Studies on Production of Bacterial Xylanases

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SUBRAMANIYAN, S.

REG. NO. 1571

Biochemical Processing and Waste Water Technology Division
Regional Research Laboratory (CSIR)
Trivandrum – 695 019, India.

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Email : root@csrritrd.ren.nic.in; Ph. EPABX : (0471) 490674, 490811, 490224

Dr. P. Prema
Scientist,
BCPWWT Division.

15-02-2000

CERTIFICATE

This is to certify that the work presented in the thesis entitled, "**Studies on production of bacterial xylanases**" is based on the original research done by Subramaniyan, S., under my guidance and supervision at the Biochemical Processing and Waste Water Technology Division, Regional Research Laboratory, Trivandrum 695019, India and no part of this work has been included in any other thesis for the award of any degree.

Dr. P. Prema

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. P. Prema, Scientist in Biochemical Processing and Waste Water Technology Division, Regional Research Laboratory, Trivandrum, India and that no part of this has been included in any other thesis submitted previously for the award of any degree.

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INTRODUCTION

1. INTRODUCTION

1.1. Preface

The present global scenario of environmental destruction demands ameliorative measures by every branch of science. The zealous anthropogenic activities lead to the destruction of delicate ecological niches. Problems like expulsion of hazardous chlorinated compounds including chlorophenols, chlorobiphenyls and other chlorolignin derivatives formed during conventional pulp production to the environment cause serious public concern. Xylan, the second most abundant polysaccharide (Christove and Prior, 1993; Ali *et al.*, 1999; Wong and Maringer, 1999) and a major component in plant cell wall is linked to lignin and cellulose (Puls, 1997; Connerton *et al.*, 1999) and its hydrolysis by xylanases eases the removal of lignin, the chromogenic precursor. The growing public concern regarding environmental impact of pollutants from paper and pulp industry was the strong driving force behind the endeavours leading to novel bleaching techniques. Chlorinated phenolic compounds and polychlorinated biphenyls produced during conventional pulp bleaching arise from residual lignin present in pulp. Several studies have been conducted to assess the deleterious effects of effluents from paper and pulp industries. Most of the chloroaromatic compounds released during the pulp bleaching process are toxic and accumulate in the biotic and abiotic components of the ecosystem (Subramaniyan and Prema, 2000). Larsson *et al.* (1988) found a negative impact of Kraft mill effluents on fish populations, even 10 km away from the plant. The use of chemical pulp paper for the manufacture of baby diapers and food packaging is also of concern since it is sometimes

associated with chlorinated compounds including the animal carcinogen dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Shoham *et al.*, 1992).

Viikari *et al.* (1986) were the first to demonstrate that xylanases could be useful in paper and pulp industry effecting delignification in bleaching process. Xylanases are of great importance to pulp and paper industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent (Shoham *et al.*, 1992).

Studies with fungal xylanases have resulted in the reduction of chlorine consumption; however, there was also an unacceptable drop in viscosity. This drop was presumably due to cellulase contamination of the xylanase preparations. Use of cellulase-free xylanases in pulp and paper industries would permit the production of rayon grade or superior quality dissolving pulp, as cellulase-free xylanases selectively remove hemicellulose components with minimal damage to cellulose (Jurasek and Paice, 1986; Srinivasan and Rele, 1995). According to Kantelinen *et al.* (1991) hydrolysis of reprecipitated and reabsorbed xylan or xylan-lignin complex is the major action in xylanase catalysed enzymatic treatment. As a result the pulp becomes more accessible to bleaching chemicals. Another, less important, effect of enzymatic pretreatment is hydrolysis of the residual, non-dissolved hemicellulose, which acts as a chromophoric xylan because the Kraft or alkaline cooking of monosaccharides leads to the production of chromophores and aromatic compounds (Ziobro, 1990). During the Kraft pulping 4-O-methyl-D-glucuronic acid chains of the fibre bound xylans are largely converted in to L-threo-4-deoxy-4-hexuronic acid groups and removal of these compound needs considerable amount of bleaching agents (Puls, 1997).

The importance of xylanases is applicable not only to the paper and pulp industry but there are also other industries with equal importance of applicability. The problems related to food industry like the processing of

agrowastes and animal food necessitate search for appropriate solutions through biotechnology. Potential applications of xylanases include bioconversion of lignocellulosic material to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock (Wong *et al.,* 1988). Application of xylanase in the saccharification of xylan in agrowastes and agrofoods adds to the need of exploiting the potential role of them in biotechnology. In all these cases xylan and its hydrolysis form a chief factor.

The Kraft pulping process at higher temperatures and pH ranges thus requires alkaline thermophilic xylanases, useful in the pre-treatment of cooked pulp. Studies have been started elsewhere for the isolation of novel strains which produce alkaline thermostable xylanases, free of cellulases, in higher levels.

1.1.1. Xylans

The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated (Puls, 1997). Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%)- a group of carbohydrates in which xylan forms the major class- and lignin (20-30%) (Browning, 1963). Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the back bone of β-1,4, -linked xylopyranose units. Xylan forms an interface between lignin and other polysaccharides. It has the binding properties mediated by covalent and non-covalent interactions with lignin, cellulose and other polymers (Kato, 1981). Lignin is bound to xylans by an ester linkage in 4-O-methyl-D-glucuronic acid residues present as substituents in xylan back bone (Roberts, 1996).

1.1.2. Xylanases

Xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8.) are endo-enzymes which release xylooligosaccharides and xylose residues from xylan, while the exoacting β-xylosidases (1,4-β-D- xylan xylohydrolase, EC. 3.2.1.37) hydrolyse xylooligosaccharides resulting in the xylose residues. Xylanases similar to the lysozymes, act by retention of anomeric carbon. There are several reports regarding the multiple forms of xylanases as isoenzymes. (Berenger *et al.*, 1985; Ohkoshi *et al.*, 1985; Kormelink *et al.*, 1992, 1993).

1.1.3 Xylanase producing microorganisms

Xylanases are produced by bacteria, fungi and actinomycetes. However there are reports of xylanases from fresh water mollusc (Yamura *et al.*, 1997) and plant fruits (Yamaki and Kakiuchi, 1979). Though high xylanase producing cultures are reported from the world of fungi, there are major problems associated with use of fungal xylanases like low pH and temperature optima. Moreover most of the fungi being acidophilic are grown in a media with initial pH of less than 7. Cellulase contamination poses an additional problem for application of the fungal enzymes in pulp industries as the former reduces the extent of cellulose recovery in the pulp. In the area of bacterial xylanases there are many reports regarding the xylanases with either high pH or temperature optima, both being optimal characteristics in pulp and paper industry application (Subramaniyan *et al.*, 1997). However, reports of the occurrence of both in the same strain are seldom seen or very rare. This necessitated the shift of attention of biotechnologists towards the isolation studies for potent organisms producing xylanases with the above mentioned characteristics.

1.1.4. Pulp and Paper industries

The environmental impact of pollutants from paper and pulp industry has drawn attention towards the application of xylanases in this industry, which needs separate consideration.

The conventional bleaching of paper pulp with chlorine results in the expulsion of effluents containing chloroaromatic compounds like chlorophenols, chlorobiphenyls and chlorolignin derivatives, and carcinogenic substances like dioxin to the environment. Kraft-pulping process involves the cooking of wood chips along with Na_2S / NaOH at 160-180^0 C. This results in incomplete degradation of lignin leading to the browning of pulp. The degradation of lignin is further impaired by the reprecipitated and relocated xylan. Thus, lignin degradation could be effected by the removal of this xylan veil which makes the lignin prone to less drastic oxidatives. The hydrolysis of xylan leads to easy acceptance of oxidative chemicals for the bleaching process.

Xylanases require some basic associated characteristics for their application in biotechnology aided paper production. The fact that the produced pulp always has high pH and temperature necessitates the use of alkaline thermostable xylanases for the pretreatment process before the bleaching step. Another important factor is, the enzyme production should be cost effective, which the industries can afford by utilising cultures producing higher levels of xylanases.

1.1.5. Optimisation of cultural and nutritional parameters of *Bacillus* SSP-34

Even though much importance has been given to purification, characterisation of xylanase proteins and elucidation of the xylanase genes, very less has been reported on optimisation of production of xylanases from bacteria including *Bacillus*. Manipulation of the cultural and nutritional conditions could effect overproduction of an enzyme from a microorganism. Experiments on the effect of pH, temperature, agitation,

aeration and inoculum concentration on the bacterial fermentation profile could be utilised for overproduction of the enzymes. The studies on nutritional parameters include effects of different carbon sources, nitrogen sources and micronutrients. There are various organic and inorganic substrates, which could be used as media for optimising the enzyme production.

1.1.6. Purification and characterisation of xylanases

The application of enzymes can be successfully completed only if the characteristics of the purified enzymes are thoroughly illustrated. There are reports dating from 1982 (Esteban *et al.*, 1982) regarding the purification of xylanases from various microorganisms. However, the purification of proteins from *Bacillus* spp. requires special consideration and integration of the various approaches. The enzymes purified are characterised and the data could be used in understanding the nature of enzymes and classifying the proteins.

1.1.7. Application of xylanases

There are several lines of evidence regarding application of xylanases in paper pulp and food industry. In paper and pulp industries they are used for the biobleaching of cooked kraft pulp which otherwise requires conventional chlorine treatment leading to environmental pollution. In food industry they can be used in juice clarification, softening of bread, treatment of food grains and in the treatment of agrowastes for successful utilisation by animals. Xylanases can also be utilised for conversion of agrowastes into single cell protein and ethanol based fuel. Pathogenic microorganisms invariably secrete xylanases, one of the major plant cell wall hydrolases. Study in this field could help elucidation of plant - pathogen relations.

1.2. Scope of the Present Study

There have been many models for solving the problems related to pulp and paper industry effluents, stretching from purely chemical methods to biotechnology-aided processes including application of fungal xylanases and even a combination of the above methods. However, the cellulase contamination, low pH and temperature optima, low stability at the required pH values and temperatures hamper the development of a biotechnological method in this area. In the meantime utilisation of microorganisms producing higher levels of xylanase is a good alternative in agrowaste treatment, silage treatment, in solving food industry related problems etc.

Search for microorganisms producing high levels of xylanase always needs more and more novel techniques for the isolation and identification of members from the existing or new organisms. Naturally high productive microorganisms have many advantages over the genetically manipulated ones because of their genetic stability and the more predictable biological nature. The manipulation of cultural and nutritional conditions alone can result in the over production of the metabolite of interest. All kind of applications of xylanases or enzymes in general, need characterisation studies of crude and purified proteins.

In the present study protocol has been formulated starting with the isolation, screening and identification of hyper producing microorganisms as the basis. After effecting this paramount target, studies on other hydrolases that influence the applications quoted earlier, like FPase, CMCase and proteases required special attention. The optimum cultural parameters of the best strain were looked into and the data on optimum nutritional parameters (including carbon, nitrogen and micronutrients) were also evaluated. Finally the determination of the molecular mass of purified xylanase was carried out which is important for its major

industrial applications. This was further supported by the characterisation studies both of crude as well as purified xylanase of *Bacillus* SSP-34.

The main objectives of the study could be summarised as following:

1. Isolation and screening of potent microorganisms which produce higher levels of endo-xylanases and identification of the selected potent cultures to their respective genera.
2. Search for hydrolases like FPase, CMCase and proteases from the selected culture, which may have marked influence in the industrial application of xylanases.
3. Define optimum cultural and nutritional parameters for enhanced production of xylanase from the potent culture. The studies on nutritional parameters included the effect of different carbon, nitrogen and micronutrients on enzyme production. The effect of carbon sources was studied using various carbohydrates including monosaccharides, disaccharides, polysaccharides and lignocellulosic substances. Induction of xylanases at different carbon sources was evaluated. The nitrogen sources studied include various organic, inorganic and complex ones.
4. Purification of xylanase from the selected potent culture. SDS-PAGE of the purified protein was conducted to determine the molecular weight and to check the purity of the protein. Zymograms were tried with substrate (oat spelts xylan) gels.
5. Kinetic studies on crude and purified xylanases. The studies cover determination of enzyme characteristics like *Km* and *Vmax*, pH and temperature optima, stability at various conditions including pH, temperature and finally influence of metal ions and metal chelators on xylanase activity. Most of the metal ions reported to be involving in

biological functions and metal ion chelators were evaluated for their influence in the activity of purified xylanase from *Bacillus* SSP-34.

6. Studies on the hydrolytic pattern of xylanases and application, which focus on the major field of endo-xylanase utilisation i.e. paper and pulp industries.

1.3. Review of Literature

1.3.1. Xylan and xylanolytic systems

Xylan, one of the major components of hemicelluloses found in plant cell wall is the second most abundant polysaccharide next to cellulose (Biely, 1985; Ali *et al.*, 1999; Wong and Maringer, 1999). The term hemicelluloses refers to plant cell wall polysaccharides that occur in close association with cellulose and glucans. In fact, the plant cell wall is a composite material in which cellulose, xylan and lignin are closely linked (Puls, 1997; Connerton *et al.*, 1999). Xylan, having a linear backbone of β 1,4-linked xyloses is present in all terrestrial plants and account for 30% of the cell wall material of annual plants, 15-30% of hard woods and 7-10% of soft woods (Sjostrom, 1981). Xylan is a heteropolysaccharide having O-acetyl, arabinosyl and 4-O-methyl-D-glucuronic acid substituents (Puls, 1997).

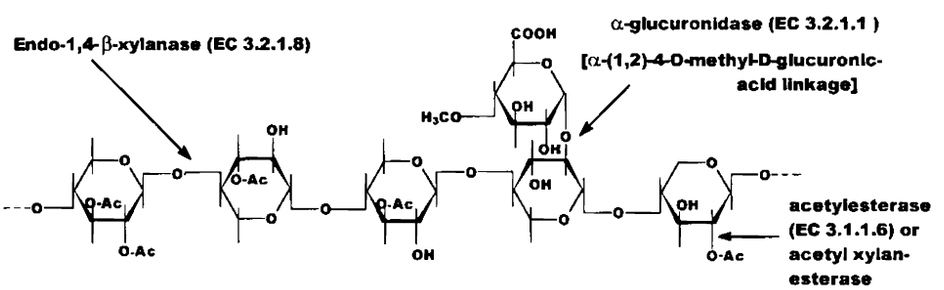


Fig. 1 Structure of O-acetyl-4-O-methylglucuronoxylan from hard wood (Puls, 1997). The side chains are 4-O-methyl-D-glucuronic acid and O-Ac (Acetyl group).

Similar to most of the other polysaccharides of plant origin, xylan also displays a large polydiversity and polymolecularity (Joseleau *et al.* 1992). It is present in a variety of plant species distributed in several types of tissues and cells. However, all terrestrial plant xylans are characterised by a β 1,4-linked D-xylopyranosyl main chain carrying a variable number of neutral or uronic monosaccharide subunits or short oligosaccharide

chains. Intra-chain hydrogen bonding also occur in unsubstituted xylan through the O-3 position which results in the helical twist to the structure. Nevertheless the acetylation and substitution disrupt and complicate this structure (Jeffries, 1996b).

Hard wood xylan is O-acetyl-4-methyl glucuronoxylan, in which the xylan chain is substituted at varying intervals with acetic acid and C-O methyl-D-glucuronic units. Approximately 60-70% of the xylose units are esterified with acetic acid at the hydroxyl group of carbon 2 and/or 3 and on an average every tenth xylose unit carries an α 1,2-linked uronic acid side group (Timell, 1967; Lindberg *et al.*, 1973) (Fig. 1).

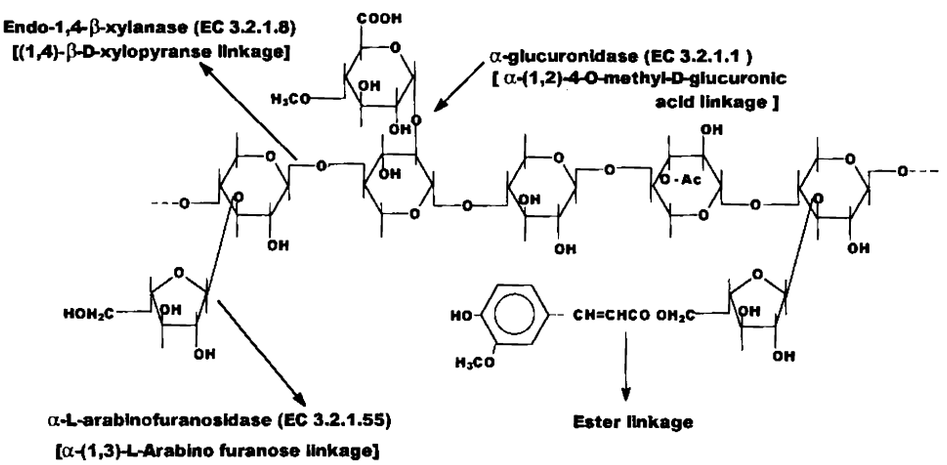


Fig. 2. Structure of arabinoxylan from grasses. The substituents are: arabinose, 4-O-methyl-D-glucuronic acid, O-Ac (acetyl group) and there is also ester linkage to phenolic acid group (Puls, 1997).

In the case of soft wood plants xylan is mainly arabino-4-O-methyl glucuronoxylan which in addition to 4-O-methyl glucuronic acid is also substituted by α-arabino furanoside units linked by α-1,3-linkage to the xylan backbone and the ratio of arabinose side groups to xylose residue is 1:8 (Timell, 1967) (Fig. 2). Rarely, acetyl groups are attached to the softwood xylan. The reducing ends of the xylan chains are reported to be linked to

rhamnose and galacturonic acid in order to make alkali resistant end groups of xylan chain (Andersson *et al.*, 1983).

There are reports regarding covalent lignin carbohydrate bonds by means of ester or ether linkages to hemicelluloses (Jeffries, 1990,1996b; Puls, 1997) but the covalent attachment to cellulose is less certain (Fig. 2). Finally the hemicelluloses are further associated with pectins and proteins in primary plant cell walls and with lignin in secondary walls, exact composition of which varies between organism and with cell differentiation (Jeffries, 1990).

1.3.2. Why xylanases

Xylans do not form tightly packed structures hence are more accessible to hydrolytic enzymes. Consequently, the specific activity of xylanase is 2-3 times greater than the hydrolases of other polymers like crystalline cellulose (Gilbert and Hazlewood, 1993). In the pulping process, the resultant pulp gets a characteristic brown colour due to the presence of residual lignin and its derivatives. The intensity of pulp colour is a function of the amount and chemical state of the remaining lignin. In order to obtain white and bright pulp suitable for manufacturing good quality papers, it is necessary to bleach the pulp to remove the constituents such as lignin and its degradation products, resins and metal ions (Singh, 1979). Biobleaching of pulp is reported to be more effective with xylanases than with lignin degrading enzymes. This is because the lignin is cross-linked mostly to the hemicellulose and the hemicellulose is more readily depolymerised than lignin (Jurasek and Paice, 1988).

Removal of even a small portion of the hemicellulose can be sufficient to open up the polymer and facilitate removal of the residual lignin by solvents. The principal objective behind application of biotechnological methods is to effect selective hemicellulose removal without degrading cellulose. Degradation of cellulose is the major problem associated with conventional pulping process, which invariably affects the

cellulose fibre, and thus the quality of paper (Shoham *et al.*, 1992). Removal of xylan from the cell walls by biotechnological methods leads to decrease in energy demand during bleaching (Noe *et al.*, 1986). Therefore enzymatic treatments of pulp using xylanases have better prospects in terms of both lower costs and improved fibre qualities.

1.3.3. Xylanolytic enzymes

The complex structure of xylan needs different enzymes for its complete hydrolysis. Endo-1,4-β-xylanases (1,4-β-D-xylan xylanohydrolase, E.C.3.2.1.8) depolymerise xylan by the random hydrolysis of xylan back bone and 1,4-β-D-xylosidase (1,4,β-D-xylan xylo hydrolase E.C.3.2.1.37) split off small oligosaccharides (IUB 1982). The side groups present in xylan are liberated by α-L-arabinosidase, α-D-glucuronidase, galactosidase and acetyl xylan esterase.

Endo-xylanases are reported to be produced mainly by microorganisms (Table1); many of the bacteria and fungi are reported to be producing xylanases (Wong *et al.*, 1988; Eriksson *et al.*, 1990). However, there are reports regarding xylanase origin from plants i.e. endo-xylanase production in Japanese pear fruit during the over ripening period (Yamaki and Kakiuchi, 1979) and later Cleemput *et al.* (1997) purified one endo-xylanase with a molecular weight of 55 kDa from the flour of Europian wheat (*Triticum aestivum*). Some members of higher animals, including fresh water mollusc are able to produce xylanases (Yamura *et al.*, 1997). There are lots of reports on microbial xylanases starting from 1960 (Gascoigne and Gascoigne, 1960). Nevertheless these reports have given prime importance to plant pathology related studies (Dekker and Richards, 1976; Takahashi and Kutsumi, 1979). Only during 1980's the great impact of xylanases has been tested in the area of biobleaching (Viikari *et al.*, 1986; Jurasek and Paice, 1986).

Exo-1,4-β-D-xylosidase (EC 3.2.1.37) catalyses the hydrolysis of 1,4-β-D-xylo-oligosaccharides by removing successive D-xylose residues

from the non-reducing end (IUB, 1982). The endo-xylanases reported to release xylose during hydrolysis of xylan do not have any activity against xylobiose, which could be easily hydrolysed by β-xylosidases (Panbangred *et al.*, 1984). There are reports regarding *Bacillus* sp. (Panbangred *et al.*, 1984) and different fungi (Poutanen, 1988) producing intracellular β-xylosidases. Many β-xylosidases are coupled with transferase activity, which is helpful for the organism to regulate xylanolytic systems. (Rodionova *et al.*, 1983; Conrad and Noethen, 1984).

α-Arabinofuranosidases (EC 3.2.1.55) hydrolyse the terminal, non-reducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans (IUB, 1982). A number of microorganisms including fungi, actinomycetes and other bacteria have been reported to produce α-arabinosidases (Kaji, 1984). *Bacillus polymyxa* – producing two different polypeptides with α-arbinofuranosidase activity - (Morales *et al.*, 1995a), *Streptomyces lividans* – single gene product - (Vincent *et al.*, 1997) and *Cytophaga xylanolytica* – two different polypeptides - (Kim *et al.*, 1998) are the examples of prokaryotic cultures characterised at the gene level for the production of α-arabinofuranosidases.

α-D-glucuronidases (EC 3.2.1.1) are required for the hydrolysis of the α-1,2-glycosidic linkages between xylose and D-glucuronic acid or its 4-O-methyl ether (Figs. 1 and 2). The hydrolysis of the far stable α-(1,2)-linkage is the bottle neck in the enzymatic hydrolysis of xylan and the reported α-glucuronidases are having different substrate requirements. Similar to lignin carbohydrate linkage, 4-O-methyl-glucuronic acid linkage is forming a barrier in wood degradation (Puls, 1992). There are number of microorganisms reported to be producing α-glucuronidases (Puls, 1992).

The complete hydrolysis of natural glucurono xylans requires esterases to remove the bound acetic and phenolic acids (Fig. 1, 2). Esterases break the bonds of xylose to acetic acid [acetyl xylan esterase

(EC 3.1.1.6)], arabinose side chain residues to ferulic acid (feruloyl esterase) and arabinose side chain residue to p-coumaric acid (p-coumaroyl esterase) (Christove and Prior, 1993). Hydrolysis of acetyl xylan and removal of feruloyl and p-coumaroyl groups from the xylan are helpful in the removal of lignin. They may contribute to lignin solubilisation by cleaving the ester linkages between lignin and hemicelluloses. If used along with xylanases and other xylan degrading enzymes in biopulping and biobleaching of pulps the esterases could partially disrupt and loosen the cell wall structure (Christove and Prior, 1993).

1.3.4. Xylanase producing microorganisms

Several microorganisms including fungi and bacteria have been reported to be readily hydrolysing xylans by synthesising 1,4-β-D endo-xylanases (E.C. 3.2.1.8) and β-xylosidases (EC.3.2.1.37). Reports regarding xylanases producing microorganisms date back to 1960s. Gascoigne and Gascoigne (1960) reported the synthesis of xylanases from the fungus *Fusarium roseum*. According to many of the early reports pathogenicity of xylanase producers in plants was an unifying character and it was thought that β-xylanases together with cellulose degrading enzymes play a role during primary invasion of the host tissues (Esteban *et al.*, 1982). There are reports regarding the induction of biosynthesis of ethylene (Fuchs *et al.*, 1989; Hanania and Avnni, 1997) and two of three classes of pathogenesis related proteins in tobacco plants by the microbial xylanases (Lotan and Fluhr, 1990). Thus these points reveal that certain xylanases can elicit defence mechanisms in plants. These actions may be mediated by specific signal oligosaccharides, collectively known as oligosaccharins or it may be due to the functioning of enzymes themselves or their fragments as the elicitors (Dean and Anderson, 1991; Dean *et al.*, 1991).

Most of the fungal plant pathogens produce plant cell wall polysaccharide degrading enzymes (Heiler *et al.*, 1993; Mendgen and

Deising, 1993). These enzymes result in the softening of the region of penetration by partial degradation of cell wall structures. A soft-rot producing bacteria *Erwinia chrysanthemi* was reported to be producing an array of plant cell wall degrading enzymes including endopectate lyase, exopolygalacturonase, pectin methyl esterase, endoglucanase and protease. Xylanase was produced in substantially greater amounts (Braun and Rodrigues, 1993). Xylanases have been reported in *Bacillus*, *Streptomyces* and other bacterial genera that do not have any role related to plant pathogenicity (Esteban *et al.*, 1982). Since the introduction of xylanases in paper and pulp and food industries (Viikari *et al.*, 1986; Biely, 1985), there have been many reports on xylanases from both bacterial and fungal microflora.

1.3.4.1. Panorama of bacterial xylanases

A search for microorganisms producing high levels of xylanase activity at alkaline pH and high temperature resulted in the isolation of several *Bacillus* spp. *Bacillus circulans* (Ratto *et al.*, 1992) produced 400 IU/ml of xylanase with a pH optimum of 7 and 40% of this activity was retained at pH 9.2. Importantly, the culture supernatant contained low levels of cellulolytic activities, with 1.38 IU/ml of endoglucanase (CMCase EC 3.2.1.4) and 0.05 U/ml of cellobiohydrolase (FPase - EC 3.2.1.91). *Bacillus stearothermophilus* strain T6 produced xylanases; however, the absolute levels were low and there was the associated production of detectable cellulase activity (0.021 IU/ml) (Shoham *et al.*, 1992; Khasin, *et al.*, 1993; Lundgren *et al.*, 1994). Lundgren *et al.* (1994) even tried a Mill trial TCF (total chlorine free) bleaching of pulp with xylanase from *Bacillus stearothermophilus* strain T6 which has optimum activity at pH 6.5 (Lundgren *et al.*, 1994; Khasin *et al.*, 1993). *Rhodothermus marinus* was found to produce thermostable xylanases (approximately 1.8-4.03 IU/ml) but there was also detectable

amounts of thermostable cellulolytic activities (Dahlberg *et al.,* 1993; Hreggvidsson *et al.,* 1996). Most of the other bacteria which degraded hemicellulosic materials are reported to be potent cellulase producers including *Cellulomonas* sp. (xylanase, 9.33 U/ml and CMCase, 0.94 U/ml), *Micrococcus* sp. (xylanase, 3.33 U/ml and CMCase, 3.11 U/ml) (Saxena *et al.,* 1991) and *Streptomyces roseiscleroticus* NRRL-B-11019 (xylanase 16.2, IU/ml and cellulase 0.21 IU/ml) (Grabski and Jeffries, 1991). The strict thermophilic anaerobe *Caldocellum saccharolyticum* possess xylanases with optimum activities at pH values 5.5-6.0 and at temperature 70°C (Luthi *et al.,* 1990). Mathrani and Ahring (1992) reported xylanases from *Dictyoglomus* sp. having optimum activities at pH 5.5 and 90°C, however merits the significant pH stability at pH values 5.5-9.0. A detailed description of all other organisms producing cellulases along with xylanases are given in Table 1.

1.3.4.2. Fungal xylanases and associated problems.

The 15 leading companies manufacturing xylanase preparations invariably use fungal xylanases, all of which have an optimum pH $\leq$ 5.5 (Haltrich *et al.,* 1996). The optimum pH for xylan hydrolysis is about 5 for most of the fungal xylanases, which are normally stable at pH 2 –9. Most of the fungal xylanases tolerate temperatures below 50^{0}C. Application of fungi in paper and pulp industries is further impaired by the growth requirement of the fungi themselves. However, this is not the case with bacteria (Table 1)

The optimal pH for bacterial xylanases in general, is slightly higher than the pH optima of fungal xylanases (Okazaki *et al.,* 1985; Honda *et al.,* 1985a; Khasin *et al.,* 1993). In most industrial applications, especially paper and pulp industries, the low pH required for the optimal growth and activity of xylanase necessitate additional steps in the subsequent stages which make fungal xylanases less suitable. Although high levels of xylanase activity are produced by several fungi, the presence of

considerable amount of cellulase activity and lower pH optimum, make them less suitable for pulp and paper industries. Gomes *et al.* (1992) reported xylanase activity (188.1 U/ml, optimum pH 5.2) and FPase activity (0.55 U/ml, optimum pH 4.5) from *Trichoderma viride*. *Trichoderma reesei* has been shown to produce even higher levels of xylanase (approximately 960 IU/ml for 180 U/ml of culture filtrate) but this was associated with a corresponding increase in cellulase production (9.6 IU/ml) (Bailey *et al.*, 1993). *Thermomyces lanuginosus* under submerged fermentation showed 2172 U/ml of xylanase yield after 7 days (Purkarthofer *et al.*, 1993). Like *Thermomyces lanuginosus*, *Schizophillum commune* is also one of the high xylanase producers with a xylanase activity of 1244 U/ml, CMCase activity 65.3 U/ml and FPase activity 5.0 U/ml (Steiner *et al.*, 1987). Among white rot fungi, a potent plant cell wall degrading fungus, *Phanerochaete chrysosporium* produced a xylanase activity of 15-20 U/ml in the culture medium, but the medium contained high levels of cellulase activity measuring about 12% of maximum xylanase activity (Copa-Patino *et al.*, 1993). Reports of fungal isolates with negligible cellulase activity (0.01 U/ml) like *Thermomyces lanuginosus* are very rare (Gomes *et al.*, 1993). All other fungal strains were showing considerable levels of cellulase activities (Table 1).

Another major problem associated is the reduced xylanase yield when fungi are grown in a fermentor. The shearing forces in fermentor originating from agitation cause disruption of fungal biomass (Taguchi, 1971; Palma *et al.*, 1996).

Even though there are differences in the growth conditions including pH, agitation and aeration, and the optimum conditions needed for xylanase activity (Taguchi, 1971; Honda *et al.*, 1985a; Okazaki *et al.*, 1985; Steiner *et. al.*; 1987; Bailey and Poutanen, 1989; Grabski and Jeffries, 1991; Ratto *et al.*, 1992; Copa-Patino *et al.*, 1993; Gomes *et al.*; 1992,1993; Bailey *et al.*, 1993; Haltrich *et al.*, 1996; Palma *et al.*, 1996;

Subramaniyan *et al.*, 1997; Subramaniyan and Prema, 1998) there are little differences regarding molecular biology and biochemistry of prokaryotic and fungal xylanases (Gilbert and Hazlewood, 1993).

1.3.5. Mode of fermentation for xylanase production.

Both submerged and solid state fermentations have been exploited for the xylanase production from microorganisms. Several workers have reported the production of xylanases from · fungi and bacteria. *Schizophillum commune*, 1244 U/ml xylanase activity (Steiner *et al.*, 1987), *Trichoderma reesei*, 960 IU/ml xylanase activity (Bailey *et al.*, 1993) and *Thermomyces lanuginosus*, 2172 U/ml (Purkarthofer *et al.*, 1993), showed higher levels of production in submerged fermentation. In the case of bacteria *Bacillus circulans,* 400 IU/ml xylanase activity (Ratto *et al.*, 1992) and *Bacillus* SSP-34, 379 IU/ml xylanase activity (Subramaniyan *et al.* 2000) were the good producers of xylanases. There are several reports regarding the production of xylanases under solid state fermentation by fungi. Xylanase produced by *Thermomyces lanuginosus* was extracted by using 500 ml of distilled water and the diluted filtrate showed a xylanase activity of 172 U/ml (Hoq *et al.*, 1992). A comparative study on the production of xylanases by *Thermomyces lanuginosus* under submerged and solid state fermentations showed 2172 U/ml of xylanase yield after 7 days and 20220 U/g dry solid within 9days respectively (Purkarthofer *et al.*, 1993). But Kalogeris *et al.* (1998) reported a xylanase activity of 6193 U/g from *Thermoascus aurantiacus* Miehe IMI 216529 under solid state fermentation. There are other reports regarding microorganisms producing higher levels of xylanase activities like *Aspergillus niger* A3 (5147 U/g dry mycelium) (Cai *et al.*, 1997), *Aspergillus tamarii* (Ferreira *et al.*, 1999) and a list of fungi producing xylanases by solid state fermentation was made by Haltrich *et al.* (1996). Reports regarding the production of xylanases under solid state fermentation by bacteria are rare. Archana and

Satyanarayana (Arcahna and Satyanarayana, 1997) reported xylanase production of 19.13 U/g dry bacterial bran by *Bacillus licheniformis* A99.

Table1. Xylanase and cellulase production from microorganisms.

Microorganism	Initial pH of the culture mediu m	Xylan ase IU/ml	Cellulase (IU/ml)		Reference
			FPase	CMCase	
FUNGI					
Arthrographis sp.strain F4	5.5	8.95	2.54	13.37	Okeke and Obi (1993)
Aspergillus awamori VTT-D-75028	5.5	12.0	0.1	3.2	Poutanen, *et al.* (1987)
Aspergillus niger KKS	7.0	138	3.9	1.2	Kang *et al.* (1995)
Chaetomium globosum 11-Ch.g./5	4.8	65.3	1.5	2.2	Zychlinska *et al.*, (1992)
Fusarium oxysporum VTT-D-80134	5.5	3.7	0.1	0.7	Poutanen, *et al.* (1987)
Irpex lacteus KY 2902	4.5	353	9.8	252	Kawai *et al.* (1978)
Pencillium pinophilum NTG III/6	-	27.3	8.14	165	Brown *et al.*(1987)
Phanerochate chrysosporium	4.5	15-20	-	1.8-2.4	Copa-Patino *et al.*, (1993)
Piromyces sp.strain E 2	-	7.96	0.009	0.77	Tenuissen *et al.* (1992)
Schizophyllum commune	5-5.5	1244	65.3	5.0	Steiner *et al.* (1987)
Schizophyllum radiatum	6	5.7	2.3	2.4	Cvazzoni *et al.* (1989)
Sclerotium rolfsii	5	267	1235	10	Sachslehner *et al.* (1997)
Sporotrichum pulverulentum	4.5-5.0	20.4	0.13	9.48	Eriksson and Johnsrud (1983)
Talaromyces emersonii CBS 814.70	4.5	56	26.7	2.41	Tuohy *et al.* (1990)
Thermomyces lanuginosus [a]	6.0	650-780	0.01	0.01	Gomes *et al.*(1993)
Thielvia terrestris ATCC 26917	-	25.5	0.11	8.7	Merchant *et al.* (1988)
Tiarosporella phaseolina	5.5	5.09	0.04	1.49	Gomes *et al.* (1989)
Trichoderma harzianum	-	450	2.8	66.0	Saddler *et al.* (1985)

Microorganism	Initial pH of the culture mediu m	Xylan ase IU/ml	Cellulase (IU/ml)		Reference
			Fpase	CMCase	
Trichoderma reesei RUT C-30 ATCC 56765 [a]	5.0	400	-	6.0	Gamerith *et al.* (1992)
Trichoderma reesei [b]	4.4	960	0.7	9.6	Bailey *et al.*(1993)
Trichoderma reesei PC-3-7	4.0	1.67	-	1.76	Xu *et al.* (1998)
Trichoderma viride	5.5	188.1	0.55	-	Gomes *et al.* (1992)
BACTERIA					
Bacillus circulans	-	400	0.05	1.38	Ratto *et al.* (1992)
Bacillus stearothermophilus StrainT6[a]	7.0	2.33	-	0.021	Shoham *et al.* (1992), Khasin *et al.* (1993) Lundgren *et al.* (1994)
Bacillus sp.	9.0	120	-	0.05	Balakrishnan *et al.* (1992)
Bacillus sp.	7.2	11.5±	-	1.2±0.13	Paul and Varma. (1993)
Cellulomonas sp. ATCC 21399	-	0.301-0.888	-	0.013-0.117	Poulsen. And Petersen (1988)
Cellulomonas flavigena NIAB 441	7.3	16			Rajoka *and* Malik (1984)
Cellulomonas sp. (GS2)	-	9.33	-	0.72	Saxena *et al.* (1991)
Micrococcus sp. (DG10)	-	3.33		3.11	Saxena *et al.* (1991)
Rhodothermus marinus [a]	7.1	1.8-4.03	0.05	0.025	Dahlberg *et al.* (1993), Hreggvidsson *et al.* (1996)
Streptomyces roseiscleroticus NRRL-B-11019 [a,c]	7.0	16.2	-	0.21	Grabski and Jeffries (1991)

a. Microorganisms reported to be producing `virtually` cellulase-free xylanases

b. Cellulase assay was performed using hydroxyethyl cellulose.

c. Cellulase assay carried out using 1 % acid swollen cellulose prepared from Solka floc SW 40 wood pulp cellulose (Brown Co. Berlin, N.H.).

1.3.6. Classification of xylanases

Wong *et al.* (1988) classified microbial xylanases into two groups on the basis of their physiochemical properties such as molecular mass and isoelectric point, rather than on their different catalytic properties. While one group consists of high molecular mass enzymes with low pI values, the other has low molecular mass enzymes with high pI values, but exceptions are there.The above observation was later found to be in tune with the classification of glycanases on the basis of hydrophobic cluster analysis and sequence similarities (Gilkes *et al.*, 1991a,b; Henrissat and Bairoch, 1993).

The high molecular weight endo-xylanases with low pI values belong to glycanase family 10 (Henrissat and Bairoch, 1993) formerly known as family 'F' while the low molecular mass endo-xylanases with high pI values are classified as glycanase family 11 (formerly family G). A number of xylanases belonging to the family G/11 are having single domain β-sheet proteins while the high molecular weight family F/10

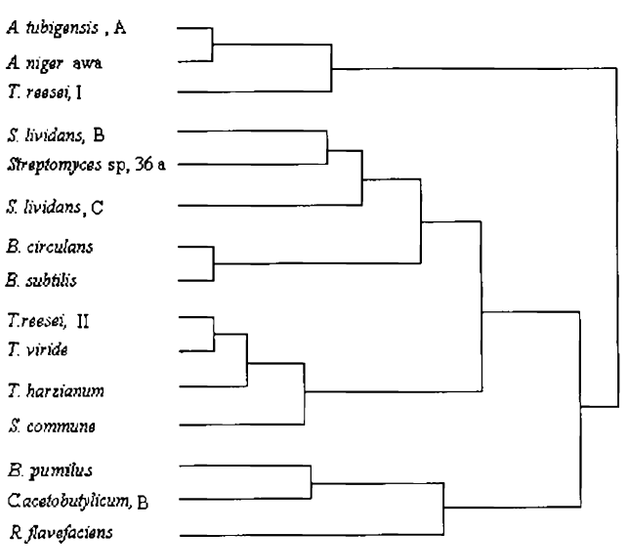


Fig. 3 Evolutionary relationship among low molecular weight xylanases based on the multiple alignments of amino acid sequencing (Courtesy Oku *et al.* 1993).

xylanases, (>35 kDa) are a having an eight fold α/β barrel structure (Connerton *et al.,* 1999). Recently there has been the addition of 23 endo-xylanases in family 10 and 17 in family 11 (Henrissat and Bairoch, 1996). Biely *et al.* after extensive study on the differences in catalytic properties among the xylanase families concluded that endo-xylanases of family10 in contrast to the members of family 11 are capable of attacking the glycosidic linkages next to the branch and towards the non-reducing end (Biely *et al.,* 1997). Endo-xylanases of family 10 require two unsubstituted xylopyranosyl residues between the branches while the endo-xylanases of family 11 require three unsubstituted consecutive xylopyranosyl residues. According to them endo-xylanases of family 10 possess several catalytic activities which are compatible with β-xylosidases. They liberate terminal xylopyranosyl residues attached to a substituted xylopyranosyl residue, but they also exhibit aryl-β-D-xylosidase activity.

From a comparison of the amino acid sequence a dendrogram has been constructed to study the possible evolutionary relationships among these xylanases (Oku *et al.*, 1993). The results showed that these low molecular mass xylanases could be classified into three major groups. The first group consists of *A. tubigensis*, *A. niger* awa and *Trichoderma reesei* I xylanases. Apparently this group diverged from the others before other bacterial and fungal xylanases were separated to form their own groups. *Schizophyllum commune* xylanase is highly similar to *Trichoderma harzianum*, *T. reesei* and *T. viride* xylanases (Oku *et al.* 1993).

1.3.7. Multiple forms of xylanases

Ohkoshi *et al.* (1985) reported the occurrence of three types of xylanases from the alkalophilic *Aeromonas* sp. *Streptomyces* sp. B-12-2 produced five endo-xylanases when grown on oat spelt xylan (Elegir *et al.,* 1994). Kormelink *et al.,* (1993) obtained two types of xylanases, endo-xylanase I and III from *Aspergillus* sp., of which endo-xylanase I could degrade xylan to a higher extent than endo-xylanase III. John *et al.* (1979)

reported the occurrence of five endo-xylanases from *Aspergillus niger*. The culture filtrate of *Aspergillus niger* was composed of 15 xylanases and *Trichoderma viride* of 13 (Biely *et al.*, 1985), while *Butyrivibrio fibrisolvens* (Lin and Thomson, 1991) and *Talaromyces emersoni* (Coughlan *et al.*, 1993) produced 11 and 11-13 xylanases respectively. The most outstanding case regarding multiple forms of xylanases was production of more than 30 different protein bands separated by analytical electrofocusing from *Phanerochaete chrysosporium* grown in Avicel (Dobozi *et al.*, 1992). There are several reports regarding fungi and bacteria producing multiple forms of xylanases (Ohkoshi *et al.*, 1985; Berenger *et al.*, 1985; Kormelink *et al.*, 1992, 1993). The filamentous fungus *Trichoderma viride* and its derivative *T. reesii* produce three cellulase free β-1,4-endo-xylanases (Biely, 1985; Lappalainen, 1986). Due to the complex structure of heteroxylans, all of the xylosidic linkages in the substrates are not equally accessible to xylan degrading enzymes. Because of the above hydrolysis of xylan requires the action of multiple xylanases with overlapping but different specificities (Wong *et al.*, 1988). Recently Wong and Maringer (1999) after extensive statistical analyses concluded that there might not always be the synergy of different xylanases. They were also of the opinion that the substrate used for assay was hydrolysed differently by the multiple forms of xylanases (Wong and Maringer, 1999).

1.3.8. Origin of multienzymes

The fact that protein modification (e.g. post transcription cleavage) leads to the genesis of multi-enzymes has been confirmed by various reports (Leathers, 1988; Li and Ljungdahl, 1994). Leathers (1988) identified one xylanase, APXI with a molecular weight of 20 kDa and later another xylanase APX II (25 kDa) was purified by Li *et al.* (1993) from the same organism *Aureobasidium.* However, according to Li and Ljungdahl (1994), APXI and APXII are encoded by the gene xyn A. This

suggestion was based on their almost identical N-terminal amino acid sequences, immunological characteristics and regulatory relationships and the presence of a single copy of the gene and the transcript (Li and Ljungdahl 1994). Purified APX I and APX II from *Aureobasidium pullulans* differ in their molecular weights. Post-transcriptional modifications such as glycosylation, proteolysis or both could contribute to this phenomenon (Leathers, 1989; Li *et al.*, 1993; Li and Ljungdahl 1994). Therefore several factors could be responsible for the multiplicity of xylanases. These include differential mRNA processing, post-secretional modification by proteolytic digestion, and post-translational modification such as glycosylation and autoaggregation (Biely, 1985; Coughlan *et al.*, 1993). Multiple xylanases can also be the product from different alleles of the same gene (Wong *et al.*, 1988). However, some of the multiple xylanases are the result of independent genes (Hazlewood and Gilbert, 1993). Finally the functional purpose of multiple xylanases necessitate a detailed description which remains unclear to date (Wong and Maringer, 1999).

1.3.9. Purification of xylanases

Column chromatographic techniques, mainly ion exchange and size exclusion are the generally utilised schemes for xylanase purification, but there are also reports of purification with hydrophobic interaction column chromatography (Wong and Saddler, 1992). There are several reports regarding the purification of xylanases to electrophoretic homogeneity. However, the yield and purification fold varies in different cases. (Table 2). In all the cases the culture supernatants are initially concentrated using precipitation or ultrafiltration techniques. Use of cellulose materials as the matrix in column chromatography is impaired by the fact that certain xylanases are having cellulose binding domains, which will interact with the normal elution process (Gilkes *et al.*, 1991a,b).

Table 2. Characterisation of xylanases from different microorganisms

Microorganisms	Mol. Wt. (kDa)	Purification fold	Yield (%)	Optimum pH and Temperature		Stabilities at		pI	K_m (mg/ml)	V_{max} (µmol / min / mg)	Reference
				pH	Temperature	pH	Temp.				
FUNGI											
Aureobasidium pullulans Y-2311-1	25	5.8	10.3	4.8	54	4.5	50	9.4	7.6	2650	Li *et al.* (1993)
Aureobasidium pullulans ATCC 42023	21	38	6.3	3-4.5	35	-	-	-	2.93	866	Vadi *et al.* (1996)
Cephalosporium sp.strain RYM-202	35	17.3	9.9	7.5-8.0	50	-	-	6.3	5.26	118.4	Kang *et al.* (1996)
	24	22.9	15.0	7.5-8.0	50	-	-	4.4	4.16	145.2	,,
Fusarium oxysporum F3	20.8	3.96	19.2	6.0	60	9-10	-	9.5	0.41	-	Christakopoulos *et al.* (1996)
	23.5	0.54	1.3	6.0	55	7-9	-	8.45-8.7	0.37	-	,,
Humicola insolens	6.0	-	-	6-6.65	55-60	-	-	9.0	-	-	Dusterhoft *et al.* (1997)
	21	-	-	6-6.5	55-60	-	-	7.7	-	-	,,
Irpex lacteus	38	-	-	4.6-5.2	-	-	-	7.6-8	-	-	Haoebler and Brillouet, (1984)
Lentinula edodes	41	8.9	0.3	4.5-5	60	-	-	3.6	0.66	-	Mishra *et al.* (1990)
Aspergillus awamori	39	-	-	5.5-6	55	-	-	5.7-6.7	1.0	10000	Kormelink *et al.* (1993)
	23	-	-	5.0	50	-	-	3.7	0.33	3333	,,
	26	-	-	4.0	45-50	-	-	3.3-3.5	0.09	455	,,
Aspergillus nidulans	34	24	7.5	6	56	4.0-6.7	56	3.4	0.97	1091	Fernadez-Espinar et al (1994)
Aspergillus sojae	32.7	8.2	9	5.5	60	5-8	50	3.5	-	-	Kimura *et al.* (1995)
	35	4.6	5	5.5	50	5-8	35	3.75	-	-	,,
Neurospora crassa	33	-	-	4.8	-	-	-	4.8	-	-	Mishra *et al.* (1984)
	30	-	-	4.8	-	-	-	4.5	-	-	,,
Pencillium purpurogenum	33	15.8	5.7	7.0	-	4.5-5.5	-	-	-	-	Belancic *et al.* (1995)
	23	5	4.3	3.5	-	4.5-5.5	-	-	-	-	,,

Table Continued..

Table 2. Continuation

Microorganisms	Mol. Wt. (kDa)	Purification fold	Yield (%)	Optimum pH and Temperature		Stabilities at		pI	K_m (mg/ml)	V_{max} (µmol / min / mg)	Reference
				pH	Temperature	pH	Temp.				
Schizophyllum commune	21	-	-	5	-	-	-	4.5	-	-	Jurasek and Paice, (1988)
Thermoascus aurantiacus	32	-	-	5.1	-	-	-	7.1	-	-	Tan *et al.* (1987)
Trichoderma koningii	18	-	-	4.9-5.5	-	-	-	7.3	-	-	Wood and McCrace (1986)
	29	-	-	4.9-5.8	-	-	-	7.2	-	-	,,
Trichoderma longibrachiatum	37.7	55.8	5.1	5-6	45	5	-	-	10.14	4025	Chen *et al.* (1997)
Trichoderma viride	22	16	12.5	5	53	-	-	9.3	4.5	160	Ujiie *et al.* (1991)
Trichoderma harzianun	20	7.5	-	5.0	50	-	40	-	0.58	0.106	Tan *et al.*(1985)
Bacteria											
Aeromonas caviae ME1	20	-	-	7	50	30-40	6.5-8	7.1	9.4	4330	Kubata *et al.* (1992)
Bacillus amyloliquefaciens	18.5-19.6	7.3	53.9	6.8-7.0	80	9	50	10.1	-	-	Breccia *et al.* (1998)
Bacillus circulans WL-12	85	-	-	5.5-7	-	-	-	4.5	8	-	Esteban *et al.* (1982)
	15	-	-	5.5-7	-	-	-	9.1	4.0	-	,,
Bacillus sp. 11-1S	-	57	-	4	80	2-6	80	-	1.68	-	Uchino and Nakane (1981).
Bacillus sp. W1 (JCM2888)	21.5	24.6	25	6	65	4.5-10	-	8.5	4.5	-	Akiba and Horikoshi (1988).
	49.5	9.6	2.6	7.9	70	4.5-70	-	3.7	0.95	-	,,
Bacillus strain XE	22	1.86	62	6	75	4.8-	60	7.7	0.6	-	Debeire-Gosselin *et al.* (1992b)
Bacillus stearothermophilus T-6	43	38.9	46	6.5	75	7	65	9	1.63	288	Khasin *et al.* (1993).
Bacillus sp.strain 41-1(36)	36	3.6	15.3	9	50	-	-	5.3	3.3	1100	Nakamura *et al.* (1993b).
Bacillus sp.strain TAR-1	40	-	-	6	75	-	-	4.1	-	-	Nakamura *et al.* (1994).
Bacillus sp. strain K-1	23	-	-	5.5	60	12	50	-	-	-	Ratanakhanokchai *et al.* (1999)

Table Continued..

Table 2. Continuation

Microorganisms	Mol. Wt. (kDa)	Purification fold	Yield (%)	Optimum pH and Temperature		Stabilities at		pI	K_m (mg/ml)	V_{max} (µmol / min / mg)	Reference
				pH	Temp.	pH	Temp.				
Cryptococcus flavous	31	55	-	4.5	55	3.0-8.0	45	10	3.1	-	Nakanishi *et al.*, (1984).
Chainia sp.	5.5-6.0	75	-	6.0	60	4.5-7.5	50	8.5-9.0	5.0	-	Bastawde (1987).
Clostridium thermolacticum	39	66	70	6.5	80	-	-	4.45	0.4	3300	Debeire-Gosselin *et al.* 1992a
	55	66	70	6.5	80	-	-	4.55	0.53	3140	,,
	65	66	70	6.5	80	-	-	4.65	0.48	2950	,,
Erwinia chrysanthemi	42	19.9	3.12	5.5	55	4-7	35	8.8	-	-	Braun and Rodrigues (1993).
Microtetraspora flexuosa S 11X	26.3	16.8	8.3	6	80	-	-	8.4	2.44	1537	Berens *et al.* (1996)
	16.8	15.7	23.8	6	80	-		9.45		353	,,
Streptomyces sp.	50	3.9	21	5.5-6.5	60-65	3-10.5	55	7.1	9.1	-	Marui *et al.* (1985).
	25	2.6	7	5.0-6.0	60-65	1.5-11.5	55	10.06	-	-	,,
	25.68	3.8	23	5.0-6.0	60-65	1.5-11.5	55	10.26	11.2	-	,,
Streptomyces T-7	20.643	41.3	6.7	4.5-5.5	60	5	50	7.8	10 '	7600	Keskar *et al.* (1989).
Streptomyces sp. No 3137	50	48	33	5.5-6.5	60-65	5.5-6.5	55	7.1	9.1[a]	-	Nakanishi *et al.* (1992).
	25	2.85	2	5.0-6.0	60-65	5.0-6.0	55	10.06	-	-	,,
	25	3.6	8	5.0-6.0	60-65	5.0-6.0	55	10.26	11.2 [a]	-	,,
Streptomyces lividans 66	22	4.5	27.3	6	57	-	-	>10.25	4.1	3.0	Kluepfel *et al.* (1992).
Streptomyces sp. Strain C5-A13	19.5	13.8	12	8.5-9.5	55	-	-		2.7X10^{-5}	2.0	Vinci *et al.* (1993).
Thermoanaerobacterium sp. Strain JW/SL-YS485	350 [b]	2.7	6.2	80	6.5	-	-	-	4.37	26680	Shao *et al.* (1995).
Thermotoga thermarum	266 [c]	22.5	16.2	6	80	-	-	-	0.36	1.18	Sunna *et al.* (1996).
	35 [d]	1.9	1.5	7	90-100	-	-	-	0.24	19.5	,,

a. Km estimated on xylotetrose. b. Total mass of heterosubunits. c. Dimer of 105 Kda and 150 Kda. d. Monomer

1.3.10. Structure of xylanases

The differences in catalytic activities of endo-xylanases of family 10 and 11 can be attributed to the differences in their tertiary structure. The family 11 endo-xylanases are smaller and are well packed molecules with molecular organization mainly of β-pleated sheets (Wakarchuk *et al.*, 1994; Torronen *et al.*, 1994). These enzymes appear very small in native state (Grabski and Jeffries, 1991). The catalytic groups present in the cleft accommodate a chain of five to seven xylopyranosyl residues. The substrate binds to the shallow groove at the bottom of the 'bowl'. The (α/β) barrel appears to be the structure of endo-xylanases of family 10. The substrate binding sites of the family 10 endo-xylanases are apparently not in such deep cleft as the substrate binding sites of family 11 endo-xylanases. This fact together with a possible greater conformational flexibility of the larger enzymes compared to the smaller ones may account for a lower substrate specificity of family 10 endo-xylanases (Jeffries, 1996a,b). Another important point is that two glutamic acid residues, Glu78 and Glu172 are conserved in the entire family of G/11 xylanases. These glutamic acid residues are pointing to the active site. The Glu 78 and Glu 172 are acting as a nucleophile and an acid catalyst respectively (Wakarchuk *et al.*, 1994). (Fig. 4 and 5). Connerton *et al.* (1999) found an unusual occurrence of aromatic residues (eg. Trp 5) on the surface of family G/11 xylanase from *Bacillus* D3. According to Connerton *et al.*, (1999) these aromatic residues (11 in total) forming inter-molecular clusters or sticky patches could be responsible for thermostability of the enzyme. This observation is important because careful genetic manipulation could result in the conversion of unique xylanases into highly thermostable forms.

1.3.11.Catalytic sites

The structure of *Bacillus* 1,4-β-xylanases as mentioned earlier, have a cleft which according to Torronen *et al.* (1994) can be the active site. There are two members of the family 11 xylanases, (XYNII from *Trichoderma harzianum* and 1XNB from *Bacillus circulans*) which clearly show this kind of catalytic sites (Torronen *et al.,* 1994; Davoodi *et al.,* 1995). The *Bacillus circulans* xylanase has two proximal carboxylates, Glu 172 and Glu 78, which act as an acid catalyst and nucleophil respectively (Torrenen *et al.,* 1994; Wakarchuk *et al.*, 1994). The abnormally high p*K*a of Glu 172, the character which enabled it to act as acid catalyst is resulting from the electrostatic interactions with neighbouring groups like the Arg 112 (Davoodi *et al.,* 1995) (Fig. 4 and 5). Endo-1, 4-xylanases of the F10 xylanases is having a cylindrical [(α)/ (β)] barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the barrel (Derewenda *et al.,* 1994; Ali *et al.*, 1999) and there are five xylopyranose binding sites. The high molecular weight F10 xylanases tend to form low DP oligosaccharides. Xylanase cex from *Cellulomonas fimi* has a catalytic (N-terminus) region and a cellulose-binding domain (C-terminus), the former resembling the head and the latter the tail of a tadpole structure (White *et al.,* 1994). Wakarchuk *et al.* (1994) observed that Tyr 69 of *Bacillus circulans* xylanase makes 2 hydrogen bonds, one with C_2OH groups of the non reducing xylose ring and the second with Glu 78. This hydrogen bond to Glu 78 possibly help the orientation of nucleophile for reaction while the other one helps to position the substrate (Wakarchuk *et al.*, 1994; Fig. 5). Tyr 80 of *Bacillus circulans* was suggested to have role in hydrogen bonding to longer substrate molecules.

The members of family F11 have catalytic domains formed from β-pleated sheets which form a two layered trough surrounding the catalytic site (Withers and Aebersold, 1995) (Fig. 5). Törrönen and Rouvinen have

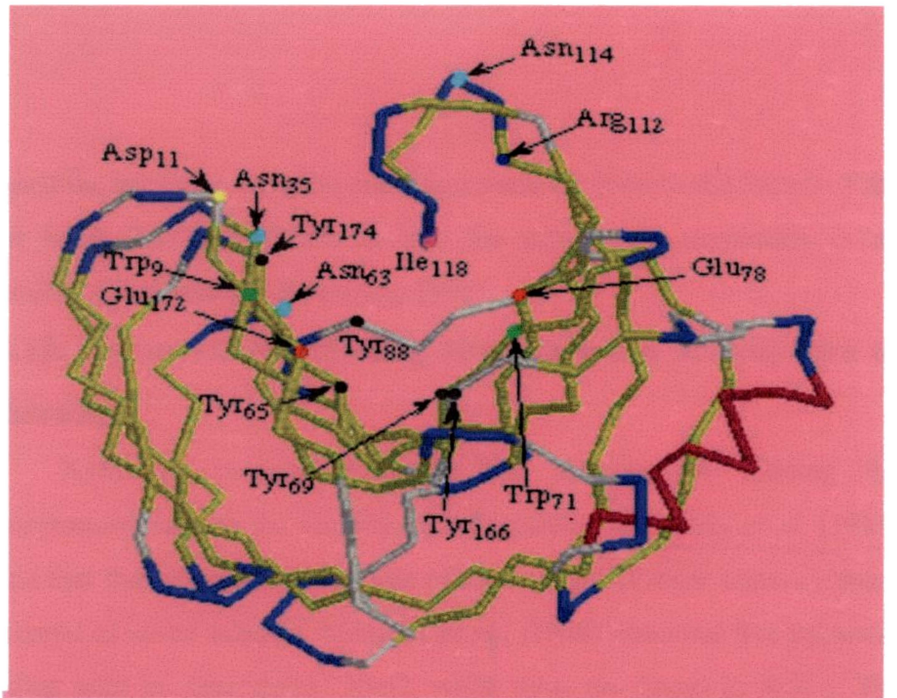


Fig. 4 The three-dimensional structure of the Family 11 xylanase (1XNB) from *Bacillus circulans*. Yellow colour denotes β-pleated structure while blue and red corresponds to bends and α-helical structures. (Courtesy Jeffries, 1996a,b).

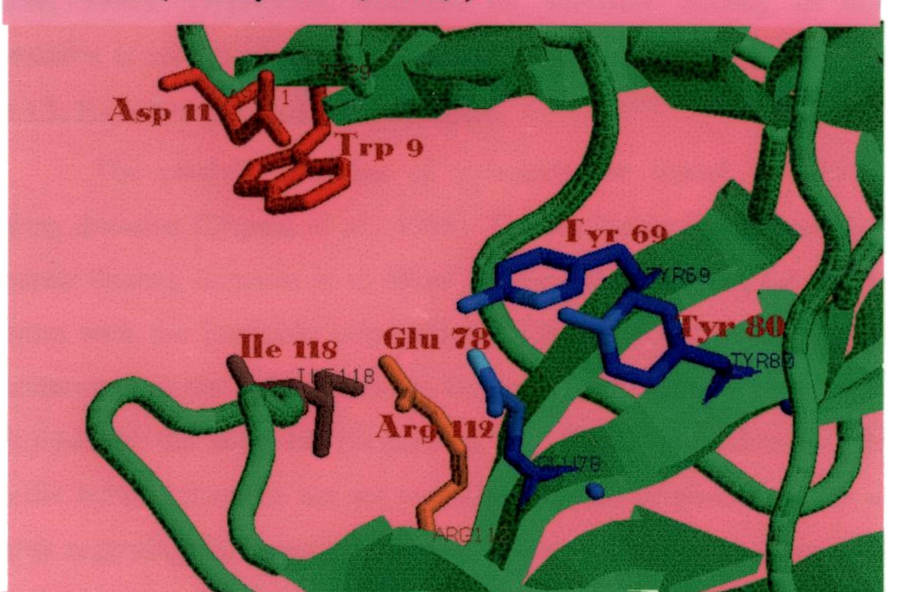


Fig. 5 The backbone of *Bacillus circulans* xylanase with side chains of the active site. The protein's structure (code: 1XNB) was retrieved from SCOP's (Structural Classification of Proteins) data bank deposited by Campbell *et al.* (1994). The three dimensional structure was generated with the help of X-Ray diffraction at resolution 1.49 Å. Molecular structure was generated with the help of RasWin and Corel Photo-Paint.

likened the trough to the palm and fingers and the loop to the thumb of the right hand. The loop protrudes into the trough and terminates in an isoleusin. (Törrönen and Rouvinen, 1995).

1.3.12. Xylopyranose binding sites near the activity site of xylanases

Xylanases possess three to five subsites for binding the xylopyranose rings in the vicinity of the catalytic site. Biely *et al.* (1983) found that the substrate-binding site of an *Aspergillus niger* endo-xylanase consisted of seven subsites. Meagher *et al.*, (1988) observed five pyranose binding sites in *Aspergillus* Xyn2, while three are found in Xyn I. The subsites for binding xylopyranose residues are defined by the presence of tyrosine as opposed to tryptophan (Wakarchuk *et al.,* 1994; Bray and Clarke, 1995). Tryptophan, essential for substrate binding in most glycosides, is not reported to have a role in xylanase action.

1.3.13. Substrate-binding domains

The catalytic domains of xylanases are linked to substrate binding domains (Dupont *et al.,* 1998). The justifiable function of these substrate-binding domains is to allow unerring alignment of the soluble enzyme with the insoluble polysaccharide, there by increasing enzyme concentration at the point of attack. However, they are not essential for hydrolysis of the substrate (Dupont *et al.,* 1998). Even though xylan specific binding domains are not common in xylanase proteins, there are reports regarding the same eg. endo-xylanase of *Bacillus* sp. Strain K-1 (Ratanakhanokchai *et al.*, 1999), the xylanases STX I and II of *Streptomyces thermoviolaceus* OPC-520 and Xyl D of *Cellulomonas fimi* (Black *et al.*, 1995). Dupont *et al.* (1998) corroborated the inclusion of a new family of substrate binding glycanases i.e. the family including members with xylanase binding domains. Substrate binding domains are more common in F10 than in F11 xylanases. Cellulose-binding domains are found in several xylanases. (Hazlewood and Gilbert, 1992; Sakka *et*

al., 1993, 1996; Millward-Saddler *et al.*, 1994; Christakopoulos *et al.*, 1996; Ruiz-Arribas *et al.*, 1997; Black *et al.*, 1997; Sun *et al.*, 1998, Ali *et al.*,1999). The reason for the presence of CBD on plant cell wall hydrolases is possibly due to the performance of cellulose as a general receptor of plant cell wall hydrolases (Black *et al.*, 1997). It is the only non-variable structural polysaccharide in the cell wall of all plant species, although there are some marginal changes in the degree of crystallinity of cellulose (Purkarthofer *et al.*, 1993). Two enzymes such as arabinofuranosidase (Kellett *et al.*, 1990) and acetyl xylan esterase from *Pseudomonas fluorescens* subsp. *cellulosa* (Ferreira *et al.*, 1993) were having CBDs. *Thermomonospora fusca* xylanase (T fx A) and *Streptomyces lividans* xylanase (XylB) are the only members of family 11 known to be possessing substrate binding domains (Irwin *et al.* 1994). T fx A binds both to cellulose and xylan. Recently there are information regarding the xylan binding domains in family 11 (family G) xylanases. The STX I and STX II xylanases from *Streptomyces violaceus* OPC-520 are having xylan binding domains (Tsujibo *et al.*, 1997).

1.3.14. Mode of action of xylanases

Several models have been proposed to explain the mechanism of xylanase action. Xylanase activity leads to the hydrolysis of xylan. Generally hydrolysis may result either in the retention or inversion of the anomeric centre of the reducing sugar monomer of the carbohydrate. This suggests the involvement of one or two chemical transition states (Sinnot, 1990). Glycosyl transfer usually results in nucleophilic substitution at the saturated carbon of the anomeric centre which takes place by either retention or inversion of the anomeric configuration (Sinnot, 1990). Most of the polysaccharide hydrolyzing enzymes like cellulases, cellulohydrolases and xylanases are known to hydrolyse their substrates with the retention of the C1 anomeric configuration (Gebler *et*

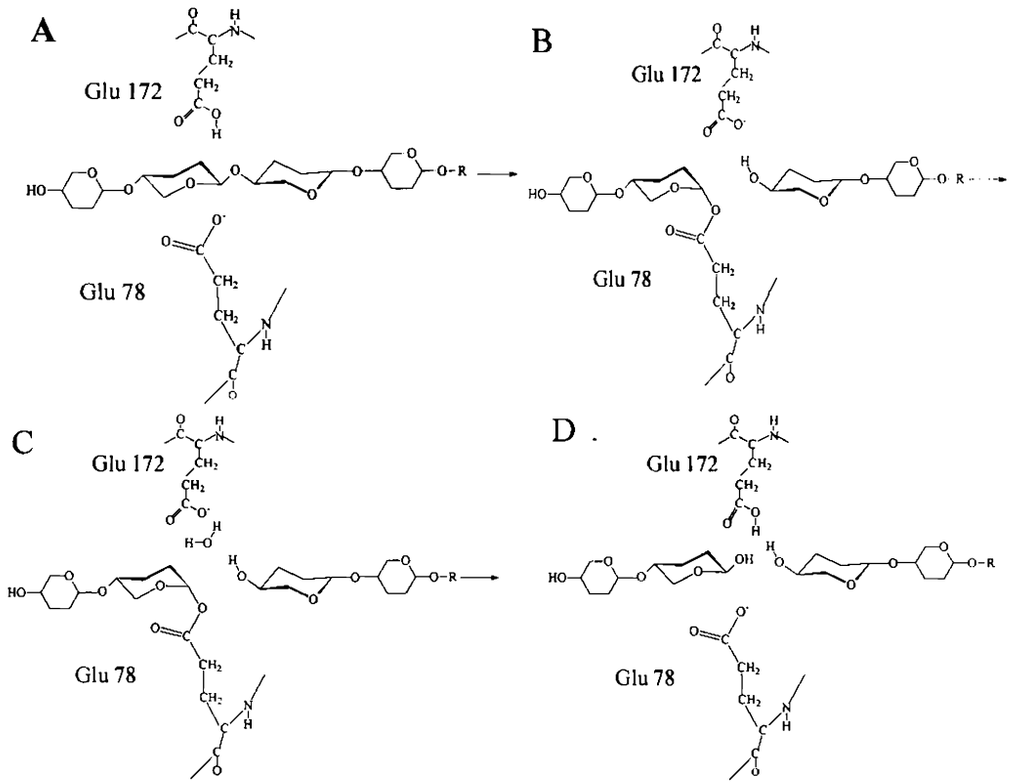


Fig. 6. Reaction mechanism by *Bacillus circulans* xylanase (1XNB). A) The helical xylan structure is positioned in the trough formed between Tyr 65 and Tyr 69. Glu 72 is the acid/base catalyst and Glu 78 is the nucleophile. B) The glycone is bound to Glu 78. This intermediate is retained during transglycosylation reactions. C) Water displaces the nucleophile. D) Dissociation and diffusion of the glycone (xylobiose) allow movement of the enzyme to a new position on the substrate. Xylanases of family 11 exhibit a random endo-mechanism rather than progressive cleavage. This is because the aglycone is released in step B and the glycone in D. (After Jeffries, 1996a, b).

al., 1992). Koshland (1953) proposed involvement of double displacement mechanism for the anomeric retention of product, confirmed later by other reports (Sinnot, 1990; Clarke *et al.*, 1993). The double displacement mechanism involves the following features (Clarke *et al.*, 1993)

(i) an acid catalyst which protonates the substrate

(ii) a carboxyl group of the enzyme positioned on

(iii) a covalent glycosyl enzyme intermediate with this carboxylate in which the anomeric configuration of the sugar is opposite to that of the substrate.

(iv) this covalent intermediate is reached from both directions through transition states involving oxo carbonium ions.

(v) various non-covalent interactions providing most of the rate enhancement.

1.3.15. Hydrolytic pattern of xylanases

There are several reports regarding the hydrolytic pattern of xylanases from *Bacillus* spp. and most of them are mainly releasing xylobiose, xylotriose and xylotetraose while formation of xylose occurred only during prolonged incubation. Xylanase II of *Bacillus circulans* WL-12 (pI 9.1) hydrolysed xylan principally to xylobiose, xylotriose and xylotetraose. This enzyme was shown to be requiring a minimum of four xylopyranoside residues to form the productive complex, thus xylotetraose out of other substrates tried was the most preferred substrate to saturate all binding sites of the enzyme. While Xylanase I from the same source degraded xylan rapidly to xylatetraose and prolonged incubation resulted in xylose, xylobiose and xylotriose as the main end products (Esteban et al., 1982). Another endo-xylanase from *Bacillus* sp. (Horikoshi and Atsukawa, 1973) resulted in the release of xylobiose and xylotetraose as the main products with the production of xylanase on prolonged incubation. Recently a xylanase (Xyn X) from *Aeromonas caviae* has been reported to be release only xylobiose and xyloteraose from xylan (Usui *et al.*, 1999).

1.3.16. Xylanase gene regulation

In most of the reports regarding xylanases there is the occurrence of constitutive enzyme production (Wang *et al.*, 1992; Haltrich *et al.*,

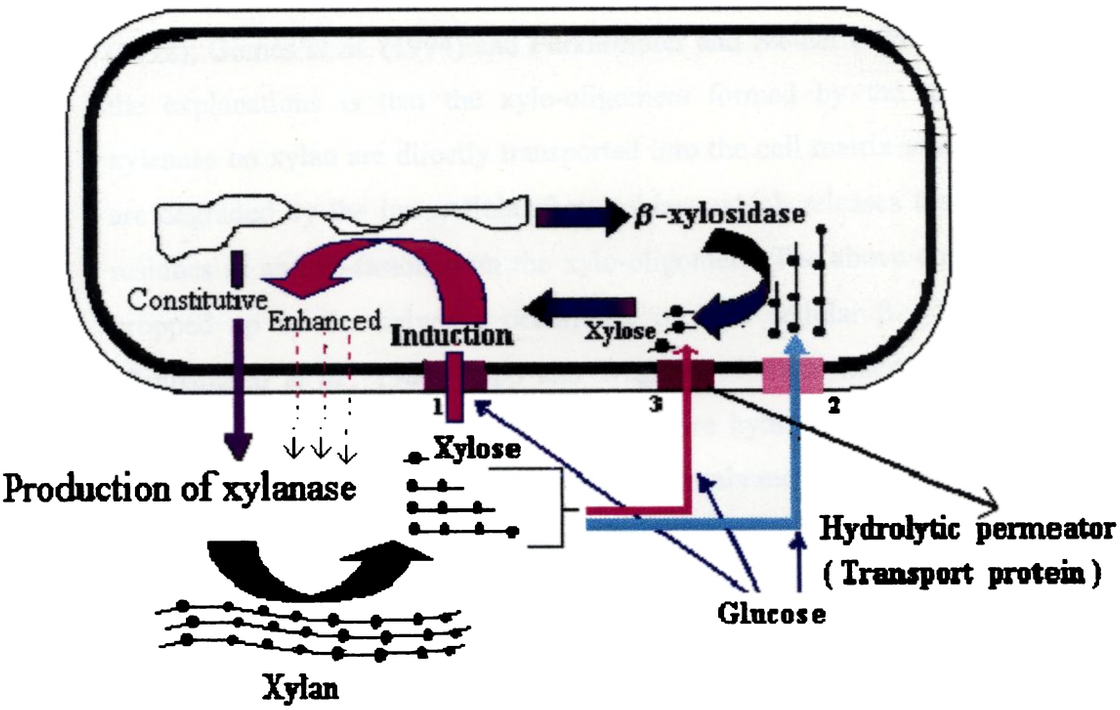


Fig. 7 Hypothetical model for xylanase gene regulation in bacteria based on the reports of Wang *et al.* (1992), Gomes *et al.* (1994) Zhao *et al.* (1997) and Ali *et al.* (1999). 1. Xylose monomers can be easily transported through the cell membrane which induce the enhanced xylanase synthesis. 2. The action of constitutively produced xylanases results in xylooligosaccharides eg. xylotriose (Zhao *et al.*, 1997), the transportation of which in to the cell later cause the enhanced synthesis. 3. The hydrolytic permeator can result in the transportation-coupled hydrolysis of xylooligomers from the constitutive xylanase action Ali *et al.* (1999). All the cases could be affected by the presence of glucose

1995; Zhao *et al.*, 1997). Xylanase attacks xylan, comparatively a large heteropolysaccharide, which is prevented from entering the cell matrix by the cell membrane. The products of xylan hydrolysis are small molecular weight xylose, xylobiose, xylotriose and other oligosaccharides. These molecules easily enter the microbial cells and sustain the growth by acting as energy and carbon source. The products of hydrolysis can stimulate xylanse production by different methods. Xylose being a small pentose molecule can enter the bacterial and fungal cells easily and induce xylanase production (Biely, 1985; Haltrich *et al.*, 1995; Zhao *et al.*, 1997). However, the larger molecules are posing problem in transportation, which questions the direct induction role of these macromolecules on enzyme synthesis (Zhao *et al.*, 1997). There are two plausible explanations for the

inductive role of larger molecules based on the reports of Wang *et al.* (1992), Gomes *et al.* (1994) and Purkarthofer and Steiner (1995). One of the explanations is that the xylo-oligomers formed by the action of xylanase on xylan are directly transported into the cell matrix where they are degraded by the intracellular β-xylosidase which releases the xylose residues in an exo-fasion from the xylo-oligomers. The above concept is propped up by the universal occurrence of intra cellular β-xylosidases (Panbangred *et al.*, 1984; Rapp and Wagner, 1986) in microorganisms. The other possibility is that the oligomers are hydrolysed to monomers during their transportation through the cell membrane in to cell matrix by the action of hydrolytic transporter having exo β-1,4- bond cleaving proteins like the β-xylosidases. The above idea was stemmed from the reports on β-xylosidases with transferase activity (Rodionova *et al.*, 1983; Conrad and Noethen, 1984). Recently a Family F/10 xylanase from *Clostridium stercorarium* was found to associate with one ORF1 (open reading frame) (Ali *et al.*, 1999). According to them polycystronically derived proteins are responsible for the hydrolysis of xylan and transport of xylo-oligomers. In both the ways the resulting xylose molecules as mentioned earlier effect in the enhanced production of xylanase. However, there are rare cases where the xylose molecules repress the xylanase production (*Bacillus thermoalkalophilus*, Rajaram and Varma, 1990; *Bacillus* sp., Uchino and Nakane, 1981; *Bacillus* sp. Strain 41M-1, Nakamura *et al.*, 1993a) where the inducer may be yet another derivative from the xylan hydrolysates. If glucose, the most effective carbon source, is present in the growth medium there is repression of synthesis of catabolic enzymes, which may be at the transcriptional level or by mere inducer exclusion of the respective inducers of these enzymes. The first one i.e. the catabolic repression at the transcriptional level has been clearly explained by Saier and Fagan (1992). The second possibility of catabolite inhibition may be inducer exclusion at the level of inducer transport across

the cell membrane (Mc Ginnes and Paigen, 1973; Moat and Foster, 1995a). An example of inducer exclusion is the fact that glucose will prevent the uptake of lactose, the inducer for the *lac* operon of *E. coli* (Moat and Foster, 1995a). The xylanase inducer proteins resulting in the transcriptional activation have recently been elucidated by Peij *et al.* (1998). The xln R gene of *Aspergillus niger* controls all the xylanolytic enzymes and other two endoglucanases suggesting the occurrence of common regulatory systems in microorganisms (Peij *et al.*, 1998) (Fig. 7).

1.3.17. Xylanase gene cloning

There are several reports regarding genetic manipulation of xylanase producing microorganisms (Panbangred *et al.*, 1985; Bernier *et al.*, 1985; Honda *et al.*, 1985b; Sung *et al.*, 1993; Jung and Pack, 1993; Gat *et al.*, 1994; Ethier *et al.*, 1994; Arhin *et al.*, 1994; Mazy-Servais *et al.*, 1996; Okada *et al.*, 1999). During the early periods of xylanase research, lack of hyper producing potent culture resulted in the taming of xylanase genes from the already available cultures. Gene manipulation has the advantage of producing microbial strains with selected enzyme machinery. According to Biely (1985) the main objectives of gene cloning are: 1.) Construction of producers of xylanolytic systems free of cellulolytic enzymes and 2.) Improvement of fermentation characteristics of industrially important xylose fermenting organisms by introducing genes for xylanase and xylosidase so that the direct fermentation of xylan would be possible. The early studies on cloning of xylanase gene include the works on *Bacillus* spp. (Biely, 1985). In addition to permitting the introduction of novel genes, cloning techniques could enable amplification of the expression of genes already present. For instance, the production of xylanase in *Bacillus subtilis* was enhanced successfully using a plasmid vector carrying the *Bacillus pumilus* gene. The transformant produced approximately three times more extracellular xylanase than the donor strain. More over, the enzyme was produced constitutively, suggesting that

regulatory elements of the donor organism were absent in the vector used for the transformation (Panbangred *et al.*, 1985). The xylanase genes xyn *A* and *B* of *Bacillus subtilis* were cloned in *Escherichia coli* (Bernier *et al.*, 1985). An alkalophilic *Bacillus* sp. strain C125 produced two types of xylanases (N and A) having molecular weights 43 and 16 kDa respectively. The xyn *A* gene located on a 4.6 kbp DNA fragment was cloned in *E. coli*, and more than 80 % of the activity could be detected in the culture medium (Honda *et al.*, 1985b). Sung *et al.* (1993) successfully completed the over expression of *Bacillus subtilis* and *Bacillus circulans* genes from *E.coli* by constructing synthetic genes with multiple unique restriction sites. The synthetic genes encoded only the mature enzymes and the results were 10-100 folds more than all previous experiments. According to them the repeated usage of degenerate codons in the *Bacillus* derived genes if present in *E.coli* may deplete the supply of specific tRNA thus limiting the expression.

Gat *et al.* (1994) using *E. coli* cloned the 1236 bp open reading frame *of Bacillus stearothermophilus* T-6 xylanse gene. They also found that the β-xylosidase gene was present 10 kb down stream of the xylanase gene, but it is not a part of the same operon. Despite the future role of *Bacillus* expression system there are few reports regarding the xylanase gene cloning using *Bacillus* spp. Jung and Pack (1993) cloned the *Clostridium thermocellum* xylanase gene in *Bacillus subtilis*. They constructed the vector pJX18 by inserting a Bam HI 1.6 kb DNA fragment of pCX18, which contained the xylanase structural gene. However, the glycosylation of the over expressed protein was not considered in this case which resulted in the proteolytic degradation leading to the formation of different bands of proteins with hydrolytic nature (Jung and Pack, 1993). Use of *Bacillus* as the host cell in rDNA technology is a novel method and there are few reports regarding the same especially in the case of xylanase genes. Cho *et al.* (1995) tried to validate this aspect by using a protease-

deficient *Bacillus subtilis* DB104 for cloning endo-xylanase (I) from *Clostridium thermocellum*. The transformed cells successfully secreted xylanases into the culture broth and this technique is highly valuable considering the problems associated with intra-cellular production of proteins. There are reports regarding the cloning of xylanases from organisms other than *Bacillus* spp., like *Streptomyces thermoviolaceus* OPC-520 (Tsujibo *et al.*, 1997), *Actinomadura* sp. strain FC7 (Ethier *et al.*, 1994), *Streptomyces lividans* (Arhin *et al.*, 1994) and *Streptomyces* sp. strain EC3 (Mazy-Servais *et al.*, 1996). In the last case the xylanase gene of one *Streptomyces* sp was cloned in another mutant species. Recently Okada *et al.* (1999) was successful in cloning two xylanase genes (*xyn1* and *xyn2*) from *Trichoderma reesei* using the fission yeast *Schizosaccharomyces pombe* as the host.

1.3.18. Application of xylanases

Potential application of xylanases in biotechnology include biopulping, wood pulp bleaching, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels.

1.3.18.1. Paper industry

Chlorinated phenolic compounds as well as polychlorinated biphenyls, produced during conventional pulp bleaching being toxic and highly resistant to biodegradation, form one of the major sources of environmental pollution.

Viikari *et al.* (1986) initially demonstrated the use of xylanases for the selective removal of hemicelluloses from Kraft pulp prior to pulp bleaching.

1.3.18.1.1. Kraft Process :

Removal of residual lignin from Kraft pulp is physically and chemically restricted by hemicelluloses. Lignin has been reported to be linked to hemicelluloses (Puls, 1997) and there are reports regarding the

isolation of lignin carbohydrate complexes from the Kraft pulp (Iverson and Wannstrom, 1986). Further hemicelluloses seem to physically restrict the passage of high molecular mass lignin out of the pulp fibre cell wall (Scallan, 1977).

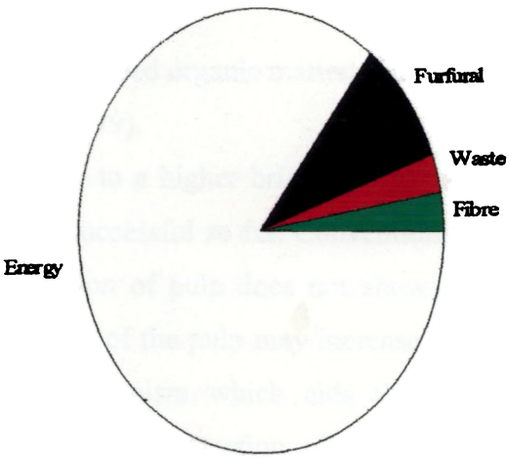


Fig.8. Distribution of xylans in the different parts/stages of pulp manufacture. Apart from those present as Furfural, waste and those used fore energy production 3.3 % was present on the fibres (Gamerith and Strutzenberger, 1992).

The most common pulping process is the Kraft process or Sulphate process where cooking of wood chips is carried out in a solution of Na_2 S/NaOH at about 170°C for two hours resulting in the degradation and solubilisation of lignin (Sanyer and Chidester, 1963). The resulting pulp has a characteristic brown colour which is primarily due to the presence of residual lignin and lignin derivatives. The intensity of pulp colour is a function of the amount and chemical state of the remaining lignin. To obtain pulp of very high brightness and brightness stability, all the lignin must be removed from the pulp. For that, chemical pulping is more effective than mechanical pulping. However, there is the formation of residual lignin, which has to be removed by bleaching process. The residual lignin in chemical pulp is dark in colour because it has been extensively oxidized and modified in the cooking process. This residual lignin is difficult to be removed due to its covalent binding to the hemicellulose and perhaps to cellulose fibres. The bleaching of the pulp can be regarded as a purification process involving the destruction,

alteration or solubilization of the lignin, coloured organic matters and other undesirable residues on the fibres (Singh,1979).

Bleaching of chemical pulp to a higher brightness without complete removal of lignin has not been successful so far. Conventionally chlorine is used for bleaching. Chlorination of pulp does not show any decolourising effect, and in fact, the colour of the pulp may increase with chlorination and it is the oxidative mechanism which aids the pulp bleaching (Loras, 1980). At low pH the main reaction of chlorine is chlorination rather than oxidation. Thus chlorine selectively chlorinates and degrades lignin compounds rather than the carbohydrates (e.g. hemicelluloses – xylan) moieties in the unbleached pulp. The dominant role of chlorine in bleaching is to convert the residual lignin in the pulp to water or alkali soluble products. The effluent that are produced during the bleaching process, especially those following the chlorination and the first extraction stages are the major contributors to water pollution from the pulp paper industry (Erikson and Kirk, 1985).

1.3.18.1.2. Biobleaching

During the Kraft process part of the xylan is relocated on the fibre surfaces (Yllner and Enstrom., 1956). Considerable amount of xylan is present in the fibres after pulping process. Enzymatic hydrolysis of the reprecipitated and relocated xylans on the surface of the fibres apparently renders the structure of the fibre more permeable. One of the leading producers of chemical pulp manufacturers, (Lenzing AG, Austria) has recovered fibers containing 3.3% of total xylan. (Fig, 8) (Gamerith and Strutzenberger, 1992). The increased permeability allows the passage of lignin or lignin-carbohydrate molecules in higher amounts and of high molecular masses in the subsequent chemical reactions (Kantelinen *et al.*, 1993).

Ligninases and hemicellulases (xylanases) have been tested for biobleaching. Use of hemicellulases was first demonstrated by Viikari

et al., (1986). Use of xylanase resulted in the reduction in chlorine consumption.

It was suggested by Kantelinen *et al.* (1991) that two types of phenomena are involved in the enzymatic pretreatment. The major effect is due to hydrolysis of reprecipitated and readsorbed xylan or xylan-lignin complexes that are separated during the cooking process. As a result of the enzymatic treatment, the pulp becomes more accessible to oxidation by the bleaching chemicals. A minor effect is due to the enzymatic hydrolysis of the residual non-dissolved hemicellulose by endo-xylanases. Paice *et al.* (1988) reported that residual lignin in unbleached pulp (Kraft pulp) is linked to hemicellulose and that cleavage of this linkage will allow the lignin to be released.

Two xylanases from *Trichoderma reesei* Rut-30 could effectively hydrolyse the xylan isolated from wood (Tenkanen *et al.*, 1992). But after cooking the xylans become clearly less susceptible to one of the xylanase with pI value 5.5 (Tenkanen *et al.*, 1992).

Milagres and Duran (1992) showed that concentrated hemicellulosic hydrolysate can be used for xylanase synthesis from *Penicillium janthinellum* for the purpose of bleaching pulp. They found high levels of xylanase activities in the shake flasks when subjected to low agitation. This enzyme was applicable in pulp pre-treatment, however, during alkali extraction the potentiality in decreasing Kappa number was found lowered. More over, even the treatment with high concentration of *P. janthinellum* xylanase failed to improve the condition

Highest relative decrease of the Kappa number (21.5%) was observed after xylanase treatment and alkali extraction, while in the sulphate soft wood pulp it was only 12.12% (Pekarovicova *et al.*, 1992).

1.3.18.1.3. Need for cellulase free xylanase

The public concern over the impact of pollutants from paper and pulp industries which use chlorine as the bleaching agent act as strong driving force in developing biotechnology aided techniques for novel bleaching i.e. biobleaching. As mentioned earlier (section **1.3.2.**) xylanases are more preferable to ligninases. However the occurrence of cellulase contamination in most of the reported fungi (Table 1) is posing a major threat in applying the xylanases in biobleaching. The cellulases easily result in the hydrolysis of cellulose which should be the main recovered product in paper industry. Most of the reported cases are having significant cellulase activity (Table 1). However, the enzyme preparations from microorganisms producing higher levels of xylanases with tenuous or no cellulase activity can be applied in paper industry because the loss of pulp viscosity will be at minimum level.

1.3.18.1.4. Pulp fibre morphology

After comparing SEM micrographs of soft wood sulphate pulp with that of the same pulp after xylanase pre-bleaching and alkali extraction, Pekarovicova *et al.* (1992) found that there is no change in the shape of fibre after xylanase pre-bleaching. However, flattening of the fibre arises after alkaline extraction, confirming that the lignin extraction from the cell wall results in its collapse. The fibre surface after xylanase treatment is less wrinkled than the original one. Perhaps this can be explained as resulting from the digestion of the readsorbed linear xylan from the pulp fibre surface (Kantelinan *et al.*, 1991).

1.3.18.2. Other applications of xylanolytic enzymes

The xylanolytic enzymes are also employed for clarifying juices and wines (Biely, 1985, 1991; Zeikus *et al.*, 1991), for extracting coffee, plant oils and starches (Biely 1991), for improving the nutritional properties of agricultural silage (Zeikus *et al.*, 1991) and grain feed (Campbell *et al.*, 1991). Xylanases are also having application in rye baking where the addition of xylanase make the doughs soft and slack (Puls, 1997). Sugars like xylose, xylobiose and xylooligomers can be prepared by the enzymatic hydrolysis of xylan (Pellerin *et al.*, 1991). Bioconversion of lignocelluloses to fermentable sugars has the possibility to become a small economic prospect. It is because massive accumulation of agricultural, forestry and municipal solid waste residues create large volume of low value feedstock (Eriksson, *et al.*, 1990). If the feed stock is variable, a complete xylanolytic system would appear desirable to ensure maximal hydrolysis. Such an enzyme system would include xylanases, β-xylosidases and the various debranching enzymes.

Production of environmentally friendly fuel is gaining great importance as the energy sources are shrinking. There are reports regarding production of ethanol from the agrowastes by incorporating xylanase treatment (Ahring *et al.*, 1999).

1.3.19.The merits of *Bacillus* spp. in industrial biotechnology

The genus *Bacillus* is an optimal host for a number of applications in industry and research. The important feature of all *Bacillus* spp. used in practical applications is their apathogenesity and the well-proved safety of appropriate industrial processes using them. They secrete degradative enzymes in large amounts, which make them very attractive for commercial exploitation as well as for basic research. The secretion of proteins is the most important feature of *Bacillus* spp. that has attracted attention in Biotechnology and this feature also point towards their possible dominant role as hosts for the production of heterologous proteins

by genetic manipulation. There are several cases of *Bacilli* secreting multitude of proteins including xylanases. (Uchino and Nakane, 1981; Esteban *et al.*, 1982; Akiba and Horikoshi, 1988; Debeire-Gosselin *et al.*, 1992b; Khasin *et al.*, 1993; Nakamura *et al.*, 1993a,b; Nakamura *et al.*, 1994; Breccia *et al.*, 1998; Ratanakhanokchai *et al.*, 1999).

The secretory production could be advantageous for industrial production. Purification of a secreted protein is simpler and more economical than that of a product produced intracellularly, the prevalent mode of production in most microbial production systems. The secreted protein could be expected to adopt its native conformation even at a high level of secretion. In the meantime the overproduction of an intra-cellular protein often leads to its aggregation resulting in denatured condition. Most *Bacilli* used in industry and research are non-toxic and have the generally recognised as safe (GRAS) status (Bron *et al.*, 1998). This is due to the fact that they lack the cellular components of metabolic products toxic to human being and animals, an important feature facilitating the production of proteins of food and medical interest with an additional element of safety in the technology of genetic engineering. This fact is obvious because the members of the genus *Bacillus* are gram-positive organisms and do not contain endotoxins (lipopolysaccharide), which are ubiquitous in all gram-negative bacteria including *Escherichia coli*. The endotoxins from gram-negative bacteria are difficult to remove from many proteins during the process of purification. *Bacilli* have long been used as a source for industrial production of enzymes like xylanases, amylases and proteases. This has contributed much to the basic knowledge and practical technology for the culture, fermentation and downstream processing of products. *Bacilli* have many features attractive for a microorganism to be used as a host for the production of heterologous proteins. The long availability of genetic exchange methods, including physiological transformation, has provided an ideal system for the study of a broad range

of genetic, biological and physiological properties of *Bacillus*. This has generated a wealth of knowledge concerning the genetic map, the expression and regulation of Bacillar genes and the regulatory mechanisms associated with the adaptation to environmental changes, including sporulation (Sonenshein *et al.*, 1993; Sarvas, 1995).

METHODOLOGY

2. METHODOLOGY

2.1. Isolation and Screening of Microorganisms

Isolation of alkaline thermostable xylanase producing microorganisms has been carried out in three major steps and emphasis was given to the selection of high xylanase-producing bacteria. Possibility of getting a potent organism with the required characters depended on the inclusion of maximum initial number of microorganisms for screening.

2.1.1. Primary screening

Initial screening was conducted on media rich in hemicelluloses. Forest soil samples from Kallar and Ponmudi, Kerala (India), riverbed soil, alluvial soil and various hemicellulose containing substrates (wheat bran, bagasse and rice straw) exposed to atmosphere at different pH values (5, 7 and 10.5) were suspended in sterile water. Suspensions (0.1 ml) after serial dilution in sterile saline or water were spread onto wheat bran agar plates.

Initial media for isolation studies contained (g/L):

Wheat bran	50.0
Peptone	5 .0
NaCl	5.0
Yeast extract	3.0
Agar	20.0
Media pH	5, 7 and 10.5.

1% Na_2CO_3 and 1 N HCl were used to adjust the pH of the media at required levels. Unless and otherwise specified all the sterilisation steps were performed at 121^0 C, 15 lb pressure for 20 minutes. Organisms showing good

growth in all pH values were collected and maintained in appropriate media corresponding to their isolation conditions.

2.1.2. Secondary screening

The cultures isolated in the previous step were spread onto xylan agar plates containing 0.5% xylan (Oat spelts xylan, Sigma Chemicals Co.) instead of wheat bran as the carbon source while keeping all the other components of media the same. After six days of incubation, colonies that showed areas of clear zones with a minimum radius of 1 cm were selected for further screening in liquid medium where oat spelts xylan was the main carbon source.

2.1.3. Tertiary screening

The number of cultures thus narrowed down during the secondary screening were further screened in xylan liquid medium (XLM) with xylan as the main carbon source. The high productive organisms were finally identified by the submerged fermentaion studies using the modified Horikoshi Basal medium II, which had been routinely used for the isolation of xylanase producing microorganisms (Horikoshi, 1991a; Nakamura *et al.,* 1993a).

The composition of modified Horikoshi basal medium II (g/L) :

Xylan	5.0
Peptone	5.0
Yeast extract	5.0
K_2HPO_4	1.0
$MgSO_4$. 7 H_2O	0.2

pH 7 or 10.5

Fermentation studies with the selected 40 isolates were carried out in 200 ml of this medium in 500 ml Erlenmayer flasks subjected to shaking for a period of 144 hours on a rotary shaker (120 rpm) at ambient temperature. Samples were taken every 24 hours and used for measuring pH, optical

density, viable count, dry weight etc. and the cell free supernatant was used for xylanase assay, protein estimation and reducing sugar quantification.

2.1.4. Inoculum used for the fermentation studies

The inoculum was raised in the same medium under similar conditions in 250 ml Erlenmayer flasks and 18 hour old inoculum was used to initiate growth. The properly inoculated fermentation media were incubated for a period of 144 hours on a rotary shaker (120 rpm) at ambient temperatures (30 ± 2^{0} C).

2.1.5. Production of xylanases

Production of xylanases from the selected isolates was studied in correlation with the growth profile of the cultures. Fermentation experiments were carried out using conditions already mentioned. Cells were separated by centrifugation (10,000 g 15 min, at 4^{0} C, HITACHI Himac CR 20 B2 centrifuge) and the cell free supernatant was used as the extracellular crude enzyme preparation.

2.1.6. Endo-xylanase (1,4 – β - D - xylan xylanohydrolase) assay

Endo-xylanase (1,4-β-D-xylan xylanohydrolase EC 3.2.1.8.) was assayed by the method of Bailey *et al.* (1992) with some modifications using 0.5% oat spelts xylan (Sigma Chemicals Co.). The substrate (xylan) was homogenised in 0.2 M phosphate buffer ($NaHPO_4$ / Na_2HPO_4), 0.2 M carbonate buffer (Na_2CO_3 / $NaHCO_3$) and 0.2 M acetate (CH_3COONa / CH_3COOH) buffer at 70^{0} C and subjected to boiling point on a heated magnetic stirrer. The solution was cooled and kept overnight with slow stirring which was then made up to the required volume using appropriate buffers. The reaction mixture containing 1.8 ml of preincubated oat spelts xylan suspension and 200 µl of suitably diluted enzyme preparation was

incubated for 10 minutes at 50 ^{0} C. Enzyme blanks were required if the dilution was rather small and/ or if the sample contained high level of reducing sugar. Enzyme blanks were prepared adding the DNS reagent prior to the enzyme addition so that only the reducing sugars present in the enzyme preparations would be answering. The reagent blank was prepared in the same manner but 200 µ ml phosphate buffer (0.2 M pH 7) was used instead of enzyme. The reaction was terminated by adding 3:0 ml of dinitrosalicylic acid reagent, which was then kept in boiling water bath for 5 minutes. The concentration of reducing sugars released was estimated against xylose standard by noting the absorbance at 540 nm. The stock solution for xylose standard (Xylose, Merk) was prepared in 10 µmol/ ml concentration and appropriate dilutions were used as the standard. One unit of endo-xylanase activity was defined as µ mols of xylose liberated per minute per ml of enzyme preparation.

2.1.7. Cellulase assays

All the selected cultures were screened for cellulases during the fermention period. Absence of cellulases in the xylanase preparation is crucial in selecting a xylanolytic strain applicable to paper and pulp industry. Cellulase assays including both CMCase and FPase assays were carried out using cell free supernatant from samples collected at an interval of 24 hours of growth.

2.1.7.1. CMCase (1,4-(1,3:1,4)-β-D-glucan-4-glucano hydrolase (E.C.3.2.1. 4):

Cellulase (1,4-(1,3:1,4)-β-D-glucan glucanohydrolase, EC 3.2.1.4) or carboxy methyl cellulase assay was carried out by estimating the reducing sugars liberated from 1% sodium salt of carboxymethyl cellulose in 0.2 M sodium phosphate buffer, pH 7 (Dahlberg *et al.*, 1993). To 1.0 ml of

preincubated substrate kept at 50^0 C, 0.5 ml of suitably diluted crude enzyme was added and incubated for 15 min. The reaction was stopped by adding 3.0 ml DNS reagent and heating in boiling water bath for 5 minutes. The absorbance of the solution was measured at 540 nm using glucose as the standard. CMCase activities were expressed as μ mols of glucose liberated per minute per ml of culture supernatant.

2.1.7.2. FPase (1,4-β-D- glucan cellobiohydrolase (E.C.3.2.1.91):

FPase or cellulose-1,4-β-cellobiosidase (EC 3.2.1.91) activity was determined by using modified method of Mandels *et al.* (1976). Whatman No.1 filter paper was taken as the substrate. To 1.0 ml of pre incubated 0.2M phosphate buffer (pH 7.0) containing filter paper strip (1 X 6 cm) was added 0.5 ml of crude enzyme solution and incubated for 15 min. at 50^0C. The reaction was stopped by boiling with 3 ml DNS reagent for 5 min. The absorbance of the solution was measured directly at 540 nm. Glucose was used as the standard and the reducing sugar liberated was expressed as μ mols of glucose produced per min. per ml of culture supernatant.

2.1.8. Analytical Methods

2.1.8.1. Monitoring of Bacterial growth:

a. By measuring the optical density of the suitably diluted culture broth at 600 nm
b. Viable count method. The cells were serially diluted in sterile saline and 0.1 ml of random samples were plated onto nutrient agar plates. Number of cells was counted in Plate counter and mean of the data was taken.
c. Growth was also determined by dry weight methods.

2.1.8.2. Identification of bacteria:

The selected microorganisms were identified using the procedures given in Bergey's Manual of Systematic Bacteriology. Microbiological tests for observing the growth patterns are studies on fermentation mode i.e. whether aerobic or anaerobic, detection of spores using the stain Malachite Green, motility test for the vegetative bacteria, and Gram staining helped the classification of the isolates to respective genera. The biochemical characterisation studies include catalase test, VOGES-PROSKAUR test, M.R. test, urease test, nitratase test, indole test, starch hydrolysis test and gelatin liquifaction test. All the tests were carried out using the procedure given in standard microbiological text (Collins *et al.*, 1989) and for identifying the bacteria Bergey's Manual of Systematic Bacteriology (for *Bacillus* spp. Claus and Berkeley, 1986 and for *Streptomyces* spp. Holt *et al.*, 1994) was followed.

2.1.8.3. Reducing Sugar:

Reducing sugar in the culture supernatant was estimated by dinitosalicylic acid method (Miller, 1959).

2.1.8.4. Protein:

Soluble protein was estimated by Lowry's method (Lowry *et al.*, 1951).

2.2. Optimisation of Cultural Parameters

2.2.1. The selected microorganism used for the optimisation studies.

Bacillus SSP-34 used in the present study was isolated following an extensive screening for xylanase producers described in section **2.1.** The culture was periodically subcultured and stored in xylan agar slants with a composition of (g/L): xylan (Oat spelts xylan, Sigma chemicals Co.) 5.0; peptone 5.0; yeast extract 5.0; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.2 and agar 20.0 (pH 7.0).

2.2.2. Culture medium used for optimisation studies

The organism was grown in liquid medium (Horikoshi II basal medium with modifications) where oat spelts xylan was the carbon source. The basal xylan liquid medium (XLM) contained (g/L): xylan, 5.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0 and $MgSO_4.7H_2O$, 0.2 (pH 7.0).

2.2.3. Inoculum

The inoculum was raised in the basal medium. The cultures were grown in an environmental shaker (New Brunswick) at 120 rpm. Unless otherwise specified, the inocula of age 18 hrs were used at 10% v/v.

2.2.4. Production of xylanases

Production of xylanases was studied in correlation with the growth profile of the culture. Fermentations were carried out using XLM in 250 ml Erlenmeyer flasks with a working volume ratio of 0.4 (100 ml medium in 250 ml flasks) at 120 rpm in a rotary shaker. Cells were separated by centrifugation (12000 g, 20 minutes, 4°C Hitachi CR 20 B2 Centrifuge) and the supernatant served as the extracellular crude xylanase preparation. Growth of the cells was monitored by optical density measured at 600 nm with a dilution factor of 10.

2.2.5. Xylanase assay

Endo-xylanase [1,4-β-D-xylan xylanohydrolase (E.C.3.2.1.8)] was assayed by the method of Bailey *et al.* (1992) with some modifications which is described in section **2.1.**

2.2.6. Analytical methods

All the analytical methods including the reducing sugar and soluble protein estimations were carried out as described earlier in section **2.1.**

2.2.7. Effect of pH on the growth and xylanase production of *Bacillus* SSP-34

In order to study the effect of initial pH of the culture medium on the formation of extracellular endo-xylanases, *Bacillus* SSP-34 was grown in Horikoshi II basal medium with the initial media pH adjusted within a range of 5 to 10 at 0.5 unit increment (after autoclaving). In all cases the initial pH values have been uncontrolled through out the fermentation period and the data of variation in pH value was recorded every 24 hours. The fermentation was carried out for a period of 144 hours. Samples were used for analysing pH, OD, reducing sugar estimation, protein determination and xylanase assay. Growth was correlated with the xylanase production.

2.2.8. Effect of temperature on the growth and xylanase production of *Bacillus* SSP-34

Bacillus SSP-34 was grown in Horikoshi II basal medium at varying temperatures ranging from 30-60^{o}C (30, 35, 40, 45, 50, 55 and 60 ^{0}C). The temperatures were set at different shakers including G24 environmental incubator shakers (New Brunswick Scientific Edison, N.J. USA) and water bath shaker (LAB-LINE Instruments Incorp. Orbit water bath shaker). The fermentation was carried out for a period of 144 hours. Samples were used for analyses as described earlier (**2.1.**).

2.2.9. Effect of inoculum concentration

In order to minimize the time lag in fermentation process (Lincoln, 1960), inocula were raised in the medium with a composition similar to that of the fermentation medium. The inocula were transferred at the logarithmic stage of growth. The effect of inoculum concentration on the production of xylanases by *Bacillus* SSP-34 was studied by using different concentrations such as 1%, 2.5%, 5%, 7.5% and 10%. Concentrations above 10% were not preferable in industrial fermentation (Lincoln, 1960). The fermentation was

carried out for a period of 144 hours. Samples were taken every 24 hours for all the routine analyses as discussed earlier.

2.2.10. Effect of agitation and aeration

Agitation and aeration were carried out in three separate experiments. In the first batch, fermentation media with a working volume ratio (wv ratio) of 0.2, 0.4 and 0.6 were taken in Erlenmeyer flasks and incubated at 100 rpm. In the second batch, different wv ratios (0.2, 0.4 and 0.6) were grown at 200 rpm. Finally, all the three wv ratios (0.2, 0.4 and 0.6) were incubated at 300 rpm. Samples taken every 24 hrs were used for different estimations as described previously.

Table 3. Experimental set up to detect optimum agitation and aeration condition of *Bacillus* SSP-34

		Agitation (rpm)		
		100	200	300
Working volume ratio. (W V ratio) Vol. of medium/ Vol. of flask	0.2	0.2+100 rmp	0.2+200 rpm	0.2+300 rpm
	0.4	0.4+100 rmp	0.4+200 rpm	0.4+ 300 rpm
	0.6	0.6+100 rpm	0.6+200 rpm	0.6+300 rpm

2.3. Optimisation of Nutritional Parameters

The optimisation studies were conducted to evaluate the higher production environment using three different strategies i.e. studies on the optimisation of carbon sources, nitrogen sources and mineral ions for the enhanced production of xylanases from *Bacillus* SSP-34.

2.3.1. Effect of carbon source on xylanase production

2.3.1.1. Carbon sources used for the enhancement of xylanase production

Optimisation experiments were carried out in Horikoshi basal medium II as mentioned earlier. Different carbon sources were added to the basal medium replacing xylan. In the first set of experiments production of xylanases by the culture *Bacillus* SSP-34 was monitored using various carbon sources. The aim was to identify the carbon source effecting the highest xylanase production. All the experiments were conducted for a period of 144 hours.The carbon sources studied include:

1. Monosaccharides
   - i.) Xylose
   - ii.) Glucose
   - iii.) Fructose
   - iv.) Galactose
2. Disaccharides
   - i.) Sucrose
   - ii.) Lactose
   - iii.) Maltose
3. Sugar Alcohol
   - i.) Sorbitol
4. Polysaccharides
   - i.) Xylan
   - ii.) Carboxymethyl cellulose
   - iii.) Starch
   - iv.) Inulin

2.3.1.1.1. Fermentation requirements

The microorganism tried was *Bacillus* SSP–34, isolation procedure of which was discussed in section **2.1.** Inoculum used for fermentation was 5 % (age 18 hrs). Basal media with different carbon sources were incubated at 300 rpm for 120 hours at 35 ^{0} C (pH 8.5). Cell free culture supernatant obtained by centrifugation (at 4^{0} C and 10,000 g using Hitachi Himac CR 20 B2 Centrifuge) served as the crude xylanase preparation.

2.3.1.1.2. Endo-xylanase assay

Endo-xylanase assay was carried out using 1.8 ml of oat spelts xylan suspension and 0.2 ml of suitably diluted enzyme with DNS as the stopping reagent. The detailed description could be found in the section **2.1.**

2.3.1.1.3. Analytical Methods

pH variation and OD of the culture broth were monitored periodically. The determination of soluble protein and reducing sugar were carried out as described in the section **2.1.** Cell protein was determined by using 0.1N NaOH, which was added to the centrifugally separated cell pellets and incubated for 18 hours. The incubated NaOH preparation was centrifiuged and supernatant was subjected to protein determination using Lowry's method.

2.3.1.2. Effect of different levels of xylan on xylanase production

The selected carbon source, xylan was tried at different concentrations and the subsequent effect on the fermentation profile of *Bacillus* SSP-34 was monitored. Cellulase activity by each concentration was evaluated to assist the selection of optimum carbon source at the required levels. This is because the cellulase free xylanases are having major biotechnological applications in the pretreatment of paper pulp (Chapter 1.2). The different concentrations tried were (% w / v):

(i.) 0.25 (ii.) 0.5 (iii) 1.0 (iv) 1.5 (v) 2.0 and (vi) 2.5.

Samples were taken every 24 hours and used for checking pH, OD, cell protein, reducing sugar, soluble protein, and enzyme assays.

2.3.1.2.1. Assay procedures:

Xylanase assay: Carried out as described in section **2.1.** with 0.5% oat spelts xylan suspension .

CMC ase assay: 1% carboxymethyl cellulose was used as the substrate (CDH India) and DNS as the stopping reagent. (Section **2.1.**).

FPase : Filter paper strips (1x6 cm) in phosphate buffer was the substrate and the enzyme reaction stopped by DNS reagent (Section **2.1.**)

2.3.1.2.2. Analytical methods: Conducted as described in the section **2.1.**

2.3.1.3. Induction of xylanases

The carbon sources identified earlier were evaluated for xylanase production from the culture in order to elucidate their inductive influence. The inductive effect of carbon sources was characterised and studies were also conducted with glucose, the well-known carbon catabolite repressor.

In order to study the induction, the following combinations were tried and the individual members were also studied separately.

i.) Xylan (0.5%)+ Glucose (0.5%)

ii.) Xylose (0.5%) + Glucose (0.5%)

iii.) Xylan (0.5%) + Xylose (0.5%)

iv.) Xylan (1%)

v.) Xylose (1%)

vi.) Glucose (1%)

Samples taken in 1hour intervals up to 6^{th} hour and in 6 hour intervals afterwards were analysed for pH, OD, cell protein, RNA and xylanase activity.

2.3.1.3.1. RNA estimation

Bacterial cells were separated by centrifugation (Hitachi Himac CR 21 centrifuge at 4^0 C and 10000 g for 20 minutes). To a known quantity of cells was added 2.5 ml 0.1 N NaOH and incubated for 18 hours. The solution was centrifuged to remove the insoluble parts. To the supernatant 2.5 ml of

perchloric acid was added and the optical density was measured at 260 nm. The concentration of RNA was estimated using the standard rRNA (Sigma Chemical Co. USA).

2.3.2. Effect of nitrogen source on xylanase production

The production of xylanase was analysed in different nitrogen sources. The nitrogen source giving highest enzyme yield in comparison with the control was treated as the potent one and used for further studies. All the experiments were conducted for a period of 120 hours.

2.3.2.1. Nitrogen sources studied for the enhancement of xylanase production.

A. Complex nitrogen sources:

1. Beef extract
2. Yeast extract
3. Peptone
4. Peptone (0.5%) + Yeast extract (0.5%) (Control.)
5. Peptone (1.0%) + Yeast extract (1.0%)

B. Organic nitrogen source:

1. NH_2-CO-NH_2

C. Inorganic Nitrogen sources:

1. KNO_3
2. NH_4HPO_4
3. $(NH_4)_2SO_4$
4. NH_4NO_3
5. NH_4Cl

2.3.2.1.1. Xylanase production

The microorganism tried was *Bacillus* SSP–34, isolation procedure of which has been discussed in section **2.1.** Inoculum used for fermentation was 5 % (age 18 hrs). Basal media with different nitrogen sources were incubated at 300 rpm for 120 hours at 35 ^{0} C (pH 8.5). Cell free culture supernatant obtained by centrifugation (at 4^0 C and 10,000 g using Hitachi Himac CR 20 B2 centrifuge) served as the crude xylanase preparation.

2.3.2.1.2. Endo-xylanase (xylan xylanohydrolase EC 3.2.1.8.) assay

Endo-xylanase assay was carried out using 1.8 ml of oat spelts xylan suspension and 0.2 ml of suitably diluted enzyme with DNS as the stopping reagent. The detailed description could be found in the section **2.1.**

2.3.2.1.3. Analytical methods

Conducted as described in the section **2.1.**

2.3.2.2. Effect of different levels of optimum nitrogen source on xylanase production

Yeast extract and peptone combination identified as optimum nitrogen source were tried at different levels (Table 4). This is because the response by complex nitrogen sources vary in different cases due to the change in the components (Kelly, 1983). Optimum level producing minimal protease induction was also identified.

Table 4. Different combinations of optimum nitrogen source studied

Yeast ext.(%)	+ Peptone(%)	Final concentration
0.05	0.05	0.1
0.1	0.10	0.2
0.25	0.25	0.5
0.5	0.5	1.0
1.0	1.0	2.0
2.0	2.0	4.0

2.3.2.2.1. Protease assay

Protease activity was assayed according to Tsuchida *et al.* (1986) using 2% casein as the substrate. Preincubated 0.5 ml of the buffered substrate (phosphate buffer, pH - 8) was digested with 0.5ml of suitably diluted enzyme for 10 minutes at 40 ^{0}C and the reaction was terminated by the addition of 1ml 10% TCA. TCA soluble fragments were estimated using Folin's reagent at

660 nm. Protease activity was expressed as micromoles of tyrosine released per minute per ml under the assay conditions.

2.3.3. Effect of metal ions on xylanase production

Studies on the influence of different metal ions using NaCl, KCl, Na_2HPO_4, $MgSO_4$, $MnSO_4$, $CaCl_2$, $CoCl_2$, $FeSO_4$ and $ZnCl_2$ were carried out at 0.02% concentration replacing the minerals in the basal medium. All other parameters were kept constant.

2.4. Purification and Characterisation of Endo-xylanases

2.4.1. Purification of endo-xylanases

Xylanase from the crude enzyme preparation obtained after the fermentative production by the culture was subjected to the purification scheme given in Fig. 9.

2.4.1.1. Crude enzyme preparation

Bacillus SSP-34 was grown in the optimised medium as mentioned in Section **2.3.1.1**. after including the optimum carbon and nitrogen sources. Cell free supernatant was obtained by centrifugation (Hitachi Himac CR 20 B2 at 10000 g and 4 ^{0} C for 20 minutes) of the culture broth grown for a period of 102 hours and served as the crude xylanase preparation.

2.4.1.2. Concentration of the enzyme

The cell free culture supernatant (1000 ml) was concentrated by ultrafiltration using pellicon- Millipore membrane system with 10000 kDa molecular weight cut off. The volume reduced to 200 ml was then used for subsequent steps.

2.4.1.3. Fractional ammonium sulphate precipitation

Solid ammonium sulphate for the required saturation level was slowly added with mild stirring to crude enzyme at 0^0 C. The mixture was kept at 4^0 C for 1 hour and centrifuged (Hitachi Himac CR 20 B2) at 10000 g for 20 minutes at 4^0 C.

After removing the precipitate for the 1 fraction, the supernatant was again subjected to further precipitation to the next level by adding the required amount of solid ammonium sulphate. This process was repeated up to 100% fraction with gradual 10 % increase between the consecutive fractions.

The recovered precipitates were dissolved in double the volume of phosphate buffer (0.05 M Na_2HPO_4 / Na_2HPO_4, pH 7) and dialysed against the same buffer at 4^0 C for 18 hours with buffer changes at every 4 hours.

Protein content and endo-xylanase levels were monitored in the dialysed samples obtained from the precipitates. Initial experiments showed that endo-xylanase activities were distributed between 30-60 % fractions with maximum at 50-60%.

2.4.1.4. Chromatographic separations

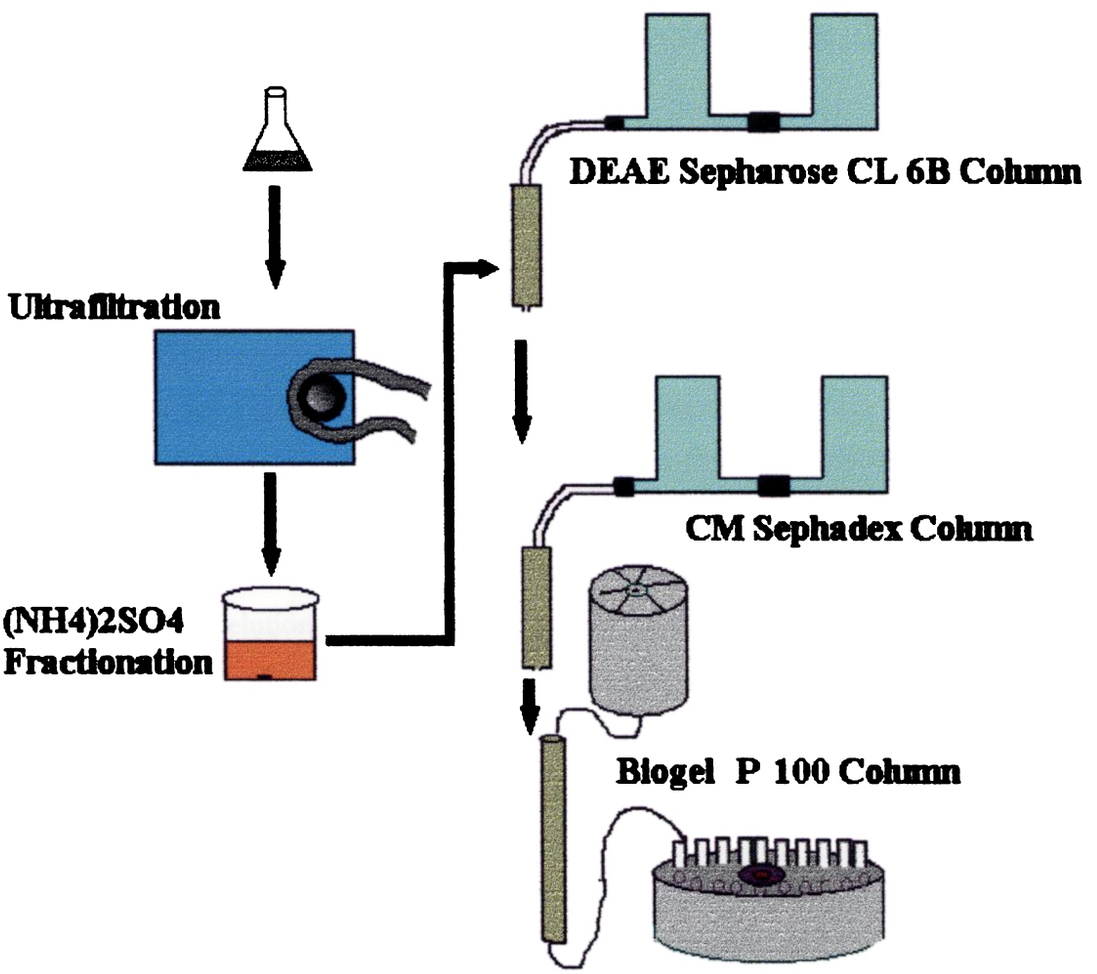


Fig. 9. Flow chart of the steps conducted to purify xylanases from *Bacillus* SSP-34

The saturated enzyme from the previous step was subjected to chromatographic separations including DEAE, CM and Bio Gel P 100 columns.

2.4.1.4.1. DEAE Sepharose CL 6B column chromatography

The dialysed fractions from the previous step was loaded on to DEAE Sepharose CL 6B column preequilibrated with phospate buffer (0.05 M and pH 7). In all the steps the buffer pH and composition have been kept constant. The matrix used was DEAE Sepharose CL 6B (Sigma chemicals Co.). The binding of enzyme protein to the matrices has been checked previously. After loading, wash was given to the column with twice the bed volume. Flow rate was adjusted to have 4.5 ml in each fraction. Adsorbed proteins were eluted using gradient elution system and the gradation ranged from 0.0 to 0.5 M NaCl. Each fraction was monitored for xylanase activity and protein concentration. The assay for the xylanase has been given in section **2.1.** The optical density of the samples at 280 nm in comparison with protein standard gave the protein concentration.

The molarities of the NaCl in the fractions were determined by the ORION Ion Analyser. Fractions showing endo-xylanase activity were pooled and dialysed to remove the NaCl. To the dialysate was added solid ammonium sulphate of 60 % concentration. After 1 hour of mild stirring in ice bath it was then centrifuged to collect the protein of interest. The dialysate from this experiment was subjected to further purification.

2.4.1.4.2. CM Sephadex column chromatography.

The precipitate obtained in the previous step was collected and dialysed against phosphate buffer (0.05 M, pH 7). The CM sephadex column was initially equilibrated with 5 times the volume of column using the same buffer. The treatment of the matrix, CM Sephadex (SRL, India) was carried

out according to standard protein purification literature. The concentrated dialysate was loaded to the column after centrifugation to remove the insoluble materials. Adsorbed proteins were eluted using gradient elution system and the gradation ranged from 0.0 to 0.5 M NaCl. The elution was carried out at a flow rate of 90 ml / hour with fraction volume of 4.5 ml.

Xylanase and protein assays were monitored in each fraction. The molarity of NaCl in each fraction was determined by using ORION Ion Analyser. Active fractions were pooled and then loaded to Bio Gel P100 column.

2.4.1.4.3. Biogel P 100 column chromatography – Size exclusion chromatography

Precipitates obtained in the CM Sephadex column chromatography were collected, centrifuged and dissolved in phosphate buffer. This solution was loaded to the Pharmacia X 16 Column system containing Biogel P 100 (BioRad) as the matrix which was pre-treated according to the manufacturer's instructions. Elution was carried out using 0.05 M PO_4 buffer at a flow rate of 30 ml / hour. The activity occurred in the 40 – 50 fractional range which was then pooled (30 ml) for the next step. Protein concentration of the individual fractions was determined by checking OD at 280 nm and xylanase activity of each fraction was estimated. The pooled fractions were centrifuged through amicon miniprep tubes.

2.4.1.5. Electrophoresis

2.4.1.5.1. Electrophoresis under non denaturing conditions

Polyacrylamide gels were prepared according to the method of Laemmli (1970). Electrophoresis was carried out in slab gel of 1.5 mm thickness. The separating gel was of 12 % concentration, while 5% stacking gel was found to be the optimum.

The samples loaded were prepared from the crude enzyme, $(NH_4)_2 SO_4$ fraction, DEAE fraction, CM fraction and Bio Gel P 100 fraction. The electrophoresis was carried out along with the protein markers. After solidification of the separating gel, the staking gel was poured on the surface of separating gel, which was then inserted with comb to make wells. Water saturated iso-butanol was used to aid the polymerisation process of the separating gel. In to the wells were added 40 μ l samples which were then subjected to electrophoresis using a power pack with a potential difference of 150 Volts which was then raised to 200 Volts when the protein bands reached the bottom of stacking gel. The electrophoresis was allowed to run for the completion of movement of proteins along with the tracking dyes towards the bottom of the gel. The gel was stained with Coomassie Brillliant blue for 20 minutes. The excess stain was washed in de-staining solution containing methanol, acetic acid and deionised distilled water.

2.4.1.5.2. Electrophoresis under denaturing conditions

Sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12% slab gels having a thickness of 1.5 mm. The separating gel and the stacking gel were of 12% and 5% concentrations respectively. The preparation of gel casting either separating or stacking were carried out as described for the non-denaturing conditions but the deionised water was replaced with 0.1% SDS. The samples loaded were crude enzyme preparation, $(NH_4)_2 SO_4$ fraction, DEAE fraction, CM fraction and Biogel P100 fraction. The samples were dissolved in sample buffer containing, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% of bromophenol blue, 25 % glycerol, 60 mM Tris HCl (pH 6.8), and 0.9 ml of water. The electrophoresis was conducted at a potential difference of 200 Volts and was stopped when the tracking dye reached the bottom of the

gel. Coomassie brilliant blue was used to stain the protein bands separated. The samples were run along with the protein markers in order to detect the molecular mass of the proteins. The proteins markers used were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), alkaline phosphatase (97 kDa), β-galactosidase (116 kDa) and myosin (200 kDa).

2.4.1.5.3. Zymogram

The zymogram analysis was performed by the method of Morag *et al.* (Morag *et al.*, 1990) with some modifications. Samples were electrophoresed on a 12 % SDS polyacrylamide separating gel under the conditions described in section **2.4.1.5.2.** Then the gel was washed five times with phosphate buffer (pH 7, 0.05 M). The first two washes contained 25% (Vol / Vol) iso-propyl alcohol. This removed the SDS present in the gel and also renatured proteins in the gel. A substrate gel was prepared containing 0.1% oat spelts xylan. The substrate gel was then overlaid on to the electrophoresed gel and incubated in phosphate buffer for 30 minutes at 37^0 C. It was then immersed in 0.1% Congo red solution taken in a wash tray for fifteen minutes at ambient temperature. The stained gel was then washed in excess with 1 M NaCl until the excess dye was removed. In order to complete the zymogram preparation the stained gel was treated with 0.5% acetic acid to turn the substrate containing background into dark blue while the active bands were represented by the clear zones of xylan hydrolysis.

2.4.2. Characterisation studies

The temperature optimum pH optimum and stabilities were determined using both crude and purified xylanases. The characterisation of the crude enzyme helps the purification procedure. The purified protein was then used for studying the kinetic properties.

2.4.2.1. Effect of temperature on activity and stability of the crude and purified xylanases

The effect of temperature on enzyme activity was determined by performing the standard assay procedure as mentioned earlier for 10 minutes at pH 9.2 within a temperature range of 30-65^0 C.

Thermostability was determined by incubation of the crude and purified enzyme at temperatures ranging from 25-65^0 C. The residual enzyme activities were measured at every 10 minutes interval for a period of two hours.

2.4.2.2. Effect of pH on crude and purified xylanases

The optimum pH of the xylanase activity was determined by incubating the crude and purified xylanases from *Bacillus* SSP-34 with the oat spelts xylan in appropriate buffers. Effect of pH on the activity was measured by essentially adopting Bailey's method using the following buffer systems: 0.2 M acetate buffer for pH 4-5, 0.2 M phosphate buffer for pH 6-8 and 0.2 M carbonate buffer for pH values 9 to 11. Stability of the purified enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 4-11 for two hours and then estimating the residual activity.

2.4.2. 3. Effect of substrate concentration on purified xylanase.

The Michaelis-Menton constant (K_m) and maximum velocity (V_{max}) were determined by using oat spelts xylan as the substrate at concentrations ranging from 1.8-18.0 mg/ml in phosphate buffer (0.05 M, pH 7). Initial reaction rate was measured for each condition according to the standard assay procedures mentioned earlier. The values of the Michaelis-Menton constant (K_m) and the maximum velocity (V_{max}) were calculated from Lineweaver-Burk plots (Lineweaver and Burk, 1934).

2.4.2. 4. Effect of metal ions and enzyme effectors on purified *Bacillus* SSP-34 xylanases

Various salts at concentrations ranging from 0.01 to 1.0 mM were added to the standard enzymatic reaction mixtures and the xylanase activity was estimated as mentioned earlier.

2.5. Application of *Bacillus* SSP-34 Xylanase

2.5.1. Hydrolytic experiments

Xylan (Oat spelts) was hydrolysed with purified xylanase from *Bacillus* SSP-34 at 50^0 C for 30 minutes. The reaction was stopped by incubating the reaction mixture for 10 minutes in a boiling water bath. After this the samples were centrifuged and filtered through Millipore filtering unit. The products were analysed by HPLC using Shim-pack-CLC-NH_2 (M) column (Shimadzu Co. Ltd.). Sugars were identified by comparing retention times measured by a refractive index detector (RID-6A – Schimadzu Co. Ltd.)

2.5.2. Application of xylanases in paper and pulp industry.

2.5.2.1. Xylanase Pretreatment

Crude xylanase obtained by the growth of *Bacillus* SSP-34 in xylan containing liquid media was used in the pretreatment of paper pulp.

Pulp samples were kindly provided by Hindustan Newsprint Ltd. Velloor. The pulp from the cooking plant was washed thoroughly. The pH of the pulp was first adjusted to 8.0. It was then subjected to chelation treatment with 0.01 % EDTA and incubated for 30 minutes. Pulp samples from the above treatment were washed well and the pH adjusted to 7. The pulp was maintained at 5% consistency (w/100 ml) for enzyme treatment. Crude xylanase preparation was then added to the pulp sample and incubated for 2 hours for the completion of pretreatment. Enzyme pre-treated samples were washed thoroughly and 0.5 ml of 25% NaOH (high alkalinity) was then added for alkali extraction of the

chromophores released during the pretreatment process. After 30 minutes the solution was washed well and subjected to oxidising agents.

2.5.2.2. Bleaching of the xylanase pretreated pulp with oxidising agents

The alkali extracted sample was then subjected first to H_2O_2 treatment while maintaining the conditions and components for super bleach (Rydholm, 1965b). The other components added during bleaching process were:

H_2O_2	-	1.5 % (w/w of pulp)	
NaOH	-	0.2%	,,
$MgSO_4$	-	0.02%	,,
Sod. Silicate	-	2.0%	,,

The bleaching process was continued for 2 hours at 50^0 C. The peroxide bleached samples were washed thoroughly and subjected to NaClO (sodium hypochlorite) treatment at 70^0 C. The conditions were favouring the oxidation process by hypo on the lignin content of pulp (Rydholm, 1965a). The active chlorine available for oxidation was 5%.

Thus 1.5 ml NaClO (5 % sol.) ≡ 0.075 gm Cl_2 added, which is equal to 0.125 g / 5 gm of pulp (2.5 %).

The samples after 3 hours of incubation were washed thoroughly and pressed to form round sheets. These sheets then analysed for brightness studies. Observations were made against MgO (Color touch Spectrophotometer, Technidyne Corporation, USA.). The sequence for the entire process of pretreatment and bleaching are given below.

ε / E/ P / H / P / H

ε - Enzyme treatment.

E - Alkali extraction.

P - Hydrogen peroxide treatment.

H – Hypochlorite treatment.

RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Microorganisms

The importance of xylanases in paper, pulp and food industries initiated the search for microorganisms producing higher levels of xylanases. Only a few bacterial and actinomycete xylanases have been reported earlier with pH optima in the neutral or alkaline ranges (Nakamura *et al.*, 1994; Duarte *et al.*, 1999; Ratanakhanokchai *et al.*, 1999). In search of novel thermostable alkaline xylanases suitable for industrial applications, studies were initiated for isolation of microorganisms that could produce xylanases. Various methods have been adopted for isolation of microorganisms degrading lignocellulosics. This screening was complicated by the fact that each of the main components in the lignocellulosic substrates such as cellulose, hemicellulose and lignin required two or more enzymes which were controlled by intricate pathways of biochemical and genetic regulation (Kluepfel, 1988).

Isolation of microorganisms having higher competence and higher production of xylanases was the major goal in the present study. Soil samples collected include soil from forest regions (Kallar forest, Thiruvananthapuram), riverbed soil and alluvial soil. Moreover, screening was also done using different lignocellulosic substrates that have been exposed to decomposing conditions. The lignocellulosic substances like wheat bran, bagasse and rice straw are having hemicelluloses among the other components and are frequently used as the media component for xylanase production (Vyas *et al.*, 1990; Liu *et al.*, 1998). These substrates were subjected to rotting conditions

at different scales of pH values i.e. 5.0, 7.0 and 10.5 standing for acidic, neutral and alkaline conditions respectively.

As the nutritional requirements of microorganisms vary greatly, a single medium component may not be adequate for the growth of all microorganisms present in a natural population (Stanier *et al.*, 1987). Thus there was the necessity for a preliminary stage in isolation studies with nutrient rich medium such as the wheat bran medium. The samples collected were suspended in sterile water or saline and subjected to serial dilution. 0.1 ml each of different dilutions of the sample was inoculated to nutrient medium plates containing wheat bran as given earlier. Wheat bran is a rich source of carbohydrate, however, it also contains certain nitrogenous components (Andersson *et al.*, 1994). This medium was supplemented with peptone, yeast extract and NaCl. The cultures that showed good growth on wheat bran medium were selected for further work. The selected cultures in the media obviously include those producing enzymes degrading substrates other than xylan. Due to this, steps were taken later for weeding out the organisms producing hydrolases other than xylanases.

In the second stage of screening, the selected 200 ~~cultures~~ were grown in media where wheat bran was replaced with xylan as carbon source, to eliminate the isolates those were not specific for xylanases. The potent xylanase producers were differentiated by observing areas of clear zone production on xylan agar plates. Since xylan, a large molecular weight polymer, could not enter the microbial cells, the endo-xylanases produced by most of the microorganisms were extracellular in nature (Biely, 1985). The cultures those showed clear zones of minimum 1 cm diameter were selected for further studies. Secondary screening resulted in the selection of 40 isolates and they were subjected for further screening. However, all the clear zone producers might not be the potent producers of endo-xylanases. During the

growth of an isolate on solid agar plates, the presence of membrane bound hydrolases possibly caused clear zones by good xylan hydrolysis. This may be due to the regional presence of enzymes secreted near the growing colony in the agar plates. However, this effect was not expressed during liquid culture cultivation (Aunstrup, 1974). Hence the above selected strains were subjected to submerged fermentation using xylan as the carbon source to have a clear picture of xylanase producers.

The 40 ~~cultures~~ selected were grown in liquid medium for a period of 144 hours. Culture broth pH and biomass were monitored at every 24 hours and the cell free culture supernatant was used for the estimations of xylanase activity (Bailey *et al.*, 1992), soluble protein (Lowry *et al.*, 1951) and reducing sugar (Miller, 1959). The data at the hour of maximum enzyme production for each culture is given in the Table 5. At the hour of maximum enzyme production, the reducing sugar levels were low indicating the onset of release of catabolite repression on xylanase production. There are reports regarding the catabolite repression of xylanase in bacteria and fungi (Samain *et al.*, 1992; Nakamura *et al.*, 1993a; Fernandez-Espinar *et al.*, 1992).

Table 5. Growth profile and xylanase production by cultures from the initial screening.

Isolate No	Period of maximum enzyme production. (hrs)	pH	Biomass mg/ml at the hour of maximum enz. prodn.	Composition and xylanase activity of cell free culture supernatant harvested at the hour of maximum enzyme production.			
				Soluble Protein mg/ml	Reducing Sugar µg/ml	Xylanase Activity IU /ml	
						pH 7	pH 9
SSP 1	96	9.11	2.0 ±0.5	2.917 ±0.8 2	314.28 ±0.86	0.12± 0.01	1.02 ±0.01
2	96	9.18	1.2 ±0.5	2.448 ±0.04	349.9 ±0.86	0.174 ±0.012	0.91 ±0.11
3	96	9.08	2.0 ±0.4	2.524 ±0.04	346 ±1.2	1.26 ±0.105	1.16 ±0.08
4	72	10.07	2.1 ±0.55	2.662 ±0.07	149.48 ±1.24	0	0.01 ±0.001
5	144	9.15	1.6 ±0.3	1.647 ±0.13	163.33± 0.57	0.065 ±0.013	0.1 ±0.01
6	120	9.37	2.1 ±0.5	1.342 ±0.02	148.98±1.2	0	0.004 ±0.003
7	144	9.28	1.1 ±0.5	2.368 ±0.07	99.4 ±1.26	0.144 ±0.022	0.1 ±0.002
8	72	9.57	2.3 ±0.25	2.162 ±0.02	99.6 ±1.1	0.09 ±0.01	0.65 ±0.03
9	72	10.63	2.6 ±0.5	2.764 ±0.03	125.48 ±2.74	0.071 ±0.01	0.06 ±0.001
10	96	9.16	1.4 ±0.75	2.582 ±0.01	340.8 ±2.1	0.1 ±0.05	0.26 ±0.03
11	96	10.26	3.78 ±0.44	2.256 ±0.13	125 ±1.1	0	0.033 ±0.001
12	120	10.09	4.34 ±0.125	2.119 ±0.001	204.7 ±1.5	0.09 ±0.01	0.07 ±0.01
13	24	10.56	3.72 ±0.31	2.675± 0.013	24.05± 0.48	0.1 ±0.03	0.07 ±0.015
14	96	10.37	4.58 ±0.39	2.084 ±0.01	9.7 ±0.35	0	0.03 ±0.01
15	96	9.76	6.0 ±0.25	2.527 ±0.09	31.33± 0.66	0.004 ±0.003	0.026 ±0.01
16	96	9.22	7.5 ±0.25	1.912 ±0.04	844.53± 2.27	0	0.016 ±0.01
17	24	10.35	6.84 ±0.42	2.562 ±0.03	127.74 ±0.84	0	0.016 ±0.002
18	24	10.24	4.860 ±0.48	2.636 ±0.02	168.11 ±1.56	0	0.0144 ±0.01
19	72	6.70	3.58 ±0.29	2.828 ±0.04	667.81 ±0.6	0.026 ±0.002	0
20	144	8.36	3.88 ±0.44	1.639± 0.08	1310.9 ±3.0	1.37 ±0.065	0.0168 ±0.002

Table 5 (Contd....)

Isolate No	Period of maximum enzyme production. (hrs)	pH	Biomass mg/ml at the hour of maximum enz. Prodn.	Composition and xylanase activity of cell free culture supernatant harvested at the hour of maximum enzyme production.			
				Soluble Protein mg/ml	Reducing Sugar µg/ml	Xylanase Activity IU /ml	
						pH 7	pH 9
21	144	8.42	4.76 ±0.38	2.507± 0.004	454.6± 2.3	1.75 ±0.03	0.05 ±0.01
22	72	8.20	4.1 ±0.05	1.978 ±0.04	542 ±6.0	0.0711 ±0.02	0.024 ±0.003
23	72	7.51	4.58± 0.29	2.07± 0.02	423.15 ±2.1	0.09 ±0.01	0.09 ±0.003
24	72	8.40	3.48 ±0.24	0.003 ±0.0001	542.94± 2.47	0.09 ±0.01	0
27	48	7.90	4.62 ±0.17	1.525 ±0.04	368 ±4.0	0.42 ±0.09	0.42 ±0.06
28	96	8.64	5.38± 0.44	1.441 ±0.08	732.45 ±1.23	0.04 ±0.03	0
29	72	9.13	8.04 ±0.52	3.873± 0.01	155.5 ±5.25	0.852 ±0.02	1.41 ±0.46
30	48	9.06	5.18 ±0.14	2.906 ±0.05	542.5± 1.25	0.07 ±0.01	1.04 ±0.03
31	72	9.06	4.2 ±0.16	3.092 ±0.01	268.05 ±4.53	0.05 ±0.02	0.3 ±0.05
32	120	8.25	8.02 ±0.24	2.393 ±0.004	22.88 ±1.44	2.4 ±0.25	0.51 ±0.05
33	120	8.00	4.40 ±0.24	2.830 ±0.17	360.93 ±5.5	2.95 ±0.28	0.58 ±0.04
34	102	8.55	2.50 ±0.3	3.37 ±0.03	58.1 ±1.44	100.72 ±0.64	4.44 ±0.22
35	96	8.70	4.82 ±0.11	1.291± 0.05	1238.5± 6.75	0.78 ±0.09	0.03 ±0.01
36	96	8.85	4.7 ±0.1	0.0182 ±0.02	654.8 ±2.4	0.06 ±0.01	0
37	144	9.24	5.4 ±0.22	1.31± 0.06	47.0 ±1.5	0.15 ±0.04	0
38	144	9.05	5.52 ±0.26	1.764 ±0.3	466.2 ±3.6	0.05 ±0.02	0
39	96	8.68	4.44 ±0.22	1.878 ±0.02	303.5 ±2.25	0.011 ±0.01	0.021 ±0.01
40	24	7.56	1.5.22 ±0.21	2.287 ±0.31	21.80 ±0.9	0.4 ±0.05	0

Data at the hour of maximum enzyme activity were taken from the periodically studied samples, which represented mean value of 5 separate analysis with standard deviations

Growth profile study of the 40 strains indicated that xylanase production seldom occurred at the early stages of growth (Table 5). Most of the organisms displayed maximum xylanase expression at the post-exponential or stationary phase of growth (Samain *et al.*, 1992; Nakamura *et al.*, 1993a; Fernandez-Espinar *et al.*, 1992). Culture broth pH of all the microorganisms studied varied during the fermentation period and for *Bacillus* SSP-34 enzyme production occurred at pH value of 8.55 (Table 5). Among the 40 strains isolated, only 10 showed potent xylanase production, confirming the earlier assumption that the regional presence of xylanases and membrane bound xylanases might be effecting good clear zone production on xylan agar solid plates (Aunstrup, 1974). The isolate SSP-34, which earlier showed good clear zone on the xylan agar plates (Fig. 10), resulted in the highest xylanase activities of 100.72±0.64 IU/ml and 4.4±0.22 IU/ml at the reaction pH of 7 and 9.2 respectively.

3.1.1. Identification of the bacterial cultures.

Since strain number 34 was the most potent culture expressing the xylanolytic activity 100 times over the remaining ones, it was subjected to identification by biochemical and morphological studies according to Bergey's Manual of Systematic Bacteriology.

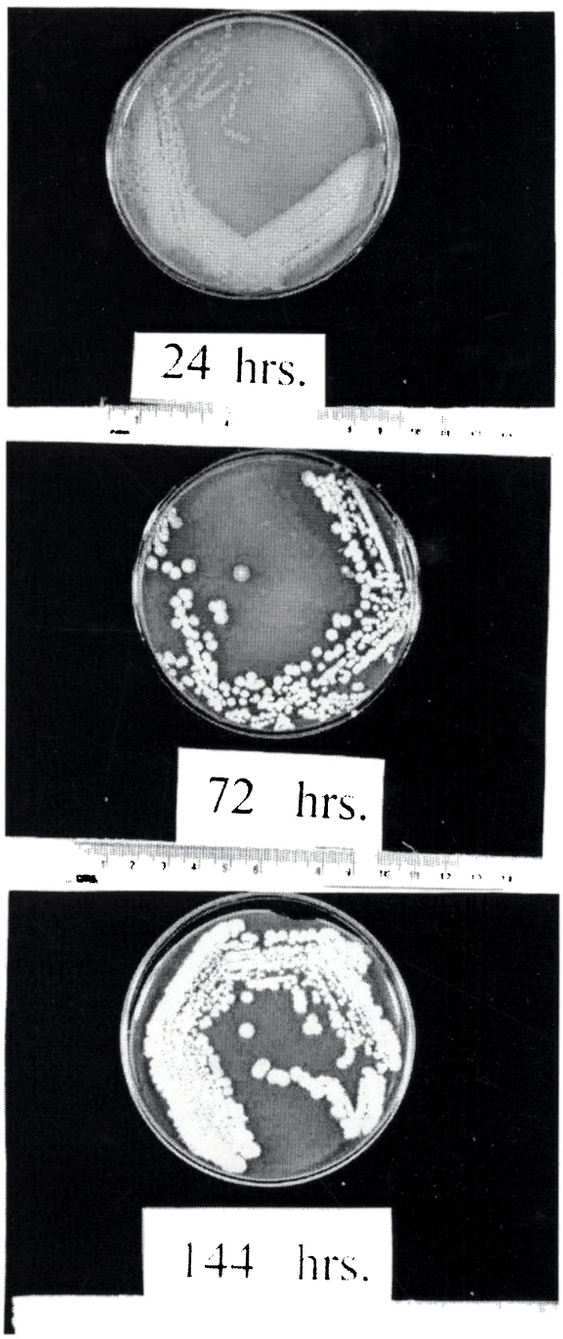


Fig. 10 Growth of *Bacillus* SSP-34 on xylan agar plates at 24, 72 and 144 hours. Circular clear zone region could be identified around colonies grown for 24 hours. After 144 hours the opaque xylan agar medium was completely made transparent due to hydrolysis of insoluble xylan by extracellular xylanase. Circular clear zones and colony morphology were prominent in the separately prepared xylan agar plate with *Bacillus* SSP-34 grown for 72 hours.

Results indicated that the above strain was an aerobic, Gram-positive (during the initial hours of growth) and motile rod producing endospores. Biochemical characterisation showed that isolate SSP-34 was positive to catalase test, VOGES-PROSKAUR test, M. R. test, and gelatin liquifaction, while it was negative to urease test, nitratase test, indole test and starch hydrolysis. The characteristics inferred that the culture SSP-34 belonged to the genus *Bacillus* (Claus and Berkeley, 1986) and it was named as *Bacillus* SSP-34 (Subramaniyan *et al.*, 1997). Identification studies were also carried out for the other potent cultures SSP-20, SSP-32, SSP-35 and identified them belonging to

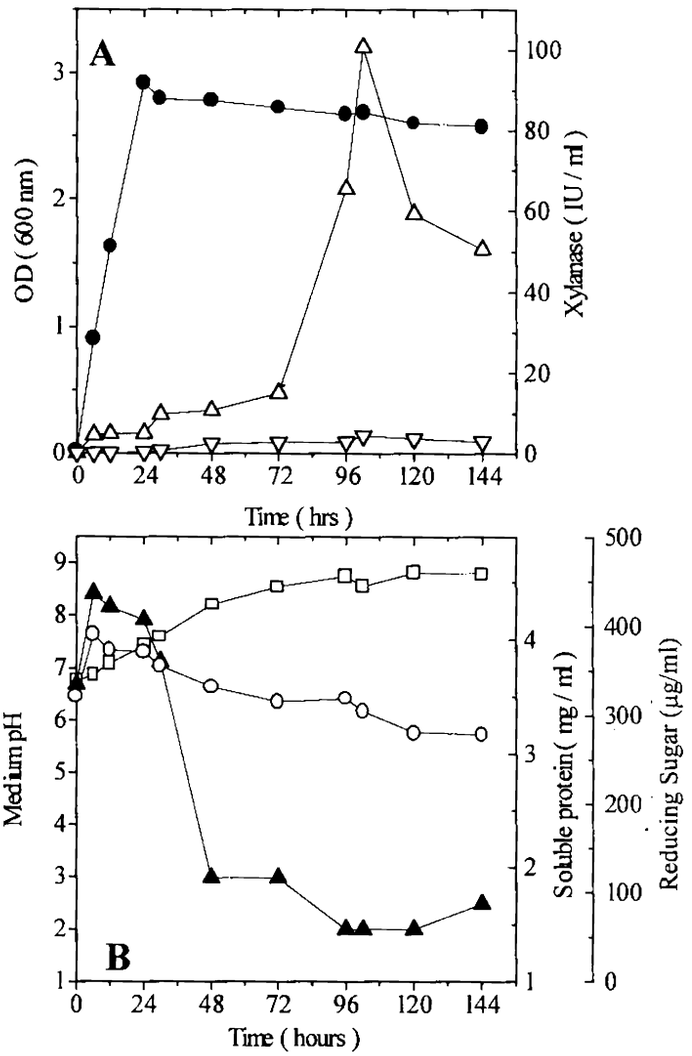


Fig. 11. **A**. Fermentation profile of *Bacillus* SSP-34. **B**. The variation of pH (□) and the other compounds. Growth (●) was observed by measuring the optical density at 600 nm. Soluble protein (○), reducing sugar (▲) and xylanase activities both at pH 7 (Δ) and pH 9.2 (∇) were monitored using the procedures discussed in materials and methods

Bacillus spp. while SSP-30 identified as *Streptomyces* sp. (Holt *et al.*, 1994).

3.1.2. Growth characteristics of *Bacillus* SSP-34

The culture was grown in liquid medium containing xylan at 0.5% level as the carbon source. The endo-xylanase activities were studied in association with pH change, growth, soluble protein and reducing sugars in the medium (Fig.11.A and B). During the logarithmic phase of growth, low amounts of extracellular xylanases were detected. Though the culture growth attained stationary phase by the 36^{th} hour, there was still an increase in enzyme production to the end of stationary phase. Similar lack of xylanase activity during logarithmic phase has been reported in *Bacillus* spp. (Samain *et al.*, 1992; Nakamura *et al.*, 1993a) and *Aspergillus nidulans* (Fernadez-Espinar *et al.*, 1992). Low free xylanase activity might be due to the adsorption of xylanases on the surface of insoluble xylan particles present in the culture medium (Rozie *et al.*, 1992; Fernandez-Espinar *et al.*, 1992; Irwin *et al.*, 1994; Subramaniyan *et al.*, 1997). The higher xylanase activity of *Bacillus* SSP-34 during later stages of growth could be the reflection of small amounts of xylanase liberated from cells undergoing autolysis (Fernandez-Espinar *et al.*, 1992; Subramaniyan *et al.*, 1997) as well as the non availability of insoluble particles in the medium which if present might have bound the xylanases (Subramaniyan *et al.*, 1997; Connerton *et al.*, 1999). After 120 hours of growth there was a slight decline in xylanase production suggesting the possible action of intracellular proteases from the autolysed cells.

Reducing sugar levels showed steep increase in the early hours of growth which was presumed to be due to the xylanases present in considerable amounts in the inoculum causing the hydrolysis of xylan in the medium (Fig.11 B). Concentration of reducing sugars at the hour of maximum enzyme

production was lower than that at log phase. These effects might have been due to (1) the depletion of reducing sugars by the growth of bacteria causing the reversion of catabolite repression (2) the lack of xylan particles at the later phase of growth which can adsorb xylanase molecules and (3) autolysis of cells (Fernandez-Espinar *et al.*, 1992; Subramaniyan *et al.*, 1997) releasing xylanases.

Protein concentration in the culture medium did not fluctuate much during the fermentation period and slightly decreased at 144 hours of growth (Fig.11 B.), while pH changed towards alkaline side by 144 hours of growth. SSP-34 with maximum activity 100 IU / ml (basal medium) was found to be a potent producer of xylanases than most of the reported cases. Earlier reports from many workers are listed below - Duarte *et al.* (1999) reported an activity of 2.6-4 U/ml. Ratanakhanokchai *et al.* (1999) observed an activity of 4.8 U/mg protein by a *Bacillus* sp. strain K-1 while the production of xylanases by *Bacillus* SSP-34 (30 IU/mg protein- in the non-optimised medium) is 6.23 times more than the *Bacillus* sp. K-1. Liu *et al.* (1998) observed an activity of 74 IU/ml from *Trichosporon cutaneum* SL409. Other reports include the observations of Nakamura *et al.* (1994) – 10 IU/ml, Dhalberg *et al.* (1993) 3 – 4 IU/ml, Keskar (1992) 70-72 IU/ml, Zychlinska *et al.* (1992) - 70.4 IU/ml etc. Thus the production of xylanases by *Bacillus* SSP-34 is comparable with the earlier reports. The production could be further increased by the optimisation of fermentation conditions.

3.1.3. Comparison of the cellulolytic activities of potent xylanase producers

Ten xylanase producing cultures were grown for a period of 144 hours and the samples were analysed for soluble protein, reducing sugar, biomass,

pH, xylanases and cellulases. Filter paper assay was used to detect the activity of cellobiohydrolase (FPase or cellulose 1,4-β-cellobiosidase, E C 3.2.1.91) (Mandels *et al.*, 1976) and carboxymethylcellulose was used to detect endocellulase activity (CMC ase or 1,4(1,3:1,4)-β-D-glucan-4-glucano hydrolase E.C 3.2.1.4) (Dahlberg *et al.*, 1993). Endocellulase or CMCase activity of *Bacillus* SSP-34 was very low. 0.43 IU/ml at reaction pH 7 (Table 6) and no activity was observed at pH 9.2. The order of magnitude of CMCase activity at pH 7 was: SSP-32 - 1. 04 IU/ml > S SP-35 > SSP-29 > SSP-2 > SSP-34 > SSP-1 > SSP-20 > SSP-27 > SSP-30 > SSP-3. Out of the ten isolates SSP-35 and SSP-29 exhibited FPase (cellobiohydrolase) activity of 1.38 IU/ml and 1.2 IU/ml respectively. Further more, while the FPase activity of strain *Bacillus* SSP-34 was 0.24 IU/ml at pH 7, it was undetectable at pH9.2. The above indicated that *Bacillus* SSP-34 has negligible amounts of cellulolytic activities. Order of magnitude of FPase was SSP-35 (1.38IU/ml)> SSP-29> SSP-20> SSP-27> SSP-32> SSP-30> SSP-2> SSP-1> SSP-34> SSP-3.

Thus in general, most of the cultures had low levels of cellulolytic activity justifying the quest for cellulase free bacterial xylanases (Ratto *et al.*, 1992; Subramaniyan *et al.*, 1997; Subramaniyan and Prema, 1998) rather than fungal xylanases which were associated with higher levels of cellulases (Table 1). Moreover most of the fungal xylanases showed optimum temperatures below 50^0C and pH optimum towards acidic range with rare exceptions (Haltrich *et al.*, 1996). In certain industrial applications especially related to paper and pulp industries, usage of low pH enzymes necessitated additional steps in subsequent stages which make the fungal xylanases less suitable for practical applications owing to cost increase. Occurrence of cellulases in the crude enzyme preparations (Table 1) usually effected serious viscosity drop of paper pulp due to cellulose hydrolysis (Srinivasan and Rele, 1995).

Table 6. Comparison of cellulase and xylanase production by the selected 10 cultures.

Microorganism	Initial pH of the culture medium	Xylanase IU/ml (pH7)	Cellulase (IU/ml)	
			Fpase	CMCase
SSP-34	6.74	100.72±0.36	0.24±0.02	0.43±0.02
SSP-32	6.77	2.38±0.04	0.52±0.02	1.04±0.02
SSP-35	6.63	0.78±0.02	1.38±0.01	0.88±0.01
SSP-20	6.85	1.4±0.05	0.66±0.02	0.28±0.04
SSP-27	6.86	0.43±0.02	0.54±0.01	0.23±0.01
SSP-1	10.3	0.12±0.01	0.25±0.01	0.34±0.02
SSP-2	10.35	0.17±0.01	0.44±0.02	0.46±0.03
SSP-3	10.35	1.26±0.01	0.12±0.01	0.08±0.02
SSP-30	10.58	0.07±0.01	0.51±0.06	0.10±0.01
SSP-29	10.64	0.85±0.03	1.2±0.01	0.81±0.01

3.1.4. Exposition of trace cellulolytic activities associated with xylanases

Although *Bacillus* spp. were identified as industrially important producers of hemicellulases, little information was available on the interrelationship regarding their cellulolytic and xylanolytic activities. The low amounts of cellulolytic activities in the culture supernatants for most of the bacteria may be due either to the presence of traces of cellulases or to the hydrolysis of xylan present in commercial preparation of cellulosic substrates used for cellulase assays (Bailey and Poutanen, 1989; Royer *et al.*, 1992; Subramaniyan and Prema, 2000).

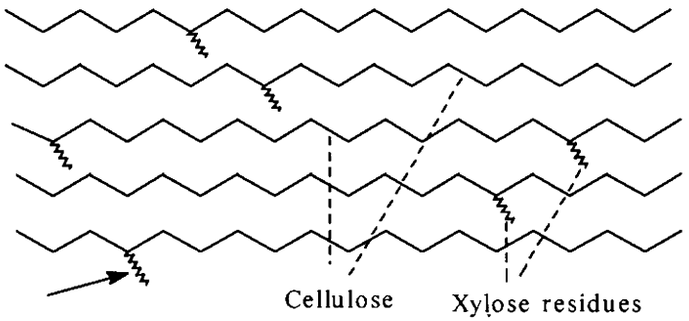


Fig. 12. Proposed mechanism of reducing sugar release from impure cellulose substrate by xylanase based on the reports of Bailey and Poutanen (1989); Royer *et al.* (1992).

⟶ Point of attack of xylanase on xylose residues present in impure cellulose resulting in the 'pseudo-cellulase' activity.

It has been observed that commercial celluloses like solka floc cellulose contain approximately 10% w/w xylose residues (Bailey and Poutanen, 1989; Royer *et al.*, 1992) which supports the present experimental observations (Fig. 12). Thus trace cellulolytic activity may be due to the possible release of xylose residues (owing to 'pseudo-cellulase activity') from the samples of commercial cellulose substrates used for cellulase assays. There could be other possibilities for the trace amounts of cellulolytic activities by *Bacillus* SSP-34 culture supernatant. Since the cellulose binding domains (CBDs) present in some endoglucanases play an important role in cellulose hydrolysis, negligible cellulolytic activities by some xylanases can also be attributed to the presence of CBDs in these xylanases. (Sakka *et al.*, 1996; Black *et al.*, 1997; Sun *et al.*, 1998). Although it is quite unnatural for one enzyme to have a high affinity towards a non-specific substrate, CBDs are widely distributed in xylanases (Black *et al.*, 1997, Millward-Sadler *et al.*, 1994). Apart from the hydrolytic activity against xylan, some of the microbial xylanases produced from *Clostridium stercorarium* (Sakka *et al.*, 1993),

Cellulomonas fimi (Millward-Sadler *et al.,* 1994), *Pseudomonas fuorescens* (Hazlewood and Gilbert, 1992)), *Streptomyces halstedii* J M8 (Ruiz-Arribas *et al.,* 1997) and *Fusarium oxysporium* F3 (Christakopoulos *et al.,* 1996) are also reported to contain cellulose binding domains. The reason for the presence of CBDs on plant cell wall hydrolases is possibly due to the performance of cellulose as a general receptor for plant cell wall hydrolases (Black *et al.,* 1997). It is the only non-variable structural polysaccharide in the cell wall of all plant species, although there are some marginal changes in the degree of crystallinity of cellulose (Gilbert and Hazlewood, 1993). Some exo-β-1,4-glycanases (e.g. exoglucanase/xylanase cex from *Cellulomonas fimi*) hydrolyze not only cellulose but also xylan since they share a common catalytic mechanism as evidenced by the sequence homology of the family of β-1, 4-glycanases showing the conservation of Glu 127, the acid/base catalyst in *C. fimi* glycanase (MacLeod *et al.,* 1994). It is conceivable that some of the xylanases in the family retain glycosidase activity, which could be attributed to a common catalytic mechanism (MacLeod *et al.,* 1994). The cloning of cellulase and xylanase genes into non-cellulolytic/xylanolytic backgrounds confirmed that they were also capable hydrolyzing both polymers i.e. cellulases hydrolysing xylan and vice versa (Wakarchuk *et al.,* 1994). For special applications with reference to pulp and paper industries, the nature of xylanases used were preferred to be cellulase free one in order to avoid any cellulose damage. In general it is evident that fungal xylanases with rare exceptions are less suitable than bacterial xylanases, especially with respect to cellulases, operational pH and growth requirements. The strain *Bacillus* SSP-34 selected from the screening procedure satisfied two criteria of selection - free of cellulase and production of high levels of xylanase activity.

3.2. Optimisation of Cultural Parameters

Improved industrial fermentation could be ascribed to the development of superior strains by genetic manipulation. However, other parameters like nutritional and physical environments to which an organism was exposed were also known to significantly influence the yield (Greasham and Inamine, 1986). Xylanase activity by *Bacillus* SSP-34 grown in Horikoshi II medium (Horikoshi, 1991a) containing 0.5% oat spelts xylan as the carbon source was maximum at the late stationary phase. There has been lot of emphasis given to purification, characterisation and elucidation of the xylanase genes. However, not much importance has been given for the optimisation of cultural and nutritional conditions for the production of xylanases from bacteria including *Bacillus* sp. (Nakamura *et al.*, 1993a) which could have influenced the production. Investigations were undertaken to study the influence of cultural parameters on enhanced production of xylanases from *Bacillus* SSP-34. The cultural parameters studied include pH, temperature, inoculum concentration, agitation and aeration, which have effects on xylanase production by *Bacillus* SSP-34.

3.2.1. Effect of pH on xylanase production

The initial pH of the media strongly influences many enzymatic systems and transport of several species of enzymes across the cell membrane (Moon and Parulekar, 1991). In order to study the effect of initial pH of culture (pH being uncontrolled during the fermentation process) on the formation of endo-xylanases, *Bacillus* SSP-34 was grown in Horikoshi basal medium with the initial media at different ranges - pH 5 to 10, adjusted after autoclaving. The culture showed maximum xylanase production at pH 8.5; but maximum growth was observed at pH 9 (Fig. 13). A significant increase in the pH was observed for media having an initial pH $\leq$8, while in the case of

samples having initial pH values 8.5, 9.0 and 9.5 there was a decline of pH during the early growth phase (Fig. 14). This was followed by an increase towards pH upto 9.0 after 96 hours of growth, at which maximum xylanase production occurred. Similar pattern of enzyme production has been reported earlier (Horikoshi, 1991b). Irrespective of the initial pH value, the media pH shifted towards a pH range of 8.5-9.0, indicating that the culture was alkalophilic. Similar observation on pH adaptation by alkalophiles was reported earlier (Horikoshi, 1991a). The results showed that the enzyme production in the medium with an initial pH of 9.0 was 62% of that at pH 8.5. Higher production of xylanases was noticed when the pH of the medium was in the range of 7-9 (Subramaniyan and Prema, 1998). Moreover, most of the reported

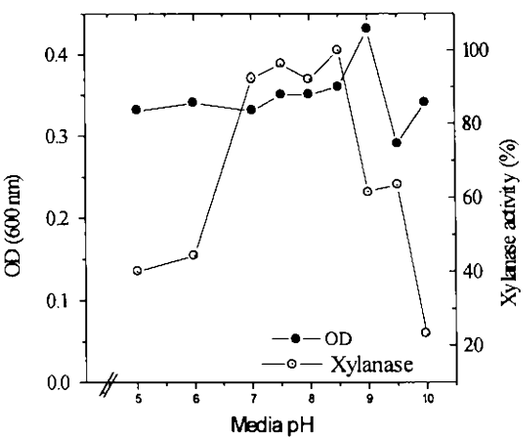


Fig.13. Growth profile of *Bacillus* SSP-34 in media with different initial pH values. OD was monitored after 24 hrs and xylanase activity after 96hrs.

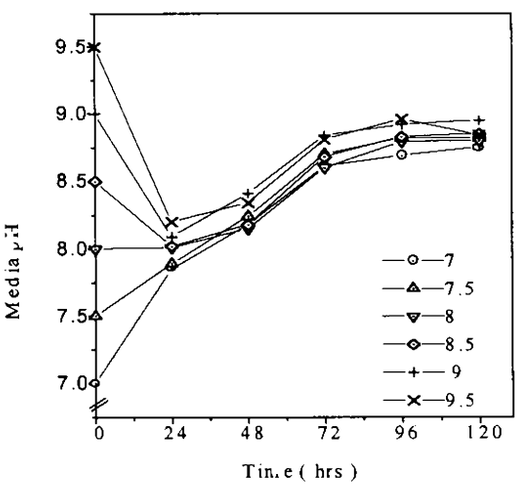


Fig.14. Pattern of pH change in basal media with different initial pH values (pH 7 - 9.5) during growth of *Bacillus* SSP - 34

xylanases from alkalophilic organisms have a similar optimum pH in the range of pH 5-8 (Krulwich and Guffanti, 1989). The optimum pH (pH 9) of *Bacillus* SSP-34 was higher than that of many bacteria reported earlier (Krulwich and Guffanti, 1989; Lopez *et al.*, 1998).

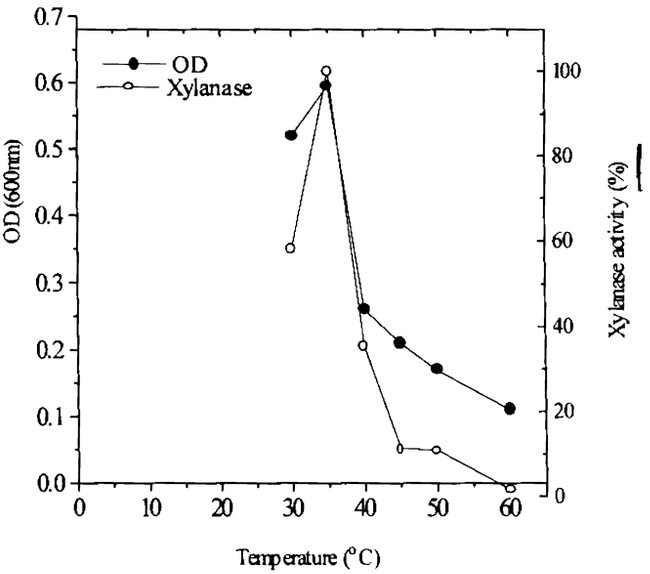


Fig.15. Growth profile of *Bacillus* SSP-34 at different temperatures.

3.2.2. Effect of temperature on xylanase production

Bacillus SSP-34 was grown in Horikoshi II basal medium at temperatures ranging from 30-60°C. Optimal production of xylanases was detected at 35°C and it was near the optimum temperature of other *Bacillus* spp. like *Bacillus* sp. BP-7 (Lopez *et al.*, 1998). At growth temperatures of 30° C and 40°C xylanase production was functionally 58% and 35.34% respectively of that produced at 35°C. Increase of growth temperature to 45° C and 50°C lowered xylanase production to a level of ~10% of that occurred at optimal temperature (Fig. 15).

At 60°C negligible amount of xylanase was recorded. Maximum growth was observed at 35°C with an absorbance of 0.595 at 600 nm, which was followed by 30°C (0.52), 40°C (0.26), 45°C (0.21) and 50°C (0.17). Thus, *Bacillus* SSP-34 could grow at temperatures near 50° C and it indicated that

the organism could be classified under thermo-tolerant bacteria (Dubeau *et al.*, 1987).

3.2.3. Effect of inoculum concentration

The fermentation profile of an organism is usually affected by the size of the inoculum and its physiological conditions (Meyrath and Suchanek, 1972). In order to minimise the time lag in fermentation process (Lincoln, 1960), inocula were raised in the same medium with a composition similar to that of fermentation medium discussed under Methodology (Chapter 2). However, the above has resulted in the transfer of small quantities of xylanases synthesised in the xylan containing seed culture causing an initial increase in reducing sugar concentration of the fermentation medium (Subramaniyan *et al.*, 1997). They were transferred to the fermentation medium during the logarithmic stage of growth. This is because the age of inoculum of a sporulating organism like *Bacillus* SSP-34 was an important factor as it would have resulted in the transfer of high quantities of spores if transferred after metabolically active stages (i.e. during the stationary phase or death phase). Presence of higher percentage of spores in the inoculum has been reported to result in the long lag phase of the fermentation profile (Meyrath

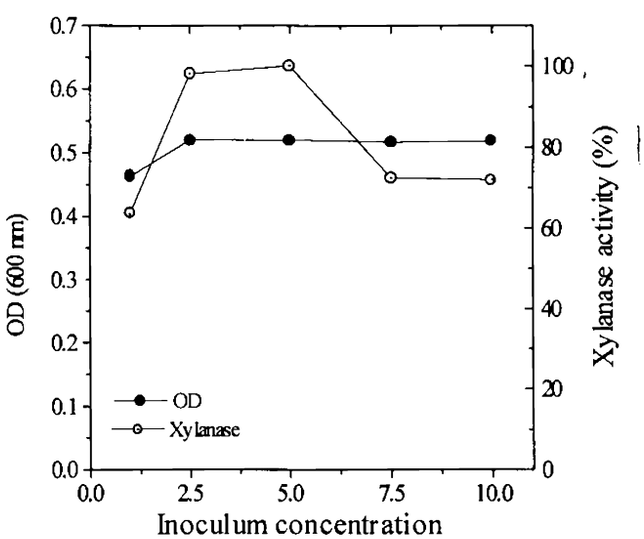


Fig. 16 Effect of inoculum concentration on growth and xylanase production by *Bacillus* SSP-34.

and Suchanek, 1972). The effect of inoculum concentration on the production of xylanases by *Bacillus* SSP-34 was studied at levels of 1%, 2.5%, 5%, 7.5% 10% respectively and concentrations above 10% were not preferable in industrial fermentation (Lincoln, 1960).

Except for 1%, biomass production remained more or less similar at most of the concentrations (Fig. 16) and at 1% concentration lower levels of xylanase production resulted. Highest xylanase production was effected by 5% inoculum concentration which was closely followed by 2.5% (with 98% of xylanase activity of 5% inoculum concentration) while 7.5% and 10% inocula produced a xylanase activity of 72%. There were many reports regarding the higher production of hydrolases from *Bacillus* spp. at lower inoculum concentrations (Lincoln, 1960; Sen and Satyanarayana, 1993). In the present study, it was noticed that 1% inoculum was not efficient in enzyme production which could be due to low cell density. Higher concentrations of 7.5% and 10% might have transferred the compounds which would have affected the higher production of xylanases (Lincoln, 1960) (Fig. 16).

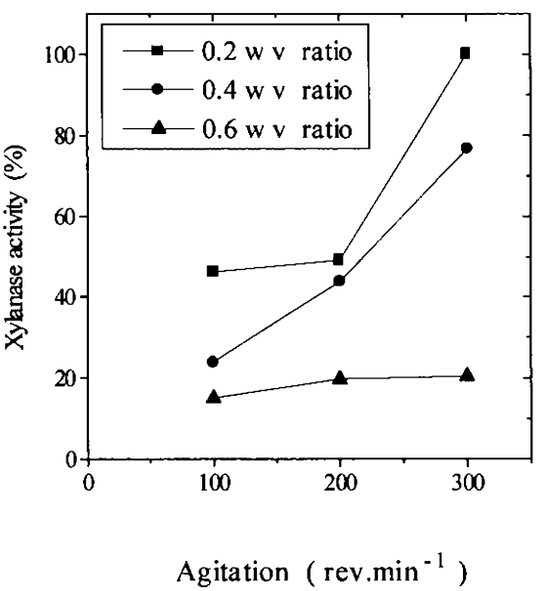


Fig.17. Effect of agitation (100, 200 and 300 rev.min^{-1})) and working volume ratio (0.2, 0.4 and 0.6) on xylanase production by *Bacillus* SSP-34.

3.2.4. Effect of agitation and aeration

The process of agitation and aeration is generally used to meet the demand of oxygen during fermentation process.

An important aspect of aeration in the fermentation process is the resistance offered to the transfer of dissolved oxygen through the medium into the microbial cell, which was generally overcome by increased agitation (Bartholomew *et al.*, 1950). The transfer of oxygen from air to solution formed the second factor; which could be solved by modelling the working volume ratios. Providing large area of gas-liquid inter-phase could increase the rate of formation of dissolved oxygen in the fermentation broth and a number of means for obtaining large surface areas have been tried which include: (i) thin layer cultures (maintenance of restricted volume of liquid in the fermentation vessel), (ii) agitation of the liquid by shaking (reciprocal or rotary shakers) (iii) forced aeration of a liquid with air under pressure through a gas distributor. Of the above, former two could be utilised for a batch experiment in shake flasks. Agitation and aeration experiments were carried out in three separate batch experiments at ambient temperature. The working volume ratios (0.2, 0.4 and 0.6) were studied with different agitation rates at constant temperature (ambient i.e. 30±2). In the first batch, fermentation media with the three wv ratios (0.2, 0.4 and 0.6) were taken in Erlenmeyer flasks and incubated at 100 rpm. In the second batch, the three wv ratios were grown at 200 rpm. Finally, all the three wv ratios were incubated at 300 rpm. Of all the aeration conditions, the cases with 0.2 w/v ratio was found to be having the highest xylanase production followed by 0.4 and 0.6 (Fig.17).

At a working volume ratio of 0.6 all agitation rates (100, 200 and 300 rpm) expressed low levels of xylanase activities, almost equal to each other (Fig.17). This might be due to the fact that irrespective of the agitation, the higher quantities of fermentation volume restricted the two factors regulating the aerating process of the fermentation as discussed earlier. The increased agitation facilitates transfer of dissolved oxygen to the microbial cell as well as transfer of oxygen from air into the medium. Thus, at 0.2 wv ratio and 300

rpm, xylanase production was higher than that of 0.4 wv ratio and 300 rpm (with 76% xylanase production) and in the remaining cases the xylanase production was less than 49%. The later effects might have been due to the low levels of oxygen supply, which would have negatively influenced the enzyme production. The adverse effect of reduced oxygen supply on the enzyme production has been reported earlier (Bartholomew *et al.*, 1950; Palma *et al.*, 1996).

3.2.5. Comparison of xylanases production in the optimised cultural condition with non optimised medium

Bacillus SSP-34 produced more than 100.72 IU/ml of xylanase activity, which was higher than most of the reported cases (Subramaniyan *et al.*, 1997) and traces of cellulases were secreted by the culture (Section 3.1). Optimisation studies on overproduction of the xylanases by the bacterium enabled the selection of favourable parameters. Even though optimum growth occurred at initial medium pH of 9.0, initial medium pH of 8.5 was selected as the latter favoured highest xylanase production. The optimum temperature of 35°C and an inoculum concentration of 5.0% were suggested for the enhanced production of xylanases. 300 rpm agitation and a working volume ratio of 0.2

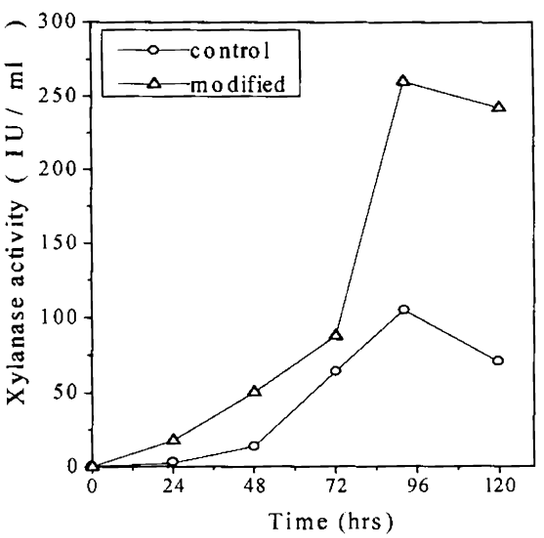


Fig. 18 Xylanase production in control and modified cultural conditions by *Bacillus* SSP-34.

were found to be the optimal agitation and aeration conditions. The above combination of parameters were compiled and a comparative study was carried out against a control i.e. the basal medium with an initial pH of 7 at room temperature ($30\pm2^{o}C$) using 10% inoculum concentration and 0.4 wv ratio with 120 rpm as agitation rates (Horikoshi, 1991a), the conditions used for the final screening of potent cultures. It was found that the modified condition practically resulted in 2.5 fold higher production of xylanase (260 IU/ml) than that the control (Fig. 18). The alkalophilic thermo-tolerant *Bacillus* SSP-34 with its higher productivity of xylanases, which had significant properties would be a promising alternative in the present scenario of biobleaching of Kraft pulps. The favoured working volume ratio (0.2) for higher xylanase production could be used for enhanced production of hydrolases from *Bacillus* spp.

3.3. Optimisation of Nutritional Parameters

By careful manipulation of the growth environments, overproduction of metabolites in microorganisms could be elicited. For rapid growth during fermentation process, the media should contain sufficient and essential nutrient sources such as carbon, nitrogen, phosphate, trace elements and other specific growth factors.

3.3.1. Effect of Carbon source on Xylanase Production

The rate of assimilation of a carbon source could often influence the formation of biomass and production of primary or secondary metabolites. Rapid growth resulted from high concentrations of readily metabolised sugars were often associated with low productivity of metabolites (Inamine *et al.*, 1969). Another important point to be considered during the selection might be that a single carbohydrate material may act as both carbon and energy source.

The main product of a fermentation process would often determine the choice of carbon source and in the present study, xylan, xylose or xylan containing materials were taken for xylanase production. However, a search for other substrates is necessary which would induce the production of xylanases from the microorganisms, leading to efficient processes.

Optimisation experiments were carried out in the basal medium. Different carbon sources at a concentration of 1% were added to the basal medium replacing xylan. The carbon sources selected for the study were as given in Section 2.3.1.

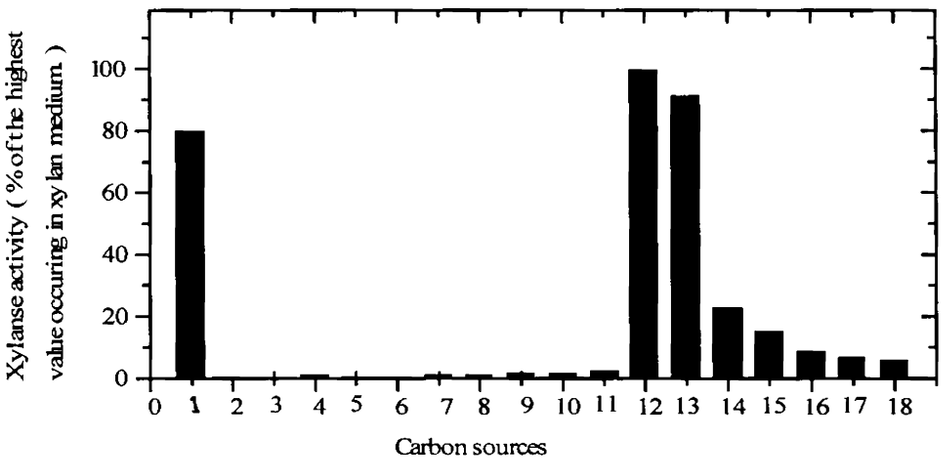


Fig. 19. Effect of different carbon sources on xylanase production by *Bacillus* SSP-34. The culture was grown with different carbon sources and the maximum enzyme production occurred in each case was compared with that of xylan, the most effective carbon source. The carbon sources studied are:

1. Xylose
2. Glucose
3. Fructose
4. Galactose
5. Sorbitol
6. Sucrose
7. Lactose
8. Maltose
9. Starch
10. CMC
11. Inulin
12. Xylan
13. Wheat bran
14. Rice straw
15. Bagasse
16. Rice bran
17. Coir
18. Rice husk

The carbon sources were screened initially for their suitability to promote culture growth and high xylanase yields. They had varying effects on the xylanase production by *Bacillus* SSP-34. The culture produced xylanases by assimilating all of the lignocellulosic materials (Fig. 19). Monosaccharides except xylose, disaccharides, starch, CMC, inulin, etc. resulted in very low

production of xylanases. Results shown in the fig. 19 indicated that xylan was the optimum carbon source inducing the highest level of xylanase production.

Xylanase production by wheat bran and xylose media showed 91% and 80% respectively of that in xylan medium. The xylanase production in rice straw (23%) and bagasse (15%) were much lower when compared to the xylan-containing medium. (Fig. 19)

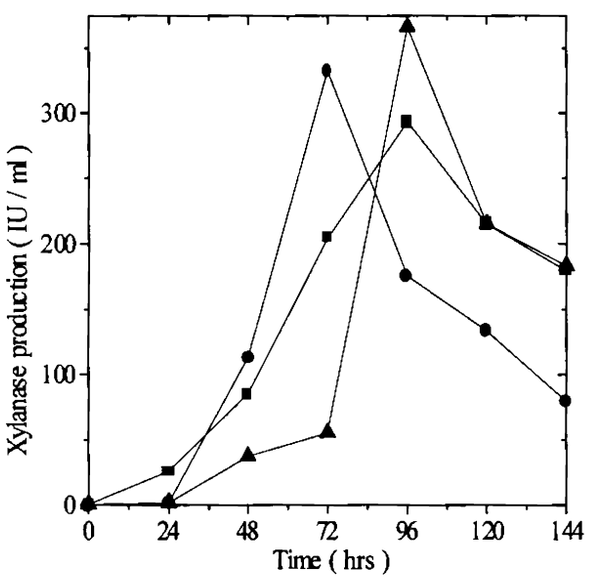


Fig. 20 Xylanase production by *Bacillus* SSP-34 in different media (1%). ▲ Xylan medium ■ Xylose medium ● Wheat bran medium.
Xylan resulted in the highest production followed by wheat bran (91%) and xylose (80%). Hour of maximum production is 96 hrs except for wheat bran (72 hrs).

. Similar to xylan, xylose (80% of xylanase production in xylan medium) also induced production of xylanases. There are ample reports regarding the inducing effect of xylan and xylose on xylanase production (Table 7). During the stationary phase of the growth of *Bacillus* SSP-34, the availability of inducers and the lack of other sugars or carbon sources, ensued the elevated xylanase production. However, this is not the case when the cell death phase commences after the depletion of substrates. At this stage lack of xylan molecules depletes the cells of energy source and the biomass production decreases. However, there is the turnover process for energy by degrading the molecules like protein, RNA, cell wall, lipid and finally DNA. Therefore the cessation of synthesis of these molecules is not accompanied by the stoppage

of metabolic turnover resulting in the net degradation of the molecules starting with RNA, then protein and so on (Daves, 1976). This fact is clearly illustrated by the decrease in the xylanase activity in the culture supernatant from 120 hrs onwards (Fig. 20).

One of the complex lignocellulosic materials such as wheat bran showed 91% of xylanase activity shown by xylan medium within 72 hours (Fig. 20). Similar reports regarding higher production of xylanases from lignocellulosics have appeared earlier in many organisms like *Bacillus thermoalkalophilus* utilising bagasse (Rajaram and Varma, 1990), *Streptomyces chattanoogensis* UAH 23 (Fernandez *et al.*, 1995) and *Streptomyces* sp. (Vyas *et al.*, 1990) utilising wheat bran, *Thermomonospora curvata* using bagasse (Stutzenberger, 1994), *Trichoderma reesei* using wheat bran (Bailey *et al.*, 1993) and *Melanocarpus albomyces* IIS-68 consuming wheat bran (Saraswat and Bisaria, 1997). The period of maximum xylanase activity occurred at 48 hrs for fructose, maltose, rice straw and coir while it was 72 hrs for sorbitol, starch and wheat bran. Other carbon sources showed maximum activity at 96-102 hrs of growth. The protein concentrations in all these fermentations never showed drastic fluctuations. Depletion of reducing sugars during the course of time was recorded along with increase in enzyme levels.

Similar to *Bacillus thermoalkalophilus* (Rajaram and Varma, 1990), *Bacillus* SSP-34 also failed to induce xylanase production in the presence of carboxymethyl cellulose. The possible small quantity of xylan residues present as contaminants in the commercial preparations of celluloses (Fig.12) failed to induce enzyme production as the sugars formed by the constitutive xylanases were consumed by the organism rather than rendering them as inducers. Similar to CMC, other polysaccharides, starch and inulin showed negative results. All sugars except xylose failed to induce xylanases (Fig. 19).

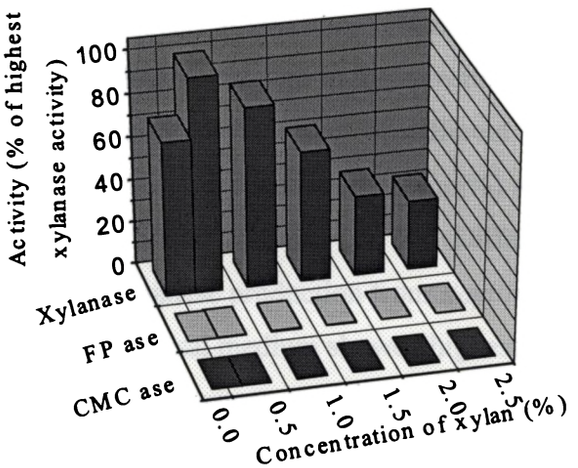


Fig. 21 Production of xylanases and cellulases in different levels of xylan, the optimal carbon source. The highest activities from the fermentation profile of *Bacillus* SSP-34 were considered here. Xylanase at 0.5% conc. is the maximum (100%).

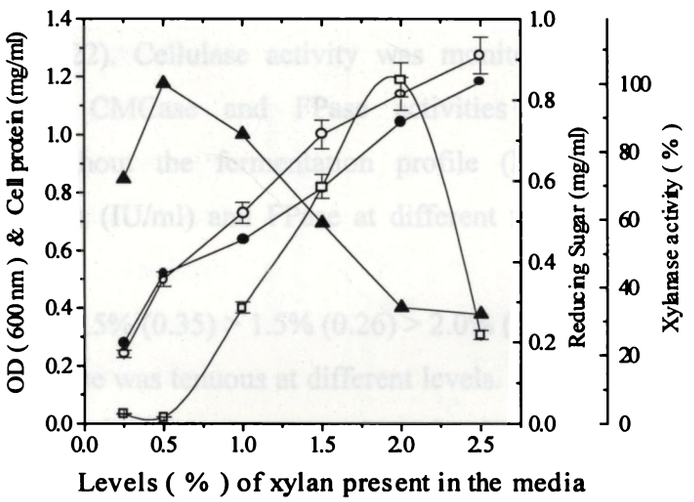


Fig.22 Effect of different levels of xylan (%) on the growth profile of *Bacillus* SSP-34. Optical density and cell protein (after 24 hrs) were used for comparing growth. The reducing sugar concentration and xylanase production in each media after 96 hrs were analysed which showed that the least presence of RS can trigger better xylanase production. Symbols used are (●) OD (600 nm), (▲) xylanase (%), (○) cell protein (mg/ml) and (□) reducing sugar mg/ml.

3.3.1.1. Effect of different levels of xylan on xylanase production

Optimisation experiments resulted in the selection of xylan as the best carbon source. In order to study the effect of different levels of xylan on xylanase production, the following concentrations (gm%) were tried.

(i) 0.25, (ii) 0.5, (iii) 1.0, (iv) 1.5, (v) 2.0, (vi) 2.5. Different levels of xylan were clearly affecting the xylanase production from *Bacillus* SSP-34 (Fig. 21). 0.5% xylan present as in the basal medium showed highest enzyme production followed by 1% (with approximately 84% of that at 0.5%). Higher concentrations gradually declined xylanase yield (59%, 39% and ~ 32% for 1.5%, 2% and 2.5% xylan media respectively). Reduced synthesis of the enzymes in high concentrations of xylan may be due to the accumulation of high concentration of reducing sugars (Gomes *et al.*, 1994). This was clearly supported by increasing concentration of reducing sugars occurring even after 96 hrs of growth of *Bacillus* SSP-34 in the media with higher xylan concentrations (Fig. 22). Cellulase activity was monitored simultaneously which showed that CMCase and FPase activities were low in all concentrations throughout the fermentation profile (Fig. 21). Order of magnitude of CMCase (IU/ml) and FPase at different xylan concentrations are:

CMCase 1% (0.51) > 0.5% (0.35) > 1.5% (0.26) > 2.0% (0.0) and 2.5% (0).

The production of FPase was tenuous at different levels. There is reduction of the tenuous levels to zero as the concentration of xylan decreases resulting from the action of xylanases present in the inoculum (Fig. 21).

Growth pattern of *Bacillus* SSP-34 (Fig.22) at different levels of xylan showed the maximum growth at 2.5% xylan and the minimum at 0.25 %. The magnitude of influence is 2.5% > 2% > 1.5% > 1.0 > 0.5% > 0.25%. However, the highest xylanase production was effected in lower levels of xylan showing the relationship between growth and enzyme production. If the

medium supports fast and higher growth there is little need for xylanase production. If the growth is limited by the medium, the culture will produce xylanase at higher levels to meet the needs of the bacteria. In all the levels, soluble protein and pH showed no drastic fluctuation.

3.3.1.2. Induction of xylanase from *Bacillus* SSP-34

In order to study the influence of catabolites including glucose on enzyme production, *Bacillus* SSP-34 was grown in media containing (1) glucose (2) xylan, (3) xylan+glucose (4) xylose (5) xylose + glucose and (6) xylan + xylose. Analyses carried out include the estimation of OD, cell protein, RNA concentration, reducing sugar, xylanase, soluble protein and cellulolytic activities.

Even though most of the monosaccharides failed to induce xylanase production by *Bacillus*

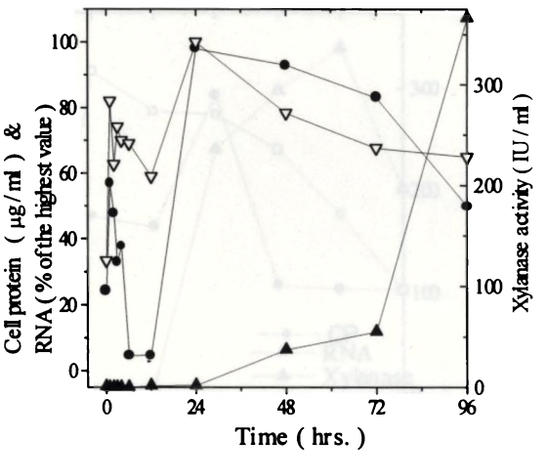


Fig. 23 Xylanase production profile in 1 % xylan medium. Enzyme activity was measured in cell free culture supernatant as indicated in the materials and methods. RNA and cell protein were estimated as in the materials and methods. ▲ Xylanase activity, ● Cell protein, ∇ RNA.

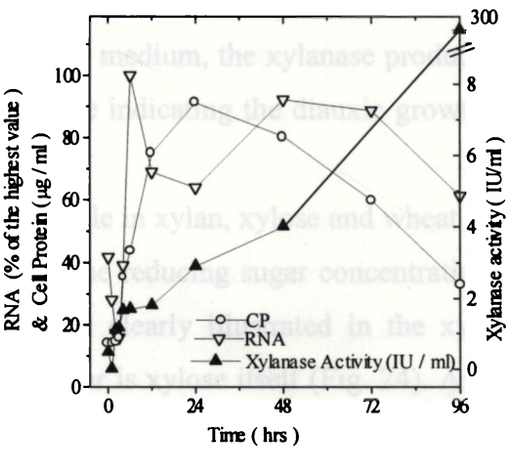


Fig. 24 Induction of xylanases by the 1 % xylose medium. The cell growth indicated by cell protein and RNA concentrations decrease after 48 hrs. The xylanase production in the later period is high. RNA and cell protein were estimated as in the materials and methods.

SSP-34, xylose stood as a distinct exception (Fig 19, 24). Similar strong induction by the xylose has been reported earlier (Table 7).

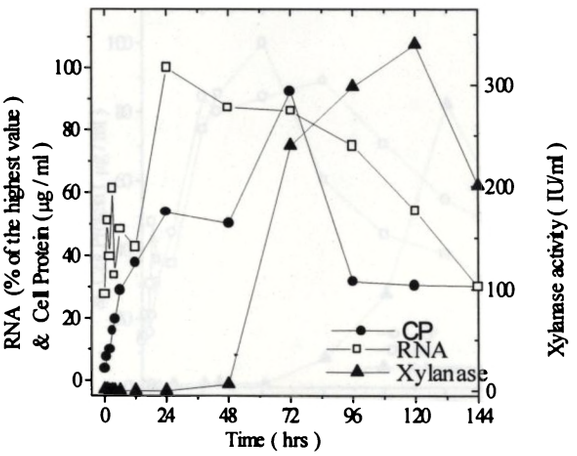


Fig. 25 The production of xylanase by *Bacillus* SSP-34 in medium containing 0.5 % xylan and 0.5% glucose. The maximum production delayed and there is the indication of diauxic growth. RNA and cell protein were estimated as in the materials and methods.

From the results, it is clear that the xylan and xylose were good inducers (Fig. 23, 24). However, when xylan was present along with second carbon source, xylose, there is slight decrease in xylanase production (Fig. 27). In the glucose + xylan medium, the xylanase production occurred only after the depletion of glucose indicating the diauxic growth or catabolite repression (Fig.25).

By comparing the fermentation profile in xylan, xylose and wheat bran media it was evident that the decrease in the reducing sugar concentration is the key factor for enzyme induction. It is clearly illustrated in the xylose medium where the prominent reducing sugar is xylose itself (Fig. 24). As the reducing sugar decreased to a minimum level after 24 hrs of active growth, the xylanase production started reaching its peak value. This indicates that xylanase production occurs at lower level of xylose. Kyu *et al.* (1994) earlier reported similar observation using *Bacillus circulans* grown in the xylan medium supplemented with xylose. There are other reports regarding the delayed production of xylanases by the xylose induction metabolism in the case of other *Bacillus* spp. (Lopez *et al.*, 1998; Okada and Shinmyo, 1988).

The role of xylose in various microorganisms is not consistent. It can act both as an inducer and a repressor (Table 7) of enzyme production. Nakanshi and Yasui (1980a, b), Gomes *et al.* (1994) and Purkarthofer and Steiner (1995) reported an interesting feature in the xylanase induction from *Streptomyces* sp., *Thermoascus aurantiacus* and *Thermomyces lanuginosus* respectively (Table 7). In the first two cases, the inducers were xylan and β-methyl xylopyranoside (BMX) while in the latter it was induced by xylan and xylose. All the three cultures required longer availability of inducer molecules, that is the continuous synthesis of xylanase required the presence of an appropriate external inducer concentration. When the organism is separated from the inducing culture medium, the xylanase production comes to a halt. The above factors may be probable reasons for the delayed maximum production of xylanase from *Bacillus* SSP-34 (ie. 102 hrs) and other microorganisms [*Bacillus* sp. strain 41 M-1 – Nakamura *et al.* (1993b); *Bacillus* sp BP-7 – Lopez *et al.* (1998); *Bacillus subtilus* CD-4 - Srivastava and Srivastava (1993) and *Bacillus thermoalkalophilus* - Rajaram and Varma (1990); (Table 7)].

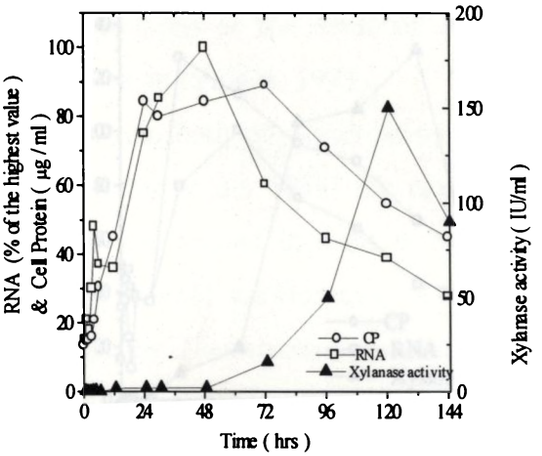


Fig. 26 Growth of *Bacillus* SSP-34 in the medium supplemented with 0.5 % xylose and 0.5 % glucose. The xylanase activity, RNA and cell protein were estimated as in the materials and methods.

Xylanase activity resulting from the constitutive production results in the slow liberation of xylooligomers from xylan and xylanase formation is extended. This leads to delay in higher or maximum level of xylanase production. Purkarthofer and Steiner (1995) observed pronounced xylanase

production by the fungus *Thermomyces lanuginosus* when xylose was added to the culture medium in several doses. This was in contrast to the lesser amounts of enzyme production obtained when the same amount was added in total in the beginning of fermentation.

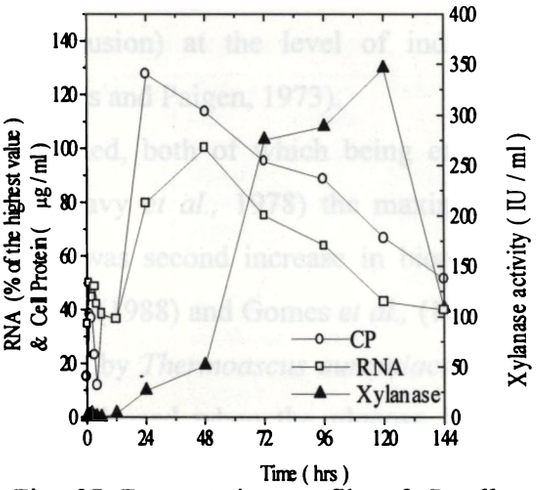


Fig. 27 Fermentation profile of *Bacillus* SSP-34 in medium containing 0.5 % xylan and 0.5 % xylose as the carbon source. Xylanase activity, RNA and cell protein were estimated as in the methodology.

Use of xylan and xylose, as carbon source in the medium resulted in the induction of xylanase production and maximum production occurred at 96 hrs hour of growth while the maximum biomass production occurred after 24 hrs. However, when glucose was mixed with xylan and xylose there was the preferential use of carbon sources i.e. glucose was used first resulting in a diauxic growth (Fig.25, 26). In the xylan + glucose medium (Fig. 25) the concentration of RNA is also showing double growth maximum, first one corresponding to the glucose. Glucose is the most preferred substrate than any other carbon source (Moat and Foster, 1995 b).

There was an indication of repression of xylanase production by glucose. There are several reports regarding the negative action of glucose on the xylanase synthesis (Nakanishi and Yasui, 1980b; Esteban *et al.,* 1982; Marui *et al.,* 1985; Nakamura *et al.,* 1993a; Gomes *et al.,* 1994; Purkarthofer and Steiner; 1995; Flores *et al.,* 1996; Saraswat and Bisaria, 1997; Siedenberg *et al.*, 1997; Lopez *et al.,* 1998). Glucose executes this negative effect through both catabolite repression at the transcriptional level (Saier and Fagan, 1992)

and by catabolite inhibition (inducer exclusion) at the level of inducer transport across the cell membrane (McGinnes and Paigen, 1973).

When glucose and xylose were mixed, both of which being easily transportable sugars, (Kornberg, 1976; Silhavy *et al.,* 1978) the maximum growth occurred after 12^{th} hour and there was second increase in biomass indicating the diauxic growth. Feldman *et al.,* (1988) and Gomes *et al.,* (1994) reported the repression of xylanase synthesis by *Thermoascus aurantiacus* in the inducing culture medium, which was released when the glucose level decreased. They are of the opinion that glucose caused only transient repression of enzyme synthesis in those organisms. Gomes *et al.* (1994) confirmed the above hypothesis using different concentrations of cAMP added concomitantly with glucose, which failed to relieve the repression of xylanase synthesis caused by glucose.

One interesting fact noted in the present study was that in xylan medium supplemented with xylose, xylanase production was comparable with that in media containing the individual components (Fig.27). This illustrates that xylose is not a repressor for xylanase production from *Bacillus* SSP-34.

In all the above cases, there was no indication of cellulase production, which confirms that *Bacillus* SSP-34 xylanase is cellulase free. From the observations made in the present study it could be concluded that xylan and xylose are good inducers of xylanase production, while glucose is not. Addition of xylose did not stop the xylanase production from the xylan medium, while glucose did so during the early hours of growth. However, the decrease in level of glucose after some period may reverse the xylanase production.

Table 7. Inducers and repressors of xylanase production in reported cases.

Sl. No.	Microorganism	Xylanase inducing Nutritional sources	Carbon sources that repress strongly	References
1	*Bacillus* sp. BP-7	Xylose, xylan	Glucose	Lopez *et al.* (1998)
2	*Micrococcus* sp. AR-135	Xylose, xylan	Glucose and other sugars.	Gessesse, and Mamo (1998)
3	*Bacillus subtilis* CD-4	Xylan Xylose	-nd- [a]	Srivastava and Srivastava (1993)
4	*Clostridium stercorarium*	Xylan, cellulose	Xylose, glucose, cellobiose and lactose	Berenger *et al.* (1985)
5	*Pseudomonas fluorescens* subsp. *cellulosa*	Xylan	Glucose	Gilbert *et al.* (1988)
6	*Trichosporon cutaneum* SL409	Xylan, wheat bran, xylose	Glucose, sucrose, other sugrs	Liu *et al.* (1998)
7	*Bacillus thermoalkalophilus*	Bagasse, rice husk Xylan	Xylose	Rajaram and Varma (1990)
8	*Bacillus* sp. Strain 41 M-1	Xylan	Glucose, xylose Arabinose	Nakamura *et al.* (1993a)
9	*Bacillus circulans* WL-12	Xylan Xylose	Glucose	Esteban *et al.* (1982)
10	*Bacillus stearothermophilus* T-6	Xylan, xylose	Glucose +sugars	Gat *et al.* (1994)
11	*Bacillus* sp.	Xylan	Glucose, xylose	Uchino and Nakane (1981)
12	*Cellulomonas uda*	Xylan, xylotriose	Glucose, xylose	Rapp and Wagner (1986)
13	*Streptomyces* sp. No.3137	Xylan, methyl β-xyloside	Glucose [b]	Marui *et al.* (1985)
14	*Streptomyces* sp.	Xylan, BMX	Glucose	Nakanishi and Yasui (1980a,b)
15	*Streptomyces* sp. CH-M-1035	Xylan, D-xylose, D-Arabinose, Sucrose	Glucose, glycerol, Succinic acid	Flores *et al.* (1996)
16	*Thermoascus aurantiacus*	Xylan, methyl-β-D-xylo pyranoside	Glucose, arabinose Lactose, xylose	Gomes *et al.* (1994)
17	*Aspergillus awamori*	Xylan	Glucose	Siedenberg *et al.*(1997)
18	*Melanocarpus albomyces* IIS-68	Xylan, wheat bran, xylose, solka floc, methyl β-xyloside	Glucose, cycloheximide	Saraswat and Bisaria (1997)
19	*Aspergillus tamarii*	Xylan methyl-β-D-xylose	Glucose, cyclodeximide	Simao *et al.* (1997)
20	*Thermomyces lanuginosus*	Xylan,Xylose, lyxose, ribose arabinose	Glucose	Purkarthofer and Steiner (1995)

nd = not detected

a. Even hough the glucose did not enhanced the xylanase production, the repression is absent i.e. about 46% of that of Xylose medium
b. Only used in the proliferating medium

3.3.2.Effect of Nitrogen source on Xylanase Production

Lignocelluloses in nature are degraded by microorganisms with their different types of cell wall hydrolysing enzymes. Enzyme production from an organism could be enhanced by the manipulation of its nutritional requirements and similar to carbon sources, nitrogen sources also have major role in microbial growth and metabolism. There are several reports regarding the selection of nitrogen sources for maximum xylanase expressions from bacteria including *Bacillus* spp. (Gomes *et al.*, 1993; Purkarthofer *et al.*, 1993; Christakopoulos *et al.*, 1996; Kuhad *et al.*, 1998). However, none were giving

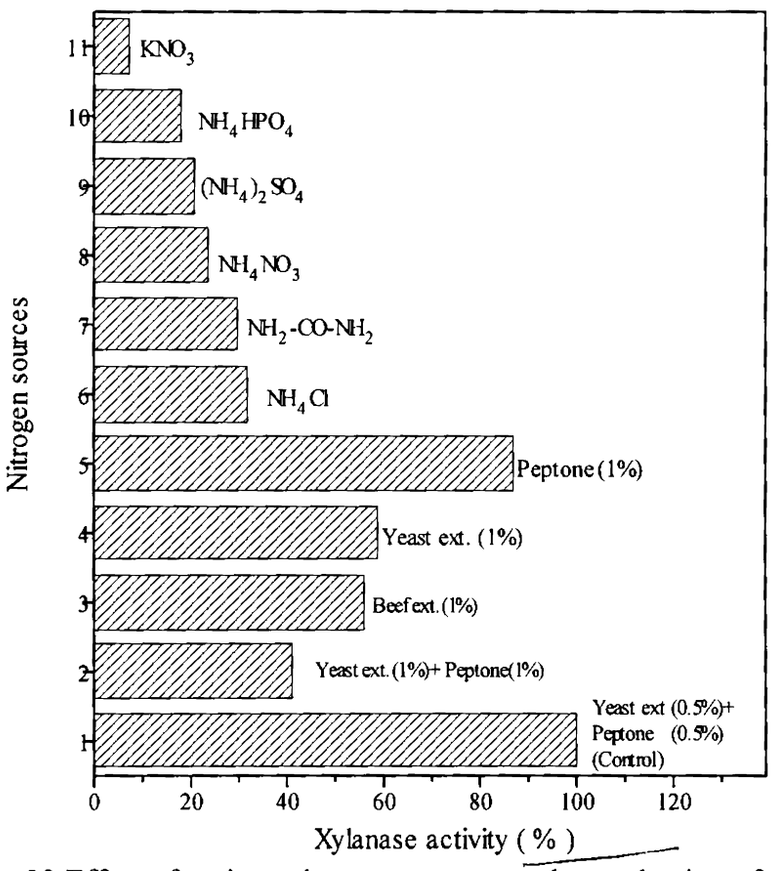


Fig. 28 Effect of various nitrogen sources on the production of xylanase by *Bacillus* SSP-34 grown in shake flasks. Maximum enzyme production occurring during the fermentation period by different nitrogen sources were compared with that of control.

much importance to the proteolytic inductive property of the selected nitrogen source. In the present study the effect of nitrogen source on the production of xylanases by *Bacillus* SSP-34 in comparison to protease production was analysed. Studies on the effect of nitrogen sources on xylanase production along with their influence on proteases were carried out using basal medium II, replacing peptone and yeast extract with different nitrogen sources including complex, organic and inorganic ones (Section 2.3.2). The results are given in

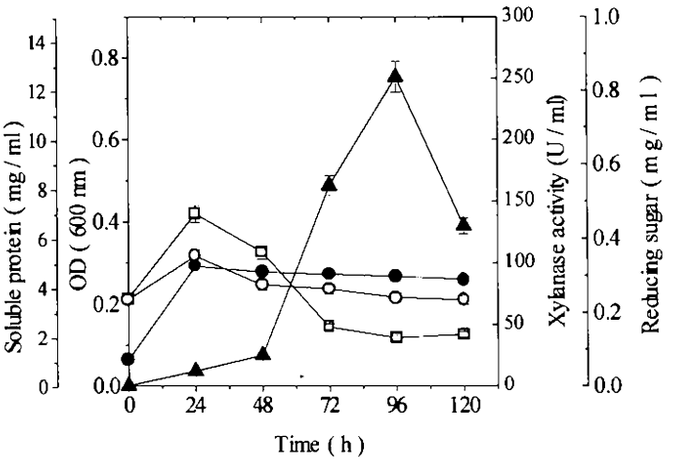


Fig. 29 Fermentative production of xylanase by *Bacillus* SSP-34 on control medium with 0.5 % yeast extract and 0.5 % peptone (1 % final conc.). Time cource of xylanase production was compared with growth profile. The cultures were grown for a period of 120 hours and samples were taken every 24 hours. ●-Optical density at 600 nm, ▲-Xylanase activity, O-Soluble protein, □-Reducing Sugar.

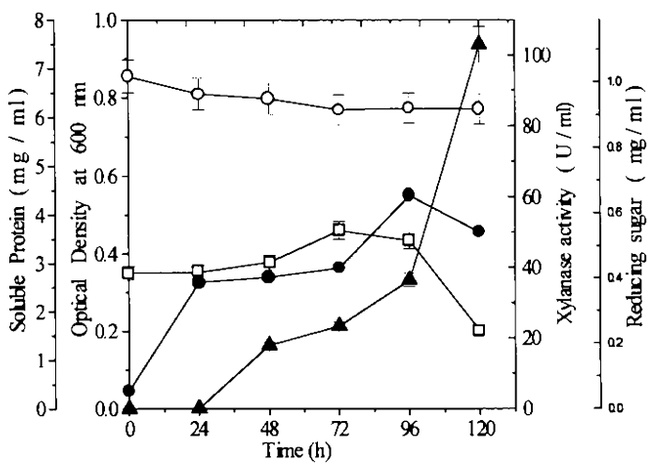


Fig. 30 Xylanase production in the medium containing 1% peptone and 1% yeast extract as nitrogen source. Fermentations were carried out for a period of 120 hrs and samples were taken at 24 hrs interval. ●-Optical density at 600 nm, ▲-Xylanase activity, O-Soluble protein, □-Reducing Sugar.

Fig. 28. The highest enzyme production (251±0.05 IU/ml) was observed in control with nitrogen source of 0.5% peptone and 0.5% yeast extract. When yeast extract and peptone were tried separately at 1 % concentration, 60 and 87% production of xylanases was expressed respectively in comparison to the control (Fig. 28).

Peptones, the intermediate products of protein hydrolysis by enzymes are composed of proteoses, peptones, peptides and amino acids. They usually have 10-15% of total nitrogen while the available α- amino nitrogen is approximately 2% (Cowan, 1983). The important function of peptone is to furnish an available source of nitrogen. Yeast extract containing approximately 37-44% of protein content (Kelly, 1983) is also used as a nutrient in natural and semisynthetic media. In addition to proteins, it also has approximately 650 μg / g yeast extract of vitamin B contents, 10-11% NaCl , 6-17 % carbohydrates, amino acids and nucleic acids (Kelly, 1983; Shin *et al.,* 1997). Even though both are having growth stimulatory components, when compiled together they result in higher levels of enzyme production in *Bacillus* SSP-34 possibly due to the compensation effect by both the nutrients. Stokes (1944) has suggested that the growth factor deficiencies in culture media could be remedied by proper combination of ingredients like peptone and yeast extract. However, a pure mixture of amino acids as a source of nitrogen was not satisfactory for the growth of all bacteria even though the culture medium was optimum in all respects (Daniels, 1966). Contrary to the medium containing yeast extract and peptone at 1% final concentration, the 2% level resulted in a shift in the period of maximum enzyme production towards 120 hours. Here the growth involved two phases similar to diauxic growth (Fig.30). This was in correlation to the presence of fermentable carbohydrates in yeast extract (Kelly, 1983), sufficient concentration of which resulted in the delayed usage of xylan (Fig. 30). The

microorganisms degraded xylan only in the absence of other simpler carbohydrates like sugars, oligomers and other carbon containing organic compounds, which could afford the growth. This might have risen from the general catabolic repression pattern, and most organisms regulate xylanase synthesis as follows. The constitutive production of xylanase resulted in the production of monomers and oligomers, which in turn caused the induction of xylanases. But in a medium with high concentration of monomers, the polymerase production is delayed. The growth pattern also showed the two sigmoid curves of diauxic growth.

Other nitrogen sources [beef extract, NH_4Cl, urea, NH_4NO_3, $(NH_4)_2SO_4$, NH_4HPO_4 and KNO_3 all at 1.0% concentration] were producing xylanase activities lesser than 60% as compared to the control (Fig. 28). Use of complex nitrogen sources like peptone and yeast extract lead to rise in pH due to the release of excess nitrogen as ammonia (Forage *et al.*, 1985), consequently the presence of ammonium ions stimulate growth rate. This can be an explanation for higher enzyme production in the case of a mixture of yeast extract and peptone. Similar observation was also made in *Bacillus* SSP-34. Lounes *et al.* also reported the extraction of ammonium ions in the bacterium *Streptomyces ambofaciens* while using amino acids as the nitrogen source. According to them the catabolic pathways of the amino acids like valine and isoleusine were repressed in the presence of ammonium ions. Both urea and ammonia produced approximately 30% of xylanase production within 24 hours of growth in comparison to the control (Fig. 28). Use of urea as the nitrogen source resulted in raising the initial culture pH to 9.0. According to Forage *et al.* when urea was used as the nitrogen source there is the formation of ammonia and CO_2 causing initial rise in pH, which gradually falls as the ammonia was utilised (Forage *et al.*, 1985). Microorganisms usually produced sufficient urease so that the urea added to

the nutrient medium would be cleaved completely and ammonia appears (Schlegel, 1987). KNO_3 resulted in the least production of xylanase (less than 10%), which is far different from other NH_4^+ ion forming media (i.e. urea, NH_4Cl and complex nitrogen sources) (Fig.28.). The reason might be due to the low efficiency of KNO_3 to form biomass, unlike NH_4^+ ion containing media. The amount of cell material that can be formed per mol of ATP is much lower with NO_3^- as the inorganic nitrogen source when compared to NH_4^+ (Southamer, 1979). Therefore the nature of the nitrogen source in the medium has considerable influence on the ATP requirement for the biomass formation.

3.3.2.1. Effect of different levels of optimum nitrogen source on xylanase production

The best nitrogen source for xylanase production by *Bacillus* SSP-34 was a combination of yeast extract (YE) and peptone (P). This combination was tried at different levels in the basal medium. Maximum xylanase production occurred with the concentration of 0.5% (0.25% YE + 0.25% P) with an activity of 379±0.2 IU/ml (Fig. 31), followed by 1.0%, 0.1% and 0.2% having the xylanase activities of 251±0.05 IU/ml 220±0.54 IU/ml and 208±0.3 IU/ml respectively. However other concentrations failed to produce higher enzyme yields and were less than 45% of the highest activity noted in media with 0.5% nitrogen source (Fig. 31). This was in contrast to the growth pattern in different nitrogen sources as evidenced by the absorption at 600 nm and cell protein estimation. The highest growth occurred at highest nitrogen level (4% i.e. 2% YE + 2%P) while the lower levels of nitrogen sources like 0.1 to 0.5% resulted in comparatively lower growth rate. The presence of higher amount of complex nitrogen source for a carbohydrate hydrolysing enzyme like xylanase could result in faster growth of the bacterium suggesting that the medium enhanced the growth rate than the enzyme production in the

initial level due to the presence of simple sugars (Kelly, 1983). The availability of non-polymeric carbohydrates from the complex nitrogen source like yeast extract (Kelly, 1983) increases with increase in total concentration thus the need for polysaccharase is delayed. The pH pattern of the culture also exhibited similar effect. Even though different nitrogen levels in the media resulted in changing media pH towards alkaline side at the end of fermentation, there was a delay in the pH shift towards alkaline side by higher levels of nitrogen source (Fig. 33). This might have been due to

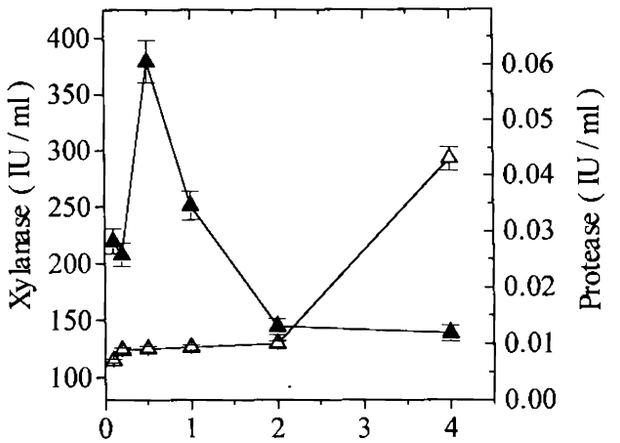


Fig. 31. Production of xylanase at different levels of the selected nitrogen source, (combination of yeast extract and peptone). The final concentration of the combination ranges from 0.1% to 4% (w/ v). xylanase activity (IU/ml) (▲), protease(IU/ml)(△).

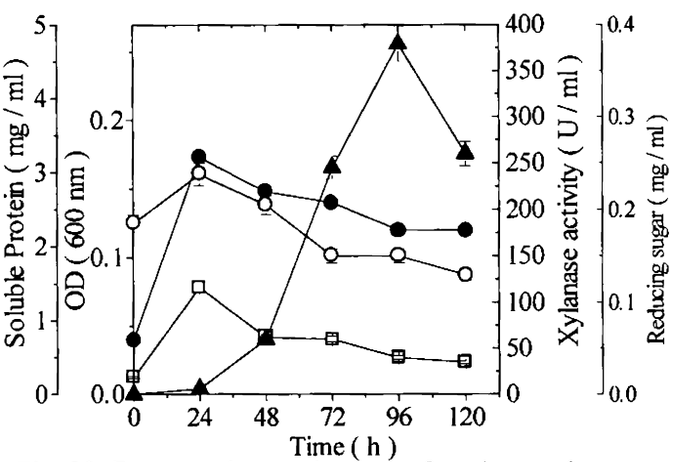


Fig. 32. Fermentative production of xylanase by *Bacillus* SSP-34 in the optimised nitrogen source containing medium with a final concentration of nitrogen source as 0.25% yeast extract and 0.25% of peptone. ●-O D at 600 nm, ▲-Xylanase activity, O-Soluble protein, □-Reducing Sugar.

the positive effect of the simple sugars present in the YE, which could have reduced the pH by providing acids thereby neutralising the liberated ammonia within the medium (Forage *et al.*, 1985). The neutralisation of ammonium ions, which normally maintained the culture medium pH near 9, resulted in the initial lowering of pH values and once the positive effect was removed, the organism started synthesising large quantities of xylanase for hydrolysing xylan. However, by that time there was good growth of bacteria. Wagner *et al.* (1995) have explained that the concentration of yeast extract in the media must not be higher than that required to provide growth factors. The proteolytic activities of the *Bacillus* SSP-34 at different levels of optimum nitrogen source were also evaluated. Most of the nitrogen concentrations resulted in very low levels of proteolytic enzyme production, except at 4% final concentration, which was possibly due to the higher concentration (i.e. 2%) of peptone, a proteolytic inducer (Fig. 31). There are lots of references regarding the induction of proteases by peptone (Moon and Parulekar, 1991).

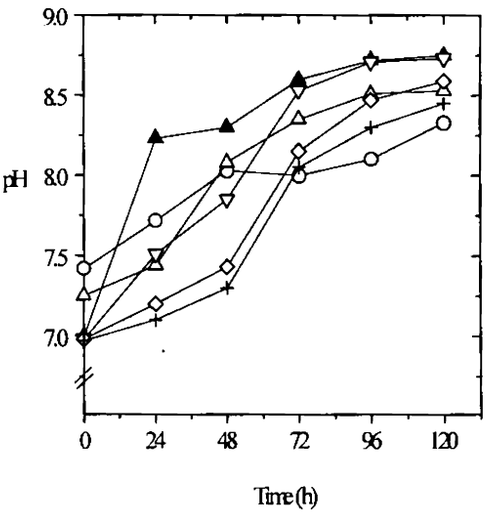


Fig. 33 pH variance at different levels of optimum nitrogen source (combination of yeast extract and peptone) for xylanase production. Symbols used (final concentration (g%) of Yeast extract and peptone) are (○) 0.1 %, (△) 0.2%, (▲) 0.5%, (▽) 1.0%, (◇) 2.0% and (+) 4.0%.

The selected nitrogen source (0.25% YE + 0.25% P) produced only 0.009 IU/ml of protease activity. Order of magnitude of protease activity by different nitrogen sources is 0.1 < 0.2 <0.5 <1.0 < 2.0 < 4.0. Ammonium ions

if not neutralised by the acids of positive effect turn the medium pH towards alkaline side, and exert a negative effect on proteases (Untrau *et al.*, 1994). Thus the lower concentrations reaching alkalinity with out any difficulty (Fig. 33) are well in accordance with low protease production as the ammonium ions, the protease inhibitor, produced from the nitrogen metabolism are not neutralised by acids of positive effect. Period of maximum xylanase production was the same both in control (0.5 YE + 0.5% P) and optimised medium (0.25% + 0.25%) (Figs. 29 and 32). However, initial concentration of reducing sugar was lesser for the 0.25 YE + 0.25 P media than the control. This also indicates the minimum availability of carbohydrates from the lower level of yeast extract.

The above results indicated that during lignocellulosic degradation studies, care should be taken to incorporate the best nitrogen source in its optimum level, which might effect lesser growth or slow growth at a restricted time of a particular organism. This depended on the easily assimilating carbohydrates derived from yeast extract (Kelly, 1983) which should be kept low. The carbon compounds from the yeast extract could interfere the production pattern of enzymes from an organism. The complex nitrogen sources like peptone and yeast extract were having more effect on the xylanase production than the inorganic ones. Kuhad *et al.* (1998) observed similar results using *Fusarium oxysporum*. The optimum enzyme production conditions are desirably coupled with lower production of protease in order to avoid activity loss. Storage of cell-free crude preparation of xylanases will be difficult in the presence of proteases (Whelan and Pembroke, 1989; Pembroke *et al.*, 1992; Balakrishnan *et al.*, 1997). The production of considerable level of protease could lead to the loss of xylanase activity during storage. According to Balakrishnan *et al.* (1997) and Ikura and Horikoshi (1987)

xylanase production could be enhanced by the presence of protease inhibitors like glycine. A combination of yeast extract and peptone was having advantage over other nitrogen sources for the production of polysaccharases. This fermentation leads to the formation of ammonium ions, which being a known protease inhibitor minimises the proteolytic degradation of xylanase formed under inducing conditions. The presence of ammonium ions also helps in the long time storage of xylanases in the absence of proteolytic activity.

3.3.3. Effect of metal ions on xylanase production

Different metal ions are clearly influencing the production of xylanase from *Bacillus* SSP-34. Metal ions are having different roles on the growth of a microorganism. Studies on the influence of different metal ions were carried out using NaCl, KCl, Na_2HPO_4, $MgSO_4$, $MnSO_4$, $CaCl_2$, $CoCl_2$, $FeSO_4$ and $ZnCl_2$. All the metal ions tried at levels as mentioned in methodology gave varying results. Except for cobalt, all the other ions supported growth. The xylanase production was met with drastic reduction with Co^{2+} and unexpectedly at Ca^{2+} also. Co^{2+} was inhibiting both growth and xylanase production. Ca^{2+} did not affect the growth of the organism. Highest xylanase production occurred with Na_2HPO_4 (103 % of xylanase production occurring at control) which was closely followed by $MnSO_4$ (101 %). $CaCl_2$, $FeSO_4$ and $CoCl_2$ were producing low levels of xylanases, all less than 35%. Gomes *et al.* (1994) reported similar results with *Thermoascus aurantiacus* except for KCl which is not inhibitory in the case of *Thermoascus aurantiacus*, but resulted in reduction of xylanase production by *Bacillus* SSP-34. K_2HPO_4, $MgSO_4$, NaCl and $ZnCl_2$ did not inhibit both the biomass and xylanase

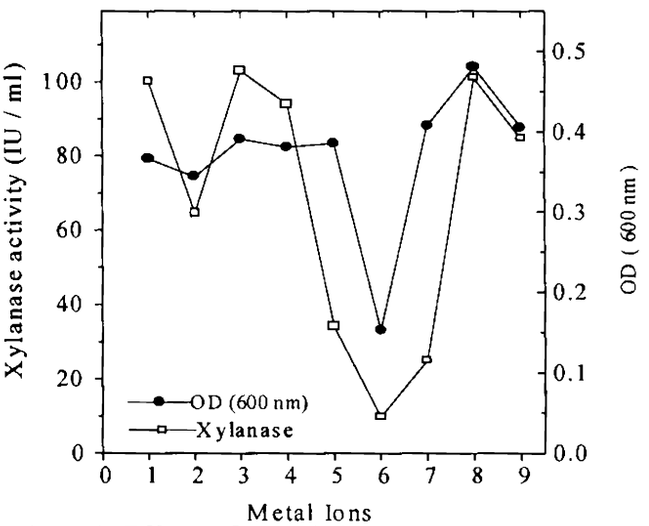


Fig. 34 Effect of metal ions on xylanase production and growth by *Bacillus* SSP – 34

1. Control
2. KCl
3. Na_2HPO_4
4. NaCl
5. $CaCl_2$
6. $CoCl_2$
7. $FeSO_4$
8. $MnSO_4$
9. $ZnCl_2$

production while Na_2HPO_4 and $MnSO_4$ resulted in higher levels of both than the control (Fig. 34).

Metal ions have been classified as hard and soft acids and bases (Pearson, 1973) and Class A, Class B and border line ions (Nieboer and Richardson, 1980). Some class A ions (eg. Mg, Ca, Na) are essential for the growth of microorganisms. Some border line ions (eg. Cu,Fe, Ni, Zn) are required as micronutrients and some class B ions (eg. Hg, Pb) are toxic and not necessary for biological function (Collins and Stotzky, 1996). The effects of metal ions on the surface of prokaryotic cells are influenced by the physiochemical characters like pH. Microorganisms are negatively charged owing to the various structures on their cell surfaces (Collins and Stotzky, 1992). In general, their anionic character arises from carbohydrates or carbonyl moieties and also from peptidoglycan, teichoic acid and teichuronic acids which are reported to be influencing the metal-cell surface interaction in *Bacillus subtilis* (Mc Lean *et al.*, 1996). One possible explanation for metal ion interaction is that the microorganisms may get more energy from the reduction of metal ions [eg. Fe(III) to Fe(II)] than the other sources like SO_4^{2-} to S^{2-} (McLean *et al.*, 1996). Bacteria adopt various methods to cope with the metal ion environment like the plasmid-encoded resistance, which may provide them with efflux and bypass mechanisms. They produce enzymes which catalyse the transformation of metals to variable forms or make the bacterial cell impermeable to the metals (Trevors *et al.*, 1985). The problem regarding the concentration to be used to detect the metal resistant or metal sensitive bacteria is under controversy and standard concentration has not been universally proposed and/or accepted by the scientific community (Trevors *et al.*, 1985). This task was complicated by the various forms of metals used, the effect of media components, pH and culture conditions which

have the capacity to influence the effect of metals and the results by different sources may not be identical (Trevors *et al.*, 1985).

3.3.4. Comparison of xylanase production in the optimised condition with non-optimised medium i.e. Horikoshi Basal medium II

All the optimised cultural and nutritional parameters were added together and comparative studies for xylanase production were made against Horikoshi basal medium discussed in methodology. The cultural conditions changed include initial pH 8.5, growth temperature 35^0 C. 18 hours old 5% inoculum was used for starting fermentative production at 0.2 working volume and the operational agitation was 300 rpm. The optimum conditions resulted in increase of five times i.e. from 100.2 IU/ml occurring in non-optimised medium to 506 IU/ml (Fig. 35). Present study showed that *Bacillus* SSP-34 xylanase activity was one of the highest amount of xylanase produced by bacteria. Ratto *et al.* (1992) reported the higher levels of xylanases ranging up to 400 IU / ml.

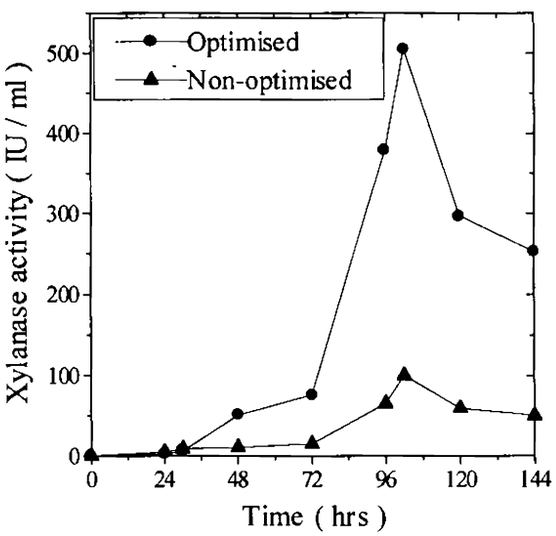


Fig. 35 Comparison of xylanase production in the non-optimised and optimised medium. All the optimal conditions for cultural and nutritional parameters were integrated for the overproduction.

3.4. Purification and Characterisation of Endo-Xylanases

Purification and characterisation of enzymes are important prerequisites for the successful application of enzymes in industries. There were reports dating from 1982 (Estaban *et al.*, 1982) regarding the purification of xylanases from various microorganisms. However, the purification of proteins from *Bacillus* spp. requires special consideration and integration of various approaches. The enzymes purified were characterised and the data could be used in understanding the nature of enzymes and classifying the protein. Cell free culture supernatant (1000 ml) obtained after centrifugation of the culture broth (10000 g, Hitachi Himac CR 20 B2 Centrifuge) served as the crude xylanase preparation with total activity of 419050 units. The specific activity of crude xylanase preparation was 281 units per milligram of protein.

3.4.1. Purification of Endo-xylanases from *Bacillus* SSP-34

3.4.1.1. Concentration of xylanase from *Bacillus* SSP-34 culture broth

The crude xylanase obtained from the culture broth after centrifugation was concentrated prior to the chromatographic separations. Ultrafiltration and ammonium sulphate fractional precipitations were the techniques successively used for concentration of xylanases from *Bacillus* spp. (Table 9).

3.4.1.1.1. Ultrafiltration

The crude preparation was concentrated by ultrafiltration using millipore tangential flow filtration unit with a membrane having molecular weight exclusion limit of 10,000 daltons. The xylanase activity in the ultrafiltered solution (200 ml) was 314290 U with 75% recovery and the purification fold was 1.1. The specific activity was 311 U/mg of protein.

3.4.1.1.2. Ammonium sulphate fractionation

Protein solution concentrated by ultrafiltration was subjected to ammonium sulphate fractional precipitation and unlike the earlier reports with *Bacillus* xylanase purification (Okada and Shinmyo, 1988), the above effected in significant concentration of proteins. The activity could be recovered from the ammonium sulphate fractional range of 30 – 60 % with maximum at 50-60%. There were sufficient reports regarding the inclusion of ammonium sulphate fractionation in the purification procedures: Honda *et al.* (1985a) using *Bacillus* sp. no. C-125; Akiba and Horikoshi (1988)- *Bacillus*; Okada and Shinmyo (1988) – *Bacillus pumilus*; Li *et al.*, (1993) – *Aureobasidium pullulans* Y-2311-1; Sakka *et al.* (1994)- *Clostridium stercorarium*; Kubata *et al.*, (1992) - *Aeromonas caviae*. Similar to the present study, there are other reports regarding the low molecular weight xylanases retrieved within approximately 20 to 60-70 % fractional range (Okada and Shinmyo, 1988; Honda *et al.*, 1985a). The specific activity of the concentrated preparation was 493 U/mg protein with a purification fold of 1.8 and the yield as 44%.

3.4.1.2. Chromatographic separations

3.4.1.2.1. DEAE Sepharose column chromatography

The dialysed enzyme solution was applied to a DEAE Sepharose column. During gradient elution using NaCl (0.0 to 0.5M) xylanase activity appeared in the void volume while most of the other proteins and coloured materials were adsorbed to the gel (Fig.36). Similar kind of elution was reported for other organisms (Okada and Shinmyo, 1988; Yamura *et al.*, 1997; Pembroke *et al.*, 1995). The specific activity of DEAE Sepharose purified xylanase was 1218 units/mg protein and purification fold was 4.33 with the yield as 21.2%. There were four major peaks for other proteins, all being eluted at different molar levels of NaCl (Fig.36). The active fractions were pooled (130 ml) which was then subjected to ammonium sulphate

precipitation for 60% concentration followed by dialysis in the sodium phosphate buffer (0.05 M, pH7) for 20 hours at 4^0C.

3.4.1.2.2. CM Sephadex column chromatography.

The active fractions from DEAE column were pooled, concentrated and then applied to CM Sephadex column that was pre-equilibrated with sodium phosphate buffer (pH 0.05 M). The enzyme was eluted with a linear gradient of NaCl (0.0 to 0.5 M) and xylanase activity occurred within the fractional range of 22-30 at about 0.03-0.125 M NaCl concentration. There were 7 peaks of which 3 major peaks and 3 minor ones were proteins without xylanase activities (Fig. 37). The specific activity was 1451 U/mg protein with a purification fold of 6.13 while

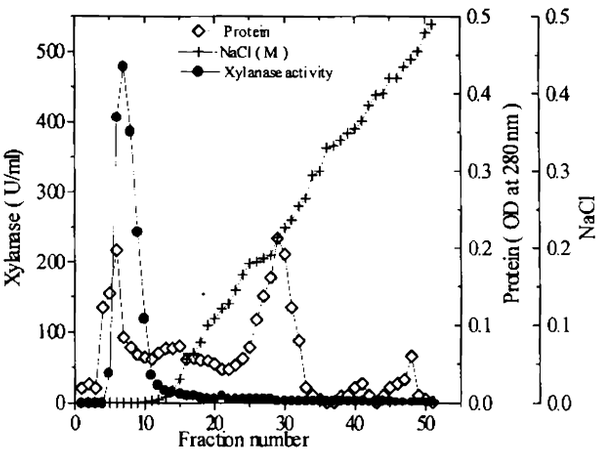


Fig. 36 Anion exchange chromatography, using DEAE-Sepharose CL 6B.
The 30 - 60 % fraction of $(NH_4)_2 SO_4$ precipitation was concentrated and dialysed and applied to the DEAE-Sepharose CL 6B column. The flow rate was 1.5 ml / min and fractions of 4.5 ml were collected.

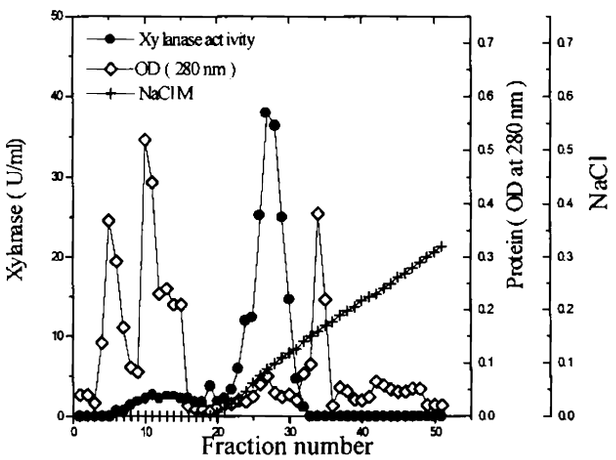


Fig. 37 Cation exchange chromatography using CM Sephadex.

Active fractions from the DEAE Sepharose CL 6B column were pooled and loaded to the CM Sephadex column. The flow rate was 1 ml / minute and fractions of 5 ml were collected.

the yield was 10.86%. The pooled fractions were concentrated with 60% solid ammonium sulphate addition and the resultant precipitate was used for subsequent stage.

3.4.1.2.3. Size exclusion chromatography (Bio Gel P100 Column chromatography)

The fraction from the CM column was then subjected to gel permeation chromatography with Biogel P-100 using Pharmacia X 16 column. Xylanase activity was eluted at about 40-50 fractions using the sodium phosphate buffer (0.05 M, pH 7) as the eluent. There were two prominent protein peaks in the elution pattern with the major one corresponding to the xylanase. The smaller one was not separated during the CM sephadex chromatography. This was confirmed by the occurrence of 2 clear bands when the CM Sephadex fraction was subjected to electrophoresis (Fig. 39).

The active fractions were pooled and concentrated by ammonium sulphate (60% precipitation) and dialysed for 20 hrs and considered as pure xylanase for further studies. The purification fold was 33.3 and the yield 2.5%. But the specific activity was 1723 U/mg protein. Parallel to the present study the report regarding the purification of low molecular weight xylanases from *Bacillus*

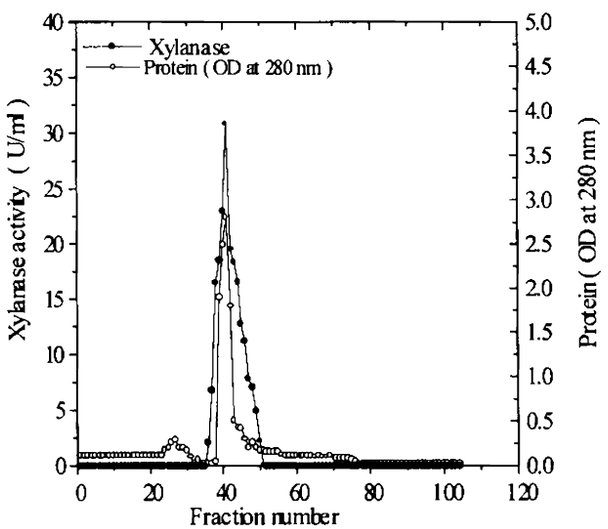


Fig. 38 Bio GelP-100 Gel Filtration Chromatography.

The Active fractions from the CM Sephadex cation exchange chromatography were pooled, concentrated, and loaded to the Bio Gel P - 100 column. The flow rate was 30 ml / hr and fractions of 2 ml were collected.

polymyxa resulted in an yield of less than 1% and purification fold of 2-12 (Morales *et al.*, 1993). Low purification yields were also reported for xylanases from (1) *Streptomyces cyaneus* (Wang *et al.*, 1992) (2) *Cellulomonas fimi* (Khanna and Gauri, 1993) and (3) *Trichoderma harzianum* (Tan *et al.*, 1985). However, the purification procedures of *Bacillus* SSP-34 xylanase resulted in a preparation of pure xylanase without contamination suitable for further characterisation (Table 8).

Table 8. Purification of xylanase from *Bacillus* SSP-34

	Volume (ml)	Total Activity [a] (U)	Total Protein [a] (Mg)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude	1000	419050	1489	281	1	100
Ultrafiltration	200	314290	1012	311	1.1	75
$(NH_4)_2SO_4$ Precipitation	40	184380	374	493	1.8	44
DEAE Sepharose –CL6B	130	88972	73	1218	4.33	21.2
CM Sephadex	100	45505	31.35	1451	6.13	10.86
Bio Gel P 100.	30	10476	6.1	1723	33.3	2.5

[a] Xylanase activity was assayed using oat spelts xylan using the standard procedures mentioned in the methodology. Protein was measured by Lowry's method

There are several cases for the microbial xylanases purifications using anion or cataion exchange chromatography. Reports regarding xylanases from *Bacillus* spp. are given in Table 9. Ishihara *et al.* (1997) used DEAE Sephadex A-50 and CM Sephadex C-50, ultrafiltration and Sephadex-150 column chromatography for the purification of xylanases from thermophilic fungus strain HG-1. Pembroke *et al.* (1995) purified endo-xylanase from *Cellulomonas flavigena* using ammonium sulphate precipitation, gel filtration and fractionation using DEAE Sepharose CL-6B. Renner *et al.* (1994) used DEAE-Cellulose, phenyl Sepharose CL-6B, hydroxyapatite and Superose 12 for the purification of 3 major endo-xylanase activities from *Cytophaga*

xylanolytica. The protocol used for the purification of one endo-xylanase with a molecular mass of 70 kDa from *Penicillium purpurogenum* was ammonium sulphate fractionation, Gel filtration on Bio-Gel P10, DEAE cellulose and CM sephadex chromatographies (Eyzaguirre *et al.*, 1992).

Table 9. Different techniques applied in the purification of xylanases from *Bacillus* spp.

Microorganisms	Purification steps	Mol.Wt (Kda)	Reference
Bacillus circulans WL-12	DEAE-Biogel A	85	Esteban *et al.* (1985)
	CM-Biogel A	15	,,
Bacillus spp	$(NH_4)_2SO_4$, chromatography on DEAE-Toyopearl 650 M. Gelfiltration on Toyopearl HW 55 S	21.5 49.5 22.5 50.0	Okazaki *et al.* (1985)
Bacillus sp. no. C-125	$(NH_4)_2SO_4$, DEAE-Cellulose, Sephadex G-75	43 16	Honda *et al.* (1985 a)
Bacillus sp. strain C-125	$(NH_4)_2SO_4$, DEAE-Cellulose, Sephadex G-75	43	Honda *et al.* (1985 c)
Bacillus pumilus	$(NH_4)_2SO_4$, DEAE-Sephadex A-50, CM-Sephadex C-50, TSK HW-65 (Poly vinyl gel)	20-24	Okada and Shinmyo (1988)
Bacillus sp. YC-35	Ethanol precipitation, DEAE-Cellulose, Sephadex G-100, Hydroxylapatite, Sephadex G-75	40-41	Park *et al.* (1992)
Bacillus polymyxa	DEAE-Biogel A, FPLC on Sephadex G-100	34 22	Morales *et al.* (1993)
Bacillus sp. 41 M-1	$(NH_4)_2SO_4$, DEAE-Toyopearl 650 M	36	Nakamura *et al.* (1992)
Bacillus subtilis	Acetone precipitation, Sepharose 6B, DEAE Sepharose		Cho *et al.* (1995)
Bacillus polymyxa	DEAE – Biogel A Chromatofocusing, Anionic FPLC on Mono-Q column	61	Morales et al. (1995b)
Bacillus sp. (NCIM 59)	Stage 1. Polyethylene glycol (PEG)-8000 and K_2HPO_4 Stage 2. 12% K_2HPO_4 and 8 %PEG	-	Gaikaiwari *et al.* (1996)
Bacillus sp. strain K-1	Affinity adsorption-desorption on insoluble xylan	23	Ratnakhanokachai *et al.* (1999)

a.) Genetically engineered gene of *Clostridium thermocellum* xylanase expressed in *Bacillus subtilis*.

Belancic *et al.* (1995) separated two xylanases from *Penicillium purpurigenum* using ultrafiltration with a membrane of molecular weight cut off 10,000, $(NH_4)_2SO_4$ precipitation (20-70 % fractional range) and gel filtration chromatography on Bio Gel P - 100 column. The two forms of

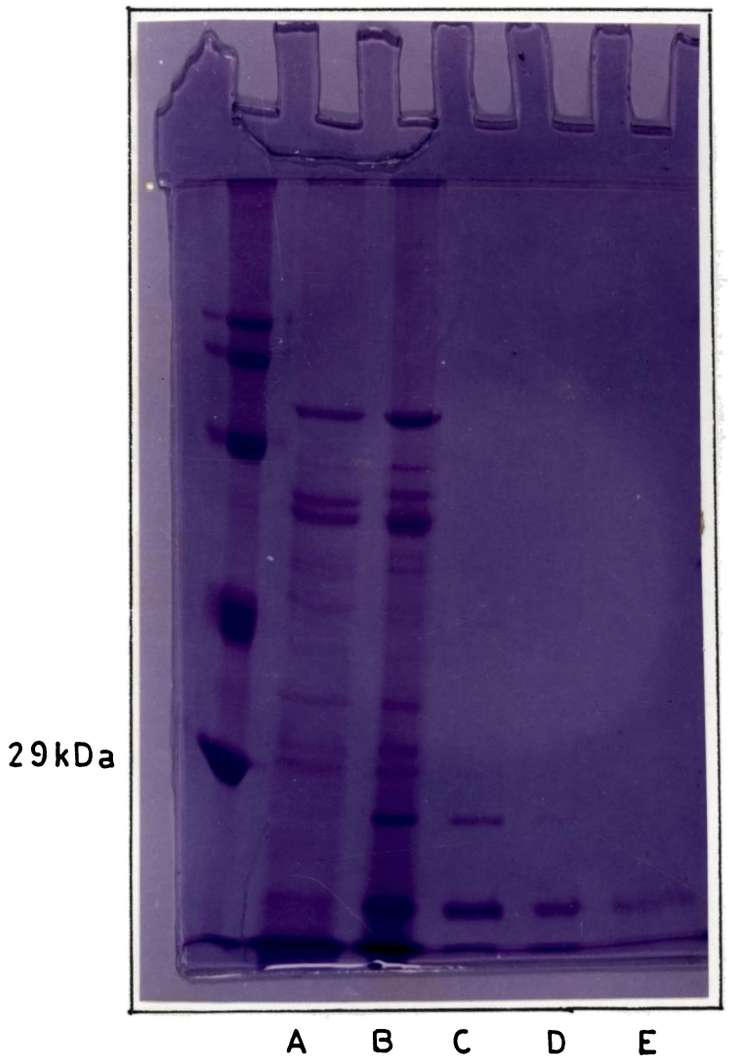


Fig. 39 SDS-PAGE of xylanase prepared at different stages of purification. Fractions used were A. Crude, B. $(NH_4)_2SO_4$, C. DEAE Sepharose, D. CM Sephadex and E. BioGel P100.

xylanases thus separated were then subjected to 1). DEAE cellulose DE-32 chromatography, hydroxyapatite chromatography on a Bio Gel HPT column and CM Sephadex C-25 chromatography and 2). DEAE cellulose and CM Sephadex chromatography. In all the cases mentioned for *Bacillus* spp. (Table 9) and other microorganisms there were the usage of simplest to complicated procedures but neither of them corresponds the protocol adopted for the purification of *Bacillus* SSP-34 xylanases. This method successfully isolated xylanase from other proteins to homogeneity. The specific activity of the purified protein i.e. 1723 U/mg was very high and one xylanase from *Bacillus* sp. with a specific activity of 2000 IU/mg protein (Debeire-Gosselin *et al.*, 1992b) and another from *Thermatoga maritima* (DSM3109) with a specific activity of 4600 U/mg protein (Winterhalter and Liebl, 1995) surpassed this data.

3.4.1.3.Molecular weight determination - SDS-PAGE

SDS-PAGE was carried out to determine the purity and molecular weight of the enzyme in quest. In order to determine the molecular weight, the purified sample was run in polyacrylamide gel (12%) containing sodium dodecyl sulfide. Nature of proteins present in the culture supernatant and purity was analysed using samples from the crude xylanase preparation, $(NH_4)_2$ SO_4 fraction, DEAE sepharose fraction, CM sephadex fraction and finally the GPC fraction. The markers added were (1) carbonic anhydrase (29 kDa) (2) chick albumin (45 kDa) (3) bovine serum albumin (66 kDa) (4) alkaline phosphatase (97 kDa) (5) β-galactosidase (116 kDa) and (6) myosin (205 kDa). The bands appearing in the crude and $(NH_4)_2SO_4$ fractions were

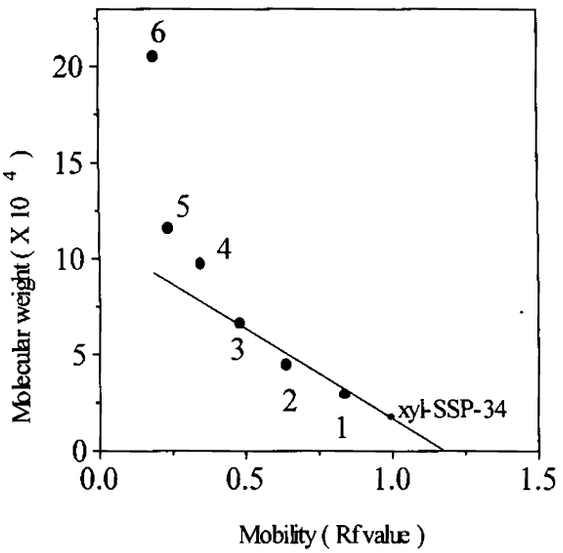


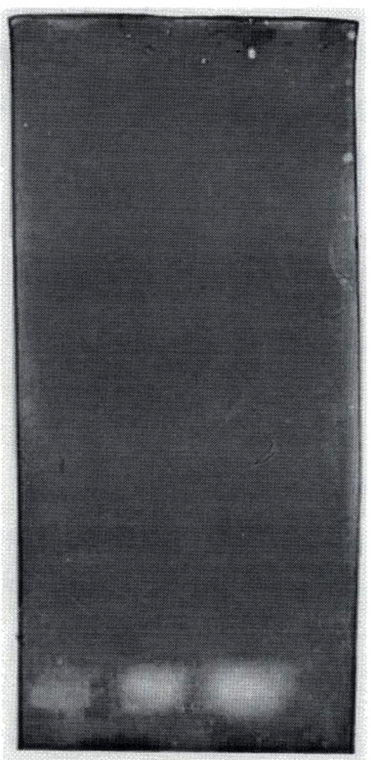
Fig. 40 Determination of molecular weight of *Bacillus* SSP-34 xylanase. Samples were loaded on to a Slab Gel of 12% acryl amide. The proteins were allowed to separate at a voltage of 200 V. Proteins were stained with Coomassie brilliant blue. The marker proteins (Sigma Chem. Co.) used along with the *Bacillus* SSP-34 xylanase (xyl-SSP-34) are: i) Carbonic anhydrase (29), ii) Ovalbumin (45), iii) Bovine serum albumin (66), vi) Alkaline phosphatase (97), v) β-Galactosidase (116), vi) Myosin (205).

having the same pattern. The culture medium contained other proteins although the xylanase protein was the prominent one.

The proteins were concentrated during the ultrafiltration and ammonium sulphate fractional precipitation. All the other proteins except for a single band were separated during the DEAE Sepharose and CM Sephadex column chromatography (Lanes 4 and 5 of Fig. 39). The second band was well separated in the GPC as shown in the lane 6 of plate (Fig. 39) showing the purity of the protein. DEAE column eliminated the colour pigments from

the broth and the purification was almost complete with the CM sephadex stage. Similar results have been reported earlier (Okada and Shinmyo, 1988; Pembroke *et al.,* 1995).

The molecular weight of the xylanase protein calculated from the electrophoretic mobility was found to be 20-22 kDa (Fig. 40). There are few reports on the low molecular weight xylanases, which are finding important application in paper and pulp industry. The small molecules can easily penetrate the holes of hydrolysis formed in the reprecipitated xylan on the surface of Kraft cooked pulp. This alleviates the problem of xylan barrier on the surface of lignin containing pulp to the bleaching chemicals.



1 2 3

Fig. 41 Zymogram for Crude, CM and BioGel xylanase fractions purified from *Bacillus* SSP-34 culture broth. Clear zones developed by overlaying the elctrophoretically moved protein onto substrate gel corresponds to xylanase activity. 1.) Crude 2.) CM Fraction 3.) BioGel Fraction

3.4.1.4. Zymogram

The xylanase activity of the purified protein was

confirmed by using zymograms. The proteins were separated by electrophoresis and the substrate gel was then overlaid onto the electrophoretic gel. Congo red staining turned the colour of substrate gel to black and the clear zones represented the areas of xylanase activity (Nakamura *et al.*, 1993b). The three clear zones in the Fig. 41 correspond to the active fractions contained in crude, CM Sephadex and GPC samples respectively.

Thus the purified protein was having xylanase activity which was proved by the zymogram study. There are several reports regarding the verification of xylanase activity of the purified protein using zymogram (Nakamura *et al.*, 1993b).

3.4.2. Characterisation of kinetic properties of xylanases

Industrial application of most of the enzymes is based on their crude forms. However, the acceptance of the enzymes for industrial application has to be corroborated with sufficient data regarding the enzyme characteristics. In order to understand the kinetic properties of xylanases from *Bacillus* SSP-34, crude enzyme kinetics were studied initially to reveal the extremes of pH and temperature affecting the activity. Purified enzymes were further characterised in detail.

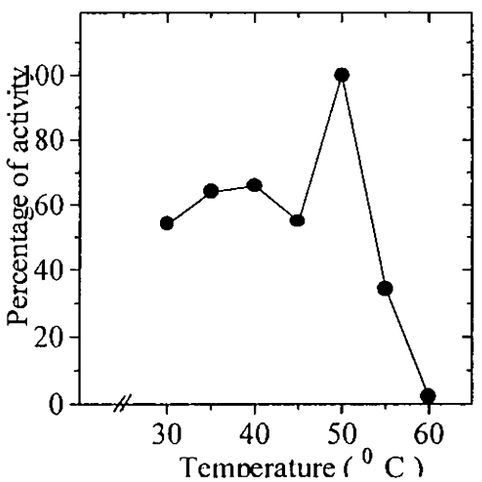


Fig. 42 Effect of temperature on crude xylanase activity. The reaction was carried out for 10 minutes at various temperatures (30-60^0 C) using 0.2 M Na_2CO_3 / $NaHCO_3$ buffer (pH 9) and enzyme activity was expressed as percentage of highest activity (50^0 C).

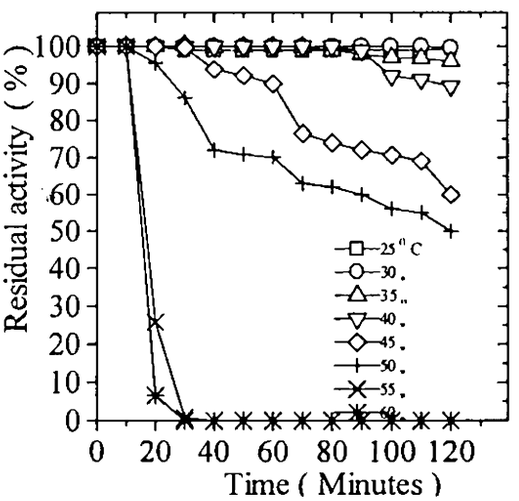


Fig. 43 Effect of temperature on stability of xylanase. Thermal stability was determined by preincubating the crude xylanases for two hours at 25^0 C (□), 30^0 C (O), 35^{0}C(△), 40^{0}C (▽), 45^{0}C (◇), 50^{0}C (+), 55^{0}C (X) and 60^{0}C (✳). The residual activities were monitored at 10 minutes intervals under the standard assay conditions

3.4.2.1. Effect of temperature and pH on crude xylanase from *Bacillus* SSP-34

The crude enzyme was produced as described before and harvested at the hour of maximum enzyme activity.

3.4.2.1.1. Effect of temperature on activity and stability of crude xylanase

Enzyme activities were assayed at different temperatures ranging from 30-65°C at a constant pH of 9.2 and a substrate concentration of 0.5%. The enzyme showed optimum activity at 50°C (Fig. 42). Reports of alkaline xylanases that showed higher temperature optima were scarce (Nakamura *et al.*, 1992). Emphasis was given to the higher pH and temperature even though they act as drastic conditions for enzyme activity. The residual activities of crude xylanase incubated at different temperatures for a period of 2 hours were estimated at optimum temperature. The enzyme was stable at temperature ranging from 25-40°C while at 45 and 50°C, 40% and 50% of the original activity was lost within 2 hrs (Fig.43). The enzyme retained 100% activity for 20 minutes at 50°C. At 60 and 65°C, almost complete inactivation was observed by 20 minutes. The effect of temperatures was studied at pH 9.2 unlike most of the previous reports (Dahlberg *et al.*, 1992, 1993; Shoham *et al.*, 1992; Arase *et al.*, 1993) where the reaction was monitored at pH 7 or at low values. The reaction pH has an important influence on thermal stability and rate of reaction owing to the effect of pH on the protein structure. Irreversible thermal inactivation at high temperature was facilitated by the pH extreme (Kristjansson and Kinsella, 1991).

3.4.2.1.2. Effect of pH on activity and stability of crude xylanase

The pH optimum of the xylanase at a constant reaction temperature (50°C) and substrate concentration was in the range of 6-8. The enzyme activity at pH values 7, 8 and 9 were 84, 72 and 20% of that at pH 6 respectively (Fig. 44). Thus SSP-34 xylanase has pH optima near the neutral range similar to most of the alkalophilic bacterial xylanases described earlier (Ohkoshi *et al.*, 1985; Dey *et al.*, 1992; Nakamura *et al.*, 1993a,b,1994). The pH optima of xylanase SSP-34 i.e. pH 6-8 (with 84 % and72 % of activity at pH 6 for pH 7 and 8 respectively) was in support of the general observation that *Bacillus* spp. xylanases were having broad pH optima ranging from pH 5

to 9.5 with peak at pH 6 (Nakamura *et al.*, 1994). The xylanase was found to be stable at pH values ranging from 4.5-9.0. There was complete loss of activity at pH values of 4, 9.5, 10 and 11 after two hours (Fig. 45). Thus the xylanase from *Bacillus* SSP-34 has a very broad pH stability similar to most of the reported bacterial xylanases (Schofield, and Daniel, 1993), however, with higher xylanase activity.

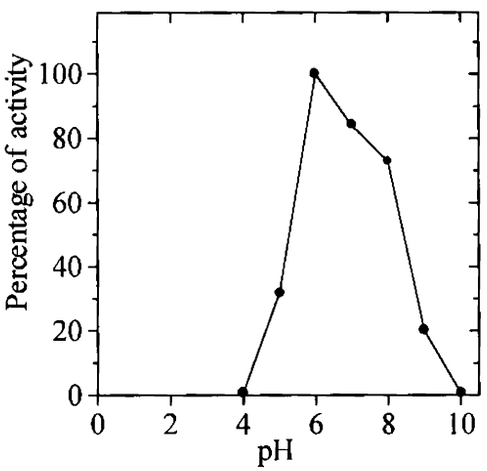


Fig. 44 Effect of pH on xylanase activity. The enzyme was buffered with 0.2 M acetate buffer (pH 4-5), 0.2 M phosphate buffer (pH6-8) and 0.2 M carbonate buffer (pH 9-11) and incubated for 10 minutes.

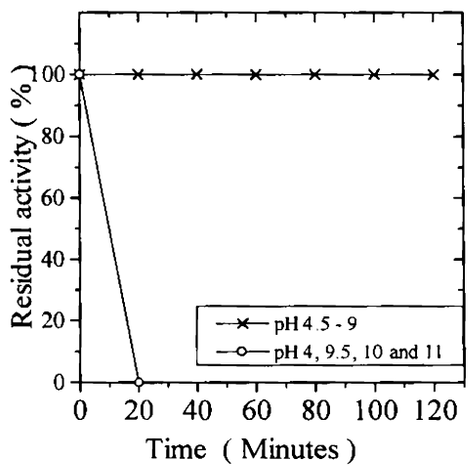


Fig. 45 Effect of pH on stability of xylanase. Stability studies were performed by incubating crude enzyme solution for two hours at different pH. For pH values of 4.5-9 (x) there was no loss of activity after two hours while pH values of 4, 9.5, 10 and 11 (o) showed drastic loss of activity within 20 minutes.

3.4.2.2. Effect of temperature and pH on the purified xylanase from *Bacillus* SSP-34

The purified xylanase fraction from BioGel P-100 was characterised for the properties.

3.4.2.2.1. Effect of temperature on the activity and stability of purified xylanase

In order to detect the optimum temperature the purified xylanase was incubated along with oat spelts xylan (0.5%) at a pH value of 9.2 for 10 minutes at various temperatures ranging from 30-60^{0}C. The temperatures

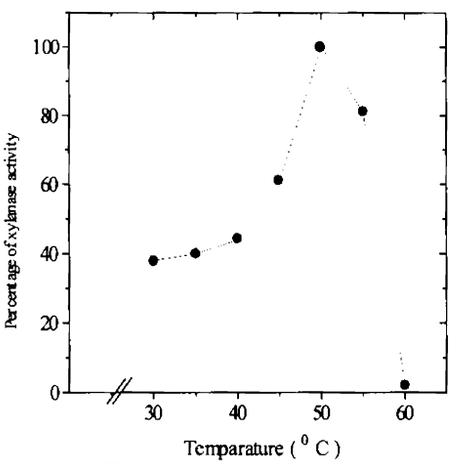


Fig. 46 Effect of temperature on purified xylanase.The reaction was carried out for 10 minutes at various temparatures. The enzyme activities were expressed as the perccentage of highest activity (50^{0}C).

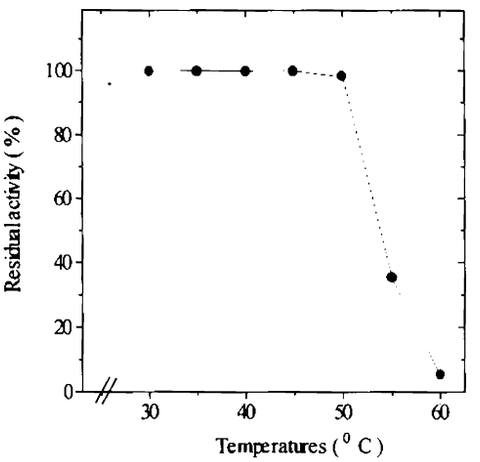


Fig. 47 Effect of temperatures on stability of purified xylanase. Thermal stability was determined by preincubating the GPC fraction for 30 minutes at different temperatures ranging from 30 to 60 with 5^0 incriment. The residual activities were determined according to the standard assay conditions.

lesser than 30^{0}C and above 60^{0}C were not taken considering the results with crude xylanase. Maximum activity was obtained at a temperature of 50°C (Fig. 46), a result similar to that of the crude xylanase. Here also the pH of the reaction was 9.2, unlike in most of the previous reports where the reaction was monitored at pH 7 or below (Subramaniyan *et al.*, 1997). As cited for the crude xylanases the optimum studies were conducted at higher pH values by considering the application part. The reaction pH either at different sides of neutrality has an important influence on thermal stability and rate of reaction owing to the irreversible thermoinactivation of the protein in these conditions. Irreversible thermal inactivation at higher temperatures was facilitated by the

pH extremes (Kristjansson and Kinsella, 1991). At 55^{0}C, 80% of activity of that at 50°C could be observed. Other temperatures i.e. 30, 35 and 40°C resulted in an activity lesser than that of maximum (50^{0}C) (Fig. 46). The xylanase was stable for 30 minutes up to 50^0 C (Fig. 47).

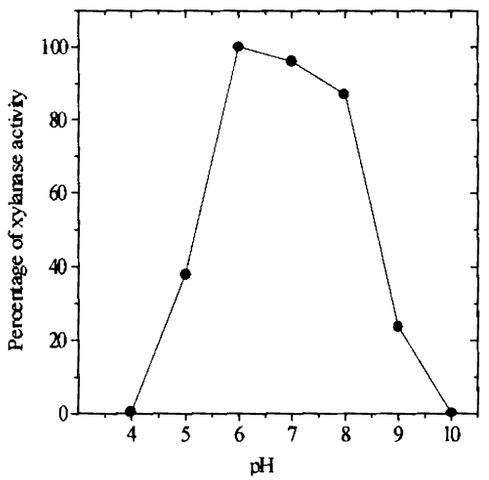


Fig. 48 Effect of pH on purified xylanase. The enzyme was buffered with 0.2 M acetate buffer (pH values 4-5), 0.2 M phosphate buffer (pH values 6-8) and 0.2 M carbonate buffer (pH values 9-11) and incubated for 10 minutes.

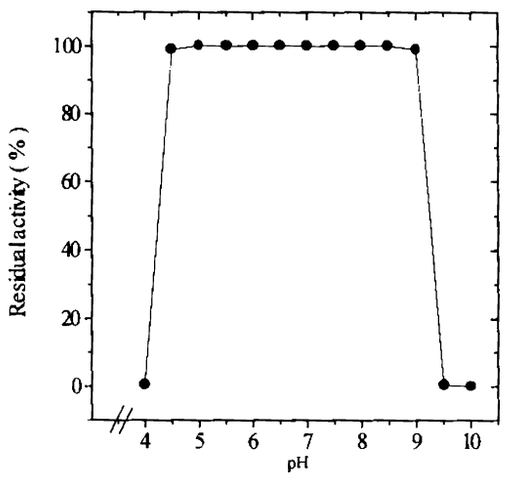


Fig. 49 Effect of pH on the stability of purified xylanase from *Bacillus* SSP-34. The stability studies were performed by incubating the active fractions from GPC at different pH values (4-10) for two hours.

3. 4.2.2.2. Effect of pH on the activity and stability of purified xylanase

Effect of pH on the purified xylanase has a close similarity to that of crude xylanase. The pH optimum of the xylanase was measured at constant reaction temperature (50°C) and substrate concentration. The Biogel P-100 fraction of xylanase was incubated with substrate solutions prepared in appropriate buffers [0.2 M acetate buffer (pH values 4 - 5), 0.2 M phosphate buffer (pH values 6-8) and 0.2 M carbonate buffer (pH values 9-11)] for 10 minutes. The results showed the bell shaped graph for xylanase activity with the optimum pH ranging from 6-8 (Fig. 48). Similar to the observations with

crude xylanase here also the peak resides at pH value 6. Such reports of xylanases especially from *Bacillus* spp. having alkaline pH optima range for enzyme activity with peak at pH 6 has been reported elsewhere (Subramaniyan *et al.*, 1997; Nakamura *et al.*, 1994). Xylanase activities recorded at pH values 7 and 8 were 96% and 89% respectively of that at pH 6. But the activities at pH 5 (40%) and pH 9 (25%) were comparatively low. Such reports as mentioned in the case of crude xylanases are in support of the general observation that *Bacillus* spp. xylanases were having broad pH optima ranging from 5-9 with a peak at pH 6 (Nakamura *et al.*, 1994; Subramaniyan *et al.*, 1997). The purified xylanase like the crude enzymes showed stability at pH values ranging from 4.5 to 9.0. There was complete loss of activity at pH values 4, 9.5 and above after 2 hrs of incubation (Fig. 49). This broad pH stability is similar to most of the reported bacterial xylanases (Schofield and Daniel, 1993).

3.4.2.3. Effect of substrate concentration on purified xylanase.

Purified xylanase from *Bacillus* SSP-34 had a K_m of 6.5 mg of oat spelts xylan per ml and V_{max} of 1233 μ mol/min/mg protein under the standard assay conditions described in the methodology (Fig. 50).

Similar to the present study, most of the reported xylanases from the different species of *Bacillus* are having a K_m value between 3-8 mg of substrate (oat spelts xylan). Only few exceptions are there for the *Bacillus* spp. with lower K_m values like *Bacillus* sp. 11-15 (1.68 mg/ml), *Bacillus* strain XE (0.6mg/ml) and *Bacillus stearothermophilus* (1.63 mg/ml) (Table 2). *Trichoderma longibrachiatum* (10.14), *Aeromonas caviae* (9.4) and *Streptomyces* T-7 (10) were the ones with higher K_m values (Table 2). The species *Streptomyces* sp. No.317 was producing the highest activity with 40 mg/ml but here the substrate used was xylotriose which is far smaller than the

xylan molecules with which the same xylanase (Xyn-2-b) showed a K_m of 11.2 (Nakanishi *et al.*, 1992). Extremely thermophilic xylanases from *Thermotoga thermarum* were larger molecules with mol wt. of 266 kDa (dimer of 105 kDa and 150 kDa) having surprisingly low value 0.36 for the K_m. The other synergistic xylanase with comparatively low molecular weight (33 kDa) also had low K_m value of 0.24 (Sunna *et al.*, 1996). Low K_m values have also been reported for purified xylanases from *Aspergillus awamori* (Kormelink *et al.*, 1993).

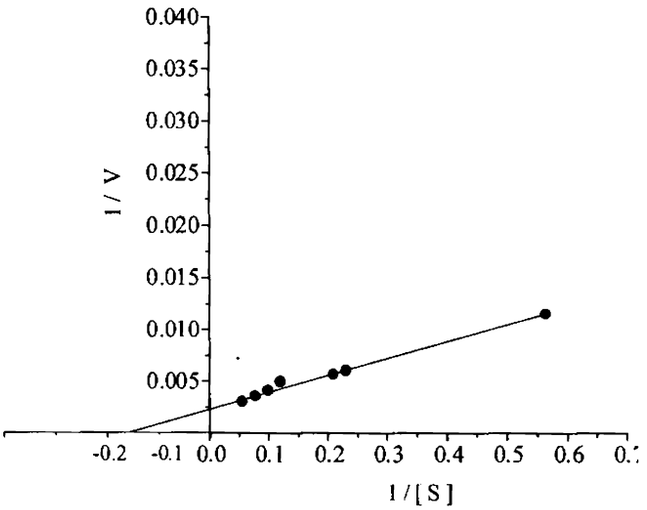


Fig. 50 Lineweaver-Burk Plot for determining K_m value of xylanase from *Bacillus* SSP-34. The substrate used was oat spelts xylan taken as mg/ml composition. S-substrate concentration. V-initial rate of reaction, K_m 6.5 mg / ml. V_{max} - 1233 μ mol / min, / mg protein.

3.4.2.4. Effect of metal ions on purified xylanase activity

In order to study the nature of protein, different metal ions and chelators were incubated separately with purified *Bacillus* SSP-34 xylanase. Hg^{2+} showed a drastic inhibition, Cd^{2+}, Cu^{2+}, Ba^{2+}, Sn^{2+}, Mn^{2+} and CO^{2+} showed considerable inhibitory effects at the experimental level tried i.e.1.0 mM. However, the inhibition was lesser at a low level (0.01mM) but the pattern of inhibition was obvious. 2-Mercaptoethanol resulted in 10% loss of activity. Inactivation by Hg^{++} and mercaptoethanol (4-10% loss) suggests a role of sulphydral groups in enzyme activity (Capalash *et al.*, 1991). The metal ions

Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+} and chelater - EDTA had no inhibition effect on the enzyme. Apart from attacking the sulphydral groups of xylanase, Hg^{2+} and group II b metals may also have high affinity for reactive groups. For example the affinity of Hg covered reactive groups such as SH > $CONH_2$ > >NH_2 > COOH > PO_4 (Venugopal and Luckey, 1978). There are several lines of evidence for the inhibitory effect of Hg^{2+} (Liu *et al.*, 1998).

Monovalent cations Na^+ and K^+ had small stimulating effect on the activity while the lack of inhibition by EDTA suggests the non-metalic nature of this xylanase from *Bacillus* SSP-34. Similar xylanases have also been reported in *Bacillus* spp. (Liu *et al.*, 1998; Khasin *et al.*, 1993; Akiba and Horikoshi, 1988) and in *Streptomyces* sp. (Marui *et al.*, 1985).

3.5 Application of *Bacillus* SSP-34 Xylanase

3.5.1. Hydrolysis of xylan

Endo-xylanases from the bacterial culture *Bacillus* SSP-34 was subjected for its ability to cleave xylan. The hydrolysis of oat spelts xylan was carried out with purified xylanase from *Bacillus* SSP-34 and the results are shown in the Fig. 51. The main products of hydrolysis were mainly xylobiose, xylotriose, and xylopentose. The hydrolysis pattern indicated that the enzyme was an endo-xylanase. Similar to the present study xylanases from *Bacillus flavothermus* strain LB3A produced xylotriose, xylotetraose and xylopentose as the hydrolytic products (Sunna *et al.*, 1997). According to Biely *et al.* (1993), there is relation between the molecular weight and enzyme action. The high molecular weight endo-xylanases (Generally Family 10/F) exhibit greater catalytic versatility than Family 11/G xylanases. However there are rare exceptions for this hypothesis i.e. xylanase from *Aeromonas caviae* ME-1 liberated only xylobiose and xylotetraose (Usui et al.1999). Xylanase (xylanase C, molecular weight is 22 kDa) from *Streptomyces lividans* resulted mainly in the release of xylobiose, xylotriose, xylotetraose and xylopentose (Kluepfel *et al.*, 1992). Three types of xylanases from *Streptomyces* sp. were able to convert xylotriose, xylotetraose and xylan to xylose and xylobiose (Marui *et al.*, 1995). However the enzymes were non-active on xylobiose.

Biely *et al.* suggested the relationship between the molecular size and hydrolytic properties. The members of family 10 liberated more efficiently the terminal xylopyranose residues than family 11, as they have smaller substrate binding sites in comparison to endo-xylanases of 11 (Biely *et al.*, 1997). This factor alone allowed a possible conformational flexibility of the larger enzymes than of the smaller ones. Substrate binding sites of family 10 endo-xylanases are not in such deep clefts as the substrates binding sites of family

11 endo-xylanases (Fig. 4,5). From the catalytic properties and molecular weight measurements it was assumed that the *Bacillus* SSP-34 was having family G/11 characters.

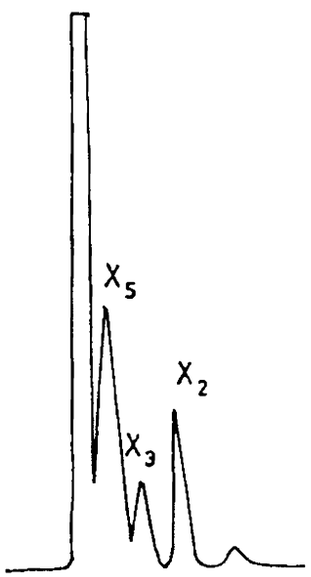


Fig 51. HPLC hydrolysis patterns of oat spelts xylan by *Bacillus* SSP-34 xylanase. X_2, X_3 and X_5 are xylobiose, xylotriose, and xylopentose sugars. Purified xylanase was incubated with oat spelts xylan for 30 minutes.

3.5.2. Application of *Bacillus* SSP-34 Xylanases

Biobleaching with xylanases from microorganisms

Kraft cooking involved the usage of Na_2S and NaOH to process the wood chips to partially remove lignin and the hemicelluloses. The residual lignin, covalently bound to carbohydrate moieties in the pulp (Yamasaki *et al.*, 1981) would be removed later by bleaching agents. During the final stages of cooking, xylan removed earlier would be reprecipitated on the surfaces of cellulosic fibres (Gierer and Wannstrom, 1984; Yamasaki *et al.*, 1981).

The reprecipitated and relocated xylans trap the degradation products in the matrix, resulting in the characteristic brown colour of pulp. Even though lignin was the main contributor of pulp colour, there were other compounds, which according to Ziobro (1990) could add to the colour of the Kraft pulp. Sugars cooked under Kraft conditions gave rise to compounds with absorption spectra similar to those present in Kraft liquors. According to Kantelinen *et al.* (1993), during the pretreatment with xylanases, they act primarily by hydrolysing the reprecipitated xylan located on the surface of the pulp fibres. The average pore size in kraft pulp would be about 5-10 nm allowing the penetration of enzymes with a molecular mass of 40 kDa or less, assuming a spherical conformation (Stone and Scallan, 1968). This necessitated search for xylanases like the one from *Bacillus* SSP-34 with a molecular mass of 20-22 kDa (Fig. 39).

The strongly alkaline Kraft pulping process usually traps the remaining alkali in the fibres even after extensive washing resulting in the pH drift to higher ranges during the enzyme treatment (Patel *et al.*, 1993). Thus it becomes necessary to have enzymes with higher pH optima and wide range of pH stability. Temperature stability would also be desirable as the pulps are warm when they come out of the Kraft cook and washing steps. Number of xylanases have been reported earlier to be applicable in paper and pulp industries, but met with a little success in achieving the goal. The application of xylanases was pioneered by Viikari *et al.* (1986) and Jurasek and Paice (1986), however the earlier studies were conducted with xylanases obtained from fungi. Those enzymes with considerable cellulase activity have effected viscosity loss of pulp. More over most of the enzymes were having low pH, temperature optima and the stability was not satisfying the requirements of the pulp biobleaching. Later there were few reports regarding the alkaline

thermostable xylanases from fungi (Purkarthofer *et al.*, 1993 - *Thermomyces lanuginosus*).

Table. 10 Biobleaching experiments with *Bacillus* SSP-34 xylanases on Kraft pulp

Sl no;	Xylanase/control	Xylanase Unit / g	% of H_2O_2 treated	Brightness % ISO
1	SSP-34 Xylanase	5	1.5	44.5±0.2
2	„	5	2.0	48.5±0.2
3	„	5	3.0	48.05±0.08
4	„	10	1.5	45.09±0.05
5	„	10	2.0	47.11±0.06
6	„	10	3.0	46.98±0.24
7	„	15	1.5	44.4±0.2
8	„	15	2.0	45.1±0.08
9	„	15	3.0	45.01±0.03
10	„	20	1.5	44.57±0.24
11	„	20	2.0	44.62±0.11
12	„	20	3.0	44.8±0.05
13	Control	0	1.5	40.2±0.05
14	„	0	2.0	41.1±0.08
15	„	0	3.0	40.9±0.05

Pulp samples treated with EDTA, Xylanase, alkali extraction, Hypochlorite, H_2O_2 and Hypochlorite. Brightness measured against MgO_2 (ISO units).

Bacillus SSP-34 xylanase resulted in the increase of 7.4 units of ISO brightness than the control (from 41.1% to 48.5%) without xylanase pretreatment (Table 10). In all the experiments, concentration of H_2O_2 higher than 2.0% failed to yield significant result i.e. the optimum bleach effect was attained in the 2.0% level (Table 10). Of the different concentrations of enzymes applied like 5, 10, 15 and 20 U/g pulp, the 5 U/g pulp resulted in higher brightness. In all these cases, the alkali treatment was applied as per previous reports (Rydholm, 1965a, b) because during treatment of pulp with

high concentration alkaline solution (Alkalisation), the low molecular components are removed (Gamerith and Strutzenberger, 1992). This high alkaline treatment was thus useful in xylanase pre-bleaching as the hydrolysed products of the reprecipitated and relocated xylans could be easily removed during the alkalisation process.

The increase in brightness of the *Bacillus* SSP-34 xylanase pretreated pulp was comparable with most of the earlier reports. Ratto *et al.* (1994) tried the applicability of xylanases from *Dictyoglomus* sp. in the biobleaching of kraft pulps and found minor increase, from 46.9 ISO units to 48.9 ISO units in brightness. While xylanases from *Streptomyces thermoviolaceus* (Garg *et al.*, 1996) resulted in an increase up to 5 ISO units, from 60.2±0.2 to 67.2±0.1. Garg *et al.* (1998) later reported the comparison of pretreatment effects of *S. thermoviolaceus* xylanases with two commercially available enzymes, Pulpazyme and Cartazyme and found that all the cases were having an 6-7 ISO unit increase in brightness. The white rot fungus *Trametes* (*Coriolus*) *versicolor* effected biobleaching effect by changing the brightness change from 36.8 to 50.2 ISO units under immobilisation conditions (Kirkpatrick *et al.*, 1990). While Tenkanen *et al.* (1992) in a study using chlorine as the bleach sequence agent found that when xylanase was used, chemical consumption of active chlorine was reduced by 7% for achieving the same brightness obtained in the reference. The xylanase from *Aureobasidium pullulans* resulted in brightness increase of 2 ISO units (Yang *et al.*, 1992). Thus from the present study it is clear that the *Bacillus* SSP-34 xylanase was having characters suited for the application in pulp and paper industries.

SUMMARY

SUMMARY

- Isolation and screening for bacterial xylanases conducted at three stages, including wheat bran agar plates, xylan agar plates and xylan liquid medium resulted in the selection of 10 strains from a collection of 200 isolates.
- SSP-34, the most potent one from 200 isolates was identified and named as *Bacillus* SSP-34, which produced 100 times more activity than the other isolates.
- *Bacillus* SSP-34 showed maximum xylanase activity at 102 hours and growth at 30 hours. The culture was having only trace levels of cellulase activity in the cell free culture supernatant than the other nine isolates. It was proposed to be due to the action of xylanases on small chains of xylose residues present as contamination in the commercial celluloses. The bifunctional nature could also effect the cellulase\ xylanase activity.
- Growth optimum of *Bacillus* SSP-34 was at pH 9.0 while maximum xylanase production occurred at pH 8.5.
- In studies with different initial pH values, all the media reached pH value 8±0.5 by 96th hour when the highest production occurred. When this alkaline condition was provided highest enzyme expression was the result. This kind of xylanase production has been seldom reported.
- Optimum xylanase production as well as highest growth occurred at a temperature of 35^0 C. The culture showed thermotolerant nature.
- Highest xylanase production occurred at 5% inoculum concentration. When the case was 1%, both biomass and xylanase production were low which could be due to the low cell density. Low xylanase production at higher concentrations was apparently due to the transfer of metabolically active compounds affecting the higher production of xylanases.
- Highest enzyme production occurred at the agitation of 300 rpm and at an aeration of 0.2 wv ratio, which was followed by 0.4 wv ratio and 300 rpm (76% xylanase production of that occurring at the highest case). Other

agitation and aeration conditions resulted lower xylanase production, all lesser than 49% possibly due to low efficiency in the transfer of dissolved oxygen to the microbial cell and transfer of oxygen from air into the medium.

- Of the 18 different carbon sources studied including monosaccharides, disaccharides, polysaccharides and lignonocellulosics, xylan was the optimum carbon source inducing highest levels of xylanase expression.
- Wheat bran and xylose induced xylanase production amounting 91% and 80% of that occurring at xylan. Lignocellulosics such as rice straw, bagasse, rice bran, coir and rice husk showed low levels of production (less than 23%).
- Growth in the CMC containing medium could be attributed to the constitutively expressed enzymes acting on the xylose residues present as contaminant in commercial celluloses, the utilisation of which maintained the growth rate of *Bacillus* SSP-34.
- Glucose resulted in the inhibition of xylanase production. The effect of glucose could be attributed to two mechanisms, first the catabolite repression occurring at the transcriptional level and second by inducer exclusion.
- Study with different concentrations of xylan showed maximum xylanase production was at 0.5% xylan while higher concentrations resulted in poor enzyme production. Maximum growth occurred at a level of 2.5%.
- Even though xylan and xylose resulted in xylanase expression, each when supplemented with glucose resulted in catabolite repression. But xylan along with xylose showed no inhibition of xylanase production confirming the inductive effect of xylose.
- The present study showed that the kind and level of nitrogen source used in the medium influenced xylanase production from microorganisms. The optimum nitrogen source was a combination of yeast extract and peptone,

each at a concentration of 0.25%. This medium resulted in low level of protease production i.e. 0.009 IU/ml.

- The reason for incorporating ammonium ion forming medium (with complex nitrogen sources) is to minimise the protease induction.
- When the level of nitrogen source increased (e.g. 2.0% YE and 2.0% Peptone) there occurred positive growth by the utilisation of minor carbohydrates found in the complex medium and xylanase production delayed up to 120 hours.
- From the present study it becomes evident that the nitrogen source in the medium should be optimum and promote growth as well as enzyme production.
- Inorganic nitrogen sources failed to effect good xylanase production.
- Different metal ions classified as Class A, Class B and borderline ions markedly differed in their influence on growth and enzyme production by *Bacillus* SSP-34. K_2HPO_4, $MgSO_4$, NaCl and $ZnCl_2$, Na_2HPO_4 and $MnSO_4$ did not inhibit both the biomass and xylanase production while $CoCl_2$ did. Co^{2+} was inhibiting both growth and xylanase production. Class A ions (eg. Mg, Ca, Na) are essential for the growth of microorganisms.
- Unique purification procedure was employed for purifying the xylanases from *Bacillus* SSP-34. After ultrafiltration and ammonium sulphate fractionation of the cell free culture supernatant, column chromatography was employed for further purification.The first stage in this was the gradient elution using DEAE Sepharode CL 6B column. In the second stage CM Sephadex column was used. Partially purified xylanase from the ion exchange column chromatographic analyses was further purified in size exclusion column chromatography with BioGel P100 (BioRad) column. Specific activity of the purified xylanase protein, i.e. 1723 U/mg was one among the highest values. The purification fold was 33.3 while the yield was 2.5%.

- Electrophoretic analysis revealed the pattern of proteins present in crude and fractions from different stages of purification. The band pattern illustrates the justification of the incorporation of two ion exchanges (anion and cation) prior to GPC. The purification was almost 90% in the first two levels which minimises the task of GPC column considerably. The molecular weight of the single xylanase produced from *Bacillus* SSP-34 was 20-22 kDa and Zymogram analysis confirmed the xylanolytic nature of the purified low molecular weight protein.
- The *Bacillus* SSP-34 xylanase was having optimum activity at a temperature of 50^0 C and pH of 6-8. The enzyme showed considerable thermostability (30 minutes at 50^0 C) and pH stability (4.5 to 9 for two hours).
- The *Bacillus* SSP-34 xylanase was not a metalloenzyme and Hg^{2+} was a strong inhibitor.
- The K_m value of the xylanase was 6.5 mg of oat spelts xylan per ml and V_{max} was 1233μ mol/min/mg protein.
- Hydrolytic pattern of the purified *Bacillus* SSP-34 showed the formation xylobiose, xylotriose and xylopentose. The hydrolysis pattern indicated that the enzyme was an endo-xylanase.
- The prebleaching of pulp sample with *Bacillus* SSP-34 was highly effective with an ISO % brightness increase from 41.1% to 48.5%. This increase in brightness (7.4 %) was comparable to most of the reported cases.

Conclusive Remarks

Xylanases with hydrolytic activity on xylan, one of the hemicellulosic materials present in plant cell walls, have been identified long back and the applicability of this enzyme is constantly growing. All these applications especially the pulp and paper industries require novel enzymes. There has been lot of documentation on microbial xylanases, however, none meeting all the required characteristics. The characters being sought are: higher production, higher pH and temperature optima, good stabilities under these conditions and finally the low associated cellulase and protease production. The present study analyses various facets of xylanase biotechnology giving emphasis on bacterial xylanases. Fungal xylanases are having problems like low pH values for both enzyme activity and growth. Moreover, the associated production of cellulases at significant levels make fungal xylanases less suitable for application in paper and pulp industries.

Bacillus SSP-34 selected from 200 isolates was clearly having xylan catabolizing nature distinct from earlier reports. The stabilities at higher temperatures and pH values along with the optimum conditions for pH and temperature is rendering *Bacillus* SSP-34 xylanase more suitable than many of the previous reports for application in pulp and paper industries.

Bacillus SSP-34 is an alkalophilic thertmotolerant bacteria which under optimal cultural conditions as mentioned earlier, can produce 2.5 times more xylanase than the basal medium.

The 0.5% xylan concentration in the medium was found to the best carbon source resulting in 366 IU/ml of xylanase activity. This induction was subjected to catabolite repression by glucose. Xylose was a good inducer for xylanase production. The combination of yeast extract and peptone selected from several nitrogen sources resulted in the highest enzyme production (379 ± 0.2 IU/ml) at the optimum final concentration of 0.5%. All the cultural and nutritional parameters were compiled and comparative study showed that

the modified medium resulted in xylanase activity of 506 IU/ml, 5 folds higher than the basal medium.

The novel combination of purification techniques like ultrafiltraton, ammonium sulphate fractionation, DEAE Sepharose anion exchange chromatography, CM Sephadex cation exchange chromatography and Gel permeation chromatography resulted in the purified xylanase having a specific activity of 1723 U/mg protein with 33.3% yield. The enzyme was having a molecular weight of 20-22 kDa. The K_m of the purified xylanase was 6.5 mg of oat spelts xylan per ml and V_{max} 1233μ mol/min/mg protein.

Bacillus SSP-34 xylanase resulted in the ISO brightness increase from 41.1% to 48.5%. The hydrolytic nature of the xylanase was in the endo-form.

Thus the organism *Bacillus* SSP-34 was having interesting biotechnological and physiological aspects. The SSP-34 xylanase having desired characters seems to be suited for application in paper and pulp industries.

Further work is necessary to enhance xylanase production and protein stabilities to still higher values. This requires genetic changes caused either by conventional mutation studies or gene manipulation.

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