EXPERIMENTAL RESULTS

The present study was done under five phases

Phase I

4.1.1Collection of different lignocellulosic biomass for a suitable Substrate

4.1.2 Isolation of xylanolytic bacteria from five different soil samples for the maximum xylanase activity

4.1.3 Identification of isolates based on a molecular level

Phase II

a. Selection of suitable substrate using *Bacillus subtilis* for xylanase production

b. SEM analysis for treated and untreated rice bran

Phase III

Optimization of cultural conditions for the xylanase production from rice branusing*Bacillus subtilis*

Phase IV

Purification and characterization studies on the purified xylanase from *Bacillus subtilis*

Phase V- Application of xylanase in various industries

PHASE I

4.1.1Collection of different lignocellulosic biomass for a suitable substrate

Xylan is widely distributed in lignocellulosic wastes including corncob, rice husk, wheat bran, wheat straw since xylan derivatives are frequently used to induce the production of xylanase by microorganisms. Xylanase hydrolyzes the major hemicellulosic polysaccharide xylan and randomly cutting the arabinoxylan backbone produces a wide range of arabino xylan fragments. In enzyme production cost of enzyme is very important for their industrial production.30-40% of the costs is usually affected by the substrate. Thus by the utilization of low cost substrates as agricultural wastes in the fermentation study.

Therefore attempt was made to study the lignocellulosic biomasses and were collected from Coimbatore district of Tamil Nadu and to suggest their possible applications. Nearly forty five lignocellulosic biomasses namely, Bamboo sheath, Bamboo leaf, *Sanseiveria,* Almond shell, *Delonix regia* pod, Pomegranate peel, Drum stick fruit pod, Palm fruit peel, Palm fruit calyx, *Agave* sp 1, *Banana* leaf, Banana flower bract, *Cyanodon* grass,*Cissus* stem, *Sorghum* stem and leaf, Coir pith, Turmeric leaf, Orange peel, *Agave* sp 2, Sugarcane bagasse, Corn cob, Corn leaf, Corn stover, Rice bran, Wheat bran, Saw dust, Tea waste, Sugarcane leaf, Wheat straw, Paddy straw, Groundnut shell, Cotton shell, Pine apple peel, pine apple crown, *Typha* leaf and stem, *Eucalyptus* leaf and bark, Water hyacinth leaf and stem, *Parthenium* stem, Onion peel, *Mimosa* sp fruit pod, Red gram leaf were collected. Plate I shows the lignocellulosic biomass used as substrates in the present study.

Plate 1

The lignocellulosic biomasses used as substrates in the present study

Bamboo Sheath

Bamboo leaf

Almond shell

Sanseiveria

***Delonixregia* pod**

Pomegranate peel

Drum stick pod

Palm fruit peel

Palm fruit calyx

Agave sp

Banana leaf

Banana flower bract

***Cyanodon* grass**

Cissus

Sorghum stem and leaf

Coir pith

Turmeric leaf

Orange peel

Agave sp

Sugarcane bagasse

Corn cob

corn leaf and corn stover

Wheat bran

Rice bran

Saw dust

Tea waste

Sugarcane leaf

Wheat Straw

Paddy Straw

Groundnut shell

Cotton Shell

Pine apple peel

Pine apple crown

Typha leaf and Stem

Eucalyptus leaf& bark

Water hyacinth leaf & stem

Parthenium stem

Onion peel

Mimosa sp pod

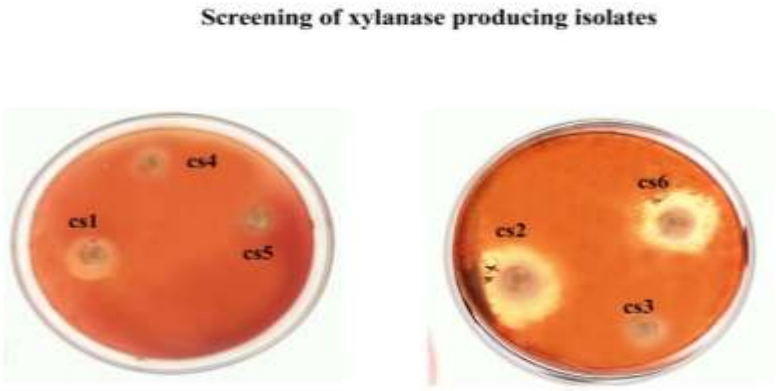
Red gram leaf

4.1.2 SCREENING OF XYLANASE PRODUCE BACTERIAL ISOLATES

A total of five soil samples (Gardensoil, Saw dust soil, Coir pith rich soil, Paddy field soil and Forest soil) from different areas of Coimbatore district were collected. Isolation and screening of potentiisolates were done in two distinct stages using Nutrient Agar medium for primary screening. In the primary screening, the sample suspensions were serially diluted and 0.5ml of the aliquots was spread into the medium (Gupta *et al.,* 1992).

The bacterial colonies obtained from the first screening procedure were then subjected to secondary screening.The cultures were plated on Nutrient Agar medium containing oat spelt xylan (1%) as carbon source. After four days of incubation, the xylanolytic property of the colonies was assessed by Congo red assay method which was done by flooding the plates with 1% (v/v) Congo red for 15 minutes (Carlos *et al.,* 2002; Kumar *et al.,* 2008) followed by destaining with 1M NaCl. The best isolate was selected based upon the zone formation and then it was subjected for sequencing using 16S rRNA techinique.

PLATE II



TheTable 4.1 shows the number of bacterial species from the soil samples and zone of inhibition.

Table 4.1

Zone of inhibition

Isolates name	Zone of inhibition (mm)
Cs1	08
Cs2	**14**
Cs3	-
Cs4	-
Cs5	-
Cs6	11

Out of six, three isolates were showed maximum xylanolytic activity. The strain which showed maximum clearing zone of 14mm was selected as the best for further studies.

4.1.3 IDENTIFICATION OF ISOLATES BASED ON A MOLECULAR LEVEL

Six isolates were isolated and identified and the best xylanolytic bacteria were selected based on the zone of inhibition and then subject to 16Sr RNA gene sequence.

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - C9007H45013**

Formatting options

Download

Blast report description

Nucleotide Sequence (826 letters)

RID	C9007H45013 (Expires on 02-18 21:13 pm)		
Query ID	lcl|Query_79743	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.3.1+
Query Length	826		

Graphic Summary

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis strain SWI3 16S ribosomal RNA gene, partial sequence	1495	1495	99%	0.0	99%	JF834077.2
Bacillus subtilis strain 55AA2-2 16S ribosomal RNA gene, partial sequence	1495	1495	99%	0.0	99%	JN366718.1
Bacillus subtilis strain F3-2 16S ribosomal RNA gene, partial sequence	1495	1495	99%	0.0	99%	EU882850.1
Bacillus subtilis strain M6-7 16S ribosomal RNA gene, partial sequence	1495	1495	99%	0.0	99%	EU882847.1
Bacillus subtilis strain MSEB 34 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	99%	KP261061.1
Bacillus subtilis strain MSEB 29 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	99%	KP261057.1
Bacillus subtilis strain RC28 16S ribosomal RNA gene, complete sequence	1491	1491	99%	0.0	99%	KJ669213.1
Bacillus subtilis strain C5-16 16S ribosomal RNA gene, partial sequence	1491	1491	99%	0.0	99%	HQ236380.1

Based on BLAST results, the organism identified as*Bacillus subtilis.*

PHASE II

Selectionof suitable substrate using *Bacillus subtilis* for xylanase production

4.2.1 PRODUCTION OF XYLANASE FROM BACILLUSSUBTILIS IN LIQUID STATE FERMENTATION IN DIFFERENT SUBSTRATES

Various lignocellulosic biomass have been screened for xylanase production. Rice bran enhanced maximum xylanase production in *Thermomyces aurantiacus* (Santos *et al.*,2003), rice bran in *Bacillus* sp (Virupakshi *et al*., 2005) *Bacillus pumilus* (Battan *et al*., 2007), corn cob in *Bacillus lichniformis* (Gupta and Kar, 2008), cassava bagasse in *Bacillus subtilis* (Sugumaran *et al.*, 2013), sugarcane bagasse in *Bacillus safensis* (Rahmani *et al*., 2014).

Hence an experiment was carried out to findout the effect of various lignocellulosic biomass namely Bamboo sheath, Bamboo leaf,*Sanseiveria*, Almond shell, *Delonix regia* pod, Pomegranate peel, Drum stick fruit pod, Palm fruit peel, Palm fruit calyx, *Agave* sp 1, *Banana* leaf, Banana flower bract, *Cyanodon* grass,*Cissus* stem, *Sorghum* stem and leaf, Coir pith, Turmeric leaf, Orange peel, *Agave* sp 2, Sugarcane bagasse, Corn cob, Corn leaf, Corn stover, Rice bran, Wheat bran, Saw dust, Tea waste, Sugarcane leaf, Wheat straw, Paddy straw, Groundnut shell, Cotton shell, Pine apple peel, pine apple crown, *Typha* leaf and stem, *Eucalyptus* leaf and bark, Water hyacinth leaf and stem, *Parthenium* stem, Onion peel, *Mimosa* sp fruit pod, Red gram leaf on xylanase production by *Bacillus subtilis* in liquid state fermentation.

The results are presented in Table 4.2

Xylan from all these agro residues were extracted and used as substrate, for liquid state fermentation. In liquid state fermentation, conical flasks containing xylanase production medium with lignocellulosic biomasses as a carbon source.Enzyme was extracted and assayed as mentioned in Materials and Methods.

Table 4.2

Xylanase production by *Bacillus subtilis* in xylanase production medium using different lignocellulosic biomasses

S.No	Lignocellusic biomasses	Xylanase Production (U/ml)
1	Bamboo sheath	0.15 ± 0.03
2	Bamboo leaf	0.13 ± 0.03
3	*Sanseiveria* sp	0.21 ± 0.01
4	Almond shell	0.14 ± 0.06
5	*Delonixregia* pod	0.14 ± 0.03
6	Pomegranate peel	0.13 ± 0.03
7	Drumstick fruit pod	0.14 ± 0.07
8	Palm fruit peel	0.19 ± 0.04
9	Palm fruit calyx	0.20 ± 0.06
10	Agave sp 1	0.15 ± 0.05
11	Banana leaf	0.15 ± 0.02
12	Banana flower bract	0.17 ± 0.02
13	*Cyanodon* grass	0.17 ± 0.09
14	*Cissus* stem	0.18 ± 0.06
15	Sorghum stem	0.28 ± 0.05
16	Sorghum leaf	0.19 ± 0.04
17	Coir pith	0.14 ± 0.03
18	Turmeric leaf	0.15 ± 0.04

19	Orange peel	0.14 ± 0.02
20	Agave sp2	0.23 ± 0.03
21	Sugarcane bagasse	0.19 ± 0.08
22	Corn cob	0.17 ± 0.04
23	Corn leaf	0.18 ± 0.06
24	Corn stover	0.19 ± 0.04
25	**Rice bran**	**1.84 ± 0.13**
26	Wheat bran	0.28 ± 0.04
27	Saw dust	0.17 ± 0.05
28	Tea waste	0.14 ± 0.02
29	Sugarcane leaf	0.16 ± 0.06
30	Wheat straw	0.15 ± 0.02
31	Paddy straw	0.19 ± 0.02
32	Groundnut shell	0.20 ± 0.05
33	Cotton shell	0.16 ± 0.07
34	Pineapple peel	0.15 ± 0.07
35	Pineapple crown	0.14 ± 0.04
36	Typha leaf	0.14 ± 0.05
37	Typha stem	0.15 ± 0.03
38	*Eucalyptus* leaf	0.14 ± 0.02
39	*Eucalyptus* bark	0.15 ± 0.04
40	Water hyacinth leaf	0.16 ± 0.03

41	Water hyacinth stem	0.17 ± 0.04
42	Parthenium stem	0.17 ± 0.01
43	Onion peel	0.15 ± 0.03
44	*Mimosa* fruit pod	0.19 ± 0.09
45	Red gram leaf	0.20 ± 0.04

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

From Table 4.2, it is inferred that outof forty five various lignocellulosic biomasses was tested, rice bran was found to support maximum xylanase production in *Bacillus subtilis.*

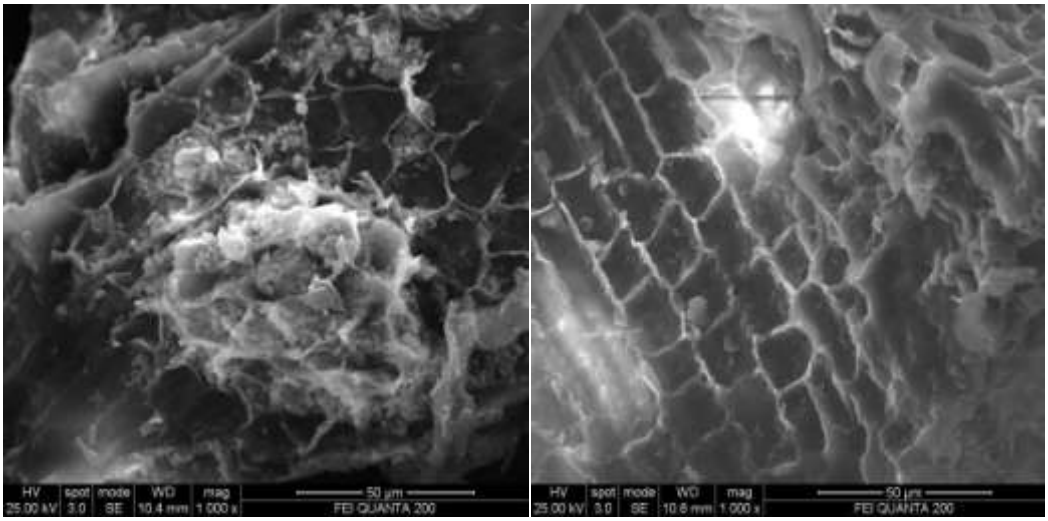
4.2.2 SURFACE ANALYSIS OF TREATED AND UNTREATED RICE BRAN XYLAN

Out of the forty five substrates tried rice bran enhanced the enzyme production. Hence, SEM analysis was done for rice bran study the surface profile. The results of SEM analysis showed changes on surface profiles of rice bran before and after treated with alkali. There were changes on rice bran surface structure. The microphotographs were presented in Plate III

Plate III

SEM analysis for the morphology of treated and untreated rice bran xylan

A B



A-Untreated rice bran

B- Alkali (3% NaOH) treated rice bran

From Plate III- It can be noticed that there was removal of impurities and partial cracking of surface layers compared to untreated rice bran.

Scanning electron microscope images of rice bran before and after alkali treatment showed dissociation of biomass, resulted in improved production of reducing sugar. SEM observations of untreated rice bran showed a continuous smooth surface, whereas the roughness of the surface increased following alkali treatment.

PHASE III

Optimization of cultural conditions for the xylanase production from rice bran using *Bacillus subtilis*

4.3.1DETECTION OF CARBOXY METHYL CELLULOSE ACTIVITY IN THE CULTURE FILTERATE OF BACILLUSSUBTILIS:

Cellulase activity was studied in *Sclerotium rolfsii* (Haltrich *et al*., 1994) in *Bacillus licheniformis* (Archana and Satyanarayana 1998), in *Rhizopus oryzae* (Bakir *et al*., 2001) and in *Trichoderma reesei* (Kar *et al*., 2006). In order to determine the presence of cellulase along with the xylanase in the culture filterate of *Bacillus subtilis*, the culture filterate was examined for Carboxy Methyl Cellulase activity as mentioned in Materials and Methods. The results are presented in Table 4.3

Table 4.3

Detection of carboxy methyl cellulose activity in the culture filtrate of *Bacillus subtilis*

S.No	Cellulase Activity (U/ml)		
1	0.18	0.15	0.16

From Table 4.3 it is noticed that only a low cellulase activity was found in the culture filtrate.

4.3.2 EFFECT OF DIFFERENT CONCENTRATION OF THE SUBSTRATE ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Each organism prefers its own substrate concentration for maximum enzyme production. In *Sphinogobacterium* sp, 0.9% wheat bran was most effective for the production of xylanase (Ghasemi *et al*., 2014), 2% wheat bran xylan in *Bacillus pumilus*, (Battan *et al*., 2007), 1.5% sugarcane bagasse in *Bacillus safensis* (Rahmani *et al*., 2014), Hence the effect of different concentration of rice bran xylan on xylanase production by *Bacillus subtilis* was studied.

Conical flasks containing rice bran xylan at a concentration of 0.5%,1%,1.5%,2%,2.5%,3% were inoculatedwith 24hrs growth culture as inoculumand incubated for five days. On the sixth day, the enzyme was extracted, assayed and estimated for the enzyme production as mentioned in Materials and Methods. The results are presented in Table 4.4 and Figure 4.1

Table 4.4

Effect of different concentration of the substrate on xylanase production by *Bacillus subtilis*

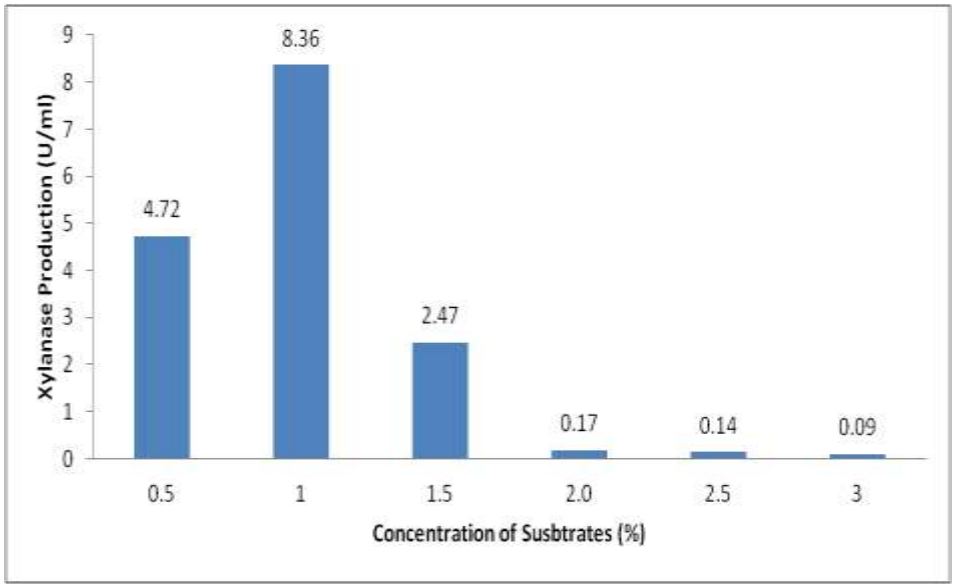
Concentration of substrate (%)	Xylanase production (U/ml)
0.5	4.72. ± 0.44
1	**8.36 ± 0.43**
1.5	2.47 ± 0.52
2	0.17 ± 0.05
2.5	0.14 ± 0.07
3	0.09 ± 0.01

Values given in each cell is the mean ± SD of three replicates.

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.1

Effect of different concentration of the substrate on xylanase production by *Bacillus subtilis*



From Table 4.4 and Figure 4.1, it is noticed that 1% concentration of rice bran xylan showed maximum xylanase production followed by 0.5 % concentration.

4.3.3. EFFECT OF pH ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Each organism has its own pH range for growth and activity. The optimum pH for xylanase production was 6-7 in *Strptomyces* sp (Sivakumar and Sharmal kumar, 2016), pH 7 in *Bacillus safensis* (Rahmani *et al*., 2014), pH 8 in *Bacillus subtilis* (Sugumaran *et al*., 2013), pH 8.5 in *Bacillus* sp (Subajini Mahilrajan *et al*., 2012), pH 9 in *Paenibacillus* sp (Pathania *et al*., 2012).

Hence an experiment was carried out to find out the effect of pH of culture medium on xylanase production by *Bacillus subtilis*. The pH of xylanase production medium was studied using buffers with a pH range of 5-10. Conical flasks containing 50ml of medium of each pH was inoculated with 24hrs grown bacterial cultures as inoculum and incubated for five days. The enzyme was extracted, assayed and estimated as mentioned in Materials and Methods. The results are presented in Table 4.5 and Figure 4.2

Table 4.5

Effect of pH on xylanase production by *Bacillus subtilis*

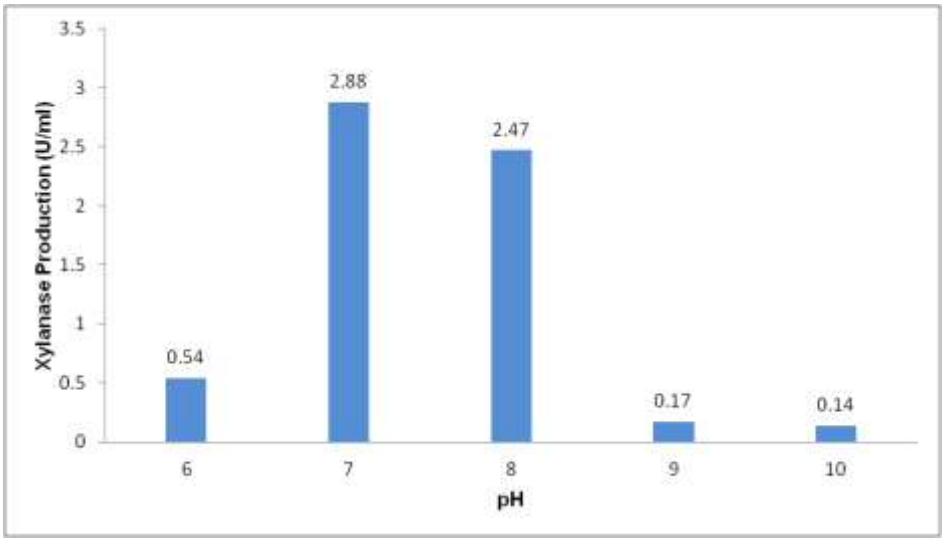
pH	Xylanase production (U/ml)
6	0.54± 0.05
7	**2.88± 0.40**
8	2.47 ± 0.52
9	0.17 ± 0.05
10	0.14 ± 0.07

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.2

Effect of pH on xylanase production by *Bacillus subtilis*



From Table 4.5 and Figure 4.2, it is observed that the xylanase production was found to be maximum at pH7 followed by pH 8.

4.3.4 EFFECT OF TEMPERATURE ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

The optimum temperature for xylanase production was reported for a number of microbes. Each organism prefers to have its own temperature optima for maximum enzyme production. Xylanase production was more in *Bacillus subtilis* at 37°C (Irfan *et al*., 2012) and *Bacillus pumilus* (Adhyaru *et al*., 2014), at 40°C in *Bacillus* sp (Chi *et al*., 2012), at 45°C in *Bacillus* sp(Subajini and Mahilarajan, 2012), at 50°C in *Paenibacillus* sp (Pathania *et al*., 2012), At 60°C *Geobacillus thermodenitrificans* (Anandkumar and Satyanarayana, 2013), *Strptomyces* sp (Sivakumar and Sharmalkumar, 2016).

Hence an experiment was carried out to find out the effect of temperature of culture medium on xylanase production by *Bacillussubtilis*. Conical flasks containing 50ml of the medium was inoculated with bacterial inoculums of *Bacillus subtilis* and incubated at different temperatures of 17°C,28°C,37°C,47°C for five days.

The different temperature regimes were maintained in an incubator shaker. After five days, the enzyme was extracted, assayed and tabulated as mentioned in Materials and Methods. The results are presented in Table 4.6 and Figure 4.3

Table 4.6

Effect of Temperature on xylanase production by *Bacillus subtilis*

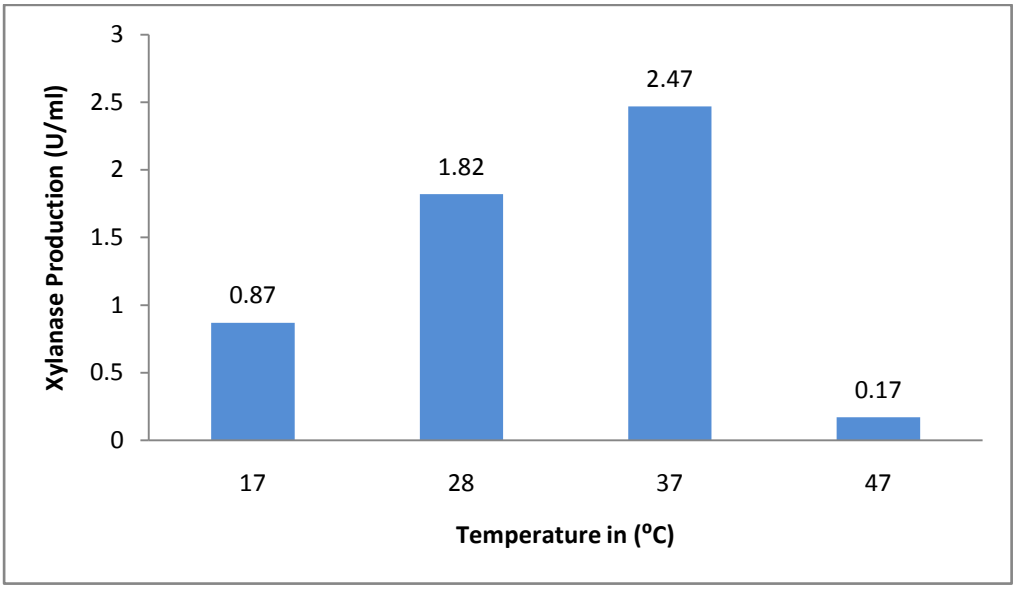
Temperature (degree Celsius)	Xylanase productioin (U/ml)
17	0.87± 0.48
28	1.82± 0.41
37	**2.47 ± 0.52**
47	0.17 ± 0.05

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.3

Effect of Temperature on xylanase production by *Bacillus subtilis*



From Table 4.6 and Figure 4.3, it is inferred that 37°C temperature was found to be suitable for maximum production of xylanase by *Bacillus subtilis*.

4.3.5 EFFECT OF INCUBATION PERIOD ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Incubation period plays an important role in the production of xylanase and it varies from organism to organism. Maximum enzyme production was noticed after 24hrs in *Bacillus* sp (Chi *et al*., 2012), at 48hrs in *Bacillus subtilis* (Irfan *et al*., 2012), 56hrs in *Bacillus pumilus* (Adhyaru *et al*., 2014) 72hrs in *Bacillus pumilus* (Battan *et al*., 2006).

The effect of different growth periods of xylanase production by *Bacillus subtilis* was studied. The conical flasks containing medium were inoculated with bacterial inoculums and incubated for different periods. The filterates in the conical flasks were regularly tested for their xylanase production from 24hrs to 72hrs. The results are presented in Table 4.7 and Figure 4.4

Table 4.7

Effect of incubation period on xylanase production by *Bacillus subtilis*

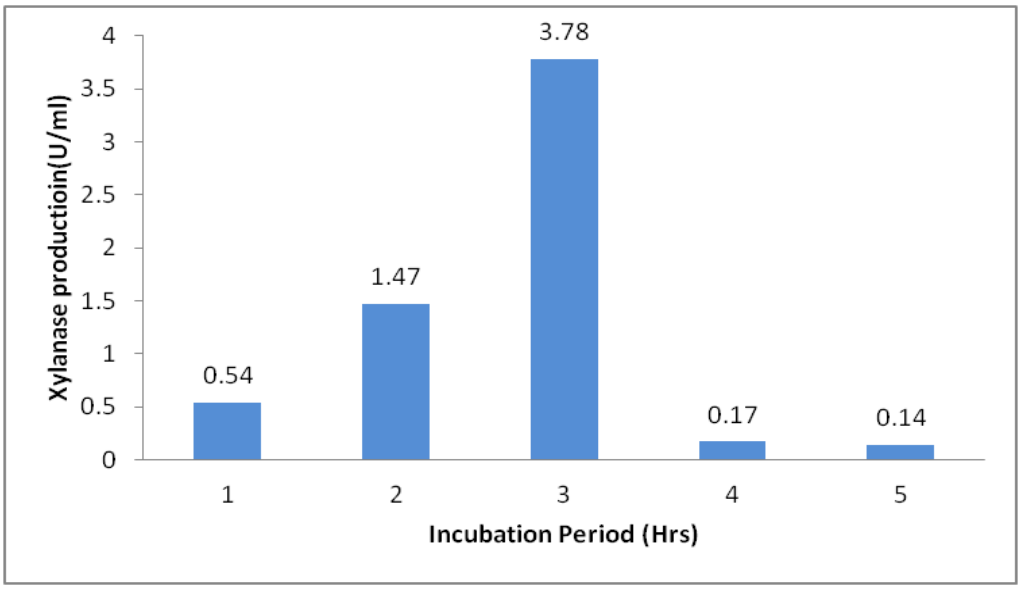
Incubation Period (Hrs)	Xylanase production (U/ml)
24	0.54± 0.05
36	1.47± 0.45
48	**3.78± 0.42**
60	0.17 ± 0.05
72	0.14 ± 0.07

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.4

Effect of incubation period on xylanase production by *Bacillus subtilis*



From Table 4.7 and Figure 4.4, it can be observed that maximum xylanase production was noticed at 48hrs.

4.3.6 EFFECT OF AGITATION RATE ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Agitation rates from 50rpm to 390rpm on xylanase production have been reported. In *Streptomyces* sp 150 rpm enhanced maximum enzyme production (Techapun *et al*., 2003), 200rpm in *Streptomyces cyaneus* (Ninawe and Kuhad, 2006) and *Bacillus pumilus* (Kapoor *et al*., 2008), 250 rpm in *Bacillus* sp (Pham *et al*., 1998).

Hence an experiment was carried out to find out the effect of agitation rate on xylanase production by *Bacillus subtilis*. Conical flasks containing 50ml of the medium were inoculated with bacterial inoculum of *Bacillus subtilis.* The conical flasks were incubated and kept in an orbital shaker incubator for five days. After five days the enzyme was extracted and estimated as mentioned in Materials and Methods. The agitation rates tested were 50rpm,100rpm,150rpm,200rpm and 250rpm. The results are presented in Table 4.8 and Figure 4.5

Table 4.8

Effect of Agitation rate on xylanase production by *Bacillus subtilis*

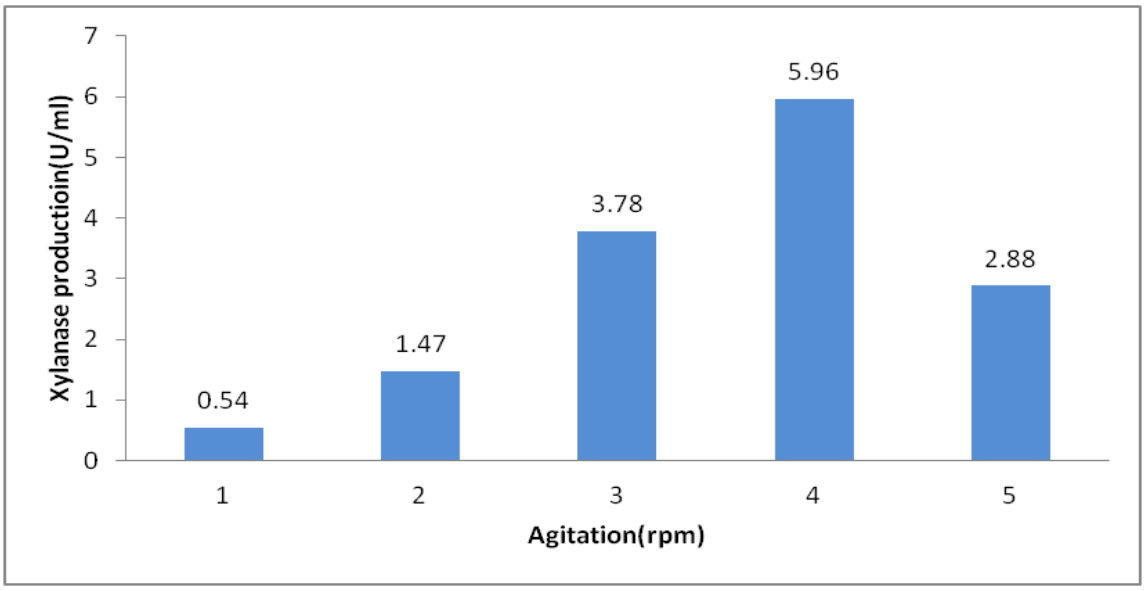
Agitation (rpm)	Xylanase production (U/ml)
50	0.54± 0.05
100	1.47± 0.45
150	3.78± 0.42
200	**5.96 ± 0.31**
250	2.88 ± 0.40

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.5

Effect of Agitation rate on xylanase production by *Bacillus subtilis*



From Table 4.8 and Figure 4.5, it is noticed that agitation rate 200rpm showed maximum enzyme production.

4.3.7 EFFECT OF CARBON SOURCES ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Carbon sources in the culture medium plays an important role in xylanase production. Maximum xylanase production was achieved by the medium supplemented with Beechwood xylan in *Geobacillus stearothermophilus* (Bibi *et al*., 2014), xylose in *Trichoderma viride* (Irfan *et al*., 2014), lactose in *Bacillus* sp (Bhakyaraj, 2014), sucrose in *Achromobacter xylosoxidans* (Mahalakshmi and Jayalakshmi, 2016).

In the present study the effect of different carbon equivalents on xylanase production was studied. The carbon source in the medium was replaced with the equivalents of fructose, lactose,maltose, sucrose, glucose and xylose. The conical flasks containing 50ml of the medium were incubated for five days. After five days, the enzyme was extracted and assayed as mentioned in Materials and Methods. The results are presented in Table 4.9 and Figure 4.6

Table 4.9

Effect of carbon sources on xylanase production by *Bacillus subtilis*

Carbon Sources	Xylanase production (U/ml)
Fructose	0.11 ± 0.02
Lactose	0.15 ± 0.02
Maltose	**0.19 ± 0.02**
Sucrose	0.14 ± 0.01
Glucose	0.17 ± 0.05
Xylose	0.06 ± 0.02

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.6

Effect of carbon sources on xylanase production by *Bacillus subtilis*

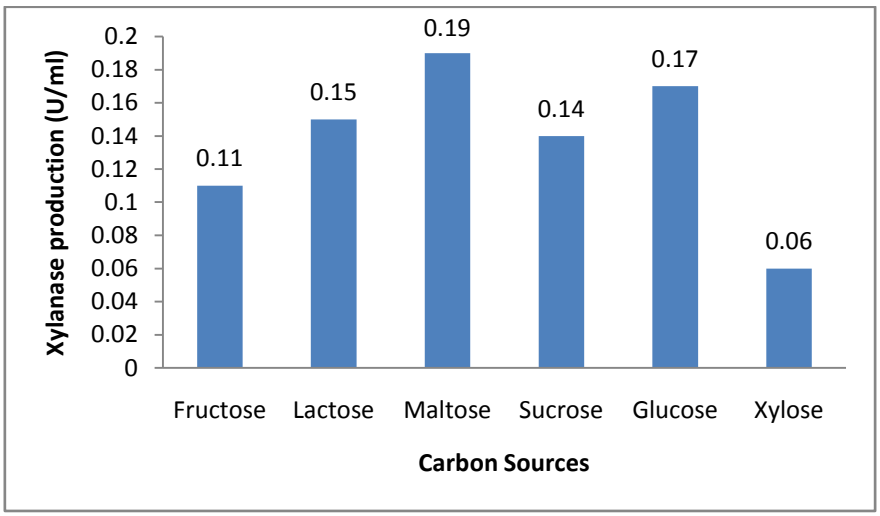


Table 4.9 and Figure 4.6, it is obseved that among the carbon equivalents tested, maltose supported the maximum xylanase production.

4.3.8 EFFECT OF NITROGEN SOURCES ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

The effect of various inorganic and organic nitrogen sources on xylanase production has been reported in many organisms. Ammonium sulphate showed maximum production in *Sphingobacterium* sp (Ghasemi *et al*., 2014), Combination of yeast extract, meat extract, peptone enhanced maximum enzyme production in *Geobacillus stearothermophilus* (Bibi *et al*., 2014), sodium nitrate in *Achromobacter xylosoxidans* (Mahalakshmi and Jayalakshmi, 2016), urea and peptone in *Streptomyces* sp (Sivakumar and Sharmal kumar, 2016).

The effect of various nitrogen sources on the xylanase production by *Bacillus subtilis* was studied by replacing with equivalent amounts of nitrogen sources. An equal amount of organic sources namely,yeast extract, malt extract, beef extract, pepton was added.

The results are presented in the Table 4.10 and Figure 4.7

Table 4.10

Effect of nitrogen sources on xylanase production by *Bacillus subtilis*

Nitrogen sources	Xylanase production (U/ml)
Yeast extract	0.14 ± 0.01
Malt extract	0.41 ± 0.55
Beef extract	0.19 ± 0.02
Peptone	**1.78 ± 0.42**
Sodium nitrate	0.02 ± 0.00
Potassium nitrate	0.06 ± 0.02

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.7

Effect of nitrogen sources on xylanase production by *Bacillus subtilis*

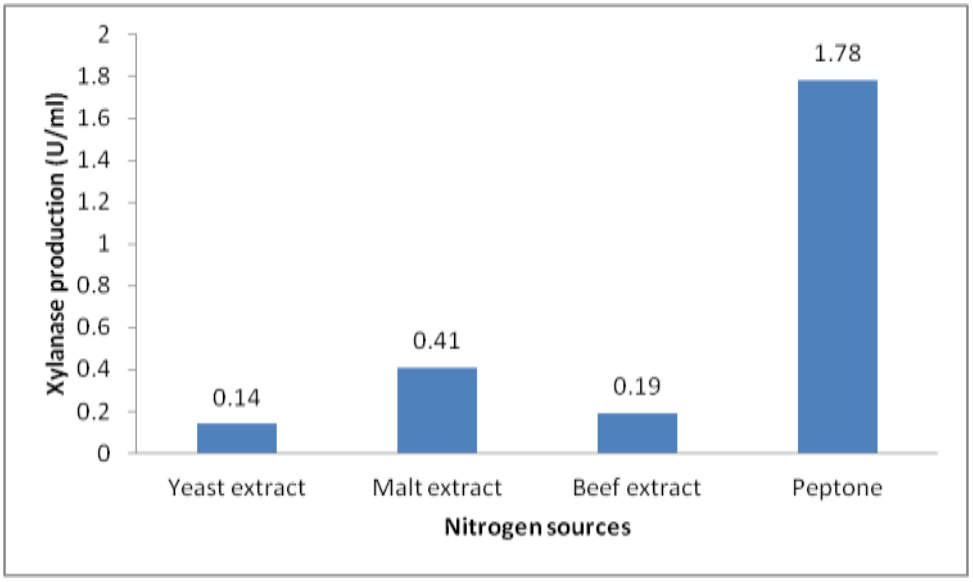


Table 4.10 and Figure 4.7, it is inferred that among the nitrogen equivalents tested, peptone supported maximum xylanase production.

4.3.9 EFFECT OF METAL IONS ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Various metal ions were reported to inhibit xylanase production. Kaur *et al*. (2015)reported that Mg^{2+} influence enzyme productionin *Bacillus* sp.

Conical flasks containing xylanase production medium were supplemented with the metal ions such as Magnesium, Mercury, Copper, Zinc, Iron at the concentration of 1ppm. The chemicals were mixed thoroughly inoculated with 24hrs grown bacterial inoculums for five days. The Enzyme was extracted, assayed and estimated as mentioned in Materials and Methods. The results are tabulated in the Table 4.11 and Figure 4.8

Table 4.11

Effect of metal ions on xylanase production by *Bacillus subtilis*

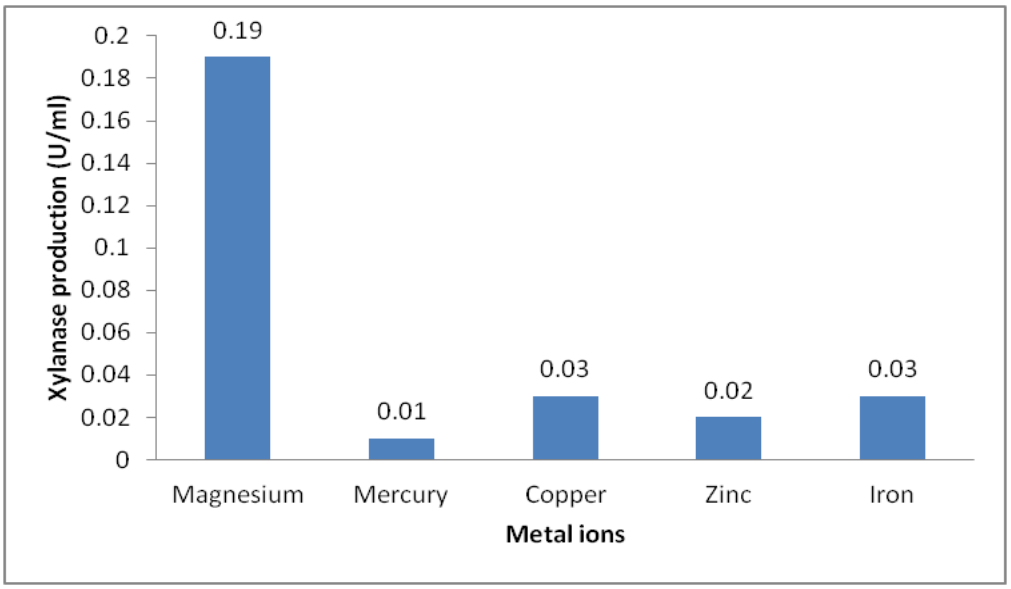
S.No	Metal ions (1ppm)	Xylanase production (U/ml)
1	**Magnesium (control)**	**0.19 ± 0.01**
2	Mercury	0.01 ± 0.00
3	Copper	0.03 ± 0.01
4	Zinc	0.02 ± 0.01
5	Iron	0.03 ± 0.00

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.8

Effect of metal ions on xylanase production by *Bacillus subtilis*



From table 4.11 and Figure 4.8, it is inferred that all the metal ions inhibited the enzyme production whereas magnesium supported enzyme production.

4.3.10 EFFECT OF BIVALENT IONS ON XYLANASE PRODUCTION BY BACILLUSSUBTILIS

Effect of different chemicals on xylanase production was reported by many workers. In *Streptomyces cyaneus,* Ninawe and Kuhad. (2005) studied the effect of bivalent ions on xylanase production.

Therefore, the effect of bivalent ions on xylanase production by *Bacillus subtilis*was studied. In xylanase production medium was replaced with equivalents of $CaCl_2$, $ZnCl_2$, $NiCl_2$, $SiCl_2$, $CuCl_2$, $BaCl_2$, $CoCl_2$at 1 mM concentration. The conical flasks were inoculated with the bacterial inoculums and incubated for five days. After five days the enzyme was extracted, assayed and estimated as mentioned in Materials and Methods. The results are presented in the Table 4.12 and Figure 4.9

Table 4.12

Effect of bivalent ions on xylanase production by *Bacillus subtilis*

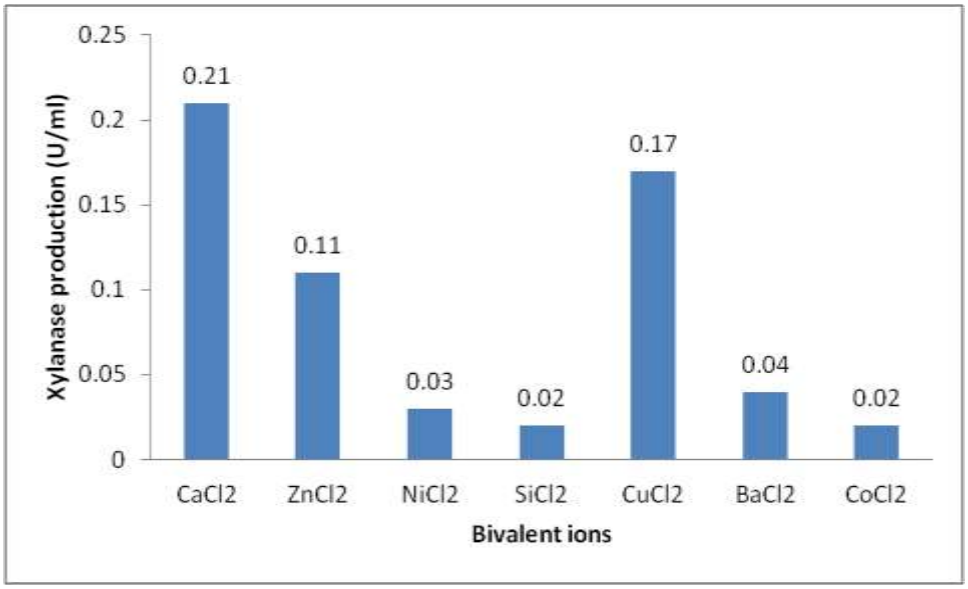
Bivalent ions	Xylanase productioin(U/ml)
$CaCl_2$(control)	**0.21 ± 0.01**
$ZnCl_2$	0.11 ± 0.01
$NiCl_2$	0.03 ± 0.01
$SiCl_2$	0.02 ± 0.00
$CuCl_2$	0.17 ± 0.02
$BaCl_2$	0.04 ± 0.01
$CoCl_2$	0.02± 0.00

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.9

Effect of bivalent ions on xylanase production by *Bacillus subtilis*



From Table 4.12 and Figure 4.9, it is observed that among the bivalent ions $CaCl_2$ supported to maximum enzyme production.

PHASE-IV

4.4 PURIFICATION AND CHARACTERIZATION OF XYLANASE

4.4.1Purification of xylanase enzyme from *Bacillus subtilis*

In the present study *Bacillus subtilis* showed maximum crude enzyme production unde the conditions employed. Hence, an attempt was made to purify the xylanase of *Bacillus subilis*.

Xylanase have been purified to homogeneity in *Bacillus circulans* (Estaban *et al*., 1982),*in Bacillus brevis* (Gowsami *et al.,* 2013), in *Caldicoprobacter* algeriensis sp nov. strain. TH7C1 (Amel *et al*.,2016), in *Thielaviopsis basicola* MTCC 1467(Goluguri *et al.,* 2016).

Purification was carried out as mentioned in Materials and Methods. The crude culture filterate of *Bacillus subtilis* showed a specific activity of 13.99 (U/ml)Table-4.13.

The crude culture filterate after precipitated with ammonium sulphate, the purification fold 1.36 with specific activity 19.5(U/ml). The contentrated protein after precipitation was loaded in Sephadex G-200. The fractions showing high xylanase activity yielded a single band with a molecular weight of 29 kDa(Plate-IV).

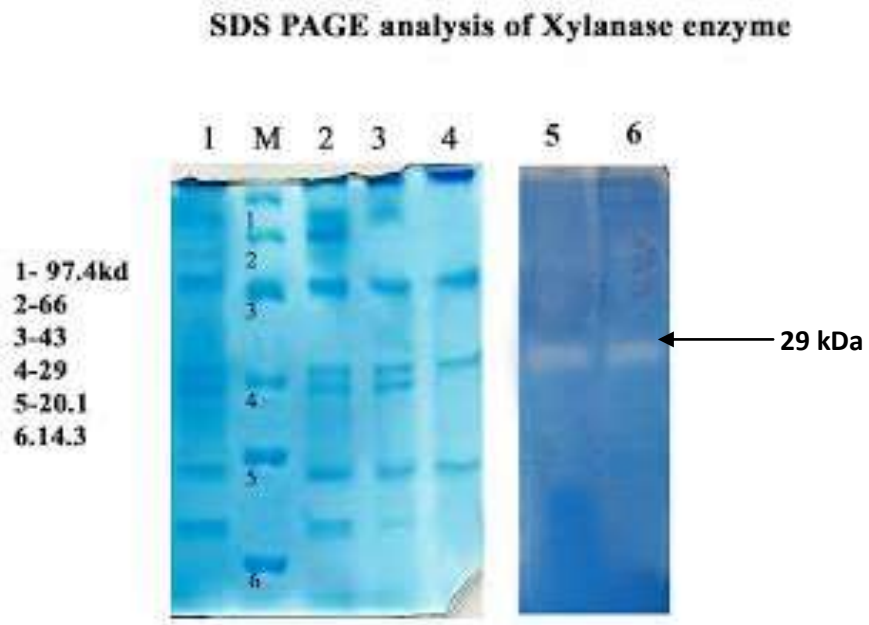
Table-4.13

Summary of purification of xylanase from *Bacillus subtilis*

Steps of Purification	Enzyme Activity (mg)	Protein value (mg)	Specific activity (U/ml)	Purification folds
Crude broth	3.078	0.22	13.99	1
Ammonium sulphate	3.510	0.18	19.5	1.36
Dialysis	4.158	0.12	34.65	1.77
Sephadex G 200	5.130	0.08	64.12	1.85

Plate IV

Purification of xylanase enzyme from *Bacillus subtilis*



Lane:1- Crude, Lane:M- Medium range protein marker
Lane:2- A.sulphate precipitate enzyme
Lane:3- Dialysis enzyme
Lane:4- Column purified enzyme
Lane :5 and Zymogram sample

Molecular markers

Lysozyme	-	14.3 kDa
Trypsin inhibitor	-	20kDa
Carbonic anhydrase	-	29kDa
Ovalbumin	-	45 kDa
Bovine serum albumin	-	66kDa
Phosphorylase b	-	97kDa

4.4.2 CHARACTERIZATION STUDIES ON THE PURIFIED ENZYME FROM BACILLUSSUBTILIS

Characterization of purified xylanase of *Bacillussubtilis*

After purification, the enzyme was characterized for pH stability and temperature stability. The effect of metal ions and inhibitors on enzyme activity also studied.

4.4.2.1 EFFECT OF pH ON THE STABILITY OF PURIFIED XYLANASE FROM BACILLUSSUBTILIS

Stability of the purified enzyme at different pH was studied by many workers. Many of the xylanases were stable at 5-8 in *Eschericia coli* (Yin *et al.,* 2007),at pH 4.5-10 in *Bacillus* sp (Avcioglu *et al.,* 2005),at pH 6-12.5 in *Bacillus stearothermophilus* (Dhiman *et al.,* 2008), at pH 5-9 in *Paenibacillus campinasensis* (Dheeran *et al.,* 2012).

Hence an experiment was carried out to find out the effect of pH on the stability of purified xylanase from *Bacillus subtilis.* The purified xylanase was studied by incubating the enzyme with buffer of pH 5-9 for 30minutes. The reaction mixture was assayed as mentioned in Materials and Methods. (Table 4.14 and Figure 4.10)

Table 4.14

Effect of pH on the stability of purified xylanase from *Bacillus subtilis*

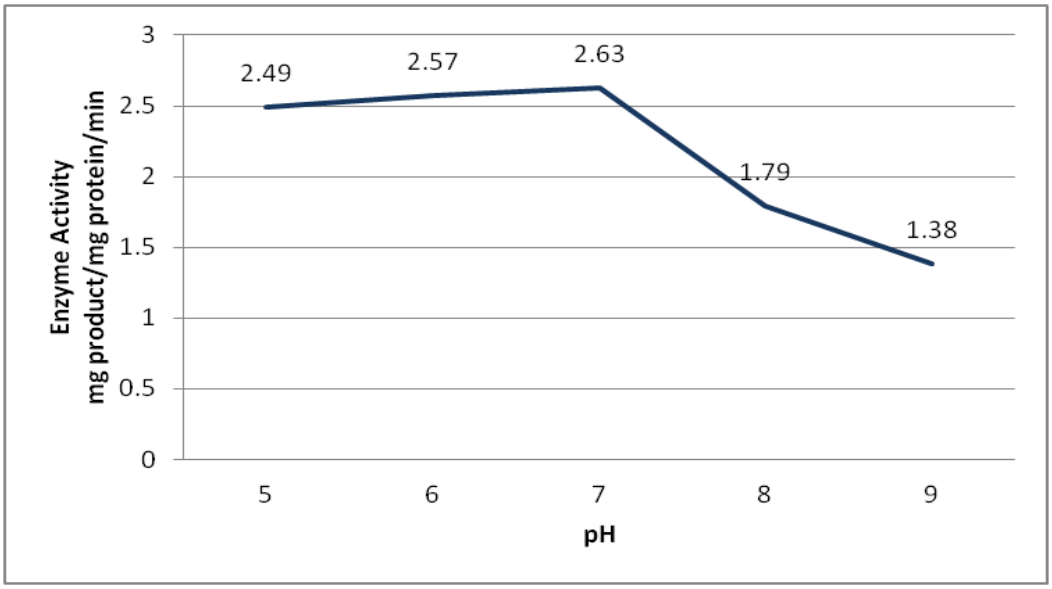
pH	Enzyme Activity mg product/mg protein/min
5.0	2.49±0.22
6.0	2.57±0.27
7.0	**2.63±0.20**
8.0	1.79±0.07
9.0	1.38±0.14

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.10

Effect of pH on the stability of purified xylanase from *Bacillus subtilis*



Fromthe Table 4.14 and Figure 4.10, it was noticed that the enzyme was more stable at pH 7

4.4.2.2 EFFECT OF TEMPERATURE ON STABILITY OF THE PURIFIED XYLANASE OF BACILLUSSUBTILIS

Temperature stability of the purified enzyme was studied in number of organisms. In *Bacillus* sp the stability of the enzyme was at 50°C (Avcioglu *et al.*,2005), at 60°C in *Bacillus* sp (Azeri *et al.*, 2010),at 70-80°C in *Paenibacillus campinasensis* (Dheeran *et al.*, 2012).Temperature stability of the purified xylanase from *Bacillus subtilis* was studied by incubating the enzyme alone in 0.05 M sodium acetate buffer at pH 7 in the absence of substrate in sealed tubes at 50°C, 60°C and 70°C for 3 hours. Samples were withdrawn and relative activity was assayed at 60°C.

Temperature stability of the purified xylanase from *Bacillus subtilis* was studied by incubating the enzyme alone in 0.05M sodium acetate buffer at pH 5.5 in the absence of substrate in sealed tubes at 40°C,50°C and 60°C for 3 hours. Samples were withdrawn and relative activity was assayed at 40°C. The results are presented in Table 4.15 and Figure 4.11

Table 4.15

Effect of temperature on stability of the purified xylanase of *Bacillus subtilis*

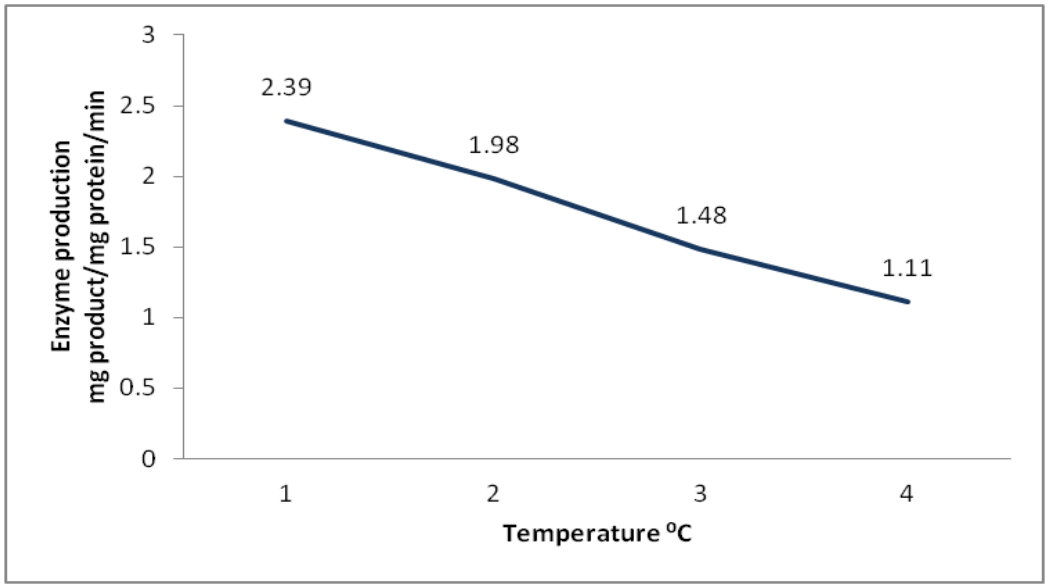
Temperature ^{o}C	Enzyme activity mg product/mg protein/min
40	**2.39±0.07**
50	1.98±0.11
60	1.48±0.03
70	1.11±0.15

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

Figure 4.11

Effect of temperature on stability of the purified xylanase of *Bacillus subtilis*



From Table 4.15 and Figure 4.11, it was noticed that the enzyme was stable at 40^{o}C.

4.4.2.3 Effect of inhibitors on purified enzyme of *Bacillus subtilis*

Effect of inhibitors on enzyme activity was studied by number of authors. Latif *et al.,* (2006) in *Chaetomium thermophile* studied that the xylanase activity was inhibited by all the concentrations of EDTA. Table 4.16

Table 4.16

Effect of inhibitors on purified enzyme of *Bacillus subtilis*

Inhibitors(2mM)	**Enzyme activity mg product/mg protein/min**
Control	**2.57±0.11**
EDTA	1.08±0.13
SDS	1.33±0.14
Sodium azide	1.23±0.07
2-Mercaptoehtanol	1.13±0.11

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

The Enzyme was incubated at room temperature for 30minutes with various inhibitors like EDTA (Ethylene Diamine Tetra Acetic Acid), SDS (Sodium Dodedyl Sulphate), Sodium azide, 2 Mercaptoethanol at a final concentration of 2mM and 2 Mercaptoethnol at different (%) were used. After incubation the reaction mixture was assayed for enzyme activity and the residual activity was calculated. Our inhibitors served as control. The results shown in Table 4.16. Table 4.16, it was observed all the inhibitors inhibit thexylanase production.

4.4.2.4 EFFECT OF METAL IONS ON PURIFIED XYLANASE OF BACILLUSSUBTILIS

The effect of metal ions namely Cu^{2+}, $Zn^{2+,}$ Fe^{2+}, Hg^{2+}on xylanase activity was studied. The enzyme was incubated at room temperature for 30 min with various

metal ions at a final concentration of 2ppm. The substrate was then added and reaction was allowed to proceed for 30min. The reaction mixture was assayed and the residual activity was calculated (Table 4.17).

Table 4.17

Effect of metal ions on purified enzyme of *Bacillus subtilis*

Metal ions (2ppm concentration)	Enzyme activity mg product/mg protein/min
Mg^{2+} (Control)	**2.87±0.15**
Cu^{2+}	1.23±0.07
Zn^{2+}	1.33±0.14
Fe^{2+}	0.61±0.13
Hg^{2+}	1.08±0.11

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

From Table 4.17 it was found that Mg^{2+} enhanced the maximum enzymeactivity among all other metal ions.

PHASE IV

4.5APPLICATION OF XYLANASE IN VARIOUS INDUSTRIES

4.5.1 EFFECT OF ENZYME CHARGE ON WASTE PAPER PULP

To determine the optimum enzyme charge for the pulp treatment the pulp was incubated with various charges of enzyme viz 6.93,9.24,11.55,13.86,16.17, 18.48 and 20.79 U/gpulp at a standard temperature 60°C for 3hrs. The results are presented in the Table 4.18.

Table 4.18

Effect of enzyme charge on waste paper pulp

Enzyme charge U/mL	Reduction in kappa number (%)	Reducing sugar released (mg/g/pulp)
Control	16.52±0.01	1.94±0.01
6.93	15.63±0.01	2.23±0.00
9.24	14.58±0.04	2.44±0.02
11.55	13.56±0.01	2.57±0.01
13.86	**9.12±0.01**	**3.93±0.01**
16.17	9.87±0.01	2.77±0.01
18.48	10.37±0.11	1.73±0.01
20.79	11.44±0.01	1.33±0.01

Values given in each cell is the mean ± SD of three replicates

Means followed by a common letter are not significantly different at the 5% level by DMRT

From Table 4.18, it was observed that 13.86 IU was found to be the best for the maximum reduction of kappa number.

4.5.2 EFFECT OF XYLANASE IN THE BREAD MAKING

Xylanases are widely used as additive in the baking industry to improve processing and production quality. They have enhanced the dough quality, machinability and stability, prolonged shelf life and improved crumb structure (Bailey *et al.*, 2003). In the present study, an experiment was carried out to find out the efficiency of purified xylanase of *Bacillus subtilis* on wheat bread as mentioned in Materials and Methods. These results are presented in the Table 4.19 and Plate V

Table 4.19

Physical analysis of dough and bread attributes

Attributes	Control	Purified xylanase
Dough		
Water absorption (%)	100	70
Dough rising(%)	75	100
Bread		
Volume of Bread (ml)	550	700
Weight of the loaf(g)	140	120
Density (g/ml)	0.37	0.18

Table 4.20

Determination of loaf volume and weight of the bread treated with xylanase

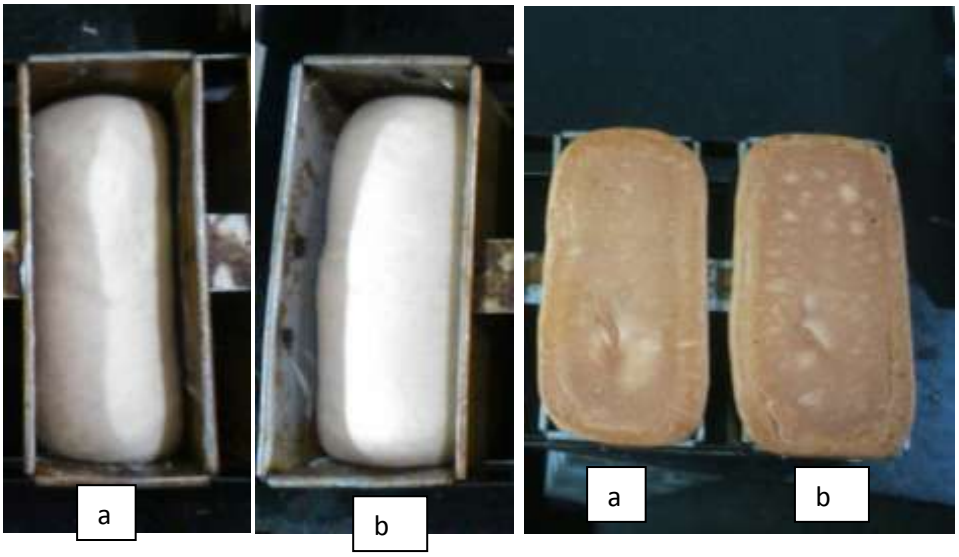
Enzyme charge(U/mL)	Loaf volume (mL)
Distilled water (control)	300
11.55	450

From the Table 4.19, 4.20, it was observed that bread made with the addition of purified xylanase showed increase in the volume, density and dough rising. It is also noted that loaf volume of bread also increased than control.

Plate V

Effect of xylanase in bread making

A- Control (unbaked) B.Baked bread



a-Dough without enzyme

b- Dough with enzyme

4.5.3 CRUDE XYLANASE OF BACILLUSSUBTILISAS DETERGENT

Leisola *et al*. (1996) and Showell *et al*. (1999) reported that vast pools of enzymes from microorganisms are the most widely exploited enzymes in the detergent industries worldwide. Xylanases from alkaliphilic *Bacillus* sp are not only compatible with a wide variety of commercial detergents of different formulas, but can also be used as a detergent additive (Kumar *et al*., 2004). Vishalakshi *et al*. (2009) studied the production of alkaline protease from *Streptomyces gulbargenesis* and its application in the removal of blood stains. An experiment was carried out in a similar way to find out whether the crude enzyme obtained from *Bacillus subtilis* can be used as an detergent additive.

The stained cloth from various stains such as coffee and tea powder (10mg/mL), Tomato, Mango and Pomegranate (1% extract) was treated with crude

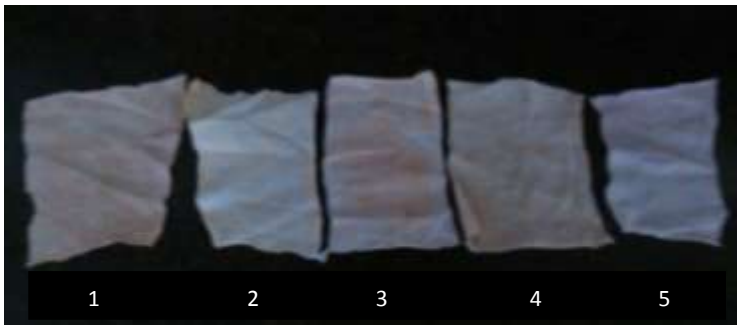
enzyme (10% v/v), mixture of crude enzyme (10%v/v) and 1% (w/v)commercial detergent, as mentioned in Materials and Methods. The stained cloth after the treatment was analyzed visually.

From Plate VI, it is evident that the combined action of enzymes along with the detergent gave a boost to the bleaching performance of the stained clothes. Amongthe various stains, the cloth stained with the mango extract was completely bleached in all the two conditions studied.

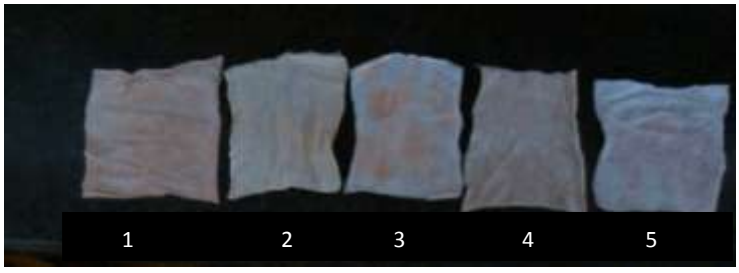
Plate VI

Crude xylanase of *Bacillus subtilis* as detergent

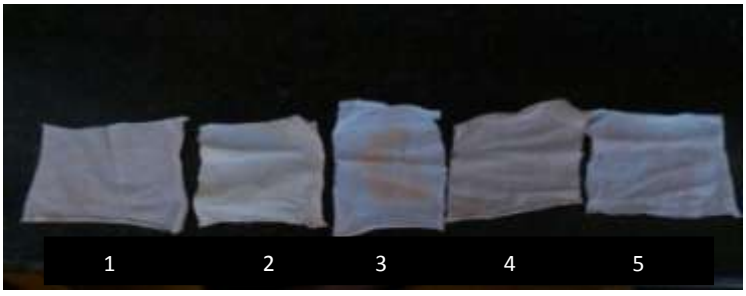
A-Control



B- Treated with crude enzyme



C- Crude enzyme+Detergent



1.Tea strain 2.Mango strain 3.Tomato strain

4.Coffee strain 5. Pomengrante strain

4.5.4 EFFECT OF CRUDE XYLANASE OF BACILLUSSUBTILISAND EFFECTIVE MICRORGANISMS (EM) ON COMPOSTING

Enzymatic parameters also reflect the activity of the microbial community and indicate the ability of composting to degrade a wide range of common organic substrates. Bacterial and fungal feruloyl and p-coumaroyl esterases are relatively novel enzymes capable of releasing feruloyl and p-coumaroyl play an important role in biodegration of recalcitrant cell walls in grasses (Kuhad *et al*., 1997). These enzymes are synergistically with xylanases to distrupt the hemicellullose-lignin association, without mineralization of the lignin. Therefore, hemicellulose degradation is required before efficient lignin removal can commence. In recent years agro wastes were converted to Biofertilizers using several microbes and their enzymes synthesized during solid state fermentation (Kanmani *et al*., 2009).

The present study biowaste including kitchen wastes, dry leaves and cow dung was subjected to composting using crude xylanase from *Bacillus subtilis* and with Effective Microorganisms (EM). The compost was tested for various parameters and the results are tabulated Table 4.17 and Plate VII

Table 4.21

Effect of crude xylanase of *Bacillus subtilis* and Effective Microrganisms (EM) on composting

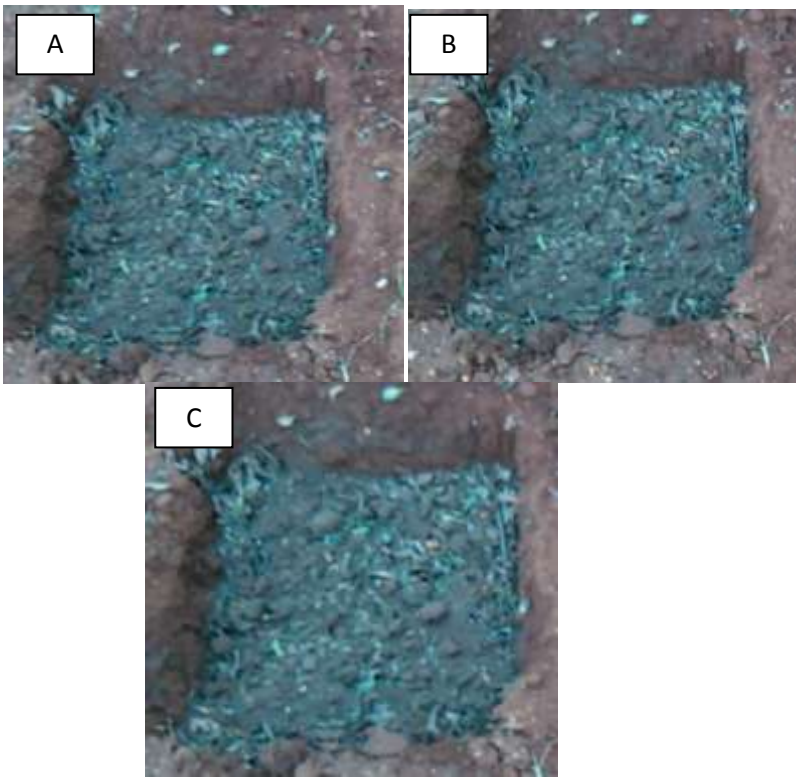
Parameters	Results		
	Control	Crude enzyme	Crude enzyme+ Effective Microorganisms
pH	6.0	7.0	6.7
Nitrogen	11.7%	11.2%	12.1%
Phosphorous	0.74%	0.84%	0.74%
Potassium	24.0%	22.0%	22.1%

From the Table 4.17, it was observed that the crude enzyme+ EM treated, in this samples nitrogen, phosphorous increased than the control and crude enzyme treated samples.

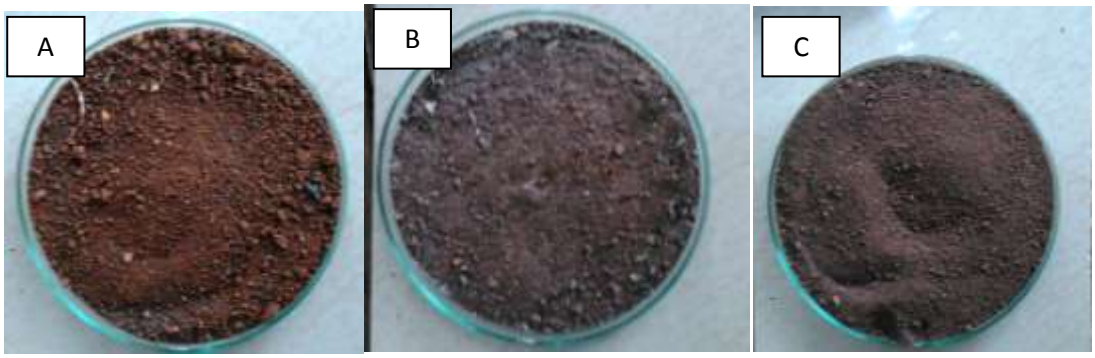
Plate VII

Effect of crude xylanase in bio composting

a. Before composting



b. After composting



A-Control **B- Crude enzyme**

C- Crude enzyme + EM (Effective Microrganisms)

4.5.5 FTIR ANALYSIS OF TREATED AND UNTREATED FIBRES AFTER 45 DAYS OF BIO-SOFTENING

The fibers of treated and untreated fibers of coir were characterized after 45 days of their with *Bacillus subtilis* for their chemical composition by FTIR method as mentioned in Materials and Methods.

The value of the peak position of the absorption bands corresponding tothe vibrating groups was presented in plate VIII and Figure 4.12

Plate VIII

Treated and untreated fibers after 45 days of Bio-softening

A. Untreated coir pith

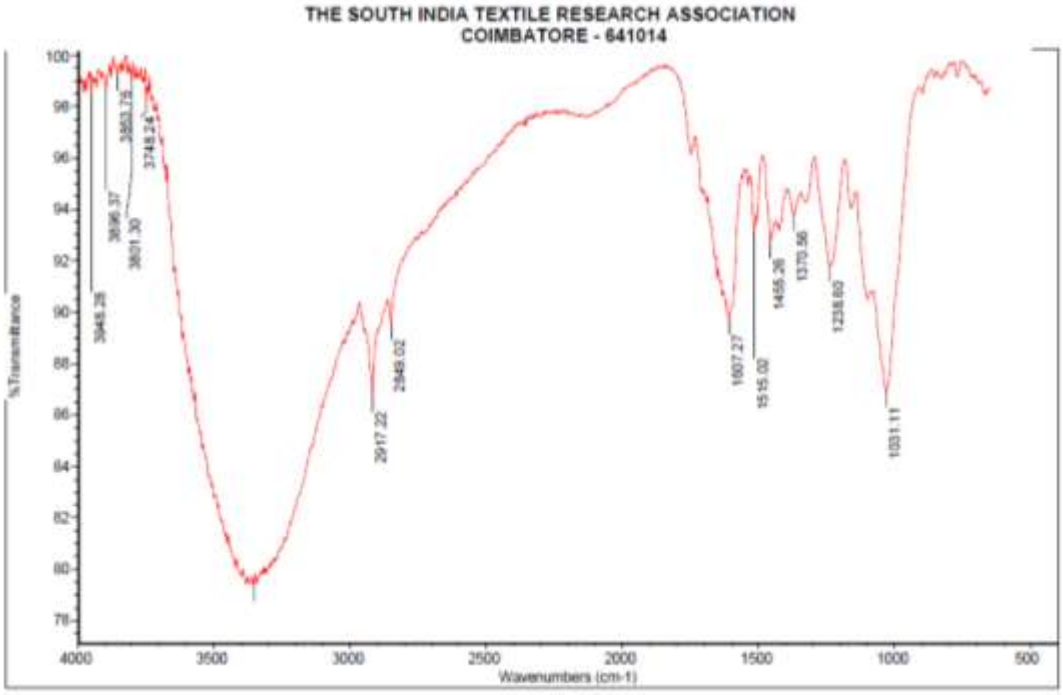
B. Treated coir pith

The value of the peak positions of the absorption bands corresponding groups. Figure 4.12 showed the various peak positions of absorption bands between 4000cm-1 and 400cm-1. The intensity of the band around 3588.71cm-1 to 3422.65 cm-1 and 1607.27 to 1515.02 were respectively. Corresponding to the O-H stretching of a cellulose and C=O stretching of hemicelluloses.

Figure 4.12

FTIR Analysis of treated and untreated fibers after 45 days of Bio-softening

Sample A



Sample B