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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ALKALINE β-MANNANASE FROM *Bacillus brevis*

PROJECT REPORT

Submitted by

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PROJECT WORK -PHASE II

MAY 2010

This is to certify that the project entitled **"PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ALKALINE β-MANNANASE FROM *Bacillus* brevis"** is the bonafide record of project work done by **R.RAMYA Register No: 0820203010** of M.Tech during the year 2009-2010.

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DECLARATION

I affirm that the project work titled **"Production, Purification and Characterization of alkaline β-mannanase from *Bacillus brevis*"** being submitted in partial fulfilment for the award of **M.Tech. (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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TABLE OF CONTENTS

ABSTRACT

Alkaline β-mannanase producing *Bacillus brevis* was isolated from soil exhibited maximum activity. Different parameters for the production of β-mannanase were studied. Locust bean gum (LBG) and sodium nitrate as carbon and nitrogen source supported the maximum enzyme secretion. The enzyme production was growth associated. Alkaline β-mannanase (Man b) was purified to homogeneity by ammonium sulphate precipitation followed by DEAE-Sephadex column chromatography. The molecular weight of the purified enzyme (Man b) in non-denaturating PAGE (Native-PAGE) was estimated to be 15 kDa and was confirmed by activity staining. Optimum pH and temperature was found to be 9.5 and 50°C, respectively. The enzyme was most stable between pH 8.5 and 10.5 for 2h. The enzyme retained 60% of its original activity at 40-50°C for 2h and also retained 50% stablity at 60°C for 1h. N-Bromosuccinimide and $HgCl_2$ inhibited the enzyme activity completely. Silver nitrate, copper sulphate and zinc sulfate also inhibited the enzyme activity. But $CaCl_2$ and $FeSO_4$ enhanced the enzyme action. No α-galactosidase and cellulase activity was detected in the culture filtrate, but an appreciable amount of xylanase activity was found. Alkaline β-mannanase from *Bacillus brevis* can be exploited for its biotechnological applications in paper and pulp industry and animal feed industry.

Keywords: Alkaline β-mannanase, *Bacillus brevis,* Xylanase, Locust bean gum

LIST OF TABLES

LIST OF FIGURES

ABBREVATIONS

Abbreviation	Explanation
µg	Microgram
mg	Milligram
ml	Millitre
µl	Microlitre
h	Hour
L	Litre
Min	Minutes
M	Molar
nm	Nanometer
EDTA	Ethylenediamine tetracetic acid
PNPG	p-Nitrophenyl-α-D-galactopyranoside

Introduction

Enzymes are the catalytic keystone of metabolism and are the hub of concentrated worldwide research. The advent of enzymology represents an important breakthough in the biotechnology industry, with the worldwide increase in usage of enzymes. The latter half of the twentieth century saw an unparalleled expansion in our knowledge of the use of microorganisms, their metabolic products, and enzymes in a broad area of basic research and their potential industrial applications. Hydrolases such as proteases, amylases, amidases, esterases and carbohydrolases such as cellulases, hemicellulases and pectinases occupy the major share of the industrial enzyme market. Cellulases and hemicellulases have numerous applications and biotechnological potential (Bhat, 2000; Sun and Cheng, 2002; Wong and Saddler, 1992; Beauchemin *et al.,* 2001). It is estimated that approximately 20% of the more than one billion US dollars of the world's sale of industrial enzymes consist of cellulases, hemicellulases and pectinases (Bhat, 2000).

Mannan and heteromannans are a part of the hemicellulose fraction in plant cell walls. Structural analysis of plant cell wall polysaccharides has revealed that the cell wall of a dicot sp. contains three major classes of polysaccharides viz. cellulose, hemicellulose and lignin (Dekker *et al.,* 1985). Hemicelluloses are defined as those plant cell wall polysaccharides that are not solubilized by water or chelating agents but are solubilized by aqueous alkali (Selvendran and O'Neill, 1985). According to this definition, hemicelluloses include mannan, xylan, galactan and arabinan. Hemicelluloses are also defined chemically as cell wall polysaccharides that are structurally homologous to cellulose because they have a backbone composed of 1,4-linked β-D- pyranosyl residues (O'Neill and York, 2003). Hemicellulose is abundant in primary walls but is also found in secondary walls (Puls, 1997).

The major hemicelluloses in softwoods are acetylated galactoglucomannans. These consist of a backbone of β-1,4-linked mannose and glucose residues substituted with α-1,6-linked galactosyl side groups. Hardwoods also have, besides the major hemicelluloses which are substituted xylans, a minor glucomannan content. Other types of heteromannans can be found in plant seeds, e.g., locust bean gum, a galactomannan

polymer with a main chain of β -1,4-mannan with α-1,6-bound galactosyl side groups. Mannan is one of the major hemicellulose constituents of plants. Some kinds of plants such as endosperus of copra and ivory palm nuts, beans of guar, locust and coffee, and roots of konjak contain a lot of mannan. Potential uses of β-mannanase have been shown to promote pulp bleaching ability in the manufacture of kraft pulp to produce oligosaccharides from hemicellulose as one of the best growth factors for *Bifidobacterium* sp. to hydrolysis galactomannans in the massive hydraulic fracturing of oil well and to provide excellent cleaning performance on food and cosmetic stains in detergent industry.

In recent years, hemicellulases have emerged as key enzymes in the rapidly growing biotechnology industry, owing to their multifaceted properties, which find usage in a wide array of industrial applications (Hongpattarkere *et al.,* 2002). Most studies on hemicellulases have focused until now on enzymes that hydrolyse xylan, the primary constituent of hemicellulose in grasses. Enzymes that hydrolyse mannan have been largely neglected, even though it is an abundant hemicellulose, therefore the application of mannanase for catalyzing the random hydrolysis of beta-D-1,4 mannopyranoside linkages in β-1,4 mannans is as important as the application of xylanases.

1.1 Mannanases:

β-mannanase (endo-1,4- β -mannanase; mannan endo-1,4-β -mannosidase EC 3.2.1.78) is an hemicellulase enzyme that cleaves the β-1,4-mannosidic linkages in mannan, glucomannan, galacto-mannan, and galacto-glucomannan (Tipson et al., 1976). Mannan degrading enzymes are found in multiple enzyme forms (www.brenda.com, 2003). This multiplicity might result from the requirements to bind and to degrade substrates of varying complexity. Mannanases are enzymes that degrade 1,4-β and 1,6-α -glycosidic bonds of mannans. Mannanase enzymes involved in the degradation of mannans are: Mannan 1,4-β-mannobiosidase(EC 3.2.1.100), mannan endo-1,6-α-mannosidase (3.2.1.101), mannosyl- oligosaccharide 1,2-α–mannosidase(EC 3.2.1.113), α–mannosidase(EC 3.2.1.24), β-mannosidase(EC 3.2.1.25) and mannan endo-1,4-β-D-mannosidase (EC 3.2.1.78). Enzymatic degradation of the mannan backbone is affected

by endo-1,4-β-mannanases, which cleaves the chain randomly, producing mannobiose and longer manno-oligosaccharides from which mannose is released by exo-acting β-mannosidase. α-galactosidase and β-glucosidase are required for the complete breakdown of galactomannan (Halstead *et al.,* 1999).

Since mannans are found in softwood hemicellulose and in seeds as storage carbohydrates, microorganisms growing in these environments are able to produce mannan degrading enzymes. β-mannanase are produced by a number of fungi, yeasts, bacteria, marine algae, germinating seeds of terrestrial plants (Feng *et al.,* 2003; Nonogaki *et al.,* 2000; Viikari *et al.,* 1993) and by vertebrates (Yamasura & Matsumoto 1993). In fruits, β-mannanase enzyme is well associated; its activity increases during ripening, and is concentrated in the skin and outer pericarp of the fruit. In contrast, the seeds produce active enzymes, especially following germination (Banik *et al.,* 2001)

β-mannanase have numerous applications in food industry, which include fruit and vegetable maceration, brewery and wine, juice clarification, oil extraction from legume seeds and instant coffee production, in textile and laundry, animal feed, pulp and paper industry (biobleaching in soft wood kraft pulp), etc. The use of mannanases in the paper and pulp industry increased significantly with the discovery of Gubitz et al. (1997). Alkaline enzymes play a significant role in bleaching process. The term"alkaline mannanase enzyme"is meant to encompass enzyme having an enzymatic activity of at least 10%, preferably at least 25%, more preferably at least 40% of its maximum activity at a given pH ranging from 7 to 12, preferably 7.5 to 10.5.

β-mannanase are available commercially, e.g. endo-β-mannanase from megaenzyme (*Aspergillus niger*), Hemicell Feed enzyme from Hubbard Feeds Inc. (*Bacillus lentus*) or as one component of an enzyme powder such as in Mannaway from Novozymes and Procter & Gamble (recombinant *Bacillus* strain). The most successful bacterial genus used for the industrial purpose is *Bacillus* sp. *Bacillus subtilis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens Bacillus pumilus, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis, Bacillus licheniformis, and Bacillus agaradherens*

The researchers worldwide have focused their attention towards the newer microbial isolates for mannanases. Despite the availability of several reports on mannolytic microorganisms, there are only a few reviews available on alkaline mannanases.

In the present study, alkaline β-mannanase was isolated from the soil, purified and characterized to check its suitability for paper and pulp bleaching process.

Objectives

- To screen and isolate the alkaline β-mannanase producing bacteria from soil.
- To purify the β-mannanase enzyme.
- To characterize the β-mannanase enzyme

Literature Review

3.1 Mannan

Mannans and heteromannans are widely distributed in nature as part of the hemicellulose fraction in hardwoods and softwoods (Capoe *et al.*, 2000), seeds of leguminous plants (Handford *et al.*, 2003; Buckeridge *et al.*, 2000) and in beans (Lundqvist *et al.*, 2002). Hemicelluloses are copolymers of both hexose and pentose sugars. The branched structure allows hemicellulose to exist in an amorphous form that is more susceptible to hydrolysis. Within biomass, mannans or the hemicelluloses are situated between the lignin and the collection of cellulose fibres underneath. Consistent with their structure and side group substitutions, mannans seem to be interspersed and covalently linked with lignins at various points while producing a coat around underlying cellulose strands via hydrogen bonds, but as few H-bonds are involved they are much more easily broken down than cellulose. The mannan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose may be important in maintaining the integrity of the cellulose in situ and helps to protect the fibers against degradation to cellulases (Puls and Schuseil, 1993).

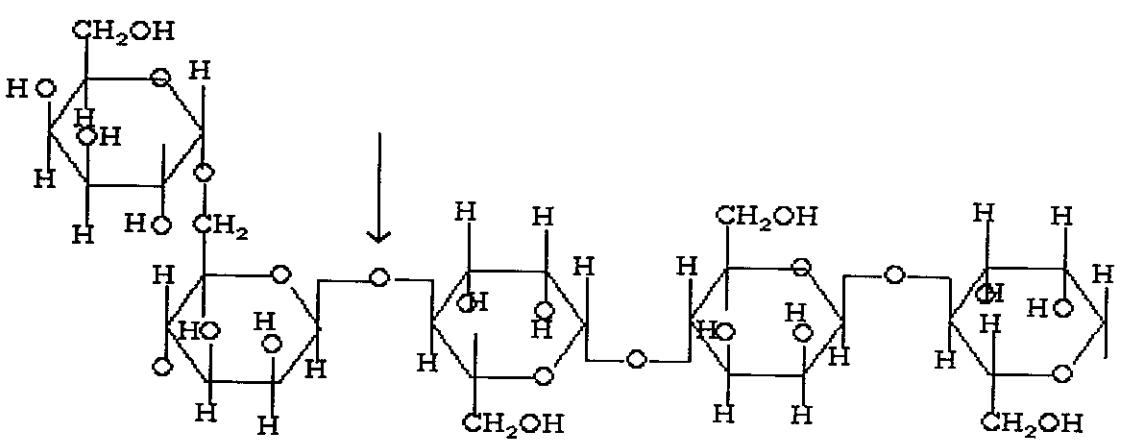


Fig. 2.1: Diagram of a mannan chain. Mannans are 6-carbon sugars which form long polysaccharide chains. The mannans are held together by β -(1,4) bonds (arrow) which connect carbon 1 of one mannan to carbon 4 of an adjacent mannan. It is this bond that is the target of endo-β-mannanase (Bewley, 1997)

Mannan is the predominant hemicellulosic polysaccharide in softwoods from gymnosperms, but is the minor hemicellulose in hardwood from angiosperms (Puls, 1997). Unsubstituted β-1,4-mannan composed of a main chain of β-mannopyranose residues(Fig. 1), is an important structural component of some marine algae (Yamasaki etal., 1998) and terrestrial plants such as ivory nut (Chanzy *et al.,* 2004) and coffee bean (Nunes *et al.,* 2006). It resembles cellulose in the conformation of the individual polysaccharide chains, and is water insoluble.

Hardwood mannans are composed of β-1,4-linked mannopyranose and glucopyranose units, whereas softwood contains two different types of acetylated galactoglucomannans. These consist of glucose, mannose and galactose in the ratio 1:3:1 and 1:4:0.1 respectively (Lundqvist *et al.,* 2002). In leguminous seeds, water soluble galactomannan is the main storage carbohydrate, comprising up to 20% of the total dry weight (McCleary, 1988). It has a α-galactose linked at the O-6 position of some mannose residues and may also have some β-D-glucose residues incorporated in the backbone. Furthermore, the mannose residues can be acetylated to various degrees at the C-2 and C-3 positions. The galactomannans from different leguminous taxonomic groupings differ in their degrees of galactose substitution and M:G ratios between 1.1:1 (high galactose) and 3.5:1 (low galactose) are encountered. The high degree of galactose substitution of the (1-4) β-D-mannan in galactomannans is clearly sufficient to prevent the chain aggregation that leads to insolubility and crystalline order in the mannans and glucomannans. The mannose: galactose ratio (M:G) is key in determining the amount of intermolecular association called hyperentanglements. Without any galactose side chains, mannan backbone will aggregate due to intermolecular interaction between the unbranched parts of heteromannans.

The substrate used routinely for the study of mannanases is galactomannan from locust bean gum (*Ceratonia siliqua*) with a mannose: galactose ratio of 4:1 (De Nicolas-Santiago *et al.,* 2006). In addition, ivory nut (Phytelephas macrocarpa) mannan, an unbranched β-1,4-linked mannan homopolymer and manno-oligosaccharides (mannobiose, mannotriose, mannotetraose and mannopentoase), can also be used as substrates in the hydrolysis (Stoll *et al.,* 2005).

3.2 Enzymatic Hydrolysis of Mannan

The main component of mannan is D-mannose, a six carbon sugar, but due to the heterogeneity and complex chemical nature of plant mannans, its complete breakdown into simple sugars that can be readily used as energy sources by particular microorganisms, the synergistic action of endo-1,4-β-mannanases (E.C 3.2.1.78, mannan endo-1,4-β-mannosidase) and exoacting β-mannosidases (E.C 3.2.1.25) is required. Additional enzymes, such as β- glucosidases (EC 3.2.1.21), alpha-galactosidases (EC 3.2.1.22) and acetyl mannan esterases (Tenkanen *et al.,* 1998) are required to remove side chain sugars that are attached at various points on mannans. A galactomannan structure whose glycosidic bonds are hydrolyzed by two enzymes viz. endo-1,4-β-mannanases and alpha-galactosidases is shown in Figure 2. FIGURE 2 Scheme of Enzymatic Action on Galactomannan. The β-(1-4) linked polymannose chain is substituted with alpha-(1-6) linked galactose (GAL) residues. The arrows represents the glycoside links recognized by β-mannanase. B-1,4-mannanases are endohydrolases that cleave randomly within the 1,4-β-D mannan main chain of galactomannan, glucomannan, galactoglucomannan, and mannan (McCleary and Matheson, 1986). Apart from their ability to hydrolyze different mannans, some β-D-mannanases have also been reported to transglycosylate manno-oligosaccharide substrates (Harjunpaa *et al.,* 1995; Schoder *et al.,* 2004).

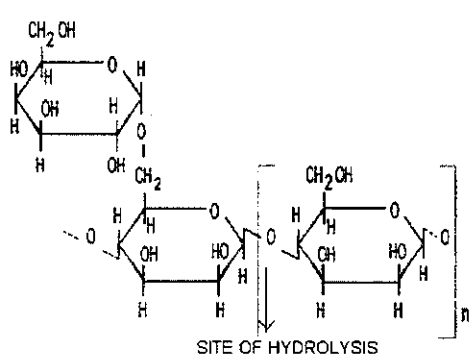


Fig. 3.2: Hydrolysis Site in mannan

Hydrolysis of these polysaccharides is affected by the degree and pattern of substitution of the main chain by β-D-galactosyl residues in galactomannan and

galactoglucomannan (McCleary, 1979) and by the pattern of distribution of D-glucosyl residues within the main chain in glucomannan and galactoglucomannan. In glucomannan, the pattern of distribution of O-acetyl groups may also affect the susceptibility of the polysaccharide to hydrolysis. Like β- glucosidases in the cellulase system, β-mannosidases are essential for the complete hydrolysis of plant heteromannans. They convert the manno-oligosaccharides produced by β-mannanases to mannose (Franco *et al.,* 2004). The galactose release from softwood pulp is enhanced by the presence of mannanase in combination with alpha-galactosidase (Clark *et al.,* 2000). The main products obtained during the hydrolysis of mannan by β-mannanases are mannobiose and mannotriose. β-mannanases from *Aspergillus tamarii*(Civas *et al.,* 1984), *Trichoderma reesei* (Stalbrand *et al.,* 1993) and *Aspergillus niger* (Ademark *et al.,* 1998) all produced mainly mannobiose and mannotriose and traces of higher oligosaccharides.

The pattern of galactomannan hydrolysis and the apparent subsite- binding requirements have been interpreted from structural analysis by NMR of isolated hydrolysis products from glucomannan and galactomannan incubated with *Aspergillus niger* β-mannanase. It is suggested that the enzyme has five substrate binding subsites- alpha, β, gamma, delta and epsilon, which is equivalent to -3, - 2, -1, +1, and +2, according to the nomenclature given by Davies (1998). Binding to at least four subsites is required for efficient hydrolysis. Substitution of the substrate monomers at two of the subsite positions restricted hydrolysis, most likely by preventing binding (McCleary and Matheson, 1986). The hydrolysis of the pine craft pulp by the *Trichoderma reesei* β-mannanase was also studied (Tenkanen *et al.,* 1997). Judging from this study, *Trichoderma reesei* enzyme also appears to be restricted by galactosyl residues in a similar way to the *Aspergillus niger* enzyme.

3.3 Sources of Mannanases

Mannanases are ubiquitous in nature and are elaborated by a compendia of microorganisms largely isolated from natural environments. A vast variety of bacteria, actinomycetes, yeasts and fungi are known to be mannan degraders (Talbot and Sygusch,

1990; Puchart *et al.,* 2004). Among bacteria, degradation is mostly confined to gram-positives, including various *Bacillus* species (Yanhe *et al.,* 2004; Sun *et al.,* 2003) and *Clostridia* species (Kataoka et al. 1998; Perret *et al.,* 2004; Nakajima and Matsuura, 1997). *Bacillus* sp. include *Bacillus subtilis* KU-1 (Zakaria *et al.,* 1998), *Bacillus subtilis 5H* (Khanongnuch *et al.,* 1998), *Bacillus subtilis* (Mendoza *et al.,* 1994) , *Bacillus* sp.W-2(Ooi *et al.,* 1995), *Bacillus stearothermophilus* (Ethier *et al.,* 1998), *Bacillus amylolequifaciens* (Mabrouk *et al.,* 2008), *Bacilllus pumilus* (Alberto *et al.,* 1990) However, a few strains of gram negative bacteria, viz. *Vibrio* (Tamaru etal., 1997, *Pseudomonas* (Braithwaite et al., 1995) and Bacteroides (Gherardini and Salyers, 1987) have also been reported. In addition, a few thermophiles and extremophiles belonging to genera *Bacillus, Caldocellum, Caldibacillus, Rhodothermus*, have also been described (Hatada et al., 2005; Morris *et al.,* 1995; Sunna *et al.,* 2000; Politz *et al.,* 2000). Besides these, actinomycetes from the Streptomycetes group, viz. *Streptomyces galbus* (Kansoh and Nagieb, 2004), *Streptomyces lividans* (Arcand *et al.,* 1993), Actinobacteria group, viz. *Cellulomonas fimi* (Stoll *et al.,* 1999) and the Actinoplanetes group viz. *Thermomonospora fusca* KW3 (Hilge *et al.,* 1996) are described as mannan degraders with an ability to act on a wide variety of mannan substrates. The most mannolytic group among fungi belongs to genera *Aspergillus, Agaricus, Trichoderma, Sclerotium* (Huang *et al.,* 2007; Chen *et al.,* 2007; Tang et a., 2001; Franco *et al.,* 2004; Sachslehner *et al.,* 2000).

Thus the property of mannolysis is widespread in the microbial world. Mannanases of microbial origin have been reported to be both induced as well as constitutive enzymes and are usually being secreted extracellularly into the medium in which the microorganism is cultured. The bacterial mannanases produced by *Sporocytophaga coccoids* and *Aerobacter mannolyticus* were found to be intracellular (Dekker and Richards, 1976). Extracellular mannanases are of considerable commercial importance, as their bulk production is much easier. Mannanases are also produced in higher plants (Shimahara *et al.,* 1975; Marraccini *et al.,* 2001) and animals (Yamaura *et al.,* 1993, 1996). Recently, β-mannanase from *Cocos nucifera* isolated (Soumya *et al.,* 2010) Although a number of mannanase-producing bacterial sources are available, only a

few are commercially exploited as wild or recombinant strains, of these, the important ones are: *Bacillus sp., Streptomyces, Caldibacillus cellulovorans, Caldicellulosiruptor* Rt8B, *Caldocellum saccharolyticum*. (Zhang *et al.*, 2006; Hatada *et al.*, 2005; Morris *et al.*, 1995; Sunna *et al.*, 2000).

3.3.1 Alkaline β-mannanases from *Bacillus* sp.

Most of the β-mannanases were neutral or acidic. Alkaline β-mannanases have not been intensively investigated, although the properties of alkaline β-mannanase provide obvious advantages for enzyme to be used in the applications such as the manufacture of kraft pulp and detergent industry. In addition, hemicellulose swells better in alkaline condition, in that hydrolysis of hemicellulose using alkaline enzyme is more effective. Only β-mannanase from *Bacillus* sp. are alkaline. Some of the alkaline β-mannanases are shown in Table 3.1 .

S.No.	*Bacillus* sp.	Optimum pH	References
1.	Thermostable alkaline *Bacillus* sp. JB-99	10	Virupakshi *et al.*, 2005
2.	Alkaline *Bacillus* sp. N16-5 (Man A)	9.5	Ma *et al.*, 2004
3.	*Bacillus licheniformis* THCM3.1 (ManBL3.1)	9.0	Kanjanavas *et al.*, 2009
4.	Alkaline *Bacillus* sp. (M1, M2, M3)	9, 9, 8.5	Akino *et al.*, 1988
5.	Thermostable *Bacillus* sp. AM001	8-9	Akino *et al.*, 1996

Table 3.1: Alkaline β-mannanases sources

3.4 Family Classification and Modularity of Mannanases

Mannanases are glycoside hydrolases that degrade mannans and heteromannans. Glycoside hydrolases (EC 3.2.1.78) are a widespread group of enzymes that hydrolyze the glycosidic bonds in oligo- and polysaccharides. Due to the complicated structures of the carbohydrates with different combinations in nature, a large number of enzymes with different substrate specificities are required. Glycoside hydrolases from various sources were classified into different families based on their amino acid sequence similarities (Henrissat and Bairoch, 1993, 1996). The basic principle behind the family classification is that the family membership of a particular enzyme can be defined from its sequence alone and there is a direct relationship between sequence and folding similarities. Structure conservation was shown to be much stronger than amino acid conservation. Families can thus be grouped into "clans" according to their three-dimensional structure (Henrissat, 1990). So far there are a total of 110 families and some of them are well studied. The β-mannanase sequence comparison studies permit assignment of these enzymes to either glycoside hydrolase family 5 or 26.

Family 5 comprises several bacterial mannanases including *Caldocellum saccharolyticum*, *Caldibacillus*, *Vibrio* species, fungal mannanases from *Aspergillus aculeatus, Trichoderma reesei and Agaricus bisporus* and eukaryotic mannanase from *Lycopersicon esculentum* and *Mytilus edulis*. Family 26 comprises mannanases from *Bacilli* sp., *Cellvibrio japonicus*, *Pseudomonas fluorescens* and *Rhodothermus marinus*. Thus both bacterial and eukaryotic mannanases have been annotated to family 5. With the exception of a few anaerobic fungi, the mannanases in family 26 are of bacterial origin. In some cases, mannanases from the same genus have been classified in different families; β-mannanases from different strains of *Caldocellulosiruptor saccharolyticus* have been classified in both families 5 and 26 (Gibbs *et al.*, 1996), and multiple β-mannanases in *Cellvibrio japonicus* have been classified in both families 5 and 26 (Hogg *et al.*, 2003). Besides this, β-mannanases from different Bacilli species are also found in both families (Sygusch *et al.*, 1998; Hatada *et al.*, 2005). Some glycoside hydrolases are multifunctional enzymes that contain catalytic domains that belong to different GH families like *Paenibacillus polymyxa*. A cel44C-man26A gene was cloned from this

endophytic strain. This gene encodes for a protein that contains a glycoside hydrolase family 44 (GH44) catalytic domain and a glycoside hydrolase family 26 (GH26) catalytic domain. The multifunctional enzyme domain GH44 possesses cellulase, xylanase, and lichenase activities, while the enzyme domain GH26 possesses mannanase activity (Han *et al.*, 2006).

Most mannanases often display a modular organization and usually consist of two-domain proteins (Henrissat etal, 1995; Perret *et al.*, 2004). These proteins generally contain structurally discrete catalytic and non-catalytic modules (Warren *et al.*, 1996). B-mannanases in both families belong to the 3-D structure group (β/alpha) fold catalytic module characteristic of clan Glycoside hydrolase-A (Stoll *et al.*, 2005). The most important non- catalytic module consist of carbohydrate binding module (CBM) which facilitate the targeting of the enzyme to the polysaccharide, By analogy to the glycoside hydrolases, CBMs are classified into families based on sequence similarities and *t*hee dimensional structures (Boraston *et al.*, 2004). Presently, 49 families of CBMs have been reported and a number of these families are closely related and consequently grouped into superfamilies or clans. A comprehensive classification of mannanases and other hydrolases is available at http://afmb.cnrs-mrs.fr/CAZY. Amongst β-mannanases from aerobic fungi, the enzymes from *T. reesei* and *A. bisporus* are composed of a family 5 catalytic module linked to a family 1 CBM (Tang *et al.*, 2001; Yague *et al.*, 1997).

Some bacterial β- mannanases from families 5 and 26 have more complex structures: the C. fimi β-mannanase Man26A contains both a mannan binding family 23 CBM, a putative SLH-module and a module of unknown function (Stoll *et al.*, 1999, 2000; Stalbrand *et al.*, 2006). The family 5 β-mannanase from *Thermoanaerobacterium polysaccharolyticum* also contain a SLH-module and, in addition, two internal family 16 CBMs (Cann *et al.*, 1999). Several β-mannanases from anaerobic bacteria and fungi contain dockerin modules, which attach the β- mannanases either to the microbial cell surface or to multienzyme complexes such as the cellulosome (Shallom and Shoham, 2003; Halstead *et al.*, 1999; Tamaru and Doi, 2000; Perret *et al.*, 2004).

The family 26 β-mannanases from *Caldocellulosiruptor saccharolyticus* contains two family 27 mannan-binding modules (Sunna *et al.,* 2001) and from *Paenibacillus pofymyxa* contain a fibronectin domain type 3, CBM family 3 in addition to two catalytic domains (Han *et al.,* 2006). A comparison of the modular structure of different mannanases reveals CBMs have been predicted for several β-mannanases from family 5 and family 26. However, only in a few cases has the binding been experimentally confirmed. In addition to Man5A, family 1 CBMs have been indicated in die fungal β-mannanases from *Agaricus bisporus* (Tang *et al.,* 2001; Yague *et al.,* 1997). However, the β-mannanases from *Aspergillus aculeatus* and *Aspergillus niger* appear to lack CBMs (Ademark et al., 1998; C*h*istgau *et al.,* 1994).

Some other family 5 β-mannanases have CBMs that have been classified into family 3, 16 and family 35. Moreover, some family 26 β-mannanases have family 23, 27 and 35 CBMs, which have been shown to bind to mannan (Stoll *et al.,* 2000; Sunna *et al.,* 2001). However, many β-mannanases from both families 5 and 26 appear to be sole catalytic modules. Thus, at present, it is difficult to establish any obvious patterns in modularity among β-mannanases. It clearly appears as if several different strategies in mannan-hydrolysis subsist. Henrisat *et al.,* (1998) proposed a scheme for designating enzymes that hydrolyze the polysaccharides in cell walls of plants.

3.5 Purification of β-mannanases:

Most purification schemes have been made use of traditional biochemical methods to purify β-mannanases. This includes precipitation, ion exchange, size exclusion, c*h*omatofocusing and hydrophobic interaction c*h*omatographies. Other works separate the proteins by their isoelectric point, and most recently recombinant fused β-mannanases have been purified using immobilized metal affinity c*h*omatography (IMAC).

A purified thermostable β-mannanase from *Bacillus stearothermophilus* enzyme was a dimmer of 162kDa with identical subunits (Talbot & Sygusch 1990). β-mannanase from *A. oryzae* was purified to about 90.7 fold with an activity yield of 10.2% (Regalado

et al., 2000). Low molecular weight β-mannanase was purifed among three β-mannanases from *Pencillium occitanis* to 25 fold and molecular weight was 18kDa (Blibech *et al.,* 2009). β-mannanase from *Aspergillus oryzae* was purified to about 10 fold (Abdel *et al.,* 2009). β-mannanase of *Bacillus licheniformis* was purified to about 33 fold with the recovery of 47% (Zhang *et al.,* 2000). β-mannanase from Hyperthermophilic Eubacterium *Thermotoga neapolitana* 5068 was purified with cumulative fold 4.8 with cumulative yield of about 13.6% (Duffaud *et al.,* 1997). 4000 fold purification was done in β-mannanase from *Aeromonas* sp.F-25 (Araki *et al.,* 1982).

3.6 Characterization of β-Mannanases:

3.6.1 Physicochemical characterizations:

Fungal β-mannanases generally have acidic isoelectric points and molecular masses of about 40-110kDa. Optimum pH of all enzymes reported until now range from 2.9-10 which is related to natural habitat of the organism studied. Optimal temperature reported for β-mannanase ranges from 40° to 90°C. No archeal β-mannanase has been described so far (Table 2)

3.6.2 β-mannanase enzyme activity inhibition:

Generally metal ions have been shown to be relevant in enzyme activity. It has been reported (www.brenda.com) that some metal cations like Ag^{+}, Cr^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+} inhibited β-mannanase activity. Also Cu^{2+}, Fe^{2+} and Mg^{2+} enhanced the enzyme activity (Table 2).

Organism	MW (kDa)	Optimum temperature (°C)	Thermal stability (°C)	Optimum pH	pH stability	References
Vibrio sp.(MA-138)	49	40		6.5		Tamaru *et al.*, 1995
C.cellulovorans	30.7	80		6.0		Sunna *et al.*, 2000
E.coli (recombinant)	116	65		5.8		Cann *et al.*, 1999
S.cerevisieaei (recombinant)	50	60	<50	2.9	4.0-6.0	Guibitz *et al.*, 2000
A.niger	46.5	40	50	3.0	3.5	Megazyme
A.oryzae	110	50-55	<70	6.0	3.5-4.5	Regalado *et al.*, 2000
M.edulis	39	45	<40	5.2	7.8	Xu *et al.*, 2002
Bacillus licheniformis THCM3.1 (ManBL3.1)	40	70	45(48*hs*)	9	7(48*hs*)	Kanjanavas *et al.*, 2009

Alkaline *Bacillus* sp. N16-5 (ManA)	55	60	60 (48*h*s)	9.5	9(48*h*s)	Ma *et al.*, 2004
Bacillus sp. M1	58	60	60(30min)	9	8-9	Akino *et al.*, 1998
M2	59	60	60(30min)	9	8-9	
M3	42	65	65(30min)	8.5	7-9	
Bacillus subtilis	38	55	55(10min)	5	4-9	Mendoza *et al.*, 1994
Bacillus sp. W-2	40	70	60(1*h*)	7	5-10	Ooi *et al.*, 1995
Rhodothermus marinus (Man26A)	113	85	70(1*h*)	5.4	-	Politz *et al.*, 2000
Thermatoga neapolitana 5068	65	92	90-92 (34*h*s)	7.1	-	Duffaud *et al.*, 1997
Enterococcus casseliflavus(M-2)	142	50	40 (30min)	6	4-7	Oda *et al.*, 1993
Bacillus subtilis KU-1	39	50-55	60(1*h*)	7	4.5	Zakaria *et al.*, 1998
Bacillus subtilis 5H	37	55	45(1*h*)	7	6-7.5	Khanongnuc *et al.*, 1998

Table 3.2: Enzyme characterization studies

3.7 Detection of β-Mannanase

There are a variety of methods, such as viscometry, spectrophotometry and gel diffusion, that have been used in the past to detect the presence of endo-β-mannanase in an extract (Downie *et al.,* 1994). Each of these assays' methods have advantages and disadvantages, which were assessed before commencing this research.

a) Viscometry method

The viscometry assay assumes that galactomannan substrates suspended inbuffer form viscous solutions, particularly at higher concentrations of substrate. Theviscometry assay then measures changes in flow rate of viscous galactomannan solutions over time, relying on the action of the endo-β-mannanase to cleave the internal bonds in the galactomannan chains. As the enzyme cleaves the mannan bonds, the viscosity of the solution decreases and the time taken for a specific volume to drain from a pipette also decreases. It is this change in flow rate, when compared to a standard curve, which can be used to quantitatively determine the activity of the enzyme in solution (Tampion, 1972). While this assay is able to detect very low levels of activity, it has the disadvantage of being very laborious and leading to low *th*oughput. Also, because the enzyme activity is being expressed in arbitrary units there is no direct connection between the measured values and the quantity of substrate which has been hydrolyzed (Downie *et al.,* 1994).

b) Spectrophotometry method

Spectrophotometry is another common method to determine the activity of endo-β-mannanase, either measuring an increase in reducing units over time or the increase of an ethanol-soluble dye released from high *Mr* coloured substrates (Downie *et al.,* 1994). The ethanol-soluble dye method involves dyeing a galactomannan substrate with Remazol Brilliant Blue then adding the enzyme to the dyed product. After a specific incubation period, the soluble dye is separated from the insoluble dyed substrate by filtration, and the amount of dyestuff liberated in the separated solution is determined spectrophotometrically (McCleary, 1978). However,the enzyme activity (Downie *et al.,* 1994).

Another type of spectrophotometric assay measures the increase in reducing units over time. An enzyme extract sample is mixed with a galactomannan substrate and

allowed to react for a set amount of time. During this time, any endo-β-mannanase present in the extract hydrolyzes the mannan chains into smaller mannose fragments. These mannose fragments are reducing sugars and will reduce an oxidizing agent, often 3,5-dinitrosalicylic acid. The mannose fragments react to produce 3-amino-5-ditrosalicylic acid which is a dark red-brown colour. This colour is measured spectrophotometrically (McCleary, 1988). The darker the colour, the more reducing sugars are present, and therefore the greater the enzyme activity. The major disadvantage with this assay is that it yields nonlinear results and is prone to interference from other enzymes found in crude extracts, which may also yield reducing sugars (Downie *et al.*, 1994).

c) Diffusion method

More recently, the most common method of determining mannanase activity in a crude extract has been the Congo red dye diffusion assay. It is possible to do many assays simultaneously, is quantitative and there is no interference by other enzymes. The Congo red dye gel diffusion assay relies on the specificity of Congo red dye in binding to a range of mannan polysaccharides. The galactomannan substrate iss uspended in a 0.7% agar gel and extracts are inoculated into wells bored into the gel. As the extract in the wells diffuses out *th*ough the gel, the galactomannan substrate ish ydrolyzed. Congo red dye binds to intact mannan chains and not to the hydrolyzed mannose fragments. This creates a clear zone, the diameter of which is proportional to the enzyme activity present. This assay can be used to quantify endo-β-mannanase activity from a wide range of sources, is extremely sensitive and produces linear results (Downie *et al.*, 1994). These advantages, as well as the ability to perform many assays simultaneously, made this method of quantification ideal for this research.

3.8 Applications:

There are many applications for mannanases in the industrial processes. The worldwide requirement for enzymes for individual applications varies considerably. Mannanases are used mainly for improving the quality of food, feed and aiding in enzymatic bleaching of softwood pulps in the paper and pulp industries. There is a

paucity of knowledge about the roles that govern the diverse specificity of these enzymes. (Howard *et al.,* 2003; Galbe *et al.,* 2002; Comfort *et al.,* 2004).

a) Paper and Pulp Industry

The most potent application of mannanase is consistent with its potential use in enzymatic bleaching of softwood pulps. The extraction of lignin from wood fibers is an essential step in bleaching of dissolving pulps. Pulp pretreatment under alkaline conditions hydrolyzes hemicelluloses covalently bound to lignin and thus facilitates subsequent removal of lignin. There is a drawback to alkaline treatment of wood pulps, however, in that it creates an environmental pollution problem (Hongpattarakere, 2002). The alternate use of mannanases equally facilitates lignin removal in pulp bleaching and yields results comparable to alkaline pretreatment (Cuevas et al., 1996). Consequently, to substitute within a pulp-bleaching sequence an enzymatic pretreatment for the ultrahot alkaline extraction stage (Clarke *et al.,* 2000) offers the possibility of significant reduction in environmental pollution and thus is of considerable interest to the pulp and paper industry. To be feasible, however, enzymatic bleaching requires that hemicellulase treatment not impair pulp quality by attacking cellulose fibers. Softwoods from which the majority of pulps are derived contain as much as 15 to 20% hemicellulose in the form of galactomannan (Suurnakki *et al.,* 1996). Mannanases having substrate specificities for galactomannan constituents would make excellent candidates for use in enzymatic bleaching of softwood pulps (Gubitz *et al.,* 1997). Moreover, pulping is best carried out at elevated temperatures, thermophilic mannanases could offer significant advantages over mesophilic mannanases in terms of their higher intrinsic stability and catalytic efficiencies at such elevated temperatures (Yanhe *et al.,* 2004). Mannanase is useful in chlorine- free bleaching processes for paper pulp (chemical pulps, semichemical pulps, mechanical pulps or kraft pulps) in order to increase the brightness, thus decreasing or eliminating the need for hydrogen peroxide in the bleaching process (Tenkanen *et al.,* 1997).

b) Hydrolysis of Coffee Extract

Different mannanase preparations are used for the hydrolysis of coffee mannan, thus reducing significantly the viscosity of coffee extracts. Mannan is the main polysaccharide component of these extracts and is responsible for their high viscosity, which negatively affects the technological processing of instant coffee. Mannanase may also be used for hydrolyzing galactomannans present in a liquid coffee extract, preferably in order to inhibit gel formation during freeze drying of the (instant) coffee. Coffee mannan is isolated from green defatted beans by delignification, acid wash and subsequent alkali extraction with a yield of 12.8%. Additionally, coffee extract polysaccharides are separated by alcohol precipitation and are found to form nearly half of the coffee extract dry weight. These isolated mannans as well as the mannans in the coffee extract are efficiently hydrolyzed by the mannanase, which resulted in significant viscosity reductions (Sachslehner *et al.,* 2000; Nicolas *et al.,* 1998). Concurrently, the reducing sugar content increased continuously due to the release of various mannooligosaccharides including mannotetraose, mannotriose, and mannobiose. Both a partially purified, immobilized and a soluble, crude mannanase preparation are successfully employed for the degradation of coffee mannan (Nunes *et al.,* 1998, 2006).

c) Use in Detergent Industry

Application of carbohydrases in detergents is well known. Amylases and cellulases being the most common enzymes. Recently, alkaline mannanases stable to constituents of detergents have found application in certain laundry segments as stain removal boosters. Mannanases hydrolyze mannan containing materials like gums (galactomannans, glucomannans and guar gum). These gums are used worldwide as a thickener or stabiliser in many types of household products and foods including ice-creams, BBQ sauces, hair styling gels, shampoos, conditioners and toothpaste (Wong and Saddler, 1992). Stains containing mannan are generally difficult to remove because mannans have a tendency to adsorb to cellulose fibers and therefore bind to cotton textiles. Further, mannan also has a "glue effect." This gluing effect means that particulate soils released during the wash cycle or from subsequent wear can bind to

invisible residual mannan. In other words, not only many mannan stains reappear, but mannan can also be transferred to otherwise clean fabrics during washing and result in fabric greying. Mannanases cleave the β-1,4-linkages of mannans *th*ough hydrolysis, thus breaking down the gum polymer into smaller carbohydrate fragments. These smaller, more water-soluble polysaccharide fragments remain free from the fabric and are siphoned out of the wash. Mannanases can thus prevent redeposition of soil released during washing.

The cleaning compositions must contain at least one additional detergent component. These compositions can also be formulated as sanitization products, contact lens cleansers, dishwashing, hard surface cleaner, and health and beauty care products (Bettiol *et al.,* 2000). Such additive products are intended to supplement the performance of conventional compositions and can be added at any stage of the cleaning process (McCoy, 2001). Treatment with cleaning or detergent compositions comprising the mannanase can improve whiteness as well as prevent binding of certain soils to the cellulosic material. Accordingly, mannanase are used in cleaning compositions, including laundry, personal cleansing and oral/dental compositions, Such cleaning compositions comprising a mannanase and an enzyme selected from cellulases, proteases, lipases, amylases, pectin degrading enzymes and xyloglucanases, are prepared to provide superior cleaning performance.

d) To Improve the Nutritional Value of Poultry Feeds

Reduction in weight gain and feed conversion efficiency in poultry birds has been associated with intestinal viscosity due to different feeds. β-Mannan is a polysaccharide commonly found in feed ingredients such as soybean meal (SBM), palm kernel meal (PKM), copra meal (CM), guar gum meal and sesame meal. There is almost universal use of soybean meal and full fat soy as protein sources in poultry feeds, PKM and CM as ruminant feed (Moss and Givens, 1994; Chandrasekariah *et al.,* 2001) pig diets (Pettey *et al.,* 2002) and rabbit diets (Aganga et al., 1991). All these meals have some common properties: high fiber content, low palatability, lack of several essential amino acids and high viscosity coupled with several anti- nutritional properties such as mannan,

galactomannan, xylan and arabinoxylan, their utilization in the intestine is very limited. β-Mannans have been found to be highly deleterious to animal performance, severely compromising weight gain and feed conversion as well as glucose and water absorption. Incorporation of β- mannanase into these diets results in decreased intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency. More recently, the beneficial effect of enzymatic degradation of β-mannan by addition of β-mannanase to diets containing SBM has been documented in broilers (Jackson et al., 2004; Daskiran *et al.*, 2004; Lee *et al.*, 2003), layers (Wu *et al.*, 2005), turkeys (Odetallah *et al.*, 2002) and swine (Pettey *et al.*, 2002). Using endo-mannanase alone may only produce a small proportion of mannose and thus only a small amount of mannan is likely to be absorbed in the intestine of broilers (Saki *et al.*, 2005).

A combination of endo-mannanase from different strains may have a complementary ability to cleave sugars, the use of a combination of endo-mannanase from different strains of organisms may be helpful and that is more possible in the poultry industry. A study of endo-mannanases has been done by Tamaru et al. (1997), who found that endo-mannanase from *Vibrio* sp. could not hydrolyse mannotriose but produced mannotriose, while endo-mannanase from *Streptomyces* could hydrolyse mannotriose to form mannose and mannobiose (Kusakabe and Takashi, 1988). Due to the fact that most of mannanase in the market is in form of endo β-mannanase (e.g. gamanase and hemicell mannanase), it can be speculated that manno- oligosaccharides, mannotriose and mannobiose as well as a small amount of mannose are generated when this type of enzyme is included in the diet. Since only mannose can be absorbed in the intestine, the production of mannobiose and mannooligosaccharides are, to some extent, useless. So these components of carbohydrates may not be absorbed and are therefore unable to supply energy to the host. However, in the sense of the health of the poultry, the production of mannooligosaccharides can improve a chicken's health (Laere *et al.*, 1999), either by increasing the population of specific bacteria such as *Bifidobacteria* because the supply of these types of carbohydrates are a source of feed for bacteria in the caeca and thus suppressing the pathogenic ones, or by flushing out the pathogenic bacteria which attach to the mannooligosaccharides. In fact, mannooligosaccharides are added to the

poultry diets for this effect (Lyons and Jacques, 2002). The use of mannanase in poultry research has been widespread and proven to be successful (Sundu *et al.*, 2006).

Recently acidic stable β-mannanase from *Phialophora* sp. p13 was isolated which involved in high mannan degrading activity under stimulated gastric condition which proves its suitability to use as a feed additive (Zhao *et al.*, 2010). Also the effect of mannanase on nutrient digestability was analysed (Yoon *et al.*, 2010).

e) Use in Oil Drilling

Mannanases can also be used to enhance the flow of oil or gas in drilling operations. Practically, product flow to the well bore is stimulated by forcing out open crevices in the surrounding bedrock, which is done by flooding the well with a natural polymer (guar gum) solution and sand particles, capping the well and then pressurizing the bedrock until it fractures. The viscous polymer solution carries the sand though the fractures, propping open cracks for oil and gas flow. To facilitate product flow, the polymer solution is thinned (Adams *et al.*, 1995; Christoffersen, 2004). As mannanases are able to hydrolyze guar gum at elevated temperatures (Politz *et al.*, 2000), so implementation of oil production by these enzymes could be feasible.

f) In oil Extraction of Coconut Meats

Mannanases can be used in enzymatic oil extraction of coconut meat. The main components of the structural cell wall of coconut meat are mannan and galactomannan (Saittagaroon *et al.*, 1983). A high oil yield is achieved in this process. In the traditional method of oil extraction by expeller, unsanitary handling, drastic processing of copra for oil extraction, refining of the product, the presence of polycylic aromatic hydrocarbons (PAH) retained in the coconut oil not only lessen the quality of the by-product like protein but also render the product susceptible to aflatoxin contamination and oxidative rancidity. The enzymatic oil extraction process not only eliminates such problems but also improves the sensory qualities of the products. In addition, the improvement of the quality of coconut oil minimizes the need for refining the oil as it is already comparable to the semi-refined coconut oil. This enzymatic process gives three valuable products:

coconut oil, an aromatic protein containing liquid that may be used as a beverage base and a coconut protein isolate (Chen *et al.*, 2003).

g) In the Textile and Cellulosic Fiber Processing Industries

The mannanase can be used for preparation of fibers or for cleaning of fibers in combination with detergents in the textile and cellulosic processing industry. The processing of cellulosic material for the textile industry into a material ready for garment manufacture involves several steps: spinning of the fiber into a yarn, construction of woven or knit fabric from the yarn and subsequent preparation, dyeing and finishing operations. Woven goods are constructed by weaving a filling yam between a series of warp yarns; the yarns could be of two different types. Mannanase is useful in an enzymatic scouring process and in desizing (removal of mannan size) during the preparation of cellulosic material for proper response in subsequent dyeing operations (Palackal *et al.*, 2006).

h) For Degradation of Thickening Agents

Galactomannans such as guar gum and locust bean gum are widely used as thickening agents, e.g. in food and print paste for textile printing such as prints on T-shirts. Mannanase can be used for reducing the viscosity of residual food in processing equipment and thereby facilitate cleaning after processing. The enzyme or enzyme preparation is useful for reducing viscosity of print paste, thereby facilitating wash out of surplus print paste after textile printings (Ademark *et al.*, 1998).

i) As Non-Nutritional Food Additives

Mannanases also contribute to the human health as they degrade mannans, which otherwise are resistant to mammalian digestive enzymes in the small intestine but are readily fermented in the large intestine, particularly by probiotic bacteria belonging to the genera *Bifidobacteria* and *Lactobacillus*. Prebiotic oligosaccharides including mannooligosaccharides, i.e. hydrolysis products of mannan degradation, are believed to

promote the selective growth and proliferation of human beneficial intestinal microflora (Kobayashi *et al.*, 1984, 1987).

Thus, mannanases recently only attracted and increased the scientific and commercial attention due to potential applications in several industries.

Materials and Methods

4.1 Chemicals:

Locust bean gum (Galactomannan) was obtained from Sigma-Aldrich, USA. Molecular weight markers for PAGE were purchased from Genei, Bangalore. All other reagents used were of analytical grade.

4.2 Sample collection:

Soil samples were collected from Polavakalipalayam, Gobichettipalayam taluk of Erode district. Soil sample was crushed, mixed thoroughly and sieved to get rid of large debris, and the sieved soil was used for the isolation of β-mannanase producing organism.

4.3 Bacterial Isolation:

Soil samples of 1 g were suspended in 100 ml sterile distilled water and vortexed intermittently for 5 min. Then the mixture was allowed to settle down and serial diluted up to 10^{-7}. From 10^{-6} & 10^{-7} dilutions, 0.1 ml was taken and spread on Petri plates containing Akiba-Horikoshi agar (AHMA) using L-shaped glass rod and the inoculated plates were kept in an incubation chamber at 40°C for 2 days. Plated dilutions that gave 30 colonies were chosen for further studies.

4.4 Preliminary screening of mannan degrading enzymes in solid media:

Primary screening was performed in a Akiba-Horikoshi media plate containing 5g/l Locust bean gum at 37°C. After 24*hs*, mannanase activity was detected on the cultures by staining it with 0.05%(w/v) congo red solution for 15 min and thereafter by destaining with 1M NaCl (Copa-patino *et al.,* 1993). Locust bean hydrolysis was detected by the appearance of clear zones around the growth of the strains assayed. The culture producing β-mannanase was identified and stored at 4°C.

4.5 Enzyme production:

Isolated β-mannanase producing strain was grown in a chemically defined media (Virupakshi *et al.,* 2005) in a 250ml Erlenmeyer flask containing (g/L) Locust bean gum(LBG) 10.0, $NaNO_3$ 10.0, K_2HPO_4 5.0, $MgSO_4$ 0.3, $CaCl_2$ 0.2, and NaCl 5.0 (about

100ml). Sodium carbonate (10g/l) was added separately after autoclaving. Then the flasks were incubated at 37°C on a shaker for 24*hs* at 150rpm. After 24*hs*, the culture was centrifuged at 8,000 rpm for 10 min (4°C) and the supernatant was used for the further studies.

4.6 Enzyme assay:

4.6.1 β-mannanase assay:

β-mannanase activity was measured at 50 °C using 3,5-dinitrosalicylic acid (DNS) method (Table 4.1).

S.No	0.1% Locust bean gum (ml)	50mM glycine-NaOH Buffer(pH 10.0) (ml)	DNS solution (ml)	Enzyme (ml)		DNS solution (ml)		Absorbance at 540nm
1	0.5	0.4	1.0	0.1	Tubes were incubated at 50°C for 10min	0	Tubes were kept in a boiling water bath for 5 min	
2	0.5	0.4	0	0.1		1.0		
3	0.5	0.4	0	0.1		1.0		

Table 4.1: Assay for β-mannanase (Virupakshi *et al.*, 2005)

The absorbance was measured at 540nm after diluting the coloured complex with 8ml of distilled water. One unit of enzyme was defined as the amount of enzyme that could release 1μmol of D-mannose per minute under the above standard conditions.

4.6.2 Xylanase assay:

4ml reaction mixture containing 2ml buffer (Glycine-NaOH buffer, pH 9.5), 1ml of 1% xylan solution (w/v) and 1ml of enzyme were incubated at 50°C for 15 min. Then the reaction was arrested by adding 1ml of DNS solution followed by boiling for 5 min. The absorbance was measured at 540nm. One unit of enzyme was defined as the amount of enzyme that could release 1µmol of xylose per minute under the above standard conditions.

4.6.3 α-galactosidase assay:

1ml reaction mixture containing 0.1ml enzyme, 0.8 ml of glycine NaOH buffer (50mM, pH 10.0) and 0.1ml pNPG(10mM) substrate were incubated at 45°C for 15 min. Then the reaction mixture was terminated by adding 3ml of 0.2M sodiumcarbonate solution and absorbance was measured at 405nm. One unit of enzyme was defined as the amount of enzyme that could release 1µmol of p-nitophenol from p-nitrophenyl-α-D-galactopyranoside(pNPG) per minute under the above standard conditions.

4.6.4 Cellulase assay:

0.5ml reaction mixture containing 0.45ml of 1% CMC solution prepared in glycine NaOH buffer (50mM, pH 10.0) and 0.05ml enzyme solution were incubated at 50°C for 15 min. Then the reaction was stopped by adding 0.5ml of DNS solution followed by boiling for 5 min. The absorbance was measured at 540nm. One unit of enzyme was defined as the amount of enzyme that could release 1µmol of cellulose per minute under the above standard conditions.

4.7 Protein Estimation:

A Standard protein curve was constructed by the method described by Lowry et al (1951). Bovine serum albumin (BSA) 1mg/ml was used as standard protein. Different concentrations of BSA solutions were pipetted out in the test tube and the total volume was made up to 1ml with distilled water.5ml of alkaline copper reagent was added to each tube, mixed thoroughly and allowed to stand at room temperature for 10 min. Then to each tube 0.5ml of 1N Folin-Ciocalteau's reagent was added with immediate mixing.

The extinction was read after 30 min incubation at 660nm in dark in ELICO spectrophotometer. A standard graph was constructed.

4.7.1 Alkaline copper reagent:

- Reagent A was prepared by dissolving 2% sodium carbonate in 0.1N sodium hydroxide.
- Reagent B was prepared by dissolving 0.5% copper sulfate in 1% sodium potassium tartarate.
- Reagent C was prepared by mixing 50 ml of reagent A and 1ml of reagent B just before use.

4.7.2 Folin-Ciocalteau's reagent:

2N Folin Ciocalteau reagent commercially available was diluted with distilled water in the ratio 1:1(v/v) just before use.

4.8 Production of β-mannanase using different carbon sources:

Various carbon sources, locust bean gum, lactose, glucose, xylan, maltose, raffinose and fructose were examined in the above used chemically defined media where Sodium nitrate served as the sole carbon source. Logarithmically grown culture were used as inoculum.

4.9 Production of β-mannanase using different nitrogen sources:

Various nitrogen sources, ammonium sulphate, urea, potassium nitrate, beef extract, yeast extract, peptone, casein, sodium nitrate were examined in the above used chemically defined media where locust bean gum served as the sole carbon source. Logarithmically grown culture were used as inoculum.

4.10 Growth profile:

The growth profile and the time course of maximum enzyme production were examined in the media containing locust bean gum as carbon source and sodium nitrate as nitrogen source. Logarithmically grown culture were used as inoculum.

4.11 Purification of β-mannanase from *Bacillus* sp.:

4.11.1 Ammonium sulphate precipitation:

All the purification steps were carried out at 4°C. The centrifugal supernatant of the culture broth was used as the enzyme source. Fractional precipitation was carried out using ammonium sulphate and the resultant precipitate was collected by centrifugation at at 10,000rpm for 20min at 4°C. Then it was dissolved in a small volume of 50mM Glycine-NaOH buffer, pH 9.5. Then it was dialysed against 10mM Glycine-NaOH buffer to remove excess salts.

4.11.2 DEAE- Sephadex *c*homotogrphy:

DEAE–Sephadex matrix column was used for purification. The column was first regenerated with the following buffers.

a) 0.1M Tris-HCl buffer, pH 8.3 containing 0.5M NaCl.

b) 0.1M sodium acetate buffer, pH 4.5 containing 0.5M NaCl

c) 10mM sodium phosphate buffer, pH 8.0.

After dialysis, the enzyme solution was centrifuged and clear enzyme solution was applied to a column which was equilibrated with 10mM sodium phosphate buffer, pH 8.0. Excess unbound protein were washed out and the elution of binded protein was done with a linear gradient of 10mM sodium phosphate buffer, pH 8.0 containing 0-0.5M NaCl. The fractions were collected with a flow rate of 0.5ml/min. Fractions were then assayed for β-mannanase acivity and protein content. The fraction showing single peak was pooled, desalted by dialysis and then lyophilised.

4.11.3 Electrophoretic analysis:

4.11.3.1 Native PAGE:

Native PAGE was performed as described by Lammeli (1970) with 10% polyacrylamide gel without SDS.

a) Monomer solution:

The solution contained 29.2g of acrylamide and 0.8g of N,N-methylenebisacrylamide in a final volume of 100ml made in distilled water. The solution was filtered *th*ough Whatman No.1 filter paper and stored at 4°C.

b) 1.875M Tris-buffer, pH 8.8

22.7g of Tris dissolved in distilled water. The pH was adjusted to 8.8 with HCl and the volume was made up to 100ml with distilled water. The solution was stored in refrigerator.

c) 1.25M Tris-HCl buffer, pH 6.8

15.125g of Tris was dissolved in distilled water. The pH was adjusted to 6.8 using 1N HCl and the volume of the solution was made up to 100ml and stored at 4°C.

d) Ammonium persulfate (APS), (1% w/v)

100mg APS was dissolved in distilled water and the volume was made to 10ml. Freshly prepared APS was used every time.

e) Electrophoresis buffer (10X) (pH 8.2-8.4)

Tris base (6g) and glycine (14.4g) was dissolved in distilled water and the volume was made up to 1L and stored at room temperature.

f) Separating gel mixture (10%)

Stock acrylamide solution	13.3ml
Tris HCl (pH 8.8)	8ml
Distilled water	18.1ml
APS	200µl
TEMED	20 µl

g) Stacking gel mixture(4%)

Stock acrylamide solution	0.45ml
Tris HCl (pH 6.8)	0.33l
Distilled water	2.5ml
APS	50 µl
TEMED	10 µl

The mixture was poured into the cassette above the separating gel. A comb was introduced into the spacer gel and was allowed to polymerize in the presence of light. After polymerization the comb was removed from the cassette and the gel was inserted into the gasket of electrophoretic apparatus. The bromophenol blue containing sample buffer was mixed with protein sample and loaded in the well. Electrophoresis was performed at room temperature and at constant current of 100 volts. When the

bromophenol blue dye stopped 1cm from the bottom of the gel, electrophoresis was stopped. The gel was removed from the glass plate and was stained.

4.11.3.2 Activity staining:

Activity detection of β-mannanase in gel was done by equilibrating the acrylamide gel in 50mM Glycine-NaOH buffer, pH 9.5 for 30 min. Then it was overlaid on the agar plate containing 0.5% LBG and 1.5% agar and was maintained at 50°C for 30 min. After 30 min, the acrylamide gel was removed and the agar replica plate was stained in a congo red solution for 30 min at 50°C. It was then destained with 1M NaCl. Clear areas in the dark red background indicate the β-mannanase activity.

4.12 Characterization of β-mannanase:

4.12.1 Optimum pH:

The pH profile of β-mannanase was evaluated by incubating the enzyme at 50°C for 10min in appropriate buffers [Glycine-HCl buffer (pH 3.0, 3.5), Sodium acetate buffer (pH 4.0-5.5), Sodium phosphate buffer (pH 6.0-8.0), Tris HCl buffer (pH 8.5-9), Glycine-NaOH buffer (9.5-10.5).

4.12.2 Optimum temperature:

The optimum temperature for β-mannanase activity was determined by incubating the enzyme for 10 min at temperatures ranging from 30°-100°C using Glycine-NaOH buffer (50mM, pH 9.5).

4.12.3 pH stability:

pH stability was determined by incubating the enzyme at pH 4.0 to 10.5 in the absence of substrate for 2*h*s and then the residual activity was determined under standard conditions for every 1*h*.

4.12.4 Temperature stability:

The enzyme was incubated at 40°, 50°, 60° and 70°C in the absence of substrate for 2*h*s and then the residual activity was determined under the standard conditions for every 1h at pH 9.5 and 7.0.

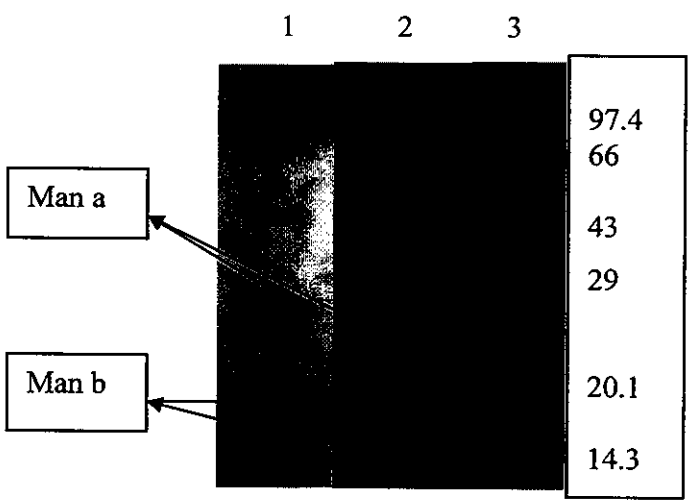
4.12.5 Effect of metal ions, sugars and some reagents:

The effect of various metal ions and inhibitors on the enzyme activity were examined by incubating 100µl of enzyme solution with each of the metal ions and inhibitors at a final concentration of 10^{-3} in 50mM glycine–NaOH buffer (pH 9.5) for 30min at room temperature. The residual activity was determined under the above standard conditions.

Results and Discussion

The mannan degrading organism was screened and isolated from the soil and sent for culture identification. The bacterial culture showing mannanase activity was identified as *Bacillus brevis*. Only one paper has been reported on β-mannanase from *Bacillus brevis* which has maximum activity at pH 6.8.(Araujo *et al.*, 1990). But no purification and application studies were found on β-mannanase from *Bacillus brevis*.

In this *Bacillus* sp., two forms of β-mannanase were identified in the crude extract by Zymogram technique and the molecular weight was found to be 28 and 15kDa approximately in Native PAGE (Fig. 5.1). So the attention has been focused on small enzymes. Because of their low molecular weight, these enzymes penetrate easily within the lignocelluloses system and cleave the mannan efficiently. So in this study, Man b enzyme was focused for purification.



Lane 1 - Crude enzyme proteins stained with Coomassie brilliant blue,
Lane 2 - Activity staining by Zymogram technique
Lane 3 - Molecular Marker

Fig 5.1: Native PAGE to determine the presence of two enzymes in the crude extract

5.1 Purification of alkaline β-mannanase:

5.1.1 Ammonium sulphate precipitation:

Partial purification of β-mannanase enzyme was carried out by fractional precipitation with ammonium sulphate. 35% and 75% saturation were done. Of which 75% ammonium sulphate concentration showed the highest mannanase activity with good recovery. Therefore this fraction was further purified by DEAE- Sephadex column c*h*omatography.

5.1.2 DEAE-Sephadex C*h*omatography:

Stepwise elution was performed with sodium phosphate buffer (10mM, pH 8.0 with 0-0.5M). The results in the elution profile (Fig. 5.2) showed 2 peaks a and b, of which peak b showing comparatively high activity was selected and Zymogram technique was carried out to find the molecular weight of β-mannanase (Fig 5.3).

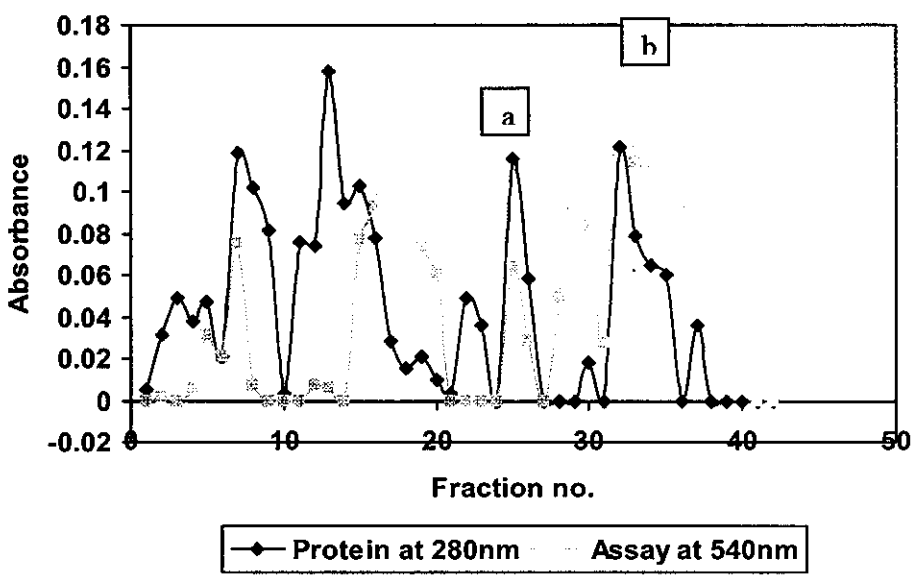
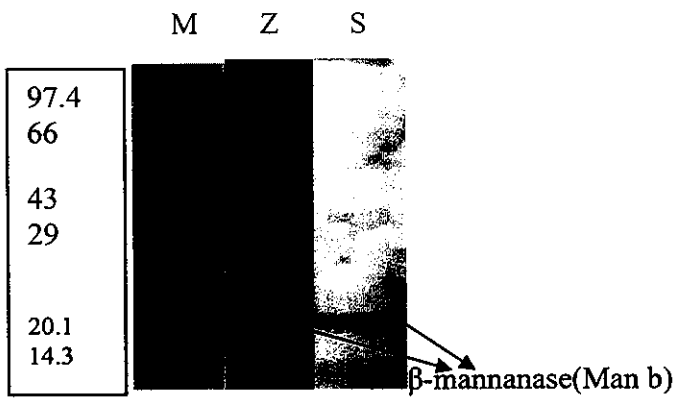


Fig. 5.2: Elution profile of β-mannanase on DEAE -Sephadex



Lane M - Molecular Marker,

Lane Z - Activity staining by Zymogram for peak b (Man b)

Lane S - Protein stained with Coomassie brilliant blue.

Fig. 5.3: Native PAGE for the peak b (Man b) after DEAE column *ch*omatography

Mannanase (Man b) was purified and the molecular weight was estimated to be 15kDa approximately in the Native PAGE (Fig. 5.3). Thus the low molecular weight mannanase protein was separated and purified. Purification chart was shown in table 5.1. Approximately, 1.34 fold purification with low yield (0.8%) was obtained. Since these enzymes acted synergistically for the complete hydrolysis of mannans, the yield and fold values were probably underestimated. Low molecular weight β-mannanase from *Pencillium occitanis* Pol6 was 18kDa (Monia *et al.*, 2010). But the optimum pH and temperature was found to be 4.0 and 40°C. No reports were found in low molecular weight alkaline β-mannanase and is unique so far. Molecular weight of β-mannanase

from alkaline *Bacillus* sp. N16-5, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus subtilis*-5H were found to be 55, 38, 40 and 39 kDa respectively. This low molecular protein molecule can penetrate easily though the pulp and make the bleaching process more efficient. So β-mannanase (Man b) from *Bacillus brevis* can be used in paper and pulp industries for bleaching purpose.

S.No	Purification Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
1.	Crude	500	2500	200	12.5	1	100
2.	Ammonium sulphate precipitation(70% saturation)	30	208.4	14.4	14.45	1.156	8.3
3.	DEAE Sephadex Chomatography	2	20	1.2	16.67	1.34	0.8

Table 5.1: Purification chart for alkaline β-mannanase from *Bacillus brevis*

5.2 Enzyme activity for xylanase, cellulase and α-galactosidase:

No cellulase and α-galactosidase activity were found in the crude extract at pH 9.5, but xylanase activity was found at pH 9.5. Cellulase free xylanase and mannanase enzyme have a wide range of biotechnological applications particularly in paper and pulp industries (Techapen *et al.,* 2002). This is also one of the advantage of using *Bacillus brevis* in bleaching process other than low molecular weight property, since both xylanase and β-mannanase are found, bleaching process will be more efficient.

5.3 Effect of various carbon sources on enzyme production;

β-mannanase activity was found maximum in LBG carbon source(Fig 5.4) using sodium nitrate as nitrogen source. LBG is a galatomannan containing 23% galactose and 77% mannose. Nearly 70% mannanase activity was found using xylan as carbon source. pH used for the production of β-mannanase was pH 10.5. LBG carbon source was involved in maximum β-mannanase production in *Bacillus brevis* (Araujo e al, 1990).

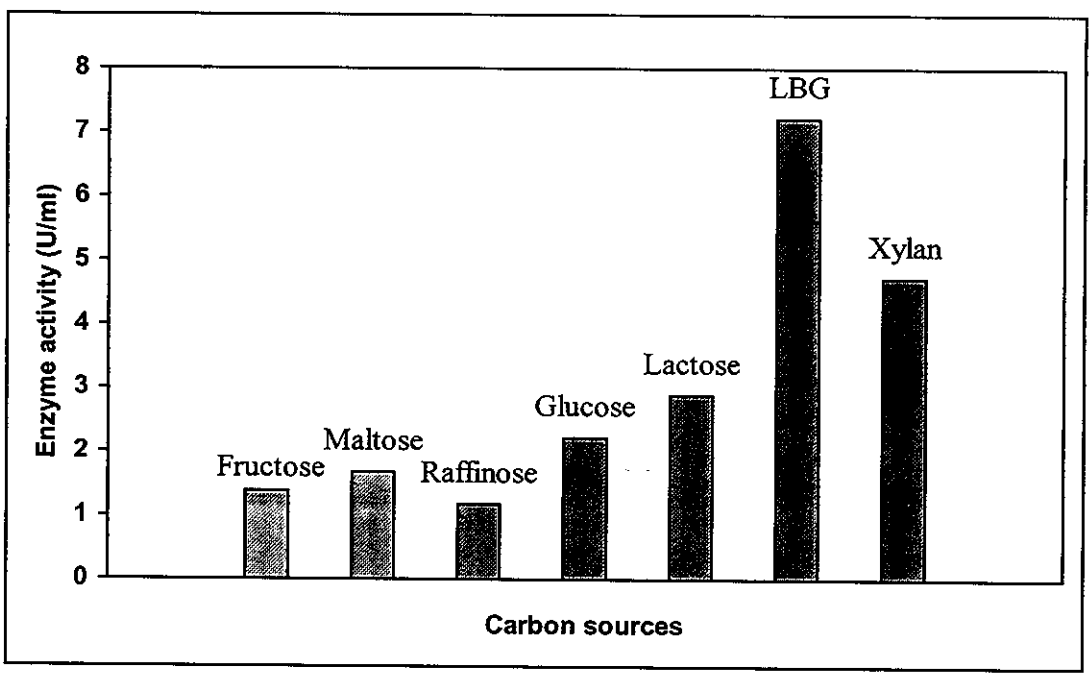


Fig. 5.4: Effect of different carbon sources on enzyme production.

5.4 Effect of various nitrogen sources on enzyme production:

Fig. 5.5 shows the β-mannanase activity for various nitrogen sources. The highest activity was obtained with sodium nitrate as nitrogen source, low activity with urea and no activity with ammonium sulphate. pH used for the production of β-mannanase was pH 10.5.

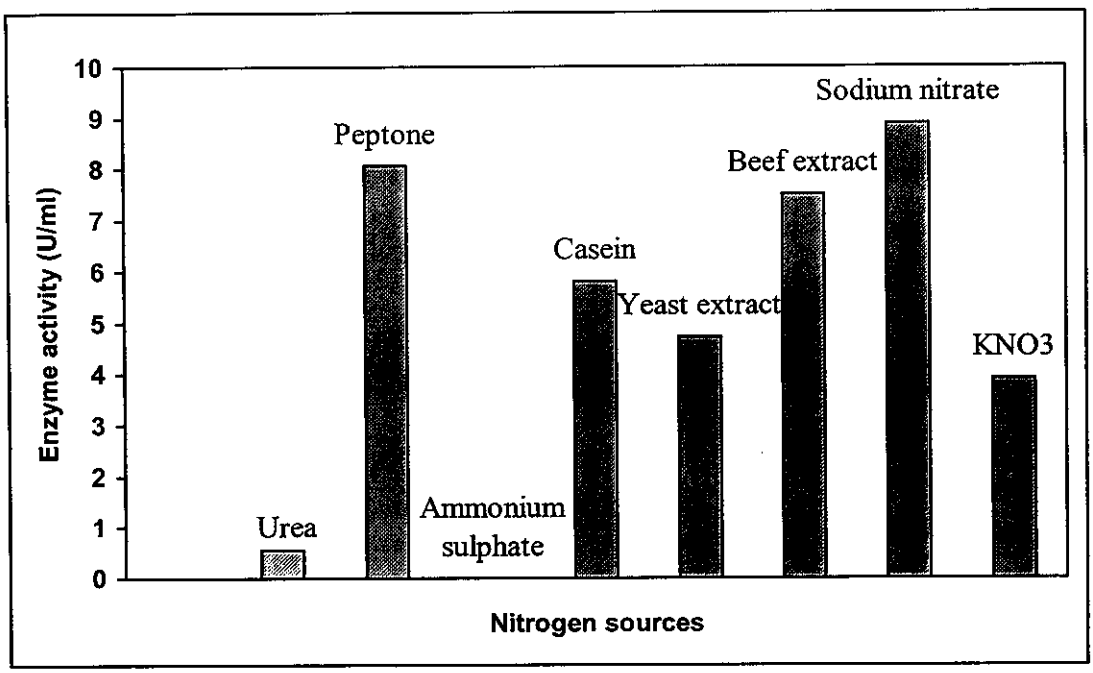


Fig. 5.5: Effect of different nitrogen sources on enzyme production

5.5 Effect of incubation period on enzyme production:

The fermentations were monitored at defined time intervals, from 12 to 48 *h*s. As shown in Fig 5.6. It was evident that the enzyme production depended on the growth of the bacteria. Maximum enzyme production was achieved at 24*h*s and after that activity declined with the growth.

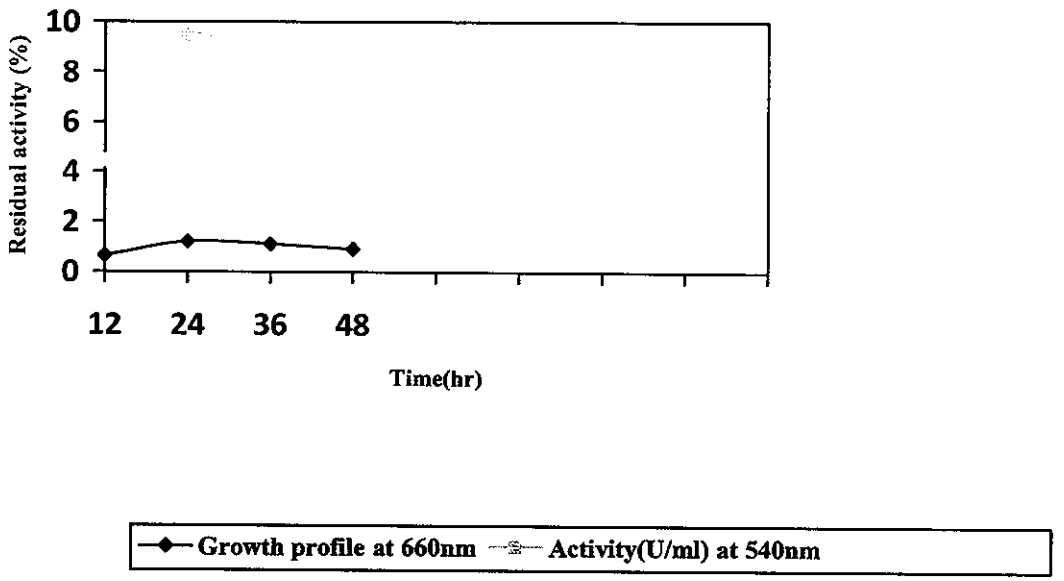


Fig. 5.6: Growth profile and time course of enzyme production

5.6 Characterization of β-mannanase from *Bacillus brevis*

5.6.1 Optimum pH

β-mannanase from *Bacillus brevis* was active from 3.5 to 10.5 (fig. 5.7) and maximum activity was found at two pH 7.0 and 9.5(Table 5.2). β-mannanase from *Bacillus brevis* reported showed maximum activity at pH 6.8(Araujo *et al.*, 1990). Since the present study focus on the alkaline β-mannanase suitable for paper and pulp bleaching, all characterizations studies were carried out at pH 9.5. β-mannanase from *Bacillus* sp. N16-5 (ManA) showed optimum activity at pH 9.5(Ma *et al.*, 2004) and more than 70% of the maximum activity was detectable between pH 7 and 11. β-mannanase from thermostable *Bacillus* sp. JB-99 showed maximum activity at pH 10.0 (Virupakshi *et al.*, 2005) and were active from 8.0 to 10.5. Bacillus sp. AM001 produced three extracellular mannanases with optimal activity at pH 8-9 (Akino *et al.*, 1989). From the reports, it was inferred that only the β-mannanase from *Bacillus* sp. possessed a higher optimum pH 8.0 to 10 (Jigyodan *et al.*, 1996). Very few reports were available in alkaline β-mannanase.

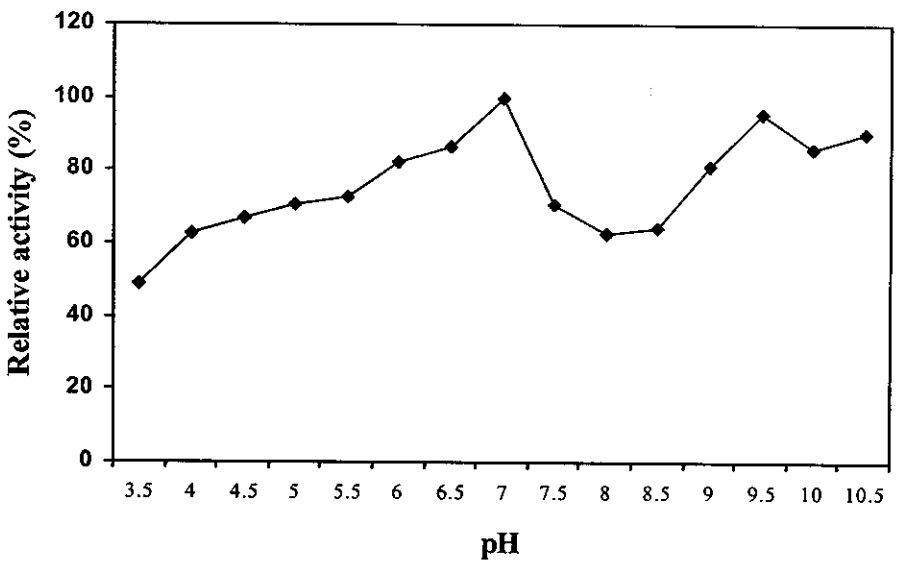


Fig. 5.7: Effect of pH on the activity of β-mannanase from *Bacillus brevis*

pH	Relative activity (%)
3.0	49
3.5	62.4
4.0	67
4.5	70.46
5.0	72.5
5.5	73.8
6.0	82.55
6.5	86.5
7.0	100
7.5	70.46
8.0	62.41
8.5	64.1
9.0	81.2
9.5	95.57
10	85.97
10.5	94.63

Table 5.2: Effect of pH on the activity of β-mannanase from *Bacillus brevis*

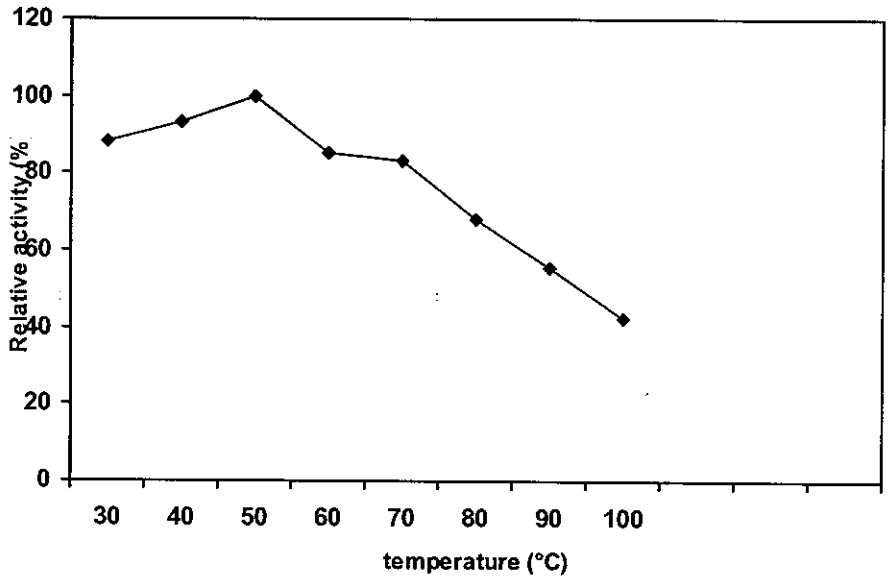


Figure 5.8: Effect of temperature on the activity of β-mannanase from *Bacillus brevis*

Temperature (°C)	Relative activity at pH 10.0 (%)
30	88
40	93.5
50	100
60	85.32
70	83.02
80	67.88
90	55.5
100	42.2

Table 5.3: Effect of temperature on the activity of β-mannanase from *Bacillus brevis*

5.6.2 Optimum temperature:

Maximum activity was attained at 50°C (Fig. 5.8) but the enzyme was active from 30-90°C(Table 5.3). β-mannanase from *Enterococcus casseliflavus* (M1 and M2) showed maximum activity at 50°C(Oda *et al.,* 1993). β-mannanase from *Bacillus subtilis* KU-1, *Bacillus subtilis* 5H, *Bacillus subtilis* was found to be 50-55°C (Zakaria *et al.,* 1998; Khanongnuch *et al.,* 1998; Mendoza *et al.,* 1994). β-mannanase from *Bacillus licheniformis* THCM3.1 (ManBL3.1) showed maximum activity at 45°C.

5.6.3 pH stability

β-mannanase from *Bacillus brevis* was found to be stable at pH 6.5 to 10.5 (Fig. 5.9) for about 2*h*s, but more stable at pH 8.5 to 10.5 nearly 60%(Table 5.4). β-mannanase from alkaline *Bacillus* sp.(M1, M2, M3) was found to stable at pH 7-9 for 30 min(Akino *et al.,* 1989). β-mannanase from *Bacillus* sp. W-2 reported stability to about 1*h* at pH 5-10 (Ooi *et al.,* 1995). β-mannanase from *Bacillus* sp.N16-5 was stable at pH 9 and retained almost 90% of its original activity between pH 8.5 an 10.0. From the earlier reports, it was found that only β-mannanase from *Bacillus* sp. have a greater stability from pH 5.0 to 10 (Duffaaud *et al.,* 1997).

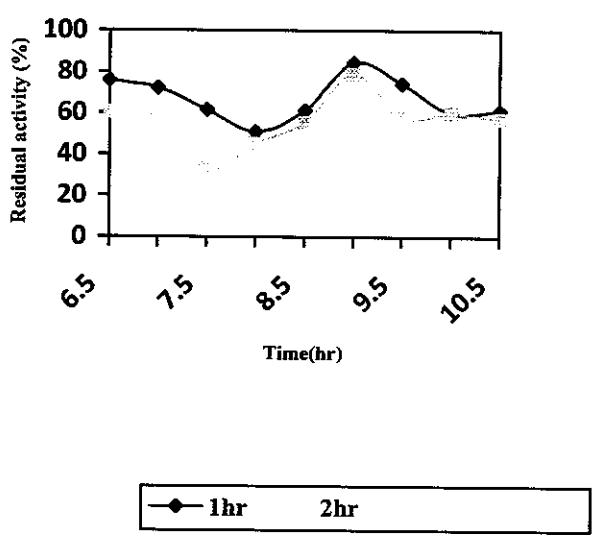


Fig. 5.9: pH stability of β-mannanase from *Bacillus brevis*

pH	Residual activity at 0 *h* (%)	Residual activity after 1*h* (%)	Residual activity after 2*h*s (%)
6.5	100	75.8	60.5
7.0	100	72	57.3
7.5	100	61.48	32.4
8.0	100	50.72	44.9
8.5	100	61.2	55.4
9.0	100	84.4	78.57
9.5	100	74.1	57.8
10.0	100	60	59.7
10.5	100	61.4	57.2

Table 5.4: pH stability of β-mannanase from *Bacillus brevis*

5.6.4 Thermal stability:

β-mannanase from *Bacillus brevis* was stable at 40-50°C for 2*h*s(Fig.5.10) and retained about 60% of its original activity at pH 9.5(Table 5.5). At 60°C, the enzyme was 50% stable for 1*h* but at 70°C, activity get decreased and lost completely after 2*h*s.

β-mannanase reported on *Bacillus brevis* showed thermal stability for 3*h*s at 65°C (Araujo *et al.*, 1990). β-mannanase from alkaline *Bacillus* sp. (M1, M2, M3) showed stability at 60-65°C for 30 min. β-mannanase from *Bacillus* sp. N16-5 and *Bacillus* sp. W-2 reported thermal stability at 60°C for 2*h*s and 1*h* respectively (Ma *et al.*, 2004, Ooi

et al., 1995) at pH 9.5. β-mannanase from *Bacillus subtilis* showed stability for about 10 min at 55°C(Mendoza *et al.*, 1994).

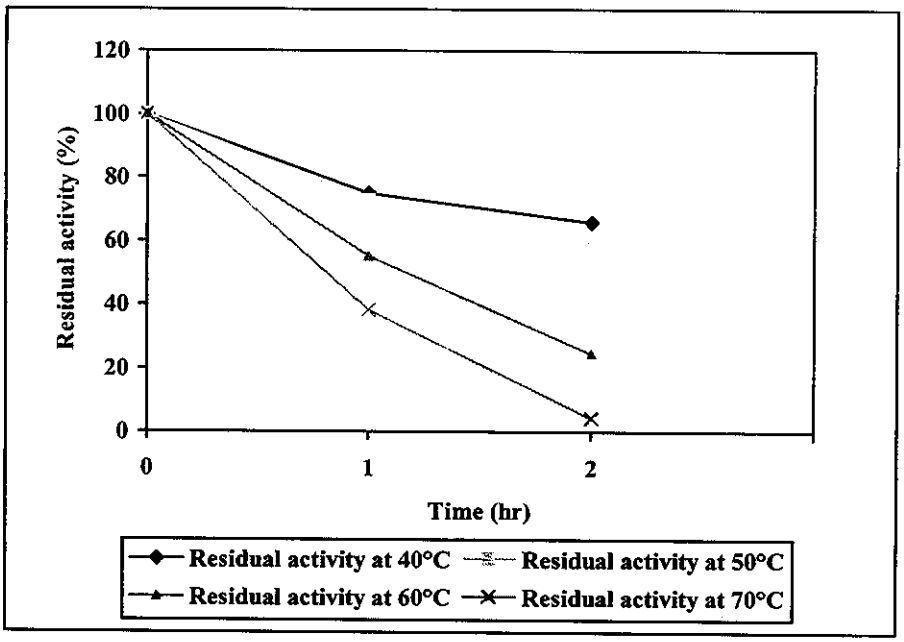


Fig. 5.10: Thermal stability for β-mannanase from *Bacillus brevis* at pH 9.5

Temperature for pH 9.5 (°C)	**Residual activity at 0 *h* (%)**	**Residual activity after 1*h* (%)**	**Residual activity after 2*hs* (%)**
40	100	74.89	65.9
50	100	72.76	60.42
60	100	55.3	24.68
70	100	38.29	4.25

Table 5.5: Thermal stability for β-mannanase from *Bacillus brevis* at pH 9.5

5.6.5 Effect of metal ions and chemical reagents:

The effect of metal ions and reagents (at 1mM concentration) were determined using LBG as substrate (Table 5.6). Hg^{2+} and N-bromosuccinimide inhibited the enzyme completely. Cu^{2+}, Ag^{2+}, Zn^{2+}, 1,10-Phenant*h*oline and EDTA inhibited the activity comparatively(Table. 5.6). The loss of enzyme activity by the Cu^{2+}, Ag^{2+}, Hg^{2+} and Zn^{2+} might be due to the oxidation of aminoacid residues essential for the enzyme activity. Complete inhibition by N-bromosuccinimide denoted the role of tryptophan residue in β-mannanase activity. Additionally, the loss of activity in the presence of chelating agents like EDTA and 1,10-Phenant*h*oline also suggests the tryptophan presence at or near the active site of the β-mannanase and divalent and cation dependent property of β-mannanase enzyme.

Metal ions and chemicals (1mM)	**Residual activity (%)**
Control	100
$HgCl_2$	0
$CaCl_2$	102.5
$AgNO_3$	27.63
$CuSO_4$	12.6
$ZnSO_4$	7.8
$MnCl_2$	56.47
$MgSO_4$	41.96
$FeSO_4$	131.57
EDTA	1.3
N-bromosuccinimide	0
1,10-Phenant*h*oline	9.02

Table 5.6: Effect of metal ions(1mM) and chemicals(1mM) on the β-mannanase activity from *Bacillus brevis*

Conclusion

Alkaline β-mannanase producing *Bacillus* sp. was isolated from the soil and was identified as *Bacillus brevis*. Enzyme production was done at pH 10.5. No reports were found for alkaline β-mannanase from *Bacillus brevis*. The enzyme isolated from this *Bacillus strain* exhibited unique properties like cellulase free xylanase and mannanase, stability at pH 8.5-10.5 and low molecular weight about 15kDa. All these unique properties suggests that the effective bleaching process can be done in Paper and Pulp industries using this β-mannanase from *Bacillus brevis*, thereby reducing the use of chlorine and environmental pollution. Also the enzyme was stable from pH 6.5 to 10.5 and 30-40°C. So it can also be applied in animal feed as additive as the physiological conditions of the unit animal's small intestine is pH 6.5 and 37°C.

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