DISCUSSION

Lignocellulose, the most abundant natural biopolymer on earth is an important source for the production of various industrially useful materials. Enzymatic and chemical methods can be used for the degradation of these materials. Chemical methods are performed at high temperature and alkaline conditions which produce toxic by product as compared to the enzymatic methods. Chemical degradation of these materials is toxic to the environment which can be replaced by the enzymatic processes or can be merged with enzymatic methods so as to reduce the concentration of toxic chemicals being used conventionally in chemical processes. Various industrial processes involving the use of microbial enzymes are less polluting, highly efficient and energy saving and also in lower disposal problems (Kaur *et al.,* 2015; Singh *et al.,* 2015).

Xylanases are of increasing interest for their potential applications in the industry for the enzymatic conversions of agro industrial residues to pentose fermentation substrates, the selective degradation of xylan in food, pulp and paper industries and in fiber technology (Biely, 1985; Wong *et al.,* 1998).

Endo 1,4β- xylanase has been produced by different microorganisms, but bacterial isolates are widely used for the commercial production of endo 1,4 β-xylanase due to high metabolic diversity (Bajaj and Singh, 2010).

Microorganisms are regarded as a good source of useful enzymes because they multiply at extremely high rates and synthesize biologically active products that can be controlled by humans. These enzymes offer more advantages than chemical catalysts, because they exhibit high catalytic activity and high degree of substrate specificity, produced in large amounts, highly biodegradable, they pose no threat to the environment and they are economically viable.

There is an urgent need to manage the lignocellulosic biomasses effectively and economically. At the same time, it is also necessary to generate value added products from this biomass. Lignocellulosic wastes due to their similar structure polymers as xylan, these lignocellulosic wastes are suitable to use as the prime carbon source for xylanase production.

Coimbatore is at an attitude of 467m MSL, between 10°55': 11°10N and 76°50 and 77°10E. The city is determinant with 75% of red soil and 25% of black soil and geography diverse with rivers, wetlands and dry lands. The floristic wealth of Coimbatore has been recorded b the Botanical Survey of India (BSI), Coimbatore. The city is rich in biodiversity of trees, shrubs, herbs and aquatic plants. Therefore an attempt was made to study the lignocellulosic biomasses were collected from Coimbatore district of Tamil Nadu and to suggest their possible applications.

In the present study the lignocellulosic biomasses used in the extraction of xylan were collected in Coimbatore district of Tamil Nadu State in India Nearly forty five lignocellulosic biomasses were collected, powdered and treated with alkali (3%NaOH) (Plate I).

Xylanolytic bacteria in this present study were isolated from various soil samples in and around Coimbatore. Habitats with degrading lignocellulosic materials are considered to be the best niches for isolating xylanolytic microorganism, as the xylanases are induciable enzymes. Many researchers have isolated xylanase producing bacteria from natural habitats rich in degrading organic matter. Ghasemi *et al.* (2014) isolated *Sphingobacterium* sp from agriculture wastes in farmland area in Iran. Shanthi and Roymon, (2014) isolated a xylanase producing alkaline thermostable strain isolated from lignocellulosic rich forest soil in Durg region. Mahalakshmi and Jayalakshmi,(2016) isolated *Achromobacter xylosoxidans* from marine environment in South East Coast of India.

In the present study there are six bacterial isolates were isolated and selected after preliminary screening for xylanolytic activity using Congo-red staining were subjected to liquid state fermentation in xylanase production medium. The organisms used in this investigation were chosen on the basis of their ability to hydrolyze the commercial xylan (Plate II)

Among the potential xylanase producers, strain CS2 showed the maximum xylanolytic activity.Therefore, strain CS2 was opted for future study and identification through 16Sr RNA sequencing analysis. Further 16S rRNA sequence analysis also confirmed that the bacterium is *Bacillus subtilis.*Many researchers have isolated xylanase producing bacteria from soil and identified with the technique 16S

rRNA technique, in *Sphingobacterium* sp (Ghasemi *et al.* 2014), in *Achromobacter xylosoxidans* (Mahalakshmi and Jayalakshmi, 2016).

Maximum enzyme production by the organism largely depends upon the type of microbial strains, their genetic makeup and cultural/environmental conditions during cultivation of enzymes.Rani and Nand,(2000)found birchwood and larchwood xylan support maximum enzyme production in *Clostridium absonum* CFR 702. However it is uneconomical to use purified xylan as substrate for large scale production. Therefore several natural lignocellulosic biomasses were tested as substrates (Materials and Methods).

Among forty five lignocellulosic substrates were tested. Rice bran was found to be the best followed by wheat bran (Table 4.1). Similar results were also obtained by Virupakshi *et al.*(2005) in *Bacillus* sp and Pater and Prajapati. (2014) in *Bacillus aerophilus* KGJ2. These results prove that agricultural wastes can serve as inexpensive and reliable substrates for xylanase production.

Iloduba *et al.* (2016) reported that xylanase preparations which are used in the paper industries should be free from cellulolytic activities, since they damage the cellulose fibers and paper quality. In the present study, the *Bacillussubtilis* was studied for its cellulose production. It was noted that only negligible amount of the production was present (Table 4.2). The result was in accordance with the findings of Haltrich *et al*., 1994; Archana and Satyanarayana, 1998.

The most important factor that influences the fermentation process is the substrate concentration.Carbon source and concentration in the medium formulation appear to exert a profound effect on the xylanase production behavior of bacteria as it is one of the crucial elements of the microbial fermentation medium with major role on the overall growth and metabolism.

In the present study an attempt was made to investigate the concentration of rice bran. In enzyme production 1% concentration of rice bran was found to enhance the maximum production of 8.36U/ml of xylanase when compared to all other concentration studied. When concentrations increased or decreased, a drastic effect on

the enzyme production was noted (Table 4.3 and Figure 4.1). Thus each organism prefers its own substrate concentration for the maximum production of xylanase.

Each organism has its own pH range for growth and activity. One of the factors which play a crucial role in transportation of nutrients across the membrane and the functioning of enzyme systems within an organism is pH. The enzyme production of the bacteria was studied at various pH values ranging from 6-10. The organism showed maximum production of 2.88U/ml at pH 7 (Table 4.4 and Figure 4.2). Adhyaru *et al.*(2014) also reported maximum enzyme production in *Bacillus altitudinis* at pH 7 andin *Streptomyces* sp (Sivakumar and Sharmalkumar, 2016).

Many of the xylanases are produced by alkalophilic organisms (Okazaki *et al.,* 1984). Yasinok *et al.*(2010) obtained maximum xylanase production at pH 7.5 using *Bacillus* sp. While Gupta *et al.*(2000) found that *Staphylococcus* sp showed a better xylanase production at pH 9. Prakash *et al.*(2012) obtained maximum xylanase production at pH 11. Briggs *et al.* (2005) reported that if the pH of the medium is unfavorablexylanase production may be limited due to substrate inaccessibility.

In any fermentation process, the temperature is one of the important factors that influence the enzyme production. In the present study, the maximum production was noticed at 37°C (Table 4.5 and Figure 4.3). Ghasemi *et al*. (2014) also observed an optimum temperature of 37°C in *Sphingobacterium* sp SAH-05 and at 50°C in *Paenibacillus macerans* (Dheeran *et al.,* 2012). Lin *et al.* (2013) also obtained an optimum temperature of 70°C in *Bacillus halodurans.* Driss *et al.* (2012); Singh *et al.,* (2015) reported that in their study, the organism showed enzyme production at 50°C in xylano-pectino-cellulolytic microbes.

Incubation period plays an important role in the production of xylanase and it varies from organism to organism. Xylanase production reached its maximum after 24hrs in *Bacillus* sp MX 47 (Chi *et al.,* 2012), after 42hrs in *Bacillus altitudinis* DHN8 (Kumar *et al.,* 2014), after 56hrs in *Bacillus pumilus* VLK 1(Adhyaru *et al.,* 2014), after 72hrs in *Streptomyces violascences* (Saulpi *et al.,* 2015), after 96hrs in *Bacillus subtilis* (Ho *et al.,* 2015).The reduction in xylanase yield after optimal period was probably due to the depletion of nutrient available to the microorganism or due to proteolysis. In the present study, a sharp increase in the xylanase production was

noticed after 48hrs (Table 4.6 and Figure 4.4) as obtained by Chaturvedi *et al.*(2015) in *Bacillus licheniformis.*

Agitation is normally used to maintain the medium homogeneity. In the present study higher rates of agitation, decreased the xylanase production. The lower enzyme level under low agitation conditions may be attributed to the dissolved oxygen limitation for cell growth, improper mixing of media components and cell clumping. Agitation rate on xylanase production has been reported for few organisms. Techapun *et al.* (2003) supported maximum enzyme production in *Streptomyces* sp at 150rpm. In the present study maximum xylanase production was achieved at an agitation rate of 200 rpm (Table 4.7 and Figure 4.5). Ninawe and Kuhad in *Streptomyces cyaneus,* and Lalitkumar *et al.*(2014) in *Bacillus pumilus* VLK-1 they also could notice an increase in enzyme production at 200 rpm.

A cheap and readily metabolizable carbon source is one of the key parameters of the economy of the process. So it is imperative that the best carbon source and concentration in the media formulation appear to exert a profound effect on the xylanase production, behavior of bacteria as it is one of the crucial elements of the microbial fermentation medium with the major role on the overall growth and metabolism.The effect of carbon sources enrichment on xylanase production media with different easily metabolizable sugars in the production of xylanase was studied. Xylose induced maximum production in *Paenibacillus* sp (Pathania *et al.,* 2012). Lactose enhanced xylanase production in *Bacillus* sp (Bhakyaraj, 2014), and sucrose in *Acromobacter xylosoxidans* (Mahalakshmi and Jayalakshmi, 2016).

The results obtained from the present study indicate that among six different carbon sources tested, the medium supplemented with maltose supported maximum xylanase production (Table 4.8 and Figure 4.6).

Various nitrogen sources, including both organic and inorganic were added to the xylanase production medium to determine the best nitrogen source supporting maximum xylanase production. Peptone like yeast extract is a complex organic nitrogen source which might be stimulating growth by releasing NH_{4+} and improving the expression of nitrogen assimilating enzymes. Yeast extract supported maximum enzyme production in *Bacillus subtilis* (Annamalai *et al.,*2009 and Sukumaran *et al.,* 2013). Battan *et al.,* 2007 found that a combination of yeast extract and peptone gave

better results in *Bacillus pumilus* and *Bacillus subtilus* (Ho *et al.,* 2015). Kaur *et al.,* (2015) found 1% of yeast extract to be the best nitrogen source for xylanase production from bacteria *Bacillus lpuarvinder* st. lpu002. Bibi *et al.*(2014) reportedthat *Geobacillus stearothermophilus* KIBGE-IB29 in the combination of peptone, yeast extract and meat extract produced the maximum amount of xylanase.

In the present study, the effect of various inorganic and organic nitrogen sources on xylanase production was studied. Among the six nitrogen sources tested peptone supported maximum enzyme production (Table 4.9 and Figure 4.7).

Metal ions namely $Hg^{2+}Ag^{2+}$, Cd^{2+}, Cu^{2+}, $KMnO_4$were inhibiting the xylanase production in *Bacillus* (Koki Horikoshi and Yoko Atsaikawa, 1973). Ca^{2+}, Mn^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}these metal ions inhibit the xylanase activity in *Bacillus* sp (Kaur *et al.,* 2015).

Theexcretion of xylanase and its extracellular performance also depend directly on the type of ions present in the solution. In the present study, the effect of metal ions and bivalent ions were tested on xylanase production. Metals like mercury, copper, zinc, iron and magnesium were found to inhibit the growth of bacteria and very low effect on xylan activity. When compared to other metals. (Table 4.10 and Figure 4.8)Magnesium in the medium was found to be the best for enzyme production. Various bivalent ions like, $CaCl_2$, $ZnCl_2$ $NiCl_2SiCl_2$ $CuCl_2$, $BaCl_2$, and $CoCl_2$ were tested, $CaCl_2$ supported maximum enzyme production (Table 4.11 and Figure 4.9).

Characteristics of biotechnological systems make purification is the most expensive part of biomaterial process production. Thus the development of new and economically advantageous purification method is a challenging area. Purification of target enzymes requires the separation from the media or from the raw extract used for the maintenance of the bio molecules (Bim and Franco, 2000). The column chromatographic techniques are generally employed for xylanase purification Kuno *et al.*(2000) reported that there are two families of xylanases depending upon on the molecular weight and pI values. Wong *et al.* (1998) had also classified microbial xylanase into two groups on the basis of their physicochemical properties.

In the present study, the enzyme was isolated, purified and characterized at 4°C. The extracellular xylanase was purified to homogeneity from the culture filtrate. The crude culture filtrate was precipitated with Ammonium sulphate (80%) saturation at 4°C and left over night. While 1.85 fold purification of xylanase was achieved with specific activity of 64.12 U/ml (Table 4.13) and the molecular weight is 29 kDa. In favour of the above findings there are several reports in the purification of xylanase with different molecular weight. Suna *et al*.(1997) reported four activity bands with molecular masses ranging from 810 to 130 kDa from *Bacillus thermoleovarans* strain K3D and *Bacillus flavothermus* strain LB3A. Battan *et al*.(2006)*Bacillus* sp produced a high level of an extracellular and thermo stable enzyme when grown on wheat brawn as substrate. Dheeran *et al.* (2012) purified xylanase with the molecular weight of 205 kDa in *Paenibaccillus macerans.* Zhang *et al.* (2014) purified xylanase with the molecular weight of 37 kDa in*Paenibaccillus* sp.

The effect of pH stability and temperature stability was also studied on the purified enzyme (Table 4.14 and Figure 4.10). The enzyme was found to be stable at pH 7 with the raise in pH the enzyme got inactivated which is in accordance with the result of Zhang *et al.* (2014) and reported pH 7 as the optimum pH for xylanase activity

Optimum pH for purified enzyme has been reported in number of organisms in many of the researches. The optimum pH for xylanase activity was 4.5 in *Paenibaccillus macerans*(Dheeran *et al.,* 2012), 5–9 in *Paenibaccillus campinasensis* (Dheeran *et al.,* 2012), pH 6 in *Paenibacillus* sp(Zhang *et al.,* 2014), 7 in *Bacillus brevis* (Gowswamy *et al.,* 2015).

In the present study, purified xylanase of *Bacillus subtilus* stable at 40°C for one hour. The optimum temperature for xylanase activity for bacterial species was reported by many of the researchers; at 70°C in *Bacillus halodurans* (Lin *et al.,* 2013), at 50°C in *Paenibaccillus macerans* (Dheeran *et al.,* 2012), at 45°C to 90°C in *Bacillus brevis* (Zhang *et al.,* 2014)

The effect of inhibitors on the purified enzyme was studied. When compared to control, EDTA strongly inhibited the activity. (Table 4.15) Latif *et al*. (2006) also

reported that the enzyme activity was inhibited by all the concentration of EDTA in *Cheatomium thermophile*. In the present study EDTA inhibit the xylanase activity.

The effect of different metal ions on the purified xylanase were studied (Table 4.16) Wu *et al.* (2006) reported that the xylanase obtained from thermophilic *Geobacillus* sp showed stronger inhibition towards Co^{2+}, Ni^{2+v}, Mn^{2+}, Cu^{2+v}, Hg^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+}. Gupta *et al.*(2000) in his study also stated that Ca^{2+}, Ba^{2+} have stronger inhibition towards xylanase activity.Mohana *et al.*(2008) reported that in *Burkholderia* sp metal ions such as Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+}increased the enzyme activity, where as strong inhibition of enzyme activity was observed in the presence of Cu^{2+}, Ag^{2+} and Fe^{2+}. Sugumaran *et al.*(2013) reported in *Bacillus subtilis,* metal ions such as Mg^{2+}, Ca^{2+}, Fe^{2+} and Mn^{2+}, increased the enzyme activity whereas as Cu^{2+}, Hg^{2+} inhibit the xylanase activity.

The most promising application of xylanases is in the prebleaching of kraft pulps in order to reduce the consumption of chlorine (Viikari *et al.*,1986; Bajpai, 1999). Environemental regulations have also put a restriction on the usage of chlorine in the bleaching process in paper and pulp industry.

Enzyme application improves pulp fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibres and selective removal of xylans from dissolving pulps. Xylan glucomannan form the basic polymers of the wood hemicellullose backbone. Treated of pine pulp with xylanase enriched with endo-β-mannase of *Bacillus subtilis* resulted in only a slight increase in delignification compared with xylanase alone (Beg *et al.,* 2001).

During conventional bleaching, the alkaline –stable lignin carbohydrate complexes are cleaved by acidic bleaching stages Eg. Chlorine and Chlorine-di-oxide. However the degradation products adversely contribute to the effluent. In contrast, hemicellulose-degrading enzymes selectively hydrolyze polysaccharide chains attached to lining, therebydecreasing the amount of chemicals required for pulp bleaching. The xylanases assume special importance in the paper and pulp industry as they replace toxic chemicals (Messener *et al.,* 1994).

In the present study, the waste paper pulp was pretreated with crude xylanase from *Bacillus subtili*s. The effect of the treatments on paper quality was analyzed by estimating the amount of reducing sugars released and kappa number reduction. It was observed that 13.86 IU was found to be the best for the maximum reduction of kappa number.

FTIR spectroscopy is a powerful and widely used method in cellulose research from which the direct structural information or any changes can be obtained (Wang *et al.,* 2007). From the Figure 4.13 the appearance of new peak positions for lignin indicates that the removal of lignin content and improvement in the cellulose structure in the fibre.

CONCLUSION

The judicious selection of strain and nutritional supplements may regulate the described enzyme levels and enzyme composition. This could be targeted for various industrial applications. In the global context of switch over to biotechnology, pulp bleaching using chlorine is viewed with positive disfavour. Therefore it become essential that paper industry in India too should opt for enzyme alternative at the earliest. If we have to develop indigenous enzyme technology to suit the indigenously available raw material for paper manufacture, we have to evolve strategies that generate viable technologies for xylanase production based on original discoveries.

Lignocellulosic agricultural and forestry waste materials largely accumulate and cause environmental degradation. The hemicellulose content of lignocellulosic waste can be efficiently used as xylan substrate for xylanase production. This will reduce the chlorine compounds in the bleaching process of the paper and pulp industry. Thereby reducing the cost of production and environmental pollution.Xylanases are also finding its application in bakery, fruit juice, animal feed and textile industries. For the successful production of xylanase, substrate should be inexpensive. Thus rice bran is an attractive alternative for the xylanase production used in the present study. In order to make the application of xylanases more realistic in industries, awareness should be created to reduce the emission of greenhouse gases and environmental regulations should be well maintained in all industry.