DISCUSSION

4.1. Isolation, screening and identification of xylan-degrading fungi

Seventy fungal species were isolated from 25 soil samples collected from different parts of Southern Kerala. Several workers had reported the isolation of xylanolytic fungi from soil (Mukhopadhyay et al. 1997; Kvesitadze et al. 1999). The Czapek's medium containing 0.5% birch wood xylan was used for the initial screening. The suitability of Czapek's medium for the screening of xylanolytic fungi was reported by several workers (Bhalla and Joshi, 1993; Kvesitadze et al., 1999; Abdel-Sater and El-Said, 2001). Eventhough a clearing zone was obtained, after 7 days of incubation the clarity of the clearing zone increased when the medium was stained with 0.1% Congo red, followed by washing with 1M NaCl. Pajni et al. (1989) demonstrated the carboxy methyl cellulase activity with 0.1% Congo red. According to Capalash et al. (1990) Congo red plate assay (Teather and Wood, 1982) was the most widely used method for the screening of hemicellulase activities. The detection of clearing zone with 0.1% Congo red in Streptomyces sp. was done by Techapun et al. (2001). Bhalariao et al. (1990) detected the xylanase positive colonies by using 0.1% Congo red, followed by washing with 1M NaCl.

Among the 34 selected fungal species, Aspergillus was the most common genus and constituted 55% of the selected strains. Carmona et al. (1997) made an extensive study on xylanolytic microorganisms and observed that species of Aspergilli were efficient producers of xylanases. Penicillium was the second most common genus. Trichoderma represented the third common genus. Abdel-Sater and El-Said (2001)
reported that *Trichoderma* species were often cited as high cellulose decomposers and xylan degraders. Species of *Aspergilli, Penicillium* and *Trichoderma* were the best xylanase producers among ten fungal species isolated from decomposing wood (Medeiros et al., 2003).

4.2. **Xylanase production of selected strains by solid state fermentation (SSF) and submerged fermentation (SmF)**

Solid state fermentations are characterized by the complete absence of free liquid. These cultivation conditions are especially suitable for the growth of fungi which are known to be able to grow at relatively low water activities.

The xylanase production in SSF was much higher than that of SmF. In several studies, liquid and solid state cultivation procedures have been compared. Deschamps and Huet (1985) reported that solid state fermentation gave a higher productivity per unit volume. For *Cheatomium cellulolyticum* an increase in xylanase production in solid state fermentation by more than two fold has been reported at longer incubation times (Dubeau et al., 1986) and similar results obtained for *Aspergillus ochraceus* (Biswas et al., 1988 b). Malarvizhi et al. (2003) observed that 30 fold enhancement of xylanase production was obtained in solid state fermentation than liquid culture when wheat bran was used as the substrate in *Ganoderma lucidum*. The results obtained in the present study well agreed with this observation.

Xylanase is an inducible enzyme and the xylan present in the wheat bran as well as the birch wood xylan acted as good inducer of the enzyme production. The concomitant cellulase production with xylanase also monitored during the solid state fermentation and all the 34 fungal strains produced cellulase along with xylanase during SSF. This might be because of the presence of cellulose in the wheat bran, the substrate for
Previously it was reported that both xylanase and cellulase was produced when cellulose and hemicellulose were used together as the carbon source (Kulkarni et al. 1999). Haltrich et al. (1996) reported that xylan-degrading organisms are often cellulolytic and secrete complex mixtures of xylanases and cellulases concurrently.

Wheat bran proved to be a suitable substrate along with 0.1% birch wood xylan for the production of xylanase during SSF. Several workers reported the suitability of wheat bran for xylanase production by SSF (Shamala and Sreekantiah, 1986; Gawande and Kamat, 1999; Malarvizhi et al., 2003). Commercial wheat bran consists of 30% cellulose, 27% hemicellulose, 21% lignin and 8% ash (Gawande and Kamat, 1999).

Most of the research workers used submerged cultures for xylanase production, which allowed control over the degree of aeration, pH and temperature of the medium and control over other environmental factors required for the optimum growth of the organisms. In the present study the cellulase production was absent in 70% of the strains under submerged fermentation (SmF) conditions. In submerged fermentation the only carbon source available was xylan. Haltrich et al. (1996) reported that purified xylans can be excellent substrates for xylanase production and are frequently used for small-scale experiments. In a number of organisms these pure and defined substrate increased the yield of xylanase, caused a selective induction of xylanase, either with complete absence or with low cellulase activities (Yu et al., 1987; Hrmova et al., 1989; Biswas et al., 1990; Gilbert et al., 1992).
4.3. Effect of different physico-chemical parameters on the production of xylanase from *Aspergillus sydowii* SBS 45

The basic factors for efficient production of xylanolytic enzymes depends on inducing substrate and optimum composition of the medium. Other bioprocess parameters that could affect the activity and productivity of xylanase in a fermentation process include the pH, temperature etc.

Environmental temperature will not only affect the growth rates of an organism, but it can also have a marked effect on the levels of xylanase formed. Maximum xylanase production was obtained at 30°C in *Aspergillus sydowii* SBS 45. The growth of the organism as well as the synthesis of xylanase was highly reduced when temperature increases from 30°C to 35°C. Kuhad et al. (1998) reported that maximum xylanase production by *Fusarium oxysporum* NTG-19 was observed at 30°C. At 26°C and 37°C xylanase production was reduced by 25% and 80% respectively. In the present study at 25°C, 15% of the enzyme activity and at 40°C, 38% of the enzyme activity was obtained when compared to normal. Shah and Madamwar (2005) reported that xylanase production in *Aspergillus foetidus* under SmF was increased with increase in temperature from 20°C to 30°C. Maximum production of xylanase was obtained at 30°C. At 37°C a significant decline (36%) of xylanase activity was observed. Growth and xylanase production were totally ceased a higher temperature (42°C). Similar effects have been reported by Suh et al. (1988) in *Trichoderma reesei*, Biswas et al. (1988 b) in *Aspergillus ochraceus* and *Aspergillus versicolor*, Smith and Wood (1991 b) in *Aspergillus awamori*, and Malarvizhi et al. (2003) in *Ganoderma lucidum*.

Bhatt et al. (1994) studied the effect of temperature on xylanase production in *Flavobacterium* sp. and observed that maximum xylanase was produced at 28°C and 30°C. However, an abrupt decline in enzyme yield was observed at temperatures above and below this range. This
behavior of the bacterial strain is similar to the usual response of the mesophilic organisms where the metabolic activities get slowed down below and above the optimum temperature which results in the denaturation of certain essential enzymes involved in various metabolic pathways.

An important environmental factor which significantly affects the production of xylanase is the pH during cultivation. A 16.98 % increase of enzyme production was obtained at pH 7 compared to control (pH 5). At pH 6, 12.27 % increase, at pH 8, 11.71 % increase and at pH 9, 8.79 % increase of xylanase production was obtained. The final pH of the medium did not deviated much from the initial pH after the fermentation.

Malarvizhi et al. (2003) reported that in *Ganoderma lucidum* maximum xylanase production was occurred at neutral pH range (6.0-7.0). Production of xylanase by *Chaetomium cellulolyticum* was maximum in the neutral pH (Dubeau et al. 1987). Ruckmani and Rajendran (2001) reported that the production of xylanase was comparatively higher at alkaline pH than acidic pH of the medium in *Aspergillus flavus* under SmF. Abdel-Sater and El-Said (2001) reported that maximum xylanase production by *Trichoderma harzianum* was obtained when the initial pH of the medium was adjusted between 6-8. But considerable amounts of xylanase were also produced at lower and higher pH values.

Rani and Nand (2000) studied the influence of pH on xylanase production in *Clostridium absonum* CFR – 702 and stated that the xylanase activity was dependent on the pH of the growth medium, the highest xylanase activity (2600 nkatals/ml) was observed at pH 8.5 while at lower and higher pH ranges (4-5.5 and 9-10) the growth and xylanase production were very poor. Although *C.absonum* CFR-702 was able to grow at acidic and neutral pH, the highest xylanase activity was observed at alkaline pH. In the present
study *Aspergillus sydowii* SBS45 was able to grow at pH ranging from 3-10, the highest xylanase production obtained at pH ranging from 6-9.

The time taken for maximum production of xylanase varies with strains selected for the fermentation process. The influence of incubation time on xylanase production was studied by different researchers, the best time for xylanase production for *Aspergillus versicolor* was 5 days (Carmona et al. 1997) and for *Aspergillus niger* and *Aspergillus terreus*, it was 4 days (Gawande and Kamat, 1999). Seyis and Aksoz (2005) studied the effects of incubation time on xylanase production in *Trichoderma harzianum* and the maximum production was obtained on the 7th day. In a similar study maximum activity was obtained on 13th day of incubation (Seyis and Askoz, 2003). In *Trichoderma harzianum* the highest yield of xylanase was achieved after 8 days (Abdel-Sater and El-said, 2001). In the present study maximum xylanase was produced on the 9th day of incubation.

Reis et al. (2003) observed that in *Aspergillus nidulans* the maximum peak of xylanase production occurred on the 6th day of cultivation. The xylanase activity remained stable up to 8th day of cultivation, suggests that the enzyme produced by the fungus was stable and no inactivation due to simultaneous production of protease was observed in the medium. These results agreed with the present study that no drastic reduction of xylanase was observed after 9th day of incubation, rather it became more or less steady.

Thaker et al. (1986) studied the secretion of xylanase upto 14th day of incubation and from the 12th day onwards the production of xylanase remains in a steady state. In the present study biomass production increases with increase in incubation time and attained a more or less steady state after 9th day of incubation.
The choice of an appropriate substrate is of great importance for the successful production of xylanases. The substrate not only serves as carbon and energy source, but also provides the necessary inducing compounds for enzyme production. When various concentrations of birch wood xylan and oat spelt xylan were tested for the xylanase production in *Aspergillus sydowii* SBS 45, higher xylanase activities were detected at lower substrate concentrations and lower xylanase activities obtained at higher substrate concentrations.

When *Aspergillus nidulans* grown on birch wood xylan and oat spelt xylan, and when the concentration was increased from 1% to 3% a decrease in the enzyme activity was observed (Reis et al., 2003). According to them the decrease in the production of xylanase when high amounts of xylan was offered as carbon source, may be due to catabolite repression, as described for other xylanolytic microorganisms (Kadowaki, 1997). Of the various birch wood xylan concentrations tested (0.5, 1, 2 and 4% w/v), 1% (w/v) xylan was optimum and induced 24 U/ml xylanase after 5 days of incubation in *Streptomyces cuspidosporus*. Higher concentrations of birch wood xylan and oat spelt xylan (2% and 4%) were inhibitory (Maheswari and Chandra, 2000).

Rani and Nand (2000) in *Clostridium absonum* CFR-702 studied the influence of different concentrations of birch wood xylan on xylanase production and most effective induction was achieved at 1% level in the cultivation medium. When the birch wood xylan concentration was increased further, a drastic decrease in the production of xylanase was obtained. Thaker et al. (1986) tested the effect of different concentrations (0.5%, 1% and 2%) of oat spelt xylan on xylanase production in *Trichoderma longibrachiatum* and find out that level of xylanase was highest when the concentration of xylan was 1%.
Mukhopadhay et al. (1997) tested the effect of increasing concentration of oat spelt xylan (1 to 4% w/v) in the production medium of *Aspergillus flavipes*. The xylanase activity was increased with increasing concentration of xylan in the medium up to 2.5%, then the enzyme production decreased gradually, especially above 3% xylan. Decrease in enzyme activity with increase in substrate concentration may be due to binding of enzyme with unutilized excess xylan in the medium (Panda, 1989). Kulkarni et al. (1999) also mentioned that xylanases bind tightly to the substrate. A part of the enzyme produced during the fermentation is often lost, as bound enzyme, along with insoluble substrate.

In the anaerobic rumen fungus *Neocallimastix frontalis* the xylan was found to be the most effective of all the substrates tested for the production of xylanase. But at elevated xylan concentrations, xylanase production decreased and this was accompanied by the accumulation of xylose and to a lesser extent, arabinose in the culture medium. No accumulation of xylose or arabinose occurred in cultures which were actively xylanolytic, thus a regulatory role for these sugars on xylanase production was considered (Mountfort and Asher, 1989).

Since the biomass increased with increase in concentration of xylan, some kind of inhibition might have occurred in xylanase production at higher substrate concentrations. Previous reports (Kulkarni and Rao, 1996) shows that the substrate derivatives and the enzymatic end products may often play a key positive role in the induction of xylanases; they can also act as the end product inhibitors, possibly at much higher concentrations. Wiseman (1995) described the substrate inhibition, which is a non-competitive type of inhibition in which substrate inhibition caused by the formation of non-productive enzyme-substrate complexes. These can be decreased by using a lower concentration of substrate.
In the present study, among the different sugars and nonsugars tested for xylanase production in glucose, maltose and sucrose completely suppressed the xylanase production but the biomass production was not affected. Similar results were reported by Shah and Madamwar (2005) in *Aspergillus foetidus*. They reported that pure sugars gave good growth but xylanase production was very poor. With 1% glucose and 1% lactose the xylanase production was 2.9 and 3.5 U/ml respectively Christakopoulou et al. (1996) in *Fusarium oxysporum* also reported the same phenomenon.

Jorgensen et al. (2004) reported that xylose is a less repressing sugar and cultivation on xylose revealed detectable amounts of xylanase in *Penicillium* sps. as was seen in the present study. The inhibitory activity of glucose and sucrose was also reported by Seyis and Aksoz (2005 b) in *Trichoderma harzianum* 1073 D3.

Srinivasan et al. (1984) reported that in *Chainia* sp. glucose, xylose, lactose and maltose were appeared to be a poor inducer of xylanase as compared to xylan. Fernandez – Espinar et al. (1992) tested the influence of arabinose, glucose, lactose and xylose on the production of xylanases in *Aspergillus nidulans* and found out that no extracellular xylanase was produced with any of the monosacharides tested.

Berenger et al. (1985) studied the effect various carbon source at 0.5% level in *Clostridium stercorarium* and shown that no measurable amounts of xylanase could be detected on glucose, maltose and galactose, and low specific activities of these enzymes were found on lactose, cellobiose, xylose and arabinose. Eventhough the enzyme production was very low in xylose, lactose, cellobiose and arabinose the fact that xylanases could be formed in the absence of xylan which seems to indicate that these enzymes were formed constitutively. The strong inhibition of xylanase synthesis caused by glucose and other readily
metabolizable substrates can be interpreted in terms of catabolite repression.

Rani and Nand (2000) examined the effect of different sugars on the growth and enzyme formation by *Clostridium absonum* CFR-702. They reported that growth was not observed in the cultivation medium containing lactose, starch, pectin, cellulose, carboxymethyl cellulose (CMC) etc. Among the other carbon sources comprising monosaccharides, disaccharides, trisaccharides, and polysaccharides, the maximum growth of bacterium was attained with birch wood xylan, larch wood xylan, oat spelt xylan which was followed by xylose. The highest xylanase activities were obtained with birch wood xylan, larch wood xylan and oat spelt xylan. Reduced enzyme activity was obtained in xylose, fructose, galactose, lactose, mannose, maltose, sucrose etc. whereas no activity was found in starch, pectin, cellulose and CMC. The present study agrees with the earlier findings.

Catabolite repression by glucose is a common phenomenon observed in xylanase biosynthesis (Kulkarni et al., 1999). De Graaff et al. (1994) reported that the catabolite repression of xylanase gene appeared to be controlled at two levels, directly by repression of gene transcription and indirectly by repression of transcriptional activator.

Among the various concentrations of wheat bran tested, at 1% concentration, maximum production of xylanase was obtained. Kuhad et al. (1998) reported that the maximum amount of xylanase was produced at 4% concentration of wheat bran. Increasing the concentration further to 5% (w/v) a significant decrease in xylanase activity was observed. According to them visual observation showed that at high concentration of wheat bran, especially at 5% (w/v), the substrate form a very thick suspension and did not mix freely in shake flasks. Similar situations have been encountered by other researchers while using high concentration of
Discussion

lignocellulosic material as substrate for enzyme production (Wase et al. 1985b; Singh et al. 1991). Ruckmani and Rajendran (2001) reported that the optimum concentration of wheat bran was 2% for maximum xylanase production. When concentration was increased from 2% to 7% a reduction of xylanase production was obtained. Smith and Wood (1991b) reported that when the concentration of oat straw raised more than 2% in the medium, no significant increase in xylanase titer was obtained. According to them either a nutrient other than the carbon source was limiting the production of xylanase enzyme or the higher concentrations of carbon sources causes repression of enzyme synthesis, due to negative feedback control from the breakdown products of straw.

Since the cost of the substrate plays a crucial role in the economics of an enzyme production process, different lignocellulosic substrates were used for the xylanase production by several workers (Stewart et al., 1983; Smith & Wood, 1991b; Gomes et al., 1992; Haltrich et al., 1993; Purkarthofer et al., 1993a). In these studies, several inexpensive substrates, such as corn cobs, wheat bran and barley husks were tested for xylanase production.

Alam et al. (1994) studied the profile of xylanase production using two organisms Thermomyces lanuginosus and Thermoascus aurantiacus and reported that when substrates like wheat bran, rice bran, sugarcane bagasse, sawdust, sulfite pulp, jute dust and rice straw were used for the production of xylanase in SSF, wheat bran proved to be the best for xylanase production followed by sugarcane bagasse and rice bran. Similar results were obtained when the substrates were added at 1% concentration in the present study.

Techapun et al. (2001) tested the suitability of cane bagasse, corn cobs, rice bran, wheat bran and oat spelt xylan along with several other
lignocellulosics in a Streptomyces sp. and reported that corn cobs and cane bagasse were appeared as suitable substrates for xylanase production. Rice bran found to be a moderate inducer for xylanase production. This results were well matching with the present study.

Rani and Nand (2000) reported that maximum amount of xylanase was produced in corn cobs (3%) when anaerobic bacterium Clostridium absconum CFR-702 when allowed to grow in agricultural wastes such as wheat bran, rice bran, corn cob, corn stalk etc. In the present study, among the different lignocellulosic materials tested maximum production was obtained in soyabean waste (at 1% concentration).

Nitrogen sources have a dramatic effect on the production of xylanolytic enzymes by fungi and organic nitrogen sources were generally better than inorganic nitrogen sources for Chaetomium cellulolyticum (Dubeau et al., 1987), Aspergillus awamori (Smith and Wood, 1991 b), Fusarium oxysporum (Kuhad et al., 1998).

Typical media for the production of xylanases by fungi often contain complex nitrogen sources. Haltrich et al. (1994) studied the effects of individual components on xylanase formation by Sclerotium rolfsii and stimulatory effects were found when concentrations of organic nitrogen source was increased. It has also been reported for several fungal organisms that the addition of a complex supplement was advantageous, resulting in higher xylanase production than when employing inorganic nitrogen sources, even when these gave similar concentrations of nitrogen in the medium (Brown et al., 1987; Smith and Wood, 1991b; Fernandes – Espinar et al., 1992). Haapala et al. (1994) reported that nitrogen source can significantly influence the pH of the medium during the course of a fermentation.
In the present study soyabean meal had given the maximum xylanase titer. Haltrich et al. (1996) reported that soyabean meal have been successfully used in certain organisms as a relatively cheaper complex N source. Miller and Churchill (1986) and Atkinson and Mavituna (1991 a.) analysed corn-steep liquor, soya meal, peanut meal, cotton-seed meal etc. and found that these proteinaceous nitrogen compounds serve as sources of amino acids, vitamins, and minerals. So the cumulative effect of all these can increase the xylanase yield. These findings supported the increase in xylanase yield in the present study when soyabean meal and casein were added to the medium.

All microorganisms require certain mineral elements for growth and metabolism. The elements such as cobalt, copper, iron, manganese, molybdenum and zinc are some essential minerals needed for the normal growth of microorganisms (Stanbury et al.,1995). Haltrich et al. (1996) reported that typical media for the production of xylanase by fungi contain some metallic ions such as Fe²⁺, Co²⁺ and Zn²⁺.

In the present study Al³⁺, Ba²⁺, Cu²⁺, Co²⁺, Cd²⁺, Fe³⁺, Mn³⁺, Na⁺ and Hg²⁺ and Zn²⁺ enhanced the xylanase production when they were given at 10 ppm concentration. Only Ca²⁺, Pb²⁺ and Ni²⁺ were found to be inhibitory for enzyme production. Biomass production was more or less similar in all the tested conditions. Rani and Nand (2000) reported that when 10 mM concentration of Zn²⁺, Mn²⁺, Co²⁺, Cu²⁺, Fe²⁺, Ag⁺ and Hg²⁺ were given in the cultivation medium to find out their influence in xylanase production by anaerobic bacterium *Clostridium absonum* CFR-702, maximum activity of enzyme was recorded with zinc followed be ferrous ion. In the present study both Zn²⁺ and Mn²⁺ shown maximum influence on xylanase production.

Stanbury et al. (1995) reported that in many instances growth will be faster when organic nitrogen was supplied in the form of amino acids.
Gupta et al. (1999) tested the effect of various amino acids (0.25% w/v) on the production of xylanase from *Staphylococcus* sp. SG-13 and found that in presence of DL-alanine, DL-2-amino-n-butyric acid and L-lysine monohydrochloride a decrease in the cell density was accompanied by an increase in xylanase production, which indicated that xylanase production is not a direct function of cell growth, instead there might be some switching on and off phenomenon present in the organism for xylanase production which is operating not only in presence of xylan but also in presence of some specific amino acids. They concluded that the carbon chain length and the position of –CH₃ and –NH₂ groups in amino acids have a significant role in the stimulation of xylanase production.

Berg et al. (2002) stated that 7 out of the 20 amino acids have readily ionizable side chains. These 7 amino acids are able to donate or accept protons to facilitate reactions as well as to form ionic bonds. These amino acids include lysine, aspartic acid and cysteine. The cysteine in the present study could not impart much influence in the production of xylanase while aspartic acid and lysine enhances the xylanase production.

The inositol stimulated the xylanase production by 8.96% when it was added at 0.1% (w/v) concentration. The ascorbic acid and riboflavin inhibited the xylanase production. Previously it was reported that vitamins in some cases added as specific growth factors to the medium, for example, calcium pantothenate in the media formulation for vinegar production (Stanbury et al., 1995).

In addition to the medium components different surfactants or fatty acids are frequently added to the medium to enhance the yields of xylanases. Surfactants and fatty acids have been used for enhancing the production of lignocellulolytic enzymes (Asther et al. 1987; Deshpande et al. 1987, Singh et al. 1991). It was assumed that these compounds increase the
permeability of cell membranes and thus affect the secretion of certain proteins. Tween 80 which is added at 0.5 – 3 g/l could increase xylanase titres by 12-60 % in certain organisms (Dubeau et al. 1987; Okeke & Obi, 1993; Singh et al., 1995). However it apparently had no effect on xylanase induction (Fernandez – Espinar et al. 1992; Gomes et al. 1993 a).

Kuhad et al. (1998) studied the effect of surfactants and fatty acids at 0.2% level. The addition of oleic aid and olive oil stimulated enzyme production by about 40%. Similarly tween – 80 considerably enhanced the xylanase production, while sodium dodecyl sulphate (SDS) considerably reduces the xylanase production. SDS had earlier been reported to be a potent inhibitor of glycanases from different origins (Bastawde, 1992).

4.4. Purification of xylanases from Aspergillus sydowii SBS45

Generally most of the xylanolytic micro-organisms produce multiple xylanases. The production of multi-enzyme system of xylanases in which each enzyme has a specific function, is once strategy for a micro-organism to achieve effective hydrolysis of xylan (Beg et al. 2001).

Different workers adopted different strategies for the purification of xylanases from different organisms. Some researchers used only a single step for the purification of xylanases. Sandrim et al. (2005) purified 2 xylanases, Xyl I and Xyl II by gel filtration on Sephadex G-100 from Aspergillus caesipitosus while Khasin et al. (1993) used SE-52 cation exchanger for the purification of xylanase from Bacillus stearothermophilus T-6.

Some researchers used two steps for the purification of xylanases. Christakopoulos et al. (1997) employed two steps i.e. SP – sepharose and Sephacryl S-200 for the purification of xylanase from Fusarium oxysporum F3. Ximenes et al. (1999) first used ultrafiltration followed by gel filtration on Sephacryl S-100 for the purification of xylanase from Acrophialophora nainiana.
More than two steps were used by some workers for the purification of xylanases. Chen et al. (1997) purified a xylanase from *Trichoderma longibrachiatum* CS – 185 by following ammonium sulphate precipitation (30-50%), cation exchange chromatography on CM-Sepharose CL-6B and gel filtration on Sephacryl S-200 HR. A xylanase from *Aspergillus terreus* was purified by ammonium sulphate fractionation (65%), anion exchange chromatography on DEAE –Bio-Gel A followed by gel filtration on Sephadex G –75 (Ghanem et al., 2000). Kolenova et al. (2005) purified two extracellular xylanases (Xyn B and Xyn C) from *Schizopyllum commune*. The culture filtrate was first ultra-filtered and then subjected to DEAE-Sepharose chromatography followed by purification on Mono Q HR 5/5 column which was further purified on a Phenyl Sepharose column. Belancic et al. (1995) purified xylanase A and xylanase B from the culture filtrate of *Pencillium purpurogenum* by using the following steps, ultrafiltration, ammonium sulphate precipitation (20-70%) and size exclusion chromatography in Bio-Gel P-100. Xylanase A was further purified by means of DEAE-cellulose, hydroxyapatite and CM-Sephadex C-25 while xylanase B was further purified by DEAE-cellulose and CM-Sephadex C-50.

In the present study, a three step purification strategy had been adopted ie. ammonium sulphate precipitation (30–90 %), gel filtration on Sephadex G-200 followed by anion exchange chromatography on DEAE Sephadex A-50. After the 3rd step, the two isozymes designated as xylanase I and xylanase II get separated.

Different workers purified xylanases with different purification factor and yield. A purification factor of 251 with 11% yield was reported for a xylanase purified from *Fusarium oxysporum f. sp. melonis* by Alconada and Martinez (1994). Chen et al. (1997) purified a xylanase from
Trichoderma longibrachiatum CS-185 and obtained a purification factor of 55.8 with an yield of 5.1%.

A purification factor of 378 and an yield of 34% was obtained for the purification of xylanase from Fusarium verticillioides (Saha, 2001). Bakir et al. (2001) reported 53.6 fold purification with 9.73 % yield for the purified xylanase from Rhizopus oryzae ATCC 9363. Li et al. (2005) purified a xylanase from Thermomyces lanuginosus CBS 288.54 with a purification factor of 33.6 and an yield of 21.5%.

In the present study 93.41 and 77.4 fold purification was achieved for xylanase I and xylanase II, while 4.49 % and 10.46 % yields were obtained for xylanase I and xylanase II from Aspergillus sydowii SBS 45.

A single band of protein was obtained for both xylanase I and II during the SDS-PAGE on a 12.5% acrylamide gel shows the purity of the enzyme. Sunna and Antranikian (1997) reported that microbial xylanases are single subunit proteins with molecular masses in the range of 8-145 kDa. In the present study it was observed that xylanase I had a molecular mass of 20,100 Da (20.1 kDa) and xylanase II had a molecular mass of 43,000 Da (43 kDa). Several workers isolated isozymes with different molecular masses. Rana et al. (1996) isolated two xylanases, xyl I and xyl II having molecular masses of 95 kDa and 13 kDa respectively from Humicola grisea var. thermoidea. Segura and Fevre (1993) purified xylanase I and II from rumen fungus Neocallimastix frontalis having molecular masses of 45 kDa and 70 kDa respectively. Two isozymes designated as xylanase A and xylanase N were isolated from Bacillus sp. No. C-125 by Honda et al. (1985 a) and found out their molecular masses as 43 kDa and 16 kDa respectively. Xylanases I having a molecular mass of 105 kDa and xylanase II having a molecular mass of 35 kDa were isolated from Thermotoga thermarum by Sunna et al. (1996).
4.5. Characterisation studies of purified xylanase I and Xylanase II

The optimum temperature for endoxylanase from bacterial and fungal sources varies between 40 and 60°C. Fungal xylanases are generally less thermostable than bacterial xylanases. There are several reports where fungi which were mesophilic in origin but producing thermostable xylanases (Kulkami et al., 1999).

Xylanase I and xylanase II isolated from *Aspergillus sydowii* SBS 45 showed maximum activity at 50°C. Several workers reported that the xylanase isoymes shows identical temperature optima. Duarte and Costa-Ferreira (1994) reported that xylanase isolated from the members of the genus *Aspergillus* show maximum activity at 50°C. Kolenova et al. (2005) purified the two xylanases Xyn B and Xyn C, both were most active at 50°C from *Schizophyllum commune*. Sandrim et al. (2005) purified xylanase I (XI) and xylanase II (XII) from *Aspergillus caespitosus* and both xylanase exhibited the same optimum temperature of 50-55°C.

Three xylanases L, M and S were purified from *Aeromonas sp.* by Ohkoshi et al. (1985) and the properties of the three xylanases were well characterized. It was found that xylanase L and M were most active at 50°C and xylanase S was most active at 60°C. Kang et al. (1996) purified two xylanases CX-I and CX-II both of which showed identical temperature activity profiles and gave the highest activity at 50°C. They showed relatively high stabilities at temperatures up to 50°C. After 2 hr of incubation at 55°C, the enzymes retained 54% (CX-I) and 65% (CX-II) of their original activities.

In the present study xylanase I retained 50.41% activity after 1 hr. of incubation at 60°C, while xylanase II retained 94.16% activity after 4 hrs. of incubation at 40°C. 50.5% residual activity was shown by xylanase I after 4 hrs of incubation at 50°C while 0.8% residual activity was shown
by xylanase II under the same condition. From the results it was evident
that xylanase I showed more thermostability than xylanase II.

Both Xylanase I and Xylanase II purified from *Aspergillus sydowii*
SBS 45 culture filtrate showed an extra-ordinary pH activity profiles. Honda
et al. (1985 a) purified two xylanases viz. xylanase N and xylanase A from
*Bacillus* sp. No.C-125. Among these, xylanase N shows maximum activity
at pH ranging from 6-7, while xylanase A was most active at pH ranging
from 6-10 and showed some activity at pH 12 also. Mathrani and Ahring
(1992) isolated a thermophilic and alkaliphilic xylanase from *Dictyoglomus*
isolates and reported that almost 100% activity was shown by the xylanase
at pH range 5.5 to 9.0. The activities of the crude xylanases from 3 fungal
strains were tested B1, B4a and *Dictyoglomus thermophilum*.

The xylanase enzymes reported from some Bacilli such as *Bacillus*
sp. TAR-1 (Nakamura et al., 1994), *Bacillus* sp. C-125 (Honda et al., 1985
a) and *Bacillus* sp.NCL-86-6-10 (Balakrishnan et al., 1992) were optimally
active at pH 9-10. Recently an alkali tolerant xylanase from *Aspergillus*
*fischeri* (Raj and Chandra, 1996) reported to exhibit remarkable stability at
alkaline pH (pH 9.0). The xylanase from *Cephalosporium* which is an
alkaliphilic fungus shows a broad pH activity ranging from 6.5-9.0 (Bansod
et al., 1993). Many of the alkaliphilic microorganisms studied have been
found to produce xylanases with pH optima near to neutral region but with
relatively high activities being retained in alkaline conditions (Collins et al.,
2005). In addition, a number of xylanases with more alkaline pH optima
have also been isolated and one of the most alkaliphilic xylanases
reported is Xyl B from *Bacillus* sp. AR-990, which has a pH optimum of
9-10 (Gessesse, 1998). Other highly alkaliphilic xylanases include
xylanase J from *Bacillus* sp. strain 41M-1 (Nakamura et al. 1993) and a
xylanase from *Bacillus pumilus* 13a (Duarte et al. 2000) both of which have
a pH optimum of 9. In the present study the optimum activity of xylanase I and xylanase II were found to be at pH 10.0 in 50 mM glycine –NaOH buffer. A 100 % activity was retained by xylanase I after 1 hour of incubation at pH 4-11, while xylanase II retained 100% activity at pH 5-11 after 1 hr of incubation at 30°C.

Stability at the extreme pH values appeared to be characterized by a spatially biased distribution of charged amino acid residues. The enzymes stable in alkaline conditions were typically characterized by a decreased number of acidic residues and an increased number of arginines. Furthermore, a recent comparative structural study of family II enzymes suggests a correlation between pH activity/stability and the number of salt bridges, with acidophilic xylanases having much less of these interactions than their alkalophilic homologs (Hakulinen et al. 2003).

The \( K_m \) value of xylanase I for birch wood xylan was 3.18 mg/ml and for oat spelt xylan was 6.45 mg/ml. The \( K_m \) value for xylanase II for birch wood xylan was 6.51 mg/ml and for oat spelt xylan was 7.69 mg/ml. The \( V_{max} \) of xylanase I for birch wood xylan was 1191 \( \mu \)mol/min/mg protein and for oat spelt xylan was 2604 \( \mu \)mol/min/mg protein. The \( V_{max} \) of xylanase II for birch wood xylan was 1587 \( \mu \)mol/min/mg protein and for oat spelt xylan was 2381 \( \mu \)mol/min/mg protein. Bansod et al. (1993) reported that \( K_m \) values of xylanase lies in the range between 0.5 and 19.6 mg/ml. Monti et al. (1991) reported the \( K_m \) and \( V_{max} \) values of xylanase isolated from *Humicola grisea* var. *thermoidea* as 3.3 mg/ml and 229 \( \mu \)mol/min/mg protein respectively. Xylanase isolated from *Thermomyces lanuginosus* CBS 288.54 (Li et al., 2005) had a \( K_m \) value of 4 mg/ml and \( V_{max} \) value of 2402.3 \( \mu \)mol/min/mg protein. Lin et al. (1999) isolated a xylanase from *Thermomyces lanuginosus* SSBP having \( K_m \) value of 3.26 mg/ml and \( V_{max} \) value of 6300 \( \mu \)mol/min/mg protein. Sandrim et al. (2005) isolated two
xylanases (X I and X II) from *Aspergillus caesiposus* having $K_m$ values of 2.5 and 3.9 mg/ml and $V_{max}$ values of 1679 and 113 μmol/min/mg protein.

Xylanase I and Xylanase II isolated from *Aspergillus sydowii* SBS 45 showed strong specificity towards xylan. Specificity of purified xylanases towards xylan was reported by several workers. The alkaline Xylanase III from *Fusarium oxysporum F3* was most active on oat spelt xylan and the activity on carboxymethyl cellulose (CMC) and microcrystalline cellulose (Avicel) being extremely low (Christakopoulos et al. 1997). Magnuson and Crawford (1997) purified a xylanase from *Streptomyces viridosporus 77A* which shows strong specificity toward xylan preparations. No reaction was observed with other substrates tested. This was in agreement with the present study. Xylanase I and Xylanase II showed no activities towards carboxymethyl cellulose (CMC), pectin and starch. Chen et al. (1997) isolated a xylanase from *Trichoderma longibrachiatum* which specifically acts on oat spelt xylan (100%) followed by birch-wood xylan (94.8%). It did not show any activity towards pectin, potato starch and corn starch.

Kubata et al. (1994) purified a xylanase from *Aeromonas caviae* ME-1 and found out that xylanase V was about three-fold more active on birch xylan than oat spelt xylan. The xylanase was completely inactive on carboxy-methyl cellulose, and xylolbiose. Similar results were reported by Tan et al. (1987) in *Thermoascus aurantiacus*. The substrate specificity of xylanase isolated from *Pencillium chrysogenum* was studied by Haas et al. (1992) and showed that both birch wood and oat spelt xylan was hydrolysed by the enzyme. However, no detectable activity on CMC, filter paper or laminarin was detected.

Among the various metal ions tested Ba$^{2+}$ (at 10mM and 20mM) had the most enhancing effect on both xylanase I and II. Al$^{3+}$, Fe$^{3+}$ and Mn$^{2+}$ retained the activity of xylanase I at 100 % in both 10 mM and 20 mM...
concentrations, while Al^{3+}, Ca^{2+} and Na^{+} retained the activity of xylanase II at 100% in both 10 mM and 20 mM concentrations. In the present study Co^{2+} at 10 mM and 20 mM concentrations were found to be inhibitory for xylanase I, while Pb^{2+} at 10 mM and 20 mM concentrations was found to be inhibitory for xylanase II.

Sandrim et al. (2005) demonstrated that Al^{3+} and Co^{2+} enhanced the activity of xylanase II while it partially inhibits the activity of xylanase I when tested at 1 mM concentration in *Aspergillus caespitosus*. The inhibitory activity of Al^{3+}, Co^{2+} and Cu^{2+} on the activity of xylanase I and II was reported by Frederick et al. (1984) in *Aspergillus niger* at 70 mM concentration. Al^{3+} at 30 mM concentration found to exert a partial inhibitory effect on xylanase I and II in *Aspergillus sydowii* SBS 45. Okazaki et al. (1985) also reported the inhibitory effect of Cu^{2+} on the activity of xylanases purified from two *Bacillus* sp. Other metals such as Cd^{2+}, Ni^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+} and Zn^{2+} at 5 mM concentration had no inhibitory effect on any of the enzymes.

Sreenath and Joseph (1982) reported the effect of metal ions on three xylanases (Ib, Id, II) of *Streptomyces exfoliatus* and found out that Ca^{2+}, Na^{+} and Ba^{2+} stimulated the xylanase activity of II and Ib, while Ca^{2+} partially inhibited Id, while Na^{+} and Ba^{2+} completely inhibited Id. Morales et al. (1995) reported that xylanases purified from *Bacillus polymyxa* were inhibited by Cu^{2+} ions. The partial inhibitory activity of Cu^{2+} ions had reported by Tsubijo et al. (1990) in an alkalophilic actinomycete *Nocardia sp. dossorvillei*.

Gessesse (1998) reported the inhibitory activity of Pb^{2+} on xylanase activity at 1 mM concentration in an alkalophilic *Bacillus* sp. Only 8% residual activity was obtained. The enhancement effect of Na^{+}, Ca^{2+} and Zn^{2+} also reported at 1 mM concentration. Three xylanases viz. X-a, X-b-I,
X-b-II were isolated from *Talaromyces byssochlamydoides* YH-50 by Yoshioka et al. (1981) and studied the effects of various metal ions at 10mM concentration. ZnCl₂ retained 100% activity for X-b-II, CaCl₂ retained 100% activity for X-a, MnCl₂ stimulated X-a, X-b-I and retained 100% activity for X-b-II, FeCl₂ enhances the activities of all three xylanases, and CoCl₂ enhanced X-b-I and X-b-II.

Ratanakhanokchai et al. (1999) studied the effect of metals (1mM) on xylanase isolated from alkaliphilic *Bacillus* sp. strain K-1 and reported that CuCl₂, CoCl₂, MnCl₂, NiCl₂, ZnCl₂, CuSO₄, CoSO₄ and MnSO₄ partially inhibited the xylanase activity, while CaCl₂, FeCl₂, CaSO₄, FeSO₄ and MgSO₄ enhances the xylanase activity.

Biswas et al. (1990) reported that FeCl₃ (1mM), MgCl₂ (5mM), CaCl₂ (5mM), MnCl₂ (5mM), ZnCl₂ (1mM), FeSO₄ (100mM), CuSO₄ (100mM), HgCl₂ (1mM), CdCl₂ (1mM), CoCl₂ (1mM) and NiCl₂ (1mM) shows inhibition on xylanase activity, while monovalent cations such as K⁺ (50mM) and Na⁺ (50mM) stimulated the enzyme activity.

In the present study L-tryptophan stimulated the activities of both xylanase I and II. Both thiol containing reagents (dithiothreitol and β-mercaptoethanol) stimulated the activity of xylanase I. Ximenes et al. (1999) purified a xylanase from *Acrophialophora nainiana* and reported that the xylanase was activated by thiol containing reagents like L-cysteine and β-mercaptoethanol and L-tryptophan. These effects might be indicative of the presence of cysteine and tryptophan residues at the active site. Involvement of cysteine and tryptophan residues in the maintenance of tertiary structure of the active site of xylanases was reported by Kang et al. (1996).

Bastawde (1992) reviewed the effects of metal ions, activators and inhibitors on endoxylanase activity. A concentration of 5 x 10⁻³ M...
sodium dodecyl sulphate was found to be inhibitory for three different endoxylanases from *Streptomyces sp.* 3137. In the present study, sodium lauryl sulphate (30mM) strongly inhibited the xylanase I, while xylanase II was inhibited by only 3.67%. Yoshioka et al. (1981) studied the effect of various metal ions and additives at 10mM concentration on the activities of three xylanases (X-a, X-b-I, and X-b-II) from *Talaromyces byssoschlamydoides* YH-50 and found that sodium dodecyl sulphate (SDS) enhances the activity of X-a but inhibited the activities of X-b-I and X-b-II.

Belancic et al. (1995) studied the effect of various metal ions and additives (1mM) on the activity of xylanases from *Pencillum purpurogenum* and found that EDTA inhibits 30-50% of the xylanase activity. Complete inactivation of both enzymes was observed with N-bromosuccinimamide (suggesting the presence of essential tryptophan residues) and SDS.

Okazaki et al. (1985) studied the effect of EDTA on the activity of xylanases from 2 *Bacillus* sp. at 5mM concentration and reported that it was inhibitory for all the 4 enzymes isolated. Biswas et al. (1990) studied the effect of 1mM EDTA on xylanase activity and found that it was inhibitory for xylanase and only 74% residual activity was retained.

In the present study, it was appeared that both xylanase I and II were glycoproteins. The xylanase I posses 40.63% and xylanase II posses 53.67% carbohydrate content. The occurrence of glycosylated enzymes is a common phenomenon among eukaryotic xylanases (Kulkarni et al (1999). Carbohydrate moieties are covalently linked with protein as dissociable complexes with various xylanases (Wong et al., 1988). Glycosylation has been implicated in the stabilization of glycanases against extreme environments (Merivuori et al,1985).
Three thermostable endoxylanases viz. X-a, X-b-I, and X-b-II from *Talaromyces byssolamydoides* YH-50 (Yoshioka et al., 1981) possess 36.5, 31.5 and 14.2% carbohydrate residues, respectively. The glycoprotein content of endoxylanases from *Aspergillus fumigatus* was between 46.4 and 68% (Fiannigan & Sellers, 1977).

Monti et al. (1991) reported the purification of a xylanase from *Humicola grisea var. thermoidea* and studied the total carbohydrate content of the enzyme. It was estimated to be 45% with glucose as the standard.

4.6. Application of crude xylanase from *Aspergillus sydowii* SBS 45 in the pulp and paper industry

*Aspergillus sydowii* SBS 45 exhibited good xylanase production when it was grown on wheat bran moistened with distilled water, addition of organic or inorganic medium components were not necessary for its growth and xylanase production, but the enzyme titre was low (87.12 U/ml) compared to SSF conditions where mineral salt solution was added. The main advantage of xylanase from *Aspergillus sydowii* SBS 45 over other xylanases was that, no need of any pH adjustment of pulp before the addition of enzyme which in turn was the main problem in many cases.

The prebleaching of Decker pulp with xylanase showed that a considerable improvement in the release of absorbing materials at 237 nm and 280nm. Release of absorbing materials at 237 nm and 280nm can be correlated with release of lignin. Elegir et al. (1995) reported that the release of chromophores correlates well with total sugar release and this can be considered as a simple method to determine the efficacy of the enzyme treatment. They observed that in most of the cases greater chromophore release corresponds to higher brightness. So the
chromophore release might be an easy way to monitor the enzyme efficacy.

There was an increase in the liberation of absorbing materials at 237 nm, 280 nm and 465 nm with increase in the consistency, retention time and enzyme dosage. Salles et al. (2005) reported that prolonged time of incubation or higher doses of enzyme could lead to an increase in chromophore release. Li et al. (2005) reported that the amount of reducing sugars released from wheat straw pulp by the xylanase isolated from *Thermomyces lanuginosus* CBS 288.54 was significantly greater with increasing time while Elegir et al. (1995) reported that release of absorbing materials at 237 nm increased with enzyme dosage and correlated well with total sugar release.

Suurnakki et al. (1994) reported that the decrease in the lignin content of the pulp (i.e., the decrease in kappa number) did correlate with the degree of solubilisation of carbohydrates. In the xylanase treatment, pulp xylan was hydrolysed to soluble xylo-oligomers. So the increase in absorbance at 280 nm as well as the increase in the liberation of reducing sugars indicated the action of xylanase on pulp. In the present study a significant increase in the absorbance at 280nm and significant increase in the liberation of reducing sugars were obtained. The action of xylanase on the pulp significantly increased with increase in consistency, increase in retention time and increase in xylanase dose.

After the three optimization stages viz., consistency, incubation time and enzyme dose, the brightness was increased by 6.5% compared to control. Kappa number was decreased by 1 point. Li et al. (2005) reported that when wheat straw pulp was treated with 15 U/ g pulp of xylanase from *Thermomyces lanuginosus* 288.54, brightness was improved by 3.93 % ISO over control. Madlala et al. (2001) studied the
effect of two xylanases (xylanase P and crude xylanase from Thermomyces lanuginosus) in the bleaching of Kraft pulp and found that, xylanase P (at an enzyme dose of 5 U/g pulp) increased the brightness of Kraft pulp by 5.1 points while xylanase from Thermomyces lanuginosus (at an enzyme dose of 5 U/g pulp) increased 2.1 points. Bissoon et al. (2002) reported that when 5, 50, 150 U/gm pulp was added to bagasse pulp the brightness was increased by 2.28, 2.67 and 2.90 points respectively. Kulkarni et al. (1996) reported an increase in brightness of 2.5 points by a xylanase (10 U/g) from an alkaliphilic thermophilic Bacillus sp. NCIM 59 on unbleached bagasse pulp.

Li et al. (2005) reported that the xylanase pretreatment was able to lower the total charge of active chlorine on wheat straw by approximately 28.3% while still maintaining brightness at the control level. Bissoon et al. (2002) reported that it was possible to lower the total charge of active chlorine on pulp by approximately 18% while still maintaining brightness at the control level, when bagasse pulp was treated with 150 U/g pulp followed by a DED (chlorine dioxide, NaOH extraction, chlorine dioxide) bleaching sequence. Madlala et al. (2001) reported that the extent of chlorine dioxide reduction during bleaching depended on the type of pulp and the enzyme used. Xylanase P reduced the chlorine dioxide consumption on bagasse pulp by 3.5 Kg/tonne pulp thereby retaining brightness at the control level, compared to 2.5–3.0 Kg/tonne achieved with the Thermomyces lanuginosus xylanase. However the two xylanases were almost equally effective on kraft pulp, reducing the chlorine dioxide consumption between 1.5 and 2 Kg/tonne.

In the present study when 10 U/g of xylanase was given, 5 Kg/tonne elemental chlorine and 2 Kg/tonne H₂O₂ can be reduced, thereby retaining brightness at the control level. When 25 U/g of xylanase was
given 5kg/ton elemental chlorine and 4 Kg/ton H₂O₂ can be reduced. When residual activity was measured more than 60% of the original activity was retained by the filtrate. Christov et al. (1999) reported that the filtrate obtained after the enzymatic treatment possessed xylanase activity and could be reused.

The crude xylanase from the culture filtrate of *Aspergillus sydowii* SBS 45 could be used as a potential enzymatic pre-bleaching agent in the pulp bleaching process. The reduction in considerable amount of chlorine made the bleaching process more environmental friendly. Further scale up studies of this xylanase mediated pre-bleaching may help in the large scale industrial application of this enzyme.