Review Of Literature
2 REVIEW

2.1 Xylan: Occurrence

Lignocellulose constitutes the largest biomass source on earth and is mainly composed of lignin (23%), cellulose (40%) and hemicellulose (33%) with xylan as the major component of hemicellulose (Coughlan and Hazlewood, 1993). Next to cellulose, hemicelluloses are most abundantly naturally occurring polymers and are present in association with lignin and cellulose in plant cell wall. The existence of covalent linkages between carbohydrates and lignin molecule in wood tissue forming lignin carbohydrate complex (LCC), has been studied (Karlson and Westermark, 1996).

Hemicelluloses are usually classified according to the sugar residues present, for example as D-galactan, D-manan and D-xylan, the L-arabinans are often associated with the pectic polysaccharides from a number of plant sources but they are usually included in the hemicellulose group. Most of hemicelluloses, however occur not as homoglycans, but as heteroglycans containing different types of sugars residues, often as short appendages linked to the main backbone chain (Deckker and Richards, 1976). β-(1, 4) linked D-xylose residues constitute a backbone which also contains arabinose, glucuronic acid and/or mannose substitutes (Arribas et al., 1995).

After cellulose, xylan is the most abundant renewable polysaccharide in nature accounting for 20-30% of the dry weight of woody tissue. β-1, 4-xylan are heterogenous polysaccharide found in cell wall of all land plants and in all plant components. Since it has been estimated that 500 million tons of such material could be annually available from the residues of major crops (Detroy, 1981), xylan is an abundant, under utilized resource. Xylans are important not only because of their structural roles in plant cell wall (Fry, 1986) but because they are targeted as polymers to be modified in commercial application and in biomass conversion (Wong and Saddler, 1992). Underutilized lignocellulosic materials could be exploited for commercial production of enzymes and easily fermentable sugars (Ball and McCarthy, 1988).

Xylanases are enzymes which catalyse the hydrolysis of xylan the most abundant hemicellulose in nature. Xylanases are of considerable interest as catalysts in various
biotechnological applications e.g. in biobleaching, food and feed industry (Coughlan and Hazelewood, 1993). The abundance of xylan indicates that xylanolytic enzymes can play an important role in bioconversion, in the preparation of cellulose pulp and in fibre liberation technology, etc. (Biely, 1985; Wong et al., 1988). The expansion in the use of lignocellulosic agricultural and forestry waste will depend greatly on increased availability of microbial β-xylanase.

2.2 Xylan: Structure

Xylans are complex heteropolysaccharides β-linked xylose residues and are classified on the basis of the nature of linkage joining these residues. Xylans having a β-1, 3-linked backbone are found only in marine algae (Dekker and Richards, 1976) while β-1, 4-linked xylans are characteristics of hardwoods, softwood and grasses (Timmel, 1967) Figure 2.1.

2.2.1 Hardwood

The xylan of hardwood, which account for 10-35% of dry weight (Dekker and Richards, 1976) is typically acetyl 4-0-methyglucuronoxylan (DP200). Approximately 10% of the backbone xylose units are α-1, 2-linked to a 4-0-methyl-α-D-glucuronic acid residue, while 70% of the xylose residues are acetylated at C-2 or C-3 or both. Most hardwoods contain small amounts of rhamnose and galacturonic acid as integral components of main chain.

2.2.2 Softwoods

The xylan of softwoods (10-15% of dry weight) is an arabino-4-0-methyglucuronoxylan (DP>120) and differs from that of hard wood in not being acetylated. Substituents are 4-0-methylglucuronosyl residues attached to C-2, & L-arabinofuranosyl residues attached to C-3 of the relevant xylose backbone unit. The average ratio of the sugar units is 100:20:13 (xyl:4-β-Me-GlcA:Ara) (Puls & Poutanen, 1989).
Figure 2.1 Xylan structure and the sites of its attack by microbial xylanolytic enzymes.

The fragments comprise of seven D-xylose units, Ac, acetyl group, L-arabinofuranose and 4-O-methyl-D-glucuronic acid.
2.2.3 Grasses and Cereals

The xylan of grasses and cereals is also an arabino-4-0-methylglucuronoxylan (DP 70), but it has a lower 4-0-methylglucuronic acid content than does hardwood xylan, and a larger proportion of L-arabinofuranosyl side-chains linked to C-2 or C-3, or both of the xylose main chain residues (Voragen et al., 1992). These xylans also contain 2-5% by weight of O-acetyl groups linked to C-2 or C-3 of the xylose units (Bacon et al., 1975). Approximately 6% of the arabinosyl side chain residues are substituted at C-5 with feruloyl group, while approximately 3% are substituted at C-5 with p-coumaroyl residues (Mueller-Harwey et al., 1986). The relative proportions of the substituents vary from species to species. Feruloyl and coumaroyl groups have been implicated in the formation of covalent linkages between xylans, xylan and lignin and between xylan and other cell wall polysaccharide particularly galacturonans (Joseleau et al., 1992).

2.3 Classification of xylanolytic enzymes

A set of enzymes called the xylanolytic enzymes are required to hydrolyse xylan completely, which reflects the complexity of xylan. According to the mode of action these enzymes have been grouped into three classes Figure 2.1 (Chandra and Chandra, 1994).

***Group (1):*** These enzymes act on the backbone of xylan and consists of:

a) Endo-β-4-xylanases (β-1, 4-D-xylan xylanohydrolase; EC 3.2.1.8)

These act on xylan backbone randomly and release xylooligosaccharides. The degree of hydrolysis depends on the solubility of xylan, degree of polymerization nature and degree of substitution and cooperativity of other xylanolytic enzymes. Mostly the access of β-1, 4-endoxylanases has been hindered by high degree of substitution due to stearic effects.

b) β-1, 4-xylosidase (β-1, 4-D-xyloside xylohydrolase; EC 3.2.1.37)

Acts at reducing and non reducing end releasing xylose residues and the efficiency of hydrolysis increases with less degree of polymerization.
Hydrolyze xyooligosaccharides with degree of polymerisation 3-6 xylose. (Kubata et al., 1994).

c) \( \beta-1, 4\)-exoxylanase (\( \beta-1, 4\)-D-xylan xylohydrolase)

It is not clearly known whether exo-xylanase is a separate entity from \( \beta\)-D-xylosidase. These enzymes remove single xylose units. According to Kubata et al. (1994) \( \beta\)-D-xylosidases are designated as exoglycosidase, exoxylanase or xylobiase.

d) \( \beta\)-1,4-arabinoxylan xylanohydrolase

acts on xylan only if arabinose substitutions are present (Coughlan and Hazlewood, 1993).

Group (II): These are enzymes active on branching ends and consist of:

a) Acetyl xylan esterase (EC 3.1.1.6) which removes O-acetyl groups from C\(_2\) and/or C\(_3\) of xylose residues in xylan.

b) L-\( \alpha\)-arabinofuranosidase (EC 3.2.1.53) which removes L-arabinose residues substituted on C\(_3\) of xylose units of the xylan backbone.

c) 4-0-methylglucuronidase (EC 3.2.1.1) which releases \( \alpha\)-D-glucuronic acid residue from xylan backbone.

Group (III) enzymes are ferulic acid esterase and p-coumaric acid esterase and release respective phenolic acids substituted at C-5 position of arabinose. There are few report on these enzyme and are suggested to delink interaction between xylan and lignin (Christov and Prior, 1993). The sites of action of all these enzymes are shown in Figure 2.1.

Microbial degradation of xylans requires the synergistic action of several enzymes. Xylanases are the most studied because of their potential industrial application.
2.4 Areas of application

Xylanases are used in various processes where xylan is to be removed or degraded. Xylanases have major application in pulp and paper industry, bioconversion and to increase digestablitly of animal feed stock and in baking and brewing industry.

2.4.1 Pulp and paper prebleaching

In the industrially developed countries, xylanase has triggered wide spread interest in pulp and paper biotechnology involving "environmentally friendly" alternatives wherein they could be expected to play a crucial role. For applications in pulp and paper industry there is a requirement of xylanases that are almost free of cellulases since the cellulases affect cellulose the major component of paper and hence affect the quality of the product (Buchert et al., 1994). Cellulase free xylanase are currently a topic of considerable interest world wide following the realization of their potential impact in paper industry applications (Srinivasan and Rele, 1995).

Kraft pulping a process widely used in paper manufacture, removes about 95% of the lignin by alkaline sulphate cooking. The remaining lignin gives the pulp a brown colour which is removed in a multistage bleaching process with a variety of agents. The pressure to reduce the use of elementary chlorine (Cl₂) and chlorine dioxide (ClO₂) in the production of bleached sulphate pulp has led to active research concerning the use of enzymes for bleaching. Enzymes including (endo-1,4-β-xylanases, EC 3.2.1.8 ), have been shown to reduce the amount of chlorine required to achieve comparable levels of paper brightness (Tremblay and Archiebald, 1993; Viikari et al., 1994 b). Alkali extraction of chlorinated derivative of lignin comprise of dioxin and related compounds which are toxic and carcinogenic and being recalcitrant to chemical or biodegradation pose serious hazards as environmental pollution.

Xylanase pretreatment of kraft pulp has been consistently shown to reduce the brown colour of the pulp with concomitant usage of much less chlorine compounds. (Viikari et al., 1986) observed that xylanase pretreatment of pulp caused substantial reduction in residual lignin content and saving in bleach chemical, and also reduced AOX (adsorbable organic halogens and dioxins). This strategy avoids the use of chemical processes which are very expensive and polluting ( Lapidot et al., 1996).
Enzymes including xylanase (endo-1, 4-β-xylanase, EC 3.2.1.8) have been shown to reduce the amount of chlorine required to achieve comparable levels of paper brightness. However, the mesophilic enzymes currently in use have limitations because high temperatures are used in bleaching.

Faced with market, environment and legislative pressures the pulp and paper industry is modifying its pulping, bleaching and effluent treatment technologies to reduce the environmental impact of mill effluents (Samdani et al., 1991). In a report to the National Biotechnology Advisory Committee of Canada Bourbonnais et al. (1991), identified xylanase pre bleaching of kraft pulp as one of the biotechnological processes most likely to be applied in the pulp and paper industry in the near future. Xylanase preparation from *Trichoderma* sp. have been used for studying bleach boosting (Viikari et al., 1990). In Laboratory experiments, the reduction of active chlorine loading required during chlorination stage has been reported to range from 35-41% for hardwoods and 10-26% for soft woods. These xylanase pretreatment were found to be effective using enzyme leadings as low as 1-5 kg of commercial enzymes for each tonne of dry pulp and incubation time as short as 0.5 to 3 hrs.

Active pursuit of the concept of xylanase prebleaching in recent years has led to numerous mill trials (Grant, 1991). Long term use of enzymes has been reported to the Enso-Gutzeit (Korpivaaro, 1992). Metsa-Botnia and Sunila (Pearson, 1992) mills in Finland. This rapid development of xylanases prebleaching was partially due to stringent environment legislation and stringent enforcement of these legislations in Europe has led to numerous trials mainly in Europe. The Scott mill of Miranda, Spain currently utilized the xylanases prebleaching process developed by Sandoz Corporation in production of pulp. Full scale trials in Canadian Kraft Mills have been successful (Scott et al., 1993) and the commercial potential for such a product is indicated by the use of xylanase pretreatment in full scale production in 10 mills, 6 in Europe and 4 in Canada (Jurasek and Paice, 1992). Some commercial xylanases for enzyme aided bleaching are presented in Table 2.1.

Use of hemicellulase including xylanases for delignification in the paper industry has been slowed down by the lack of large scale availability of enzymes which are active at a high pH (above 8) and a high temperature (above 60°C) condition prevailing in many bleaching process. During the past years acidic neutral hemicellulases working
Table 2.1 Commercial xylanases (Viikari et al., 1994 a)

<table>
<thead>
<tr>
<th>Product</th>
<th>Application pH</th>
<th>Application Temperature (°C)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irgazyme 10</td>
<td>5-7</td>
<td>35-55</td>
<td>Genencor</td>
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<tr>
<td>Irgazyme 40</td>
<td>6-8</td>
<td>35-70</td>
<td>International</td>
</tr>
<tr>
<td>Cartazyme HS</td>
<td>3-5</td>
<td>30-50</td>
<td>Sandoz</td>
</tr>
<tr>
<td>Cartazyme HT</td>
<td>5-8</td>
<td>60-70</td>
<td>Chemicals</td>
</tr>
<tr>
<td>Ecopulp</td>
<td>5-6</td>
<td>50-55</td>
<td>Alko, Ltd.</td>
</tr>
<tr>
<td>Xylanase</td>
<td>5-6</td>
<td>55</td>
<td>Iogen Corp.</td>
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<tr>
<td>Xylanase</td>
<td>7-8</td>
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<tr>
<td>VAI-xylanase</td>
<td>6-7.5</td>
<td>65-75</td>
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<td>Pulpzyme</td>
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at temperature below 60°C were used in mill experiment. The Korsnas T6 xylanases from Bacillus stearothermophilus which is active at a pH above 9.0 and at a temperature above 65°C was produced on a large scale in collaboration with Gistbrocades and was employed on a full scale mill trial to produce a (TCF) pulp from soft wood. The bleaching sequence used was (OO) BQQPP where O stands for oxygen delignification, B for enzymatic treatment, Q for the chelating agent step and P for hydrogen peroxide step. The enzyme bleaching step was performed during a period of 4 hrs at 63°C ± 1°C and pH 8.7 ± 0. The results of the mill trial show that the TCF pulp produced had a brightness of 78% ISO and at the same time it presumed the same strength as chlorine dioxide, bleached pulp. The saving of hydrogen peroxide was 20%. The results on brightness strength and chemical saving of this first full scale trial with T-6 xylanase indicate that after optimization, a TCF bleaching sequence including an enzymatic step with a xylanase working at a high pH and a high temperature, such as T-6 xylanase, can be used to produce a high strength bleached pulp (Lundgren et al., 1994).

Successful mill trials have been carried out subsequently using Albazyme and Ecopulp. In May 1991, 35 tons of Albazyme -10 was used during a 4-week long trial at the Metsa-Sellu mill in Aanekoski (Finland) for the production of 35,000 tons of fully bleached pulps derived from soft woods and hardwoods with a total chlorine saving of 12% (Koponen, 1991). In these trials xylanase (active at acidic to neutral pH) was added before the chlorination stage after the kraft pulp had been acidified to provide conditions suitable for enzyme activity. The alkaline condition found at the prebleaching stages have prompted the search for the development of alkali tolerant xylanases by Novo Laboratories (Singh, 1979). Thermal tolerance is also a desirable characteristic as chemical prebleaching stages are run at temperature ranging from 50-100°C.

The price of the enzymatic treatment today is estimated to be about 2-5 USD per ton of pulp (Viikari et al., 1991 b). The price of the enzymes are expected to decrease as more efficient production strains and technologies are adopted.

2.4.1.1 Mechanisms of xylanase prebleaching

The way in which xylanase prebleaching affects subsequent bleaching of kraft pulp is not clear. Since little enzyme and short incubation time are required for the bleach boosting effect, one hypothesis proposed is that xylanases act on the surface of pulp fibers removing xylan that had reprecipitated after kraft pulping (Vilkari et al., 1991
b). The removal of reprecipitated xylan from the surface of kraft pulp may enhance subsequent chemical bleaching by one or more of the following mechanisms (Wong and Saddler, 1992).

1. Cleavage of the carbohydrate portion of lignin-carbohydrate complexes to produce smaller residual lignin molecules, which are easier to remove.

2. Solubilization of chromophores formed in xylan during kraft cooking.

3. Solubilization of xylan deposits that entrap residual lignin or block penetration of bleaching of chemical.

4. Swelling of the fiber matrix, by disrupting adsorptive interactions between xylan and cellulose, which in turn facilitates delignification.

The main problem with enzyme that the pulp industry is facing at present are the availability and cost of the enzymes as well as the quality standards and residual cellulase contamination. To keep enzyme production cost low agricultural wastes are frequently suggested or used (Wizani et al., 1990). These substrates contain significant amounts of cellulase inducers and therefore may not be used for culturing a cellulase secreting microorganism.

2.4.2 Saccharification of waste

Perhaps the most compelling early stimulus for saccharification of waste was the oil crisis of the seventies. This dictated the search for alternate sources of fuels and chemicals feedstock. Biomass in the form of fuel crops, agricultural and forestry residues and wastes are generated in vast tonnages annually. Since lignocellulosic materials represent the only cheap, renewable source of biomass capable of replacing petroleum as a feedstock for fuel and chemical production, biodegradation of lignocellulose is one of the nature's most important biological process. Saccharification of lignocellulose to sugars that can be used either for production of organic solvents such as ethanol (Xu and Taylor, 1993) or single cell protein (Okeke and Obi, 1992) will help solve two serious problems facing mankind today; that of energy and food shortages (Smith et al., 1985).
Production of ethanol from agricultural and forestry residues, municipal solid waste, crops and other forms of lignocellulosic biomass could improve energy security, reduce trade deficits, decrease urban air pollution and contribute little if any net carbon dioxide accumulation to the atmosphere (Wyman, 1994). The use of xylanases in conjunction with cellulases for the complete conversion of cellulosic biomass to sugars has been widely studied (Biely, 1985) and it can greatly reduce costs in processing lignocellulosic biomass (Gilbert and Hazlewood, 1993).

There has been interest in the production of xylose, xylobiose and xylooligomers (Biely, 1985). Most recent reports show that sugars can be prepared by enzymic hydrolysis of xylan (Walch et al., 1992; Okeke and Obi, 1995). Xylitol, a sugar alcohol is a naturally occurring polyalcohol. It is produced commercially and is used in some foods because of a number of advantageous and natural properties. It is a natural sweetener of higher sweetening power than common polyols (Pepper and Oligner, 1988) and is as sweet as sucrose. A further useful property is that it does not need insulin to regulate its metabolism (Emodi, 1978) and therefore can be used as a sucrose substitute in clinical diabetic foods. The most significant property of xylitol is that it is an anticarcinogenic sweetener, and can be promoted for oral health and caries prevention. Therefore, the bulk of production is consumed in various food products such as chewing gums, sweets, soft drinks and ice-cream (Emodi, 1978). Several microbial processes have been studied for xylitol production. The process optimised by (Horitsu et al., 1992) employing Candida tropicalis seems to be very economical. With the exception of xylose conversion to xylitol, the bioconversion of lignocellulosic material to fermentable sugars does not yet appear to be economic because of more competitive sugars such as starch and sucrose (Linko et al., 1989). However the massive accumulation of agricultural, forestry and municipal solid waste residue are creating a large volume of low value feed stock (Lynd et al., 1991). Alternate technologies are desirable for dealing with all these materials even if they form the perspective of waste management. One alternate is bioconversion to produce fuel ethanol, single cell, protein, xylanases and other chemicals, from xylan rich materials (Beily, 1985). The high specificity of enzyme reactions and the absence of substrate loss due to chemical modifications makes advantageous the use of microbial enzymes in the industrial hydrolysis of lignocellulose (Arribas et al., 1995).
2.4.3 Other uses

Other applications proposed for xylanases include, debarking, refining pulp fibre and preparing dissolving pulps. The major aim of enzymatic debarking and pulp refining is reduction of energy demands for these mechanical processes. For debarking a precise detachment of the bark at the cambium would also reduce the loss of wood for lumber, pulp and paper production (Viikari et al., 1991b). In enzymatic beating, the enzymes are added to bleached pulp fibers to increase external fibrillation and these improve paper making properties (Noe et al., 1986). Xylanases provide enhancement of this process.

Dissolving pulps are used to produce cellulosic materials such as acetates, cellophanes and rayon (Hinck et al., 1985). Cellulose esters, ethers and viscose rayon are manufactured from dissolving pulp that are wood derived cellulose. Commercial products made from dissolving pulp include cellophane packaging plastics, sponges, sausage casting man made fibres photographic films and cigarette filters (Hiett, 1985). Hemicellulosic contaminants lead to colour haze in products and can adversely effect the strength of end product. Their extraction from pulps requires the use of high caustic loadings and appropriate pulping conditions and is restricted to sulfite pulping and acid pretreated kraft pulping. The use of xylanase for purifying cellulose was first proposed by (Paice and Jurasek, 1984). The inaccessibility of a large portion of the xylan in pulps, however has limited the potential of the application.

The use of xylanases has also been proposed for clarifying juices and wines, for extraction of coffee, plant, oils and starch (Woodward, 1984; Biely, 1991) for improving the nutritional properties of agricultural silage and grain fuel, for macerating plant cell walls, producing food thickeners and for providing different textures to baking products (Bray and Clarke, 1995). They are already produced on an industrial scale for used as food additive in poultry, for increased feed efficiency diets (Annison, 1992; Classen, 1996) and in wheat flour for improving dough handling and the quality of baked products (Rouau et al., 1994).

2.5 Microbial biodiversity

Xylan degrading enzymes are produced by a wide variety of microorganisms, including aerobic and anaerobic mesophiles and xylan degrading thermophiles (Tan
et al., 1985) Xylanases are widely distributed and they occur in both prokaryotes and eukaryotes (Dekker and Richards, 1976) and have been demonstrated in higher eukaryotes, including protozoa, insects, snails or germinating plant seeds (Taiz and Honigman, 1976). The presence of microorganisms that degrade hemicellulose was reported over 100 years ago by Hoppe-Seyler (1889) who described gas production process using wood xylan suspension and river mud microbes. Microorganisms including bacteria fungi and yeast have also been reported to produce xylanase (Wong et al., 1988; Uffen, 1997). Some of the microbes are saprophytes, free-living soil or aquatic cells, some grow anaerobically, some grow at room temperature (mesophilic conditions), while others grow thermophilicly and some follow a host specific life style in ruminant animals or in intestines of wood eating insects. Under mesophilic growth conditions, xylanolytic activity has been reported in a wide variety of different genera and species of fungi and yeast (Wong et al., 1988). For eg. xylan degradation occurs in strains of Aspergillus niger, A. fumigatus, T. harzianum, T. reesi, T. viride, P. janthinellum, P. wortmani, P. capsulatum, Neurospora crassa (Filho, 1996), Pichia stiptis, Aurobasidium pullulans, Candida sheathe, Fusarium oxysporum (Biely, 1985). Thermophilic fungi that degrade xylan are Humicola lanuginosa, Thermoascus auranticus, Sporotrichum thermophile and Talaromyces byssochlamyroides (Shao, 1995).

Gram-positive, spore forming bacteria are ubiquitous soil microbes that play an important role in plant biomass turnover. These spore forming bacteria either respire and grow aerobically or grow under anaerobic conditions. Among aerobic or facultative anaerobic species that play an important role in plant biomass turnover, xylanolytic activity has been reported in Bacillus subtilis, B. circulans, B. pumilis, and B. polymyxa (Wong et al., 1988). Streptomyces species with xylanolytic activity include Streptomyces exfoliatu s, S. flavogriseus, S. lividans, S. xylophagus and S. halstedii (Wong et al., 1988). Strictly anaerobic, fermenting microbes which grow under mesophilic conditions have also been reported such as Clostridium acetobutylicum, C. stercorarium, C. papyrosolvens (Wong et al., 1988).

The Gram negative staining aerobic, non spore forming soil microbes, Pseudomonas fluorescens subs cellulosa has been shown to degrade xylans (Gilkes et al., 1991). Other Gram-negative fastidiously anaerobic group residing in ruminant animals is Butyri vibrio (Gilkes et al., 1991). Aeromonas caviae ME-1 was recently isolated from intestines of herbivorous insect (Kubata et al., 1995).
In addition to mesophilic temperature conditions and environment near neutral pH values a host of unidentified bacteria inhabit extreme environmental conditions where they thrive and grow at pH values 9.0 or greater (Gilead and Shoham, 1995; Yang et al., 1995). *Bacillus sterothermophilus* (Gilead & Shoham, 1995) is one aerobic thermophilic spore forming bacteria degrading xylan. Anaerobic, spore forming thermophilic cells including *C. thermocellum, C. thermosaccharolyticum* (Biely, 1985) and other are the *Thermoanaerobacterium sp.* (Biely, 1985). The use of microorganisms at temperature above 50°C and in alkaline conditions is specially desirable for Kraft pulp treatment in paper industry (Nissen et al., 1992; Yang et al., 1995).

### 2.6 Xylanases from thermophiles

#### 2.6.1 Thermostable enzymes

Thermostable enzymes constituted 90% of total enzymes sold worldwide in 1984 (Ng & Kenealy, 1986). The use of high temperature is necessitated by technical and economical reason, as reaction at higher temperature are faster thus more production and less prone to microbial contamination (Klibanov, 1983). The use of thermostable enzymes improves the process efficiency and adds to cost savings. Most of the industrial enzymatic processes are run at temperatures above 50°C (Ng and Kenealy, 1986). These include liquification and saccharification of starch, isomerization of glucose to fructose, conversion of starch to cyclodextrins, washing of clothes in laundry, hydrolysis of proteins, hydrolysis and modification of fats, biobleaching of pulp, food processing, hydrolysis and modification of fats, clarification of juices, hydrolysis of lactose, etc. (Klibanov, 1983; Ng & Kenealy, 1986; Zamost et al., 1991). In paper manufacture it is mainly lignin fraction that gives dark colour to unbleached pulp and the lignin fraction is in large part bound to cellulose and linkage formed by hemicellulose (Biely, 1985). Xylanases can be applied to unbleached pulp to hydrolyse the bond that links, the cellulose to lignin and lignin can be washed away rather than bleached. Use of xylanase for biobleaching will be most economical if enzymes are active and stable at high temperature (Upto 130°C) and pH value (>10) that occur during pulping such enzymes will allivate the necessity for cooling and subsequent reheating of the large pulp mass and save on acid required for pH adjustment (Mathrani and Ahring, 1992). Therefore enzymes tolerating high
temperature and pH values are needed in order to make the enzymatic process technically and economically more feasible.

The use of thermostable enzyme improves the process efficiency and adds to cost savings. The thermostable enzyme last longer during operation and storage because of their stability against inactivation by heat as well as other denaturants like organic, solvents, chemicals, oxidizing agents, freeze thawing and chaotropic agents (Ahern and Klibanov, 1985).

### 2.6.2 Sources of thermostable enzymes

Thermostable industrial enzymes have been traditionally obtained by screening natural microbial isolates (Ng Kenealy, 1986). The approach of screening of natural isolates is expected to gain more importance as more novel microorganism especially thermophiles are discovered (Tomazic, 1991). In general the higher the growth temperature of the organism the more stable are the enzymes (Langworthy et al., 1979). The screening process allows discovery of enzymes with better thermostability as well as with novel catalytic activities. Second approach is stabilization of existing enzymes through additives, chemical modification, immobilization of protein engineering (Janecek, 1993). Another approach is designing of new proteins (Janecek, 1993). The latter two approaches requires understanding of molecular mechanism of thermostability and catalysis and have met with limited success (Tomazic, 1991).

### 2.6.3 Xylanases from thermophiles

Interest in thermophilic microbes both aerobic and anaerobic, has increased markedly in recent years and many new and interesting bacteria have been described (Bergquist et al., 1989; Brock, 1985). The enzyme from the thermophilic microorganisms exhibit resistance to the denaturing effects of high temperature and solvents and have high activity at thermophilic temperatures (Zeikus, 1979).

To date the highest growth temperature for an organism is 110°C for archaeabacteria *Pyrodictium brockii* (Stetter et al., 1983). Until recent reports of the eubacterium *Thermotoga maritima* growing optimally at 80-85°C (Huber et al., 1986: Huser et al., 1986). All organisms known to grow optimally above 75°C were archaeabacteria.
The extremely thermophilic archaebacteria are therefore likely sources of the most thermostable enzymes. Little is known of their extracellular enzymes.

Okazaki et al. (1984) reported production of xylanases from four *Bacillus* strains with temperature optima 65-70°C and pH optima of 6.0-7.0. The maximum temperature for growth was 55°C for all the four organism. Gruninger and Fiechter, (1986) reported a novel highly thermostable D-xylanase from *Bacillus stearothermophilus* growing optimally at 65°C. The catalytic temperature optima of the enzyme was 78°C at pH 6.5-7.5. The thermophilic, strict anaerobic *Caldocellum saccharolyticum* possess xylanase with optimum between 5.5 and 6.0 and temperature optimum of 70°C and thermal half-life of 2-3 min at 80°C (Luthi et al., 1990). Rajaram and Varma (1990) reported xylanase having an optima of 60°C and 70°C and a half life of 60 min at 65°C from *Bacillus thermoalkalophilus* grown at 60°C. Simpson et al. (1991) have described an extremely thermostable xylanase with optimum temperature of 105°C with a pH optimum between 5.5 and 5.5 from *Thermotoga* and is probably the most thermostable xylanase reported. However, highly thermostable and alkaline active xylanases have not previously been described. Mathrani and Ahring (1992) isolated xylanases from thermophilic *Dictyoglomus* showing activity in the range pH 5.5 to 9.0 temperature 80-90°C and thermal half-life of over 1 hr at 90°C.

The xylanase of mesophilic fungi, *Aspergillus* when grown at 45°C produced xylanase with optimum temperature of 80°C and pH 6.5 (Castro et al., 1997). Khasin et al. (1993) reported purification of enzyme from *Bacillus stearothermophilus* grown at 60°C showing an optimal bleaching effect on pulp at pH 9 and 65°C though pH optima was 6.0 and temperature optima of 75°C. Sunna et al. (1997) reported a xylanase active between 40°C and 90°C and pH values 5.0 & 9.0 from a thermophilic xylan degrading *Bacillus thermoleovorans* grown at 70°C pH 7. The temperature and pH optima of enzyme was 70-75°C and 6.0-7.0 respectively. These properties are desirable for applications in the pulp and paper industry for enzymic pulp bleaching processes (Perrolaz et al., 1991).

2.6.4 Molecular basis of thermostability

Thermal energy unlike extremes of pH and low water activity, penetrates across the cell envelope. The cellular constituents of thermophiles have adapted to function at
high temperature (Jaenicke & Zavodszky, 1990). It is the activity and stability of cellular components, like proteins ribosomes, nucleic acids and membranes at high temperatures which forms the basis of thermophilicity. There is a positive correlation between the thermophilicity of the source, organism and thermostability of proteins (Cowan, 1992).

The stability of thermophilic proteins is intrinsic and resides in the primary structure (Bergquist et al., 1987). Thermostabilization of proteins is achieved through optimization of intramolecular interactions, packing densities, internalization of hydrophobic residues and surface exposure of hydrophilic residues (Jaenicke and Zavodszky, 1990). A clean understanding of molecular mechanisms responsible for enhanced protein thermostability is still awaited (Reus and Adams, 1995).

The thermostability of an enzyme is a function of the enzyme stabilizing forces. These include hydrogen bonding, hydrophobic bonding, ionic interactions, metal binding and disulphide bridges. Each of these stabilizing forces, either by itself or in combination has been suggested as a possibility of enhanced thermostability (Klibanov, 1983). In addition other cell components like proteins polysaccharide, lipids etc. help in forming a supra molecular structure and immobilizes the enzyme. This fixation of catalytic active centers of enzymes stabilizes and protects it against thermal denaturation, that is why certain enzymes from thermophiles have been shown to be less stable in the pure state or in cell-free extracts than in vivo, integrity being enhanced by the factors in the cellular environment. Thus only a few additional salt bridges, hydrophobic interaction or hydrogen bonds can easily account for the increase in free energy of stabilisation required to elevate the relative stability of an enzyme of several degrees centigrade. At temperatures exceeding 100°C hydrophobic bonding may not be a primary effector of protein folding or stablyt. At such temperature an increased number of internal salt bridges may be major stabilizing factor (Coolbear et al., 1992).

2.7 Xylanases from alkaliphilic organisms

Alkaline xylanases will also find a number of other applications for example because of high solubility of xylan at alkaline pH (Grant and Horikoshi, 1992) alkaline xylanases may have good potential for hydrolysis waste to fermentable sugars. Xylanase activity at alkaline pH may be advantageous in pulp applications, in which
alkaline pulping and bleaching conditions are often used. Ohkoshi et al., isolated xylanases L, M, S from alkaliphilic, *Aeromonas* sp. No. 212 TACC 31085 (Ohkoshi et al., 1985). Xylanase L was most active at pH 7-8, while M & S were active at pH 6-8 and pH 5-7 respectively. Bansod et al. (1993) isolated from soil samples an alkalitolerant *Cephalosporium* (NCL 87.11.9) strain capable of rapid growth and xylanase secretion over a wide pH range (pH 4-10). Christakopoulos et al., (1996) reported two low molecular mass alkaline xylanase I and II from *Fusarium oxysporum* F3 having pH optima of 6.0 and retaining 68% and 51% of the maximum activity at pH 8.0. The organism was cultured at pH 8.

2.7.1 Xylanase from mesophilic alkaliphilic Bacillus strains

The first paper of xylanase of alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa (1973) and purified enzyme of *Bacillus* sp # C-59-2 had pH range from 6.0-8.0. In culture broth of *Bacillus* sp # C-125 two xylanases were found, xylanase N is active at pH 6-7 and xylanase A at pH 6-10 and even had activity at pH 12 (Honda et al., 1985 b). Ratto et al. (1992) reported the production of an enzyme from an alkalitolerant *Bacillus circulans* at 30°C and pH 9. The pH optima of the overall xylan saccharifying activity was 7.0 and 40% of the maximal activity was expressed at pH 9.0. Balakrishnan et al. (1992) reported xylanase production from an alkalophilic *Bacillus* sp grown at pH 10 at 28°C. The optima was at 60°C and pH 8. Nakamura et al. (1993a) also reported an alkaliphilic *Bacillus* sp. strain 41 M-1, isolated from soil that produces multiple xylanases extracellularly when grown at 37°C and pH 10.5. One of the enzymes, *Xyl J* is most active at pH 9.0 with temperature optima of 50°C (Nakumara et al., 1993b).

An alkaline active xylanase was produced by an alkaliphilic *Bacillus* sp isolated from Kraft pulp growing at pH 9-10 (Yang et al., 1995). The pH optima was 7 at 55°C. In the absence of substrate at pH 9 it was stable at 50°C for 30 min. Gessese and Gashe (1997) reported the production of alkaliphilic and thermophilic xylanase from an alkaliphilic *Bacillus* grown at 37°C and pH 10-10.5. The pH optima of the enzyme was 9 and temperature optima was 60°C. At pH 9 it retained 80% activity after heating at 55°C for 2.5 hours.
2.7.2 Xylanases from thermophilic alkalophilic Bacillus strains

Four thermophilic alkalophilic Bacillus strain (W1 JCM 2888), W2 (JCM 2889), W3 and W4 produced xylanases (Okazaki et al., 1984). The pH optimum for enzyme action for strains W1 & W3 was 6.0 and between 6-7 for strains W2 and W4. The enzymes are stable between pH 4-5 & 10.5 at 45°C for 1 hr. The optimum temperatures of xylanases are 65°C for W1 and W3 and 70°C for W2 and W4. The degree of hydrolysis of xylan was about 20% after 24 hr incubation. After the finding of biological debleaching by xylanase, isolation of thermostable alkaline xylanases has been extensively investigated. Dey et al. (1991) isolated an alkaliphilic thermophilic Bacillus sp. (NCIM 59) that produces two types of cellulase-free-xylanase at pH 10 and 50°C both xylanases had an optima of 6 but retained 60-70% activity at pH 8.0. Khasin et al. (1993) reported thermophilic Bacillus stearothermophilus producing extracellular xylanase with pH optima was 6.5 but retained 60% activity at pH 10. Recently an alkaliphilic and thermophilic xylanase producing Bacillus sp. strain TAR-1 was isolated from soil grown at 50°C and pH 10.5. The pH optima was 6 at 50°C and retained about 80% activity at pH 9 (Nakamura et al., 1994).

2.8 Microbial sources of cellulase free xylanase

As a result of recent successes in application of xylanases in enzyme aided bleaching of kraft pulp, xylanases have acquired major potential as bulk enzymes for large industrial application. An important criterion for xylanase preparations for use in cellulase free pulp industry is very low cellulase activity. Some results have been published concerning removal of cellulase from mixed activity culture filtrates of T. harzianum (Tan et al., 1988). However, a more realistic approach would be to use microorganism producing high level of xylanase with only low or no cellulase activity. Among the reports of successful identification of cellulase-free xylanase, Chainia (NCL82-5-1) an actinomycete isolated from the desert sands of Rajasthan (Srinivasan et al., 1984) secreted 8-10 IU/ml of xylanase on media with cereal bran while higher activity of 26 IU/ml was secreted when pure xylan was used. The major component of xylanase is a low molecular weight protein (Ca 5000 dalton) with optimal activity towards xylan at pH 5-7 and 55-60°C. No detectable cellulase activity towards carboxy-methyl cellulose was exhibited by xylanases rich culture filtrate. A
thermotolerant actinomycete T-7 was identified by Keskar et al. (1989) with cellulase free xylanase showing remarkable stability at temperature of 60°C.

Search for xylanases active at and stable to highly alkaline pH conditions have intensified in more recent years in keeping with the requirements of pulping operations which are carried out at a high pH and temperature (Zamost, 1991). An obligate alkalophilic Bacillus secreting high activity xylanase was identified by Srinivasan et al. (1988) from decomposing coconut fiber collected at Calicut Kerala. The enzyme was cellulase free and in media containing wheat bran and organic nitrogen the culture secreted up to 150 IU/ml. The xylanase was optimally active at 60°C and pH 8.0. Over 75% of the activity was retained at pH 9.0 and 60°C which is a very positive and desirable attribute with regard to its application potential in paper industry (Balakrishnan et al., 1992). An alkalo thermotolerant Bacillus sp secreting cellulase free xylanase was investigated by Dey et al. (1991) who characterized the enzyme as consisting of two components active over a pH range of 6-10 and 50-60°C. Other reports of xylanases active at high alkaline pH from Bacillus strains include those of (Ratto et al., 1992) who recorded activity of 400 IU/ml in 2 days on a medium containing beech xylan and yeast extract at pH 8-8.5 from an alkali tolerant Bacillus circulans and (Nakamura et al., 1993a) who observed production of extracellular xylanase higher at pH 10.5 compared to pH 8.0 with Bacillus strain 41 M-1. The xylanase activity from B. stearothermophilus active at pH 9.0 and 65°C has been successfully tested for biobleaching and characteristics of the enzyme have already been published (Khasin et al., 1993). Samian et al. (1997) have reported a high level ie. 1000 U/ml production of cellulase free xylanase in glucose limited fed batch culture of a thermophilic Bacillus strain (mutant).

Vyas et al. (1990) reported alkalophilic Streptomyces secreting cellulase free xylanase active at pH above 9.0. Bansod et al. (1993) isolated a Cephalosporium strain growing above pH 9.0 and secreting a cellulase free xylanase active at and stable to highly alkaline conditions. This is the first report of a xylanase of fungal origin stable and active at high pH which is also cellulase free.

2.9 Regulation of xylanase synthesis

In non-cellulolytic bacteria and yeast, xylanolytic enzymes appear to be inducible: xylanase and xylosidase are produced in high amounts during growth on xylan and
Figure 2.2 The xylanolytic system

Cellular localisation of enzyme components and the regulation of their synthesis Glu, D-glucose; xyl, D-xylose; xyl2, xylobiose; xyl3, xylotriose
synthesis of the enzyme is catabolite repressed by well metabolized carbon sources such as glucose or xylose. Xylan cannot enter the cells, so that the signal for accelerated synthesis of xylanolytic enzymes must involve lower molecular weight fragments, namely xylobiose and xylotriose. The oligosaccharides are formed by hydrolysis of xylan in the medium by tiny amounts of enzymes produced constitutively Figure 2.2 (Biely, 1985). Xylanase was produced in small amounts even in the absence of the inducer, this phenomena is known as basal synthesis (Eriksson and Hamp, 1978). The low basal level of xylanase then reacted with xylan to produce a soluble molecule which entered the cell and effected induction.

In *Streptomyces* sp. the induction of xylanase with xylan was reported by Park and Toma (1974) while in another *Streptomyces* species xylanase could be induced by non-metabolizable methyl-β-D-xyloside (Nakanishi and Yasui, 1980). McCarthy et al. (1985) investigated four different thermophilic actinomycetes for xylanase secretion using xylan and oligosaccharides as inducers. They suggested that a small amount of constitutive xylanase activity could generate low molecular weight xylo-oligosaccharide from xylan which acts as inducers for further enhancement of xylanase secretion. However the monosaccharide xylose failed to induce xylanase in *Chanio*, as did glucose galactose and fructose when these were used as sole carbon sources (Srinivasan et al., 1984).

Esteban et al. (1982) showed that *Bacillus circulans* WL12 did not produce xylanase when grown on glucose medium and postulated that in this organism both induction and catabolite repression is the regulatory mechanism. The regulation of xylanase in prokaryotic organisms is not completely understood (Klupfel and Ishaque, 1982) since xylan is unable to enter the microbial cell the induction of xylanase is stimulated by low molecular weight xylan fragments which are produced in the medium by a small amount of constitutively produced enzyme. In case of *Aurobasidium pullans* it grew well in media containing glucose, fructose, xylan or xylose but β-xylanase was only produced with xylan or xylose. Lactose and maltose were poor substrates for growth (Karni et al., 1983). Xylanase production in some *Bacillus* sp. has been reported to be constitutive (Esteban et al., 1983) or be induced by xylose (Okazaki et al. 1984). In *Bacillus circulans* it was induced by xylan and on xylose and glucose low activities were detected (Ratto et al., 1992). Chandra Chandra (1995) reported *Aspergillus fischerii* produced cellulase free xylanase from arabinose, lactose, maltose and cellobiose and glucose induced low levels of xylanase.
(1.8-9.0 IU/ml) whereas xylan, xylose, wheat bran induced higher levels (34-45 IU/ml).

Purkarthofer and Steiner (1995) reported low xylanase activity in fungus *Thermomyces lanuginosus* on glucose, xylose, mannose, sucrose, arabinose compared to xylan and related that low activity constitutively formed enzyme without inducing substances. Even in the presence of easily metabolisable substances such as glucose fructose or lactose, xylanase is formed although the total activity in the presence of these repressors is low. Xylan had a dramatic effect on xylanase production as compared to D-xylose.

In case of *Bacillus circulans* B6 (Kyu et al., 1996) reported growth on D-xylose, L-arabinose, D-galactose, xylobiose, maltose, cellobiose and xylan. The highest level of xylanase was produced by xylan and low level induction with xylobiose and xylose at low concentration. The highest xylanase production was observed with 10 mg/ml xylan. At higher concentration the xylanase was repressed with the accumulated hydrolysis products resulting in catabolite repression of enzyme production. In a similar way, xylobiose and xylose had an inductive effect at the low concentration and their inductive effect were less pronounced at higher concentrations because of catabolite repression. As the type of growth substrate can effect the levels of enzyme production there are reports of agricultural residues that support xylanase production Balakrishnan *et al.* (1992) with an activity of 52 IU/ml on wheat bran as compared to 100 IU/ml on xylan.

Pham *et al.*, (1998) suggested that xylanase synthesis on xylan or xylan containing carbon sources eg. corn cobs, wheat straw, wheat bran, hardwood pulp and corn leaf is because xylan is necessary for the effective induction of xylanase by *Bacillus polymyxa*. This data may be explained not only because xylan is the main carbon source but probably also because its hydrolysis products act as inducers. According to Esteban (1982) since xylan is a large polymer which cannot be transported across the cell wall and membrane, it must be assumed that xylanases was induced by some xylan fragments from the action of low levels of extracellular enzyme. Xylanase was produced in small amounts even in the absence of the inducer (basal synthesis). This low level of xylanase then reacted with xylan to produce a soluble molecule which entered the cell and effected induction. Pham *et al.* (1998) reported higher xylanase production on treated corn cobs, xylan (birch wood), treated wheat straw, as compared to wheat straw, galactose, fructose, sorbitol, glucose and glycerol. They also
studied that when basal medium was supplemented with treated wheat straw (0.25% -2.5%) the highest levels of xylanase was detected with 0.5% wheat straw. The presence of decomposition products such as low molecular weight oligosaccharides present in treated substrates, contributes to the repression of xylanase production particularly when high substrate concentration are used (Mes-Hartree et al., 1988). Pinaga et al. (1993) and Pham et al. (1998) also reported an increase in xylanase activity when organic nitrogen ie. yeast extract was supplemented into xylanase production media stating that yeast extract is a critical component of the medium for Bacillus polymyxa growth providing a small amount of essential growth factor. Optimization studies for better xylanase yields have also been carried out by varying different parameters such as pH, temperature, aeration and medium composition and (Wood and McCrae, 1986).

2.10 Multiplicity of xylanase

The multiplicity of xylanase is of fundamental importance in the study of regulation of xylanase biosynthesis and xylan hydrolysis. The number of multiple forms differ considerably depending on source of enzyme, strain of the organism and culture conditions.

There are a number of possible explanations for these multiple forms of xylanases:(i) proteolytic modification of parental enzyme (ii) the existence of separate xylanase genes with distinct DNA sequences, or (iii) differential readout from mRNA (Yang et al., 1988). Dekker and Richards (1976) proposed that the complexity of xylans required the action of multiple xylanases with overlapping yet different specificities to effect extensive hydrolysis. These multienzymes systems suggest that every xylanase may have specialised function to perform a more effective xylan hydrolysis (Arribas et al., 1995). Some of this multiplicity has been demonstrated to be genetically determined. However, post-translational modification such as glycosylation, proteolysis or aggregation with other polysaccharides may also account for this multiplicity. Functionally identical isoforms differ in electrophoretic mobility, isoelectric point, sugar content and molecular weight.

The xylosidic linkages in lignocellulose are not equivalent and equally accessible to xylanolytic enzymes. The accessibility of some linkages also changes during the course of hydrolysis. The production of a system of enzymes, each enzyme with
specialized functions is one strategy that a microorganism may use to achieve superior xylan hydrolysis. Multiple xylanases have been reported in numerous microorganisms Dekker (1985). Five different xylanases have been purified from the culture filtrate of *Aspergillus niger* 11 and from Rhozyme a crude enzyme from *A. niger* (Wong *et al.*, 1988). The extent of xylanase multiplicity remains to be unanswered, particularly since a zymogram technique has detected five major and ten minor xylanase in the culture filtrate of *A. niger* 14 and three major and ten minor xylanase in Cellulysin a commercial enzyme from *Trichoderma viride* (Biely *et al.*, 1985). Extensive xylanase multiplicity in Cellulysin had also been reported by other workers (Labavitch and Greve, 1983). In *Bacillus* sp. Dey *et al.* (1991) reported two types of cellulase free xylanases Xyl I and II with molecular weight 35 kDa and 15.8 kDa, which exhibited immunological cross-reactivity and were glycoproteins. Xylanase I and II produced xylobiose as the major product along with xylotriose and higher xylooligosaccharides. Xylanase I released traces of xylose after 1 hour however xylanase I showed the presence of xylose only after 16 hours. An examination of xylanase multiplicity in *Bacillus* sp. suggests that these bacteria produce two xylanases. One is basic (pI 8.3-10.0) with a low molecular (16 to 22 kDa) by SDS-PAGE and other is acidic (pI 3.6-4.5) with high molecular weight (43 to 50 kDa). It appears that all xylanases isolated from *Bacillus* sp. fit into one of these two categories except for xylanase from *Bacillus subtilis* PAP 115 which has apparently intermediate molecular weight of 32 kDa. The two forms appear to be conserved in *Bacillus* sp. (Wong *et al.*, 1988). Xylanase heterogeneity may arise from posttranslational modification, such as glycosylation or proteolysis or both. Few xylanase from *Bacillus sp* are apparently glycosylated (Bernier *et al.*, 1983). Most of xylanases are apparently translated as precursors with signal peptide sequences (Fusakusaki *et al.*, 1984; Paice *et al.*, 1986; Hamamoto *et al.*, 1987). A pair of xylanases have been purified from many organisms including *Bacillus* sp (Honda *et al.*, 1985; Okazaki *et al.*, 1985) and two distinct genes have been identified in *Bacillus circulans* (Yang *et al.*, 1989).

Belanicic *et al.* (1995) reported a fungus *Penicillium purpurogenum* produces several extracellular xylanases. Two major forms xylanase A and B were purified with molecular weight of 33 kDa and 23 kDa. The antisera against both do not cross react and the amino terminal sequences of xylanase A and B show no homology. The results obtained suggest that the enzymes are produced by separate genes and that they perform different functions in xylan degradation.
One extreme is that multiple xylanases may be artifacts arising from the degeneration of microbial culture filtrates. On the other hand, each of the multiple xylanase may be a distinct gene product by a microorganism to enhance its utilization of xylan.

2.11 Purification of xylanases

In order to elucidate the mechanism of action of individual xylanase components it is necessary to use highly purified enzymes. Different methods have been reported for the purification of enzymes from culture filtrates. Mostly precipitation techniques followed by column chromatography is performed at low temperatures because most of the proteins are labile to high temperature denaturation. For purification of xylanases many methods have been employed. Uchino & Nakane (1981) reported that in Bacillus sp. 11-15 a strain of thermophilic acidophilic bacteria produced an extracellular xylanase during growth on xylan. The enzyme was purified from culture supernatant on disc-gel-electrophoresis. The molecular weight was calculated to be 56 kDa by SDS electrophoresis. The enzyme had a pH optimum for activity at 4.0 and stability range was pH 2.0–6.0. The temperature optima was 80°C (10-min assay). The enzyme retained full activity after incubation at 70°C for 15 min. The predominant hydrolysis products from larch wood xylan were xylobiose, xylotriose and xylose.

Ohkoshi et al. (1985) purified 3 xylanase from Aeromonas sp. NO. 212 ATCC 31085 from soil. DEAE cellulose ion exchange (500 g) matrix was equilibrated with 0.5% NaHCO₃ (pH 8.3) which was directly added to crude xylanase (5000 ml) and the mixture was stirred on overnight. The enzyme which was not absorbed on the DEAE-cellulose ion-exchange was precipitated with ammonium sulphate (70% saturation). The precipitate was dissolved in H₂O and dialysed against 50 mM acetate buffer (pH 5). The insoluble material formed was removed by centrifugation. The concentrated xylanase was loaded onto a CM cellulose column (2.5 x 25 cm) eluted with 50 mM acetate buffer and 0.5 M NaCl gradient, concentrated and loaded on Sephacryl S 200 column. Two (Xyl M and Xyl S) peaks were observed when column, was eluted with 50 mM PO₄ buffer (pH 7) containing 0.1 M NaCl. The enzyme that was adsorbed on DEAE cellulose column was eluted with 50 mM Phosphate buffer (pH 6.0) with 0-1 M NaCl. The active fractions were pooled and again loaded onto DEAE - cellulose column and xylanase L was thus obtained. Xylanase L & M were active at 50°C and S at 60°C. Xyl L was most active at pH 7-8 while M and S were at 6-8 and 5-7
respectively. Hydrolysis pattern after HPLC showed no significant difference. The products of hydrolysis of xylan were oligosaccharides such as xylobiose, xylotriose, xylotetraose, and high oligosaccharides.

Okazaki et al. (1985) reported purification of two xylanase from alkalophilic, thermophilic Bacillus sp. by DEAE-Toyopearl 650 M-Chromatography. Xyl I had molecular weight 21.5 kDa and pH optima of 6.0 and temperature optima 65°C and Xyl II had molecular weight 49.5 kDa and pH optima (7.0-9.0) and optimum temperature 70°C. Component I and II were inhibited by Hg** and Cu**, I hydrolyzes xylan to yield xylobiose and higher, oligomers while component II produced, xylose other than xylobiose and xylooligomers and the Km values were 4.5 and 0.95 mg/ml respectively.

Lee et al. (1987) reported the purification of two endoxylanase produced by C. acetobutylicum ATCC 824 which were purified to homogenity using ion exchange CM Sepharose, hydroxylapatite chromatography hydrophobic chromatography applied to column of Phenyl Sepharose, and Gel permeation chromatography Bio-Gel-P-150 (2.5 x 72 cm). Xylanase A with a molecular weight of 65 kDa hydrolyzed larchwood xylan randomly yielding xylohexose, xylopentose, xylotetraose, xylotriose and xylobiose as end products. Xylanase B, which has a molecular weight of 29 kDa also hydrolyzed, xylan randomly giving xylotriose and xylobiose as end products.

Grabski and Jefferies (1991) isolated one species of Streptomyces rosescleroticus (Chania rosea) NRRLB-11019 showing activity upto 16.2 IU/ml with red pigmentation in the broth. The purified xylanase enzyme had low molecular weight of 5.5 kDa by native gel filtration and denatured molecular weight was 22.6 kDa by SDS polyacrylamide. This could be that xylanase adsorbed to Superose 12 causing the xylanase to elute as if it were a smaller molecule, or the protein might be an elongated or tapered molecule allowing it to penetrate the agarose matrix and elute later. The pH optima was 6.5-7.0 and temperature optima was 60°C. The predominant products of hydrolysis included arabinose, xylobiose and xylotriose. Concentration increased the total activity by 13%. Ammonium sulphate precipitation removed most of the red pigments and resulted an 11-fold increase in purity. The pellet was dissolved in buffer and dialysed. The final steps in the purification were successive cation exchange chromatography columns. Carboxymethyl Biogel A agarose cation exchange removed the remaining pigments and acidic proteins. Xylanase proteins adsorbed to the column and eluted as a single broad peak. Fractions from this peak
were pooled, dialyzed and applied to the Mono-S column. Homogeneous xylanase was obtained by strong cation-exchange FPLC employing a Mon-S column. Four protein peak were detected via $A_{280}$. Peak II contained most of the xylanase activity and was used for characterization studies. The molecular weight was 5.5 kDa with pH optima of 6.5-7 and temperature optima of 60°C and $K_m$ 7.9 mg/ml and $V_{max}$ 305 U/min/mg.

Among fungi Ujjie et al. (1991) isolated an endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) from a commercial preparation of *Trichoderma viride*. Five gram of *T. viride* crude powder was dissolved in 100 ml of 10 mM phosphate buffer, pH 7.8 and applied to a DEAE Bio-Gel A column (2.4 x 50 cm) which was equilibrated and eluted with the same buffer. Unabsorbed fractions with xylanase activity were collected, concentrated and separated by TSK-Toyoperal HW50S gel chromatography. The column (2.5 x 90 cm) was eluted with 50 mM phosphate buffer pH 6.5). Purification steps were monitored by sodium dodecyl sulphate (SDS)-PAGE. The purified *T.viride* showed molecular weight of 22 kDa.

Tsujbo et al. (1992) purified two types of xylanase (1, 4-β-D xylan xylanohydrolase, EC 3.2.1.8) from the culture filtrate of a thermophilic actinomycete *Streptomyces thermoviolaceus* OPC-520. The enzymes (STX I & STX II) were purified by chromatography with DEAE-Toyopearl 650 M, CM-Toyopearl 650 M, Sephadex G-75, Phenyl-Toyopearl 650 M and Mono Q HR. The purified enzymes showed single bands on SDS electrophoresis. The molecular weights were 54 and 33 kDa respectively. The enzymes degraded xylan producing xylose and xylobiose as predominant end product indicating that they were endoxylanases.

Dey et al. (1994) reported an alkalophilic and thermophilic isolate *Bacillus* sp. (NCIM 59) from soil produced two types of cellulase free xylanase at pH 10 and 50°C. The two enzymes (xylanase I and II) were purified to homogeneity by ethanol precipitation, followed by Bio-Gel P 10 gel filtration and preparative polyacrylamide gel electrophoresis. The molecular weights of xylanase I and II were estimated to be 35 and 15.8 kDa respectively by SDS gel electrophoresis. The culture filtrate (100 ml) was precipitated with 3 volumes of chilled ethanol. The precipitate (15 mg) was recovered by centrifugation dried under vacuum and dissolved in 5 ml of 50 mM phosphate buffer pH 7.0, was then subjected to gel filtration on a Bio-Rad column (2.5 x 90 cm) equilibrated with 0.025 M PO₄ buffer pH 6.0. Two xylanase peaks were observed and they were designated as xylanase I and II. The enzyme had similar
temperature (50-60°C) and pH 6 optima. Both xylanases were stable at 50°C at pH 7 for 4 days. The apparent $K_m$ values using xylan as substrate were 1.58 and 3.5 mg/ml and $V_{max}$ values were 0.0172 and 0.742 umol/min/mg. The hydrolysis patterns demonstrated that xylanase were endoenzyme. Xyl I and II yielded mainly xylobiose, xylotriose and higher xylooligosaccharides with traces of xylose from xylan.

Nakamura et al. (1993 a) purified an alkaline xylanase from an alkaliphilic Bacillus sp. strain, 41M-1, which was capable of producing multiple xylanases extracellularly. One of xylanase (Xyl J) was purified to homogeneity by ammonium sulfate fractionation (20%) saturation and anion-exchange chromatography on DEAE - Toyopherol 650 M column (2.5 x 16.5 cm) and eluted with 100 mM NaCl. Zymogram analysis revealed two major activity band corresponding to molecular mass of 36 kDa and 25 kDa. Xyl J with molecular mass of 36 kDa was most active at pH 9.0 and optimum temperature was 55°C the enzyme was stable for 30 min at this pH and temperature. The predominant products of xylan hydrolysates were xylobiose, xylotriose and higher oligosaccharides.

In another report Khasin et al. (1993) from Bacillus stearothermophilus showed optimal bleaching of pulp at pH 9 and 65°C. The enzyme was purified and concentrated in a single adsorption step onto a cation exchange CM-52 and eluted with 1 M KCl and was made of single polypeptide with an apparent molecular weight of 43,000 determined by (SDS PAGE). The T-6 xylanase completely degrades xylan to xylose and xylobiose. The optimum activity was at pH 6.5, 60% activity remained at pH 10. At 65°C and pH 7 the enzyme was stable for more than 10 hrs at 65°C and pH 9 with a 1/2 life of 6 hrs. The enzyme activity was 2 U/ml with $K_m$ of 1.6 mg/ml and $V_{max}$ of 288 U/mg.

In Cellulomonas fimi (Khanna and Gauri, 1993) have reported the purification of 3 xylanases A, B, C from a 24 hrs old culture of Cellulomonas fimi in basal salt with CMC (1% w/v). After 30 hr at 37°C, the culture was harvested by centrifugation at 10,000 x g for 20 min. The culture supernatant was concentrated 10 fold by ultrafiltration on YM-10, Amicon membrane at 4°C. The crude concentrate enzyme preparation was used for column chromatography. The crude extracellular enzyme (15 ml was loaded onto a Q Sepharose column (30 x 2.5 cm) equilibrated with 10 mM sodium PO$_4$ buffer (pH 7.2) and eluted with the same buffer. After the unbound proteins were eluted the bound were eluted with a linear gradient of 500 mM NaCl and 10 mM sodium PO$_4$ buffer (pH 7.2) at a flow rate of 1 ml/hr and 6 ml fractions
were collected. The fractions showing xylanase activity were pooled and dialyzed against distilled H$_2$O for 16-18 hrs. The dialysed enzyme solution was then applied to column of Sepharose 6B (85 x 2.5 cm). The gel was equilibrated with 10 mM Sodium PO$_4$ buffer (pH 7.2) at a flow rate of 36 ml/hr. Proteins were eluted with equilibrating buffer and 6 ml fractions were collected. The molecular weights of 3 enzyme components were 13.2, 22, and 150 kDa respectively.

A 350 kDa was purified from a *Thermoanaerobacterium* sp. strain JW/SY-YS485 (Shao *et al.*, 1995). Xylanase activity was observed in only one peak during each step of purification. The enzyme was eluted as a broad protein peak from DEAE-Sepharose column together with an $\alpha$-glucuronidase and $\beta$-xylosidase. The xylanase bound weakly to Q-Sepharose and thus could be separated from other hydrolyase activities. The xylanase eluted from Mono P produce a single band when 3 $\mu$g of enzyme was applied to a native gradient gel. Subunits have M$_r$ of 180 kDa and 24 kDa by SDS electrophoresis. Few of the xylanase that have been purified have been reported to have more than one polypeptide; the exceptions are enzymes involved in the cellulase systems of *Thielamia terrestris* with the aggregated xylanase and cellulase in culture supernatants of the rumen anaerobe *Butyrivibrio fibrisolvens* (Lin and Thomson, 1991).

Belancic *et al.*, (1995) showed that fungus *Penicillium purpurogenum* produces extracellular xylanases. The two major forms (xylanase A and B) have been purified and characterized. After ammonium sulphate precipitation and chromatography in Bio-Gel P-100, xylanase A was further purified by means of DEAE-cellulose, hydroxylapatite and CM-Sephadex, and xylanase B by DEAE-cellulose and CM-Sephadex. Both xylanases showed apparent homogeneity in SDS polyacrylamide gel electrophoresis with Xyl A having molecular weight of 33 kDa with an isoelectric point of 8.6, while xylanase B (23 kDa) had an isoelectric point of pH 5.9. The amino terminal sequence of xylanases A & B show no homology. The results obtained suggest that the enzyme are produced by separate genes and they may perform different functions in xylan degradation.

Kang *et al.* (1996) reported two different xylanases CX-I and CX-II which were purified from an alkalophilic fungus, *Cephalosporium* sp. strain RYM-202. The enzymes had similar pH optima (7.5 to 8.0) and temperature optima (50°C) and were stable over a wide pH range of 5.5 to 12.0. The culture filtrate was fractionated by salting out with ammonium sulphate (30%-80%) saturation, dialyzed and
concentrated by ultrafiltration. The enzyme solution was purified by a series of column chromatography ie. DEAE-Scachryl A-50, Sephacryl S-200 HR, Superose 12HR10/30.

Gessese (1998) purified two xylanase Xyl A and Xyl B from the culture supernatant of alkaliphilic Bacillus sp. strain AR009. The cell free culture supernatant was precipitated using 70% ammonium saturation. The pellet was dissolved in 10 mM Tris-HCl buffer pH 8 and the dialysed enzyme applied to DEAE Sepharose column (2.5 x 12 cm) equilibrated with 10 mM Tris HCl buffer pH 8. The column was eluted with buffer followed by 0-0.5 M NaCl gradient. The concentrated fractions were applied to Sephadex G-75 (1.5 x 110 cm) and eluted at flow 12 l/h. Xylanase fractions were pooled and reapplied to the Sephadex column. The optimum pH for activity was 9 for Xyl A with temperature optima of 60°C. While for Xyl B it was pH 9 and 75°C. The xylanases were referred to as products of two genes as presence or absence of a protease inhibitor (2 mM phenylmethylsulphonyl fluoride) did not alter the results.

2.12 Mode of action of endoxylanases

Xylan degrading enzymes are produced by a wide variety of microorganisms including aerobic and anaerobic mesophiles and thermophiles (Biely, 1985; Wong et al., 1988). The study of several xylanolytic systems has permitted the corroboration of the fact that more than one xylanase is produced by each microorganisms (Dekker, 1985). Despite their industrial applications and their role in the bioconversion of renewable plant cell materials, little is known about the real mechanisms of xylan catalysis.

2.12.1 Bacterial endoxylanases

The endoxylanase of Bacillus and streptomyces fall under this category.

2.12.1.1 Bacillus endoxylanases

Two endoxylanase isolated from Bacillus circulans WL-12 showed different types of xylan attack. Xylanases II (pI 9.1) did not produce xylose from xylan, put principally xylobiose, xylotriose and xylotetrose. It was shown that this endoxylanase required a minimum of four β-(1-4)-D-xylopyranoside linked residues to form the productive
complex by cleaving the second $\beta$-(1-4)-D-xylosidic bond. Hence xylotetrose was the best substrate to saturate all the binding sites of the enzyme and was then immediately broken down to xylobiose. Endoxylanase I could rapidly degrade xylan to xylotetrose and with prolonged incubation gave xylose, xylobiose and xylotriose as the main end products in the hydrolysate (Esteban et al., 1982). The hydrolysate of xylan with the endoxylanases of a alkalophilic and thermophilic Bacillus sp. was analysed by TLC (Okazaki et al., 1985). The larger of xylo-oligosaccharide that were formed initially were further degraded to xylose and xylobiose on prolonged incubation. Khasin et al. (1993) reported a xylanase T-6 an endoxylanase that completely degrades xylan to xylose and xylobiose. Xylanase $X_{34}C$, $X_{34}E$ and $X_{22}$ of molecular weight 34, 34, 22 kDa were purified from Bacillus polymyxa (Morales et al., 1993). Xylose together with xylobiose was a major end product of the xylan by xylanases $X_{34}C$ and $X_{34}E$ but xylose was not an end product of xylan hydrolysis by xylanase $X_{22}$. When the endoxylanase $X_{22}$ is combined with xylanase $X_{34}E$ for xylan hydrolysis, xylose and xylobiose are released in high quantities. The $X_{34}E$ enzyme seems to have an exotype mode of action, and the action of $X_{22}$ could liberate xylooligomers increasing in this way the concentration of potential substrates of $X_{34}E$ enzyme. The hydrolysis of xyoligomers by xylanase $X_{34}E$ confirms the exotype mode of action of this enzyme. It is able to degrade almost completely xylotetrose and xylotriose with the release of xylobiose and xylose, where as $X_{22}$ enzyme does not seem to act on these xylooligomers.

2.12.1.2 Streptomyces Endoxylanase

Keskar (1990) reported that the major end product of xylan hydrolysis by Streptomyces T-7 endoxylanase was xylobiose and xylooligosaccharides with traces of xylose residues on short incubation. S. exfoliatus endoxylanase was studied for its action on xylan by paper chromatography and end product detected were xylose and xylobiose (Sreenath and Joseph, 1982). The low molecular weight (5500-6000 Da) Chainia sp. endoxylanase was studied for its mode of action on xylan, xylooligosaccharidies and tritium labelled xylohexose by HPLC (Bastawade, 1987). Xylan hydrolysis mainly produced, xylohexose and xylotriose as the major end products after a few minutes of incubation, while other xylo-oligosaccharides detected were xylobio, xylopetose and xylotetrose with traces of higher saccharides. The endoxylanase did not produce any xylose, even on prolonged incubation. Elegir et al. (1994) reported two xylanases in Streptomyces sp. Group I (Xyl I) enzyme acted on higher xylooligosaccharides as they did not fully hydrolyze $X_5$ and did not degrade $X_4$ to significant extent. The group II (Xyl II) degraded $X_4$ to various extents and
completely hydrolysed X₅ indicating that they are able to act on lower -DP substrates and produced xylobiose and xylotriose plus limited amount of X₄ and xylose from xylopentose.

2.12.2 Fungal endoxylanase

Reilly (1981) and Meagher et al. (1988) used a crude commercial preparation of Rhosyme HP-150 to isolate 5 endoxylanases with different biochemical properties. The first endoxylanase (Frederick et al., 1981) was selected for subsite mapping studies. This xylanase produced mainly xylobiose and xylose from xylan and xylo-oligosaccharide while the other four yielded longer chain length products failing to hydrolyse xylooligosaccharide less than DP4. An acidic endoxylanase from Aspergillus niger was studied for its substrate binding and subsite mapping analysis by Vrsanska et al. (1982). The bond cleavage data indicated that the substrate binding sites consisted of seven subsites. Bond cleavage frequency of this enzyme was totally dependent on its substrate concentration. The bond cleavage frequency of xylotriose to xylose was almost 90% at the first β-1-2-linkage of xylotriose, whereas xylotetrose was hydrolysed to xylobiose with 87% bond cleavage frequencies at the second β-1-4 linkage from the reducing end. The endoxylanase cleared the third β-1-4 linkage rather than the second or first xylosidic bond of xylopentose during hydrolysis. The same debranching endoxylanase has the capacity to resynthesize larger xylooligasaccharidids from xylobiose and xylose, which are further degraded to xylobiose and xylotriose.

Kang et al. (1996) reported two xylanases from an alkalophilic fungus Cehalosporium sp. Both xylanases were shown to be endotype xylanases, producing xylobiose (X₂) as the predominant end product from xylan along with X₃ and higher oligosaccharides as intermediates. Neither of the two enzymes were active on xylobiose. On the other hand, the enzyme readily attacked X₃ and finally accumulated X₂ as the major end product and X₁ as the minor product. Throughout the reaction, the amount of X₂ was much larger than X₁. The most plausible explanation for this pattern of degradation is that donor X₃ molecule was joined to an acceptor X₃ by transglycosylation activity of the enzyme to yield xylohexose (X₆), which was quite rapidly hydrolysed into X₂ molecule or into X₂ and X₄. The main hydrolysis product from X₄ were X₂ and X₃ and after prolonged reaction, the latter was further hydrolysed into X₂ without any accumulation of X₁.
2.13 Cloning of xylanase gene

The potential importance of xylanase for a variety of commercial uses has led to new approaches to develop hyperxylanolytic, fast growing industrially viable microbes capable of synthesising physico-chemically stable and end product inhibition resistant enzyme. To do this gene coding for these enzymes are presently being studied by recombinant DNA technology throughout the world. Thermoenzymes are a hot research topic because of which they are remarkable tools for developing commercial biotechnolology and for studying protein stability. Cloning of genes of thermostable enzymes into mesophilic host is a unique tool since thermoenzymes generally retain their thermal properties indicating that the unique thermal properties are genetically encoded (Zeikus et al., 1998).

Recombinant DNA technique have been used to analyse the xylan degrading enzymes of bacteria and fungi with the result that more than 70 relevant genes have been isolated from some 30 different species of bacteria and expressed for the most part in E. coli (Hazelwood and Gilbert, 1992). One obtains an idea of current impetus in this area by noting that about 75% of genes isolated were first described in paper between 1989-1991.

The fact that enzymes that depolymerize xylan by attacking the β-1, 4-linkage of the backbone are the most abundant components of xylan degrading enzyme systems (Wong et al., 1988) together with the ease with which the enzyme can be detected during the screening of gene libraries may explain why nearly 80% of the genes isolated to date encode endoxylanase while genes encoding other relevant enzymes have also been cloned. The genes encoding the full complement of enzymes involved in xylan hydrolysis have yet to be isolated from any single organism. However significant progress towards this goal has been made in studies conducted with Pseudomonas fluorescens subsp. cellulosa, Butyribrio fibriosolvens and Caldocellum saccharolyticum (Hazelwood and Gilbert, 1992). By means of sequence alignment and comparison of clustering of the hydrophobic residues that are pivotal in determining secondary structure, it has been concluded that the catalytic domains of xylanases are conserved between numerous species of bacteria and indeed between bacteria and fungi. Furthermore the fact that those sequenced to date may be assigned to only two families F & G (Gilkes et al., 1991) suggests that it is likely that
genes coding xylanases in many different species of xylanolytic microorganisms diverged from two progenitor sequences.

Several *Bacillus* sp. produce xylanases and the genes coding for these enzymes have been characterized in some instances (Fukusaki et al., 1984; Hamamoto et al., 1987; Paice et al., 1986; Yang et al., 1989). *Bacillus circulans* has been reported to produce at least two xylanases, but the xylan degrading system of this organism has not been studied at the gene level (Esteban et al., 1982).

Bernier et al. (1983) reported cloning of *Bacillus* gene in *Escherichia coli*. *PstI* cut chromosomal DNA ranging from 3-7 kb was introduced into *PstI* site of plasmid and a recombinant plasmid was obtained. The intracellular xylanase produced by transformed *E. coli* was 25% of that produced by parent and the molecular weight was 22 kDa.

Yang et al., (1988) cloned xylanase gene from *Bacillus polymyxa* in *Escherichia coli* HB101. The genomic fragments derived from separate and complete digestion by *EcoRI*, *HindIII* and *BamHI* were ligated into corresponding sites of pBR322, and the resulting chimeric plasmid were transformed into *Escherichia coli*. Of 6000 transformants screened (pBPX-277) produced a clear halo. The insert into the pBRX-277 recombinant was identified as a 8 kb *BamHI* fragment of *B. polymyxa* and was subsequently subjected to extensive mapping and a series of subclonings into pUC19. A 2.9 kilobase *BamHI-EcoRI* fragment was found to code for xylanase activity. The xylanase expressed by the cloned gene had a molecular weight of approximately 48 kDa and an isoelectric point of 4.9 and the *E. coli* product seemed to have higher molecular weight and it may be a prexylanase.

Yang et al. (1989) cloned two genes coding for xylanase in *Bacillus circulans* and expressed them in *Escherichia coli* pUC19. After digestion of genomic DNA from *Bacillus circulans* with *EcoRI* and *PstI* the fragments were ligated in component sites of pUC19 and transformed into *E. coli*. Restriction enzyme mapping of two inserts coding for xylanase activity indicated distinctly different nucleotide sequences. Cross hybridization assay showed absence of homology between two gene indicated that cloned genes encoded for proteins with molecular weights of 22 and 59 kDa are distinctly different.
It appears that these bacilli carry a common gene that codes for a 22 kDa mol wt. xylanase. Genes that code for such protein have now been identified in *B. circulans*, *B. pumilus*, and *B. subtilis* (Yang et al., 1988). A 22 kDa xylanase has also been identified in culture filtrates from fungus *Trichoderma harzianum* (Tan et al., 1985).

Kudo et al. (1985) cloned a gene with xylanase activity from alkalophilic *Aeromonas* sp. 212 (ATCC 31085) in *Escherichia coli* HB101 with pBR322 as the vector. Plasmid pAX1 was isolated from transformants producing xylanase and xylanase gene was located in a 6.0 kb *Hind* III fragment. The pAX1 encoded xylanase activity in *E. coli* HB101 was about 80 times higher than that of xylanase L in alkalophilic *Aeromonas* sp. 212. About 40% of the enzyme activity was observed in the periplasmic space of *E. coli* HB101. The pAX1-encoded xylanases had the same enzyme properties as those of xylanase L produced by alkalophilic *Aeromonas* sp. 212 but its molecular weight was lower (135 kDa VS 145 kDa as estimated by SDS PAGE). The difference might be due to the protein molecule eg. by glycosylation, that did not occur in *E. coli*.

Luthi et al. (1990) cloned a xylanase genes Xyl A of extreme thermophilic "Caldocelum saccharolyticum" over expressed in *Escherichia coli* by cloning the gene down stream from temperature inducible vector PJLA602. Induction of upto 55 times was obtained by growing the cells at 42°C, and the xylanase made upto 20% of the whole cell protein content. The enzyme was located in the cytoplasmic fraction in *E. coli*. The temperature and pH optima were found to be 70°C and pH 5.5-6.0 respectively. Xylanases was stable for 72 hrs when incubated at 60°C, with half life of 8-9 hrs at 70°C and 2 to 3 min at 80°C.

Shendye and Rao (1993 a) constructed a genomic DNA library of an alkaliphilic thermophilic *Bacillus* in *Escherichia coli* JM105 with pUC8 vector and was screened using a Congo red xylan plate clearance assay. Six xylanase positive transformants having identical inserts showed immunological reactivity towards polyclonal antibodies raised against purified xylanase (M, 15.8 kDa) from *Bacillus* were obtained. A 4.5 kb *Hind*III and *EcoRI* subfragment was found to code for two xylanases of molecular weight 14.5 and 35 kDa respectively. Equivalent amounts of xylanase activity were detected from IPTG induced and noninduced recombinants irrespective of orientation of 4.5 kb insert with respect to the lac promoter, indicating that xylanase gene expression was under the control of its own promoter. Majority (95%) of the xylanase activity (2 U/ml) was found in the extracellular culture filtrate. The hydrolysis of xylan by the recombinant xylanases yielded mainly xylobiose.
Tabernero et al., (1995) reported the cloning of genes from an alkalophilic Bacillus strain (N137) in E. coli MC 1061. It was further subcloned and sequenced and has an open reading frame of 993 nucleotides that encodes a protein of 331 amino acids. The predicted size of protein (39 kDa) is in full agreement with the molecular mass 40 kDa as determined by (SDS-PAGE). Xyl A did not have the typical N-terminal signal peptide that occurs in most of the secreted proteins.

Blanco et al., (1996) reported cloning of Bacillus sp BP-23 gene into E. coli using pBR322 vector. The intracellular enzyme in E. coli produced xylanase of 41 kDa which in the extracellular enzyme from Bacillus showed a xylanase band comigrating with it.

The expression of several Bacillus xylanases cloned in E. coli is lower than the parent organism (Yu et al., 1987; Bernier et al., 1983b) and the enzymes are intracellular in nature (Bernier et al., 1993b; Blanco et al., 1998). It is well known that most of secreted or exported proteins produced by Gram negative bacteria are trapped in the periplasmic space (Ramalay, 1979). The expression of the cloned gene can be modified by coupling the gene to a stronger promoter, eliminating the operator sensitive to repression and by increasing the efficiency of translation which is higher in the homologous host (Panbangred et al., 1985). A Bacillus pumilus gene was cloned in Escherichia coli C600 and Bacillus subtilis MI 111 using PUB110 as a vector. The B. subtilis clones produced 2.7-3 times as much xylanase as Bacillus pumilis. In E. coli it was much less than the parent. Bacillus subtilis secreted xylanase in the medium however more than 90% of xylanase activity was found in the cytoplasmic fraction of E. coli clones (Panbangred et al., 1983). Molecular weight of xylanase prepared from E. coli clones showed the same mobility as those from B. pumilis and B. subtilis culture fluid. The N-terminal amino acid sequence of xylanase in E. coli and B. pumilis suggested correct signal peptide processing in E. coli inspite of failure of secretion to the periplasm. In a homologous host system due to efficient transcription, translation and secretion, overexpression of the cloned gene product is possible. When genes from Bacillus (NCIM 59) were cloned in a B. subtilis A8 the recombinant produced xylanase which was five times as much as in E. coli recombinant, (Shend yand Rao, 1993 b).

Shend yeet al. (1994) reported the pLRX6.5 a recombinant plasmid harbouring 6.5 kB HindIII fragment of genomic DNA from alkalophilic thermophilic Bacillus NCIM
coding for xylanase was transformed into Bacillus subtilis M111. The xylanase expression in the transformant was 6-fold higher than in the host. Jeong et al. (1998) reported a high level of expression of an endoxylanase gene from Bacillus sp in Bacillus DB104. A shuttle vector pJH27Δ88 was used as an expression vector and recombinant plasmid containing 1.62 kb Smal DNA carrying the endoxylanase gene. The recombinant expressed 105 IU/ml which was 5 times higher than xylanase activity in E. coli carrying the recombinant plasmid.

Several Bacillus endoxylanase genes have been cloned and expressed in E. coli. However the expression levels were much lower than the parent organism and the expressed enzymes accumulated as inclusion bodies inside the cells, which is a limiting factor in continuous culture fermentations (Bernier et al., 1993; Panbangred et al., 1983; Shendye and Rao, 1993a). Since the discovery of the method of transforming Bacillus subtilis with plasmid DNA (Ehrlich, 1977), B. subtilis has become an attractive alternative to E. coli as a host for the expression of cloned genes because it has several advantages over E. coli. The greatest advantage is its ability to secrete proteins directly into the medium and accumulate them to a high level in a relatively pure state (Goeddle, 1990). The disadvantages are secretion of proteases (Binnie et al., 1997) and there have been numerous reports describing instability of recombinant plasmid in Bacillus and is widely known that plasmids are less stable in B. subtilis than in E. coli (Ehrlich et al., 1982; Kreft and Hughes, 1982).

In another approach Walch and Bergquist (1997) fused Xyn A structural genes from the extremely thermophilic anaerobe Dictyoglomus thermophilum Rt46B.1 in frame with the secretion signal of Kluyveromyces lactis killer toxin in the episomal expression vectors based on the Kluyveromyces plasmid pKD1. Xyn A was secreted predominantly as an unglycosylated 35 kDa protein which is comprised up to 90% of the total extracellular proteins. The protein co-migrated with Xyl A expression in E. coli and therefore was not glycosylated. This represents an improvement in the volumetric productivity of Dictyoglomus Xyl A of over 300 fold compared to that of E. coli expression system described by Gibbs et al. (1995).