"MICROBIAL ENZYME PRODUCTION UTILIZING BANANA WASTES"

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IN
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By

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CERTIFICATE

Certified that the work presented in this thesis is based on the bonafide work done by Ms. C. Krishna under my guidance in the Centre for Biotechnology, Cochin University of Science and Technology and that no part there of has been included in any other thesis submitted previously for the award of any degree.

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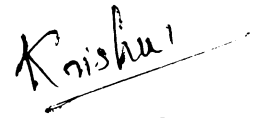
DECLARATION

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, Centre for Biotechnology and that no part of this thesis has been included in any other thesis submitted previously for the award of any degree.



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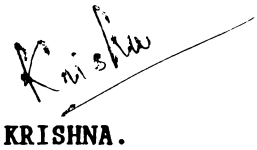


C. KRISHNA.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1. PREFACE

The essential mission of mankind can be defined as the many sided material and cultural development of each human being and the society as a whole. A healthy development can be accomplished only in a good quality environment, and thus environmental protection has become one of the primary targets of society.

Rapid industrialisation and urbanisation all over the world has not only significantly contributed to the problems of solid waste disposal but also has become a national problem of considerable magnitude, which is having significant impact on public health, natural resources availability, and economy of the country.

The relationship between public health and improper disposal of solid wastes has long been recognized. Rats, flies and other disease vectors breed in open dumps and in residential areas or other places where food and harborage are

available. An extensive search of the medical literature has indicated an association between solid wastes and 22 human diseases. Further, leachate from open refuse dumps and poorly engineered land fills contaminate surface and ground waters.

In this context exploitation of wastes through reuse or recycling processes could pave ways and means for not only the safe disposal of wastes but also resource mobilisation towards economic development of a country like India.

Solid wastes include 1. Municipal solid waste, 2. Industrial solid waste, 3. Agricultural waste.

Municipal waste is a collective term referring to residential, commercial, institutional and demolition solid wastes. The rise of certain industries and marketing techniques, have also contributed to the increase in per capita generation of solid wastes. Industrial wastes include those wastes generated from product processing, packaging and shipping. Since industries vary so much in their type and quantity of materials handled and processed, the composition of wastes from one industry to another may be completely different. Agricultural waste generation rates have been

reported on an average annual per capita basis and for specific food crops and it varies from region to region(Cheremisinoff & Morresi, 1976).

Increasingly, solid wastes are being viewed as a "resource out of place" to be recovered and reused whenever possible. As waste tonnages accumulate and greater demands are placed upon the Earth's resources, every indication is that we will run short of both disposal sites and certain mineral and forest products. Hence, it has become mandatory that the materials which have the potential of being recovered and reused from the solid waste stream may be segregated and utilised appropriately. Reuse of agro-industrial waste products is gaining great significance in developing countries due to their availability and low cost.

One of the world's most important fruit, "the banana" is consumed extensively throughout the tropics, where it is grown, and in the temperate zone, where it is popular because of its flavour, food value and availability at all times of the year (Encyclopaedia Britannica, 1964). Bananas represent 40% by weight of all world trade in fruits, either fresh or dried (Chung, 1976).

The average world production, as estimated by the Food and Agriculture Organisation of the United Nations (1971 & 1993), has gone up from 12.5 million metric tonnes in 1948-52, to 50.109 million metric tonnes in 1993. Most probably, the actual production of bananas produced certainly would place them in first place among cultivated fruits (Chung, 1976). However, most bananas are consumed locally with only about 20% being transported or exported to distant markets.

According to FAO (1993) reports banana production in India is 7.2 million metric tonnes, which accounts for about 14.37% of the world production. As the export from India is practically "nil" the entire produce is consumed within the country.

Banana wastes get generated at several stages including harvesting, packing and processing. In addition to rejected fruits, stem, pseudostem, leaves and peduncle (fruit stalk) also get accumulated and create pollution problems. In 1985-'86, a total production of about 10.6 million tonnes of stem, and 2.6 million tonnes of peelings were achieved from an area of about 0.3 million hectares in India, which was found

to be 156.4% higher than what was in 1950-51 (Agricultural situation in India, 42:240, 1987).

Industrial enzymes such as amylases, cellulases, proteases etc. are produced by both submerged and solid state fermentation. (SSF)

SSF production of industrial enzymes are known since the beginning of 20th century in the name of 'Koji', and it is becoming a desirable method of economic production of industrial enzymes now. Several organic substrates such as wheat bran, are being tested for maximizing enzyme yield. Although wheat bran seems to be a good substrate for SSF processes, still the search for better substrates is being continued. Perhaps, the use of agro-industrial wastes as substrates for fermentation processes could contribute to economic utilisation of these waste substrates.

Starch hydrolysing enzymes are among the most important enzymes that are produced on industrial scale. (Antrani kian, 1992). Conversion of starch into sugars, syrups and dextrins form the major part of the starch processing industry. Amylases have the most extensive

application, with uses in baking and brewing, production of fruit juices and starch syrups and in preparation of digestive aids. It is widely used as a desizing agent in textile industry and for sizing of paper in paper industry. The α-amylase supplied as Termamyl is used as dish washing and de-starching detergent. (Chaplin and Bucke, 1990). In the light of growing demands of this enzyme, efforts are being mobilised towards new sources and better methods of economic production of this enzyme at industrial level.

Cellulolytic organisms and their enzyme system find greatest applications in the modification of food tissues to improve the rehydratability of dried vegetables, as digestive aids, in the production of plant protoplasts, in the hydrolysis of fermentation of urban and agricultural waste residues to glucose and soluble sugars, for use in animal feed production; as raw material for fermentation industry; production of solvents, ethanol, butanol, acetone; chemical industry feed stock. Other applications include the production of cell-free high quality protein, improvement of quality in cattle feed, as a meat tenderizer, decomposition of wastes and residues, and for sewage treatment (Mandels, 1985). Considering the economic significance of this enzyme

and inadequate source and availability, several attempts are being made throughout the world for better sources and efficient and economic means of industrial production of these enzymes.

In this context, in the present investigation a detailed study was carriedout with a view to develop a bioprocess for α-amylase and cellulase production, employing bacteria, using banana fruit stalk wastes as solid substrates under solid state fermentation.

1.2.REVIEW OF LITERATURE

1.2.1.Banana Wastes

Research on banana can be seperated into pre-1960 and post-1960 eras. This is essentially the Gros Michel' era when fruit was grown in a "semi-intensive' way and shipped as bunches, and the era of Cavendish varieties when production became more intensive (high yields), and fruit was boxed for the international trade (Champion, 1964).

Pre-1960 research on banana mainly centered around nutrition (Hewitt, 1955; Murray, 1959), principles of breeding (Simmonds, 1959; 1962), and disease (Leach, 1946; Von Loesecke, 1950; Wardlaw, 1961; Stover, 1962).

The post-1960 era brought about a vast increase in banana research. Most of the papers dealt with banana nutrition (Lahav, 1980), and banana disease (Meredith, 1970; Stover, 1972). The physiology and biochemistry of storage and ripening (Marriott, 1980), in vitro growth of bananas (Folliot and Marchal, 1992), and chilling induced ethylene production in the peel and pulp of banana (Xie and Liu, 1993) are some of the other areas concentrated by scientists. Banana

production, marketing and utilisation (Stover & Simmonds, 1987) and biochemistry of banana fruit (Seymour, 1993) were extensively reviewed, whereas research carried out on banana waste utilisation was far less compared to the reports made on the production of banana.

1.2.2.Utilisation of fruit parts

Of the 50.109 million metric tonnes of banana and plantain produced worldwide (FAO, 1993), almost all are eaten either fresh or cooked including the preparation of various types of mashes.

Ripe and rejected banana were reported to be used in the preparation of alcoholic beverages like beer with low alcohol content (Acland, 1971), alcoholic fermentation of banana juice (Maldonado *et al.*, 1975; Muriel *et al.*, 1991), Vinegar using Baker's yeast (Adams, 1978), ethanol using immobilised yeast (Del Rosario and Pamatong,1985) and yeast (Zhu, 1989), wine and vinegar using *Saccharomyces cerevisiae* (Stover and Simmonds, 1987), wine using immobilised yeast (Guangying *et al.*,1992; Shizhong *et al.*, 1992); and the comparative study of mixed flora and single strain

fermentation in the production of plantain "wine" (Akinyanju and Oyedeji, 1993).

Unripe banana pulp and peel were fermented to alcohol (Pontiveros *et al.*, 1978; Iizuka *et al.*, 1985) and yeast fermentation using banana peels (Goewert and Nicholas, 1980) have also been reported.

Green fruit was stored as silage (Mallessard, 1971). The processing bananas for storage consists of canning, drying, freezing or fermentationn in the case of vinegar and alcoholic beverages (Wilson, 1975; Crowther, 1979). Aegerter and Dunlap (1980) explained a safe and economic way of preserving ripe or green banana fruits, in both large and small scales, employing five acid producing bacteria of *Lactobacillus* sp, *streptococcus* sp. *and Leuconstoc* sp. Viquez *et al.*, (1981) described a process for the production of classified banana juice using pectinolytic enzymes.

In 1974 the United Brands Company developed a pilot-scale plant for separating starch from waste green fruit. The physical and chemical properties of banana starch

were studied by Patil and Magar (1974);Kayisu and Hood (1981); Kayisu *et al.*, (1981); and Lii *et al.*, (1982).

The bio-oxidation of banana wastes, using selected strains of *A. niger* var. *tieghem* (Sassi *et al.*, 1989) and Citric acid production using *A.niger* in different types of fermentations were also reported (Sassi *et al.*, 1991).

Lefrancois (1970) designed a fermentation process to increase the protein content of banana pulp. Ffoulkes *et al.*, (1978) calculated the dry matter production of whole plant. Numerous experimental studies have been carried out using banana flour as a source of animal feed (Stover and Simmonds, 1987).

Laboratory studies have been conducted on protein enrichment of banana waste skin and pulp using *Pichia spartinae*. (Chung and Meyers, 1979); through SSF using *Aspergillus niger* (Senez, 1979; Baldensperger *et al.*, 1985). *Candida utilis* (Aker and Robinson, 1987), with fermentative and non-cellulase containing organism, *Saccharomyces uvarum* (Enwefa,1991); *Saccharomyces* sp. and *Torula* sp. (Gadgoli *et al.*, 1992).

1.2.3.Non-fruit plant parts

According to Simmonds (1966), the use of corms, shoots and male buds as food is wide spread in Africa and Asia. The inner part of a growing pseudostem is eaten in India.

The green and dried leaves and fresh sheaths are used throughout the tropics for packing of fruits, plates and wrappings. Dried banana fibres from the leaf sheaths are used throughout the tropics for making weak rope and fibre containers (Simmonds, 1966). The properties and small scale extraction and processing of banana fibre have been reviewed by Jarman *et al.*(1977).

A Dutch patent (63,937, 1949) is available on the production of fibre board from banana stalk. The stalk is used as mulch after crushing and shredding in a mill. Stalks and leaves are also used as manure (The Wealth of India, 1962).

Attempts were made to manufacture starch from pseudostem (Subrahmanyan *et al.*, 1957; Shantha and Siddappa,

1970) and to use the green banana waste through ensilaging to eliminate or reduce the negative nutritional effects (Le Dividch *et al.*, 1976; Tyagi, 1989). Utilisation of banana waste for the production of biogas (Sharma *et al.*, 1988). Lactic acid production from whole banana waste liquor using homolactic, Gram-negative amylase producing bacilli (S-9 and S-55) Lopez-Baca and Gomez,1992) were reported.

1.2.4. Production of Exoenzymes by SSF

The commercially important enzymes are produced by both submerged and solid state fermentation. In Solid State Fermentation (SSF) the moist water-insoluble solid substrate is fermented by microorganisms in the absence of any free water (Lonsane *et al.*, 1982, 1985).

Although SSF has been known to be used since ancient times to obtain fermented foods and in composting, the refinement and automation of the technique as well as its industrial exploitation have been confined mainly to the countries of the Orient (Yamda, 1977). With the advent of deep tank fermentation, SSF was neglected in the Western world and research was devoted exclusively to the design of

submerged fermentation systems (SmF). An exception to this trend was the establishment of the Kikkoman plant in Wisconsin, USA for the manufacture of soy sauce. This plant uses "Koji" prepared from wheat and soy beans inoculated with *Aspergillus oryzae* to produce enzymes (Hesseltine, 1977). Yet, it has a bright future in advanced and developing countries due to its numerous advantages over submerged fermentation systems (SmF) (Lonsane *et al.*, 1985).

SSF has recently attracted a great deal of scientific attention in view of its untapped potential for industrial exploitation (Moo-Young *et al.*, 1983; Laukevies *et al.*, 1984; Lonsane *et al.*, 1985; Raghava Rao *et al.*, 1992). In recent years many processes are being developed for the manufacture of desired products using SSF. One of the most successful exploitation of SSF techniques is for the commercial production of different exoenzymes such as pectinases, takadiastase, fungal α-amylases, amyloglucosidases, rennet and cellulase (Lonsane, 1990).

1.2.5.Bacillus sp and their α-amylases

Starch hydrloysing enzymes are among the most important enzymes that are produced on industrial scale (Antranikian, 1992). Amylolytic enzymes are widely distributed in plants, animals and in microorganism (Boyer and Ingle, 1972). Among them microbial amylases have drawn the attention of many investigations (Robyt and Ackerman, 1971; Shinke, 1975).

The α-amylases of *Bacillus* sp is known to be used in detergent, paper, brewing, starch processing and textile manufacturing industries (Ingle and Boyer 1976; Rose, 1980; Wiseman, 1985;Fogarty and Kelly, 1990, Antranikian, 1992). Of the 48 species of *Bacillus* described by Buchanan and Gibbons (1974) about 32 are reported to produce α-amylase but only a few of them are able to secrete thermostable amylases (Medda and Chandra, 1980). Most industrial applications of α-amylase require their use at high temperatures, and this sustains the ongoing research for new enzymes having increasingly better thermostability (Koch *et al.*, 1987; Fogarty and Kelly, 1990; Sunna and Hashwa, 1990; Antranikian, 1992). Production of α-amylases by different species of *Bacillus* is presented in Table-1.a

Table 1 a. distribution of α-amylases of *Bacillus* sp.

Bacillus sp.	Reference
Bacillus acidocaldarius	Buonocore *et al.*(1976)
B.acidocaldarius A-2	Kanno (1986)
B.alcalophilus subsp.*halodurans*	Yamamoto *et al.*(1972)
B.amyloliquifaciens	Granum (1979) Rajendran *et al.*,(1995)
B.caldolytius	Grootegoed *et al.*(1973); Emanuilova and Foda (1984) Ying *et al.*,(1992)
B.cereus	Yoshigi *et al.*(1985)
B.circulans	Takasaki (1983) Sata (1990)
B.coagulans	Campbell (1955) Bleismer and Hartman (1973)
B.licheniformis	Meers (1972); Saito and Yamamoto (1975); Morgan and Priest (1981); Krishnan and Chandra (1983). Takasaki (1994)
B.macerans	Depinto and Campbell (1964; 1968a 1968b)
B.megaterium	Stark *et al.*(1982) David *et al.*(1987)
B.natto	Matsuzaki *et al.*(1974)
B.stearothermophilus	Galabova (1969); Pfueller and Elliott (1969); Ogasahara *et al.*(1970) Wind *et al.*,(1994)

contd..

B.subtilis	Matsuzaki *et al.*(1974 a,b) Bailey and Markkanen (1975);Kokubu *et al.*(1978); Mitricia and Granum (1979); Robyt and French (1979); Orlando *et al.*(1983)
B.subtilis var. *amylosacchartius*	Fujimori *et al.*(1978); Matsuno *et al.*(1978); Lee *et al.*,(1993)
Bacillus spp(alkalophilic)	Boyer and Ingle (1972); Yamamoto *et al.*1972). Mc Tigue *et al.*,(1994)

Some of the properties of α-amylases produced by *Bacillus* sp. is outlined in table 1 b.

Table 1 b. Some Properties of α-amylases Produced by *Bacillus* sp.

Bacillus .sp	pH Optima	Temp. Optima (°C)	Relative molecular mass (Mr)	Isoelectric point (PI)	References
B. acidocaldarius A-2	3.5	70	66000	—	Kanno (1986)
B.alcalophilus sub sp.	10.5	—	—	—	Yamamoto *et al.*,(1972)
haloduraus	10.0	—	—	—	Mc Tigue *et al.*,(1994)
	5.5	50-70	—	—	Granum (1979)
B. amyloliquefaciens	6.0	65	—	—	Kochhar and Dua (1990)
	6.8	—	—	—	Castro *et al.*, (1993)
	—	78	—	—	Rajendran *et al.*, (1995)
B.caldolyticus	5.5	70	—	—	Heinen and Heinen (1972) Grootegoed *et al.*, (1973) Emanuilova and Toda (1984)
B. cereus	6.0	55	—	—	Yoshigi *et al.*, (1985)
B. circulans-F-2	7.0	50	—	—	Takasaki (1983)
	6.0-6.5	60	—	—	Taniguchi *et al.*, (1983)

B.coagulans	6.5-8.0	45-55	—	—	Campbell (1955)
	7.5-8.5	85	28000	—	Medda and Chandra (1980)
B.licheniformis	7.0-9.0	90	62650	—	Morgan and Priest (1981)
	9.0	90	28000	—	Krishnan and Chandra (1983)
	5.5-10.0	90	—	—	Bajpai and Bajpai (1989)
	6.5-7.0 and 9.5	90	19500 and 56000	—	Ramesh and Lonsane (1990)
	6.0-6.5	90	58000	—	Ivanova *et al.*,(1993)
	4.5-5.3	90	—	—	Yoshiyuki *et al.*, (1994)
B.macerans	6.0	—	—	—	Depinto and Campbell (1964, 1968 a,b)
B. megaterium	6.0	70	—	—	Ramesh and Lonsane (1987)
	5.5	75	55000	9.5	David *et al.*,(1987)
B. natto	6.0	—	—	—	Matsuzaki *et al.*, (1974)
B.stearothermo-philus	5.0-6.0	70-80	—	—	Manning and Campbell (1961)
					Pfueller and Elliot (1969)
					Galabova (1969)
	5.0-6.5	90	—	—	Tomazic and Klibanov (1988)
	5.0-6.0	70-80	59000	—	Vihinen and Mantsala (1990)

B.subtilis	5.9-9.5	70	—	—	Mosely and Keay (1970)
	—	75-80	—	—	Fogarty *et al.*,(1974)
	6.5	55	—	—	Mitricia and Granum (1979)
	—	—	93000	—	Robyt and French (179) Orlando *et al.*,(1983)
	6.5	60	—	—	Derkova *et al.*,(1992)
	6.0-8.5	75-80	—	—	Qian *et al.*, (1992)

The aminoacid sequences and structure of a number of α-amylases have also been reported (Sachdev and Friedberg, 1981; Kuhn *et al.*, 1982; Takkinen *et al.*, 1983; Ihara *et al.*, 1985; Yuuki *et al.*, 1985; Nakajima *et al.*, 1986; MacGregor, 1988; MacGregor and Sevensson, 1989; Yamaguchi, 1993).

Bacillus sp. have also has been reported to produce α-amylase under SSF (Ramesh and Lonsane,1987; 1989; 1990 and 1991; Gayal *et al.*,1991).

Most genetic studies of α-amylase have been conducted with the genus *Bacillus*. (Antranikian). The α-amylase gene amyE, of B.subtilis has been mapped on the chromosome (Yuki, 1968; Yamaguchi *et al.*, 1969; Piggot and Hoch, 1985). The effect of different mutation on α-amylase production was investigated by many early workers (Lepesant *et al.*, 1972; Yoneda *et al.*, 1973; Matsuzaki *et al.*, 1974; Sekiguchi *et al.*, 1975 and Steinmetz *et al.*, 1976). Cloning of α-Amylase genes from several *B.subtilis*, located on the chromosome, promoted α-amylase production (Thudt *et al.*, 1985; Kallio 1986; Kallio *et al.*, 1987). α-Amylase production by *B.amyloliquifaciens*, and *B.licheniformis* has been enhanced using successive mutagenesis (Bailey and Markkanen, 1975;

Maruo and Tojo, 1985). Whereas only limited knowledge exists concerning different factors affecting the α-amylase production of other exotic microbes (Antranikian, 1992).

1.2.6. Aeromonas species and their α-amylases

Aeromonas hydrophila, is reported to secrete various extracellular enzymes, amylase (Gobius and Pemberton, 1988), Chitinase (Yabuki *et al.*, 1986) and proteases (Leung and Stevenson, 1988).

Cloning of an amylase gene from *A.hydrophila* strain, JMP 636 in *E.coli*, has been reported (Gobius and Pemberton, 1988), but, whether this amylase belongs to the α-type is unkonwn. Strains of *A.salmonicida* usually produce extracellular amylases (Farmer *et al.*, 1992). A gene encoding the extra cellular α-amylase of *A.hydrophila* MCC-1 was cloned and expressed using its own promoter on the recombinant plasmid PCA 101 (Chang *et al.*, 1993). However, no reports are available on the produciton of α-amylases by *Aeromonas* sp. under SSF.

1.2.7 Cellulase production by *Bacillus* sp. and *Aeromonas* sp.

Bacillus sp, despite their ubiquity and acknowledged ability to excrete a wide variety of depolymerising enzymes, have apparently achieved only marginal status as cellulase degraders. More than one-half of *Bacillus* sp. examined were able to produce extracellular cellulases, (Zemek *et al.*, 1981; Ljungdahl and Eriksson,1985). However, Zemek *et al.*, (1981), showed that 11 out of 25 *Bacillus* sp. secrete β-1, 4-glucanses. All of the *B.stearothermophilus* strains, 85% of the *B.coagulans* strains, 50% of the *B.licheniformis* strains (Gibson and Gordon, 1974) produce thermostable cellulase components. Other cellulolytic species include *B.brevis, B.firmus, B.licheniformis, B.pumilus, B.subtilis* (Knosel, 1971), *B.polymyxa* (Greaver,1971; Fogarty and Griffin, 1973) and *B.cereus* (Thayer, 1978).

Several strains of *Bacillus* sp. that produce alkaline carboxymethyl cellulases, have the properties which fulfill the essential requirements for enzymes to be used in laundry detergents (Horikoshi *et al.*, 1984; Fukumori *et al.*, 1985; Chan and Au,1987; Horikoshi and Fukumori, 1988; Kawai *et al.*, 1988; Shikata *et al.*, 1990; Mastasuji *et al.*, 1992). A

moderately thermophilic, alkalophilic and powerful crystalline cellulose-digesting bacterium *Bacillus* K-12 was reported to hydrolyase xylan, raw starch, pullulan and β-1,3, glucan (Kim and Kim, 1992). Cellulase system of *B.circulans* F-2. (Kim and Kim 1993), an alkaline, thermophilic cellulase with pH otima at 8.5 and temperature optima at 70°C was purified and characterised from *B.thermoalcaliphilus* (Sarkar and Upadhyay, 1993); an extra cellular CMCase from *Bacillus* sp. active over a wide range of temperature (30 to 65°C) with optima at 50°C and a wide range of pH 3.0 to 11.0 with optima at 6.0 (Kricke *et al.*, 1994) have also been reported.

Despite the paucity of *Bacillus* strains producing enzyme systems active against crystalline celluloses, the genus is receiving considerable attention in the context of cellulose hydrolysis. This is due to a large extent to the potential of *B.subtilis* for the production and secretion of foreign proteins and the several advantages it enjoys over possible competition in this respect (Doi *et al.*, 1986). Among the useful properties of the *Bacillus* cellulases are that many are active and stable over a wide range of pH and temperature. This is important since biomass conversion on a

realistic scale must be able to operate efficiently under nonsterile conditions (Wood, 1985).

Much of the information on *Bacillus* sp. cellulases has been derived from recombinant DNA studies (Cantwell *et al.*, 1988, Horikoshi and Fukumori, 1988a), and genes encoding endoglucanases from the alkalophilic strain 1139 (Fukumori *et al.*, 1986 a,b & 1987a) and N-4 (Fukumori *et al.*, 1986b, 1987b & 1989; Kim *et al.*, 1987; Yu *et al.*, 1986,1987 & 1988). From thermophilic strains (Sharma *et al.*, 1987; Ishizaki and Kawauchi, 1988), from *B.subtilis* strains (Koide *et al.*, 1986; Seo *et al.*, 1986; Horinouchi *et al.*, 1987; Nakakura *et al.*, 1987; Kim and Pack, 1988; Lo *et al.*, 1988) and other *Bacillus* sp (Park and Pack, 1986) have been cloned in *E.coli* and *Saccharomyces cerevisiae* and in cellulase-negative mutants of *B.megaterium* and *Streptomyces lividans* using a variety of plasmids as expression vectors. *Aeromonas* sp. and their cellulases, a Japanese patent, (Horikoshi and Kudo, 1993) are available on cloning and expression of gene for novel cellulase AEC3 of *Aeromonas*. All these studies were reported with SmF system. However, no reports are available on the production of cellulases under SSF by *Bacillus* and *Aeromonas* sp.

Compared to fungi, cellulolytic bacteria were reported to produce lower yields of cellulases. Fungal strains which produce high titres of cellulase enzymes mainly belong to the genera of *Trichoderma* and *Aspergillus* were usually employed for SSF systems (Roussos *et al.*, 1991 a,b; Shamala and Sreekantiah, 1987 & 1988; Datta *et al.*, 1989).

1.3 Scope of the present study

A critical survey of the fruits and vegetable markets of the towns and cities in South India reveals that banana fruit stalk wastes share a dominant proportion among the solid wastes generated. In the light of the review of literature presented in the foregoing section, few reports are available on the utilisation of banana waste for the production of alcoholic beverages, biogas, and single cell protein. However, it is not yet tried for the production of industrial enzymes. Moreover, preliminary fermentation studies conducted under uncontrolled conditions revealed that banana fruit stalk could be aptly utilised as solid substrate for the industrial production of microbial amylases and cellulases at a cheaper cost.

Therefore, it was proposed to conduct a detailed study towards the development of a suitable fermentation process for the production of industrial enzymes using banana fruit stalk wastes, which is rich in carbohydrate, as solid substrate, employing bacteria, under SSF.

The specific objectives of this investigation are:—

1. Biochemical characterisation of the banana fruit stalk wastes,
2. Bioconversion of banana fruit stalk by natural micro flora,
3. Screening of potential industrial enzyme producing bacteria from the fermented waste,
4. Production of exoenzymes using banana fruit stalk wastes as substrates employing SSF using potential strains,
5. Exoenzyme production using mixed solid substrates,
6. Evaluation of Solid State Fermentation (SSF) Slurry Fermentation (SLF) and Submerged Fermentation (SmF) for exoenzyme production, and
7. Enzyme purification and characterisation.

CHAPTER II

MATERIALS AND METHODS

2.1 Banana

2.1.1 Taxonomic position

Banana belongs to the genus *Musa* comprising thirty-two or more distinct species and at least one hundred subspecies (Chung 1976). The genus is divided into two broad sections *Eumusa* with edible fruits, and *Physocaulis* with inedible fruits. On the basis of chromosome number and certain morphological features, the genus *Musa* has been divided into four sections by Simmonds (1966). (the distribution and uses of various members are given in Table 2).

1. *Eumusa* - with dull coloured bracts enclosing many flowers in two series.

2. *Rhodochlamys* - with bright red coloured bracts and a few flowers, usually in a single series.

3. *Australimusa* - with sub-globose or dorsoventrally compressed seeds.

4. *Callimusa* - with cylindrical barrel-shaped or top-shaped seeds.

The section *Eumusa*, the biggest and geographically the most wide spread and commercially cultivated, includes the majority of edible bananas, mostly of hybrid origin. The wild progenitors of edible bananas have been traced to two variable species, viz., *M. acuminata* and *M. balbisiana*, both are important varieties and represented in India.

Cultivated types of *M. acuminata* have been broadly grouped as dwarf type (*M. cavendishii*), and tall type, Gros Michel, (*M. Sapientum*), a well known clone in India and S. America.

Musa paradisiaca, the starchy, cooking banana of hybrid origin valued for their seedless fruits is included under this specific name. They comprise all the diploid, triploid or tetraploid clones, mainly hybrids of *M. acuminata*

and *M. balbisiana*. The edible clones have been grouped under the two Linnaean names *M. paradisiaca* and *M.sapientum*, the former including mainly culinary types (plantains) and the latter, the dessert types (bananas).

More number of edible clones are cultivated in India than in any other country. The great majority of them belongs to the section *Eumusa*. Many of them are mutants selected and perpetuated for certain agricultural and fruit characteristics. The clones grown in India are mostly diploid and triploid cultivars of groups (A for *M. acuminata* and B for *M. balbisiana*) AB, AAB and ABB derived as a result of hybridization between *M. acuminata* types and local *M. balbisiana* types. In addition, some diploid (AA) and triploid (AAA) clones *M. acuminata* are also grown, some of them considered as introduced from Malaysia (Simmonds 1966).

French plantain (AAB) var. Nendran is the leading commercial type grown in Kerala and this is a hybrid origin of *M. paradisiaca*.

Table 2

Distribution and uses of various members of the Banana family, *Musaceae*

(based on Simmonds, 1966)

Genus	Section	Distribution	Uses
Musa	*Australimusa*	Queensland to Philippines	Fruit, fibre eg: *M.textilis* Nee (abaca)
	Callimusa	Indochina to Indonesia	Ornamental
	Eumusa	South India to Japan and Samoa	Fruit (edible bananas (*M. acuminata* Colla and *M. balbisiana Colla*); fibre (M.basjoo Sieb); vegetable
	Rhodochlamys	India to Indonesia	Ornamental
Ensete		West Africa to New Guinea	Fibre and edible vegetables eg:*E. ventricosum*

2.1.2 Banana plant parts

The mature banana plant (Fig.1) appears superficially to consist of a 'trunk' bearing fronds and also a stalk on which the bunches of fruit are borne. The 'trunk'

known botanically as pseudostem. This is composed of a true stem which forms a central core and is tightly encircled by many long leaf sheaths which extend from the base to the top of the pseudostem where they open out into fronds. The true stem, on emerging from the leaf sheaths forms the fruit stalk or peduncle.

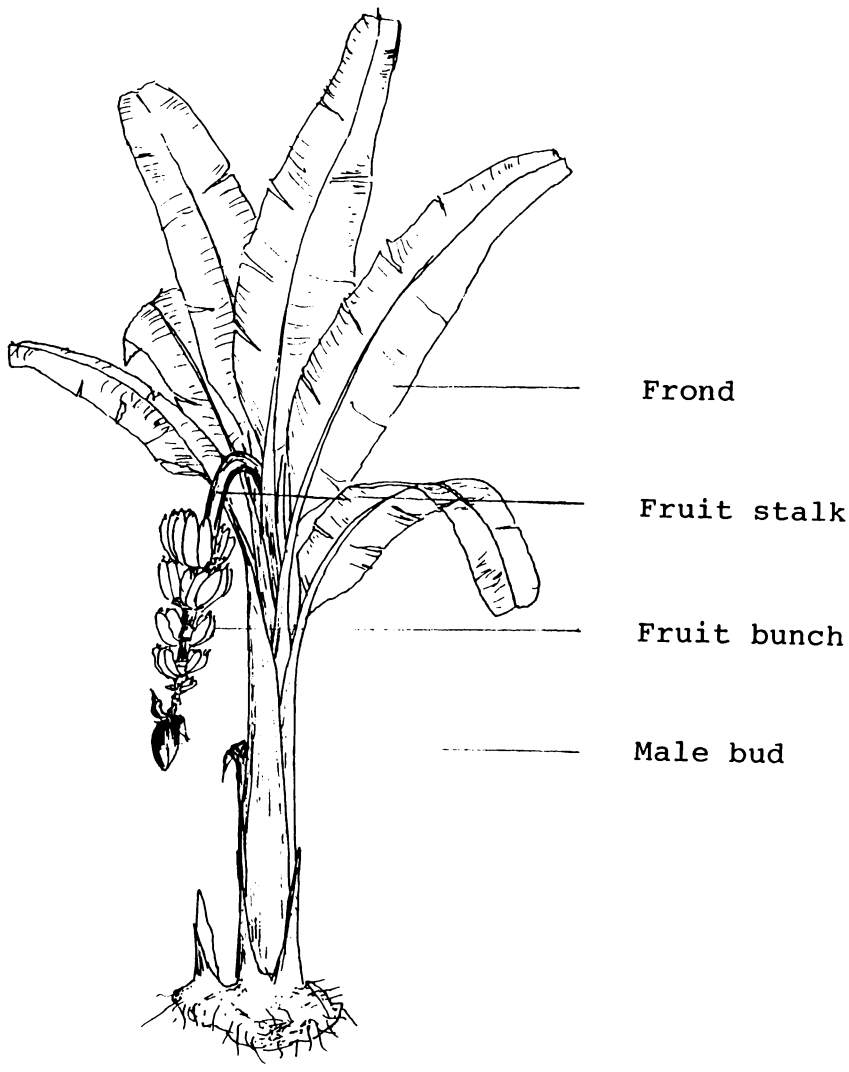


Fig. 1 Banana Plant Parts

2.2. Substrate

Fresh banana fruit stalks (peduncle) of *Musa paradisiaca* (variety - Nendran, grown in Kerala) collected from the local market were used as substrate. After removing the mud and debris adhering to it, if any, with brush and/or knife, they were weighed, cut into small pieces (0.85 x 0.35 mean size), and used for further studies.

2.2.1. Proximate analysis

2.2.1.1. Moisture content

Moisture content of the substrate was estimated as mentioned below (A.O.A.C. 1970).

1. A 5 gm sample was transferred to a preweighed dish, and dried at 105°C for 16-18 h.
2. After drying, the sample was cooled in a desiccator and reweighed.

3. Moisture content, which is the difference in weights, was calculated and expressed in terms of percentage.

2.2.1.2 Carbohydrate

Carbohydrate content in the sample was determined in terms of total sugar by phenol - sulfuric acid method (Dubois *et al.*, 1956).

1. A 100 mg sample was transferred to a boiling tube and hydrolysed with 5 ml of 2.5 N HCl in a boiling water bath for 3 h. and cooled to room temperature (30 ± 2^{O} C).
2. Neutralised with solid Na_2CO_3 and the volume made upto 100 ml.
3. Centrifuged, and the supernatant assayed for total sugar.
4. 0.1 ml of the sample was made upto 1 ml with distilled water, added with 1 ml of phenol solution followed by 5ml of conc. H_2SO_4 and the absorbance measured at 490 nm in a UV Visible Spectrophotometer (Shimadzu Model-160 A).
5. The amount of total sugar was computed from a standard curve prepared with glucose. Carbohydrate was expressed in terms of percentage.

2.2.1.3 Reducing Sugar

Reducing sugar of the substrate was estimated following the method of Miller (1959).

1. Sugar content in 100 mg of the sample was extracted with hot 80% ethanol twice.
2. Ethanol in the extract was evaporated using a water bath, at 80^{o}C and the residual sugar in the test tubes was dissolved in distilled water.
3. Pipetted out aliquots of extract varying from 0.5 to 3 ml to the test tubes and made up the volume to 3 ml with distilled water.
4. 3 ml of DNS reagent were added to the contents in the test tubes and heated in a boiling water bath for 5 min.
5. The tubes were cooled down and the absorbance measured at 510 nm in a UV Visible Spectrophotometer (Shimadzu Model - 160 A).
6. The amount of reducing sugar was calculated using a standard curve prepared with glucose and expressed in terms of percentage.

2.2.1.4 Crude Protein

Crude protein content of the sample was estimated by the Micro-kjeldahl method (A.O.A.C; 1970).

1. A 5 g sample was transferred to a 250 ml Kjeldahl flask.
2. 2 g of catalyst mixture (2.5 g of (powdered) SeO_2 plus 100 g of K_2SO_4 plus 20 g. $CuSO_4 . 5H_2O$) were added to the Kjeldahl flask.
3. Boiling chips were added to the flasks, the contents digested till the solution turned colorless, and then the digest was cooled.
4. A 5 ml sample of the digest was mixed with 30 to 40 ml of distilled water (DW), transferred to a 100 ml volumetric flask and the volume is made up to 100 ml with DW.
5. A 5 ml sample was then transferred to the distillation apparatus, added with 10 ml of 30% NaOH - $Na_2S_2O_3 5H_2O$ solution and subjected to distillation.
6. Ammonia liberated during distillation was collected in boric acid plus mixed indicator mixture (Mixed indicator preparation - 0.1% bromocresol green plus 0.1% methyl red indicator in 95% alcohol). The boric acid changes from

bluish purple to bluish green as soon as it comes in contact with ammonia.

7. Collected solution was titrated against standard 0.1N HCl until the blue color disappeared (the end point).
8. Reagent blank was also run simultaneously with an equal volume of DW.
9. The crude protein content of the sample was calculated from the Nitrogen content of the sample multiplied with 6.25 and expressed in terms of percentage.

2.2.1.5 Crude fat

Crude fat content of the sample was analysed following the method described in A.O.A.C (1970).

1. Oven-dried sample (after estimating the moisture content) was extracted with ether in a Soxhlet extraction apparatus.
2. After extraction the ether was distilled off on a steam bath at low heat, under a current of air.
3. Extract was dried at $100^{O}C$ for 1 h. cooled and weighed.

4. The crude fat content of the sample was computed from the difference in the weights and expressed in terms of percentage.

2.2.1.6 Crude fibre

Crude fibre content of the sample was determined as given in A.O.A.C (1970).

1. A 2 g dried sample (after estimating the crude - fat content) was boiled with 200 ml of H_2SO_4 for 30 min.
2. Filtered through muslin and washed with boiling water until washings were no longer acidic.
3. Boiled with 200 ml of NaOH (IN) solution for 30 min.
4. Filtered through muslin cloth again and washed with 25 ml of boiling 1.25% H_2SO_4, 150 ml of water and 25 ml alcohol.
5. The residue was removed, transferred to an ashing dish and dried at 130 ± 2^{o}C for 2 h.
6. Cooled down the dish in a desiccator and weighed.
7. Ignited the sample dish for 30 min at 600 ± 15^{o}C, cooled down in a desiccator and reweighed.

8. Crude fibre content was calculated from the difference in weights, and expressed in terms of percentage.

2.2.1.7 Mineral matter

Mineral matter content of the substrate was estimated according to the methodology given in A.O.A.C (1970).

1. A 5 g of was weighed out into previously weighed silica dishes.
2. The dish and contents were ignited, first gently, and then at 500^{O}C for 6 h.
3. Dishes were cooled and weighed.
4. The total ash content, calculated from the difference in weights, and expressed in terms of percentage.

2.3. Biodegradation of banana fruit stalk by commensal microflora

Fresh banana fruit stalks (peduncle) (100 g) were cleaned and cut into small pieces of mean size (0.85 x 0.35

cm) under aseptic conditions and subjected to biodegradation in a 1000 ml beaker. The mouth of the beaker was covered with sterile cotton cloth to prevent aerial contamination and incubated at 30^{O}C for a total period of 8 days. Samples were withdrawn at regular intervals of 24 h and subjected to various analyses.

2.3.1. Biochemical analysis

Moisture content, carbohydrate, reducing sugar and protein of the samples were estimated as described under sections 2.2.1.1 to 2.2.1.4 pH of the sample was measured using a pH meter (Systronics,digital pH meter-335) after standardisation using buffer solution of pH 4.0 and 7.0.

2.3.2. Enzyme extraction

The enzyme was extracted from fermented medium with 0.2 M phosphate buffer (pH 7.2) applying a ratio of 1:10. The extract was centrifuged at 10,000 rpm in a refrigerated centrifuge, (Kubota 6700 model) at 4^{O}C for 20 min and the supernatant was used for the assays (Ramesh and Lonsane 1991).

2.3.3. Enzyme assays

2.3.3.1. α - amylase

α - Amylase activity was determined following the method described by Medda and Chandra (1980).

1. One ml of the cell free extract was incubated with 1 ml of 1% soluble starch in 1 ml of 0.2 M phosphate buffer (pH 7.2) at 40°C for 10 min. (unless otherwise stated).
2. The reaction was arrested by the addition of 0.1 ml of 1N HCl.
3. One ml of the above sample was added with 0.1 ml of I_2-KI reagent.
4. The volume was made upto 15 ml with distilled water.
5. The blue color developed was then measured at 650 nm in a UV visible spectrophotometer (Schimadzu Model - 160A).
6. Suitable controls were also run simultaneously.
7. The concentration of residual starch was calculated from a calibration curve prepared with starch as standard.
8. One unit of α-amylase activity, expressed as dextrinizing activity, is defined as the amount of enzyme

required to bring about the hydrolysis of 0.1 mg of starch at optimal pH and temperature in 10 min. of incubation.

2.3.3.2. Cellulase

Total Cellulase activity was determined employing Filter paper assay method of Mandels *et al.* (1976) with slight modifications.

1. 0.5 ml of extract added with 1.0 ml of phosphate buffer (0.2 M, pH 7.2) was taken in a test tube.
2. Whatman No.1 filter paper, cut into 1 x 6 cm strips (50 mg), was added to enzyme extract-buffer solution and mixed well on a vortex mixer to coil the paper such that the entire paper strip was immersed in the solution.
3. The tubes were incubated for 1 h at 50°C.
4. The reaction was terminated by adding of 3 ml of DNS reagent.
5. The tubes were placed in a boiling water bath for 5 min. and reducing sugar was assayed as described under

section 2.2.1.3. Cellulase activity was expressed in terms of Filter Paper Activity (FPA).

6. Controls were included to correct for any reducing sugar present in the enzyme preparation. The concentration of glucose was calibrated from a calibration curve prepared using glucose as standard. FPA was expressed as units equal to micromoles of glucose produced per minute.

2.3.3.3. Protease

Protease was assayed by adopting the method of Kunitz (1947) with slight modifications.

1. To 2 ml of the 1% casein substrate (dissolved in 0.2 M phosphate buffer of pH 7.2) 1 ml of the extract was added and incubated at 35°C for 30 min.
2. The reaction was arrested with 2.5 ml of 0.44 M TCA solution.
3. The precipitated protein was filtered (Whatman No.1 filter paper) and the absorbance of the filterate was measured at 280 nm in a UV-Visible Spectrophotometer (Shimadzu Model-160 A).

4. Suitable controls were also run simultaneously.

5. The concentration of liberated tyrosine was calculated from a calibration curve prepared with tyrosine as standard.

6. One unit of protease activity is defined as the quantity of enzyme which liberated 1 μg of tyrosine per ml of reaction mixture per minute under standard conditions.

2.3.4. Enzyme Protein

Protein was estimated according to the methodology of Lowry *et al.* (1951).

1. A 1 ml of sample was added with 5 ml of alkaline-Copper reagent

   (Preparation of alkaline copper reagent:

   Solution A: 2% solution of Na_2CO_3 in 0.1N NaOH.

   Solution B: 0.5% of solution of $CuSO_4 . 5H_2O$ in a 1% solution of potassium sodium tartarate.

   1 ml of reagent B and 50 ml of reagent A were mixed thoroughly) and left for 10 min.

2. 0.5 ml of Folin's reagent diluted with equal volume of water was added to the contents.
3. After 40 min. absorbance was measured at 750 nm in a UV Visible Spectrophotometer (Shimadzu Model- 160 A).
4. Bovine serum albumin was used as the standard.
5. Protein expressed as percentage.

2.3.5. Bacteriological analysis

2.3.5.1 Total Heterotrophic Bacterial population (THBp)

THBp, associated with the banana waste, during the course of natural biodegradation was enumerated using nutrient agar (NA) medium (HI-Media) employing pour plate technique. Inoculated plates were incubated at 30^{o}C for a period of 2-8 days and the total viable counts were made. THBp was expressed in terms of number per gm of sample.

2.3.5.2. Isolation and maintenance of culture

After enumeration, the isolates were subcultured on nutrient agar slants. After purification, by repeated

streaking on nutrient agar, one set of subcultures was stocked under mineral oil (sterilized liquid paraffin was used) and another set, used as working cultures, maintained at 4°C. They were subcultured once in two weeks and their purity was checked at regular intervals of one month.

2.3.5.3. Identification of bacteria

All the isolates were assigned to various genera based on their morphological, physiological and biochemical characteristics outlined in the Bergey's manual of Systematic Bacteriology, (Kreig and Holt, 1984; Sneath, 1986). Following the same procedures, selected strains, after screening for enzyme production, were identified upto species level.

2.4. Screening of Potential strains for enzyme production

2.4.1. Preliminary Screening of Potential strains

Potential strains were screened for their ability to produce hydrolytic enzymes, including amylase, cellulase,

caseinase, gelatinase, lipase and pectinase on agar plates by providing respective substrates in the medium.

2.4.1.1. Amylase

Amylase production was tested on the agar medium formulated by Harrigan and McCance (1972), supplemented with starch as the substrate.

Composition of the medium:

Peptone	10 g
Beef extract	10 g
Starch (soluble)	2 g
Sodium chloride	15 g
Agar	20 g
DW	1000 ml
pH	7.2

The prepared medium was autoclaved, poured into sterile petri-plates and allowed to solidify. The cultures were inoculated by surface streaking on the media and incubated at 30°C for 7 days. The production of amylase was tested by flooding the plates with Gram's iodine solution

(KI,2 g.; I, 1 g. and DW, 300 ml). Unhydrolysed starch formed a blue color with iodine. The amylolytic colonies developed clear zones around them.

2.4.1.2. Cellulase

Cellulase production by different strains was detected using Cellulose agar medium of Riviere (1961).

Composition of the medium:

Cellulose powder	5 g
$NaNO_3$	1 g
$Na_2HPO_4 7H_4O$	1.18 g
KH_2PO_4	0.9 g
$Mg\ SO_4 7H_2O$	0.5 g
KCL	0.5 g
Yeast extract	0.5 g
Casein hydrolyzate	0.5 g
Agar	10 g
Dw	1000 ml
pH	6.8

The prepared medium was autoclaved, poured into sterile petri plates and allowed to solidify. Cultures were inoculated on these plates by surface streaking and incubated at 30°C for 7 days. Cellulolytic bacteria was identified with a tiny ring of cellulose digestion around the colonies.

2.4.1.3. Caseinase

Caseinase production by different cultures was detected by employing casein agar medium of Harrigan and Mc Mc Cance (1972).

Composition of the medium:

Peptone	10 g
Beef extract	10 g
Casein	30 g
Na Cl	15 g
Agar	20 g
DW	1000 ml
pH	7.2

The prepared medium was autoclaved, poured into sterile plate and allowed to solidify. Cultures were inoculated on these plates by surface streaking and incubated 30°C for 7 days. Caseinase enzyme production was detected by the presence of clear zones around the colonies.

2.4.1.4. Gelatinase

Gelatinase producing capacity of the bacteria was tested using Harrigan and Mc Cance's (1972) gelatin agar medium.

Composition of the medium:

Peptone	10 g
Beef extract	10 g
Gelatin	4 g
Na Cl	15 g
Agar	20 g
DW	1000 ml
pH	7.2

The prepared medium was autoclaved, poured into sterile petriplates and allowed to solidify. Isolates were inoculated by surface streaking on the solidified agar medium and incubated at 30°C for 7 days. The plates were flooded with 8-10 ml of the test reagent ($HgCl_2$, 15 gm; Conc HCl, 20 ml and DW 100 ml). Gelatin hydrolysis was identified by clear zones around the colonies.

2.4.1.5. Lipase

Production of lipase was tested on Tween agar medium of Harrigan and McCance (1972).

Composition of the medium:

Peptone	10 g
$CaCl_2$	100 g
Tween 80	10 ml
NaCl	15 g
Agar	20 g
DW	100 ml
pH	7.2

The prepared medium was autoclaved and poured into sterile plates. Isolates were streaked on the solidified media and the plates were incubated at 30°C for 7 days. Lipase production was detected by the appearance of opaque zones around the colonies. Appearance of a waxy material around the colonies was the indication of the liberation of insoluble oleic acid formed as a result of lipase action.

2.4.1.6 Pectinase

Production of pectinase was tested on pectate gel agar medium of Wieringa (1949).

Composition of the medium:

Peptone	10 g
$CaCl_2$	10 g
Pectin	10 g
NaCl	15 g
Agar	20 g
DW	1000 ml

A pectate gel was prepared by layering a solution of pectin (pH 7.4) over a layer of mineral salts agar added with $CaCl_2$ at pH 5.0 in a petri dish. The inoculated plates were incubated at 30°C for 7 days. Liquefaction of the gel, indicated pectinolytic activity of the bacteria.

2.4.2. Secondary screening

Based on the results obtained for the preliminary screening, those strains which produced α-amylase and cellulase at significant levels were subjected to secondary screening. Ability of bacteria to synthesize enzyme, maximally in enzyme production medium was determined as detailed below.

2.4.2.1. Media

Enzyme production medium (EPM) used in the present study is similar to that used by Ramesh & Lonsane (1991) added with corresponding substrate (starch/cellulose at 1% level).

Composition of the Medium:

KH_2PO_4	0.1 g
K_2HPO_4	0.25 g
NaCl	0.1 g
$(NH_4)_2HPO_4$	0.2 g
Peptone	0.2 g
$FeCl_3$	0.5 mg
$Mg\ SO_4\ 6H_2O$	0.5 mg
$CaCl_2\ 2H_2O$	0.5 mg
DW	100 ml

50 ml of the media taken in 250 ml Erlenmeyer flasks were autoclaved and used.

2.4.2.2. Preparation of inoculum, and inoculation procedures

1. Initially a loopful of 24 h old agar slope cultures was transferred to 10 ml of Nutrient Broth (NB) and grown for 24 h at 30°C.
2. One ml of the culture broth was then aseptically transferred into another 50 ml of Nutrient broth and incubated for 24 h on a rotary shaker (150 rpm) at 30°C.

3. Cells were harvested by centrifugation at 10000 rpm (Kubota 6700-Model centrifuge) for 20 min.
4. The harvested cells were washed with sterile physiological saline (0.85% NaCl) and suspended in the same after adjusting the OD to 1.0.
5. The prepared cell suspension was used as inoculum at 1% (V/V) level.
6. The inoculated flasks were incubated on a rotary shaker (150 rpm) for a period of 24 h at 30°C.

2.4.2.3. Measurement of growth

The growth in the medium was measured in terms of turbidity by quantifying the absorbance at 660 nm in a UV-Visible Spectrophotometer (Shimadzu Model-160 A).

2.4.2.4. Enzyme extraction

After incubation for 24 h (unless otherwise stated) the culture broth was centrifuged at 10000 rpm at 4°C, for 20 min in a high speed refrigerated centrifuge (Kubota Model 6700) and the supernatant was assayed for α-amylase and

cellulase as described earlier under sections 2.3.3.1, and 2.3.3.2. respectively.

2.4.2.5. Enzyme protein

Enzyme protein was determined according to the methodology of Lowry *et al.*, (1951) as described under section 2.3.4.

2.4.3. Selection of Strains

All the strains were ranked in terms of their enzyme yield and the top ranked two strains *Aeromonas caviae* (CBTK 185) and *Bacillus subtilis* (CBTK 106) were selected for further studies.

2.5. Growth Studies

2.5.1 Optimization of the growth conditions for maximal growth and enzyme production

Optimal conditions required for maximal growth and enzyme production by the selected strains were determined by subjecting them to different levels of pH (4-12), incubation temperature (15-55°C) , substrate concentration (0-3%, w/v)), NaCl concentration (0-60mM), additional Carbon (0-9mM) and Nitrogen (0-3.0 % w/v) sources and inoculum concentration (0-3.0% v/v) and incubation time (0-72hrs) in the growth media. Optimal incubation period was determined after optimising other factors.

2.5.1.1 Media

Growth characterisation studies were carried out in Nutrient Broth (NB) and Enzyme Production Media (EPM) mentioned under section 2.4.2.1.

2.5.1.2 Preparation of inoculum and inoculation procedures

The procedure described in the previous section (2.4.2.2.) was followed for the preparation of inoculum and inoculation of media. Concentration of inoculum was at 1% level unless otherwise stated.

2.5.1.3 Measurement of growth

Growth was measured according to the procedures mentioned in section 2.4.2.3.

2.5.1.4 Enzyme assay

Enzyme supernatant was prepared as per section 2.4.2.4 and assayed for α-amylase, and cellulase as per sections 2.3.3.1. and 2.3.3.2. respectively.

2.5.1.5 Enzyme protein

Enzyme protein was determined according to the methodology Lowry *et al.* (1951) as described under section 2.3.4.

2.5.2 Growth curve

Growth curve studies for the two strains were carried out in nutrient broth and Enzyme Production Media at the optimal growth conditions standardized earlier. The prepared media were dispensed as 50 ml aliquots in 250 ml conical flasks, autoclaved and inoculated with 0.5 ml of the prepared inoculum and incubated at 30°C. Samples were drawn at regular intervals and growth was determined by measuring the turbidity at 660 nm in a UV-Visible Spectrophotometer (Shimadzu Model-160 A). Using the OD values the growth curve was constructed. From the growth curve generation time and specific growth rate were determined.

2.6 Production of exoenzymes employing SSF using potential strains

2.6.1 Preparation of the substrate

Banana Fruit Stalk (BFS) substrates prepared as mentioned under section 2.2 were spread on trays and oven dried at 70°C for 24 h. The dried slices were ground and sieved through standard mesh sieves (ASTM) to obtain particles of varying sizes (ranging from 0.2-3.2 μm) and stored in polyethylene bags at room temperature (30 ± 2°C) until use.

2.6.1.1. Cellulose

Cellulose content in the prepared solid substrate was estimated according to Updegroff's (1969) methodology.

1. To 0.5 gm of sample taken in a test tube, 3 ml of acetic nitric reagent (prepared by mixing 150 ml of 80% acetic acid and 15 ml of concn nitric acid) was added and mixed well in a vortex mixer.

2. The tubes were placed in a boiling water bath for 30 min.
3. Centrifuged the sample for 5 min at 5000 rpm and the supernatant was discarded.
4. The residue was washed well with 10 ml DW.
5. Centrifuged the sample for 5 min at 5000 rpm and the supernatant was discarded.
6. Added with 10 ml of 67% H_2SO_4 (v/v), mixed well and allowed to stand for 1h.
7. 1 ml of the sample was diluted to 100 ml with DW. Centrifuged to remove the precipitate if any, present in the sample.
8. 10 ml of the above diluted sample was taken in another test tube and added with 4 ml of DW.
9. The tubes were cooled in an ice-bath to cool.
10. Added 10 ml cold anthrone reagent (0.2 gm anthrone in 100 ml concn.H_2SO_4, prepared fresh daily and chilled for about 2h in a refrigerator before use) by layering with a pipet.
11. Mixed well on a Vortex mixer and returned to ice-bath until all tubes were mixed.
12. The tubes were heated in a boiling water bath for 16 min.

13. The tubes were cooled in an ice-bath (2-3 min) and allowed to stand at room temperature (28 ± 2°C) for 5-10 min.
14. The absorbance was measured at 630 nm in a UV visible Spectrophotometer (Shimadzu Model-160 A) against a reagent blank.
15. Standard curve with cellulose was also prepared in the same manner and the cellulose concentration was estimated and expressed in terms of percentage.

2.6.1.2 Starch

Starch content in the prepared solid substrate was estimated according to the method of Clegg (1956).

1. 0.5 g. of the sample was homogenized with hot 80% alcohol to remove the sugars. Centrifuged and retained the residue. The alcohol extraction procedure was repeated till the washings did not give colour with anthrone reagent. The residue was dried well over a water bath.
2. Added 5 ml of DW to the residue of test material which has been extracted with alcohol and, while stirring,

6.5ml of 52% perchloric acid (270 ml of 72% perchloric acid plus 100 ml of DW) was added. Stirred continuously for 5 min with a glass rod and then occasionally for the next 15 min. Added with 20 ml of DW and centrifuged.

3. The supernatant was collected in a 100 ml volumetric flask.
4. Using fresh 52% perchloric acid the extraction was repeated.
5. Centrifuged and supernatant was pooled with the first extract, diluted to 100 ml with DW and filtered.
6. 1 ml of the supernatant was added with 1 ml DW and 10 ml anthrone reagent and mixed thoroughly.
7. The tubes were heated in a boiling water bath for 12 min and then cooled to room temperature (28 $\pm$ 2^{O}C).
8. Absorbance was measured at 630 nm using UV Visible Spectrorphotometer (Shimadzu Model-160 A) with a reagent blank.
9. The amount of starch was computed from a standard curve prepared with glucose. The values were corrected by multiplying with a factor of 0.9 to arrive at the starch content.

2.6.2 Media

Mineral Salts Medium (MSM) (Ramesh and Lonsane, 1989) was used for SSF studies.

Composition of Medium:-

$Na_2HPO_4.2H_2O$	1.1g
$NaH_2PO_4.2H_2O$	0.61g
KCl	0.3g
$MgSO_4.7H_2O$	0.01g
DW	100 ml
pH	7.2

2.6.3 Preparation of Solid State Fermentation (SSF) Medium

1. 10 g of banana fruit stalk substrate (section 2.6.1.), taken in 500 ml Erlenmeyer flasks was moistened with the mineral salt medium (section 2.6.2) by mixing the contents thoroughly.
2. Autoclaved for 60 min and cooled to room temperature.

2.6.4 Inoculation and incubation

The inoculum was prepared as mentioned earlier (section 2.4.2.2) was added (10% v/w) to the sterilized moist banana fruit stalk medium, mixed thoroughly and the flasks were incubated in a slanting position at 30°C in an incubator with 65-70% relative humidity (Ramesh and Lonsane, 1987) for 72 h (unless otherwise specified).

2.6.5 Enzyme recovery and Enzyme assay

After solid state fermentation, enzymes synthesized by bacteria were leached out employing the extraction strategies adopted by Kumar and Lonsane(1987) and assayed as mentioned earlier for α-amylase(section 2.3.3.1.) and cellulase(section 2.3.3.2.).

2.6.6 Effect of operational parameters on enzyme production by SSF

Effect of pretreatment of substrate, initial moisture content, particle size, incubation temperature, pH,

substrate concentration, additional nutrients, inoculum concentration and incubation period on enzyme production by bacteria under SSF was studied as detailed below:

2.6.6.1 Pretreatment of the substrate

Effect of pretreatment of the substrate on the production of enzymes by bacteria under Solid State Fermentation (SSF) was tested as follows.

2.6.6.1.1 Steaming

Effect of steaming under pressure on the banana fruit stalk particles on enzyme production was tested by subjecting the substrate particles to different temperatures (100-130^{O}C) for varied periods (0-120 min), before use in SSF studies.

2.6.6.1.2 Alkali treatment

Impact of alkali pretreatment of banana fruit stalk particles (400 μm size, unless otherwise stated) on enzyme

production was determined by treating the particles with different concentrations (0-10M) of NaOH, KOH and NH_4OH for varying soaking time at room temperature. After treatment, the particles were repeatedly washed with DW until the pH of the washed water was neutral, and dried overnight at 60°C and used (Fan *et al.*,1981).

2.6.6.1.3. Acid Hydrolysis

Impact of acid pretreatment of banana stalk particles (400 μm size, unless otherwise stated) on enzyme production was determined by treating the particles with different concentrations (0-10M) of HCl and H_2SO_4 for varying soaking time at room temperature. After treatment the particles were repeatedly washed with DW until the pH of the washed water was neutral, and dried overnight at 60°C and used.

2.6.6.2 Effect of initial moisture content of the medium

Effect of initial moisture content on enzyme production was studied by adjusting the moisture content of

the BFS (Banana Fruit Stalk) to various levels ranging from 30-90% (v/w) by varying the volume of mineral medium.

2.6.6.3 Effect of particle size of BFS

The effect of particle size of BFS on enzyme production during SSF was determined by using BFS of different particle size. The particle sizes ranging from 200 to 3200 μm were prepared using sieves of standard mesh size.

2.6.6.4 Effect of incubation temperature

Effect of incubation temperature on enzyme production by bacteria under SSF was detected by incubating the inoculated BFS medium at different temperatures viz. 20, 25, 30, 35, 40, 45, 50 and 55°C.

2.6.6.5 Effect of pH

Effect of pH on the enzyme production under SSF was ascertained by subjecting the organisms to various pH levels

adjusted in the mineral medium from pH 4-10 using 1 N HCl/NaOH..

2.6.6.6 Effect of additional substrate concentration

Effect of soluble starch/cellulose concentration as additional substrate on enzyme production by bacteria under SSF was determined at different concentrations (0-3%, w/w) in the Mineral Salt Medium.

2.6.6.7 Effects of Carbon sources

Effect of additional carbon sources on enzyme production by bacteria under SSF was detected by incorporating different carbon sources viz., glucose, galactose, maltose, lactose, sucrose and Na_2CO_3 (0.1-3.0% w/w) individually into the mineral salt medium.

2.6.6.8 Effect of nitrogen sources

Influence of additional nitrogen sources on enzyme production by bacteria under SSF was carried out by the

addition of beef extract, yeast extract, peptone, $NaNO_3$, Urea and $(NH_4)_2SO_4$ at different concentrations (0.1-3.0% w/w), individually into the Mineral Salt Medium.

2.6.6.9 Effect of Salts

Impact of additional salts on enzyme production by bacteria under SSF was determined by the addition of NaCl and KCl at different concentrations (0.1-3.0% w/w), individually into the mineral salts medium.

2.6.6.10 Effect of inoculum concentration

Effect of inoculum concentration on enzyme production by bacteria under SSF was detected by using different levels of inoculum. Inoculum to substrate ratio was adjusted to 5,10,15,20,25, 30 % v/w).

2.6.6.11 Effect of incubation time

Effect of incubation time on enzyme production by bacteria under SSF, after optimization studies, was checked by

incubating the inoculated BFS medium for a total period of 120h and estimating the enzyme yield at regular intervals of 12h.

2.6.7 Optimisation of process parameters that influence Extraction and Recovery of Enzyme

Impact of process parameters on the extraction and recovery of enzymes produced by bacteria during solid state fermentation of the banana fruit stalk medium was studied (Kumar and Lonsane, 1987). Drying temperature of the fermented medium, ratio of the fermented substrates to solvent, pH of the solvent, contact temperature and contact time were optimised with a view to recover maximal enzyme from the fermented substrates.

The enzyme was extracted before optimisation of process parameters, under arbitrarily selected conditions, using 0.2M Phosphate buffer of appropriate pH (pH 6 or 8) in a 1:10 ratio (BFS to buffer) and a contact time of 90 min at 30°C (unless otherwise specified). It was carried out in two stages. In the first stage the fermented BFS was contacted

with 100ml of buffer for 60 min with occassional stirring. Then the slurry was squeezed through a dampened cheese cloth. In the second stage the left over solids were again contacted with fresh 100 ml of the same buffer for another ·30 min and later the slurry was squeezed as mentioned earlier. The extracts were pooled and centrifuged for 20 min. at 10,000 rpm at 4°C in a Kubota 6700 Model refrigerated centrifuge (Ramesh and Lonsane, 1990). The clear supernatant obtained after centrifugation was used for different enzyme assays (section 2.3.3.1.and 2.3.3.2. respectively)

2.6.7.1. Drying Temperature

Effect of drying temperature on enzyme recovery was studied by drying the fermented Banana Fruit Stalks (BFS) at varying temperatures, i.e., 30°C, 40°C, 50°C and 60°C for 1h.

2.6.7.2. Extraction medium

Appropriate medium which shall yield maximal enzyme recovery on extraction was determined using aqueous ethanol

(80%), distilled water(DW), DW plus 1% NaCl, tap water(TW) and (0.2M phosphate buffer of pH 7.2.

2.6.7.3 pH of extraction medium

Effect of pH of the extraction media on leaching of enzymes was determined by using buffers of pH ranging from 5-8 (0.2M Acetate buffer of pH 5-6, 0.2M phosphate buffer of pH 6-8).

2.6.7.4 Ratio of solid substrate to buffer

Effect of solid substrate to buffer ratio on enzyme recovery was determined by adding buffer to fermented medium in different ratios of 1:3, 1:5, 1:10, 1:20 and 1:30.

2.6.7.5 Effect of contact temperature

Effect of contact temperature on enzyme recovery was determined by keeping the fermented medium and buffer in contact at different temperatures of 25, 35, 45 and 55°C.

2.6.7.6 Effect of contact time

Effect of contact time of buffer with fermented medium was tested by contacting the fermented medium with buffer for different time intervals of 30, 60, 90, 120 min.

2.7 Production of exoenzymes by bacteria using Wheat Bran (WB) as solid substrate under SSF

Inorder to evaluate the suitability of banana fruit stalk as solid substrate, a comparison was made with wheat bran as solid substrate added with and without soluble starch in the range of 0-3%(w/w).

The methodologies adopted for substrate and media preparation, inoculation, incubation and enzyme extraction were similar to those reported for Wheat Bran by Ramesh & Lonsane (1987 & 1989) and are mentioned under sections 2.6.4 and 2.6.5 respectively. The enzyme was assayed as per the procedures mentioned earlier under sections 2.3.3.1 and 2.3.3.2 respectively.

2.8 Enzyme production by bacteria under Slurry Fermentation (SLF)

Enzyme production by bacteria under Slurry state Fermentation (SLF) condition was evaluated for comparison with SSF process. A slurry of Banana Fruit Stalk (BFS) medium was prepared by mixing 10g of BFS in 100 ml of basal mineral salts medium (section 2.6.2). The prepared BFS slurry medium was autoclaved for 1 h, inoculated with the prepared inoculum (section 2.6.4), and incubated on a rotary shaker at 35°C for 72 h. Samples were withdrawn at regular intervals, enzyme extracted, and assayed as per the procedures described ealier under sections 2.6.5, 2.3.3.1 and 2.3.3.2 respectively.

2.9 Enzyme production by bacteria under Submerged Fermentation (SmF)

Production of α-amylase and cellulase by bacteria under submerged fermentation was carried out as detailed below:-

1. Initially BFS homogenate was prepared by adding 10 g of finely powdered BFS in 1000 ml of DW and autoclaved for 1h at 121°C.
2. The homogenate thus prepared was filtered to remove solid particles and the clear filtrate was used. Initial levels of starch and cellulose in the filtrate was determined as mentioned earlier (sections 2.6.1.1 and 2.6.1.2 respectively.
3. 100 ml of the prepared filtrate was added with all the mineral components in the same proportion as given in section 2.6.2. Final pH was adjusted to the optimal pH of the culture determined earlier. The prepared medium was autoclaved and used.
4. Inoculum preparation and inoculation were similar to that mentioned earlier (see 2.6.4). The flasks were incubated on a rotary shaker at 35°C for 72 h,samples were withdrawn at regular intervals and the enzyme extraction and assay of enzyme were done as mentioned earlier (section 2.6.5, 2.3.3.1.2 and 2.3.3.2 respectively).

2.10. Purification of Enzyme

Since α-amylase was the major fraction recovered in the enzyme leachate, obtained through SMF and SSF, it was further purified by $(NH_4)_2SO_4$ fractionation followed by dialysis, ion exchange chromatography and electrophoresis.

2.10.1 $(NH_4)_2 SO_2$ fractionation

1. $(NH_4)_2 SO_4$ (SRL-enzyme grade) was added slowly and the concentration was increased upto 30-80% saturation along with continuous stirring using a magnetic stirrer, at 4°C, in an ice - bath, until maximal precipitation of enzyme protein was achieved.
2. The precipitate obtained for each saturation was removed by centrifugation at 10000 rpm for 20 min in a high speed refrigerated centrifuge (Kubota Model - 6700) at 4°C and dissolved in 0.2M phosphate buffer (pH 7.2).
3. Enzyme activity and protein content of each fraction was determined according to the procedures mentioned in sections 2.3.3.1 and 2.3.4 respectively.

2.10.2. Dialysis

The precipitate obtained after $(NH_4)_2SO_4$ fractionation was dissolved in phosphate buffer (0.2M) pH 7.2 and dialysed against the same buffer extensively at 4°C for 24h. Enzyme activity and protein content of the dialysate were determined as mentioned earlier sections 2.3.3.1 and 2.3.4 respectively.

2.10.3. Column chromatography

The dialysate was further purified by ion-exchange chromatography using the anion exchanger DEAE - Cellulose.

1. The anionic exchanger DEAE - Cellulose (SRL) was packed in a column (2.4 x 30cm)at 30 ml bed volume.
2. The packed column was equilibrated with 0.2M phosphate buffer of pH 7.2.
3. The dialysate was applied to the column.
4. The column was washed with five bed volumes of the phosphate buffer (0.2M) of pH 7.2.

5. Elution was carried out with a linear gradient of NaCl from 0-1.0M in buffer.
6. Flow rate was adjusted to 40 ml h^{-1} and fractions of 6 ml were collected using Redifrac Fraction Collector (Pharmacia).

Each fraction was analysed for enzyme activity (section 2.3.3.1) and protein (the absorbance at 280 nm was used to monitor protein in column eluates) as mentioned by Kim *et.al.*, (1993). The fractions with higher specific activities were pooled and lyophilized in a Lyophilizer (Yamato Model).

2.10.4. SDS - Polyacrylamide Gel Electrophoresis

This was carried out according to the method of Laemmli (1970) with a 10% running gel and 4% stacking gel in SDS - discontinous buffer system at pH 8.3.

2.10.4.1.Preparation of reagents

1. Stock Acrylamide solution

Acrylamide	30 g
Bisacrylamide	0.8 g
DW	100 ml

2. Resolving Gel Buffer stock

3.0M Tris - HCl (pH 8.8)

Tris	36.3g
1 M HCl	48 ml

The total volume made upto 100 ml

3. Stacking Gel Buffer Stock

0.5 M Tris - HCl (pH 6.8)

Tris	6.0 g
1 M HCl	48 ml

The total volume made upto 100 ml

4. Reservoir Buffer stock

(0.25 M) Tris	30.3g
(1.92 M) Glycine	144.0g
SDS	10g
DW	1000 ml

All the stock solutions were prepared and stored at $4^{\circ}C$.

5. Polymerising agents

a) Ammonium per sulphate 1.5%

b) TEMED

Ammonium persulphate prepared fresh and used.

6. Sample buffer

0.0625 M Tris - HCl (pH 6.8)

SDS	2%
2-mercaptoethanol	5%
sucrose or glycerol	10%
bromophenol blue	0.002%

7. Protein stain solution

Coomassie Brilliant blue R 250	0.1 g
Methanol	40 ml
Acetic acid	10 ml
DW	50 ml

8. Destainer

Methanol	40 ml
Acetic acid	10 ml
DW	50 ml

2.10.4.2. Procedure

30 ml of 10% resolving gel mixture was prepared by mixing the components as given below:

Acrylamide-bisacrylamide (30:0.8)	10.0 ml
Resolving gel buffer stock	3.75 ml
10% SDS	0.3 ml
1.5% ammonium persulphate	1.5 ml
DW	14.45 ml
TEMED	0.015 ml

2. The resolving gel mixture was poured into the space between the glass plates assembled earlier, leaving sufficient space at the top for a stacking gel to be polymerised later. Layered DW on the top of the gel and left it to set for 10-30 min.

3. The stacking gel mixture (4%) was prepared as given below:-

Acrylamide-Bisacrylamide (30:0.8)	2.5 ml
Stacking gel buffer stock	5.0 ml
10% SDS	0.2 ml
1.5% ammonium persulphate	1.0 ml
DW	11.5 ml
TEMED	0.015 ml

4. After ploymerisation (10-30 min), as evidenced by the presence of a sharp interface between the ploymerised gel and the overlay. The overlay of DW was poured off and rinsed the top of the revolving gel with a little stacking gel mixture. This was poured off and then the remaining space between the gel plates was filled with stacking gel mixture.

6. The comb was inserted immediately into the stacking gel mixture, and allowed the gel to set for 10-30 min.

7. After ploymerination, the comb was carefully removed to expose the sample wells, which were rinsed with reservoir buffer and then filled with it.

8. Carefully installed the gel in the electrophoresis apparatus. reservoir buffer was added to the reservoirs of the electrophorsis apparatus.

9. Preparation of samples for electrophoresis was done as follows :-

The sample containing about 50-100μg protein was usually used. The protein concentration in each sample was adjusted using the sample buffer in such a way that the same amount of protein was present per unit volume. The sample solution was heated in boiling water for 3 min and then allowed to cool to room temperature (30 $\pm$ 2^{o}C).

11. The sample was loaded into the wells.

12. The electrophoresis apparatus was connected to the power pack (Hoefer Scientific Instruments,). The slab gel was electrophorsed by a voltage of 100 in stacking gel followed by 150 constant voltage for 6h.

13. The gel was removed and immersed in staining solution for overnight at room temperature .

14. The stained gel was transferred to a suitable container with at least 200-300 ml destaining solution and shaked gently and continuously until the protein bands were seen clearly.

15. The slab gel was stored at $4^{o}C$ in sealed plastic bags containing 7% acetic acid.

2.10.4.3 Molecular weight Determination

SDS - PAGE electrophoresis was used to determine the approximate molecular weight of α-amylase. Bovine serum albumin(67000) served as marker protein, (Biorad, cat no.161-0315).

2.11. Characterisation of α - amylase

The purified enzyme was characterised for its activity at various pH, temperature, substrate concentration incubation time, NaCl concentration and inhibition by cations and other inhibitors. The stability of the enzyme at different pH and temperatures was also studied.

2.11.1. Effect of pH on activity and stability of the enzyme

Effect of pH on the activity of the enzyme was studied as per the following procedures.

A mixture containing 1 ml of purified enzyme and 1 ml of 1% starch was incubated with 1 ml of buffers of different pH ranging from pH 4-10 (prepared using 0.2M acetate buffer, pH 4-5; 0.2M phosphate buffer, pH 6-8; 0.2M Tris - HCl buffer pH 8-9; and 0.2M glycine - NaOH buffer, pH 9-10) for 10 min at 40°C. The α-amylase activity and protein in the reaction mixture were determined as mentioned earlier (section 2.3.3.1 and 2.3.4 respectively).

Stability of the enzyme at various pH (viz., pH 4-10) was determined by incubating 1 ml of enzyme with 1 ml of buffers for 1h (prepared with 0.2M acetate buffer, pH 4-5;, 0.2M phosphate buffer, pH 6-8; 0.2M Tris-HCl buffer pH 8-9, 0.2M glycine-NaOH buffer, pH 9-10.

A mixture containing 1 ml of this treated enzyme solution each, was incubated with 1 ml of 1% soluble starch for 10 min at 40°C. Enzyme activity and protein were determined according to the procedures mentioned under sections 2.3.3.1 and 2.3.4 respectively.

2.11.2. Effect of temperature on activity and stability of enzyme

Effect of temperature on the stability and activity of the enzyme was estimated by the following procedures.

1 ml of purified enzyme added with 1 ml of DW and 1 ml of 0.2M Tris-HCl buffer of appropriate pH (8.6) was incubated with 1 ml of 1% soluble starch for 10 min at various temperatures such as 30,40,60,80 and 100°C. Enzyme activity and protein were analysed as per sections 2.3.3.1 and 2.3.4 respectively.

The stability of the enzyme at various temperature was determined by incubating 1 ml of purified enzyme with 1 ml of 0.2M Tris-HCl buffer (pH 8.6) at different temperatures viz,. 30,40,60,80,100 and 120°C.

The treated enzyme buffer mixture was later incubated with 1 ml of 1% soluble starch for 10 min at 40°C. Enzyme activity and protein were measured following the

procedures mentioned under sections 2.3.3.1 and 2.3.4 respectively.

2.11.3. Effect of substrate concentration on activity of the enzyme

Effect of substrate concentration on the activity of purified enzyme was determined by incubating 1 ml of enzyme with 1 ml of Tris-HCl buffer (pH 8.5, 0.2M) with different concentrations of starch (0.1-1.5%) for 10 min at 40°C. Reaction mixture was analysed for enzyme activity and protein as per the procedures mentioned under the sections 2.3.3.1 and 2.3.4. Michaelis - Menten Constant (Km) of the enzyme was determined with different concentrations of soluble starch as substrate. Values were obtained from double reciprocal plotting as described by Lineweaver and Burk (1934).

2.11.4 Effect of cations on the activity of enzyme

1 ml of purified enzyme was incubated at 40°C for 10 min.with 1 ml of Tris-HCl buffer (pH 8.6, 0.2M) and 1 ml of 1% soluble starch and 1 ml of 0.01 $moll^{-1}$ of cations

Pb^{2+},Co^{2+},Zn^{2+},Ba^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, K^{2+}, Al^{2+} and Hg^{2+} added individually. The enzyme activity and protein were determined following the procedures under sections 2.3.3.1 and 2.3.4.

2.11.5 Effect of other inhibitors on enzyme activity

1 ml of purified enzyme was incubated with 1 ml of Tris-HCl buffer (0.2M) pH 8.6, and 1 ml of 1% soluble starch and 1 ml of the following inhibitors (individually). The inhibitors used were Dimethyl sulphoxide, p-Di methylamino benzaldehyde, EDTA, Acetic acid, Iodine, Phenyl hydrazine hydrochloride and TCA at a concentration of 0.01 mol l^{-1}. The reaction mixture was incubated at 40°C for 10 min. The enzyme activity and protein in the reaction mixture were analysed according to the procedures under sections 2.3.3.1 and 2.3.4 respectively.

CHAPTER III

RESULTS

3.1 Changes in the proximate composition of Banana Fruit Stalk during fermentation under uncontrolled condition

The proximate composition of fresh banana fruit stalk, analysed, is presented in Table 3. During fermentation under uncontrolled condition, by the native microflora, the following biochemical changes were recorded (Table 4). The moisture content, pH and protein of the sample recorded a linear increase along with increase in incubation period. Whereas both total sugar and reducing sugar content of the samples were observed to decline during the course of fermentation. The pH of the sample shifted significantly to alkaline condition from neutral after fermentation. The increase in moisture content (3.9%) and protein (0.4%) were however, not of very high order compared to the levels of decrease in total sugar (7.8%) and in reducing sugar (1.5%) during the course of fermentation.

Table 3

Proximate composition of fruit stalk (Peduncle) of banana, *M.paradisiaca* var.Nendran

Expressed in terms of percentage of fresh weight of the sample	
Biochemical Parameters	Percentage Composition
Moisture content	89.3
Carbohydrate	8.7
Crude fibre	0.8
Crude fat	0.1
Mineral matter	0.6
Crude protein	0.5
* Starch	27.27
* Cellulose	23.85

(* Expressed in terms of percentage of dry weight of the sample)

3.2 Bacteriological changes in the BFS during fermentation under uncontrolled conditions

Changes in the total Heterotrophic bacterial population and generic composition of microbial flora, associated with the BFS during the course of fermentation, under uncontrolled condition, are presented in Table 5. Although the THB did not record changes in the level of population at levels of one log or more still there was significant increase in number on the 4th day of fermentation, compared to fresh condition. However, further increase in the period of incubation resulted in a decline in the level of population. It is evident from the Table 5 that there existed a clear generic diversity in the microbial flora. Species of *Bacillus, Pseudomonas, Aeromonas* and *Vibrio* were recovered as dominant groups along with some other unidentified flora. In general, Gram negative forms especially *Pseudomonas* Sp dominated during the course of fermentation. While *Pseudomonas* Sp recorded a linear increase in the percentage composition other species viz. *Bacillus, Aeromonas* and *Vibrio* declined in their number during the course of incubation period. Results presented in Table 6 indicate that of the

Table 4

Biochemical changes in the banana fruits stalk during fermentation under uncontrolled condition

Incubation Period (in days)	pH	Moisture	Total Sugar (%)	Reducing sugar (%)	Protein (%)
0	6.2	88.9	8.7	2.4	0.5
2	7.2	89.1	7.4	1.6	0.6
4	9.5	90.2	5.5	1.3	0.7
6	10.5	91.4	2.8	1.0	0.8
8	11.2	92.8	0.9	0.9	0.9

Table 5

Bacteriological changes in the banana fruits stalk during fermentation under uncontrolled condition

Incubation Period (in days)	THBp/g ($x.10^8$)	*Bacillus*.sp (%)	*Pseudomoas*.sp (%)	*Aeromonas*.sp (%)	*Vibrio*.sp (%)	Unidentified flora (%)
0	8.9	38.0	44.0	2.0	10.0	6.0
2	9.6	32.0	58.0	2.0	6.0	2.0
4	12.0	28.0	60.0	1.0	4.0	7.0
6	10.5	22.0	62.0	0.0	4.0	14.0
8	6.8	18.0	64.0	0.0	2.0	16.0

four major species of bacteria encountered, both *Bacillus* Sp and *Aeromonas* Sp could elaborate amylase, cellulase, caseinase, gelatinase, lipase and pectinase compared to *Pseudomonas* sp and *Vibrio* sp which produced, all enzymes tested except cellulase and pectinase.

3.3 Recovery of exoenzyme from fermented banana fruit stalk wastes under uncontrolled fermentation

During fermentation of banana fruit stalk under uncontrolled condition, α-amylase, cellulase and protease were recovered at significant levels. It was noted that fresh banana fruit stalks, at 0hr. of incubation contained 60 U/kg of α - amylase. Whereas cellulase and protease were absent in fresh samples. Maximal levels of each enzyme was observed on different days. Thus while maximal level of cellulase (90 U/kgs) could be recovered on the 2nd day, amylase recorded its maximum (32800 U/kg) on the 4th day. In the case of protease, it was observed to increase progressively along with increase in incubation time. It was also observed that both α-amylase and cellulase declined, after recording their maximum, during the later stages of fermentation (Table 7).

Table 6

Percentage composition of hydrolytic enzyme producing bacteria encountered during fermentation of bananas fruit stalk under uncontrolled conditions

Bacterial Cultures tested	No.of isolates tested	Amylolytic	Cellulolytic	Caseinolytic	Gelatinolytic	Pectinolytic	Lipolytic
Bacillus sp.	30	60	75	83	100	8	58
Pseudomonas sp.	48	36	Nil	53	55	Nil	27
Aeromonas sp.	10	80	60	60	100	20	80
Vibrio sp.	12	10	Nil	62	87	Nil	25

Table 7

Enzymes recovered from the banana fruit stalk during fermentation under uncontrolled conditions (Expressed as units/kg. solid substrate)

Incubation Period (in days)	Amylase	Protease	Cellulase
0	60	0	0
2	22,500	280	90
4	32,800	320	68
6	28,200	360	41
8	16,400	380	2.0

3.4 Selection of Potential Strain for Enzyme Production

Selection of potential strains for α-amylase and cellulase production, at primary screening level, was carried out on the basis of qualitative determination of growth and enzyme production in an agar media supplemented with respective substrates as the sole carbon source. All the *Bacillus* sp and *Aeromonas* sp, tested positive for α-amylase and cellulase production were subjected to primary screening and ranked in terms of maximal width of clear zones.

Secondary screening of potential strains for α-amylase and cellulase production was carried out, using the top ten ranked strains selected, after primary screening, on the basis of quantitative determination of growth and enzyme production in an Enzyme Production Media supplemented with 1% starch/cellulase, respectively, as the sole carbon source. Results obtained for the analysis of α - amylase and cellulase production by the ten strains were ranked and the top two ranked strains were selected for further studies (Table 8).

Table 8

Secondary screening for potential strains for amylase and cellulase production

Bacterial strains tested	Amylase (in mm)	Cellulase (in mm)
Bacillus sp. 106	24.0	14.0
Aeromonas sp. 185	18.0	14.0
Bacillus sp. 89	16.0	12.0
Bacillus sp. 174	14.0	10.0
Bacillus sp. 87	14.0	8.0
Bacillus sp. 116	12.0	--
Aeromonas 171	10.0	6.4
Bacillus 131	8.5	--
Bacillus 144	8.0	4.2
Bacillus 74	8.0	4.0

Table 9

Characteristics of *Aeromonas* sp.

Characteristics	*A.caviae* (CBTK 185)
Morphological	
on Nutrient Agar —	colonies are white, circular and convex with an entire margin.
Biochemical	
Gram reacion	—
Rods in singles and pairs	+
Motility	+
Monotrichous flagellation in Liquid Medium	+
Growth in Nutrient broth at 37°C	+
Indole production in 1% peptone water	+
Growth in KCN broth	+
Utilisation on	+
L-Histidine	+
L-Arginine	+
L-Arabinose	+

contd..

Fermentation of	
Sucrose	+
Mannitol	+
Salicin	+
Maltose	+
Galactose	+
Cellobiose	+
Lactose	+
Breakdown of	
Malonate	—
Nucleate	—
D-tartarate	—
Voges-Proskauer	—
Gas from glucose	—
H_2S from Cysteine	—
Oxidase	+
Catalase	+
NO_3^- reduced to NO_2^-	+
Arginine dihydrolase	+
Tryptophan and phyenylamine deaminases	—

contd..

Hydrolysis	
Urea	—
Starch	+
Gelatin	+
Casein	+
Tween 80 esterase	+
Growth in peptone water about NaCl	+

Table 10

Morphological

Colonies on Nutrient agar media	—	White, circular, convex with an entire margin.

Characteristics	*B. subtilis* (CBTK 106)
Biochemical	
Gram reaction	+
Rod-shaped	+
Endospore produced	+
Spore round	—
Sporangium swollen	—
Parasporal crystals	—
Motile	+
Catalase	+
Oxidase	+
Voges - proskauer test	+
pH in V-P broth	+
< 6	+
< 7	—

contd..

Acid from	
D-glucose	+
L-Arabinose	+
D-xylose	+
D-Mannitol	+
Gas from glucose	—
Hydroloysis of	+
Casein	+
Gelatin	+
Starch	+
Utilisation of	
Citrate	+
Propionate	—
Sulfate reduced to sulfide	—
Nitrate reduced to nitrite	+
Degradation of tyrosine	—
Deamination of phenylamine	—
Formation of indole	—
NaCl and KCl required	—
Growth at pH	
6.8, Nutrient broth	+
5.7	+

contd..

Growth in NaCl	
2%	+
5%	+
7%	+
10%	ND
Growth at	
5°C	—
10	—
30	+
40	+
50	+
55	—
65	—

They were identified as *Aeromonas caviae* (CBTK 185) and *Bacillus subtilis* (CBTK 106) based on their morphological, physiological and biochemical characteristics (Table 9 & 10) according to the schemes outlined in the Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984; Sneath, 1986).

3.5 Growth Studies

Optimal levels of environmental variables namely pH, incubation temperatures, substrate concentration, NaCl concentration, Carbon and Nitrogen sources, inoculum concentration and incubation time for maximal growth in Nutrient broth (NB) and growth and enzyme production in Enzyme Production Media (EPM) substituted with respective substrates by *A.caviae* (CBTK 185) and *B.subtilis* (CBTK 106) were determined under Submerged Fermentation (SmF) conditions.

3.5.1 pH

Results presented in Fig.2 a & b and Fig.3 a & b indicated that the optimal pH for maximal growth and enzyme production was one and the same (pH 7.0) for both the strains.

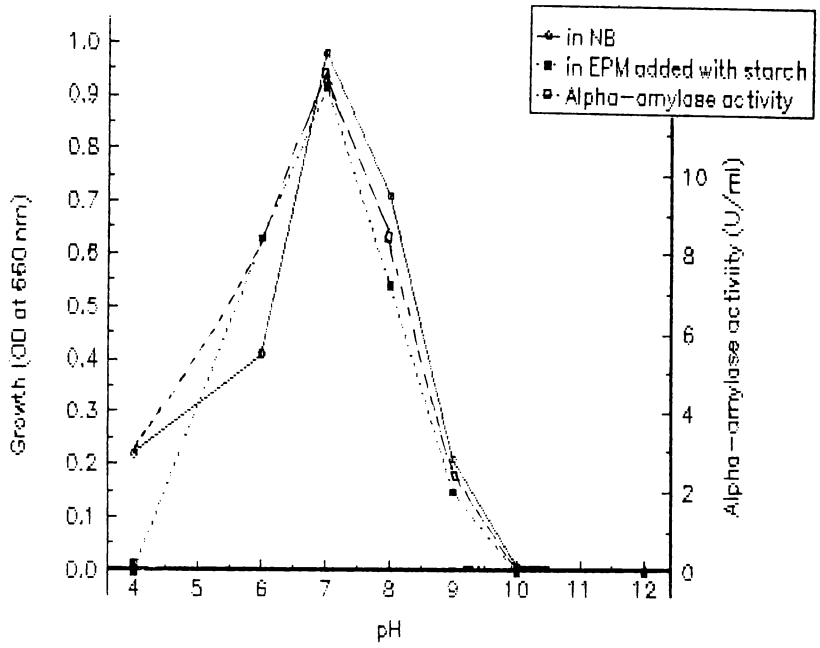


Fig.2 a Optimisation of pH for Growth and Enzyme production by *B.subtilis* CBTK 106

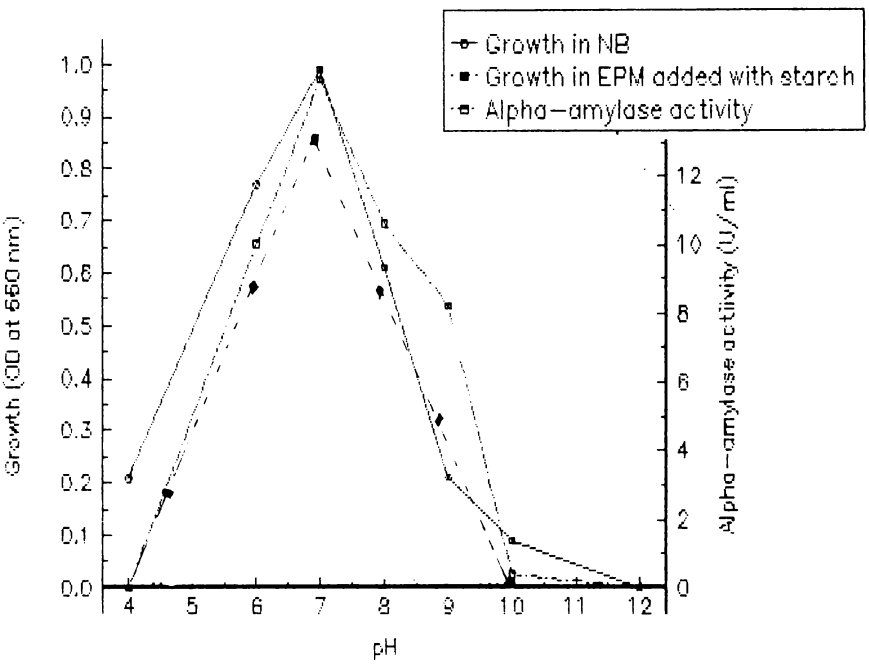


Fig.2 b Optimisation of pH for Growth and Enzyme production by *A.caviae* CBTK 185

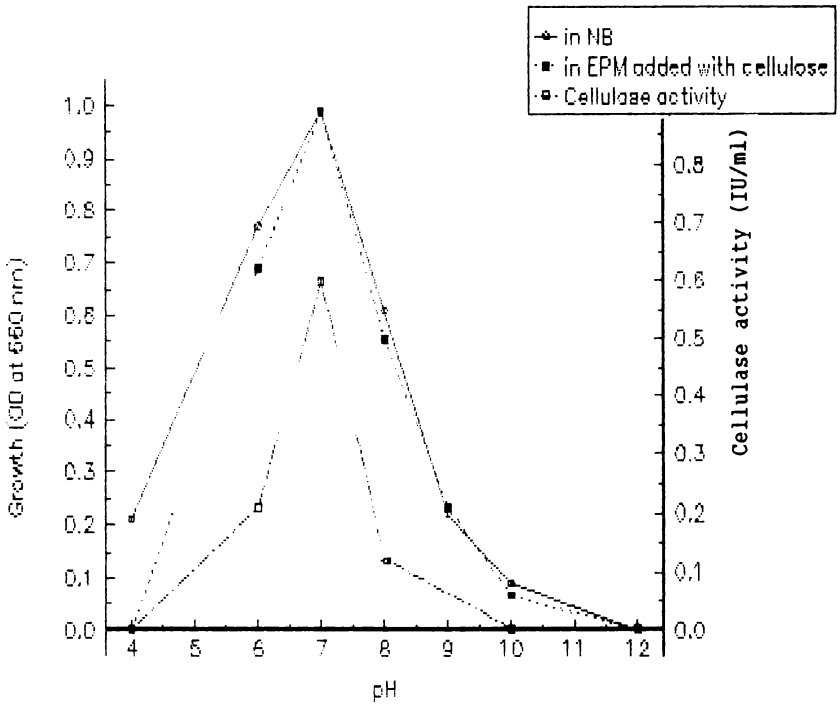


Fig.3 a Optimisation of pH for Growth and Enzyme production by *B.subtilis* CBTK 106

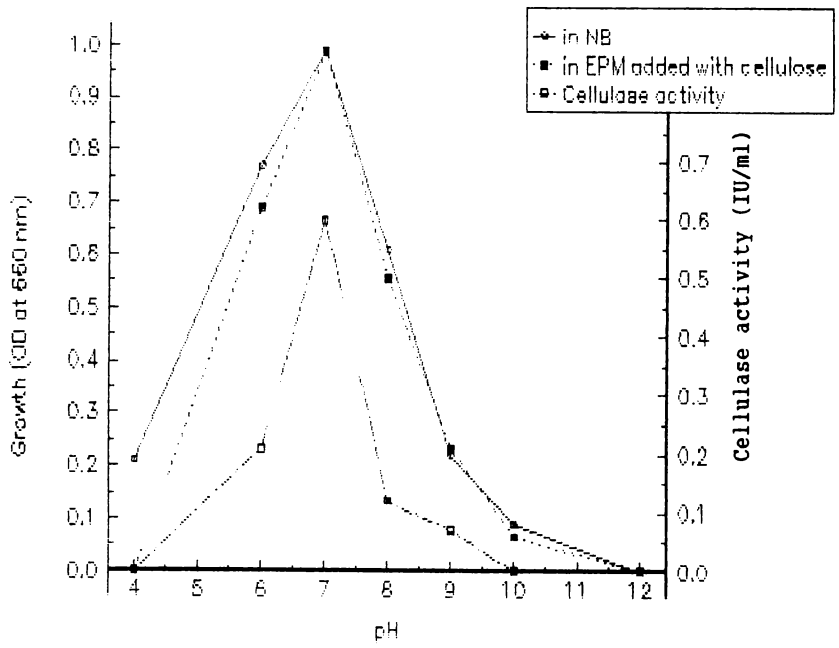


Fig.3 b Optimisation of pH for Growth and Enzyme production by *A.caviae* CBTK 185

However, while B.*subtilis* CBTK 106 recorded significant levels of growth and enzyme production over a pH range of 6-8, *A.caviae*, CBTK 185 could do the same over a wide range of pH from 6-9. At higher pH of 10-12, both species recorded insignificant growth and enzyme production.

3.5.2 Temperature

Data documented in Fig.4 a & b and Fig.5 a & b evidence that both the strains preferred 35°C to grow and produce enzyme at optimal level. However, they recorded significant levels of growth and enzyme production at other temperatures varying from 30-40°C. Incubation at temperatures above 40°C and below 30°C did not favour significant growth and enzyme production.

3.5.3 Substrate concentration

Variation in the concentration of substrate (0-3.0% w/v) influenced the growth and enzyme production by both the species of bacteria (Fig.6 a & b and Fig. 7 a & b). However the strains recorded maximal growth and enzyme production at a

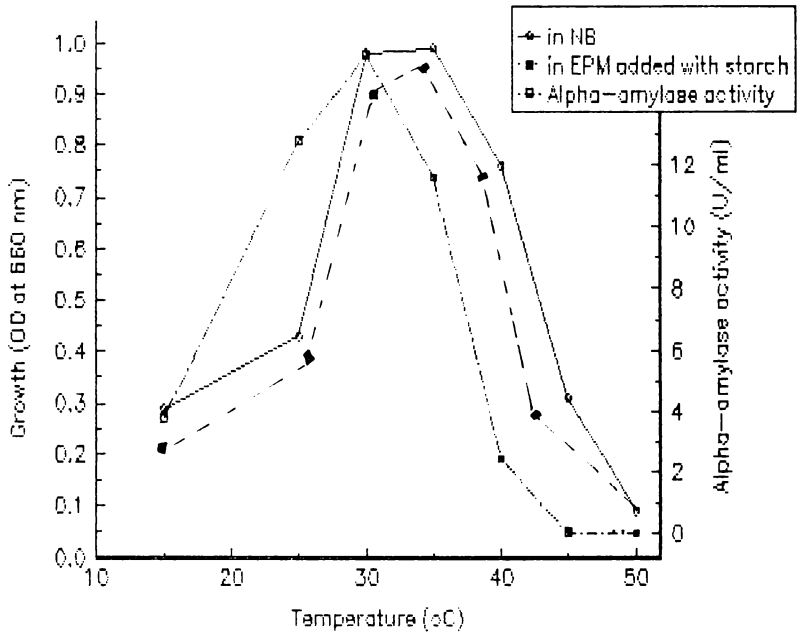


Fig.4 a Optimisation of Temperature for Growth and Enzyme production by *B. subtilis* CBTK 106.

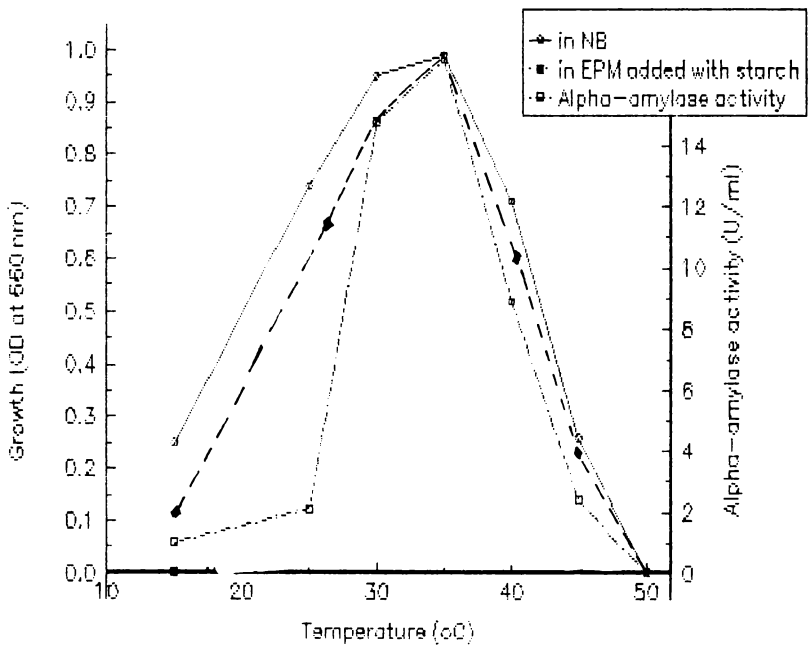


Fig.4 b Optimisation of Temperature for Growth and Enzyme production by *A.caviae* CBTK 185

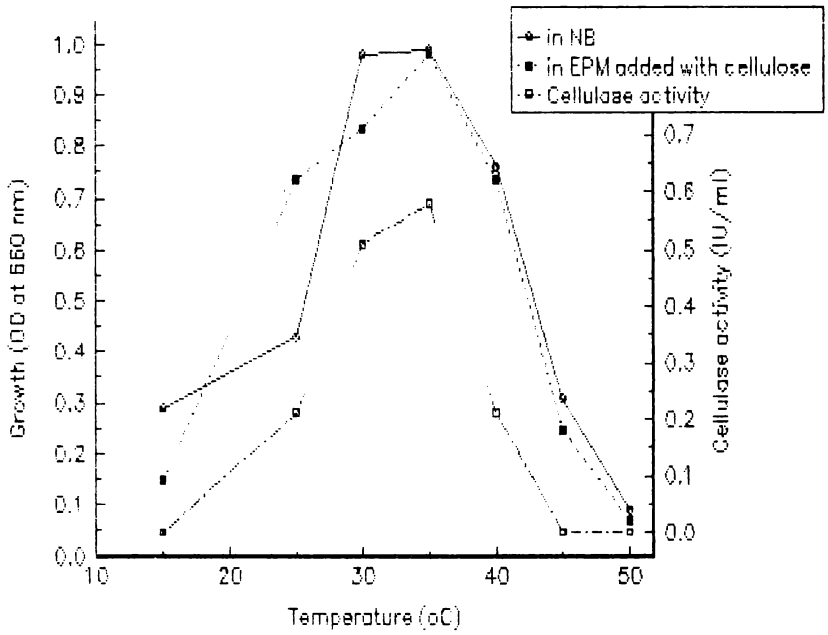


Fig.5 a Optimisation of Temperature for Growth and Enzyme production by *B. subtilis* CBTK 106.

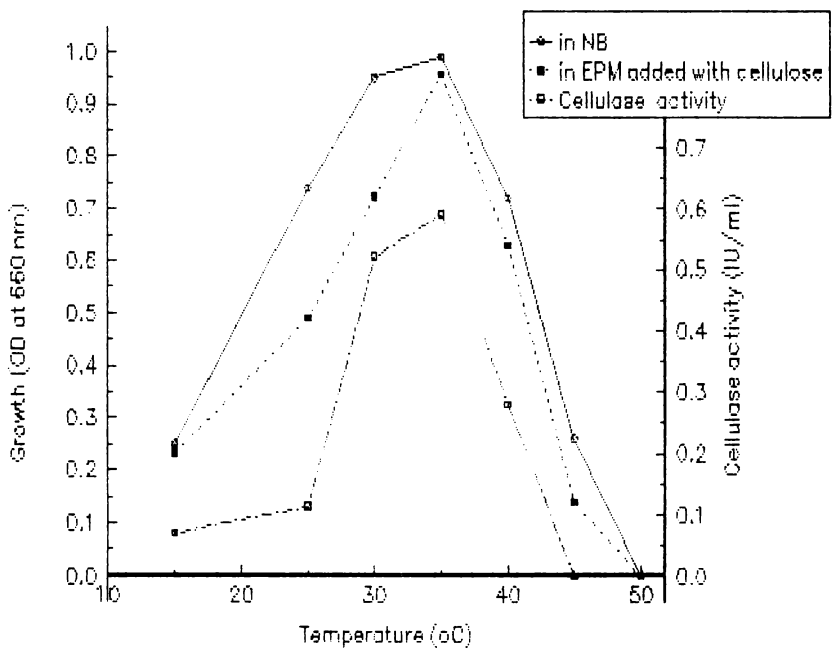


Fig.5 b Optimisation of Temperature for Growth and Enzyme production by *A.caviae* CBTK 185

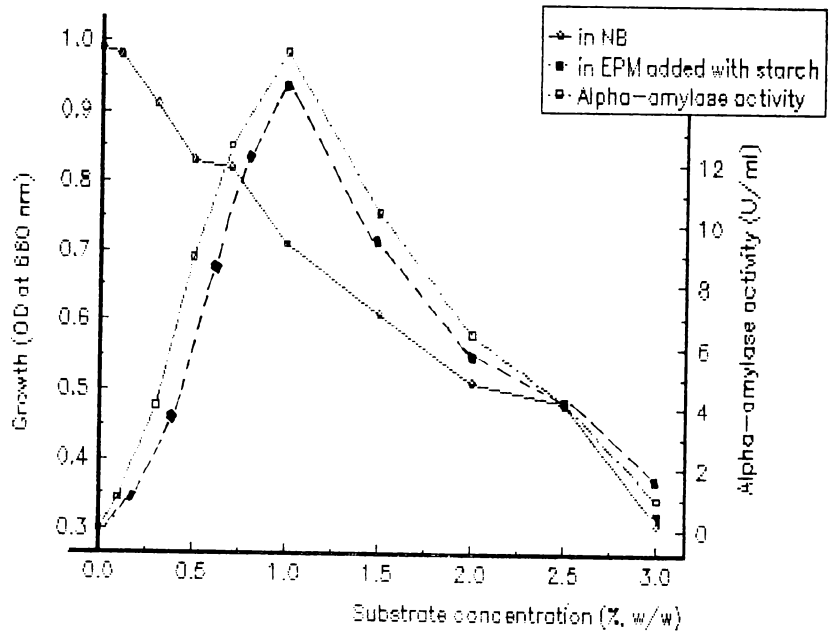


Fig.6 a Optimisation of Substrate Concentration for Growth and Enzyme production by *B.Subtilis* CBTK 106

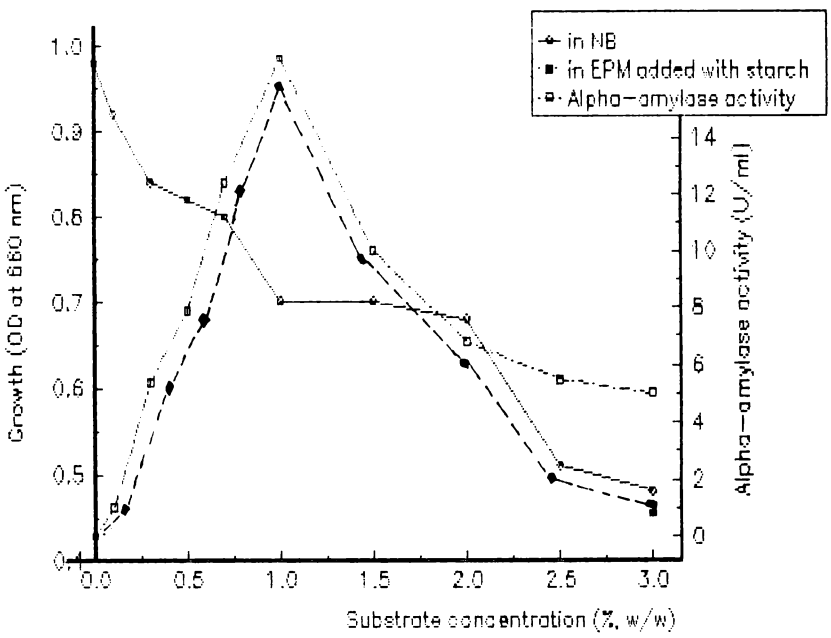


Fig.6 b Optimisation of Substrate Concentration for Growth and Enzyme production by *A.caviae* CBTK 185

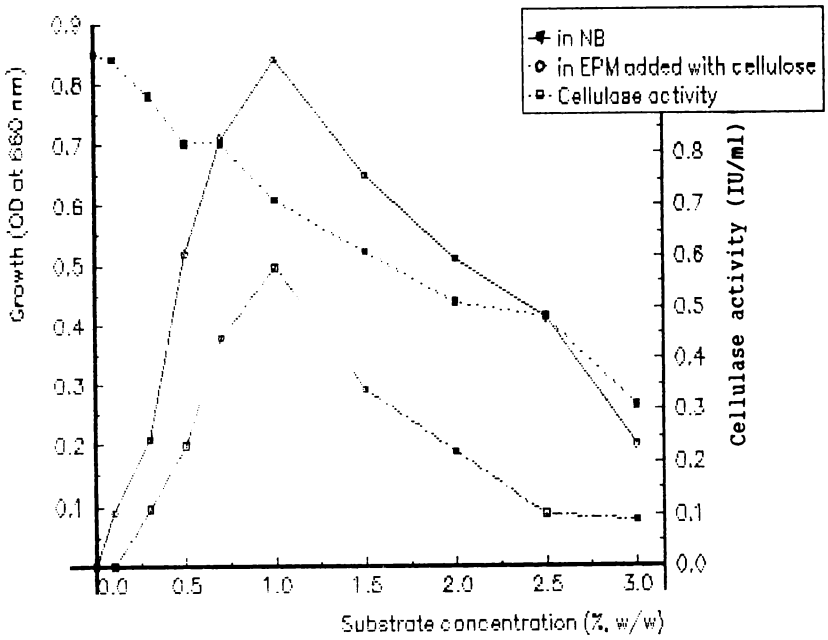


Fig.7 a Optimisation of Substrate Concentration for Growth and Enzyme production by *B.Subtilis* CBTK 106

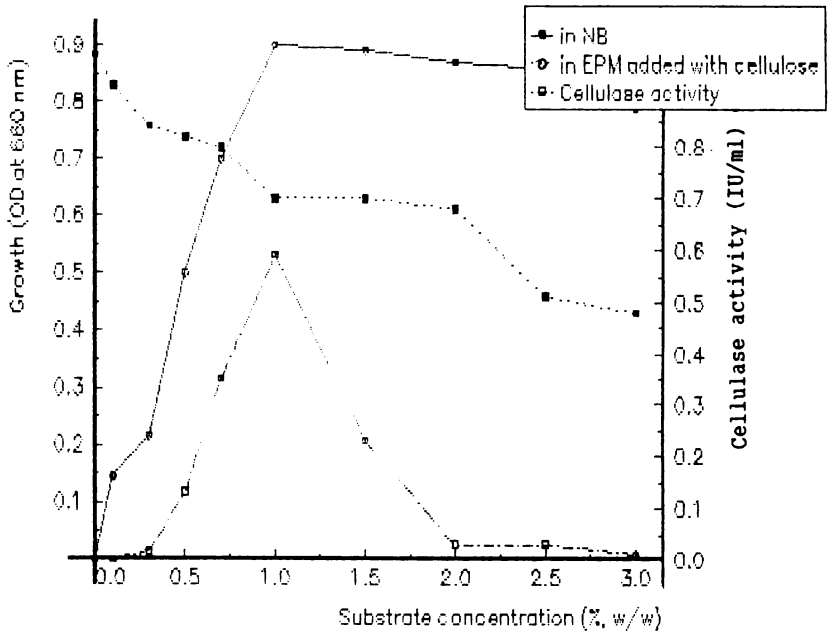


Fig.7 b Optimisation of Substrate Concentration for Growth and Enzyme production by *A.caviae* CBTK 185

substrate concentration of 1% level. It was also noted that increase in growth and enzyme production was proportionate with increase in substrate concentration from 0.1 to 1.0% and further increase in substrate conc. resulted in a decrease in the growth and enzyme production by both the strains. In general significant levels of enzyme production was observed at a wide range of substrate concentration from 0.5-1.5% for both the strains.

3.5.4 NaCl concentration

Both the strains could produce maximal level of α - amylase at 20 mM concentration of NaCl compared to other concentrations (Fig. 8 a & b and Fig.9 a & b.) In the case of cellulase, 10mM NaCl was observed as optimal requirement for both the strains (0.510IU/ml for each). Further it was noted that cellulase production was inversely proportional to further increase in NaCl concentration. The growth of both the strains in NB and EPM was inversely proportional to increase in NaCl concentration above 10mM.

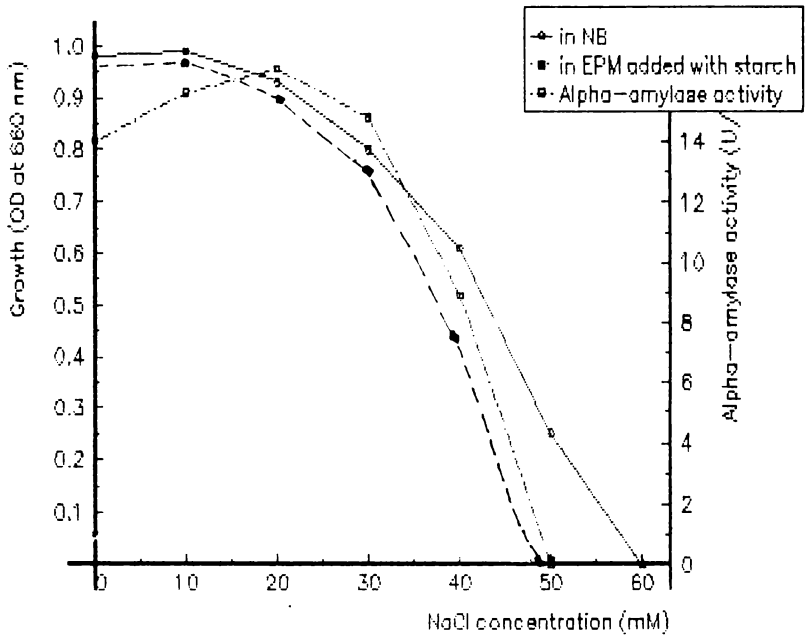


Fig.8 a Optimisation of NaCl Concentration for Growth and Enzyme production by *B.subtilis* CBTK 106

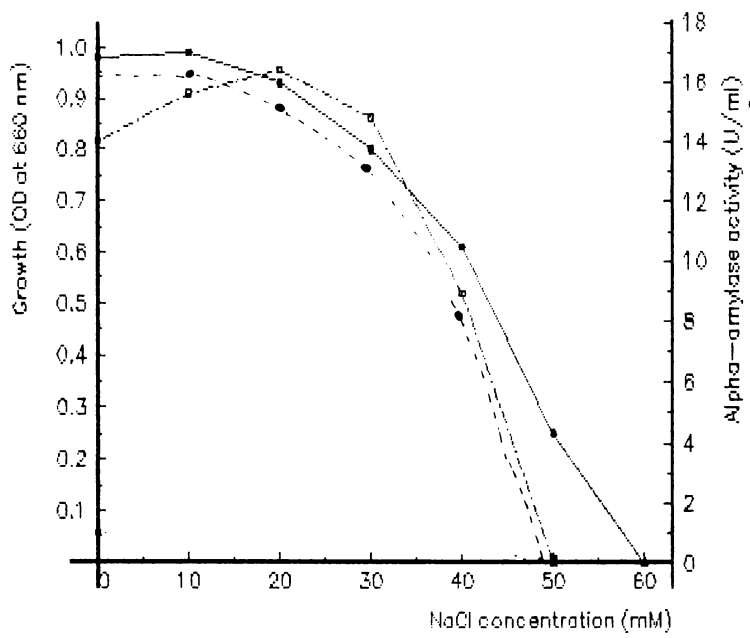


Fig.8 b Optimisation of NaCl Concentration for Growth and Enzyme production by *A.caviae* CBTK 185

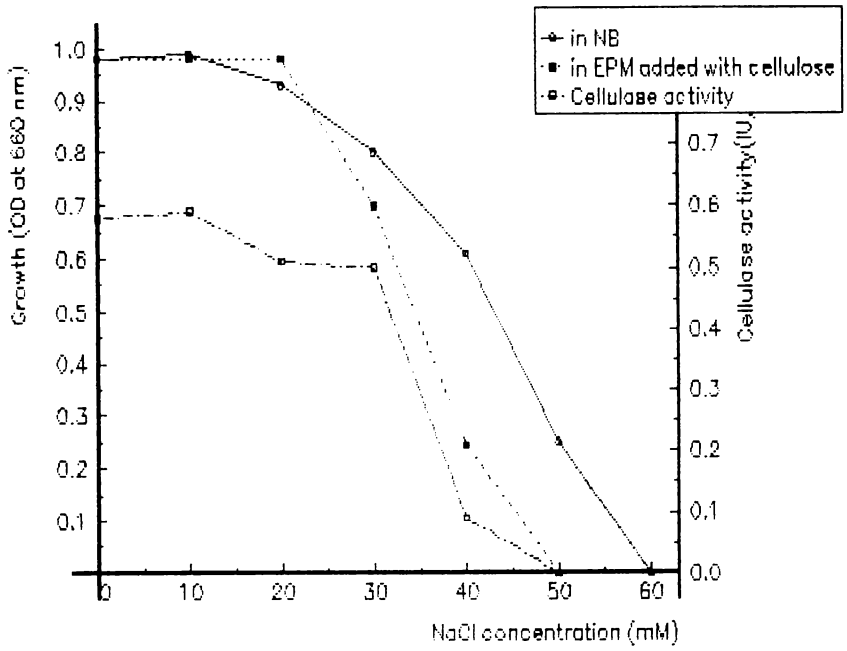


Fig.9 a Optimisation of NaCl Concentration for Growth and Enzyme production by *B.subtilis* CBTK 106

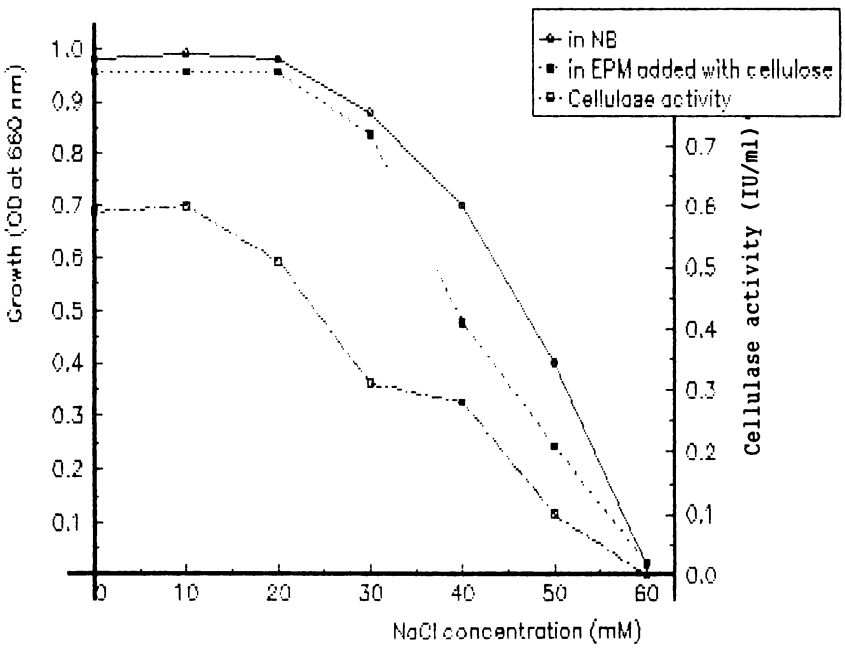


Fig.9 b Optimisation of NaCl Concentration for Growth and Enzyme production by *A.caviae* CBTK 185

3.5.5 Carbon sources

In general all additional carbon sources tested supported significant levels of enzyme production (Table 11 a & b). Among the various carbon sources tested, maltose at 1 mM level was found to be the most effective inducer for α-amylase production for both the strains even when there was low level of growth. Whereas cellulase production by both the strains, was observed to be enhanced with the addition of glucose/galactose (1 mM) into the medium. However, concentrations of above 1 mM level were inhibitory in their impact for enzyme production. An inverse relationship was observed between increase in concentration, of these carbon sources above their optimal level and enzyme production by the strains. At 1 mM concentration sucrose and lactose favoured enhanced α-amylase production by both the strains. A linear relationship between growth rate and increase in concentration of carbon sources, up to 7mM, in EPM was also recorded for both the strains . Whereas in the case of NB, concentration of carbon sources above 3 mM led to decline in growth.

Table 11 a.

Optimisation of carbon sources for growth and enzyme production by *B.subtilis* CBTK 106

Carbon sources concn. (mM)	Growth in NB (OD)	Growth in EPM + starch (OD)	α-amylase activity (U/ml)	Growth in EPM + Cellulose (OD)	Cellulase activity (IU/ml)
Glucose					
0	0.912	0.978	15.6	0.842	0.589
1	0.980	0.998	18.8	1.000	0.738
3	0.982	1.120	17.5	1.210	0.698
5	0.810	1.180	16.8	1.180	0.512
7	0.680	1.194	13.8	1.168	0.102
9	0.418	1.009	12.0	1.166	0.092
Galactose					
0	0.910	0.978	15.8	0.842	0.588
1	0.924	0.980	18.8	1.998	0.628
3	0.912	1.999	16.4	1.010	0.618
5	0.898	1.060	14.9	1.104	0.610
7	0.818	1.060	12.3	1.108	0.128
9	0.800	1.014	10.4	1.112	0.090

contd..

Sucrose					
0	0.912	0.982	15.6	0.842	0.589
1	0.928	0.999	19.8	0.998	0.710
3	0.940	1.010	16.8	1.024	0.648
5	0.896	1.090	13.0	1.100	0.656
7	0.784	1.114	12.0	1.114	0.598
9	0.720	1.010	7.8	1.084	0.420
Maltose					
0	0.910	0.980	15.6	0.842	0.590
1	0.946	0.998	22.8	1.099	0.698
3	0.968	1.060	18.4	1.110	0.640
5	0.940	1.120	14.0	1.080	0.610
7	0.728	1.164	10.4	1.040	0.410
9	0.620	1.998	9.2	1.008	0.390
Lactose					
0	0.912	0.978	15.6	0.842	0.590
1	0.946	1.082	17.8	1.984	0.616
3	0.978	1.104	16.9	1.000	0.600
5	0.910	1.110	10.0	1.112	0.580
7	0.890	1.218	8.9	1.210	0.470
9	0.889	1.240	5.6	1.240	0.320

Table 11 b.

Effect of carbon sources on growth and enzyme production by *A.caviae* CBTK 185

Carbon sources concn. (mM)	Growth in NB (OD)	Growth in EPM + starch (OD)	α-amylase activity (u/ml)	Growth in EPM + Cellulose (OD)	Cellulase activity (IU/ml)
Glucose					
0	0.980	0.896	16.8	0.820	0.598
1	0.984	1.100	18.8	1.100	0.689
3	0.998	1.218	17.2	1.218	0.668
5	0.964	1.300	14.0	1.110	0.412
7	0.940	1.410	11.4	0.998	0.364
9	0.860	1.018	9.3	0.950	0.280
Galactose					
0	0.982	0.894	16.4	0.822	0.598
1	0.986	1.018	18.8	0.868	0.660
3	0.989	1.110	17.2	1.084	0.646
5	0.918	1.140	12.4	1.090	0.410
7	0.910	1.160	8.0	1.008	0.198
9	0.820	0.978	6.0	0.940	0.170

contd..

Sucrose					
0	0.980	0.890	16.8	0.814	0.590
1	0.990	1.000	18.6	0.998	0.680
3	0.998	1.010	17.2	1.210	0.646
5	0.918	1.024	12.4	1.118	0.468
7	0.682	1.110	10.5	1.014	0.280
9	0.520	0.998	8.0	1.009	0.180
Maltose					
0	0.982	0.894	16.8	0.816	0.598
1	0.986	0.998	22.8	1.016	0.656
3	0.998	1.090	17.4	1.118	0.614
5	0.718	1.118	14.0	1.014	0.410
7	0.614	1.124	10.2	0.994	0.310
9	0.500	1.010	8.8	0.982	0.270
Lactose					
0	0.952	0.898	16.8	0.828	0.592
1	0.986	0.990	18.2	0.984	0.630
3	0.998	1.118	17.9	1.000	0.610
5	0.896	1.120	10.4	1.094	0.502
7	0.889	1.148	9.8	1.110	0.480
9	0.828	0.916	8.0	0.998	0.310

Cellulase production by both the species, was enhanced with the addition of glucose (1mM) into the medium. However, concentration of glucose above the 1mM level were inhibitory in their impact. A similar increase in cellulase production was observed with both the strains at 1mM galactose, 1mM each of sucrose, maltose and lactose. However, further increase in concentration led to decline in enzyme production to a significant extent.

3.5.6 Nitrogen Sources

Data presented in Table 12 a & b testify that both the strains could record significant levels of growth and enzyme production in the presence of all the additional, nitrogen sources, at 1% level in the medium. However, further increase in their concentration demonstrated an inhibitory effect on growth and enzyme production.

While *A.caviae*, CBTK 185 preferred 1% beef extract for enhanced α-amylase production *B.subtilis*, CBTK 106 required 1% (7mM) $(NH_4)_2 SO_4$ in the medium for maximal α-amylase production. Nevertheless other nitrogen sources

Table 12 a.

Effect of nitogen sources for growth and enzyme production by *B.subtilis* CBTK 106

Concn. (% w/w)	Growth in NB (OD)	Growth in EPM + Starch	α-amylase activity (U/ml)	Growth in EPM + Cellulase	Cellulase activity (IU/ml)
Beef extract					
0	0.996	0.960	15.6	0.842	0.589
0.5	0.962	1.058	18.2	0.998	0.642
1.0	0.989	1.120	22.4	1.000	0.700
1.5	0.990	1.080	18.4	1.100	0.680
2.0	0.940	1.000	16.8	0.998	0.598
2.5	0.930	0.998	14.2	0.990	0.516
3.0	0.928	0.996	12.0	0.980	0.406
Yeast Extract					
0	0.989	0.982	15.8	0.840	0.590
0.5	0.938	0.986	18.9	0.990	0.632
1.0	0.972	0.996	22.6	1.014	0.704
1.5	0.960	1.090	12.4	0.992	0.680
2.0	0.840	0.914	10.2	0.980	0.510
2.5	0.812	0.840	8.9	0.964	0.460
3.0	0.768	0.610	7.2	0.930	0.400

contd..

Peptone

0	0.912	0.980	15.9	0.842	0.590
0.5	0.942	0.987	18.4	0.976	0.620
1.0	0.964	1.100	23.0	0.989	0.632
1.5	0.879	1.018	18.4	0.964	0.600
2.0	0.812	0.998	14.8	0.940	0.546
2.5	0.796	0.910	10.0	0.910	0.305
3.0	0.718	0.876	6.4	0.810	0.214

$(NH_4)_2SO_4$

0	0.913	0.982	15.6	0.840	0.589
0.5	0.948	1.080	18.0	1.090	0.696
1.0	0.972	1.320	24.2	1.118	0.720
1.5	0.914	1.100	13.0	0.994	0.640
2.0	0.910	0.990	12.6	0.980	0.600
2.5	0.816	0.910	10.0	0.960	0.510
3.0	0.730	0.814	6.2	0.890	0.318

contd..

$NaNO_3$

0	0.914	0.980	15.8	0.840	0.589
0.5	0.979	0.992	18.4	0.990	0.628
1.0	0.982	1.100	20.8	1.014	0.704
1.5	0.918	1.000	12.4	0.998	0.626
2.0	0.884	0.942	8.6	0.980	0.586
2.5	0.840	0.860	7.4	0.964	0.510
3.0	0.730	0.714	6.0	0.900	0.486

KNO_3

0	0.918	0.982	15.9	0.842	0.589
0.5	0.948	1.019	18.4	0.998	0.660
1.0	0.989	1.114	21.8	1.000	0.700
1.5	0.898	0.940	11.8	0.942	0.610
2.0	0.714	0.818	9.8	0.868	0.560
2.5	0.668	0.760	7.4	0.848	0.480
3.0	0.514	0.720	4.6	0.810	0.386

Table 12 b.

Effect of nitogen sources for growth and enzyme production by *A.caviae* CBTK 185

Nitrogen Sources

Concn. (%,w/w)	Growth in NB (OD)	Growth in EPM + Starch	α-amylase activity (U/ml)	Growth in EPM + Cellulase	Cellulase activity (IU/ml)
Beef extract					
0	0.978	0.898	16.8	0.820	0.598
0.5	0.980	1.210	20.2	0.868	0.610
1.0	0.990	1.418	26.4	0.918	0.620
1.5	0.918	1.200	18.0	0.840	0.420
2.0	0.824	1.000	16.8	0.824	0.400
2.5	0.748	0.994	12.1	0.810	0.380
3.0	0.724	0.968	8.9	0.800	0.240
Yeast Extract					
0	0.976	0.898	16.6	0.826	0.598
0.5	0.982	1.100	21.6	0.900	0.621
1.0	0.992	1.210	25.8	0.920	0.630
1.5	0.884	1.100	20.1	0.906	0.498
2.0	0.780	0.994	16.9	0.890	0.450
2.5	0.642	0.960	9.4	0.878	0.400
3.0	0.519	0.918	6.2	0.870	0.390

contd..

Peptone

0	0.972	0.898	16.8	0.820	0.598
0.5	0.980	1.018	18.6	0.868	0.614
1.0	0.998	1.100	22.4	0.896	0.628
1.5	0.890	0.994	18.0	0.810	0.410
2.0	0.718	0.890	15.2	0.780	0.389
2.5	0.610	0.844	10.8	0.760	0.290
3.0	0.509	0.820	8.2	0.728	0.200

$(NH_4)_2SO_4$

0	0.978	0.910	16.6	0.826	0.598
0.5	0.982	1.009	18.2	0.920	0.618
1.0	0.990	1.188	24.8	0.941	0.632
1.5	0.890	1.078	18.0	0.900	0.504
2.0	0.828	0.946	16.2	0.896	0.476
2.5	0.798	0.880	10.4	0.820	0.410
3.0	0.629	0.720	8.2	0.810	0.380

contd..

$NaNO_3$

0	0.980	0.898	16.8	0.826	0.599
0.5	0.988	0.089	20.6	0.920	0.620
1.0	0.992	1.108	21.8	0.948	0.616
1.5	0.926	0.989	12.8	0.860	0.510
2.0	0.880	0.960	10.9	0.840	0.410
2.5	0.718	0.810	7.8	0.760	0.318
3.0	0.696	0.780	5.6	0.710	0.120

KNO_3

0	0.978	0.910	16.8	0.830	0.592
0.5	0.989	1.060	20.6	0.924	0.610
1.0	0.986	1.189	22.0	0.941	0.625
1.5	0.828	0.980	12.9	0.930	0.510
2.0	0.620	0.840	10.4	0.928	0.480
2.5	0.510	0.810	7.4	0.820	0.310
3.0	0.428	0.800	4.9	0.760	0.260

were observed to enhance the α-amylase production, to a significant level, by both the strains.

Both the species of B.*subtilis*, CBTK 106 and *A.caviae*, CBTK 185 recorded relatively higher levels of cellulase with the addition of 1% $(NH_4)_2SO_4$ into the medium. Similarly, other nitrogen sources, at 1% level, were also enhanced cellulase production to a significant level. Further increase in concentration of nitrogen sources above 1% showed inhibitory effect on growth rate and enzyme production.

3.5.7 Inoculum concentration

Though both the strains exhibited significant levels of growth and enzyme production at inoculum concentration ranging from 0.5-2.0% v/v, maximal growth and enzyme production was recorded at 1% level (Fig.10 a & b and Fig.11 a & b). While enzyme production by both the strains declined along with further increase in inoculum concentration, the growth rate exhibited a linear increase, thus recording the maximal growth at 3% inoculum concentration for both the strains.

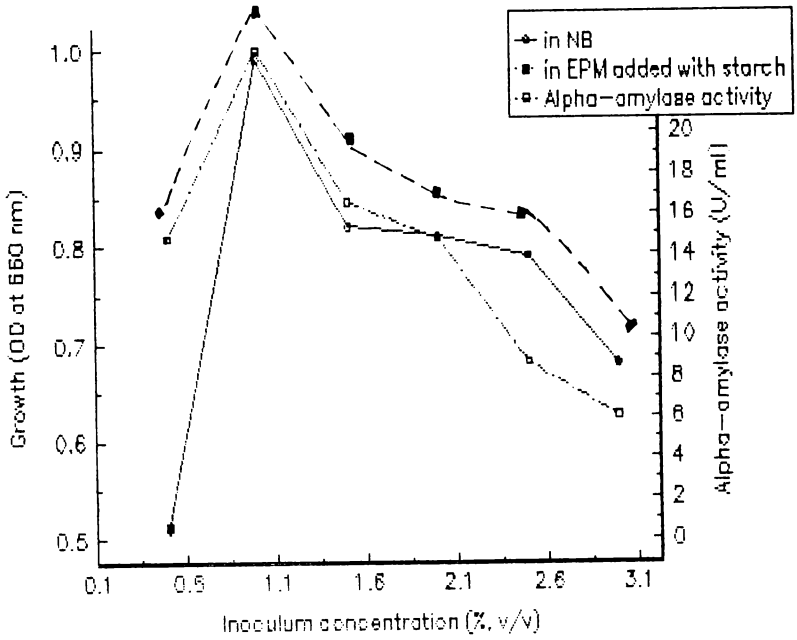


Fig.10 a Optimisation of Inoculum Concentration for Growth and Enzyme production by *B.subtilis* CBTK 106

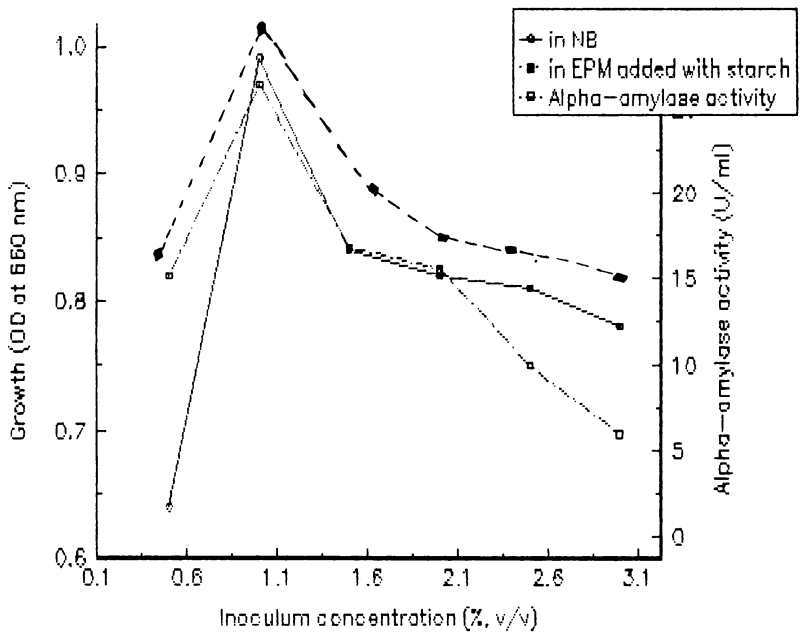


Fig.10 b Optimisation of Inoculum Concentration for Growth and Enzyme production by *A.caviae* CBTK 185

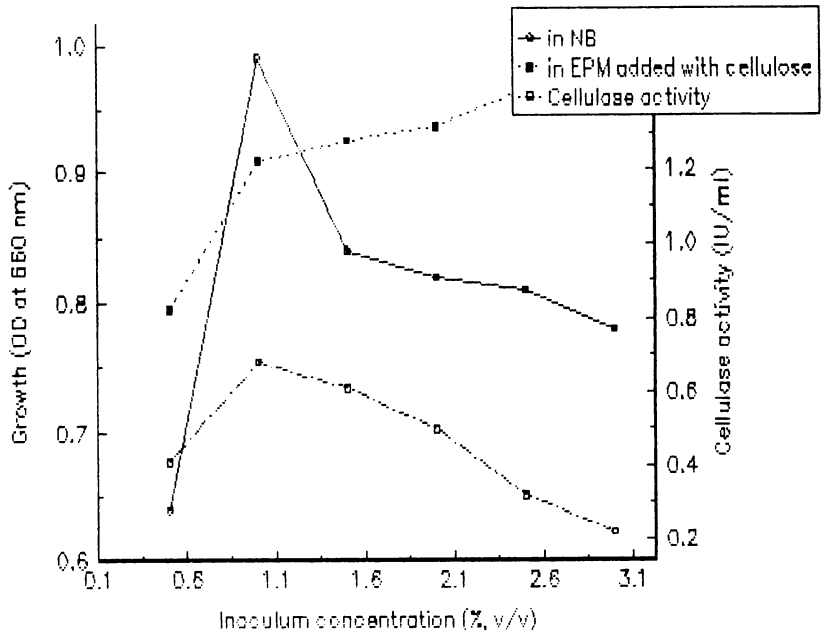


Fig.11 a Optimisation of Inoculum Concentration for Growth and Enzyme production by *B.subtilis* CBTK 106

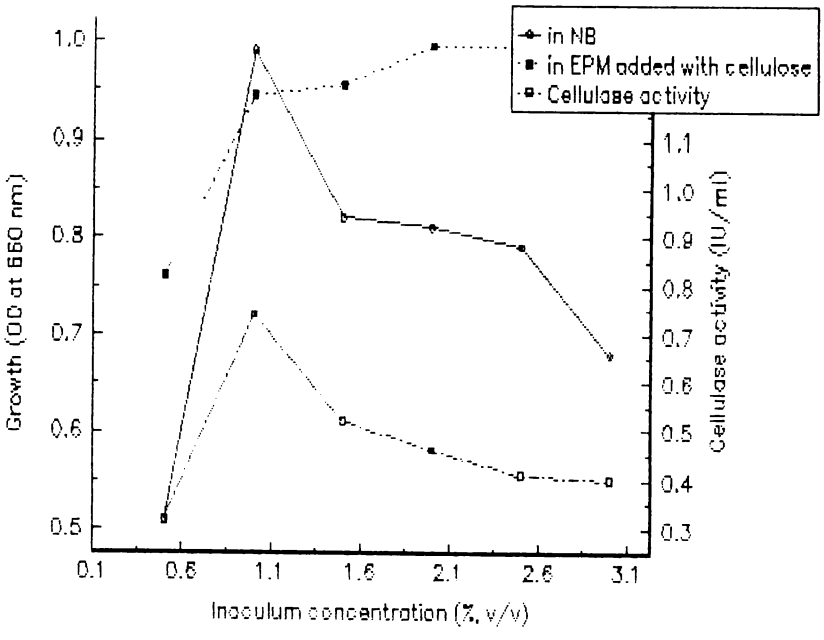


Fig.11 b Optimisation of Inoculum Concentration for Growth and Enzyme production by *A.caviae* CBTK 185

Result showed that *A.caviae* CBTK 185 produced comparatively high α-amylase at 1% (v/v) inoculum concentration than B.*subtilis* CBTK 106. Whereas, cellulase production was observed to be high with *B.subtilis* CBTK 106 than *A.caviae* CBTK 185. Both the strains exhibited significant level of enzyme production with (0.5-2.0%, v/v), inoculum concentration.

3.5.8 Incubation time

From the results presented in Fig.12 a & b and Fig.13 a & b, it is evident that both the strains could record maximal growth and enzyme production respectively at 24 hrs for α-amylase and for cellulase.

A.caviae, CBTK 185 produced comparatively higher α-amylase activity than B.*subtilis*, CBTK 106 after 24 hrs. Both the strains recorded significant levels of enzyme over a wide range of incubation period varying from 12 to 60 hrs. Further increase in incubation period led to a decrease in the enzyme yield. However, the growth rate remained static after 24 hrs of incubation for both the strains. *A.caviae* CBTK 185

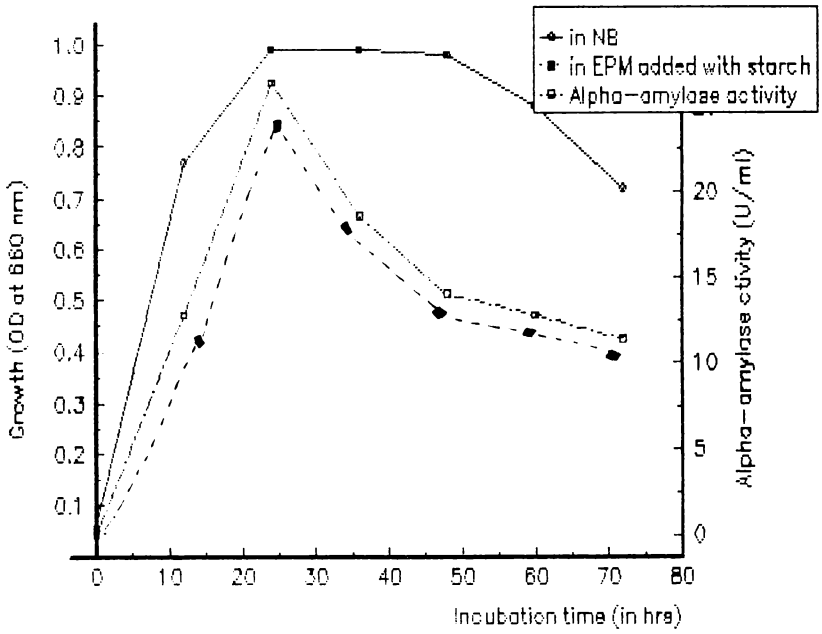


Fig.12 a Optimisation of Incubation time for Growth and Enzyme production by *B.subtilis* CBTK 106

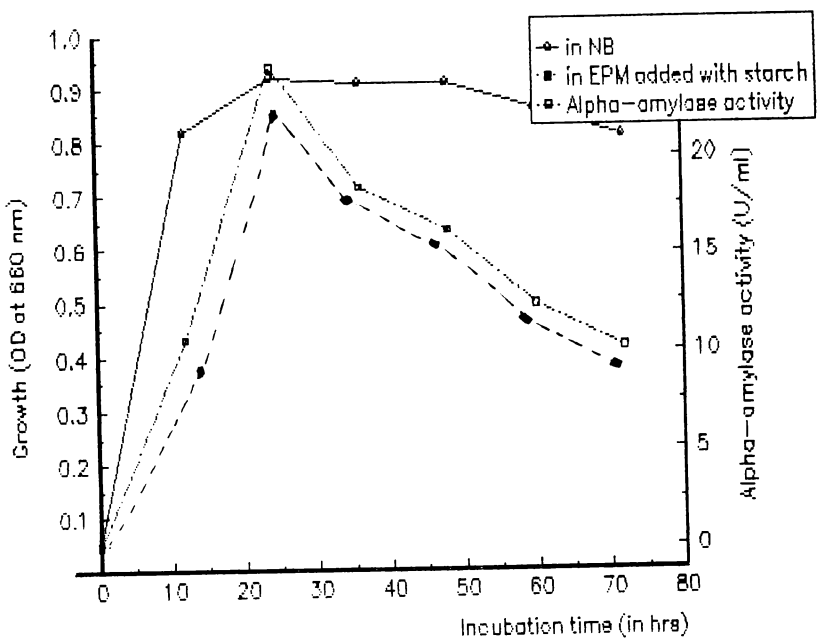


Fig.12 b Optimisation of Incubation time for Growth and Enzyme production by *A.caviae* CBTK 185

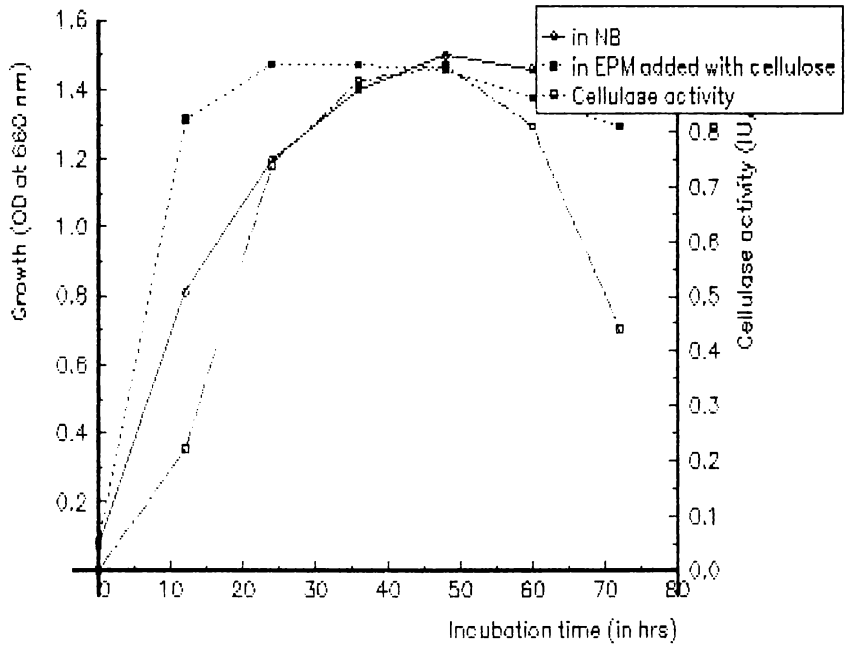


Fig 13 a Optimisation of Incubation time for Growth and Enzyme production by *B.subtilis* CBTK 106

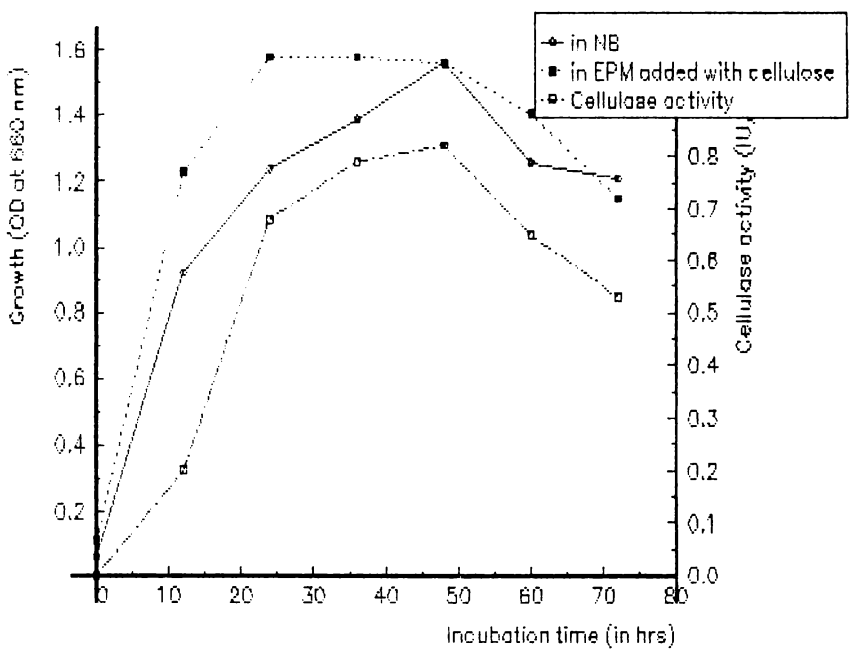


Fig 13 b Optimisation of Incubation time for Growth and Enzyme production by *A.caviae* CBTK 185

recorded high α-amylase activity than *B.subtilis* CBTK 106 at 24 hrs incubation period. Whereas *B.subtilis*, CBTK 106 recorded high cellulase activity than *A.caviae* CBTK 185 at 48 hrs incubation period. Further increase in incubation period did not favour either growth nor cellulase production.

3.5.9 Growth curve

Growth studies indicates that both the strains entered the exponential phase and extended over 2.5-10 hrs of incubation in NB and EPM (Fig 14 a & b). For *A.caviae* CBTK 185 the exponential phase was upto 12 hrs. The generation time for *B.subtilis* CBTK 106 was calculated to be 57.6 min. in NB and 60 min. in EPM added with soluble starch and 66 min. in EPM added with cellulose as substrate. The specific growth rate was calculated in NB media as 0.72. Whereas, the generation time for *A.caviae* CBTK 185 was 46.2 min. in NB and 72 minutes in EPM added with substrate (soluble starch/cellulose) and specific growth rate was determined to be 0.9 in NB media.

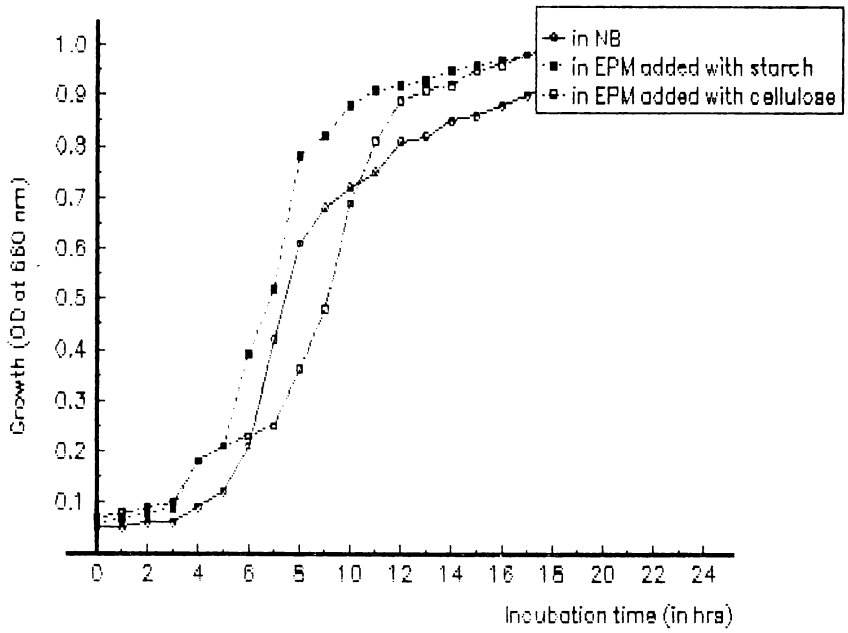


Fig.14 a Growth Curve of *B.subtilis* CBTK 106 in Nutrient broth (NB) and in Enzyme production Medium (EPM)

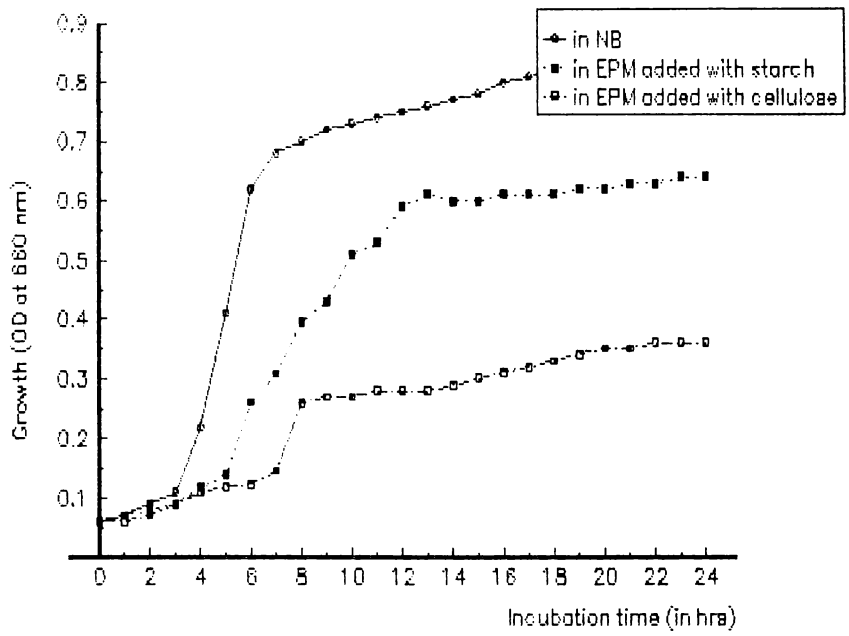


Fig.14 b Growth Curve of *A.caviae* CBTK 185 in Nutrient broth (NB) and in Enzyme production Medium (EPM)

3.6 Production of α-amylase and cellulase employing SSF by Bacteria

A bioprocess for production of α-amylase and cellulase using both *B.subtilis* (CBTK 106) and *A.caviae* (CBTK 185) employing Solid State Fermentation (SSF) was developed with Banana Fruit Stalk (BFS) as solid substrate. The strategy included selection of an ideal method of pretreatment of the substrate, and optimisation of process parameters that effect the bioprocess and identify an ideal process for extraction and recovery of the enzyme. Details of the results are presented below.

3.6.1 Pretreatment of substrate on exoenzymes production under SSF

Impact of pretreatment of substrate on enzyme production during SSF by bacteria was studied by subjecting the banana fruit stalk to steaming for various periods and with alkali and acid treatment.

3.6.1.1 Effect of steaming

From the results presented in Table 13 a & b, it is inferred that cooking the substrate at 121°C for 60 min favoured maximal enzyme production during SSF. Further increase in cooking time did not enhance the enzyme yield. It was observed that at low temperatures of cooking extended time was required for maximal enzyme production. Whereas in contrast at high temperature a short time was adequate. Enzyme production increased in a linear fashion along with the steaming time at cooking temperatures of 100 and 110°C. Whereas contrastingly, at 121°C the enzyme yield decreased for cooking periods above 60 min inspite of significant levels of production.

B.*subtilis*, CBTK 106 produced comparatively higher α-amylase, than *A.caviae*, CBTK 185 when the subtrate was cooked for 121°C for 60 min. A similar trend was also noticed with other steaming periods and temperatures for the strains.

Cellulase production by *B.subtilis* was high compared to *A.caviae* at 60 min steaming at 121°C. Interestingly,

Table 13 a.

Effect of steaming of the substrate on α-amylase production by *B.subtilis* CBTK 106 and *A. caviae* CBTK 185

Time in (min.)	α-amylase activity (U/gds)					
	100°C		110°C		121°C	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK185
15	10,100	6400	12,600	8800	24,500	10.140
30	14,600	8500	18,600	10,600	32,200	28,600
45	18,100	10,160	24,500	18,600	36,400	30,600
60	20,400	14,450	26,200	22,460	38,750	32,800
75	24,600	16,180	28,100	28,400	34,500	30,400
90	28,400	18,100	30,200	30,680	30,100	32,600
105	30,100	20,500	32,800	34,800	24,500	28,200
120	32,600	22,400	34,100	32,200	20,100	20,100

Table 13 b.

Effect of steaming of the substrate on cellulase production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

Time in (min.)	cellulase activity (IU/gds)					
	100°C		110°C		121°C	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185
15	0.00	0.00.	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00
45	0.02	0.005	0.05	0.08	0.64	0.52
60	0.08	0.05	0.14	0.18	0.72	0.78
75	0.10	0.14	0.22	0.28	0.98	0.96
90	0.14	0.18	0.44	0.40	0.98	0.96
105	0.20	0.24	0.58	0.56	0.98	0.96
120	0.24	0.28	0.68	0.62	0.98	0.96

further enhancement in cooking time at 121°C did not effect the cellulase production.

3.6.1.2 Effect of alkali treatment

Alkali treatment of the subtrate, with NaOH and NH_4OH, do not favour α-amylase production (Table 14 a & b). α-Amylase titres recorded a progressive decline along with increase in concentration from 0 to 10M, irrespective of the soaking time and the bacteria tested.

Cellulase production by bacteria during SSF was observed to be not affected by alkali treatment of the substrate, irrespective of increase in concentration as well as soaking period.

3.6.1.3 Effect of acid treatment

Table 15 a & b indicates that the acid treatment of substrates affect α-amylase production to a significant level along with increase in concentration and soaking time, for both the bacteria. Whereas cellulase production by bacteria

Table 14.a.

Effect of alkali treatment of the substrate (BFS) on α-amylase production (U/gds) by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

CBTK 106 - α-amylase activity (U/gds)									
NaOH Molar cocn.	Soaking time (in hrs)				NH_4OH Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	23650	23650	23650	23650	0	23650	23650	23650	23650
1	14200	12120	10900	9200	1	18110	16200	12100	10180
2	12600	10400	9800	8400	2	12480	10100	9800	8400
4	10000	9400	8100	6700	4	10060	8800	8000	7200
6	9800	8600	7400	5400	6	8600	6400	6200	6000
8	9100	7800	6600	4100	8	6400	4200	3800	3200
10	8200	6600	4200	2880	10	4860	2800	2600	2400

contd..

CBTK 185 - α-amylase activity (U/gds)									
NaOH Molar cocn.	Soaking time (in hrs)				NH_4OH Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	22500	22500	22500	22500	0	22500	22500	22500	22500
1	16800	14200	13800	12100	1	14800	13800	12600	10800
2	14200	12800	10800	9800	2	12180	12000	10100	8600
4	12100	11280	10400	8400	4	10400	9800	8400	6200
6	11000	10100	9200	7800	6	9800	8100	8100	4200
8	10000	9800	8600	6900	8	8400	7400	7000	3800
10	9800	8400	7800	5400	10	6200	5400	2800	2100

Table 14 b.

Effect of alkali treatment of the substrate on cellulase production

by *B.subtilis* CBTK 106 and *A.caviae*, CBTK 185 under SSF

CBTK 106 - cellulase activity (IU/gds)									
NaOH Molar concn.	Soaking time (in hrs)				NH_4OH Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	0.98	0.98	0.98	0.98	0	0.98	0.98	0.98	0.98
1	1.04	0.99	0.99	1.02	1	0.99	0.99	0.99	0.99
2	1.09	0.99	1.01	1.01	2	1.04	0.99	0.98	0.98
4	1.04	1.02	1.02	1.01	4	1.04	0.99	0.98	0.96
6	1.04	1.01	1.02	1.00	6	1.02	0.98	0.98	0.96
8	1.04	1.01	1.02	0.99	8	1.00	0.98	0.98	0.96
10	1.04	1.01	1.02	0.99	10	1.00	0.98	0.98	0.96

contd..

CBTK 185 - cellulase activity (IU/gds)									
NaOH Molar concn.	Soaking time (in hrs)				NH_4OH Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	0.96	0.96	0.96	0.96	0	0.96	0.96	0.96	0.96
1	0.98	0.99	1.02	1.02	1	0.98	0.99	0.99	0.98
2	1.02	1.04	1.02	0.98	2	0.99	1.02	1.02	0.99
4	1.04	1.02	0.99	0.98	4	1.02	1.04	1.02	1.02
6	1.02	0.99	0.98	0.98	6	1.04	1.02	1.00	1.00
8	0.99	0.98	0.98	0.96	8	1.04	1.01	0.99	0.99
10	0.99	0.98	0.98	0.96	10	1.02	0.99	0.98	0.98

Table 15.a.

Effect of acid Treatment of the substrate (BFS) on α-amylase production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

CBTK 106 - α-amylase activity (U/gds)									
HCL Molar concn.	Soaking time (in hrs)				H_2SO_4 Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	23650	23650	23650	23650	0	23650	23650	23650	23650
1	18400	16200	14200	12680	1	18110	16100	12100	10160
2	16800	14100	12100	10100	2	16420	14100	10100	9800
4	12000	9800	9000	8600	4	12140	10100	8600	8000
6	8600	7800	7200	7000	6	7800	6400	6000	5400
8	7800	6400	6200	6000	8	6100	4200	4100	8400
10	6400	5400	5000	4400	10	5800	3200	3000	2400

contd..

CBTK 185 - α-amylase activity (U/gds)									
HCl Molar concn.	Soaking time (in hrs)				H_2So_4 Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	22500	22500	22500	22500	0	22500	22500	22500	22500
1	16400	14200	12400	10800	1	16200	14200	12100	10180
2	14100	12100	10400	8800	2	14000	12100	10400	8600
4	12000	10000	8400	7600	4	12100	10400	9800	6900
6	10000	8600	7800	7000	6	9800	8600	8000	5400
8	8600	6400	4800	4600	8	8600	7200	6400	4800
10	6400	4800	2900	2800	10	6400	6000	5200	3100

Table 15 b.

Effect of acid Treatment of the substrate on cellulase production by *B.subtilis* CBTK 106 and *A.caviae*, CBTK 185 under SSF

CBTK 106 - cellulase activity (IU/gds)									
HCl Molar concn.	Soaking time (in hrs)				H_2SO_4 Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	0.98	0.98	0.98	0.98	0	0.98	0.98	0.98	0.98
1	0.98	0.99	1.04	1.09	1	0.99	0.99	0.99	0.99
2	1.04	1.04	1.09	1.04	2	1.04	1.02	1.08	1.02
4	1.08	1.09	0.99	1.00	4	1.09	1.08	1.04	1.04
6	1.09	1.00	0.96	0.98	6	1.08	0.99	1.02	1.02
8	1.09	0.98	0.96	0.96	8	1.08	0.98	1.00	0.98
10	1.09	0.96	0.96	0.96	10	1.08	0.98	0.99	0.98

contd..

CBTK 185 - cellulase activity (IU/gds)									
HCl Molar concn.	Soaking time (in hrs)				H_2So_4 Molar Concn.	Soaking time (in hrs)			
	6	12	18	24		6	12	18	24
0	0.96	0.96	0.96	0.96	0	0.96	0.96	0.96	0.96
1	0.98	1.04	1.08	1.04	1	0.98	0.98	0.99	0.99
2	1.02	1.08	1.04	1.00	2	1.04	0.99	1.04	0.99
4	1.04	1.00	0.99	0.98	4	1.08	1.04	1.02	1.02
6	1.08	0.99	0.98	0.96	6	1.04	1.04	1.00	1.00
8	1.04	0.98	0.96	0.96	8	1.02	1.02	1.00	0.99
10	1.02	0.98	0.96	0.94	10	1.02	1.00	0.98	0.98

was not influenced by the acid treatment irrespective of increase in concentration and soaking time as observed with alkali treatment.

3.6.2 Effect of moisture content

Results presented in Fig.15 a & b testify that an initial moisture content of 70% for *B.subtilis* CBTK 106 and 65% for *A.caviae* CBTK 185 are required in the solid substrate for maximal α-amylase production. Whereas, both the strains required 60% moisture content as optimum for maximal cellulase production.

Significant level of enzyme production was noticed at initial moisture content varying from 55-75%. Further enhancement in moisture content inversely influenced the enzyme production.

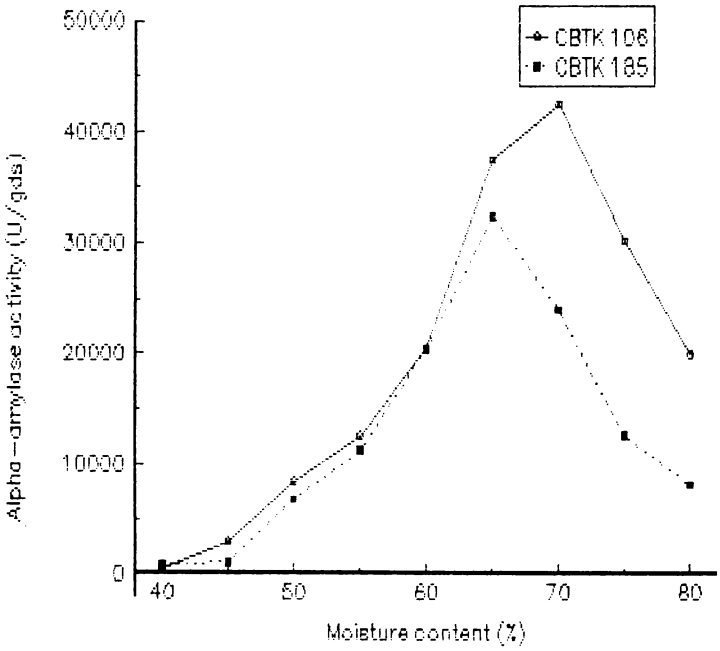
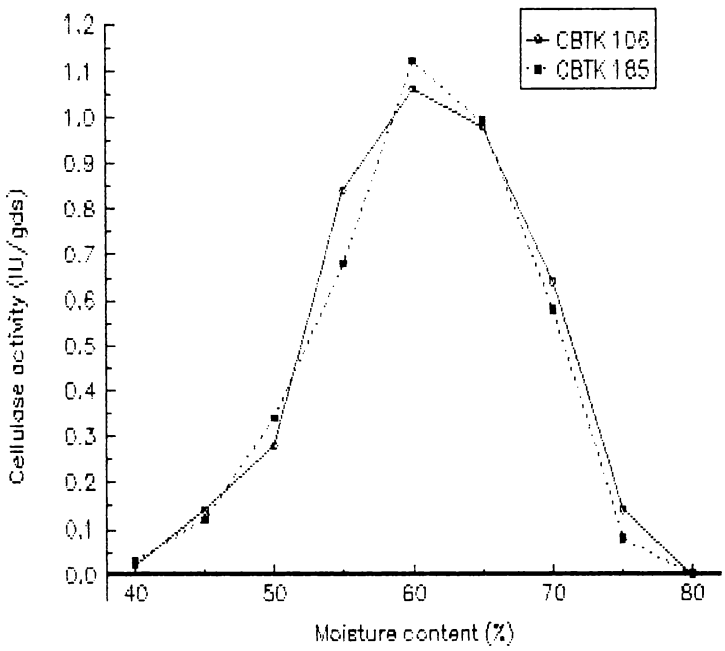


Fig.15 a&b Effect of Moisture Content of the substrate on Enzyme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF.



3.6.3 Effect of particle size

Substrate particles with a size of 400 μm supported maximal α-amylase and cellulase production(Fig 16 a & b). Further increase or decrease in particle size of the substrate led to a decline in the enzyme yield. Similarly particles with mixed size at >400 range was optimal for maximal α-amylase and cellulase production (Table 16). In this case too further increase in the range of particle slizes led to significant reduction in the enzymes yield.

3.6.4 Effect of pH

Significant levels of enzyme production, by both the strain, were observed at a wide range of pH from 6-9(Fig. 17 a & b). However, maximal α-amylase and cellulase production by both the strains were recorded to be maximal at pH 7.0. However significant level of enzyme yield was also noticed with pH 8 and 9. Cellulase production by both the strains were negligible with pH 9 and absent at pH 10.0.

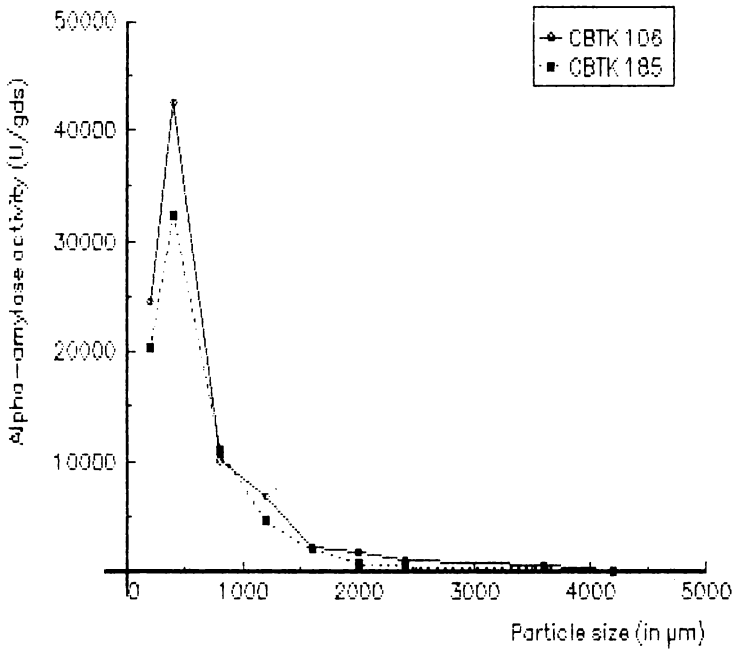


Fig 16 a&b Effect of Particle Size of the Substrate on Enzyme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF

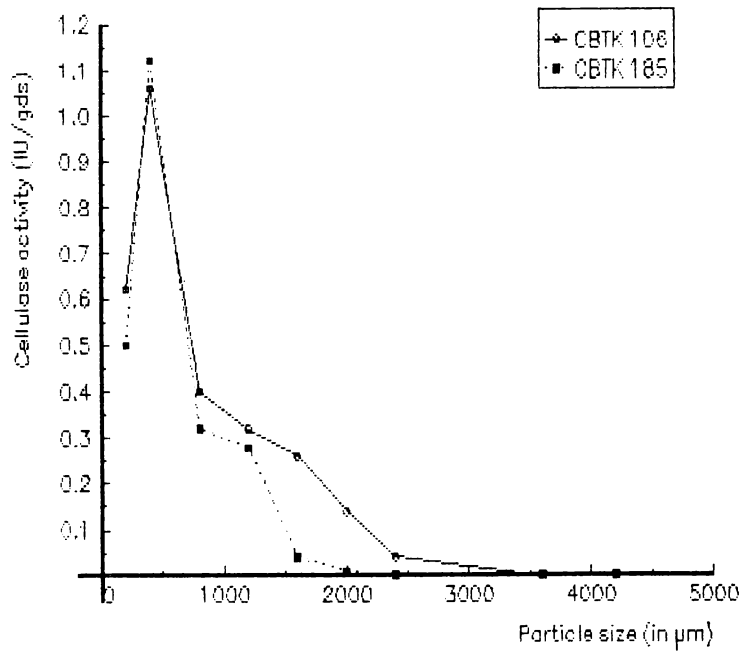


Table 16

Effect of mixed particle size of the substrate (BFS) on α-amylase and cellulase production by *B.subtilis* CBTK 106, and *A.caviae*, CBTK 185.

Mixed particle size (in μm)	α-amylase activity(U/gds)		Cellulase activity (IU/gds)	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185
> 400	14650	14860	0.60	0.49
400 -1000	7600	5600	0.48	0.42
1000-1600	3270	2200	0.30	0.14
1600-2200	1880	840	0.21	0.04
2200-2800	1010	650	0.06	0.001
2800-3200	570	340	0.004	0.00
3200-4200	420	280	0.00	0.00
< 4200	181	180	0.00	0.00

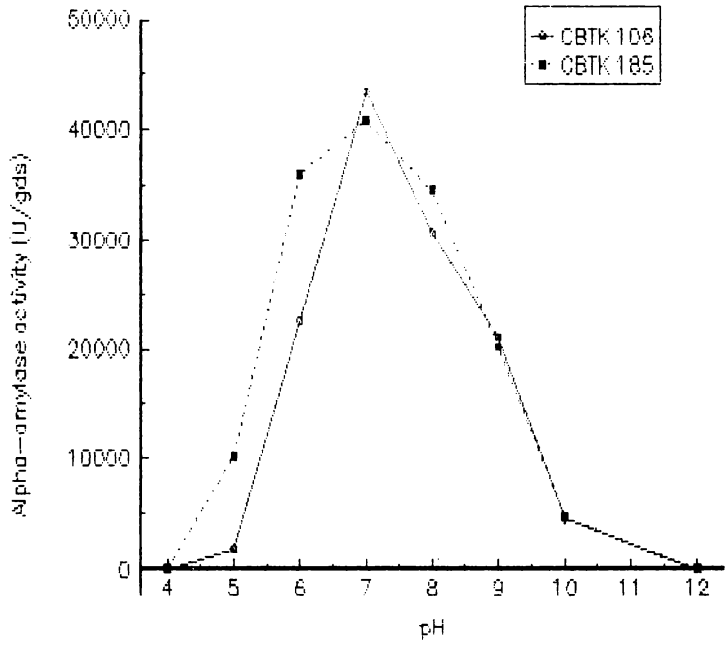
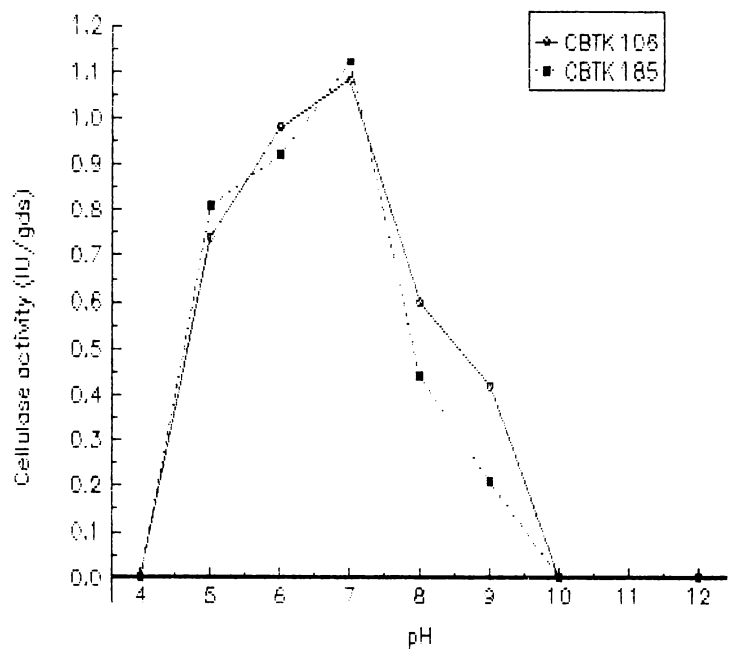


Fig.17 a&b Effect of pH of the substrate on Enyzme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF.



3.6.5 Effect of incubation temperature

Results presented in Fig.18 a & b suggest that both the strains could produce both the enzymes at significant levels at a wide range of temperature ranging from 25-45^{o}C with a maximum at 35^{o}C.

3.6.6 Effect of substrate concentration

Inclusion of additional substrate into the medium did not enhance cellulase production significantly, though α-amylase production was marginally enhanced on addition of additional substrates at low concentration (0.5%) (Fig 19 a & b). Unlike the situation in the absence of additional substrates (Fig.18 a & b), *A.caviae*, CBTK 185 recorded relatively higher levels of α-amylase when compared to *B.subtilis* (49,500 U/gds) with the addition of substrate at 0.5% concentration. In contrast *B.subtilis*, CBTK 106 yielded comparatively higher level of cellulase than *A.caviae* CBTK 185 with 0.5% substrate concentration.

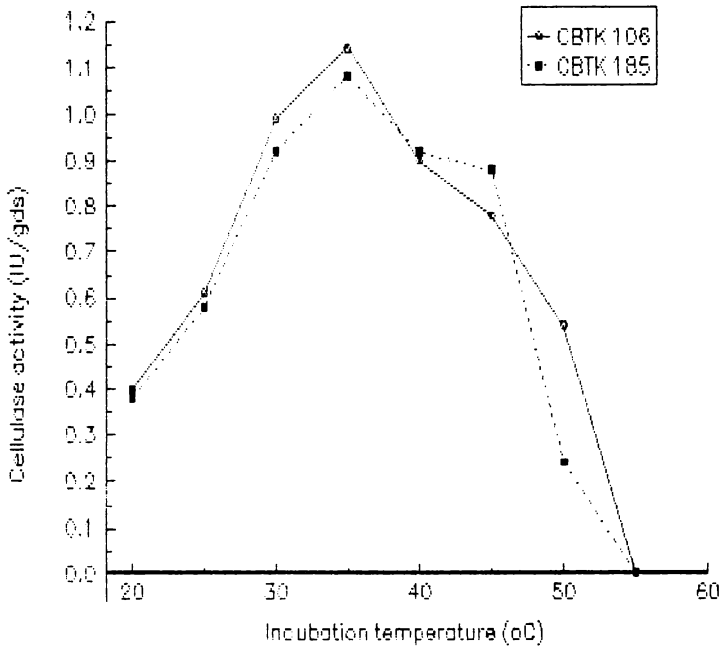
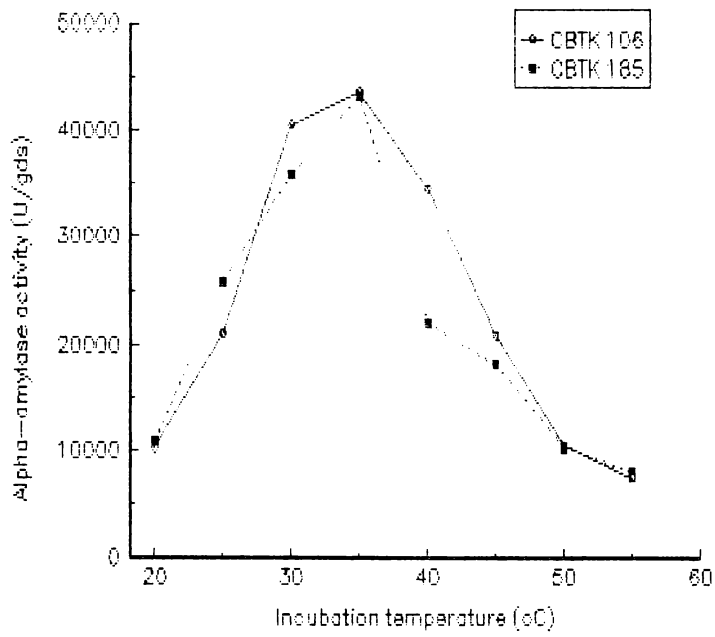


Fig.18 a&b Effect of Incubation Temperature on Enzyme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF.



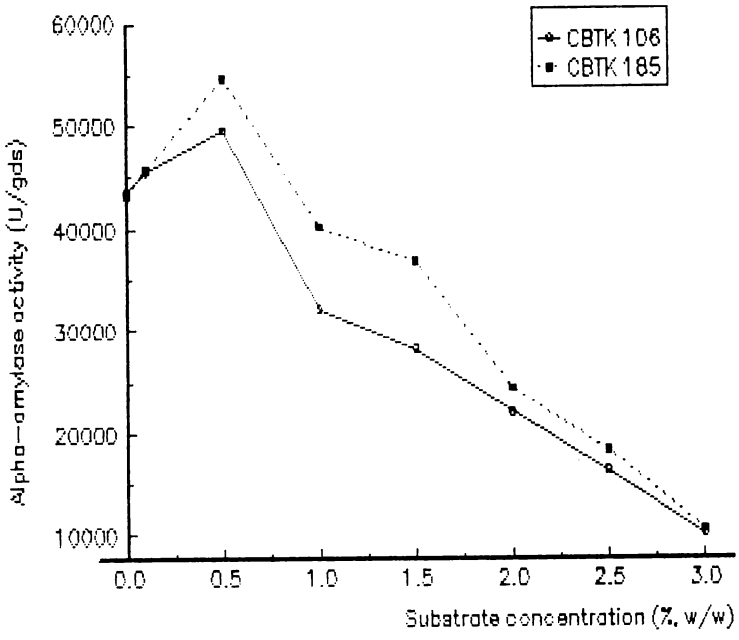
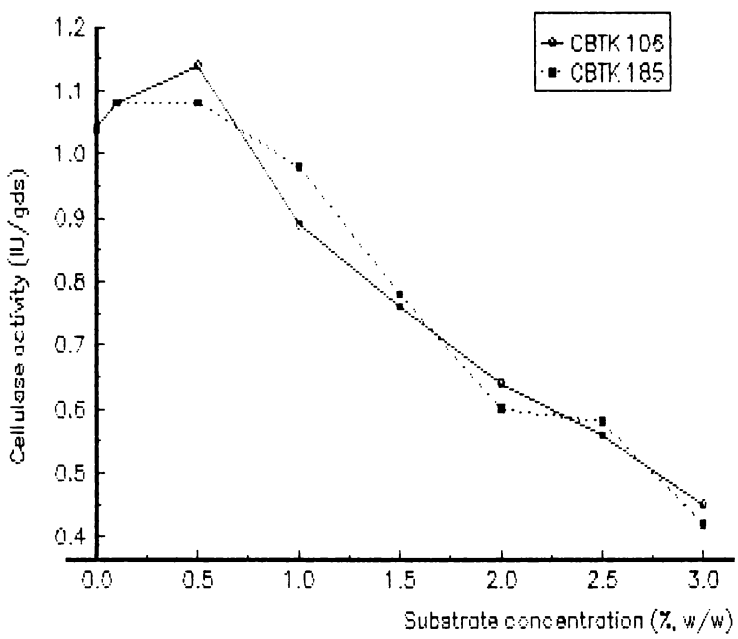


Fig.19 a&b Effect of Substrate Concentration on Enzyme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF



3.6.7 Effect of NaCl concentration

Data presented in Fig.20 a & b suggest that NaCl concentration did not favour increase in cellulase production with both the strains compared to marginal increase noticed at 20 mM NaCl concentration in the case of α-amylase. Further increase in NaCl concentration was found to be inversely proportional to enzyme yield. Whereas the optimal requirement for cellulase production was observed to be 10 mM NaCl concentration.

3.6.8 Effect of Carbon source

Addition of maltose, enhanced maximal α-amylase production by both the species. Similar effect was also noticed with glucose and sucrose. However, lactose and Na_2CO_3 were observed to have no effect on enzyme production. It was also noted that at very high concentration they were inhibitory in effect on enzyme production. *A.caviae*, CBTK 185 yielded relatively higher level of α-amylase (64,200 U/gds) compared to *B.subtilis*, CBTK 106 with the addition of 3mM maltose into the medium. Further increase in concentration

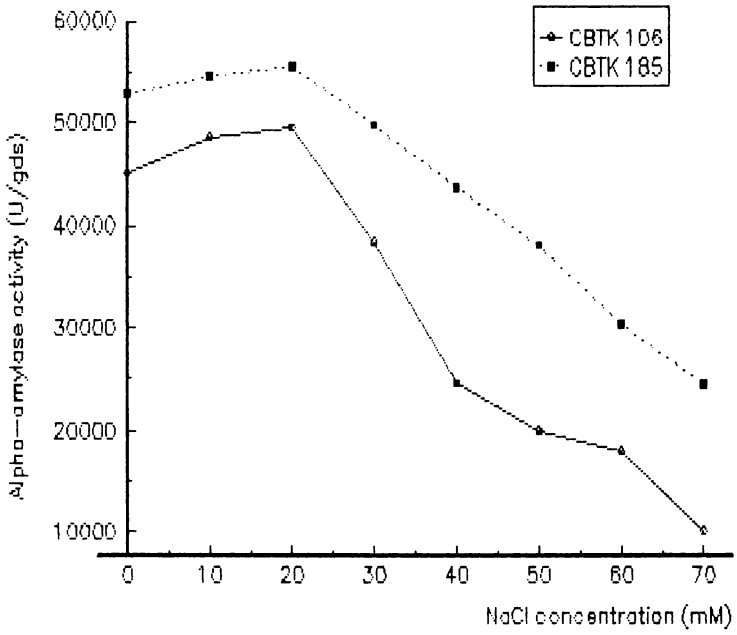
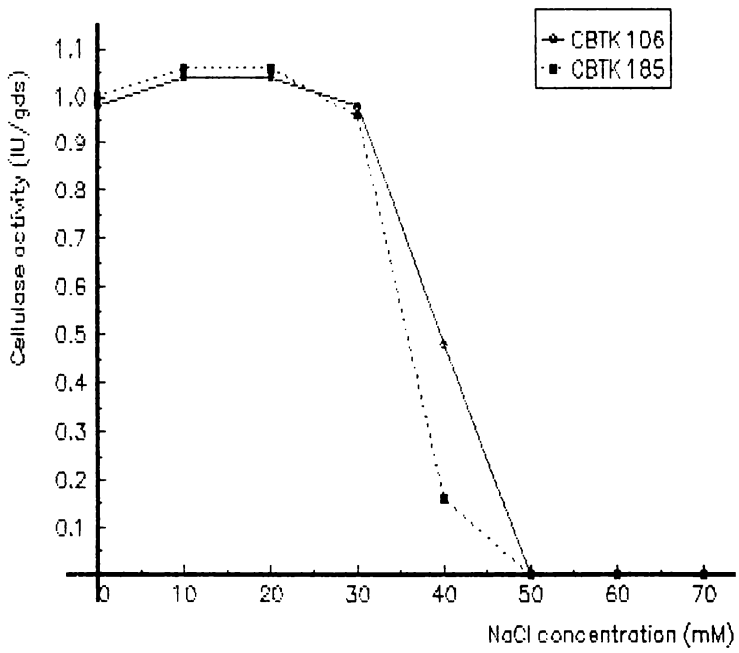


Fig.20 a&b Effect of NaCl Concentration on Enzyme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK under SSF



was found to inhibit the enzyme production. Both *A.caviae* CBTK 185 and *B.subtilis* CBTK 106 recorded maximal cellulase titres with 2mm glucose addition into the medium. Both the strain shows 26% increase in enzyme yield. Neverthless all the other carbon sources enhanced cellulase production to a significant level (Table 17 a & b).

3.6.9 Effect of Nitrogen sources

All nitrogen sources (1%) tested influenced the rate of enzyme production upto 1% level (Table 18 a & b). Further increase in concentration showed detrimental effect on enzyme yield. Maximal α-amylase was observed with 1% $(NH_4)_2SO_4$ followed by $NaNO_3$. Beef extract, yeast extract, urea and peptone.

A.caviae, CBTK 185 produced relatively higher levels of α-amylase compared to *B.subtilis*, CBTK 106 with the addition of 1% $(NH_4)_2$ SO_4 into the medium. The increase in enzyme yield obtained for *A.caviae* is 14% when compared to 5% for B.*subtilis*. Both the strains recorded maximal cellulase titres with 1% $(NH_4)_2SO_4$ added into the medium. Both the strains could record 24-26% increase in enzyme yield.

Table 17 a

Effect of Carbon Sources on ∝ - amylase production (U/gds) by B. subtilis CBTK 106 and A.caviae CBTK 185 under SSF

Concn. Milli-molar (mM)	-amylase activity (U/gds) Glucose		Galactose		Sucrose		Maltose		Lactose		Na_2CO_3	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185
0	50,400	60,200	50,400	60,200	50,400	60,200	50,400	60,200	50,400	60,200	50,400	60,200
1	50,800	60,800	51,200	61,400	51,000	60,900	51,400	60,800	50,400	60,000	50,400	60,100
3	51,400	61,400	52,400	62,200	52,600	62,500	53,400	64,200	49,200	58,000	50,200	59,000
5	52,500	62,200	52,800	62,200	52,000	62,200	53,000	64,200	42,200	52,400	50,200	59,100
7	48,700	56,400	49,800	61,000	50,800	56,400	51,400	61,000	39,400	45,600	48,200	54,100
9	45,500	50,600	47,400	60,000	48,900	52,200	48,600	58,400	32,200	40,600	42,800	48,200
12	43,100	46,400	45,600	58,400	45,600	45,100	46,500	54,600	28,500	38,600	36,400	44,100
15	38,900	42,600	43,800	54,100	42,600	40,400	44,400	50,100	22,400	32,400	32,200	38,200

Table 17 b

Effect of carbon sources on cellulase production(IU/gds) by *B.subtilis*, CBTK 106 and *A.caviae* CBTK 185 under SSF

Concn. (mM)	Cellulase activity											
	Glucose		alactose		Sucrose		Lactose		Maltose		Na_2CO_3	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185
0	1.06	1.08	1.06	1.08	1.04	1.02	1.08	1.02	1.10	1.08	1.04	1.06
1	1.10	1.14	1.10	1.09	1.12	1.14	1.10	1.14	1.12	1.14	1.04	1.04
3	1.25	1.24	1.14	1.12	1.24	1.28	1.24	1.28	1.30	1.28	1.06	1.06
5	1.32	1.34	1.16	1.14	1.22	1.24	1.20	1.28	1.21	1.24	1.04	1.04
7	1.30	1.30	0.91	0.96	1.21	0.98	1.16	1.24	1.21	1.20	1.06	1.04
9	1.28	1.28	0.88	0.94	1.14	0.96	1.14	1.08	1.08	1.18	0.96	0.98
12	1.28	1.28	0.78	0.90	1.10	0.96	1.12	0.01	0.98	1.10	0.91	1.98
15	1.21	1.26	0.54	0.86	1.02	1.01	1.01	0.98	0.86	0.98	0.89	0.96

Table 18a

Effect of Nitrogen Sources on ∝-amylase Production (U/gds) by B. subtilis CBTK 106 and A.caviae CBTK 185 under SSF

Concn. Milli-molar (mM)	-amylase activity (U/gds) Glucose		Galactose		Sucrose		Maltose		Lactose		Na_2CO_3	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185
0	50,400	60,200	50,400	60,200	50,400	60,200	50,400	60,200	50,400	60,200	50,400	60,200
0.1	51,000	60,900	50,600	62,400	50,800	60,900	51,000	60,800	50,800	60,800	50,900	60,400
0.5	51,850	65,600	51,500	64,700	51,500	62,100	51,200	62,100	51,210	62,600	51,100	62,800
1.0	53,000	68,800	52,000	66,800	52,000	64,100	51,800	64,100	52,980	66,600	52,980	66,600
1.5	49,000	64,500	48,000	64,100	50,000	60,100	48,700	58,200	49,980	60,800	50,000	58,400
2.0	46,500	60,100	46,000	60,200	48,100	58,200	47,650	48,200	47,800	58,480	48,100	54,200
2.5	44,800	58,300	42,280	57,900	45,100	54,100	45,100	35,600	45,400	46,100	45,100	50,100
3.0	38,700	50,400	38,500	55,100	38,100	52,400	40,100	30,200	40,400	39,100	38,100	41,200

Table 18 b

Effect of Nitrogen sources on cellulase production (IU/gds) by *B.subtilis*, CBTK 106 and *A.caviae* CBTK 185 under SSF

Concn. (%)	Cellulase activity (IU/gds)											
	$(NH_4)_2SO_4$		$NaNO_3$		Urea		Peptone		Beef extract		Yeast extract	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185	CBTK 106	CBTK 185
0	1.04	1.06	1.02	1.04	1.06	1.08	1.08	1.04	1.10	1.04	1.12	1.04
0.1	1.11	1.10	1.08	1.12	1.12	1.10	1.10	1.12	1.14	1.10	1.14	1.08
0.5	1.28	1.24	1.12	1.24	1.24	1.18	1.18	1.14	1.29	1.15	1.21	1.12
1.0	1.32	1.32	1.24	1.30	1.28	1.21	1.21	1.20	1.32	1.24	1.32	1.24
1.5	1.21	1.20	1.18	1.21	1.20	0.98	1.18	1.14	1.24	1.20	1.22	1.20
2.0	1.08	1.07	1.10	1.01	1.04	0.97	1.08	1.01	1.12	1.14	1.14	1.14
2.5	0.99	1.02	1.01	0.98	0.99	0.96	1.02	0.99	1.02	1.02	0.99	1.02
3.0	0.95	0.99	0.99	0.97	0.95	0.94	0.98	0.98	0.99	0.99	0.98	0.96

3.6.10 Effect of inoculum concentration

Data presented in Fig.21 a & b indicate that 5-15% inoculum concentration could promote the enzyme production. Maximal α-amylase production was recorded with 1% inoculum concentration, while maximal cellulase production was incurred with 15% inoculum concentration. Further increase in inoculum concentration found led to reduction in enzyme yield.

A.caviae, CBTK 185, produced high titres of α-amylase compared to *B.subtilis*, CBTK 106 with 10% inoculum concentration. Similarly, cellulase production was maximal with *A.caviae* CBTK 185 (1.88 IU/gds) compared to *B.subtilis*, CBTK 106 with 15% inoculum concentration. Nevertheless significant level, of cellulase production could be observed at 10-20% inoculum concentration.

3.6.11 Effect of incubation time

Maximal enzyme production was observed at 60 hr (Fig.22 a & b) of incubation for α-amylase and at 72 hrs for cellulases by both the strains, at their optimal conditions.

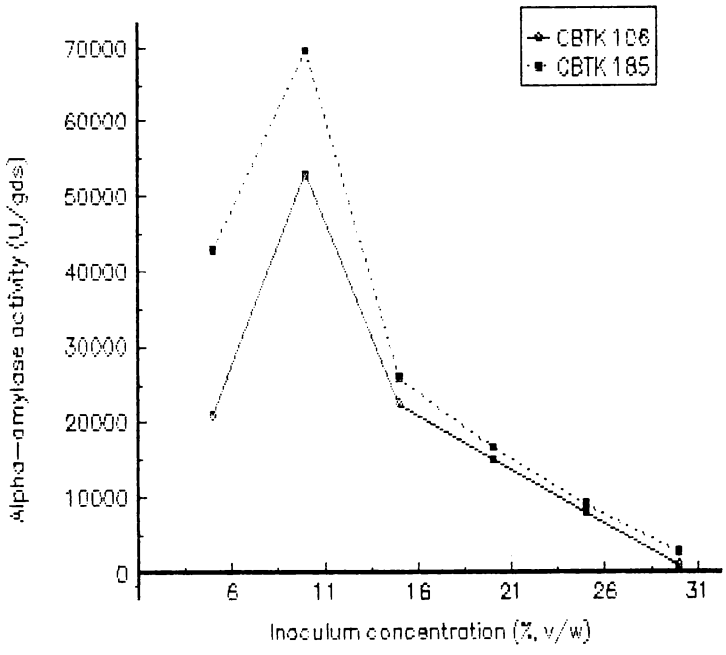
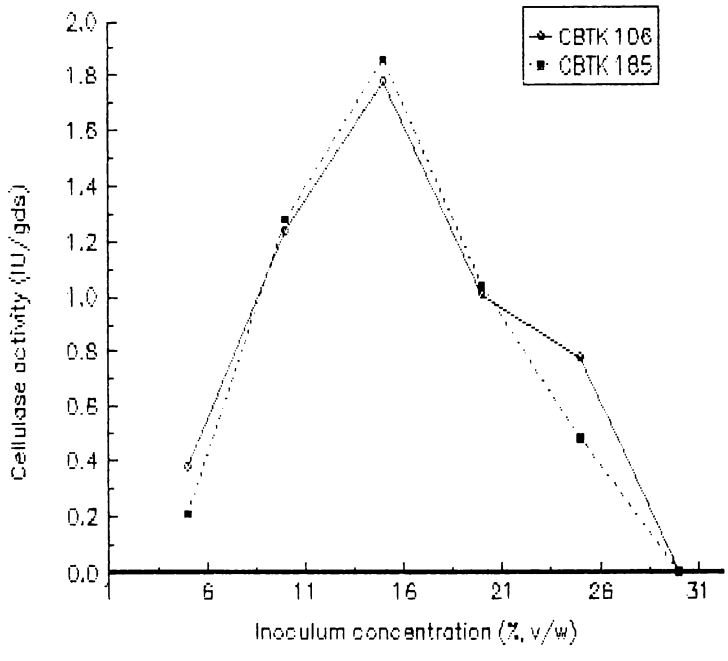


Fig.21 a&b Effect of Inoculum Concentration on Enzyme production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF



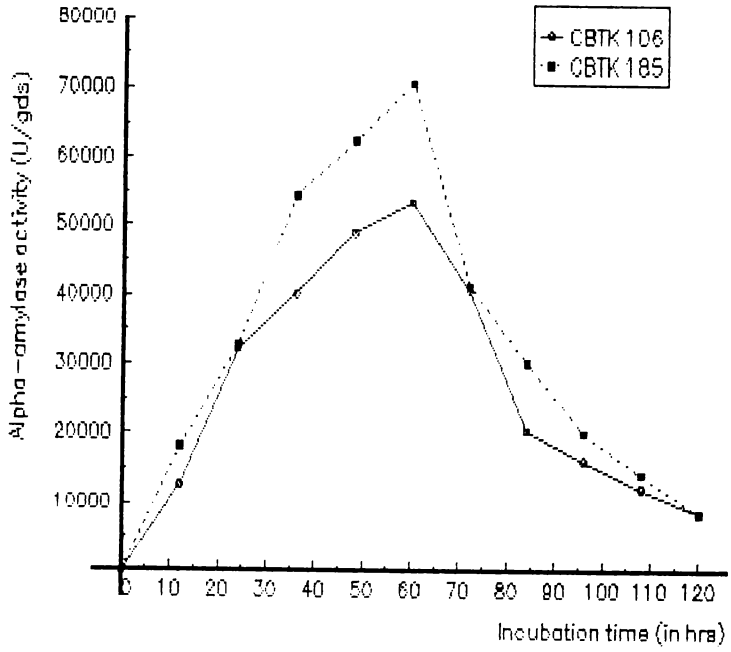
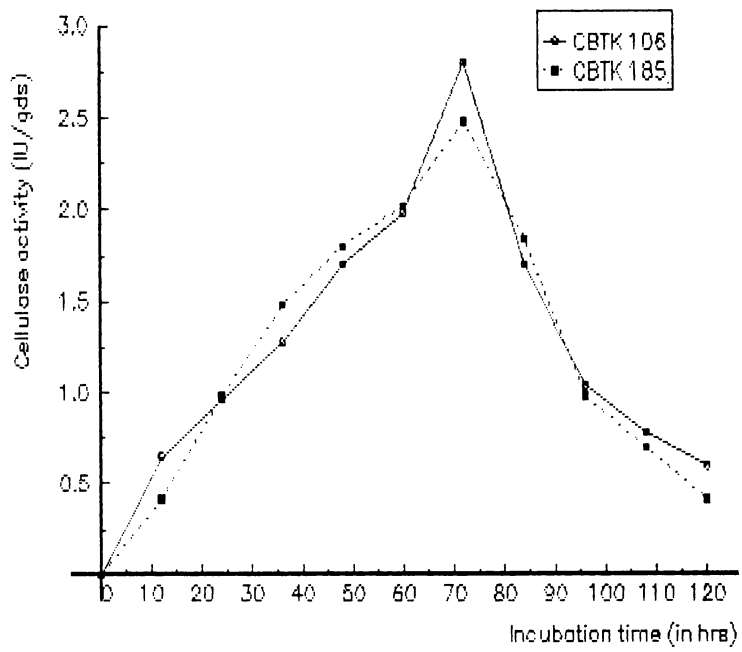


Fig.22 a&b Effect of Incubation Time on Enzyme Production by *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 under SSF



However, an appreciable level of enzyme production was also recorded at 24 hrs. An increase in incubation time above 72 hrs led to decrease in the enzyme production.

A.caviae CBTK 185 recorded maximal α-amylase (70,712 U/gds) compared to *B.subtilis* (53,450 U/gds) at 60 hrs incubation time. Maximal cellulase production was observed with *B.subtilis*, CBTK 106 (2.80 IU/gds) compared to *A.caviae*, CBTK 185 (2.48 IU/gds) at 72 hrs.

3.7 Optimisation of Extraction Parameters for Enhanced Recovery of the Enzyme Produced by SSF

Parameters that influence the process of extraction and recovery of extra cellular enzymes from fermented Banana Fruit Stalk (BFS) medium were optimised towards obtaining maximal recovery of the enzyme. They included drying temperature of fermented banana fruit stalk medium, extraction media, pH of extraction media (buffer systems), ratio of fermented medium to buffer, extraction temperature and contact time of fermented medium to buffer.

3.7.1 Drying temperature

Results presented in Table 19 indicate that drying of BFS at 30°C favoured maximal recovery of both the enzymes from both the strains. However, significant levels of enzymes could also be achieved for 40 and 50°C, for both the strains. Drying at 60°C did not support enhanced release of enzyme.

3.7.2 Extraction media

Phosphate buffer with respective optimum pH of the enzyme promoted maximum recovery for both the enzymes, followed by distilled water (DW), tap water (TW) and Dw plus 1% NaCl Table 20. When aqueous ethanol (80%) was used for extraction the α-amylase and cellulase could not be detected in the extract for both the strain.

3.7.3 pH of extraction medium

Since buffer system was identified as the most suitable extraction medium for obtaining maximal enzyme recovery from fermented BFS medium, the effect of pH of buffer

Table 19

Optimisation of drying temperature of the BFS medium for recovery of the enzyme produced by *B.subtilis* (CBTK 106) and *A.caviae* CBTK 185 under SSF

Drying temperature	α-amylase activity (U/gds)		Cellulase activity (IU/gds)	
(°C)	CBTK 106	CBTK 185	CBTK 106	CBTK 185
30	53,450	70,710	2.80	2.48
40	38,200	54,400	1.98	1.84
50	36,600	32,400	1.12	1.20
60	10,900	10,200	0.84	0.64

Table 20

Optimisation of extraction medium of the BFS for recovery of the enzyme produced by *B.subtilis* (CBTK 106) and *A.caviae* CBTK 185 under SSF

Extraction Medium	α-amylase activity (U/gds)		Cellulase activity (IU/gds)	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185
Phosphate buffer (pH 6-8)	53,450	70,710	2.80	2.48
Distilled Water (DW)	8,700	6,400	0.08	0.004
DW + 1% NaCl	242	410	--	--
Tap Water (TW)	640	900	0.01	0.005
Aqueous ethanol (80%)	--	--	--	--

system on the recovery of enzyme was tested by using buffer of various pH ranging from 4-9 for the extraction process. Extraction with phosphate buffer having pH 6 and 8 recorded maximal enzyme recoveries for both the enzymes(Table 21). Significant levels of α-amylase recovery was also observed with 0.2 M acetate buffer (4-6) and 0.2M Tris-HCl buffer (8-9) except that cellulase activity was negligible in the extract with 0.2M Tris-HCl buffer.

3.7.4 Ratio of Banana Fruit Stalk medium to buffer

Data presented in Table 22 indicate that in general, a ratio of 1:10 is optimum for effecting maximal recovery of both the enzymes from fermented BFS medium. Although significant levels of enzyme could also be recovered at 1:5 and 1:20 ratios, the enzyme recovery was very low at ratios above 1:20.

3.7.5 Effect of contact temperature

In general, enzyme recovery for both the enzymes from both the strains were maximal at 30^{o} C (Table 23).

Table 21

Optimisation of pH of extraction media (different Buffer Systems) for recovery of the enzyme produced by *B.subtilis* (CBTK 106) and *A.caviae* CBTK 185 under SSF

Buffer system	α-amylase activity (U/gds)		Cellulase activity (IU /gds)	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185
0.2 M Acetate buffer (4-6)	30,600	30,180	0.92	0.90
0.2 M Phosphate buffer (6-8)	53,450	70,710	2.80	2.48
0.2 M Tris-HCl buffer (8-9)	40,140	38,680	0.01	0.04

Table 22

Optimisation of Banana Fruit Stalk Medium to buffer ratio for recovery of the enzyme produced by *B.subtilis* (CBTK 106) and *A.caviae* CBTK 185 under SSF

BFS to buffer ratio	α-amylase activity (U/gds)		Cellulase activity (IU/gds)	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185
1:3	22,800	10,400	0.45	0.56
1:5	36,400	46,400	1.40	1.68
1:10	53,450	70,710	2.80	2.48
1:20	40,100	37,200	0.78	0.84
1:30	15,600	18,920	0.44	0.53

Extraction at higher temperatures did not enhance enzyme recovery. Nevertheless, significant level of enzymes could be recovered at 25-40°C range for both the enzymes from both the strains. Further increase in contact temperature did not favour enhancement in enzyme recovery.

3.7.6 Effect of contact time

Results shown in Table 24 suggest that α-amylases and cellulases of both the strains of *B.subtilis*. CBTK 106 and *A.caviae*, CBTK 185 could be extracted to a maximal level by providing a contact time of 60 min. In general, results indicate that contact time of 60 min is necessary to obtain significant levels of enzymes recovery from the fermented BFS medium, although 90-120 min of contact time yielded significant levels of enzyme recovery.

Table 23

Optimisation of contact temperature for recovery of the enzyme produced by *B.subtilis* (CBTK 106) and *A.caviae* CBTK 185 under SSF

Contact temperature	α-amylase activity (U/gds)		Cellulase activity (IU/gds)	
(°C)	CBTK 106	CBTK 185	CBTK 106	CBTK 185
25	28,190	28,600	1.48	1.84
30	53,450	70,710	2.80	2.48
35	40,600	58,600	0.87	0.92
40	36,400	28,160	0.78	0.81
45	30,100	18,200	0.64	0.74
50	20,600	10,700	0.50	0.48

Table 24

Optimisation of contact time for recovery of the enzyme produced by *B.subtilis* (CBTK 106) and *A.caviae* CBTK 185 under SSF

Contact time(in mins)	α-amylase activity (U/gds)		Cellulase activity (IU/gds)	
	CBTK 106	CBTK 185	CBTK 106	CBTK 185
30	28,600	26,450	0.84	0.78
60	53,450	70,710	2.80	2.48
90	40,200	40,180	0.92	0.98
120	35,600	36,400	0.88	0.89

3.8 Comparative Evaluation of Banana fruit Stalk and Wheat bran as Solid Substrates for α-amylase and Cellulase Production by Bacteria through SSF

Suitability of Banana Fruit Stalk (BFS) as solid subtrate for enzyme production through SSF was evaluated by comparing with wheat bran (WB) as solid substrate, with and without additional substrates.

Data presented in Fig 23 a & b and Fig.24 a & b for α-amylase and cellulase production with both BFS and WB clearly indicate the suitability of BFS for enzyme production by *B.subtilis* and *A.caviae* as higher levels of α-amylase (53,450 and 70,712 U/gds) and cellulase of (2.80 and 2.48 IU/gds) could be recovered with BFS compared to WB which supported relatively lesser yields of α-amylase (22760 U/gds and 15,880 U/gds) and cellulase (0.98 IU/gds and 0.82 IU/gds). *A.caviae*, CBTK 185 recorded 4.45 fold increase in α-amylase and 3.02 fold increase in cellulase with BFS as solid substrate compared to WB. Whereas *B.subtilis* could recorded 2.35 fold increase in α-amylase and 3.04 fold increase in cellulase.

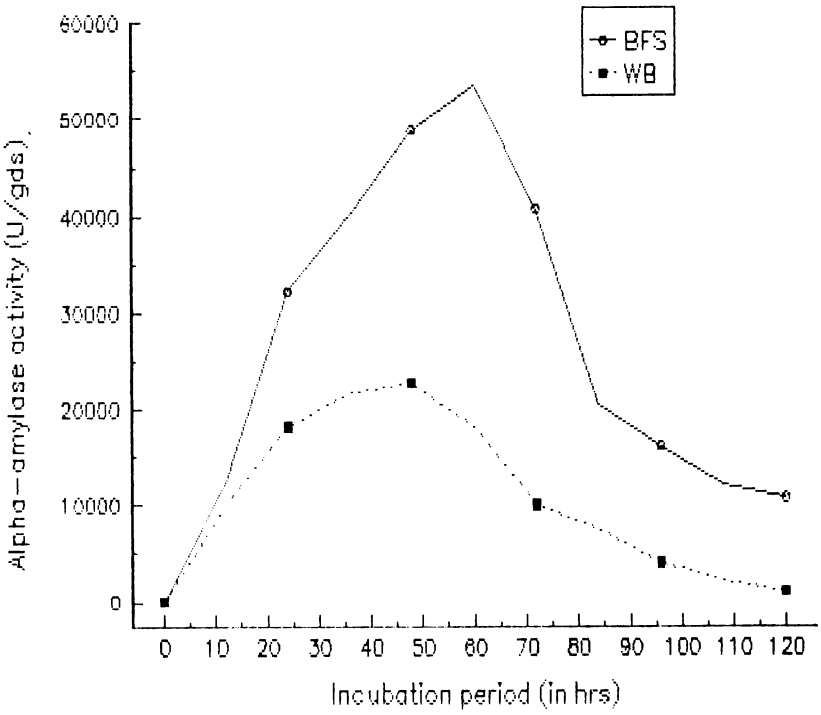
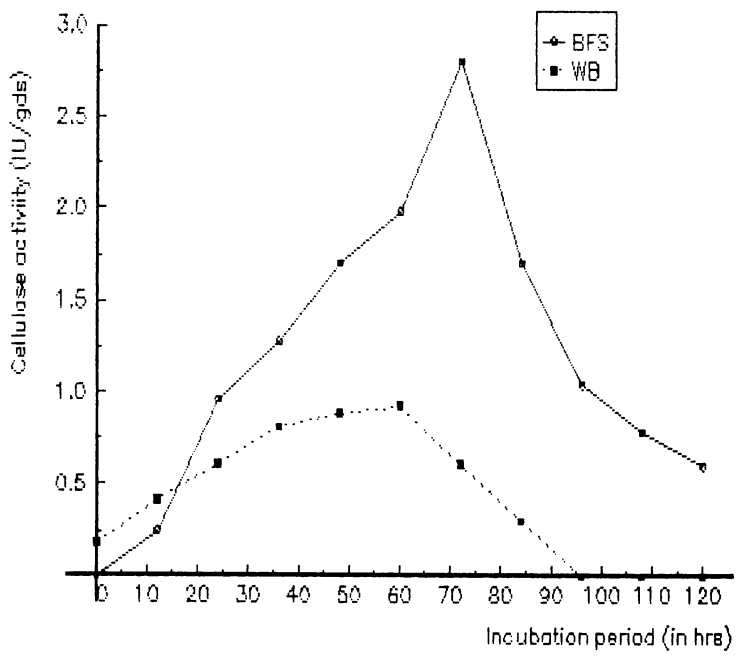


Fig.23 a&b Comparative evaluation of Banana Fruit Stalk (BFS) and Wheat Bran (WB) as solid substrates, without additional Substrates (Starch/Cellulose) for Enzyme production using *B.subtilis* CBTK 106 under SSF



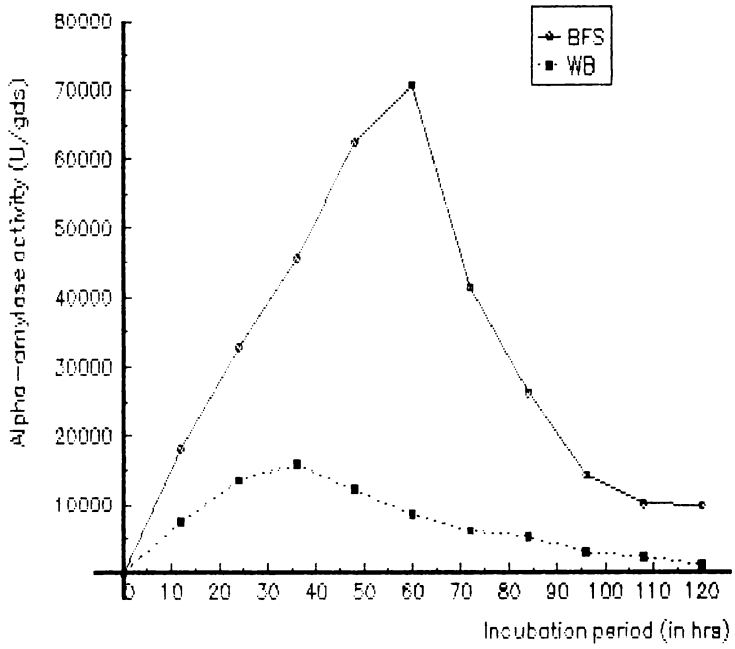
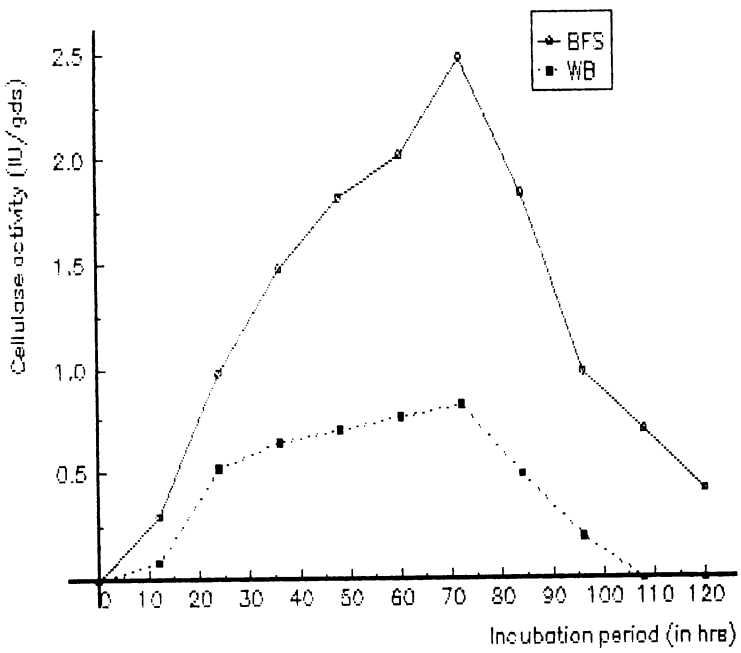


Fig.24 a&b Comparative evaluation of Banana Fruit Stalk (BFS) and Wheat Bran (WB) as solid substrates, without additional Substrates (Starch/cellulose) for Enzyme production using *A.caviae* CBTK 185 under SSF.



While maximum yield of α-amylase could be recovered at 60 hr with BFS, with WB the maximal yield was incurred at 48 hr for both the strain. However, in the case of cellulase production irrespective of the culture as well as the substrate, maximum production was observed at 72 hrs. Another interesting observation made during the course of study was that level of enzyme production declined gradually after reaching a maximum during the course of fermentation for both the strains.

Results presented in Fig 25 a & b and 26 a & b adds evidence to the earlier observation made with Fig.23 a & b and 24 a & b in confirming the suitability of BFS as good substrate for α-amylase and cellulase production compared to WB. In general, α-amylase production varied between 42,000 to 55,500 U/gds and between 36,400 to 71,800 U/gds respectively for *B.subtilis* and *A.caviae* in the presence of additional soluble starch with BFS. Whereas α-amylase production varied between 10,000 to 35,710 U/gds and 18,600 - 25,000 U/gds respectively with B.*subtilis* and *A.caviae* in the case of WB. While 0.1% of soluble starch could effect a marginal increase in α-amylase production with BFS, and 0.5% of soluble starch

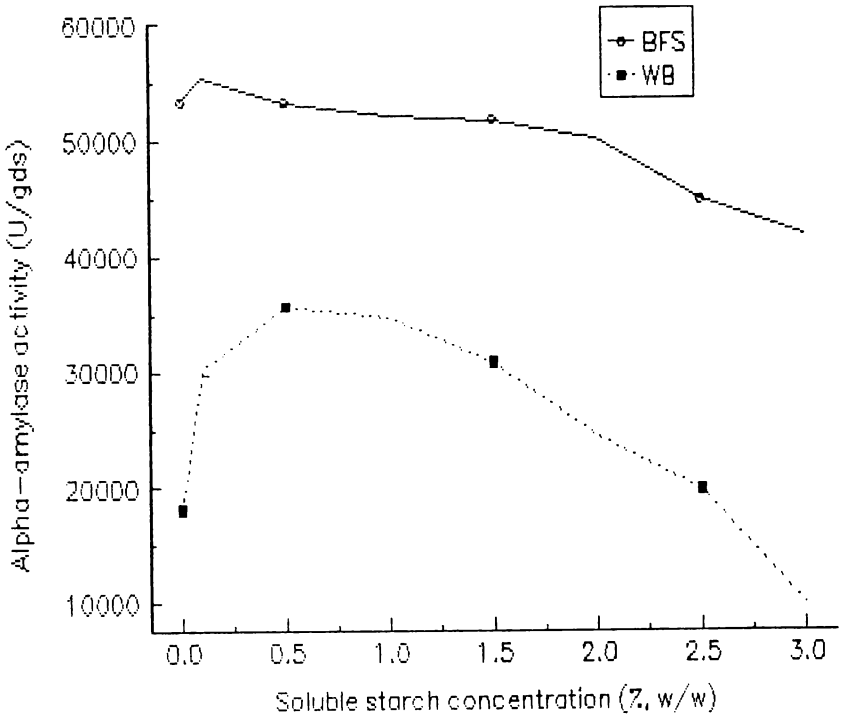
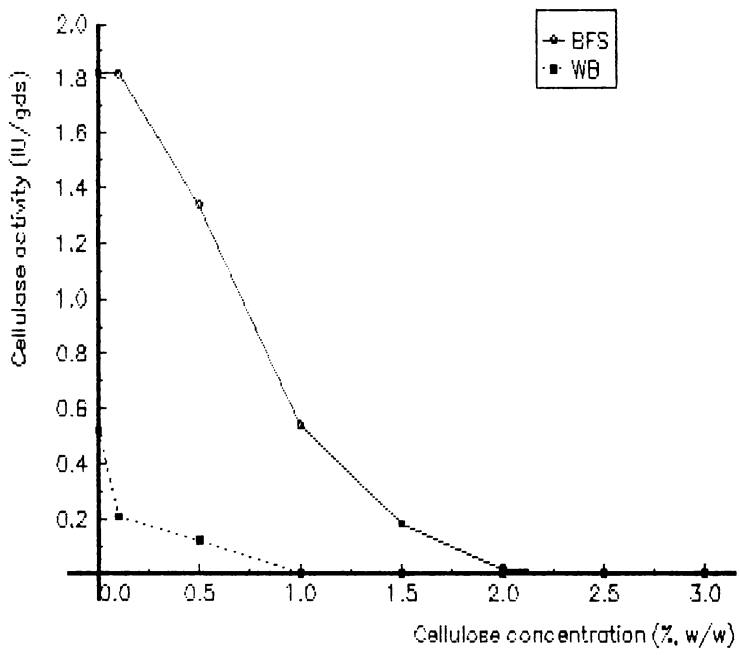


Fig.25 a&b Comparative evaluation of Banana Fruit Stalk (BFS) and Wheat Bran (WB) added with additional substrates for Enzyme production using *B.subtilis* under SSF



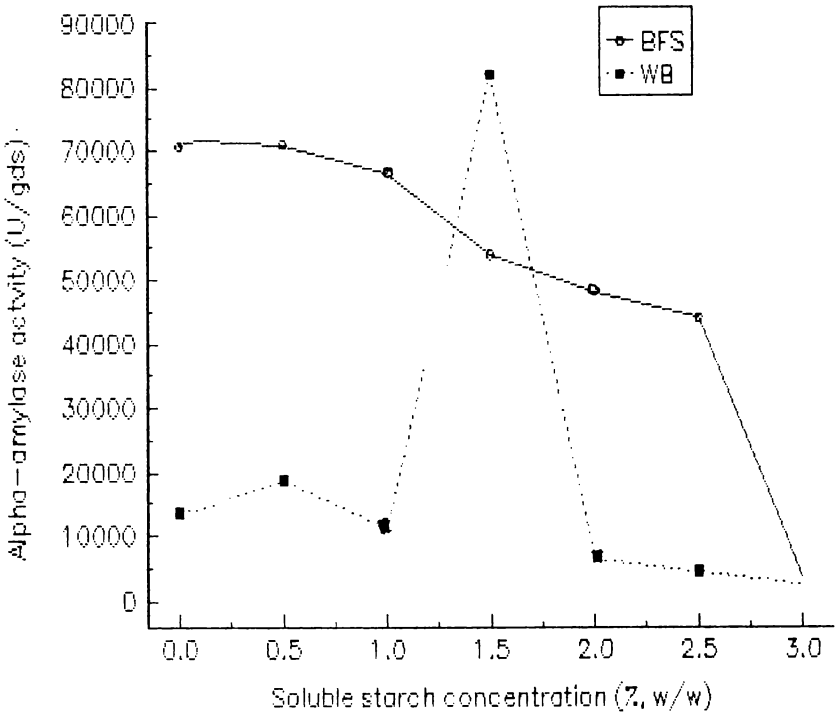
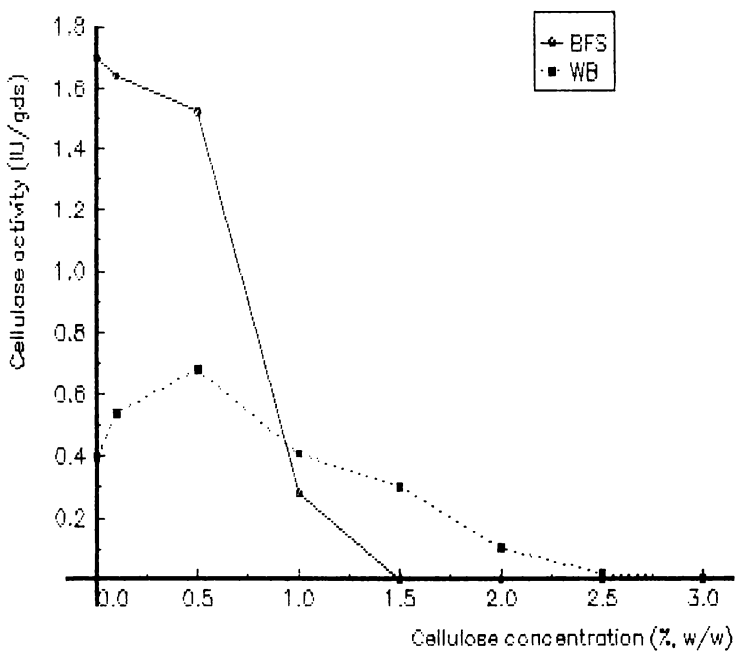


Fig.26 a&b Comparative evaluation of Banana Fruit Stalk (BFS) and Wheat Bran (WB) added with additional substrates for Enzyme production using *A.caviae* under SSF



was required with WB. In both the cases of the substrate increasing concentration of soluble starch led to decline in the case of α-amylase production. Cellulase production was not enhanced by the presence of additional cellulase with BFS substrate. While, there was a marginal increase in cellulase production at 0.5% concentration in the case of *B.subtilis*. It was noted that concentrations above 1% inhibited cellulase production irrespective of the soluble substrate and the organism.

3.9 Enzyme Production by Mixed Solid Substrates

The impact of mixed solid substrates on enzyme production by bacteria under SSF system was tested by mixing Banana Fruit Stalk (BSF) with Wheat Bran (WB) in different proportions. Results presented in Table 25 suggest that BFS and WB mixed at 4:1 ratio supported maximal α-amylase production (10 x 10^4 and 19 x 10^4 U/gds respectively for *B.subtilis* and *A.caviae*). A change in the proportion of mixing resulted in decline in enzyme yield. Cellulase production was also found to be maximal at 4:1 ratio (1.48 and 1.20 IU/gds respectively with *B.subtilis* and *A.caviae*)

Table 25

Use of mixed solid substrates for α-amylase and cellulase production using *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

Solid substrate ratio	α-amylase activity (U/gds)		Cellulase activity (IU/gds)	
	CBTK 106 (x 10^4)	CBTK 185 (x 10^4)	CBTK 106	CBTK 185
1:1	4.8	2.1	1.08	0.28
1:2	3.2	1.0	0.54	0.01
1:3	2.8	0.9	0.00	0.00
1:4	1.7	0.1	0.00	0.00
1:5	1.6	0.09	0.00	0.00
1:6	1.2	0.01	0.00	0.00
2:1	5	8	0.64	0.02
3:1	7	9	0.98	0.42
4:1	10	19	1.48	1.20
5:1	7	12	0.98	0.84
6:1	5	5	0.84	0.60
2:3	3.2	1.0	0.04	0.06
3:2	3.4	3.4	0.09	0.05
3:4	3.6	4.3	0.28	0.16
4:3	3.2	6.8	0.32	0.22
4:5	3.0	7.2	0.21	0.10
5:4	2.0	8.6	0.12	0.08
5:6	1.8	7.0	0.02	0.02
6:5	1.0	1.4	---	---

compared to other ratios. However other combinations also could effect significant levels of α-amylase production. Whereas in the case of cellulase production except for the ratios 1:1, 5:1, and 6:1 all other combinations did not support cellulase production.

3.10 Evaluation of Solid, Slurry and Submerged Fermentation for Enzyme production

Suitability of banana fruit stalk as substrate for enzyme production was also evaluated by testing enzyme production by bacteria under slurry fermentation (SLF) and Submerged Fermentation (SmF) condition. From the results presented in Table 26 a & b and 27 a & b it is inferred that the enzyme produced under slurry fermentation was less compared to SSF and higher than SmF. α-amylase yield varied between 60-1220 u/gds and 54-1680 U/gds under SLF for *B.subtilis* and *A.caviae* respectively compared to 45-820 U/l and 50-640 U/l under SmF and 7200-53450 U/gds and 5100-70712 U/gds under SSF respectively for *B.subtilis* and *A.caviae*. Maximal yield was attained at 60 hrs for SSF when compared to 48 hrs for SLF and 24 hrs for SmF. SSF system resulted in

Table 26 a & b

Evaluation of Solid, Slurry and Submerged Fermentation for α-amylase production using *B. subtilis* CBTK 106 and *A.caviae* CBTK 185

B.subtilis CBTK 106	α-amylase activity		
Incubation period (in hr)	SSF (U/gds)	SmF (U/l)	SLF (U/gds)
0	0	0	0
12	12600	200	200
24	32200	820	640
36	40200	540	950
48	48960	380	1220
60	53450	260	860
72	40800	200	460
84	20500	180	280
96	16200	100	180
108	12180	80.0	98
120	10800	45.0	60

contd..

A.caviae CBTK 185	α-amylase activity		
Incubation period (in hr)	SSF (U/gds)	SmF (U/l)	SLF (U/gds)
0	0	0	0
12	18160	280	240
24	32800	640	980
36	45500	400	1240
48	62400	380	1680
60	70712	320	1420
72	41400	280	1140
84	26200	120	810
96	14200	100	460
108	10100	70	210
120	8600	50	54

Table 27 a & b

Evaluation of Solid, Slurry and Submerged fermentation for cellulose production using *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

B.subtilis CBTK 106	Cellulase activity		
Incubation period (in hr)	SSF (IU/gds)	SmF (IU/l)	SLF (IU/gds)
0	0	0	0
12	0.25	0.004	0.02
24	0.96	0.004	0.04
36	1.28	0.007	0.05
48	1.70	0.009	0.08
60	1.98	0.004	0.06
72	2.80	0.002	0.05
84	1.70	0.001	0.03
96	1.04	0.001	0.03
108	0.78	0.00	0.01
120	0.60	0.00	0.005

contd..

A.caviae CBTK 185	Cellulase activity		
Incubation period (in hr)	SSF (IU/gds)	SmF (IU/l)	SLF (IU/gds)
0	0	0	0
12	0.30	0.02	0.01
24	0.98	0.03	0.04
36	1.48	0.05	0.05
48	1.82	0.08	0.08
60	2.02	0.04	0.05
72	2.48	0.02	0.03
84	1.84	0.01	0.01
96	0.98	0.005	0.005
108	0.70	0.00	0.00
120	0.42	0.00	0.00

43.8 and 42.1 fold increase in α-amylase production compared to SLF for *B.subtilis* and *A.caviae* respectively. Cellulase yield varied between 0.005 - 0.08 and 0.001-0.08 IU/gds for SLF for *B.subtilis* and *A.caviae* compared to 0.001-0.009 and 0.02-0.08 IU/l for SmF and, 0.28-2.8 and 0.42-2.48 IU/gds for SSF respectively for *B.subtilis* and *A.caviae*. With SmF and SLF maximum yield was attained at 48 hrs when compared to 72 hrs for SSF. SSF system resulted in 35 and 31 fold increase in cellulase production compared to SLF for *B.subtilis* and *A.caviae* respectively.

3.11 Purification of α-amylase

α-amylase isolated from *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 were purified by $(NH_4)_2SO_4$ precipitation (60-70% saturation) and dialysis followed by ion exchange chromatography and electrophoresis. Results obtained for purification steps with reference to total activity, specific activity, and yield of the enzyme are presented in Table 28 a and b.

Table 28 a.

Data on overall purification of the α-amylase from *B.subtilis*, CBTK 106

Purification step	Total enzyme units	Protein (mg)	Specific activity (units/mg Protein)	Purification factor (x)	Yield or recovery (%)
Initial crude extract	53450	178.0	300.3	1.0	100
Ammonium Sulphate Precipitation	50000	100.0	500	1.6	89.8
DEAE-Cellulose Column chromatography	27000	2.0	13500	44.95	50.5

Table 28 b.

Data on overall purification of the α-amylase from *A.caviae* CBTK 185

Purification step	Total enzyme units	Protein (mg)	Specific activity (units/mg Protein)	Purification factor (x)	Yield or recovery (%)
Initial crude extract	70712	267.3	264.5	1.0	100
Ammonium Sulphate Precipitation	63110	119.7	527.2	2.0	89.56
DEAE-Cellulose Column chromatography	40460	2.0	20230	76.48	57.2

The elution pattern of α-amylase of *B.subtilis* and *A.caviae* on DEAE - cellulose column showed two protein peaks(Fig. 27 a & b.). The first peak was without any enzyme activity.

The precipitation of the enzyme with ammonium sulphate and then ion exchange chromatography resulted in 44.95 and 76.48 fold increase in purification factor for *B.subtilis* and *A.caviae* respectively. The molecular weight of the protein was determined to be approximately for 62 kDa *B.subtilis* CBTK 106 and 58 kDa for *A.caviae* CBTK 185 respectively using SDS-PAGE. (Fig. 28 a&b)

3.12 Enzyme Characteristics

3.12.1 Effect of pH on the Activity and Stability of α-amylase

Data presented in Fig.29 a & b indicate that α-amylases of both the strains were not only maximally active and stable upto pH 9 but also could demonstrate stability and considerable activity over a wide range of pH (7-11).

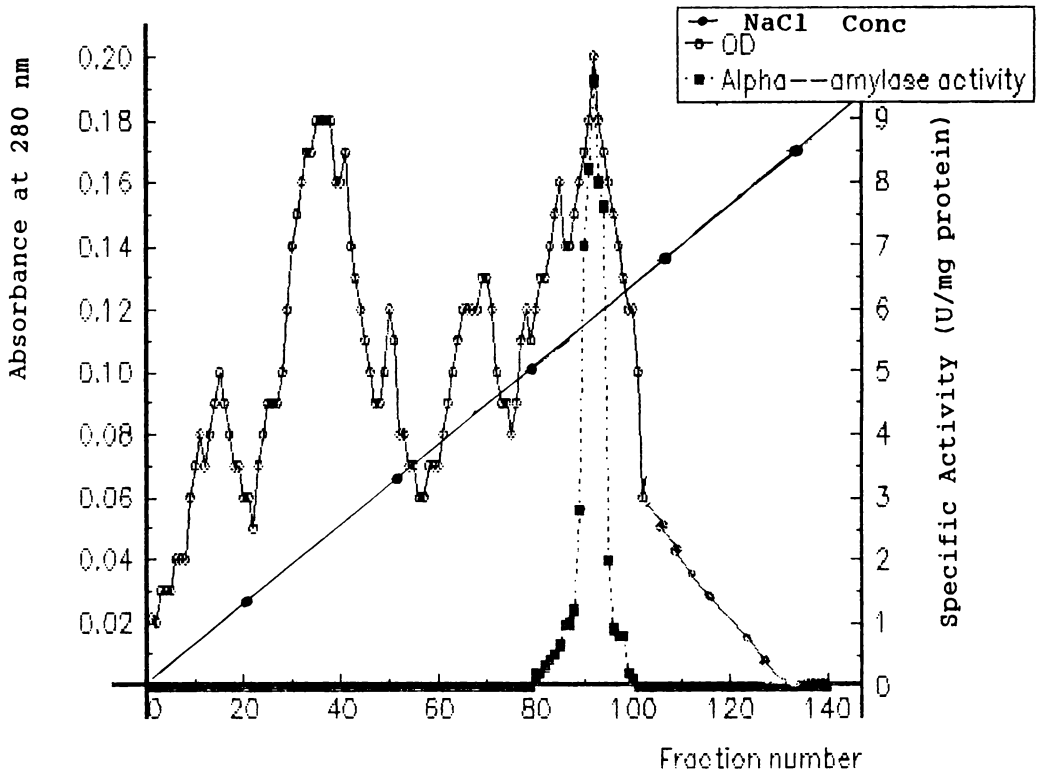


Fig.27a Elution Profile of *B.subtilis* CBTK 106 on DEAE - Cellulose Column Chromatography with linear gradient of NaCl (0-1.0 mol l^{-1}) in 0.2M Phosphate Buffer (pH 7.2)

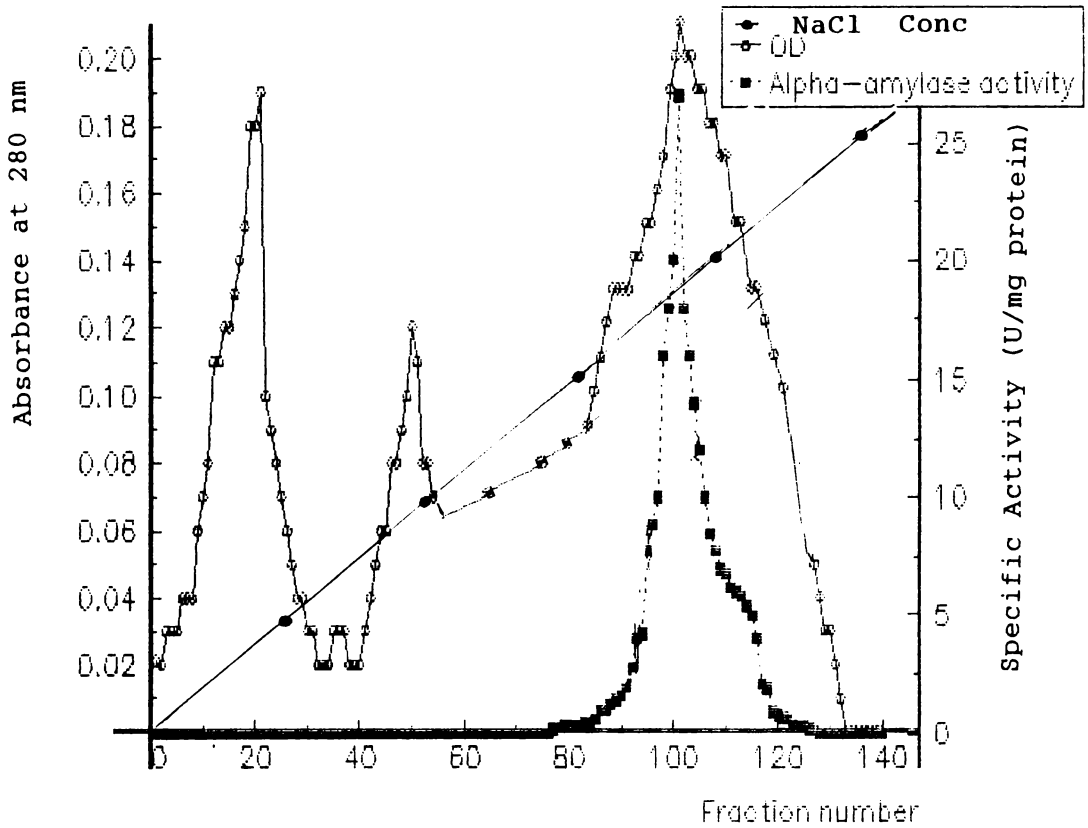


Fig.27b Elution Profile of α-amylase of *A.caviae* CBTK 185 on DEAE - Cellulose Column Chromatography with linear gradient of NaCl (0-1.0 mol l^{-1}) in 0.2M Phosphate Buffer (pH 7.2)

Fig. 28a. SDS - PAGE of purified
α - amylase of *B.subtilis*, CBTK 106
Lane 1, standard protein (Biorad)
A, Bovine Serum Albumin (MW 67000)
Lane 2, Purified α - amylase
Lane 3, Enzyme after $(NH_4)_2SO_4$ fractionation

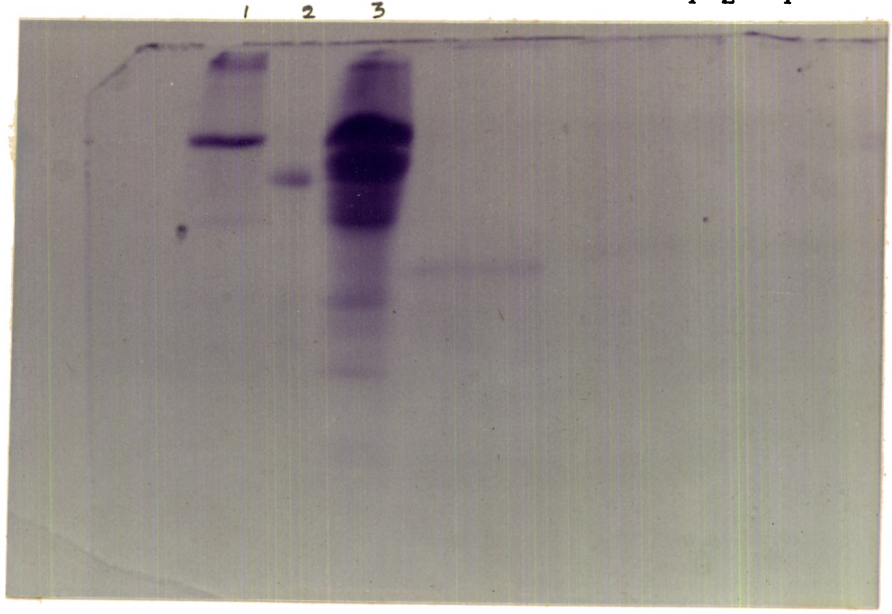
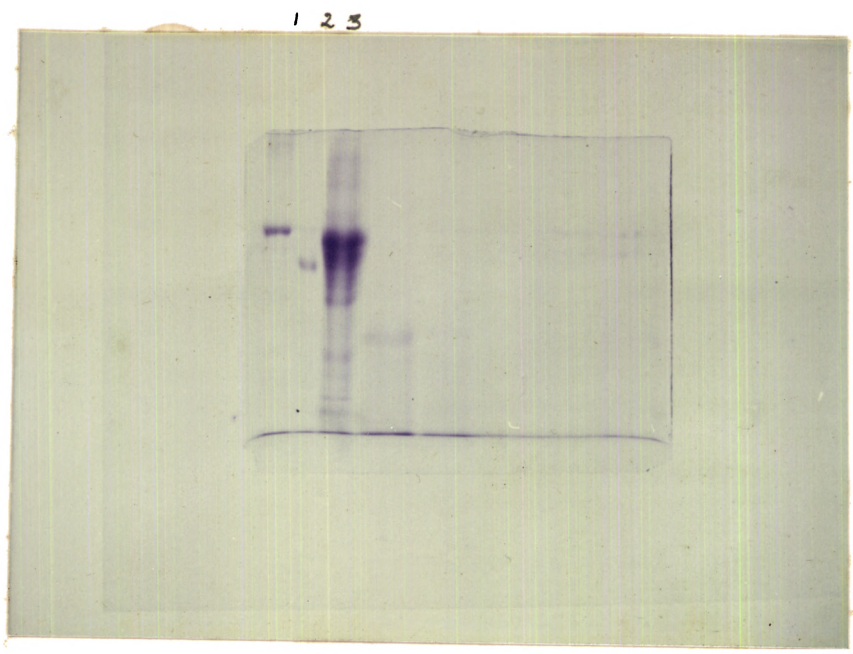


Fig. 28b. SDS - PAGE of purified
α - amylase of *A.caviae*, CBTK 185
Lane 1, standard protein (Biorad)
A, Bovine Serum Albumin (MW 67000)
Lane 2, Purified α- amylase
Lane 3, Enzyme after $(NH_4)_2SO_4$ fractionation



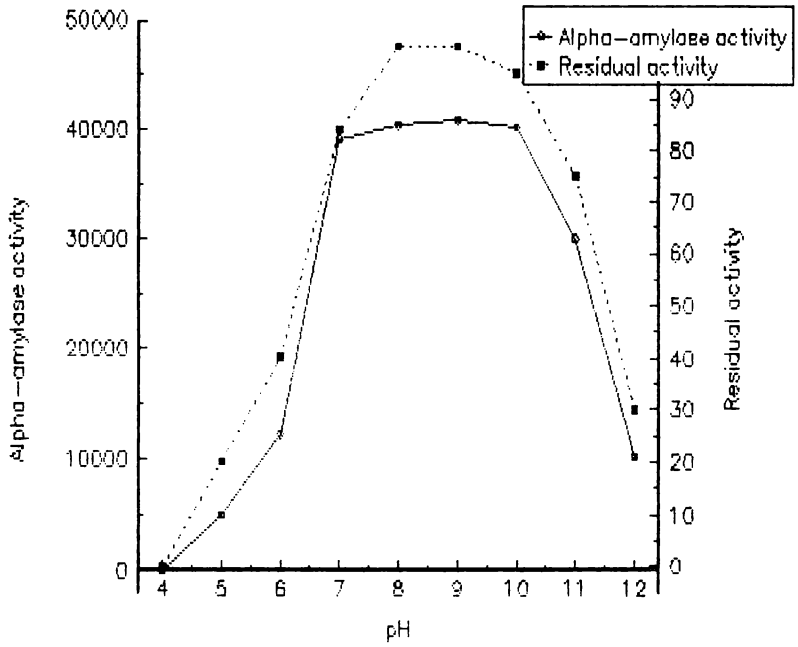


Fig.29 a Effect of pH on the activity and stability of α-amylase of *B.subtilis* CBTK 106

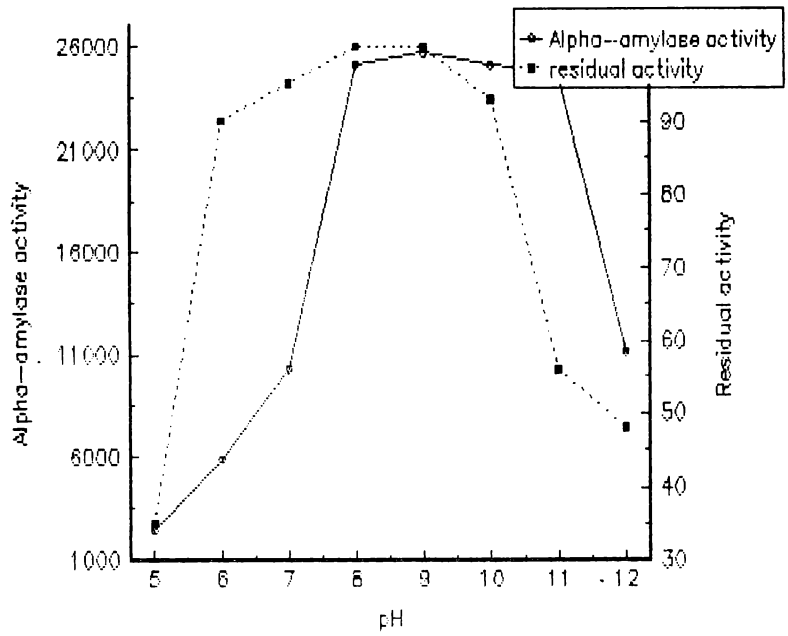


Fig.29 b Effect of pH on the activity and stability of α-amylase of *A.caviae* CBTK 185

However, enzymes from both the strains exhibited 100% stability at pH 8-9.

3.12.2 Effect of temperature on the activity and stability of α-amylase

From the results presented in Fig.30 a & b, it is evident that α-amylase of *B.subtilis* CBTK 106 was active and stable at temperatures varying from 30-100°C, with their maximal activity and stability at 80-90°C, (100% residual activity). Further increase in temperature to 100°C resulted in a decrease in the activity and stability of enzyme. Maximal activity and stability of α-amylases of *A.caviae*, CBTK 185 was recorded at 70°C, (95% residual activity). However, increase in temperature to 90°C resulted in a decrease in activity and stability of the enzyme.

3.12.3 Effect of substrate concentration on the activity of α-amylase

The results presented in Fig.31 suggested that α-amylase activity of both the strains increased progressively

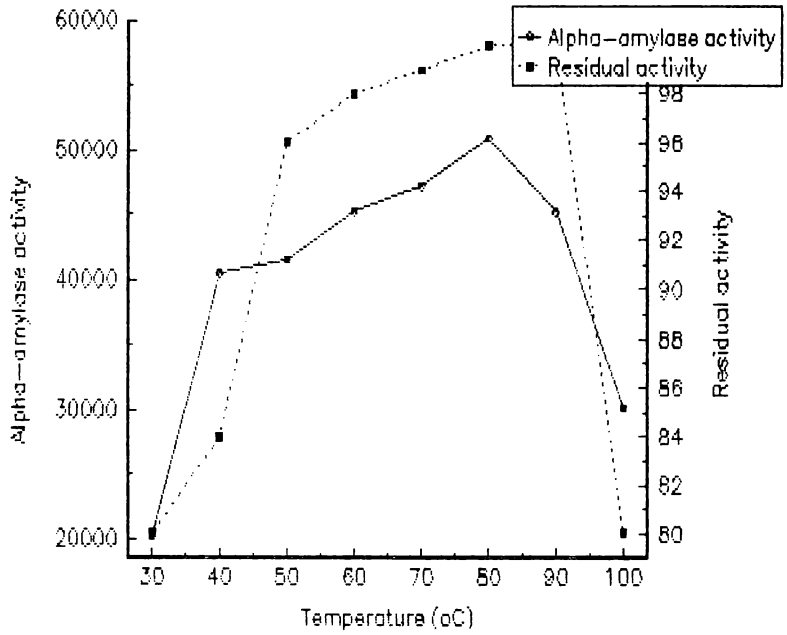


Fig.30 a Effect of Temperature on the activity and stability of α-amylase of and *B.subtils* CBTK 106

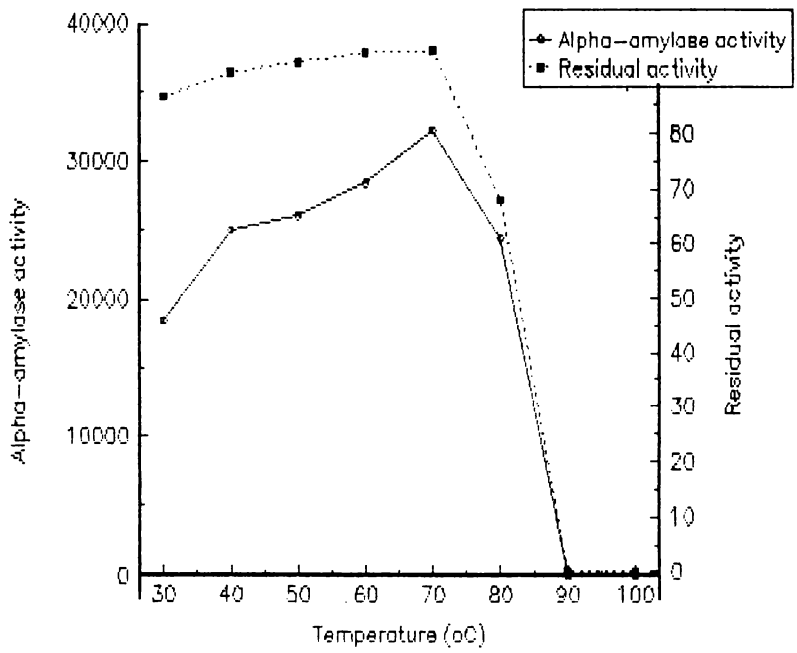


Fig.30 b Effect of Temperature on the activity and stability of α-amylase of and *A.caviae* CBTK 185

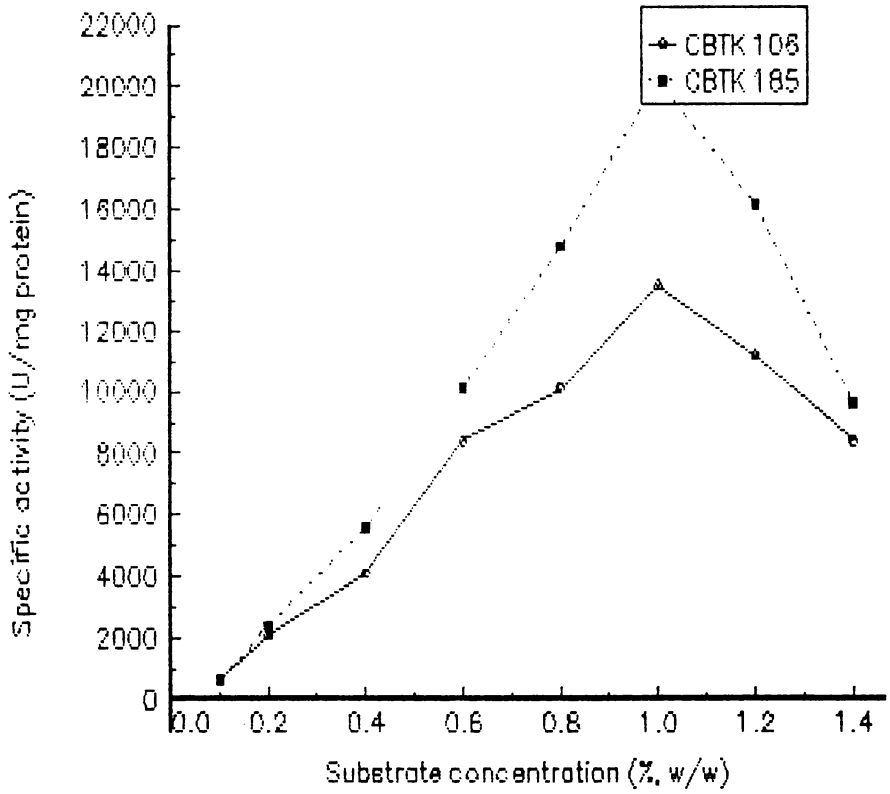


Fig.31 Effect of Substrate Concentration on the activity of α-amylase of *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

along with increase in substrate concentration from 0.1-1.0mM. Further increase in substrate concentration from 1.0-1.5% led to decline in enzyme activity. Michalis-Menten constant of α-amylase from both the strains were calculated using Lineweaver-Burk Plot. It was found that the Kn value for α-amylase of *B.subtilis* CBTK 106 was 0.95 gl^{-1} and that of *A.caviae* CBTK 185 was 0.50 gl^{-1} with soluble starch as substrate.

3.12.4 Effect of cations on α-amylase activity

Among the cations tested, Ni^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} (0.01 $mol l^{-1}$) effected 100% inhibition of the α-amylase of *B.subtilis* whereas α-amylase of *A.caviae* was inhibited by all the cations except Ni^{2+}(Table 29). Interstingly, the relative activity, of α-amylases of *A.caviae* was enhanced to 112 and 108% with $Mn^{2+}$and Ni^{2+}respectively. The α-amylases of *B.subtilis* could retain 80% of its relative activity with Ca^{2+} followed by 76 and 72% for Mn^{2+} and Mg^{2+} respectively.

Table 29

Effect of cations (0.01 $moll^{-1}$) on α-amylase activity of *B.subtilis*, CBTK 106 and *A.caviae* CBTK 185

Cation (concn.0.01 $moll^{-1}$)	Residual activity *B.subtilis* CBTK 106	*A.caviae* CBTK 185
1. $BaCl_2\ 2H_2O$	0	0
2. $CaCl_2\ 2H_2O$	80	20
3. $CuSO_4\ 7H_2O$	0	0
4. $CdCl_2\ H_2O$	13	14
5. $CoCl_2\ 6H_2O$	0	0
6. $FeSO_4\ 7H_2O$	0	0
7. $HgCl_2$	0	0
8. KCl	20	30
9. $MnCl_2\ 7H_2O$	76	112
10. $MgSO_4\ 7H_2O$	72	84
11. $NiSO_4\ H_2O$	0	108
12. $AlCl_3.2H_2O$	23	16
13. $Zn\ SO_4\ 7H_2O$	0	0

3.12.5 Effect of other inhibitors on the α-amylase activity

Results presented in Table 30 indicate that all the substances tested (0.01 $moll^{-1}$) inhibit the enzyme activity to a significant extent. The presence of TCA, Acetic acid or Iodine inhibited 100% of its relative activity of α-amylases of both the strains *B.subtilis* and *A.caviae*.

Table 30

Effect of other inhibitors (0.01 $moll^{-1}$) on α-amylase activity of *B.subtilis* CBTK 106 and *A.caviae* CBTK 185

Concn. (0.01 $moll^{-1}$)	Residual activity	
	B.subtilis CBTK 106	*A.caviae* CBTK 185
1. EDTA	60	52
2. TCA	0	0
3. P-Dimethyl amino benzaldehyde	8	12
4. Dimethyl sulphoxide	33	76
5. Acetic acid	0	0
6. Iodine	0	0
7. Phenyl hydrazine hydrochloride	6	4

CHAPTER IV

DISCUSSION

Banana fruit stalks, on fermentation by native microflora under uncontrolled environmental conditions showed significant changes in its proximate composition compared to fresh condition. Results obtained and presented in the previous sections indicate very clearly, the role of native microflora, associated with the fermented banana fruit stalk, in effecting changes in the chemical composition as well as in the accumulation of hydrolytic enzymes, viz. α-amylase and cellulase.

The increase in the initial moisture content during the course of fermentation may be attributed to the release of metabolic water during the metabolism of carbohydrates and respiration of the growing organisms (Yang, 1988). A 4—11.5% increase in moisture content has been reported during SSF of sweet potato residue inoculated with *Pichia burtonii* (Yang, 1988). Similar increase in moisture content of about 4.9% to the final product was also observed during solid state

fermentation of cabbage waste by native microflora (Krishna and Chandrasekaran, 1995). The change in pH from neutral to alkaline, after 8 days of fermentation evidences microbial utilisation of protein fractions of the banana wastes and consequent release of ammonia and some amines as by-products. Yang (1988) observed an increase in protein content on nitrogen supplementation and suggested that SSF is a method of protein enrichment of sweet potato residue with *Saccharomyces* sp. Enwefa (1991) also reported an increase in protein content in the presence of additional nitrogen sources while banana skins were fermented with *Saccharomyces uvarum*. Although, there was no similar nitrogen supplementation, a marginal rise in protein content was observed during the course of fermentation of Banana fruit stalk. This increase in protein content could be attributed to the marginal increase in THBp and consequent biomass and cell protein (Enwefa, 1991).

The decline in the initial concentration of total sugar and reducing sugar during the course of fermentation testify utilisation of carbohydrate content of the banana fruit stalk by the associated fermenting microflora as source of carbon for their proliferation. This is further evidenced

by the observed increase in THB as well as α-amylase and cellulase titres during fermentation.

The progressive increase during the initial days and later decrease in the THB level during fermentation might be due to the elimination of some groups due to the shift in pH to alkalinity, competition and consequent lack of adequate nutrients and release of toxic substances. The dominance of *Pseudomonas* sp over other species, during fermentation may be attributed to their ability to utilise non proteinaceous nitrogen such as free amines, and aminoacids released as by-products and their adaptability to survive in the alkaline pH compared to coexisting flora (Liston, 1980; Chandrasekaran, 1985).

The decline in α-amylase and cellulase titres after 4 days of incubation, might be due to inactivation by microbial proteases secreted out in the system (Zhu *et al.*,1994) or some other inhibiting substances released by the complex activities of the heterotrophic bacteria during fermentation.

Bacillus subtilis CBTK 106 and *Aeromonas caviae* CBTK 185, selected as potential strains for production of α-amylase and cellulase, on optimisation of growth characteristics for obtaining maximal enzyme production, revealed interesting observations.

Both strains showed an optimal requirement of pH 7 for maximal growth and enzyme production though the pH range over which significant growth and enzyme production occurred varied from pH 6-8 and pH 6-9 respectively for *B.subtilis* and *A.caviae*. These results are similar to those reported for maximal α-amylase production with *B. licheniformis* TCRDC B 13, at pH 6-9 (Bajpai and Bajpai, 1989) and *B.amyloliquefaciens* MIR - 41 at pH 6.8 (Castro *et al.*, 1993).

Fukumori *et al.*, (1985) reported on the alkaline cellulase production by *Bacillus* sp. which is able to grow optimally at pH 8.8-10.0. However, most of the cellulases produced by fungi and bacteria are active over a pH range from 5 to 6 and inactive at alkaline pH (Fukumori *et al.*, 1985).

Interestingly both *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 showed maximal growth and production of both α-amylase and cellulase at 35°C and at significant levels over 30-40°C. Similar observations were made earlier for *B.subtilis* at 37°C (Mitricia and Granum, 1979; Robyt and French, 1979), *B.coagulans* at 35°C (Campbell,1955), *B.licheniformis* TCRDC B 13 at 35-40°C (Bajpai and Bajpai, 1989) and *Bacillus* sp. 1139 which produced cellulase at 30-37°C (Fukumori *et al.*, 1985).

B.licheniformis TCRDC B 13 produced more amylase with 1% of corn starch than at higher concentration and an increase in the concentration of starch led to decrease in the levels of enzyme yield (Bajpai and Bajpai, 1989). *Bacillus licheniformis* M 27 produced under maximal α-amylase in submerged fermentation at 1% soluble starch concentration (Ramesh and Lonsane, 1991). In the present study, both *B.subtilis* and *A.caviae* could produce α-amylase and cellulase maximally when added with 1% soluble starch/cellolose and at significant levels over a wide range of concentrations varying from 0.5-1.5%.

Mandels and Weber, (1969) observed that *T.viride* required optimal cellulose concentration of 0.5-1.0%, for maximal cellulase production in the presence of peptone in the medium and when grown on cellulose together with nutrient salt alone, or added with small quantity of soluble carbohydrate (0.05-0.1% glucose) into the medium. Sternberg (1976) has shown that a double fold yield of cellulase could be obtained with an increase in the cellulose concentration from 0.75 to 2%. Whereas in the present study both the strains recorded maximal growth and enzyme production with the addition of 1% cellulose and at significant levels at 0.5-1.5% cellulose levels in the medium.

Horikoshi (1971) reported that an alkalophilic *Bacillus* sp. A - 40 -2 did not show enhancement in growth and enzyme production after addition of NaCl in the medium. Whereas, Medda Chandra (1980) reported that *B.licheniformis* CUMC 305 could grow in the presence of 7% NaCl. Fukumori *et al.*, 1985 reported that *Bacillus* sp., no.1139, showed CMCase activity with 5% or 7% (w/v) of NaCl in the medium.

Interestingly, in the present study both the strains *B.subtilis* and *A.caviae* preferred 20 mM (2%) and 10mM (1%)NaCl concentration for α-amylase and cellulase production respectively. However, the effect of NaCl concentration on enzyme production was found to be meagre. Results suggest that both strains being native to terrestrial environment do not require sodium chloride as an important nutrient factor for enhanced production of hydrolytic enzymes unlike other reported species of *Bacillus*.

Carbon and nitrogen sources play an important role as major nutrients, on the growth and metabolism of microorganism present in various environment. Carbon sources particularly sugars, in general, either induce or repress growth and amylase synthesis. Simple carbohydrates such as glucose and sucrose, being easily and rapidly metabolised, caused repress product formation (Demain 1971).

However in the present study the results obtained present a very different picture particularly with glucose. Both *B.subtilis* and *A.caviae* could utilise all the carbon sources provided to secrete both α-amylase and cellulase at

significant levels although maximal production was induced by different sugars for the two enzymes. Both the strains showed identical characteristics in their preference with respect to the enzyme of interest. *B.subtilis* was reported to grew well and produce α-amylase at enhanced level in the presence of starch and maltose compared to glucose, (Saito and Yamamoto, 1975). Addition of wheat bran, maltose, glucose or lactose to the culture medium stimulated the production of α-amylase by *B.subtilis* Hashem *et al.*, (1993) . *B.stearothermophilus* synthesized large amount of α-amylase in the presence of soluble starch and maltose compared to glucose and glycerol which showed a repressing effect (Welker and Campbell, 1963). Starch supported rapid growth and α-amylase synthesis by *B.amyloliquifaciens* followed by glycerol and glucose (Coleman and Grant, 1966). *B.licheniformis* TCR DC B 13 although reorded higher growth rate in the presence of glucose, their α-amylase production was more only with starch, followed by dextrin, maltose and lactose (Bajpai and Bajpai, 1989). *B.cereus* NY 14 secreted higher amounts of α-amylase in the presence of starch, dextrin and glycogen while being inhibited in the presence of glucose Yoshigi (1985). A mutant of the above mentioned starch was found to synthesize α-amylase even

in the presence of 5% glucose (Yoshigi and Kammura, 1988). In the present study also in a similar fashion, the *B.subtilis* and *A.caviae* could grow and produce α-amylase maximally with soluble starch and maltose and at significant levels in the presence of other Carbon sources.

Presence of a metabolizable carbohydrate in the growth medium stimulated the production of cellulase by *Bacillus* strain DLG, related to *Bacillus subtilis* (Robson and Chambliss, 1984). Lactose, maltose and sucrose increased the amount of TNP-CMC degrading activity roughly 1.5 to 2 fold, whereas cellobiose and glucose stimulated enzyme production approximately 3 fold. *B.subtilis* mutagenized with N-nitroso guanidine to obtain a four-fold increase in CMCase production yielded more enzyme during growth on raffinose than on classic inducers, cellobiose or insoluble celluloses (Chan and Au, 1987). The stimulation of TNP-CMC degrading activity by *B. cereus* was reported to be stimulated in the presence of glucose (Thayer, 1978). Cellulase production by *B.firmus* was strongly inhibited by 0.2% glucose. While cellobiose or CMC induced CMCase production, other Carbon sources such as maltose, sucrose, soluble starch and xylan had no effect on

enzyme production Fukumori *et al.*,(1985). When *B.licheniformis* was grown on a variety of carbon sources, glucose supported the highest production of a CMCase (Dhillon *et al.*, 1985). Present results are in agreement with these reports that glucose could induce cellulase production by *B.subtilis* and *A.caviae.*

The secretion of α-amylase was influenced by the addition of nitrogen sources into the medium. Peptone at 0.75% concentration was found to induce the growth and α-amylase synthesis of *Lactobacillus Cellobiosus* (Sen and Chakrabarty, 1984). Bajpai and Bajpai, (1989) observed that growth and α-amylase production by *B.licheniformis* TCRDC B 13 was induced by peptone followed by meat extract, beef extract and yeast extract. Whereas, Grueninger *et.al.*, (1984) reported on higher titres of α-amylases induced with malt extract, soy grist and peptone in combination with *B. stearothermophilus.* Concentrations higher than 0.5% peptone were strongly inhibitory to production of cellulase (Mandels and Weber, 1969). Two mutant strains of *T.viride.* QM 9123 and QM 9414, grow readily and produced cellulase on many subtrates when added with ammonium as the nitrogen source

Mandels *et al.*, (1975). In the present study higher levels of enzyme production with 1% $(NH_4)_2SO_4$ addition into the medium were recorded for both the bacteria.

Nystrom and Kornuta (1975) showed that fast and reproducible growth could be obtained by increasing the inoculum volume from 1% of the culture volume to 10%. However, in the present study it was observed that increasing the inoculum size above 1% do not favour enhanced growth and enzyme production by *B.subtilis* and *A.caviae*. Probably, when the inoculum concentration is increased there is a chance for competition by the cells for nutrients as food and consequent death of cells. Hence optimal concentration at 1% is satisfactory for better results.

B.subtilis has been reported earlier to produce maximal levels of α-amylase within 18 hrs of incubation (Pazlarova, 1984). *B.coagulans*, CUMC 512 and *B.licheniformis* CUMC 305 produced maximal levels of α-amylase after 24 hrs of incubation (Medda and Chandra 1980). *B.licheniformis* TCR DC N 13 recorded maximal α-amylase production at 40-70 hrs of incubation, while the maximum growth was obtained at 20-30 hrs

of incubation (Bajpai and Bajpai, 1989). Whereas, in the present study both the strains *B.subtilis* and *A.caviae* could record maximal growth and amylase production at 24 hrs of incubation time. It was also noted that further incubation, beyond 24 hrs till 60 hrs not only failed to enhance the enzyme production but also led to a decrease in enzyme titres. However, present results are quite comparable with the reports made for *B.subtilis* (Pazlarova, 1984) with regard to α-amylase production. Relatively high cellulase activity could be obtained with stationary phase cultures in 70 hrs by *T.viride* QM 9123 (Brown *et al.*, 1975). Whereas, in the present study both strains could record maximal levels of cellulase at 48 hrs of incubation.

SSF Studies

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitorgen, minerals and as well as growth factors. The bacterial and yeast culture grow by adhering to the surface of solid substrate particles (Lonsane and Ramesh, 1990) while filamentous fungi are able to penetrate deep into the solid

substrate particles for nutrient uptake (Lonsane *et al.*, 1985). A commonly used solid substrate is wheat bran which contain 2.33% total nitrogen, 14.1% starch, 7.6% sugar and 35.2% cellulose (Ramesh, 1989). Though wheat bran is desired for SSF production of industrial enzymes and other products of interest, still there is enormous scope for other organic solid substrates which are available in plenty in nature as wastes to be exploited for this purpose. The substrates traditionally used for SSF include wheat bran, rice bran, coir pith, bagasse. In this context the present study was an attempt to explore the suitability of banana fruit stalk as solid substrate.

According to Lonsane *et al.*,(1985) the substrate selection and pretreatment is critical to the success of SSF since solid substrates such as cereal grains, by-products of cereal grains, oil-seeds, lignocelluloses, and starchy substrate used in SSF are polymeric in nature and are further characterised by their insolubility in water, and inability to penetrate by microbial cells, inaccessibility to microbial attack in the initial stages of growth and the ability to

support growth with or without fortification with few additional nutrients.

Granular starch needs some pretreatment before hydrolysis with amylases (Malathi, 1991). Cooking at high temperature (121°C) for 60 min. proved to be the most effective pretreatment method for achieving higher enzyme production with BFS. Whereas cooking at high temperature (121°C) for an extended period of 90 minutes did not favour enzyme production. This might be due to gelatination of the starch granules, present in it during which the granules lost their semicrystallinity while cooking. As a result of this change, starch pastes developed high viscosity and may form a layer surrounding the substrate particles, making them less available for microbial attack and consequent decrease in growth and enzyme yield of organism (Pandey, 1992). It has been reported that steam pretreatment of lignocellulosic residues is an effective method of enhancing the enzymatic hydrolysis of cellulose component (Mackie *et al.*, 1985; Brownell and Saddler, 1987; Clark and Machie, 1987; Ramos *et al.*, 1992 a & b).

The moisture content of the substrate is one of the critical factors influencing the outcome of using SSF and is governed by the water holding capacity of substrate, the type of end-product and the requirement of the microorganism. The results of the present investigation indicated a positive relationship between moisture content and enzyme production, for both the strains, upto their optimum moisture level (70%). The optimum moisture requirement for SSF production of α-amylase was reported as 65% for *B.licheniformis* (Ramesh and Lonsane, 1990). Reduction in enzyme yield was noticed at lower and higher moisture levels. A high moisture level leads to reduction in enzyme yield owing to the decrease in porosity, lower oxygen diffusion, increased risk of bacterial contamination, enhanced formation of aerial mycelium, reduction in gas volume, decreased gas exchange and, a change in the rate of degradation of lignin (Silman *et al.*,1979; Zadrazil and Brunnert, 1981; Lonsane *et al.*, 1985; Nigam 1990; Sandhya and Lonsane, 1994). Whereas lower enzyme production with low initial moisture content might be attributed to the sub-optimal growth, a lower degree of substrate swelling and high water tension (Lonsane *et al.*, 1985; Madamwar and Patel, 1992).

The particle size (specific surface area) is another critical factor in SSF. In solid culture the size of the substrate determines the void space which is occupied by air. Since the rate of oxygen transfer into the void space affects growth, the substrate should contain particles of suitable sizes to enhance mass transfer (Pandey, 1991). With significantly smaller particles, the specific surface area is greater but the porosity is less. This leads to low enzyme production with smaller particles. With higher particle sizes, the saturated surface area for growth is less and growth and enzyme production are correspondingly less (Muniswaran *et al.*, 1994). Similar trends were also reported with mixed particle sizes (Pandey, 1991). The particle size of corn cobs was found to have a strong influence on xylanase production. A particle size of 2-7 mm was optimal. (Purkarthofer *et al.*, 1993). Wheat bran of particle size between 0.2-0.8 cm was used for the production of α-amylase by *B.licheniformis* (Ramesh and Lonsane, 1989). Whereas in the present investigation particle size of 400μm was found to be optimal for maximal enzyme production using banana fruit satlks.

Another critical factor in SSF is pH which is not usually monitored and controlled effectively. This disadvantage, is therefore, overcome by adjusting the initial pH of the mineral medium to the desired pH level while moistening the solid substrates (Lonsane *et al.*, 1985). High enzyme production was noticed with an initial pH of 7.0 using wheat bran as substrate (Ramesh and Lonsane, 1987). Carbohydrate degradation at an initial pH of 7.2 was observed using sugarcane - press mud medium under SSF employing *Aspergillus* sp. (Sandhya and Lonsane, 1994). Initial pH of banana fruit stalk medium influenced the rate of α-amylase and cellulase production. The present results clearly enunciate the fact that variation in pH influence the efficiency of the organism independent of the type of solid substrates used.

Inoculum size controls the initial lag phase (Nystorm, 1975) as smaller inoculum lengthen the lag phase. The larger inoculum size increase the moisture content to a significant extent. The free excess liquid present in unabsorbed form led to an additional diffusional barrier together with that imposed by the solid nature of the substrate and led to a decrease in growth and enzyme

production (Muniswaran *et al.*, 1994). Observations made for inoculum size in the present study using banana fruit stalk for maximal enzyme production agree well with the facts stated above.

The usual temperature maintained in SSF system is in the range of 25-32°C and depends on the growth kinetics of the microorganism employed for the fermentation purposes (Lonsane *et al.*, (1985). In the present study maximal growth and enzyme production by both the strains was recorded at 35°C. A similar trend was reported earlier B.licheniformis for maximal α-amylase production using wheat bran during SSF (Ramesh and Lonsane, 1989). Present results adds evidence to the fact that impact of temperature is independent on the SSF process, irrespective of the type of solid substrate used.

Addition of sugar viz. maltose, glucose, sucrose and lactose at concentration up to 1% in general demonstrated an enhancement in enzyme production. Results suggested Maltose as an effective inducer of α-amylase production while glucose favoured cellulase synthesis to a significant level. However addition of these sugars at concentration above 1% level led

to a significant reduction in enzyme synthesis. This could be attributed to the end product inhibition and catabolite repression caused by the excess sugar concentration resulted due to the accumulation of free sugars present already on the banana fruit stalk plus added sugars during SSF . Hence it is inferred that for enhancing enzyme production it is sufficient to add additional carbon sources at reduced concentration. Both *B.subtilis* and *A.caviae* in fact demonstrated identical response to excess concentration of glucose and maltose, irrespective of the bioprocess whether SmF or SSF.

Addition of either ammonium sulphate or sodium nitrate enhanced α-amylase production significantly. Similar increase in the carbohydrate utilisation and reduction in fermentation time was reported for the sugar-cane press mud medium enriched with 1.8% ammonium sulphate (Sandhya and Lonsane, 1994). The enrichment of cassava fibrous waste residue with urea or ammonium sulphate, at 1% level enhanced fungal pectinases production (Budiatman and Lonsane, 1987). These results advocates nitrogen enrichment of the medium for maximal α-amylase production using banana fruit stalk. It is implied that an external nitrogen source is essential for an

increased utilisation of soluble carbohydrates towards enhancement in the enzyme yield. Though addition of beef extract, yeast extract and peptone as nitrogen sources resulted in an increase in the growth and enzyme production, they were not very effective as the inorganic nitrogen sources.

The addition of NaCl and KCl at 20mM level enhanced the α-amylase production indicating their optimal requirement for amylase synthesis. Whereas lesser concentrations of 10mMthese salts could enhance cellulase production.

The time course of fermentation was observed to have a profound effect on enzyme production. The decline in enzyme activity observed on prolonged incubation might be contributed by factors such as denaturation and/or decomposition of α-amylase as a result of interaction with other compounds in the fermented medium (Ramesh and Lonsane, 1987) or due to inactivation by proteases secreted out in the system (data not given). A similar trend was also reported by Zhu *et al.*, (1994).

In order to determine the efficiency of banana fruit stalk as solid substrates for the production of α-amylase and cellulase, a comparison was made with wheat bran as solid substrates. Results obtained in the present study advocates the use of banana fruit stalk as solid substrate since 2.4 fold increase in α-amylase and 3.4 fold increase in cellulase could be recorded compared to wheat bran. This increase in α-amylase could be attributed to the higher starch content (27.27%) in banana fruit stalk compared to wheat bran (14.1%) (Ramesh, 1989). Although the cellulose content of wheat bran (35.2%) (Ramesh, 1989) is higher than banana fruit stalk (23.85%), still, the later could induce cellulase production better than the former.

Maximal cellulolytic activities were reported with combinations of either wheat straw and wheat bran or sugar cane bagasse and wheat bran at 80:20 ratio using *T.harzianum*, at 60 hr incubation (Roussos *et al.*, 1991). Maximal production of glucoamylase was observed in the case of wheat bran mixed with corn flour at 9:1 (w/w) ratio (Pandey and Radhakrishnan, 1993). In a similar fashion, in the present study, both the strains could produce maximal α-amylase and

cellulase on mixed substrates prepared with Banana fruit stalk and wheat bran in a 4:1 ratio. Results of the present study recommended the use of mixed substrates for maximal enzyme production instead of individual ones, when higher levels of enzyme production is desired.

An attempt was also made to determine the suitability of banana fruit stalk as solid substrates by evaluating its performance under SSF in comparison with Submerged Fermentation (SmF) and Slurry Fermentation(SLF). Results of the experiments very clearly testify the suitability of banana fruit stalk as substrates for simulatenous production of α-amylase and cellulase under SSF as the production under SLF and SmF were lesser than SSF. In fact increase in enzyme yield was greater in the order SmF > SLF > SSF. Similar reports were made for *T. aurantiacus* which produced a 9 fold higher cellulase activity in SSF than in submerged cultures (Grajek, 1987), and *B.cereus* which produced higher CGTase under SSF when compared to slurry and submerged fermentation (Ramakrishna *et al.*, 1994).

Precipitation of α-amylase, isolated from *B. subtilis* CBTK 106 and *A. caviae* CBTK 185, with $(NH_4)_2SO_4$ resulted in 1.6 and 2.0 fold increase in purification respectively. The increase in specific activity after ammonium sulphate precipitation was probably related to the removal of α-amylase inhibitors in the SSF system as explained by Ramesh and Lonsane (1989) for wheat bran. They reported an increase of 16% in enzyme activity after ammonium sulphate precipitation for α-amylase produced by *B. licheniformis*.

Ramesh and Losane (1989) reported on purification of α-amylases of *B.licheniformis* representing two protein peaks on CM-cellulose column chromatography. Elution pattern of α-amylase of *Lactobacillus plantarum* A6 observed two protein peaks on DEAE cellulose chromatography (Giraud *et al.*,1993). Similar reults were obtained in the present study also with α-amylases of both the strains. The first peak without any enzyme activity is probably due to the inactivated enzyme protein as explained by Ramesh and Lonsane(1989).

Results obtained in the present study for purificaton by column chromatography on DEAE cellulose column

with sodium phosphate buffer, pH 7.2, were found to be 44.95 and 76.48 fold for *B. subtilis* CBTK 106 and *A. caviae* CBTK 185 respectively. An 8 fold increase in purification was reported with α-amylase of *B. licheniformis* on DEAE - cellulose column (Chiang *et al.*, 1979) and a 33 fold increase in purification of α-amylase from *B.licheniformis* NCIB 6346 (Morgan and Priest, 1981) and 19.5 fold increase was observed by Girand *et al.*, (1993) for *Lactobacillus plantarum* strain A6 which indicated higher increase in purification fold.

The molecular weights of bacterial α-amylase from *B. subtilis* (Junge *et al.*,1959) and *B.stearothermophilus* (Pfueller *et al.*,1970) were reported to be 48000-52700 daltons. Orlando *et al.*, (1983) reported on α-amylase of *B.Subtilis* with molecular weight of 93000 and molecular weight of *B.Subtilis* was estimated to be 65 kDa by SDS-PAGE and 54 kDa by gel filtration on TSK-gel G-3000 SW (Nishimura *el al.*, 1994). Molecular weight of α-amylases of *B.Subtilis* CBTK 106 and *A. caviae* CBTK 185 recorded approximately 62 KDa and 58 KDa respectively similar to earlier reports.

The effect of pH on the reaction rates of soluble enzymes gives information about the alteration in the behaviour of the enzymes on subjection to various conditions. The α-amylases from strains of *B.subtilis* are most active in the pH range 5-7 but have been shown to be more resistant to alkaline pH than many other enzymes (Takagi and Toda (1962); Imanshi *et al.* (1964); Kennedy and White (1979). *B.subtilis was found to produce* α-amylases with a broad pH range of 5.9-9.5 (Mosely and Keay, 1970; Qian *et al.*, 1992).

Kennedy and White (1979) reported that α-amylases of *B.subtilis* was inhibited by Tris-(Hydroxymethyl)-aminomethane above pH 5.0. These properties are in direct contrast to the α-amylases isolated from *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 and it is observed that Tris buffer did not have any adverse effect on α-amylases obtained. A broad pH activity profile with a maximum activity, over a pH range from 7-11 with maximum activity at pH 9.0 was observed in the present study.

Kennedy and White (1979) reported a novel thermostable α-amylase from *B.subtilis* with an optimum

temperature of 63°C. Ramesh and Lonsane (1989) reported on α-amylase of *B.licheniformis* M27 from WB under SSF with optimal temperature at 90°C. Qian *et al.* (1992) studied on α-amylases by *B.subtilis* had optimal activity at 75-80°C. Wind *et al.* (1994) reported on α-amylases of *B.stearothermophilus* with temperature optima at 90°C.

The present reults are well in agreement with the results of Ramesh and Lonsane (1989) and Wind *et al.*, for (1994). α-Amylases of *B.subtilis* CBTK 106 was found to be active and stable at temperatures varying from 80-100°C, with their maximal activity and stability at 80-90°C where it retains 100% residual activity. While α-amylase of *A.caviae* had a temperature optima of 70°C.

The effect of substrate concentration on the purified enzyme activity was determined at 40°C, pH 8.6 using soluble starch as a substrate. Both the strains responded positively to substrate concentration upto 1.0 % level and further increase inhibited the enzyme activity.

The Km value of α-amylase from *B.licheniformis* was 0.8 mg/ml (Chiang *et al.* 1979). The Km values of three amylases of *Bacillus* H-167 was reported by Hayashi *et al.*, (1988) as 0.35, 0.43 and 0.40 mM respectively and Ivanova *et al.* (1993) determined the Km value of α-amylase from *B.licheniformis* was 0.90 gl^{-1}.

Similar results of enzyme Km values, determined with soluble starch as substrate for *A.caviae* and *B.subtilis* were 0.50 gl^{-1} and 0.955 gl^{-1} respectively.

Hayashi (1988) reported on the effect of heavy metal ions such as Mg^{2+}, Zn^{2+},Co^{2+},Ni^{2+},Pb^{2+},Fe^{2+},Mn^{2+},Cu^{2+},Ag^{2+} at 1mM level on an alkaline malto hexose forming amylases from *Bacillus* sp. H 167 and observed that Hg^{2+} and Zn^{2+} (1mM) completely inhibited the activity to 40-60%. The inhibitory action of cations was well documented by Giraud *et al.*,(1993) who reprted that the enzyme was strongly inhibited by Hg^{2+} and no other significant activation or inhibition was observed with the other cations viz. Ba^{2+}, Cu^{2+}, Mg^{2+},Co^{2+}, Ni^{2+},Hg^{2+},Zn^{2+}, Fe^{2+}, Ca^{2+}, K^{2+}, Cd^{2+},Sn^{2+}. In contrast to these results, α-amylase of *B.subtilis* CBTK 106 was inhibited

by cations such as Ni^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} and α-amylase of *A.caviae* CBTK 185 was inhibited by all the cations except Ni^{2+}. Moderate inhibition by EDTA was reported by chiang *et al.*(1979) for α-amylase from *B.licheniformis*. Strong inhibition by N-bromosuccinimide and partial inhibition with 4-dimethyl-aminobenzaldehyde with amylase from *Lactobacillus plantarum* strain A6 (Giraud *et al.*, 1993).

Whereas the presence of TCA, acetic acid or Iodine also inhibited the relative activity of amylases of both the strains of *B.subtilis* CBTK 106 and *A.caviae* CBTK 185.

Conclusion

Based on the results of the present study it is concluded that the banana fruit stalk could be utilised as solid substrate for large scale production of industrial enzyme employing solid state fermentation. This kind of reuse and recycling of wastes would to contribute safe and economic waste management in the environment, where these wastes are continuously accumulated and causes serious pollution problems. The purified α-amylases recovered from the fermented medium was found to be thermostable and able to withstand alkaline conditions. There is scope for exploitation of both the strains of bacteria, *B. subtilis* and *A. caviae* as well as their α-amylases for industrial use if scale up studies are conducted to develop an ideal biprocess.

CHAPTER V

SUMMARY

5.1 Banana fruit stalk wastes containing high carbohydrate content was used in the present study for microbial enzyme production. Banana fruit stalks contained 89.3% moisture content, 8.7% carbohydrate, 0.8% crude fibre, 0.1% crude fat, 0.6% mineral matter and 0.5% crude protein on fresh weight basis and 27.27% starch and 23.85% cellulose on dry weight basis.

5.2 Initially the biochemical and bacteriological changes occurred during fermentation under uncontrolled conditions were studied.

5.3 Increase in protein, pH and moisture content and a sharp decline in total sugar and reducing sugar were observed.

5.4 Total Heterotrophic Bacterial population showed marginal increase from 8.9×10^8 to 12.0×10^8 and there was change in generic deiversity in the microbial flora during

fermentation. Species of *Bacillus, Pseudomonas, Aeromonas* and *Vibrio* were the main groups associated with fermentation.

5.5 Significant level of exoenzymes viz α-amylase, cellulase, and protease were recovered during fermentation under uncontrolled conditions.

5.6 All the strains isolated from fermented banana wastes were subjected to screening and two potential strains, that *Bacillus sp* and *Aermonas sp* that could produce significant levels of α-amylase and cellulase were selected.

5.7 *B.subtilis* CBTK 106 and *A. caviae* CBTK 185 were selected after secondary screening for further studies.

5.8 Both the strains were initially characterised for the optimal requirements of pH, temperature, NaCl concentration, substrate concentration, additional carbon and nitrogen sources, inoculum concentration and incubation time for maximal growth and enzyme production.

5.9 Both the strains could grow and produce enzyme optimally at pH 7.0, temperature 35°C, 1% substrate (soluble starch/cellulose) concentration, and at 1% inoculum concentration.

5.10 Both the strains were able to grow and produce α-amylase optimally at 20 mM NaCl concentration and cellulase at 10 mM.

5.11 Both the strains were able to grow and produce α-amylase maximally with maltose at 1mM level and cellulase at 1 mM level of glucose.

5.12 Higher levels of growth and enzyme production were attained with the addition of 1% (7mM) $(NH_4)_2SO_4$ into the medium.

5.13 *A. caviae* produced higher α-amylase activity (26.4 U/ml) than *B. subtilis* (24.6 U/ml) at 24 hrs of incubation. Whereas *B. subtilis* recorded high cellulase activity (0.918 IU/ml) at 48 hrs of incubation than *A. caviae* (0.82 IU/ml).

5.14 Growth studies indicated that both the strains are rapid growing bacteria. The exponential phase extended over 2.5 -10 hrs of incubation in NB and EPM for *B. subtilis*. Whereas for *A. caviae* CBTK 185 the logarithmic phase was upto 8hrs in EPM added with substrates while it extended upto 12hrs.

5.15 The generation time for *B. subtilis* CBTK 106 was calculated to be 57.6 min. in NB and 66 min. in EPM added with cellulose as substrate and 60 min. in EPM added with soluble starch, and the specific grwoth rate was determined in NB media as 0.72. Whereas the generation time for *A. caviae* was 46.2 minutes in NB and 72 minutes in EPM added with substrates (soluble starch/cellulose) and specific growth rate was determined in NB media as 0.9.

5.16 α-Amylase and cellulase production, by *A.caviae* and *B. subtilis*, using banana fruit stalk as solid substrate under SSF was carried out. Initially, the process parameters that influence the SSF were optimized.

5.17 Steaming under pressure of the solid substrate at 121°C for 60 min. enhanced the enzyme production for both the strains.

5.18 Optimal requirement for enzyme production for both the strains were optimised with operational parameters viz. moisture content, particle size, pH, incubation temperature, substrate cocnentration, additional nutrients, inoculum concentration and incubation period on maximal enzyme production.

5.19 Initial moisture content of 70% for *B. subtilis* and 65% for *A.caviae* promoted maximal α-amylase production while cellulase production was maximal at 60% moisture content for both the strains.

5.20 Both the strains showed maximal enzyme activity with 400 μM particle size, at pH 7.0, incubation temperature of 35°C, 0.5% substrate (soluble-starch/cellulose) concentration.

5.21 Addition of NaCl at 20 mM and 10mM levels were required respectively for optimal α-amylase and cellulase production by both the strains.

5.22 Addtion of 3mM maltose 2mM glucose promoted maximal α-amylase and cellulase production respectively by both the strains.

5.23 Addition of 1% (7mM) $(NH_4)_2SO_4$ into the medium enhanced maximal enzyme production with both the strains.

5.24 High titres of α-amylase and cellulase were attained at an inoculum concentration of 10% and 15% respectively for both the strains.

5.25 Incubation at 60 and 72 hrs resulted in maximal α-amylase and cellulase for both the stains.

5.26 Optimal conditions for extraction of maximal enzyme from the fermented medium was carried out. The conditions optimised included drying temperature, extraction media, pH of extraction media (buffer systems), ratio of fermented BFS to

buffer, contact temperature and contact time of fermented medium to buffer.

5.27 The maximal recovery of the enzyme for both the strains were obtained at a drying temperature of 30°C, using phosphate buffer of pH 7.2 as the extraction media, at 1:10 bran to buffer ratio, a contact temperature of 30°C and with a contact time of 60 min.

5.28 A comparative evaluation of Banana Fruit Stalk (BFS) and Wheat Bran (WB) as solid substrate for enzyme production indicates the suitability of BFS as solid substrate for enzyme production without additional substrates into the medium.

5.29 *B.subtilis* produced a maximal of 53450 U/gds of α-amylase and 2.8 IU/gds of cellulase with BFS than WB (22760 U/gds for α-amylase and 0.92 IU/gds for cellulase). A.caviae produced a maximal of 70712 U/gds of α-amylase and 2.48 IU/gds of celulase for BFS medium than WB (15880 U/gds of α-amylase and 0.82 IU/gds of cellulase). Maximal enzyme production was attained when BFS and WB was mixed at 4:1 proportion for both the strains. $10x10^4$ U/gds of α-amylase and 1.48

IU/gdscellulase respectively for *B.subtilis*, and $19x10^4$ U/gds and 1.20 IU/gds of α-amylase and cellulase for *A.caviae* respectively.

5.30 Comparative evaluation of solid state, submerged and slurry fermentation demonstrated the suitability of banana fruit stalk as substrate for simultaneous production of α-amylase and cellulase under SLF (1220 U/gds and 0.08 IU/gds for the *B.subtilis* and 1680 U/gds and 0.08 IU/gds for *A.caivae* respectively), and SmF (820 U/l and 0.009 IU/l for *B.subtilis* and 640 U/l and 0.08 IU/l for *A.caviae* respectively) were lesser than SSF (53450 U/gds and 2.80 IU/gds for *B.subtilis* and 70712 U/gds and 2.48 IU/gds for *A.caivae* respectively).

5.31 The α-amylase of both the strains were purified by $(NH_4)_2SO_4$ precipitation (60-70% saturation) and dialysis followed by ion exchange chromatography and electrophoresis.

5.32 44.95 and 76.48 fold increase in purification factor for *B.subtilis* and *A.caviae* respectively were obtained with ion exchange chromatography on DEAE - cellulose column.

Specific activity of α-amylase for *B.subtilis* CBTK 106 and *A.caviae* CBTK 185 after purification were 13500 U/mg protein and 20230 U/mg protein respectively.

5.33 The molecular weight of the α-amylase was approximately 62 kDa for *B.subtilis* and 58 kDa for *A.caviae* respectively.

5.34 The α-amylases of both the strains could demonstrate stability and considerable activity over a wide range of pH (7-11) and 100% stability exhibited at pH 8-9.

5.35 The α-amylase of both the strains were active and stable at a wide range of temperatures varying from 30-100^{o}C with 100% residual activity and stability at 80-90^{o}C for *B.subtilis* and *A.caviae* recorded maximal (95% residual activity) at 70^{o}C.

5.36 Both the strains showed maximal α-amylase activity at 10% soluble starch concentration.

5.37 The Km value for α-amylases *B.subtilis* and *A.caviae* was found to be 0.955 gl^{-1} and 0.50 gl^{-1} respectively with soluble starch as substrate.

5.38 The α-amylase of *B.subtilis* was found to be inhibited (100%) with cation viz. Ni^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} at ($0.01moll^{-1}$) level whereas *A.cavaie* was inhibited by all the cations except Ni^{2+}. Interestingly the relative activity of α-amylase of *A.caviae* was enhanced to 112 and 108% by Mn^{2+} and Ni^{2+} respectively.

5.39 The presence of TCA, Acetic acid or Iodine at ($0.01moll^{-1}$) level inhibited 100% of the relative activity of α-amylases of both the strains.

BIBLIOGRAPHY

Acland, J.D., (1971) *East African Crops*. Longman, London.

Adams,M.R.,(1978) *Trop. Sci.*, 20:11-19

Aegester, P., Dunlap, C., (1980) *Appl. and Environmental Microbiol.*, Vol 39. No.5, p.937-942

Agricultural Situation in India, 42:240, (1987) *Area and production of principal crops in India*. 1981-84 (Directorate of Economic and Statistics, Min. of Agriculture Government of India, New Delhi) 294-95, 1984.

Aker, K.C., Robinson, C.W., (1987) *MIRCEN Journal*, 3: 255-274.

* Akinyanju, J.A., Oyedeji, B.M., (1993) *Chem. Mikrobiol. Technol*, Lebesm, 15 (1/2) 21-4.

Antranikian G., (1992) Microbial degradation of starch. In: *Microbial degradation of Natural Products*, Edited by Winkelmann, G. VCH Publishers, 28-37.

A.O.A.C (1970) *Official Methods of Analysis*. 11th edition. The Association of Official Analytical Chemists, Washington, D.C.

Bailey, M.J., Markkanen, P.H.,(1975) *J. Appl. Chem. Biotechonol*, (1975) 25: 73-79.

Bajapi, P., Bajpai, P.K., (1989) *Biotechnol. Bioeng.* 33: 72-78.

Baldensperger, J., Le Mer, J., Hannibal, L., Quinto, P.J., (1985) *Biotechnol Letts*. Vol.7 No:10, 743-748.

Bliesmer, B.O., Hartman, P.A., (1973) *J. of Bacteriol.* 113: 526.

Boyer, E.W., Ingle, M.B., (1972) *J. Bacteriol.*, 110: 992-1000.

Boyer, E.W., Ingle, M.B., Mercer, G.D., (1979) *Starch*. 31: 166.

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* Original not seen

Brown, D.E., Halstead, D.J., Howard, P., (1975) p.137-153 In Symposium on *Enzymatic Hydrolysis of Cellulose*. Aulanko, Finland, March 1975, eds. Bailey, M., Enari, T.M and Linka, M.

Brownell, H.H., Saddler, J.N., (1987) *Biotechnol. Bioeng.* 29: 228-235.

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

Budiatman, S., Lonsane, B.K., (1987) *Biotechnol. Letts*, 9: 597-600.

Buonocore, V., Caporale C., De Rosa, M., Gambacorta, A., (1976) *J. Bacteriology*, 128: 515.

Campbell, L.L., (1955) *Arch. Biochem. Biophys.* 54: 154-161.

Cantwell, B.P., Sharp, P.M., Gormley, E., McConnell,D.J., (1988) *Molecular Cloning of Bacillus β-glucanases*. p.181-201 In: J-P-Aubert *et al.*, (ed) FEMS Symposium. No.43, *Biochemistry and Genetics of Cellulose Degradation*. Academic Press, New York.

Castro, G.R., Ferrero, M.A., Mendez, B.S., Sineriz, F., (1993) *Acta. Biotechnol.* 13(2): 197-201.

Champion, J (1964) *The Banana Industry and Research Development in the Caribbean*. p 217-21, Caribbean Organisation, Hato Rey, Puerto Rico.

Chandrasekaran, M., (1985) *Ph.D Thesis*. Cochin University of Science and Technology.

Chan, K.Y, Au, K.S. (1987) *Antonie Leeuwenhock* J. Microbiol. 53: 125-136.

Chang, C.M., Chang, C.J. Chen, J.P., (1993) *J. Gen. Microbiol.* 139: 3215-3223.

Chaplin, M.F., Bucke, E, (1990) *Enzyme Technology*. Cambridge University Press, Cambridge, 138-166.

Cheremisinoff, N.P., Morresi C.A., (1976), *Energy from Solid Wastes. Marcel Dekker*. INC, New York, 15-18.

Chiang,J.P., Alter,J.E., Sternberg,M.E., (1979) *Starch/Starke* 31: p.86-92.

Chung, S.L., (1976) *Ph.D Dissertation.* Louisiana State University, 137pp.

Chung, S.I., Meyers, S.P., (1979) *Developments in Industrial Microbiology.* 20: 723-32.

Clark, T.A., Mackie, K.L., (1987) *J. Wood Chem. Technol.*7: 373-403.

Clegg, K.M., (1956) *J. Sci. Food Agric.* 7: 40-44.

Coleman, G., Gant, M.A., (1966) *Nature.* 211: 306-307.

Crowther, I.C., (1979) *Tropical Products Institute Publi.G.* 122. London 18p.

Datta, M., Patel, S., Parekh, H. (1989) *J. Fermn. Technol.* 67(6): 424-6.

David, M.H., Günther, H., Roper, H., (1987) *Starch.* 39: 436.

Del Rosario E.J, Pamatong .F.V (1985) *Biotechnol. Letts,* 7(11) 819-820.

Demain, A.L., (1971) *In: Advances in Biochemical Engineering.* Vol.2: p.21. Ed. by Wingard, L.B. Springer - Verlag, Berlin.

De Pinto, J.A., Campbell, L.L.,(1964) *Science.* 146: 1064-1066.

De Pinto, J.A., Campbell, L.L., *Biochemistry* (1968 a) 7: 121-125.

De Pinto, J.A., Campbell, L.L., (1968 b)*Arch. Biochem. Biophy.* 125: 253-258.

Derkova, K., Augustin J., Krajcova, D.,(1992) *Folia Microbiol* (Prague) 37(1) 17-23 (Eng).

Dhillon, N., Chibber, S., Saxena, M., Pajni, S., Vadehra, D.V., (1985) *Biotechnol. Letts.* 7: 695-697.

Doi, R.H., Wong, S.L., Kawamura, F., (1986) *Trends in Biotechnology.* 4: 232.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F.,(1956) *Anal. Chem.* 28: 350-356.

Emanuilova, E.I., Toda, K., (1984) *Appl.Microbiol. Biotechnol.* 19: 301-305.

Encyclopaedia Britannica, (1964) *Banana.*

En Wefa, O., (1991) *Appl. Microbiol. Biotechnol.* 36: 283-284.

Fan, L.N., Lee, Y.H., Beard. move, D.H., (1981) In: *Effects of pretreatments on major structural features of cellulose and on the rate of enzymatic hydrolysis.* Bioconversion and Biochemical Engineering, Vol.1: Edited by T.K. Ghose, New Delhi, 233-259.

Farmer, J.J., Arduino, M.J., Hickman-Brenner, F.W.,(1992) *The General Aeromonas and Plesiomonas In: The Prokaryotes,*IInd edition, Vol.III, Edited by Balows, A., Trůper, G.H., Dworkin, M., Harder, W., Skhleifer, K.H., Springer-Verlag 3015-3028.

*Ffoulkes, D., *et.al.*, (1978) *Trop. Anim. Prod.* (Dominican Republic) 3: 45-50.

Fogarty, W.M., Griffin, P.J., (1973) *Journal of Applied Chemistry and Biotechnolgy.* 23: 166.

Fogarty, W.M., Kelly, C.T., (1990) *Microbial Enzymes and Bitechnology.* IInd Edn. Elsevier Applied Scienes, London.

Fogarty, W.M., Griffin, P.J., Joyce, A.M., (1974) *Process Biochemistry.* July/August, 11-24.

Folliot, M:, Marchal, J.,(1992) *Fruits* 47(6): 649-55 (Jr)

Food and Agricultural Organisation (FAO), (1971) *Monthly Bulletin of Agricultural Economics and Statistics.* 20(7/8) :9-12.

Food and Agricultural Organisation (FAO),(1993) *Production Year Book* Vol.6, No.4: United Nations Organisations, Rome, Haly, p.31.

Fujimori, H., Ohnishi, M., Hiromi, K., (1978) *Journal of Biochemistry,* Tokyo, 83: 1503.

Fukumori, F., Kudo, T., Horikoshi, K., (1985) *J. General Microbiology*. 131: 3339-3345.

Fukumori, F., Kudo, T., Sashihara, N., Nagata,Y., Ito, K., Horikoshi, K., (1989) *Gene*. 76: 289-298.

Fukumori, F., Ohnishi, K., Kudo, T., Horikoshi,K., (1987) *FEMS Microbiol. Letts*. 48: 65-68.

Fukumori, F., Sashihara, N., Kudo,T., Horikoshi,K.,(1986) *J. Bacteriol*. 168: 479-485.

Gadgoli, C., Sarang, M.S., Jolly, (1992) *Research and Industry*. Vol.37, pp.18-20.

* Galabova, D., (1969) *Acta Microbiol. Bulg.* 11: 24-29.

Gayal, S.G., Khandeparkar, V.G., Rege, D.V., (1991) *J. Food Sci. Technol*.28(1): 44-5.

Gibbson, T., Gordon, R.E., (1974)1. Bacillus In: *Bergey's Manual of Determinative Bacteriology*.Buchanan, R.E. and N.E, Gibbons, N.E. Co-eds. ed. 8,pp. 529-550 Baltimore: The Williams and Wilkins Co.

Giraud, E., Gosselin, L., Martin, B., Parada J.L., Raimbault, M., (1993) *J. Appl. Bacteriol*. 75: 276-282.

Gobius, K.S., Pemberton, J.M., (1988) *J. Bacteriol*. 170: 1325-1332.

* Goewert, R.R., Nicholas, H.J., (1980) *Nutrition Reports Int*. 22: 207-12.

Grajek, W., (1987) *Appl. Microbiol. Biotechnol*. 26: 126-129.

Granum, P.E., (1979) *J. Food Biochem*. 3: 1-12.

* Greaver, H., (1971) *Aust. J. Biol Sci*. 24: 1169-1180.

* Grootegoed, J.A., Lauwers, A.M., Heinen, W., (1973) *Arch. Microbiol*. 90: 223-232.

Grueninger, H., Sonnleitner, B., Frechter, A., (1984) *Appl. Microbiol. Biotechnol*. 19: 414-421.

* Guangying, Z., Shizhong, L., Kongrong, G., (1992) *Shipin. Yu Fajiao Gongye.* (4) 32-5 (ch).

Harrigan, W. F., Mc Cance, M.E., (1972) *Laboratory Methods in Microbiology*: Academic Press, London, New York, p.362.

* Hashem, A.M., Esaway, M.A., Abdel Fattah, A.F., (1993) *Bull. Fac. Pharm.* 31(2): 125-30 (Eng).

Hayashi, T., Akiba, T., Horikoshi, K., (1988) *Applied Microbiol. and Biotechnol.* 28: 281.

Heinen, U.J., Heinen, W.,(1972)*Arch - Microbiol.*82: 1-23.

Hesseltine, C.W., (1977) *Process Biochem.* 12: 24-27.

Hewitt, C.W., (1955) *Eup J. Exp. Agric.* 23: 11-16.

Horikoshi, K., (1971) *Agricultural and Biological Chemistry.* 35: 1783-1791.

Horikoshi, K., Nakao, M., Kurono, Y., Sashihara, N., (1984) *Can. J. of Microbiol.* 30: 774-779.

Horikoshi,K., Fukumori, F. (1988) *Modification and Expression of Alkaline the cellulase genes of Alkalophilic Bacillus strains.* p. 203-217 In: J-P-Aubert P. Beguin and J. Millet (ed) FEMS Symposium No.43, *Biochemistry and Genetics of Cellulose degradation.* Academic Press, London.

* Horinouchi, S., Nishiyam, M., Nakamura, A., Beppu, T., (1987) *Mol: Gen Genet.* 210: 468-475.

Hukomori, S., (1964) *J. Biochem.* (Tokyo) 55: 205-208.

Ihara, H., Sasaki, T., Tsuboi, A., Yamagata, H., Tsukogoshi, N., Udaka, S., (1985) *J. of Biochem.* 98: 95.

* Iizuka, M., Uenakai, K., Svendsby, O., Yamamoto, T., (1985) *J. Ferment. Technol.* 63(5): 475-477.

Imanshi, A.Y.M., Isemura, T., (1964) *J. Biochem.* 55: 562.

Ingle, M.B., Boyer, E.W., (1976) In: *Microbiology.* (D. Schlessinger, ed.) 420-426, Academic Press, New York.

Ishizaki, A., Kawauchi, H., (1988) *Agric. Biol. Chem.* 52: 2937-2940.

Ivanova, V. N., Dobreva, E. P., Emanuilova, E. I., (1993)*J. Biotechnol.* 28 (2-3): 277-289.

Jarman, C.G., Mykoluk, S., Kennedy, L., Canning, A.J., (1977) *Trop. Sci.* 19(4).

Junge, J.M., Stein, E.A., Neurath, H., Fischer, E.H., (1959) *J. Biol. Chem.* 234: 556.

Kallio, P., (1986) *Eur. J. Biochem.* 158: 491-495.

Kallio, P., Palva, A., Palva, I.,(1987) *Appl. Microbiol. Biotechnol.* 27: 64-71.

Kanno, M. (1986) *Agricultural and Biological Chemistry.* 50: 23.

Kayisu, K., Hood, L.F., (1981) *J. Food Sci.* 46: 1894-7.

Kayisu, K., Hood, L.F., Vansoest, P.J., (1981) *J. Food Sci.* 46: 1885-90.

Kawai, S.H., Okoshi, K., Ozaki, S., Shikata, K., Ara, Ito, S., (1988) *Agric. Biol. Chem.* 52: 1425-1432.

Kennedy, J.F., White, C.H.,(1979) *Starch.* 31: 93.

Kim, D.S., Kim, C.H.,(1992) *J. Microbiol. Biotechnol.* 2(1): 7-13.

Kim, J.M., Pack, M.Y., (1988) *Enzyme Microb. Technol.* 10: 347-351.

Kim, J.M., Kong, S.I., Yu, J.H., (1987) *Appl. Environ. Microbiol.* 53: 2656-2659.

Kim, C.H., Kim, D.S., (1993) *Appl. Biotechnol.*42(1): 83-94.

* Knosel, *Zentralbl Bacteriol. Parasitenkd. Infektionskr.* Hyg. Abt. II. 126: 604-609.

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

Kochhnar, S., Raniyi, D.D., (1990) *Biotechnol. Letts.* 12(5): 393.

Kokubu, T., Karube, I., Suzuki, S., (1978) *European Journal of Applied Microbiology and Biotechnology.* 5: 233.

Kreig, N.R., Holt, J.G.,(1984) *Bergey's Manual of Systematic Bacteriology.*Williams and Wilkins, Baltimore.

Kriche, J., Varma, A., Mitter, D., Mayer, F., (1994) *J. Gen Appl. Microbiol.* 40(1), 53-62 (Eng)

Krishan, T., Chandra, J.K.,(1983) *Appl. and Environmental Microbiology.* 46: 430.

Krishna, C., Chandrasekaran, M., (1995) *J. Food Sci. Technol.* (in press).

Kuhn, H. Fretzek, P.P., Lampen, J.O., (1982) *Journal of Bacteriology.* 149(1): 372.

Kumar, P.K.R., Lonsane, B.K., (1987) *Process Biochem.* 22(5): 139-143.

Kunitz, M., (1947) *J. Gen. Physiol.* 30: 291-310.

Koide, Y.A., Nakamura, T., Uozumi, Beppu. T., (1986) *Agric. Biol. Chem.* 50: 233-238.

Laemmli, U.K., (1970) *Nature.* (London) 227: 680-685.

Lahav, E., (1980) *Bibliography on Mineral Nutrition of Bananas.* International Group on Mineral Nutrition of Bananas, Agricultural Research Organisation, The Volcani Centre, Israel.

Laukevics, J.J., Apsite, A.J., Viesturs, H.E.,Tengerday, R.P., (1984) *Biotechnol Bioeng.* 26: 1465-1474.

Leach, R., (1946) *Banana Leaf Spot (Mycosphaerella musicola) on the "Gros Michel" Variety in Jamaica.* The government Printer, Kingston.

* Le Dividch, J., Seve, B., Geoffroy, F., (1976) *Ann. Zootech.* 25: 313-323.

Lee, J., Satish, J., (1993) *Biotechnol. Bioeng.* 42(10): 1142-50.

Lefrancois, L., (1970) *Fruits.* 25: 112-114.

* Lepesant, J.A., Kunsf, F., Lepsant - Kejzlarova, J. Dedonder, R., (1972) *Mol. Gen Genet.* 118: 135-160.

Leung, K.Y., Stevenson, R.M.W., (1988) *Journal of General Microbiology.* 134: 151-160.

Lii, C.V., Chang, S., Young, Y.L., (1982) *J. Food Sci.* 47: 1493-7.

Lineweaver, H., Burk, D., (1934) *Journal of American Chemistry Society.* 567, 658-666.

* Liston, J., (1973) Microbial Spoilage of Fish and Seafoods In: *G.I.A.M* Iv. S. Paulo 23-28, JULHO, p.645-660.

Ljungdahl, L.G., Eriksson, K.E., (1985) *Ecology of Microbial Cellulose degradation.* p. 237-299. In: K.C. Marshall (ed), *Advances in Microbial ecology.* Plenum Press, New York.

Lo, A.C., Mackay, R.M., Seligy, V.L., Willick, G.E., (1988) *Appl. Environ. Microbiol.* 54: 2287-2292.
Lonsane, B.K., Ghildyal, N.P., Budiatman, S., Ramakrishan, S.V., (1985) *Enz. Microbiol. Technol.* 7: 258-265.

Lonsane, B.K., Ghildyal, N.P., Murthy, V.S., (1982) *Technical Bochure, Symp. on Fermented Foods, Food Contaminants, Biofertilizers and Bioenergy.* pp.12-18, Association of Micribiologists of India, Mysore.

Lonsane, B.K., Gildyal, N.P., Budiatman, S. and Ramakrishna, S.V., (1985) *Enzyme Microbiol. Technol.* 7: 258-265.

Lonsane, B.K., Ramesh, M.V., (1990) *Advances in Appl. Microbiol.* Vol. 35, Academic Press, New York, pp.1-56.

Lonsane, B.K. (1990) *General Introductionn into Commercial Exploitation of Solid Substrate Fermentation Process.* In: Specific Solid substrate processes Monograph 2(H.W. Doelle and C. Rolz Eds) Rapid Publication of Oxford Ltd., U.K. (in press)

Lonsane, B.K., (1990) *General Introduction into Commercial Exploitation of Solid Substrate Fermentation Process.* In: Specific Substrate Process Monograph 2(H.W. Dolle and C.Rolz Eds) Rapid Publication of Oxford Ltd., U.K. (in press)

Lopez Baca, A., Gomez, J., (1992) *J. Sci. Food. Agric.* 60: 85-89.

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

Mac Gregor, A., (1988) *Journal of Protein Chemistry.* 7(4): 399.

Mac Gregor, E.A., Svensson, B., (1989) *Biochemical Journal,* 259: 145.

Mac Intyre, S., Buckley, J.T., (1978) *Journal of Bacteriology.* 135: 402-407.

Mackie, K.L., Brownwell, H.H., West, K.L., Saddler, J.N., (1985) *J. Wood Chem. Technol.* 5: 405-425.

Madamwar, D., Patel S., (1992) *World J. of Microbiol. and Biotechnol.* 8: 183-186.

Malathi, S., (1991) *Growth Characteristics of SSF.* In: Short term course on SSF, CFTRI, 17.1-17.8.

Maldonado, O., Ralz. C., Cabrera, S.S., de (1975) *J. Food Sci.* 40: 265-5.

Mallessard, R., (1971) *Fruits*: 26: 20-2.

Manning, G.B., Campbell, L.L., (1961)*J. Biol. Chem.* 236: 2952-2957.

Mandels, M., Sternberg, D., Androetti, R.E., (1975) *Growth and Cellulase Production by Trichoderma In Symposium on enzymatic Hydrolysis of Cellulose.* eds., M. Baily, T.M., Enari and M. Linko Helsinki, 12-14 March pp.81-109.

Mandels M., (1985) *Biochem. Soc. Trans.* 13: 414-416

Mandels, M., Andreotte, R.E., Roche, C.,(1976) *Biotechnol. Bioeng. Symp.* 6: 21-23.

Marriott, J., (1980) *Bananas IInd edn. Interscinece Publishers Inc.* New York.

Maruo, B., Tojo, T., (1985) *J. Gen. Appl. Microbiol.* 31: 323-328.

* Mastasuji, Eiko., Sakai Nobuaki, Matsunaga, Hiroyaki, (Showa Denko, K.K) *Jpn. Kokai Tokkyo Koho.* JP 04, 104, 788 (92, 104, 788) (cl C12 N9/42) 07 Apr 1992 Appl 90/ 223, 685 24 Aug 1990 7pp.

* Matsuno, R., Nakanishi, K., Ohnishi, M., Hiromi, K., Kamikubo, T., (1978) *Journal of Biochemistry,* Tokyo 83: 859.

Matsuzaki, H., Yamane, K., Yamagushi, K., Nagata, Y., Maruo, B., (1974) *Biochem. Biophys. Acta.* 365: 235-247.

Mc Tigue, M.A., Kelly, C.T., Fogarty, W.M., Doyle, E.M., (1994) *Biotechnol. Letts,* 16, 569-574.

Meers, J.L., (1972) *Antonie van Leeuwenhock Int. J. Gen. Mol. Micro.* 38: 585-90.

* Meredith, D.S., (1970) *Phytopath Paper II Common MyCol. Instit. Kew.* 147 pp.

Miller, G.L., (1959) *Anal. Chem.* 31: 426-428.

* Mitriciah, Granum, P.E., (1979) *J. Lebensm Unters Forsch.* 169: 4-8.

Moo-Young, M., Moriera, A.R., Tengudy, R.P., (1983) Cels, J.E., Smith, D.R., Berry and B. Kristiansen Oxford IBM Publishers, New Delhi, Vol.4, pp.117-44.

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

Mosely, M. H., Keay, L., (1970) *Biotechnology and Bioengineering.* 12: 251.

Muniswaran, P.K.A., Selvakumar, P., Charyulu, N.C.L.N., (1994) *J. Chem. Tech. Biotechnol.* 60:147-151.

Muriel, C.F., Paul, P., (1991) *Rev. Boliv. Quim.* 10(1): 31-4 (Span)

* Murray, D.B., (1959) *Trop. Agricultura. Trin.* 36: 100-7.

Nakajima, R., Imanaka, T., Aiba, S., (1986) *Appl. Microbiol. and Biotechnol.* 23: 355.

Nakamura, A.T., Uozumi, Beppu, T., (1987) *Eur. J. Biochem.* 164: 317-320.

Nigam, P., (1990) *Enzyme Microb. Technol.* 12: 805-811.

Nishimura, T., Kometani, T., Takii, H., Terada, Y., Okada, S., (1994) *Journal of Fermentation and Bioenginnering.* 78: 31-36.

Nord, C.E., Sjoberg, L., Wadstrom, T., Wretlind, B., (1975) *Medical Microbiology and Immunology.* 161: 79-87.

Nystorm, J.M., Kormuta K.A., (1975) *Symposium on Enzymatic Hydrolysis of Cellulase.* Helsinki, 12-14, March pp. 181-191.

Ogasahara, K., Imanishi, A.A., Isemura, T., (1970) *J. Biochem.* (Tokyo) 67: 65-75.

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

Park, S.H., Pack, M.Y., (1986) *Enzyme Microb. Technol.* 8: 725-728.

Patil, D.L., Magar, N.G., (1974) *J. Indian Chem. Sco.* 51: 1004.

Paul, B.K., (1949) Dutch Patent - 63, 937.

Pfuller, S.L., Elliott, W.H., (1969) *J. Biol. Chem.* 244: 48-54.

Piggot, P.J., Hoch, J.A., (1985) *Microbiol. Rev.* 49: 158-179.

Pontiveros, E.R., Alcantara, J.A., del Rosario E.J., (1978) *Philipp. J. Crop. Sci.* 3: 153-158.

Pandey, A., (1991) *Biores. Technol.* 37: 169-172.

Pandey, A., (1992) *Starch/Starke.* 44: 75-77.

Pandey, A., Radhakrishnan, S., (1993) *Process Biochemistry.* 28: 305-309.

Pazlarova, J., Baig, M.A., Votsuba, J., (1984) *Appl. Micriobiol Biotechnol.* 20: 331.

Purkarthofer, H., Sinner, M., Steines, W., (1993) *Enzyme Microb. Technol.* 15: 677-682.

* Qian, J., Hua, L., Yoa, Y.,Wang, X., (1992) *Gongye Weishengwai.* 22(5): 15-19 (Ch).

Raghava Rao, K.S.M.S., Gowthaman, M.K., Ghildyal, N.P., Karanth, N.G., (1992) *Bioprocess Engineering.* 8: 1-8.

Rajendran, S., Radha, C., Prakash, V., (1995) *Int. J. Pept. Protien Res.*

Ramesh, M.V., Lonsane, B.K., (1987) *Biotechnol. Letts.* 9 (5): 323-328.

Ramesh, M.V., Lonsane, B.K., (1987) *Biotechnol. Letts.* 9(7): 501-504.

Ramesh, M.V., Lonsane, B.K., (1989) *Biotechnol. Letts.* 11: 49-52.

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

Ramesh, M.V., Lonsane, B.K., (1990) *Appl. Microbiol. Biotechnol.* 33, 501-505.

Ramesh, M.V., Lonsane, B.K., (1991) *Biotechnol Letts.* 13: 355-360.

* Ramos, L.P., Breuil, C., Kushner, D.N., Saddler, J.N., (1992a) *Holzforschung.* 46: 149-154.

Ramos, L.P., Breuil, C., Saddler, J.N., (1992b) *Appl. Biochem. Biotechnol.* 34/35: 37-47.

Riviere, J., (1961) *Ann. Inst. Paster.* 101: 253-258.

Robyt, J.R., Ackerman, R.J., (1971) *Archives of Biochemistry and Biophysics* 145: 105.

Robyt, J., French, D., (1979) *Arch. Biochem. Biophys.* 100: 451-467.

Rose, A.H., (1980) *History and Scientific basis of Commercial exploitation of Microbial enzymes and bioconversion.* In Economic Microbiology, Vol.5, *Microbial Enzymes and Bioconversion.* (A.H. Rose Ed.) pp.1-47 Academic Press, London.

Roussos, S., Raimbault, M., Saucedo-Castandeda, S., Viniegra Gonzalez, Lonsane, B.K., (1991 a) *Micol. Neotrop.* 4: 19-40.

Roussos, S., Raimbault, M., Viniegra-Gonzalez, G., Sauedo-Castaneda, Lonsane, B.K., (1991 b)*Micol. Neotrop.* 4: 83-98.

Ramakrishna, S.V., Saswathi, N., Sheela, R., Jamuna, R., (1994) *Enzyme Microb. Technol.* 16: 1-4.

Robson, L.M., Chambliss, G.H., (1984) *Appl. and Environmental Microbiol.* 47: 1039.

Sachdev, O., Friedberg, F., (1981) *International Journal of Peptide and Proteien Research.* 18: 228.

Saito, N., Yamamoto, K., (1975) *J. Bacteriol.* 121: 848-856.

Sandhya, X., Lonsane, B.K., (1994) *Process Biochem.* 29: 295-301.

Sarkar, A., Upadhyaya, S.N., (1993) *Folia Microbiol.* (Prague) 38(1): 29-32.

Sassi, G., Ruggeri, B., Speechia, V., Gianetto, A., (1991) *Bioresources Technol.* 37(3): 259-69.

Sassi, G., Ruggeri, B., Specchia, V., Scannerini, S., Gianetto, A., (1989) *Minerva Biotechnol.* 1: 23-32.

* Sata, H., (1990) *Denpun Kagaku.* 37(4): 267-76 (Jp).

Seo, Y.S., Lee, Y.H., Pek, U.H., Kang, H.S., (1986) *Korean J. Microbiol.* 24: 236-242.

Sekiguchi, J., Takada, N., Okada, H., (1975) *J. Bacteriol.* 121: 688-694.

Sen, S., Chakrabarty, S.L., (1984) *J. Ferment. Technol.* Vol.62, No.5: 407-413.

Senez Jacques, C.(1979) *Food Nutr. Bull. Suppl.* 2(Bioconvers Org. Residues Rural Communities) 127-31 (Eng).

Seymour, B., (1993) *(Hont, Res Int Little hampton/West Sussex, UK LE 12 5KD) Biochem. Fruit Ripening.* p.1-51 (Eng) Edited by Seymour Oraham, B., Taylor, Jane, E., Tucker, Gregory, A., Champman and Hall, London, U.K.

* Shizhong, L., Guangying, Z., Kongrong, G., (1992) *Biochem. Eng 2001 Proc Asia - Pac. Biochem. Eng. Cong.* 397-400 (Eng) Edited by Fursusaki, Shintaro, Endo, Isao Matsuno, Ryuichi Springer, Tokyo, Japan.

Shantha, H., Siddappa, G., (1970) *J. Food Sci.* 35: 74-7.

Shamala, T.R., Sreekantiah, K.R. (1987) *Enzyme Microbial Technol.* 9, 97-101.

Shamala, T.R., Sreekantiah, K.R., (1988) *Enzyme Microb. Technol.* 8: 178-182.

Sharma, P., Gupta, J.K., Vadehra, D.V., Dube, D.K., (1987) *Enzyme Microb. Technol.* 9: 602-606.

Sharma, S., Mishra, I.M., Sharma, M.P., Saini,J., (1988) *Biomass.* 17: 215-263.

Shinke, R., Kunimi, Y., Nishira, H., (1975) *J. Ferment. Technol.* 53: 698-702.

Silman, R.W., Conway, H.F., Anderson, R.A., Bagley, E.B., (1979) *Biotechnol. Bioeng.* 21: 1799-1808.

Simmonds, N.W., (1959) *Banana.* Ist edn. Longman, London.

Simmonds, N.W., (1962) *The Evolution of the Banana.* Longman, London.

Simmonds, N.W., (1966) *Banana.* IInd edn. Longman, London.

Sneath, P.H.A., (1986) *In Bergey's Manual of Systematic Bacteriology.* eds. Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G., Williams, and Wilkins, Baltimore, M.D, p.1104.

Stover, R.H., (1962) *Phytopath* Paper 4 Common Mycol Instit Kew.

Stover, R.H., (1972) *Banana Common Mycol: Instit. Kew.*

Stover, R.H., Simmonds, N.W., (1987) *Bananas.* Longman Group U.K. Limited England.

Subrahmanyan, V., Lal, G., Bhatia, D.S., Jain, N.L., Bains, G.S., Srinath, K.V., Anandaswamy, B., Krishna, B.H., Lakshminarayana, S.K., (1957) *J. Sci. Food-Agric.* 8: 253-261.

Sunna, A., Hashwa, F., (1990) *Biotechnol. Letts.* 12(6): 433-438.

Stark, J.R., Stewart, Priest, F.G., (1982) FEMS *Microbiology Letts.* 15: 295.

Sternberg, B., (1976) *Biotechnol. Bioeng.* Symp. 6: 35-53.

Takagi, T., Toda, H., (1962) *J. Biochem.* 52: 16.

Takasaki, G., (1983) *Agric. Biol. Chem.* 47:, 2193-2199.

Takasaki, Y., Furutani, S., Hayashi, S., Imada, K., (1994) *J. Ferment. Bioeng.* 77(1): 94-6.

Takkinen, K., Pettersson, R.F., Kalkkinen, N., Palva, I., Soderlund, H., Kaariainen, L., (1983) *J. of Biological Chem.* 258: 1007.

Taniguchi, H., Chung, M.J., Yoshigi, N. Maruyama, Y., (1983) *Agric. Biol. Chem.* 47: 511-519.

Thayer, D.W., (1978) *J. Gen. Microbiol.* 106: 13-18.

The Wealth of India, (1962) A Dictionary of Indian Raw Materials, CSIR Publications, New Delhi. 448-470.

Thudt, K., Schleifer, K.H., Gotzi, F., (1985) *Gene.* 37: 163-169.

Tomazic, S.J., Klibanov, (1988) *Journal of Biological Chemistry.* 263: 3086.

Tyagi, P.D., (1989) *Fuel From Wastes and Weeds.* Batra Book Service, New Delhi.
Vihinen, M., Mantsala, P., (1990) *Biotechnol Appl. Biochem.* 12(4): 427-35.

Viquez, F., Lwstrete, C., Cooke, R.D., (1981) *J. Food Technology*. 16: 115-45.

Von Loesecke, H., (1950) *Bananas*. IInd edn. Interscience Publishers In., New York.

Wang, F., Gu, J., You, S., Chen, A., Fu, R., Wang, X., (1991) *Weishengwaxal Zazhi*. 11(2): 47-51. (Ch)

Ward Law, C.W., (1961) *Banana Disease* Longman, London.

Welker, N.E., Campbell, L.L., (1967) *Journal of Bacteriology*. 94: 1131-1135.

Wieringa, K.T., (1949) *Proceeding of the Fourth International Congress for Microbiology*. p.482 Rosenkilde and Bagger Copenhagen.

Wilson, R.J., (1975) *Tropical Products Institute Pub. G.* 103. London.

Wind, R.D., Buitelam, R.M., Eggink, G., Huizing, H.J, Dijkhuizen, L., (1994) *Appl. Microbiol. Biotechnol.* 41(2): 155-62.

Wiseman, A., (1985) *Hand book of Enzyme Biotechnology*. John Wiley and Sons Australia.

Wood, D.A., (1985) *Ann. Proc. Phytochem. Soc. Eur.* 25: 295-314.

* Wu, L., Tiang, L., Hu, M., Yang, Y., (1992) *Zhonggao Niangzoa*. 5: 13-15 (Ch).

* Xie, H., Wang, Y., Liu Hongxian., (1993) *Zhiwu Xueban* 35(7): 526-32 (Ch).

Yabuki, M., Mizushina, K., Amatatsu, T., Ando, A., Fuji, T., Shimada, M., Yamshita, M.,(1986) *Journal of General and Applied Microbiology*. 32: 25-38.

Yamada. K., (1977) *Biotechnol. Bioeng.* 19: 1563-1621.

Yamaguchi, K., Matsuzaki, H.,, Maruo, B., (1969) *J. Gen. Appl. Microbiol.* 15: 97-107.

Yamaguchi, K., Ueda, M., Udaka, S., (1993) *Biosci. Biotechnol. Biochem.* 57(8): 1384-6 (Eng).

Yamamoto, M., Tanaka, Y., Horikoshi, K., (1972) *Agric. Biol. Chem.* 36: 1819-1834.

Yang, S.S., (1988) *Biotechnol Bioeng.* 32: 886-890.

* Ying, G.F.P., Jin, F., Shen, Z., (1992) *Huaxue Fanying Gongeheng Yu Congyi.* 8(4): 372-8 (Ch)

Yoneda, Y., Yamane, K., Maruo, B., (1973) *Biochem. Biophys. Res Commun.* 50: 765-770.

Yoneda, Y., Maruo, B., (1975) *J. Bacteriol.* 124: 48-54.

Yoshigi, N. Chikano, T., Kamimura, M., (1985) *Agric. Biol. Chem.* 49: 3369-3376.

Yoshigi, N., Kamimura, M., (1988) *Agric. Biol. Chem.* 52: 2365-6.

Yoshiyuki, T., Seigo, F., Sachio, H., Kiohisa I., (1994) *J. Ferment Bioeng.* 77(1): 94-6.

Yuki, S., (1968) *Biochem. Biophys. Res. Commun.* 31: 182-187.

Yu, J.H., Kong, S.I, Kim, J.M., Park, Y.S., (1986) *Korean J. Appl. Microbiol. Bioeng.* 14: 529.

Yu, J.H., Kong, S.I., Kim, S.U., Kim, J.M., (1987) *Korean J. Appl. Microbiol., Bioeng.* 15: 29-33.

Yu, J.H., Park, Y.S., Kong, I.S., Yum, D.Y., Lim, H.C., Kim, J.M., (1988) *Korean J. Appl. Microbiol. Bioeng.* 16: 46-50.

Zemek, J., Augustin, J., Borriss, R., Kuniak, L., Svabova, M., Pacova, Z., (1981) *Folia Microbiol.* 26: 403-407.

Zhu, Y., Smiti, J.P., Knol, W., Bol, J., (1994) *Biotechnol Letts.* 16: 643-648.

* Zhu, C., (1989) CN 1038, 305 (Cl, C12 G3/12) 27 Dec. 1989.

Zadrazil, F., Brunert, H., (1981) *Eur. J. Appl. Microbiol Biotechnol.* 11: 183-188.

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List of Publications/Paper presented in Conferences

1. Krishna,C., Chandrasekaran, M.(1995) Economic utilisation of cabbage wastes through solid state fermentation by native Microflora. *J. Food Sci. Technol.* (in press).

2. Krishna, C., Chandrasekaran, M.,(1995) Banana wastes as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid state fermentation. (Revised for publication in *Applied Microbiology and Biotechnology*).

3. Krishna,C., Chandrasekaran, M.(1992) Biodegradation of Carbohydrate market vegetable wastes. In: *Proceedings of the 8th Carbohydrate Conference.* Eds. Raja, K.C.M., Pandey, A., Trivandrum, 18-20, Nov. p.270.

4. Krishna,C., Chandrasekaran, M. (1994) Banana wastes as substrate for amylase and cellulase production by *V. costicola* under SSF. Paper presented in the *International Conference in Microbiology* (MICON - '94) held at Mysore from Nov. 9-12.

5. Krishna, C., Chandrasekaran, M. (1994) Production of α-amylase by *A.caviae* using mixed solid substrates. Paper presented in the *International Conference in Microbiology* (MICON '94) held at Mysore from Nov. 9-19.

6. Krishna, C., Chandrasekaran, M. (1995) Evaluation of SSF, SLF and SmF for α-amylase production by *A.caviae* (CBTK 185) utilising banana wastes. Paper presented in the *Keystone Symposia on Molecular and Cellular Biology,* 1995, *Conference on Environmental Biotechnology* held at Lake Tahoe, California, USA from March 16-22 and published in *Journal of Cellular Biochemistry* (1995), supplement 19C Wiley - Liss, Inc., New York.

7. Krishna, C., Chandrasekaran, M., (1995) Utilisation of banana waste for α-amylase production employing SSF. Paper presented in the International Symposium on Development of Small and Medium Enterprises for Biotechnology Commercialisation in Developing countries, held at Manila, Philippines from July 24-28.