Ilczuk, Z., Fiedurek, J. and Paszcynski, A. (1983) *Starch/Starke*, 35, 397.

Ingle, M.B. and Boyer, E.W., (1976) In Microbiology, (Ed.), Schlessinger, Academic Press, New York, p.402.

Kallio. P. (1986) *Eur. J. Biochim.*, 158, 491.

Kanno, M. (1986) *Agric. Biol. Chem.*, 50, 23.

Kimura, T., Ogata, M., Yoshida, M. and Nakakukei, T., (1988) *Biotech. Bioeng.* 32, 669.

Koch, R., Zablowski, P. and Antranikian, G., (1987) *Appl, Microbiol, Biotechnol.*, 27, 192.

Koch, R., Zablowski, P., Spreinat, A. and Antranikian, G.,. (1990) *FEMS Microbiol. Lett.*, 71, 21.

Koch, R., Spreinat, A., Lemke, K. and Antranikian, G. (1990b) Proc. Eur. *Cong. Biotechnol*, p;78.

Krishnan, T. and Chandra, J.K.,(1983) *Appl. Environ. Micribiol.* 46, 430.

Kumar, P.K.R. and Lonsane, B.K., (1987) *Process Biochem.* 22, 139.

Kunthala Jayaraman and R. Jayaraman, (1979) In Laboratory Mannual in Molecular Genetics, Wiley Eastern Ltd., New Delhi.

Laemmli, U.K., (1970) *Nature*, 277, 680.

Laiode, B.M., Chamliss, G.H. and Mc Connell, D.J., (1989) *J. Bacteiol.*, 171, 2435.

Lonsane, B.K., Ghildyal, N.P. and Murthy, V.S., (1982) In Technical Brochure Symposium on Fermented Food, Food Contaminants, *Biofertilizer and Bioenergy*, Asso. of Microbiologists of India, Mysore, p.1.

Lonsane, B.K., Ghildyal, N.P., Budiatman, S. and Ramakrishna, S.V., (1985) *Enzyme Microb. Technol.*, 7, 258.

Lonsane, B.K. and Karanth, N.G. (1990) In Proceedings, National Symposium on Current Trends in Biotechnology, Cochin University of Science and Technology, Cochin. p. 39.

Lonsane, B.K. and Ramesh, M.V., (1990) *Adv. Appl. Microbiol.* 35, 1.

Lowery, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., (1951) *J. Biol, Chem.*, 193, 265.

Modi, E. Antranikan, G., Ohmiya, K. and Gottschalle, G., (1987) *Appl. Environ. Microbiol.* 53, 1661.

Manning, G.B. and Campbell, L.L., (1961) *J. Biol. Chem.*, 236, 2952.

Markkanen, P., (1975) In Materials and Proceesing Technology Publication, Technical Research Centre of Finland, 12.

Maruo, B. and Toja, T., (1985) *J. Gen. Appl. Microbiol.*, 31, 323.

Mc Carthy, J., Kelly, C.T. and Fogarty, W.M., (1983) Biochem. Soci. Trans., 16, 1846.

Medda, S. and Chandra, A.K.,(1980) *J. Appl. Bacteriol.*, 48, 47.

Meers, J.L., (1972) *Antonie Van Leuvenhoek*, 38, 585.

Melasniemi, H., (1987) *Biochem.J.*, 246, 193.

Merkler, D.J., King Farrington, G. and Wedler, F.C., (1981) *Int. J. Peptide and Protein*, Res., 18, 430.

Mitricia, L. and Granuin, P.E. (1979) *J. Lebensm. Unters, Forsh.*, 169, 4.

Mohandass C. and Chandrasekaran, M. (1974) *Ind. J. Exp. Biol.*, 33, 912.

Morgan, F.J. and Priest, F.G., (1981) *J. Appl. Bacteriol.*, 50, 107.

Moseley, M.H. and Keay, L. (1970) *Biotech. Bioeng.*, 12, 251.

Munoz, R.G., Valencia, T.J.R., Sanches, S. and Farres, A., (1991) *Biotech. Lett.*13, 277.

Nandakumar, M.P., (1990) Ph.D Thesis, Cochin University of Science and Technology, Cochin.

Nagendra Prabhu. G. and Chandrasekaran, M., *World J. Microbiol. Biotech.*, (In press).

Nakumura, N., Sashihara, N., Nagayama, H. and Horikoshi, K., (1989) *Starch/Starch*, 41, 112.

Nicholson, W.L. and Chambliss, G.H., (1985) *J. Bacteriol.*, 161, 875.

Nicholson, W.L. and Chambliss, G.H., (1986) *J. Bateriol.*, 165, 663.

Norman, B.E., (1979) In Microbial Polysaccharides and Polysaccharases, (Eds.), R.C.W. Berkeley, G.W. Goodway and D.C. Ellwood, Academic Press, London, p.339.

Obi, S.K.C. and Odibo, F.J.C., (1984) *J. Micriobiol*, 30, 780.

Okamoto, H., Kobayashi, S., Momma, H., Hashimoto, H., Hara, K. and Kainuma, K., (1986) *Appl. Microbiol. Biotechnol.*, 25, 137.

Onishi, H. (1972) *J. Bacteriol.*, 109, 570.

Onishi, H. and Hidaka, O. (1978) *Can. J. Microbiol.*, 24, 1017.

Ortlepp, S.A., Ollington, J.H. and Mc Connell, D.J., (1983) *Gene*, 23, 267.

Orlando, A.R., Ade, P., Di Maggio, D., Fanelli, C. and Vittozzi, L. (1983) *Biochemical J.*, 209, 561.

Outtrup, H. and Aunstrup, K., (1975) In Proceedings of the First International Congress, Int. Asso. Microbiol. Soc., 5, 204.

Outtrup, H. and Norman, B.E., (1984) *Starch*, 36, 405.

Pace, C.N. and McGrath, T., (1980) *J. Biol. Chem.*, 255, 3862.

Pazloarova, J., Baig, M.A. and Votruba, J., (1984) *Appl. Microbiol. Biotechnol.*, 20, 331.

Pazur, J.H. and Okada, S., (1966) *J. Biol. Chem.*, 241, 4146.

Pfuellev, S.L. and Elliott, W.H. (1969) *J. Biol.Chem.*, 244.

Plant, A.R., Patel, B.K.C., Morgan, H.W. and Daniel, R.M., (1987) *Syst. Appl. Microbiol.*, 9, 158.

Ponnuswamy, P.K., Muthusamy, R. and Manavalen, P., (1982) *Intl. J. Biol. Macromol.* 4, 186.

Priest, F.G., (1977) *Bacteriol.* Rev., 41, 711.

Priest, F.G. (1992) In Microbial Degradation of Natural Products, (Eds.) G. Winkelmann, VCH Verlagsgesellschaft mblt, Weinhein, p.1.

Priest, F.G. and Sharp, R.J. (1989) In Fermentation Process development of Industrial Organism, (Ed.), Justin O'Neway, Marcel Dekker Inc, New York, p.73.

Priest, F.G. and Thirunavakkarasu, M., (1985) *J. Appl. Bacteriol.*, 58, 371.

Ramesh, M.V. and Lonsane, B.K., (1987a) *Biotech. Lett.*, 9, 322.

Ramesh, M.V. and Lonsane, B.K., (1987b) *Biotech. Lett.*, 9, 501.

Ramesh, M.V. (1989) Ph.D Thesis, Mysore University, Mysore.

Ramesh, M.V. and Lonsane, B.K., (1989) *Biotech. Lett.*, 11, 49.

Ramesh, M.V. and Lonsane, B.K., (1990) *Appl. Microbiol Biotech.*, 33. 501

Ramesh M.V. and Lonsane, B.K., (1991) *Appl, Microbiol. Biotech*, 35, 591.

Reichelt, J.R., (1983) In Industrial Enzymology, (Eds.), T. Godfrey and J. Reichelt, Nature Press, New York, p.375.

Renu, S. (1991) Ph.D Thesis, Cochin University of Science and Technology, India.

Robyt, J.R. and Ackerman, R.J., (1971) *Arch. Biochem. Biophys.*, 145, 105.

Robyt, J. and French, D., (1971 a) *Arch. Biochem. Biophys.*, 100, 451.

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

Saiki, T., Kobayashi, Y., Kawagoe, K. and Beppa, T., (1985) *Int. J. Syst. Bacteriol.*, 35, 253.

Sasaki, T., Yamasaki, M., Maruo, B., Yoneda, Y., Yamane, K., Takutsuki, A. and Tamuva, G., (1976) *Biochem. Biophys. Res Com.* 70, 125.

Sathyanarayana, T., (1994) In Solid State Fermentation, (Ed.), Ashok Pandey, Wiley Eastern Ltd., New Delhi, 122.

Schmid, R.D. (1979) *Adv. Biochem. Eng.*, 12, 41.

Sen, S. and Chakrabarty, S.L., (1986) *J. Appl. Bacteriol.*, 60, 119.

Shah, N.K., Nehete, P.N., Shah, V.D. and Kothari, R.M., (1989) *J. Biotechnol.*, 11, 67.

Shimazu, M., Kanno, M., Jamura, M. and Snekane, M. (1978) *Agri. Biol. Chem.*, 42, 1681.

Skyta, B. and Fend, Z., (1976) In Continuous Cultures. Applications and New Fields, Vol. 6, (Eds.), A.C.R. Dean, D.C. Ellwood, C.G.T. Evans and J.Melling, Ellis Horwood, Chichestev, 158.

Simoes - Mendes, B., (1984) *Can. J. Microbiol.*, 30, 1163.

Srivastava, R.A.K. and Baruah, J. N., (1986) *Appl. Env. Microbiol,*. 52, 179.

Stark, J.R., Stewart, T.B. and Preist, F.G., (1982) FEMS *Micriobiol. Lett.*, 15, 295.

Steinkraus, K.H. (1984) *Acta Biotechnol.*, 4, 19.

Stellwagen, E., (1984) In Enzyme Engineering 7, (Eds.) A.I Lasking, G.T. Tsao and L.B. Wingard, Jr., Annals of the New York Academy of Sciences., 434, 1.

Stefan Janecek, (1993) *Process Biochem.*, 28, 435.

Strickland, L.H., (1951) *J. Gen. Microbiol.*, 5, 698.

Stutzerberger, F. and Carnell, R. (1977) *Appl. Environ. Microbiol.*, 34, 234.

Sundaram, T.K., (1986) In Thermophiles, General Molecular and Applied Biology, (Ed.), T.D. Brock, Wiley, New York, 75.

Suzuki, Y., Nagayama, T., Nakano, H. and Oishi, K., (1987) *Starch.*, 39, 211.

Takasaki, Y., (1982) *Agic. Biol. Chem.*, 46, 1539.

Takasaki, Y., (1983) *Agic. Biol. Chem.*, 47, 2193.

Takasaki, Y., (1985) *Agic. Biol. Chem.*, 49, 1091.

Takasaki, Y., Furutani, S., Hayashi, S. and Imada, K., (1994) *J Ferm. Biotechnol.*, 77, 94.

Tanaka, T., Ishimoto, E., Shimomura, Y., Taniguchi, M. and Oi, S., (1987) *Agri. Biol.* Chem. 51, 399.

Thirunavakkarasu, M. and Priest, F.G., (1980) FEMS, *Microbiol, Lett.*, 7, 315.

Uchnio, F., (1982) *Agri Biol. Chem.* 46, 7.

Umesh Kumar, S., Fasiha Rehana and Krishna Nand, (1990) *Enzyme Microb. Technol.*, 12, 714.

Underkofler, L.A., Denault, L.J. and Hou, E.F., (1965) *Die Starke.*, 6, 179.

Upton, M.E. and Fogarty, W.M. (1977) *Appl. Environ. Microbiol.*, 33, 59.

Walker, G.T. and Whelan, W.J., (1960) *Biochem.*J., 76, 257

Wang, D.I.C., Cooney, C.L., Demain, A.L., Dunhill, P., Humphrey, A.E. and Lilly, M.D., (1979) In Fermentation and Enzyme Technology, John Wiley and Son, New York.

Ward, O.P. and Moo-Young, M., (1988) *Biotech. Adv.*, 6, 39.

Wasserman, B.P., (1984) *Food Technol.*, 28, 78.

Welker, N.E. and Campbell, L.L., (1963a) *J. Bacteriol.*, 86, 681.

Welker, N.E. and Campbell, L.L., (1963b) *J. Bacteriol.*, 86, 687.

Welker, N.E. and Campbell, L.L., (1967) *J. Bacteriol.*, 94, 1124.

Whelan, W.Y. and Nasr, H., (1951) *Biochem. J.*, 48, 416.

Wind, R.D., Buiteluav, R.M., Eggink, G., Huizing, H.J. and Dijkhuizen, L., (1994) *Appl. Micriobiol. Biotechnol.*, 41, 155.

Wiseman, A. (1978) In Topics in Enzyme and Fermentation Biotechnology (Ed.), A. Wiseman, Wiley, Chichestev, Z., 180.

Workeman, W.E., Mchindein, J.H. and Dean, D,H, (1986) Crit. *Rev. Biotech.*, 3 (3), 199.

Yamamoto, M., Tanaka, Y. and Horikoshi, K., (1972) *Agri. Biol. Chem.*, 36, 1819.

Yamaguchi, K., Nagata, Y. and Maruo, B., (1974) *J. Bacteriol.*, 119, 416.

Yamane, K. and Maruo, B. (1980) In, Molecular Breeding and Genetics of Applied Microorganisms. (Eds.) K. Sakaguchi and M. Onish, Academic Press, London, p. 117.

Yankov, D., Dobreva, E., Beschkov, V. and Emanuilova., (1986) *Enz. Microb. Technol.*, 8, 665.

Yoneda, Y., Yamane, K., Yamaguchi, K., Nagata, Y. and Maruo, B., (1974) *J. Bacteriol.*, 120, 1144.

Yoshigi, N., Chikano, T. and Kamimura, M., (1985a) *Agric, Biol. Chem.*, 49, 3369.

Yoshigi, N., Chikano, T. and Mori, Y., (1986) U.S. Patent No.4, 591, 561.

Yoshigi, N. and Kamimura, M., (1988) *Agri. Biol. Chem.*, 52, 2365.

Zhang, Q., Tsukagoshi, N. Miyushiro, S. and Udaka, S., (1983) *Appl. Env. Microbiol.*, 46, 293.