MATERIALS AND METHODS

3.1 LIGNOCELLULOSIC BIOMASS (PLANT MATERIALS)

The lignocellulosic biomasses used in the extraction of xylan were collected from the agricultural fields in Coimbatore district of Tamil Nadu State in India.

3.2 COLLECTION SPOT

Place	:	Coimbatore
Year of Collection	:	2010-2011

3.3 GENERAL MYCOLOGICAL TECHNIQUES

Mycological techniques were followed as described in the Methods in Microbiology, Vol 4 (Booth, 1971).

3.4 CULTURES USED

The bacteria used in the present study were obtained from the soil samples collected from Coimbatore.

3.5 GLASSWARES

The glasswares as used in the present study are of Borosil brand.

3.6 CHEMICALS

All the chemicals used in the present study were of analar grade. Oat spelt xylan and Sephadox G 200 were obtained from sigma chemical Co,USA, Sodium dodecyl sulphate and Bovine Serum Albumin were obtained from Sisco Research Laboratory (SRL),India.

3.7 CULTURE MEDIA

The following culture media were used

Nutrient Agar medium (Irfan *et al*., 2012)

Peptone	-	5.0 gm
Beef extract	-	3.0 gm
Sodium chloride	-	5.0 gm
Agar	-	15 gm
Distilled water	-	1000 mL
pH	-	6.8

Xylanase production medium (modified)

Yeast extract	-	2g
Peptone	-	5g
Rice bran	-	10g
Magnesium sulphate	-	0.5g
Sodium chloride	-	0.5g
Calcium chloride	-	0.15
Distilled water	-	1000mL
pH	-	7

3.8 EXTRACTION OF XYLAN FROM VARIOUS LIGNOCELLULOSIC BIOMASSES

The method of Panbangred *et al.,* (1983) was followed for xylan extraction from forty five different lignocellulosic biomasses. 100ml of 3% NaOH was added to 50gm of finely powdered lignocellulosic biomass and incubated at 121°C for one hour. 50ml of ethanol was added and mixed thoroughly with glass rod. Xylan was

precipitated and the precipitate was washed many times with tap water and dried in an oven at 50°C. The xylan thus extracted was used as a carbon source.

3.9 BACTERIAL SCREENING FOR XYLANASE PRODUCTION

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 for potent indigenous isolates were done in 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 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 (Carlos *et al.,* 2002; Kumar *et al.,* 2008) followed by destaining with 1M NaCl. The best isolate was selected based upon the zone formation then it was subjected for sequencing using 16S rRNA techinique.

3.10 STERILIZATION

Sterilization was done in an autoclave at 15psi for 15 min.

3.11 SOURCE OF INOCULUM

Twenty four hours old cultures were used as a source of inoculum. Bacteria from growing front were removed using sterile loop.

3.12 MAINTENANCE OF CULTURES

The bacterial isolates were maintained on Nutrient Agar slants

3.13 LIQUID STATE FERMENTATION (LSF)

Conical flasks (150 ml) containing 50 ml of liquid media were used for liquid state fermentation. Three replicates were maintained for each experiment.

3.14 EXTRACTION OF ENZYME

- **Liquid State Fermentation:** The method of Johri and Pandey, (1982) was followed. Bacterial cultures grown in conical flasks (150 ml) containing 50 ml of liquid media used for liquid state fermentation and it was filtered through double layered muslin cloth and the filtrate was centrifuged at 5000 rpm for 30 min. The supernatant was used as crude enzyme source. The crude enzyme was stored at 4° C till use (Shewale and Sadana, 1978).
- **Storage of enzyme:** The crude enzyme was stored at 4°C in refrigerator till use

ENZYME ASSAY:

The amount of xylanase was estimated by following the method of Bailey *et al.,* (1992)**.** Oat spelt xylan was used as the substrate for xylanase assay and the amount of xylose released was measured by DNS method of Miller (1959).

PREPARATION OF OAT SPELT XYLAN SUBSTRATE FOR XYLANASE ASSAY (Bailey *et al.,* 1992)

Onegram of Oat spelt xylan was homogenized in 50ml of 0.05M sodium acetate buffer pH 5.0. It was heated to boiling point and cooled with continued stirring. The volume is made up to 100ml with the same buffer and stored at 4°C, for a maximum period of one week.

Reaction Mixture

Thereaction mixture contained

Oat spelt xylan(1%)	-	1.8mL
Crude enzyme	-	0.2mL
Sodium acetate buffer	-	0.2mL
(0.05M pH 5.0)		

Reagent Blank

The reagent blank contained

Substratesolution	-	1.8mL
Distilled water	-	0.2mL
Sodium acetate buffer (0.05M pH 5.0)	-	0.2 mL

The reagent blank contained distilled water instead of enzyme. The test tubes containing reaction mixture andthe blank were incubated at 50°C in a water bath for 10 min. Then the reaction was stopped by adding 0.5mL of 10% Trichloro Acetic Acid (TCA). The contents were centrifuged at 3,000rpm for 10 min. To the supernatant, 3 mL of DNS reagent was added and the tubes were cooled to room temperature and the absorbance was measured at 530nm in spectrophotometer. The amount of xylose released was calculated using a xylose standard graph.

3.15 UNIT OF ENZYME ACTIVITY

Reducing sugar released was calculated using a xylose standard graph and the activity is expressed in International Units. One International Unit of xylanase is defined as the amount of enzyme required to liberate 1µ mol of D-xylose per min per mL (Bailey *et al.,* 1992).

3.16 CARBOXYMETHYL CELLULASE ACTIVITY (NELSON, 1994)

The presence of Carboxymethyl cellulase along with xylanase in the crude broth was studied. To 0.2mL of enzyme, 0.5mL of 1% Carboxymethyl cellulase in 0.1M sodium acetate buffer pH 7.0 was added and incubated for 30 min at 50°C. The amount of reducing sugars released was measured as glucose equivalents.

3.17ESTIMATION OF PROTEINS (Lowry *et al.,* 1951)

Protein Estimation

Quantitative estimation of the protein content of individual fraction obtained after different steps was done by Lowry *et al.* (1951) method.

Reagents

Reagent A	:	2% (w/v) sodium carbonate in 0.1N sodium hydroxide
Reagent B	:	Copper sulphate 0.5% in 1% sodium potassium tartarate
Reagent C	:	To 50mL of Solution A, 1mL of solution B was added.

Folin's phenol reagent

The Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. This reagent was prepared fresh at the day of use.

Procedure

About 1mL of the enzyme solution was pipetted out in a test tube and its volume was made up to 1mL with distilled water. A test tube containing 1mL of distilled water alone serves as blank. About 5mL of Reagent C was added to all test tubes including blank and allowed to stand for 10 minutes. After that 0.5ml of Folin's phenol reagent was added, mixed well and kept for incubation at room temperature in dark for 30 minutes. The colour developed after incubation was read using spectrophotometer at 660nm. Bovine Serum albumin was used as the standard.

3.18 SEM ANALYSIS

The SEM analysis for the treated and untreated rice bran was carried out at the Department of DRDO, Bharathiar University, Coimbatore, Tamil Nadu, India.

3.19 PURIFICATION OF XYLANASE COLUMN CHROMATOGRAPHY

Initially, Sephadex G 200 was soaked in Phosphate Buffer Saline (PBS) buffer pH 8.5 for overnight at 4°C. The gel slurry was slowly poured into a glass column (1.5cmX60cm) blocked with glass wool. While packing column, care was taken to avoid air bubbles. The gel was again washed with PBS buffer. Fractions of 3mL were

collected using water programmable fraction collector and the fractions were scanned for xylanase activity and protein content. The fractions showing xylanase activity were collected and concentrated by lyophilisation and stored at -20°C. This enzyme sample was subjected to gel electrophoresis for further studies.

3.20 DETERMINATION OF MOLECULAR WEIGHT

This was done by SDS-PAGE with the proteins of known molecular weight along with the protein to be characterized (Laemmli, 1970).

REAGENTS

Stock acrylamide solution

Acrylamide	-	30g
Bisacrylamide	-	0.8g
Distilled water	-	100 mL

Separating Gel buffer (pH 8.8)

Tris HCl	-	22.7g
Distilled water	-	100mL

Separating Gel (10%)

Stock acrylamide solution	-	13.3mL
Separating Gel buffer	-	8.0 mL
Distilled buffer	-	18.1mL

The gel mixture was degassed on the water pump for 3-5 minutes and then following solutions were added to it.

Ammonium persulphate solution(5%)	-	0.2mL
Sodium Dodecyl Sulphate (SDS) solution (10%)	-	0.4mL
N’N’N’N’ –Tetra Methylene Diamine (TEMED)	-	20mL

Stocking Gel Buffer (pH 6.8)

Tris HCl	-	7.6g
Distilled water	-	100mL

Stacking Gel mixture (4%)

Stock acryl amide solution	-	1.35mL
Separating Gel buffer	-	1.0mL
Distilled water	-	7.5mL

The gel mixture was degassed for 3-5 minutes on a water bath and added with the following solutions.

Ammonium persulphate solution (5%)	-	50µl
Sodium Dodecyl Sulphate (SDS) solution (10%)	-	100µl
N'N'N'N'-Tetra Methylene Diamine (TEMED)	-	10µl

Electrode buffer

Tris HCl	-	12g
Glycine	-	28.8g
SDS	-	2g
Water	-	2000mL
pH	-	8.2-8.4

Sample buffer (5X concentrations)

Stacking gel buffer	-	5mL
SDS	-	0.5g
Sucrose	-	5g
Mercaptoethanol	-	0.25mL

Bromophenol blue (0.5% w/v solution in water)	-	1mL
Distilled water	-	10mL

SDS solution (10%)

SDS	-	10g
Distilled water	-	100mL

Washing solution

1mL of formaldehyde (37%) was mixed with 40mL of methanol and 60mL of distilled water.

Staining solution

Preparation of Coomassic blue

Coomassic blue G-250	-	100mg
Ethanol 95%	-	50mL
85% w/v orthophosphoric acid	-	100mL
Distilled water	-	1000mL

Silver staining

Silver nitrate solution	-	0.1%

Developer

Sodium carbonate solution (3g in 80mL)	-	80mL
Sodium thiosulphate solution (200mg/L)	-	1mL
Formaldehyde	-	1mL
Distilled water	-	100mL

Stopper

Acetic acid - 5%

Thoroughly cleaned dried glass plates and spacers were assembled properly and held together with bulldog clips in upright position. White petroleum jelly was applied around the edge of the spacer to hold them in place and to seal the chamber in between the glass plates.

The separating gel solution was poured in the chamber between glass plates, layered on top with distilled water and left undisturbed for 30-60minutes for polymerization. After polymerization, the water layer was removed and the surface was washed with little stacking gel solution. Then the stacking gel mixture was poured on the top and comb was placed in the stacking gel. The gel was left for setting (30-60 minutes). Once the stacking gel was polymerized, the comb was removed without disturbing the well and the gel was installed in the electrophoresis apparatus after removing the clip and the agar. The tank was filled sufficiently with electrode buffer and any air trapped at the bottom of the gel was removed.

The protein content of the sample was made uniform by using 5X concentration of the sample buffer. The samples were heated in the boiling water bath for 2-3 minutes. After cooling 10µl of the sample was injected into the samples well using a micro syringe through the stacking gel and then run for 30minutes until the bromophenol blue (marker dye) reaches the bottom of the gel. After the run was complete, the gel was removed from the plates, transferred to a clean plastic container and washed with washing solution with slow shaking for 10minutes.

Staining of separated proteins

At the end of electrophoresis, the gels were carefully taken out and fixed in 10% TCA, stained immediately with 0.02% (w/v) coomassic brilliant blue (in a mixture of methanol, acetic acid and glass distilled water in the ratio of 40:10:50). After staining the gels were destained in a solvent system of the same ratio and also stained by the silver staining method to test the purity of the purified protein. After staining, the gels were stored in 7% (v/v) acetic acid at 4°C. The gel was then photographed and stored in polythene bag.

3.21 ENZYME CHARACTERIZATION

The purified enzyme of *Bacillussubtilis* was used for characterization. The physico chemical properties analyzed were molecular mass, the effect of pH, temperature, metal ions and inhibitors on xylanase activity were determined.

Effect of pH stability

pH stability was studied by incubating the reaction mixture at different pH ranging from 5.0 to 9.0 in three different buffers, (0.1M acetate buffer for acidic pH, 0.1M phosphate buffer for neutral and 0.01M citrate buffer for alkaline pH) and incubated at room temperature for 30min. Samples were withdrawn and activity was assayed in buffer of pH 7.0 for 30min at 65°C and the results are tabulated. The reaction mixture was assayed and the relative activity was calculated.

Effect of Temperature stability

Temperature stability of enzyme was investigated by incubating the enzyme reaction mixture at various temperatures ranging from 40°C to 70°C. The reaction mixture was assayed and the relative activity was calculated.

Effect of inhibitors on enzyme activity

The effect of inhibitors on enzyme activity was done with 0.2ml of enzyme and 0.2ml of Sodium acetate buffer containing 0.8ml of oat spelt xylan of inhibitor solution at 35°C for 30min. Inhibitors such as EDTA (Ethylene Diamine Tetra Acetic acid), SDS(Sodium Dodecyl Sulphate), Sodium azide (NaN3) in 1mM concentration, 2-mercaptoethanol and Dithiothreitol in 1µl concentration were used. After incubation the reaction mixture was assayed and the residual activity was calculated. The reaction mixture without inhibitors served as control.

Effect of metal ions on enzyme activity

The effect of metal ions on enzyme activity was done with 0.2 mL of enzyme and 0.2mL of sodium acetate buffer containing 0.8mL of Oat spelt xylan of metal ion solution at 35°C for 30min. Metal ions such as Magnesium, Zinc, Calcium, and Lead were used at the concentrations of 1mM. After incubation, the reaction mixture without the metal ions served as control.

3.22 APPLICATIONS OF XYLANASE IN VARIOUS INDUSTRIES

Paper pulp treatment

The method of Dhillon *et al.,* 2000 was followed for the pulp treatment and the methodology of the treatment was altered according to the lab conditions.

Kappa number

The kappa number (Kappa no) indicates the delignification level of the pulp with lower values showing higher lignification and greater Delignification Efficiency (DE) as determined according to the following equation (Bim and Franco, 2000)

$$DE = \frac{\text{Kappa no}_{\text{initial}} - \text{Kappa no}_{\text{final}}}{\text{Kappa no}_{\text{intial}}} X100$$

The Kappa number is the volume of 0.1N $KMnO_4$ solution consumed by 1g of moisture free pulp. Small pieces of sample sheets were weighed out which will consume approximately 50% $KMnO_4$ solution (0.1%). The permanganate consumption must be 30-70%. At the same time a second specimen was weighed out to determine its moisture content. The test specimen was disintegrated in 500mL or less of distilled water until free of fibre clots and undispersed fibre bundles. The disintegrated specimen was made up to 800mL to 100mL of $KMnO_4$ solution (0.1N) 100ml of H_2SO_4 (4N) was added, cooled to 25°C and immediately added to disintegrated test specimen.

After 10 minutes the reaction was stopped by adding 20mL of potassium iodide solution (1.0N) and titrated against sodium thiosulphate solution (0.2N). Starch solution (0.2%) was used as the indicator. A blank titration was carried out using the same but omitting the pulp.

1.XYLANASE PRETREATMENT ON WASTE PAPER PULP

Preparation of waste paper pulp and treatment

Waste papers such as (news papers, hard sheets, printed sheets, tickets) were collected and soaked for two days by changing the water for every 6 hours. The paper was squeezed and grinded in the mixer with water, the pulp was then defiltered and dried in the oven at 50°C and packed in a tight container for further use. The prepared pulp should be soaked in distilled water for 10 min for further experimental use.

The pulp suspension of 5% of Consistency was disintegrated in water at pH 6.5, 30°C for 15min under static conditions. In this suspension the various charge of enzyme was added and stirred for 2h at 30°C. The crude enzyme was inactivated by boiling the pulp suspension for 5min. Physical and mechanical properties of the pulp and paper were determined by preparing hand sheets, by passing the pulp through Buchner funnel which was layered with blotting paper, the water was removed by filtering and hand sheets were pressed and dried in hot air oven at 50°C.

Effect of enzyme charge on kappa nuber of waste paper pulp

To determine the optimum enzyme charge for the pulp treatment the pulp was incubated with various charges (6.93, 9.24, 11.55, 13.86, 16.17, 18.48 and 20.79 U/gpulp) at a standard temperature 60°C. The efficiency of enzyme charge for the pulp treatment was measured in terms of release of reducing sugars means reduction in kappa number.

2. EFFECT OF XYLANASE IN THE BREAD TREATMENT

The wheat bread was prepared from the wheat flour (Annapurna brand, Hindustan Lever Ltd, India), with the composition of wheat flour 250g, 3.0% (w/w);edible salt, 2.0% (w/w);wet compressed yeast, 1.5%(w/w);ghee (butter oil),1.25%(w/w) and adequate amount of water to obtain dough of optimum consistency (180mL for control and 160mL for xylanase supplemented dough) as judged by an experienced baker. Partially purified xylanase was added at the rate of 11.5U/g of wheat flour in the experimental set.

Flying ferment was prepared by mixing crumble yeast and one-fifth part of the total sugar in one-tenth part of like warm water (37°C) for 10min. The paste along with remaining ingredients and water were mixed for 15min and kneaded to form smooth dough and smeared with 3g ghee. Fermentation was carried out in a clean, glass beaker for 3 hrs in a tight container. After 30 minutes fermentation, the dough was knocked back and fermentation was allowed to continue for additional 15min. The fermented dough was equally divided into two portions, rounded, molded and placed into two greased pans (2X8X8 cm). Proofing was done at 35°C for 45min. Baking was done at 230°C for 10min. After baking, bread loaves were allowed to cool for 1h at 28°C. Slicing and packaging were carried out for the determination of loaf volume and weight.

Determination of dough and bread characteristics

The increase in dough volume during fermentation was determined using a graduated beaker. Dough rising was calculated as the ratio of increase in dough height/initial height of dough, multiplied by 100. Water absorption during mixing was calculated as the ratio of water absorbed by flour/weight of flour, multiplied by 100. Bread loaves from control and xylanase supplemented wheat flours were weighed and volumes were measured after cooling the loaves. The method of Shah *et al.*(2006) was followed for the treatment of bread.

3. COMPATIBILITY OF XYLANASES WITH LAUNDRY DETERGENTS

Various stains made from coffee and tea powder (10mg/mL), Mango, Pomegranate and tomato (1% extract) was used in the present study. A white cotton cloth of size 7X7 cm was stained with 25µl of the above stains individually and subjected to the following washing processes.

1. Washing with distilled water at 35°C for 30 min
2. Washing with crude enzyme (10% v/v) obtained from *Bacillus subtilis* for 30 min
3. Washing with the mixture of crude enzyme and (10%v/v) and 1% commercial detergent solution at 35°C for 30 min.

4. After washing, the cloth was rinsed well with water to remove excess of detergent and enzyme. It is then, dried and visualized.

4. EFFECT OF CRUDE XYLANASE OF BACILLUS SUBTILIS AND EFFECTIVE MICROORGANISMS (EM) ON COMPOSTING

The method of Kanmani *et al.*(2009) and Vidhya *et al.* (2004) was followed. The methodology of the treatment was altered according to the requirement. Four separate plots of 5mX5m were selected for composting. The material mixture consist of 6kg of vegetable wastes(fresh weight) collected from nearby area, 2kg of cattle manure and 2kg of leaf litter (dried). Prior to composting, the maximum particle size in the mixture was restricted to 1cm in order to provide better aeration and moisture control (Kalamdhad *et al.,* 2007). The contents were thoroughly mixed and placed in four plots separately. These four plots were left undisturbed for 45 days for composting

Plot 1- control

Plot 2- Crude enzyme (500ml)

Plot 3- EM Solution (500ml)

Plot 4- Crude enzyme+ EM solution (500ml+500ml)

Measurement Techniques

After 45 days, random samples were taken from several different points, mostly at the mid span and ends of the composter. Triplicates samples were collected and stored at 4°C immediately till analysis. Temperature was monitored using a digital thermometer throughout the composting period. Each sample was analyzed for the following parameters: pH, Nitrogen, Phosphorous and Potassium and the results are tabulated.

5. BIOSOFTENING OF FIBRES (Jayapriya and Vigneswaran, 2010)

The reduction of lignin content in fibres was studied by incubating fibres with microorganisms. This process is termed biosoftening and is carried out as follows

Method of inoculation

To the conical flask containing 2g of sterilized fibre (30cm) and 100ml broth medium was added and 5mm mat of 24hrs culture. The fibre in broth medium but without bacterial culture served as a control.

Incubation

The culture was incubated at 30°C±1 in an incubator for a period of 45 days

Analysis of treated fibres

Atthe end of incubation the fibres from the bacterial mat and washed thoroughly in distilled water and dried in oven at 40°C. The treated and untreated fibres were analysed for FTIR Analysis.

Fourier Transform-Infrared Spectral Analysis (FTIR) of extracted fiber

The fiber samples were cut into very fine pieces (lee than 1mm) with scissors for FTIR analysis. This powder was mixed with KBr and pellets were prepared using hydraulic press. The samples were recorded in 4000-500cm^{-1} region in a PerkinElmer 16PC FTIR instrument to observe the chemical constituents based on their wave numbers. 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).

3.23 STATISTICAL ANALYSIS

All experiments were conducted with three replicates and their mean values represented. Statistical analysis was carried out by One Way ANOVA using AGRES software and Duncan's Multiple Range Test (DMRT) (Duncan, 1955)