Chapter 2.

Selection of bacterial strain, comparison of different mode of fermentations for xylanase production and selection of suitable substrate for SSF

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Lignocellulosic biomass such as agricultural and forestry residues, municipal solid waste and dedicated crops provide a low cost feedstock for biological production of fuels and chemicals, which offer economic, environmental and strategic advantages (Someet et al., 2001). Effective utilization of these materials for the production of enzymes, biofuels and other metabolites needs extensive studies. (These materials generally contain up to 25% of lignin and 40% cellulose, which cannot be easily converted to simple monomeric form due to their non-biodegradable nature. Hemicelluloses, the second most abundant polysaccharide next to cellulose, consist of β-1, 4-linked pyranosyl backbone. The break down of hemicellulose requires the action of various enzyme of which endoxylanase is the most important one. Microbial xylanase are advantageous over their counter parts from plant and animal sources because of their easier availability, structural stability and ease of genetic manipulations (Chand and Mishra, 2003, Subramaniyan and Prema, 2002). Xylanase have been isolated from a diverse range of microorganisms including fungi and bacteria of which endo β-1-4-D-xylanase (E.C. 3.2.1.8) are mainly responsible for the random hydrolysis of xylan (Puls 1997). Only few xylanase are reported to be cellulase-free as well as active and stable at alkaline pH and high temperature (Collins et al., 2005).

In view of their potential role, cost effective development of enzymes is crucial, as this will significantly benefit the overall economics of biological processes. Agricultural residues make good substrates for fermentation, provided bioreactors are designed with suitable operational control (Pandey et al., 2000, Krishna, 2005). Earlier production of enzyme was targeted using Submerged or liquid fermentation, which is easy to handle and contamination free but the cost of production is very high. One alternative for this is use of Solid State Fermentation. These fermentation systems, which are closer to natural system, may prove more efficient in producing certain enzymes and metabolites. There are many reports related to production of endoxylanase by SmF using bacteria (Archana and Sathyanarayan, 1997, Subramaniyan et al., 1997) and SSF by fungi (Souza et al.,
2001, Someet et. al., 2001, Holker et. al., 2004, Senthilkumar et. al., 2005, Yang et. al., 2006). Fungi prefer to grow well on moist substrate, with feeble moisture content, whereas bacteria are unable to grow in these conditions, they require high moisture contents. As a result, there are only fewer reports related to successful use of bacteria for SSF (Gessesse and Mamo, 1999, Rani and Nand, 2000, Virupakshi et. al., 2005, Asha and Prema, in press). The major objective of the present study was isolation and identification of bacterial strain for the production of cellulase free endoxylanase, as well as to compare overall physiological behavior of Bacillus pumilus for endoxylanase production by liquid and solid fermentations. Also the feasibility of agro industrial residues, in fermentation process and also to evaluate their production which can lead to reduction in the cost of enzyme production. The studies also aimed at the utilization of agro industrial residues treated with alkali and acid for SSF to enhance the production of endoxylanase.

2.1.0. MATERIALS AND METHODS

2.1.1. Selection of microorganism

Xylanase producing bacterial strains were taken from RRL (TVM) culture collection, which was isolated from soil samples collected from wood mills and forest regions of South Kerala (Subramaniyan, 2000). Selection from these cultures was based on high potency for xylanase production, which was active at high alkalinity and temperature, giving importance to bacterial strains. Nine bacterial strains were selected for qualitative screening.

2.1.2. Qualitative screening by plate method for xylanase activity

Initial screening was done on Xylanase screening media (XSM), rich in oatspelt xylan (Oatspelt xylan, Sigma Chemicals Co.). The nine bacterial cultures were spot-inoculated on plates with media composition (g/L) Oat spelt xylan 5.0, Yeast extract 5.0, Peptone 5.0, K2HPO4- 0.2, MgSO4. 7H2O 0.04 and agar 20 of pH 7 and 10 (Oat spelt xylan Agar plates), the pH was adjusted after autoclaving using 10 % Na2CO3 and 1N HCl. The plates were incubated at ambient temperature for 48 hour. The plates were then flooded
with Congo red solution (0.05 % w/v) for 30 min and destained with 1N NaCl solution till a clear zone of xylan hydrolysis was visible. The colonies, which have given clear zone, were preferred as xylanolytic isolates (Wood et. al., 1988). Unless and otherwise specified all sterilization was done at 121°C at 15 lbs for 15min.

2.1.3. Quantitative screening by shake flask method

The six xylanolytic bacterial strains were selected from the preceding step were quantitatively screened for xylanase production in Xylan production media (XPM) with xylan as the main carbon sources of composition Oatspelt xylan 5.0, Yeast extract 5.0, Peptone 5.0, K₂HPO₄- 0.2 and MgSO₄. 7H₂O 0.04 (Subramaniyan, 2000, Horikoshi, 1991a). Erlenmeyer flasks (250 mL) containing 90mL XPM was inoculated with 18 hour grown inoculum (5 % v/v) and incubated in incubator shaker for 144 hour at 120 rpm at ambient temperature. Samples were taken at regular interval of 24 hour and pH, cell count as well as dry weight was estimated. The cell free supernatant was recovered by centrifuging samples at 10,000 rpm at 4 °C for 20 min (SELECTA- Cold centrifuge, Germany) and used for measuring the xylanase activity, total soluble protein and reducing sugar.

2.1.4. Pre inoculum preparation

The inoculum was raised in the same medium (Section 2.1.3) under similar conditions in 150 mL Erlenmeyer flasks and 18 hour grown inoculum was used to initiate growth. A loop full of culture was inoculated in the medium and incubated for 18 hour in rotary shaker (120 rpm) at ambient temperature (30 +/- 2 °C).

2.1.5. Production of xylanase

Production of xylanase from selected isolates was studied in correlation with the growth profile of culture in the medium of composition Wheat bran 5.0, Yeast extract 5.0, Peptone 5.0, K₂HPO₄- 0.2 and MgSO₄. 7H₂O 0.04. Fermentation experiments were carried out using conditions already mentioned. Cells were suspended by centrifugation
at 10,000 x g for 20 min at 4°C and cells free supernatant was used for extracellular crude enzyme preparations.

2.1.6. Identification of Bacteria

Following the procedure of Bergey's manual of Systematic Bacteriology the selected bacterial strain was identified (For *Bacillus* sp. Claus and Berkeley's, 1986). Growth pattern was studied by culturing in liquid fermentation media, thus identified whether aerobic or anaerobic, spore staining done using Malachite green, motility test, gram staining for classification of the isolate to specific genera. Biochemical characterization was done this includes, M.R - V.P test, catalase test, urease test, nitrates test, indole test, starch hydrolysis test, and gelatin liquefaction test (Subramaniyan, 2000). I.M. Tech, Chandigar, (India) did identification of the selected strain.

2.1.7 Enzyme profile in SmF and SSF

Selected culture was subjected to plate assay of different carbon sources to check the production qualitatively. Cellulase production during the screening procedure was checked by incorporating plate with Carboxy methylcellulose, Protease by casein, amylase by starch and pectinase by pectin. That plate which has given clear zone was checked quantitatively for the production.

2.1.8. Analytical procedures

2.1.8.1. pH

The pH of the culture filtrate was estimated using Cyber scan 1000 pH meter.

2.1.8.2. Moisture content

The moisture content of the substrate was estimated by direct dry weight method (Pandey *et al.*, 2001).
2.1.8.3. Reducing sugar

Reducing sugar of the extract was estimated by dinitrosalicylic acid method or DNS method (Miller, 1959). The reducing sugar was estimated using standard graph prepared with glucose. Reaction with glucose 3, 5 - Dinitrosalicylic acid (DNS) gets reduces to 3-amino 5- nitro salicylic acid while the sugar gets oxidized to gluconic acid.

2.1.8.4. Total soluble protein

Total soluble protein of the extract was estimated by Lowry’s method (Lowry et. al., 1951). The principle behind this method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteau phosphormolybdic /phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The protein standard was prepared using Bovine Serum Albumin (BSA).

2.1.8.5. Endoxylanase assay

Endo xylanase (1, 4, β - D - xylan xylanohydrolase- E.C. 3.2.1.8) was assayed by some modification to Bailey method (Bailey et. al., 1992). Preparation of xylan suspension or substrate - 0.5 % of oat spelt xylan (Sigma Chemicals Co.) in 0.1M phosphate buffer (Na₂HPO₄/ NaH₂PO₄), at pH -7 and 0.1 M carbonate buffer at pH- 10 (Na₂CO₃/ NaHCO₃), by warming at 80 °C and made into paste in an homogenizer, later it was boiled and made up to required volume. The solution was cooled and stored at 4 °C. The reaction mixture contains 1.8 mL of preincubated xylan suspension and 0.2 mL of suitably diluted enzyme preparation was added and incubated at 50 °C for 10 min. The reaction was terminated by the addition of 3 mL of DNS, and boiling it in boiling water bath for 5 min followed by sudden cooling in ice bath. Enzyme blank was prepared in parallel by adding DNS reagent prior to enzyme addition so that only the reducing sugar in the enzyme will be answered. This was required only when the sample contains high reducing sugar or the dilution was small. The reagent blank too was prepared in same procedure but instead of 0.2 mL enzyme buffer was added. The concentration of reducing sugar -xylose liberated was estimated against xylose standard prepared at 540 nm. The
stock solution of xylose (Xylose, Merck) standard was prepared in 10 μmole / mL concentration and appropriate dilution was taken for preparation of standard. One unit of endoxylanase activity was defined as 1μ mole of xylose liberated per minute per mL of enzyme preparation under the assay conditions.

2.1.8.6. Cellulase assay

The plates were incorporated with Carboxymethyl cellulose and cultures were spotted. Later during the fermentation studies selected culture was assayed for CMC ase and FP ase using cell free supernatant. Absence of cellulase activity was essential with high xylanase production, due to its importance in paper and pulp industry.

2.1.8.6.1. CMC ase - (1, 4-(1, 3:1, 4) - β- D- glucan-4-glucano hydrolase) 

CMC ase (1, 4-(1, 3 : 1, 4) - β- D- glucan – 4 - glucano hydrolase (E.C. 3.2.1.4) or Carboxymethyl cellulase assay was carried out using sodium salt of Carboxymethyl cellulose in 0.1 M phosphate buffer pH - 7 as substrate (Dahlberg et. al., 1993), the reducing sugar liberated was estimated by DNS method. To 1.0 mL of preincubated substrate suspension kept at 50 °C added 0.5 mL of crude enzyme suitably diluted. The reaction mixture was incubated for 15 min. The reaction was terminated by the addition of 3.0 mL of DNS reagent and heated in boiling water bath for 5 min followed by sudden cooling in ice bath. Absorbance was taken against glucose standard at 540 nm and CMC ase activity expressed as one μmol of glucose liberated per minute per mL of culture supernatant.

2.1.8.6.2. FP ase activity (1, 4-β-D-glucan cellobiohydrolase)

FP ase activity or cellobiohydrolase (E. C. 3.2.1.91) activity was estimated by modified Mandel method (Mandel et. al., 1976). Whatman No.1 filter paper (0.05g) was used as substrate; Filter paper of 1x 6 cm was cut into pieces. The reaction mixture contain 0.5 mL of preincubated phosphate buffer (pH - 7, 0.1 M) with filter paper, to this added 0.5 mL of suitably diluted enzyme and incubated at 50 °C for 1 hour. The reaction was stopped by the addition of 3 mL DNS reagent and boiled for 5 min followed by sudden
cooling. The absorbance of the reaction was taken at 540nm against glucose standard; the reducing sugar liberated was expressed as one µmol of glucose produced per mL per minute under standard assay conditions.

### 2.1.8.7. Protease assay

The protease was assayed by TCA method (Subramaniyan et al., 2001). Two percent casein was used as substrate in carbonate buffer pH - 10. The preincubated reaction mixture contain 0.5 mL of suspension of casein to this added 0.5 mL of suitably diluted enzyme and incubated at 40 °C for 10 min. The reaction was terminated by the addition of 10 % TCA (trichloro acetic acid). The mixture was centrifuged at 10,000 rpm at 4 °C for 10 min and supernatant was collected. From this supernatant 0.5 mL of reaction mixture was mixed with 5 mL of 0.5 M Na₂CO₃, and kept for 10min; to this add 1mL of 1N Folin Ciocalteau’s reagent and the tubes were incubated for 30 min in dark for colour development. Enzyme blank and reagent blank were prepared in parallel with test solution. The readings were taken at 600 nm against tyrosine standard and one unit of enzyme was expressed as one µmol of tyrosine liberated per mL per minute.

### 2.1.8.8. β- Xylosidase assay

Incubating 1 mL of 1mg/mL of O-nitro phenyl- β-D- xylopyranoside with 0.1 mL suitably diluted enzyme in 50 mM phosphate buffer pH - 7 at 50 °C for 30 min reaction was terminated by addition of 2 mL of 1 M Na₂CO₃. The liberated O-nitro phenol was measured at 410 nm (Flanigan and Sellars, 1977).

### 2.2.0. Fermentation experiments

Submerged or liquid fermentation using different agro industrial residues was studied and the results were compared with that of Oat spelt xylan as carbon sources. Optimization of culture conditions for Submerged fermentation using wheat bran was tried. Solid-state fermentation was done with different agro industrial residues and production was compared with that of acid and alkali treated residues. Comparison of SmF and SSF were done to select suitable mode of fermentation.
2.2.1 Pre inoculum preparation for SmF and SSF

The inoculum was raised in the same medium (Section 2.1.3) in 150 mL Erlenmeyer flasks and 18 hour grown inoculum was used to initiate growth. Properly inoculated media was incubated for 18 hour in water bath shaker (LAB-LINE Instruments Incorp. Orbit water bath shaker) set at 150 rpm at ambient temperature (32 +/- 2 °C). This was used as preinoculum for both SmF and SSF studies. The cell count was estimated using haemocytometer and it was estimated as ~3.6 x 10^6 cell count/mL.

2.2.2 SUBMERGED FERMENTATION

2.2.3 Effect of different carbon sources on xylanase production

Liquid mode of fermentation was carried out using media composition as follows (g/L): Yeast extract 5.0, Peptone 5.0, K2HPO4 0.2 and MgSO4. 7H2O 0.04 and Oat spelt xylan (5.0) was replaced with other simple agro industrial residues like wheat bran, rice straw, saw dust, coconut pith, sugarcane bagasse, rice bran and compared with the production related to oat spelt xylan. The flasks were incubated in a water bath shaker set at 150 rpm. Enzyme was extracted at regular interval of 24 hour and assayed for reducing sugar, endoxylanase, biomass and total soluble protein.

2.2.4 Effect of pH on xylanase production

In order to study the effect of initial media pH on enzyme production by *Bacillus pumilus* by SmF, initial media pH was adjusted. The experiment was carried out in modified Horikoshi -II basal media of composition as follows wheat bran 5.0, Yeast extract 5.0, Peptone 5.0, K2HPO4 0.2 and MgSO4. 7H2O 0.04. The initial media pH was adjusted within the range of 5 to 10 with an increment of 1.0 unit (after autoclaving). In all cases the initial pH was uncontrolled through out the experimentation. The variation in pH was also recorded through out the studies. Enzyme was extracted at regular interval of 24 hour and estimated for pH, reducing sugar, endoxylanase, biomass and total soluble protein. Growth was correlated with the enzyme production and variation in pH.
2.2.5. Effect of temperature on endoxylanase production

Effect of temperature on enzyme production by *Bacillus pumilus* was analyzed by varying the incubation temperature ranging from 20 to 65 °C (20, 30, 35, 40, 45, 50, 55 and 65 °C). The temperature was set in the environmental incubator shaker (New Bruenswik, Scientific Edison, N.J., USA). The initial pH in all case was set at 8.5 with wheat bran as substrate. The fermentation was carried out for 120 hour and samples were taken at regular interval of 24 hour for estimation.

2.2.6. Effect of inoculum sizes on xylanase production

Inoculum was raised with media composition similar to the fermentation media; this was done to curtail the lag phase in fermentation process (Lincoln, 1960). Inoculum was transferred at logarithmic phase of growth. Different concentration of inoculum was studied (%) 1, 2.5, 5, 7.5 and 10. Cell growth was monitored by measuring the optical density (OD) of suitably diluted culture broth at 600 nm. The cell count was taken by serial dilution in sterile saline and 0.1 mL was plated on nutrient agar plate. Number of cell was counted in plate counter and mean of the data was taken. Growth can also estimate by dry weight method (Pandey *et. al.*, 2000). Concentration above 10 % was not feasible to industrial process (Lincoln, 1960). Samples were taken at regular interval and estimated for 120 hour.

2.2.7. Effect of inducers on xylanase production

Different simple sugars were added to the liquid fermentation medium as carbon sources (1 %) replacing xylan from the Horikoshi basal media II. The worked aimed at the effect of different carbon sources on the enhancement of xylanase production by *Bacillus pumilus*. Different carbon sources supplemented are mono-sugars like- xylose, glucose, fructose, galactose, disaccharides like- sucrose, lactose, maltose and polysaccharides like- xylan, starch, wheat bran and rice bran.
2.3.0 SOLID-STATE FERMENTATION

Erlenmeyer flask (250 mL) containing 10 g of substrate, was mixed with basal medium of composition (g/L) Yeast extract 5.0, Peptone 5.0, K$_2$HPO$_4$ 2.0, and MgSO$_4$ 0.4, were autoclaved at 15-lbs / inch pressure for 45 min. The cooled sterilized medium was inoculated with 18 hour grown inoculum and incubated for 120 hour. The samples were taken and extracted at regular interval of 24 hour, the content was extracted with 10 fold (v/w) distilled water. The content was filtered using cheesecloth and filtrate was centrifuged at 10,000-x g for 20 min at 4 °C (SELECTA cold centrifuge, Germany), the clarified supernatant was used as crude enzyme. All experiments were done in triplicate, individually and no optimized condition of one is carried over to another.

2.3.1. Enzyme production using agro-industrial residues

The fermentation media was prepared as described earlier in Section 2.3.0. The different substrates selected for SSF studies were: - rice bran, rice husk, rice straw, sawdust, coconut pith, sugarcane bagasse, thur-dal husk, Soy meal flakes and wheat bran. The autoclaved media was cooled and inoculated with 18 hour grown culture, preinoculum prepared as mentioned in section 2.1.4. The flasks were incubated at ambient temperature, samples were taken at regular intervals and extracted as mentioned earlier. The supernatant obtained after centrifugation was used as the crude enzyme source.

2.3.2. Effect of pretreatment of agro industrial residues on xylanase production

In order to study the effect of acid and alkali treatment on xylanase production, different agro industrial residues like straw, coir pith, sawdust, bagasse, wheat bran, rice husk and rice bran were pretreated. 1 N NaOH for alkali treatment and 1 N HCl for acid treatment followed by autoclaving at 15 lbs for 30 min. It was then washed to neutral pH and dried at 60 °C before use in fermentation studies. Fermentation studies were carried out as mentioned earlier and results compared with untreated.
2.4.0. RESULTS AND DISCUSSION

2.4.1. Importance of alkaline thermostable microorganism

The significance of xylanase and its application in wide area of industries has made it a key enzyme. The interest in xylanase has been raised markedly in the recent years, mainly because of their use in various industries like bread and baking, food and feed and particularly pulp and paper industry, mainly for the bleaching process (Viikari et al., 1994, Beg et al., 2001, Subramaniyan and Prema, 2002). For such biotechnological applications xylanase should be devoid of cellulase activity as well as stable at elevated temperatures and active at alkaline pH. Manufacturers continuously search for better performing enzymes by screening different microorganisms or by developing tailor-made enzymes using molecular engineering. Only few bacterial and actinomycete have been reported earlier, which are active at high alkalinity or neutral pH (Horikoshi and Atsukawa, 1973, Nakamura et al., 1994, Durate et al., 2000, Kamal et al., 2004, Asha and Prema, in press). Various methods have been adopted for the screening of microorganism that was capable of degrading lignocellulosic matter. The screening was complicated by the fact that each main components of lignocellulosic material; requires various enzymes which were controlled by elaborate pathways of biochemical and genetic regulation (Kluepfel et al., 1986).

2.4.2 Screening for xylanolytic potential

The major aim of the study was to isolate a potent producer of xylanase form the various sources, mainly soil isolates. Of the nine bacterial isolates taken for xyloanalytic activity by spot inoculation on xylan agar plates, the plates that have shown clear zone by xylan hydrolysis were selected by measuring the diameter of clearance. Oat spelt xylan used as the sole carbon sources, this help to eliminate those strains that are not specific for xylanase. Observing the area of clear zone produced differentiated the potent xylanase producers; six of those, which have shown clear zone, were selected for further studies. Since, xylan polymer is of large molecular weight, it could not enter the microbial cell,
the endoxylanase produced by most of the microorganism were extracellular in nature (Biely, 1985). All cultures that has produced clear zone might not be good producers of endoxylanase.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>pH</th>
<th>Soluble protein mg/mL</th>
<th>Reducing sugar mg/mL</th>
<th>Biomass mg/mL</th>
<th>Maximum hour of production</th>
<th>Xylanase activity of pH-7</th>
<th>Xylanase activity of pH-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.1</td>
<td>2.9</td>
<td>0.314</td>
<td>2.1</td>
<td>96</td>
<td>0.15</td>
<td>1.01</td>
</tr>
<tr>
<td>13</td>
<td>9.1</td>
<td>2.5</td>
<td>0.346</td>
<td>2.2</td>
<td>96</td>
<td>1.34</td>
<td>1.16</td>
</tr>
<tr>
<td>20</td>
<td>9.2</td>
<td>2.4</td>
<td>0.0994</td>
<td>1.2</td>
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<td>0.18</td>
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<td>21</td>
<td>8.4</td>
<td>2.5</td>
<td>0.452</td>
<td>4.7</td>
<td>144</td>
<td>11.75</td>
<td>0.05</td>
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<tr>
<td>34</td>
<td>8.5</td>
<td>3.3</td>
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<td>2.5</td>
<td>96</td>
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</tr>
<tr>
<td>53</td>
<td>8.1</td>
<td>2.8</td>
<td>0.36</td>
<td>4.4</td>
<td>120</td>
<td>2.95</td>
<td>0.58</td>
</tr>
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</table>

Table. 2. 1. Growth profile and xylanase production of selected bacterial strains in quantitative screening

When the isolates grown on solid agar medium the presences of membrane bound hydrolyses may produces the clearing by xylan hydrolysis. This can also be due to the regional presences of enzyme secreted near the growing colony in the agar plates. This will not occur in liquid medium, so all above selected strains were subjected to liquid fermentation using oat spelt xylan, followed by xylanase assay to get a clear picture of xylanolytic enzyme produces as well as a quantitative estimation was also possible (Table. 2. 1.). Of all the isolates selected only one has highest production, indicating that all others have produced clearing due to the occurrence of regional presences of xylanase and/or membrane bound xylanase that has lead to good clearing zone on solid agar plate (Aunstrup, 1974).

2.4.3. Detection of production by plate assay

The selected bacterial strain (Fig. 2. 1.) produced clear halo of xylan hydrolysis on xylan agar plates when stained with Congo red and destained with NaCl (Fig. 2. 2.). A clear
zone of hydrolysis was noticed when the culture filtrate was dropped in wells on xylan plates stained with Congo red. I. M. Tech, Chandigar, identified the selected bacterial stain as *Bacillus pumilus*, based on the morphological and biochemical characters as given in Table. 2.2.

*Bacillus* species are dominant heterologous enzyme producing microorganism (Priest, 1992) which are very much attracted by industries for a varying reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into extracellular medium and the GRAS status (Boren *et. al.*, 1998), with food and drug administration for species such as *B. subtilis* and *B. licheniformis*. The biochemistry, physiology and genetics are well studied, facilitating further development and greater exploitation for industrial purposes (Schallmey *et. al.*, 2004). *Bacilli* are gram positive they lack lipo-polysaccharide in the cell wall, so will not produce endotoxins, which is encountered with gram negative strains. There are number of reports related to endo-xylanase production by *Bacilli* (Uchino and Nakane, 1981, Khasin *et. al.*, 1993, Subramaniyan *et. al.*, 1997, Gessess and Mamo, 1999, Dhillon *et. al.*, 2000, Virupaskhi *et. al.*, 2005, Asha and Prema, in press).

![Xylan Agar plate with 24 hour grown culture](image)

*Fig. 2.1. Xylan Agar plate with 24 hour grown culture*
Fig. 2.2. Plate assay for xylan hydrolysis

Fig. 2.3. Micrograph of *B. pumilus* culture
<table>
<thead>
<tr>
<th>Name of test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>+ve</td>
</tr>
<tr>
<td>Spore staining</td>
<td>+ve, Shape oval</td>
</tr>
<tr>
<td>Sporangia bulging</td>
<td>+ve</td>
</tr>
<tr>
<td>Position of the spore</td>
<td>Lateral</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>-ve</td>
</tr>
<tr>
<td>Voges -Proskaur</td>
<td>+ve</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+ve</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-ve</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+ve</td>
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**Acid production**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Arabinose</td>
<td>+ve, (weak)</td>
</tr>
<tr>
<td>Xylose</td>
<td>-ve</td>
</tr>
<tr>
<td>Manitol</td>
<td>+ve</td>
</tr>
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**Growth temperature (°C)**

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<table>
<thead>
<tr>
<th></th>
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<tr>
<td>4</td>
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<td>15</td>
<td>+ve</td>
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</tr>
<tr>
<td>42</td>
<td>+ve</td>
</tr>
<tr>
<td>50</td>
<td>+ve, weak</td>
</tr>
</tbody>
</table>

Table. 2.2. Identification character of selected bacterial strain provided by I. M. Tech, Chandigar, India. Based on Fatty Acid profile and few diagnostic characters of *B. pumilus* the strain has been identified as *Bacillus pumilus* – GC Sub Group- B.
2.4.4. Culture characteristics of *Bacillus pumilus*

- Colour: Creamish
- Shape: circular
- Margin: irregular
- Elevation: flat
- Opacity: opaque
- Texture: smooth, with wrinkles on aging
- Consistency: mucoid

2.4.5. Growth in liquid medium

In liquid medium, uniform growth was observed which settle to form sediment in static conditions.

2.4.6. Pigment production

No pigment productions observed on prolong incubation.

2.4.7. Morphological characteristics

Cell shape: long, slender rods (Fig. 2.3).

2.4.8. Taxonomy of *Bacillus pumilus*

- Kingdom: Bacteria
- Phylum: Firmicutes
- Class: Bacilli
- Order: Bacillales
- Family: Bacillaceae
- Genus: Bacillus
- Species: Bacillus pumilus
2.4.9. Growth profile of *Bacillus pumilus*

Growth profile of the *Bacillus pumilus* shows (Fig. 2.4.) that xylanase production seldom occur at the early hour of incubation, the activity was observed at the post exponential period of incubation or during the stationary phase (Saiman *et. al.*, 1992, Nakamura *et. al.*, 1993a). There was a shift in pH toward 8.5 during maximization of production (Table. 2.3).

![Graph showing fermentation profile of B. pumilus in liquid medium, growth and enzyme production (pH- 10 and 7).]

The culture has attained stationary phase of growth by 24 hour of incubation, but during this period low amount of xylanase was detected, whereas during the end of stationary phase there was a drastic increase in enzyme production. Similar reports were with

Low xylanase activity might be due to the adsorption of xylanase on the surface of insoluble xylan particles present in the culture medium (Espinar _et al._, 1992, Irwin _et al._, 1994). The higher xylanase activity during the lag phase of growth can be counted as the reflection of small amount of xylanase liberated by the cell undergoing autolysis (Espinar _et al._, 1992, Subramaniyan _et al._, 1997) as well as non availability of insoluble particle in the medium which if present might have bounded to the xylanase (Connerton _et al._, 1999). Decline in production after 120 hour of incubation can be considered as the result of intracellular protease from autolysed cells that inactivate xylanase.

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Total soluble protein (mg/mL)</th>
<th>Reducing Sugar (mg/mL)</th>
<th>Variation in pH of the medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.348</td>
<td>4.1</td>
<td>6.5</td>
</tr>
<tr>
<td>24</td>
<td>0.395</td>
<td>3.7</td>
<td>7.5</td>
</tr>
<tr>
<td>48</td>
<td>0.120</td>
<td>3.3</td>
<td>8.3</td>
</tr>
<tr>
<td>72</td>
<td>0.118</td>
<td>2.7</td>
<td>8.5</td>
</tr>
<tr>
<td>96</td>
<td>0.059</td>
<td>2.2</td>
<td>8.6</td>
</tr>
<tr>
<td>120</td>
<td>0.075</td>
<td>2.1</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Table. 2. 3. Fermentation profile of _B. pumilus_, the variation in pH of the fermentation medium at regular interval, reducing sugar and total soluble protein was monitored.

Reducing sugar was high during the early hour of fermentation due to the presences of xylanase in significant amount in the inoculum, which causes the hydrolysis of xylan in the medium. During maximization of production the level of reducing sugar was considerably low. This can be explained as depletion of reducing sugar related to growth.
of bacteria which causes the reversion of catabolic repression, as well as the insufficient amount of xylan at later stage of fermentation which leads to adsorption of xylanase molecules and autolysis of cell (Espinar et al., 1992).

Bocchini et al. (2005) reported that during the cultivation of *Bacillus circulans*, for xylanase production, there was a decrease of sugar concentration, concurrently with an increase of cellular growth, indicating that the sugar was used as substrate by the microorganism. The highest enzyme production coincided with the smallest sugar concentration level. Total soluble protein and reducing sugar together with variation in pH during the time course of fermentation was explained in Table 2.3. There was no considerable variation in total soluble protein and as production starts there was a slight decrease. pH of the medium has shown considerable variation during the latter fermentation hour, it has changed more towards alkaline side. Maximum xylanase production (Fig. 2.4.) was observed at 96 hour of incubation (95.3 IU/mL). Earlier similar reports were there related to xylanase production by *Bacillus* of 6.23 IU/mL (Liu et al., 1998), 2.6 - 4 U/mL (Duarte et al., 1999), 4.8 U/mL (Ratanakhanokchai et al., 1999) and 118.5 IU/mL (Shabeb, 2000). The production of *Bacillus pumilus* was comparable to earlier reports, which can be maximized by optimization of culture conditions.

**2.5.0. SUBMERGED FERMENTATION**

**2.5.1. Effect of different carbon sources on xylanase production**

To minimize the production cost various agro industrial residues were tried and the result on time course of xylanase production was given in Fig. 2.5. The results explained that wheat bran (65.5 IU/mL) as suitable sources for xylanase production but not relative to oat spelt xylan (94.9 IU/mL). Rice straw and rice bran can also be used as good sources for xylanase production where as coconut pith and sugar cane bagasse has considerable less production, the biomass as well as reducing sugar and soluble protein were also very less.
The industrial enzymes production is often limited by the costs of substrates for the cultivation of the producer microorganisms. The use of low cost substrates, such as agricultural wastes has been suggested as an alternative to reduce the production costs (Dhillon et. al., 2000, Bocchini et. al., 2005). With wheat bran high level of xylanase was reported by Bacillus sp. NCIM 59 (Dey et. al., 1992, Archana and Sathyanaarayan, 1997).

Fig. 2. 5. Time course of xylanase production by B. pumilus on various agro industrial residues at 30°C under SmF.

Subramaniyan has reported oats pelt xylan as suitable carbon sources for the enhancement of xylanase production by Bacillus SSP-34 (Subramaniyan and Prema, 1998). Wheat bran has been reported as a suitable source of xylanase production in SmF by fungus A. niger GCBMX-45 (Ikram-ul- Haq et. al., 2002) and A. nidulans KK-99 of
40 IU/mL (Taneja et al., 2002). There were reports of efficient utilization of sugar cane bagasse (8.4 U/mL) and grass hydrolysates (7.5 U/mL) as carbon sources for xylanase production by *B. circulans* by SmF (Bocchini et al., 2005). To minimize the cost of production wheat bran was taken for further optimization studies. *Thermomyces lanuginosus* IOC-4145 was able to produce a very high level of cellulase-free xylanase (266 U/mL) in shake flasks using corncob as substrate (Damaso et al., 2000).

2.5.2. Effect of pH on xylanase production

In order to study the effect of initial medium pH on endoxylanase production by *Bacillus pumilus* was grown in Horikoshi basal medium with varied initial medium pH ranging from 5 to 10 and results were given in Fig. 2. 6. The culture has shown maximum production at pH- 8.5 but maximum growth was observed pH - 9. A significant increase in pH towards 8 was observed with initial pH 5, 6, 7 and 7.5, where as medium with pH - 8.5, 9, 9.5 and 10 there was a decline in pH towards 8 during the early period of growth. Later as growth progress and enzyme production maximizes and there was considerable increase in pH towards 9. Several reports were there with similar pattern of growth and change in pH, *Bacillus* no. C-59-2 (Horikoshi and Atsukawa, 1973) *Bacillus- SSP-34* (Subramaniyan and Prema, 1998, Shabeb, 2000).

Important factor in any fermentation process is pH, and it may change in response to metabolic activities. Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. The initial pH of the medium has strong influence on enzyme production; it influences many enzymatic system and transport of several species of enzymes across the cell membrane (Moon and Parulkar, 1991). The H+ concentration of the external environment of the organism, to which it gets adapted, is an important factor that influences growth, production and stability of metabolite produced by it (Horikoshi, 1999). The most obvious reason is the secretion of organic acids that will cause the pH to drop. On the other hand, the assimilation of organic acids, which may be present in some media, will lead to an increase in pH, and urea hydrolysis will result in alkalinization (Krishna, 2005). The shift in media pH towards alkalinity during
maximization of production indicates that the organism was alkalophilic. Similarly *A. niger* has also reported to show a shift in pH towards alkalinity during maximization of production in SmF (Yuan *et. al.*, 2005).

Fig. 2.6. Influences of initial medium pH on xylanase production by *B. pumilus* with wheat bran as carbon sources at 30°C under SmF.

Xylanase production near neutral was reported in case of *B. circulans* WL-12 (Esteban *et. al.*, 1982). Many bacterial species reported to be productive at alkaline range. Similar reports related to alkalophilic organism were there (Subramaniyan, 2000, Horikoshi, 1999, Kubata *et. al.*, 1994). Most of the alkalophilic xylanolytic enzyme producers have similar optimum pH range from 5.5-9 (Kulkarni *et. al.*, 1999, Shabeb, 2000).
2.5.3. Effect of temperature on endoxylanase production

Generally microbes produce enzymes optimally during their optimum growth temperature. The enzyme activity of crude extract from *Bacillus pumilus* grown at different temperature ranging from 20 to 65 °C at a constant pH of 8.5 and substrate wheat bran 0.5 % was assayed and results were given in Fig. 2. 7.

![Graph showing the effect of temperature on endoxylanase production](image)

Fig. 2. 7. Influences of temperature on xylanase production by *Bacillus pumilus* with wheat bran as carbon sources at pH 8.5 under SmF

Optimum activity was recorded at 35 °C, it was relative to the production reported by *Bacillus* sp. BP - 7 (Lopez et. al., 1998). At temperature 30 and 40 °C almost 59 % activity was retained at 96 hour of incubation and at 45 and 50 °C 49 % of activity was retained. Increase in growth temperature above 50 °C has lowered the xylanase production, but even at 60 °C there was ~7 % of activity. Maximum growth was recorded
at 35 °C related to enzyme production. The growth and enzyme production by the microorganism indicates that this is a mesophilic culture but its production even at 50 °C could classify this organism under thermo tolerant bacteria (Dubeau et. al., 1987, Subramaniyan and Prema, 1998). There were reports of xylanase production at high temperature by *B. stearothermophilus* T-6 at 65-70 °C (Khasin et. al., 1993), whereas Esteban has reported growth and maximum enzyme production at 65 and 37 °C (Esteban et. al., 1982). There was a report related to production of alkaline xylanase from fungus *A. nidulans* KK-99 at 37 °C at pH-10 by SmF (Taneja et. al., 2002).

2.5.4. Effect of inoculum sizes on enzyme production

Several advantages have been cited in the use of spores rather than vegetative cells for inoculum. They can serve as a biocatalyst in bioconversion reactions because they are often able to carry out the same reactions as the corresponding mycelium (Larroche, 1996). The fermentation profile of an organism is affected by the inoculum concentration and physiological conditions. It also helps to minimize the time lag in fermentation process, by growing preinoculum in same fermentation medium (Krishna, 2001, Lincoln, 1960). Some organisms require vegetative inocula in which the amount of spores will be very high and young, those are highly metabolically active and can initiate the fermentation in much faster rate. The results were given in Fig. 2. 8., which show that inoculum concentration of 2.5 to 5 %, were almost equal in the production rate, where as high concentration above 7.5 to 10 % has reduced the production by 20 %. Low percentage of inoculum less than 1 % observed to be less productive and it takes more time to start the production and the biomass was also very low.

There are reports related to high production with low inocula concentration by *Bacillus* sp. (Sen and Satyanarayana, 1993, Subramaniyan and Prema, 1998). The transfer of small quantities of xylanase synthesized in the xylan preinoculum medium increases the initial reducing sugar level in the fermentation medium. Higher percentage of spore in the inoculum has reported to be a leading factor for long fermentation and thus slow down the fermentation rate (Meyrath and Suchanek, 1972). This was the reason why industries
prefer low percentage of preinoculum in fermentation process (Lincoln, 1960). It was noticed that low concentration of inocula (1 %) was efficient in enzyme production, may be due to low cell density. Higher concentration of more than 7.5 % might transfer compounds with preinocula that may resist higher enzyme production (Lincoln, 1960).

Fig. 2. 8. Influences of inoculum concentration on xylanase production by *B. pumilus* with wheat bran as carbon sources at pH - 8.5 and 35°C under SmF.

2.5.5. Effect of inducers in xylanase production

Carbon sources supplemented in the medium have a profound effect on the production and growth behavior of the organism. Some carbon sources promote good growth and production, but some promotes growth and inhibits production. The rate of incorporation
of a carbon sources could often influences the formation of biomass and production of primary or secondary metabolites.

![Graph showing xylanase and biomass production](image)

Fig. 2. 9. Effect of inducers on xylanase production by *Bacillus pumilus* with wheat bran as carbon sources at pH - 8.5 and 35°C under SmF.

Rapid growth from high concentration of easily available metabolites always affects the production (Hoq *et. al.*, 1994). Single carbo-hydrate material can act both as an energy sources and carbon sources and the main final product of fermentation help to decide the choice of carbon sources. The results of utilization of easily metabolisable sugars as additives (1 %), replacing wheat bran in the medium and combined with the production related to Oat spelt xylan as well as wheat bran in optimized condition (Fig. 2. 9).
The graph shows that xylose in low concentration have an inducing effect on xylanase production. Where as all other sugars have an inhibitory effect. There was report related to the inducing effect of xylose and xylan and inhibitory effect of glucose and other sugars xylanase production by Bacillus subtilus CD- 4 (Gessesse and Mamo, 1998). When xylanase fermentation was carried out in complex heterogeneous substrates, various factors have to be noticed for effective xylanase expression. This includes substrate accessibility and the rate of amount of xylooligosaccharides released, that act as a carbon sources and represses xylanase synthesis. Generally a slow release of inducers and the possibility of converting the inducers to its non-metabolisable forms are known to improve the level of xylanase production (Kadowaki et. al., 1997). Thermomyces lanuginosus IOC-4145, xylan or xylan containing substrates, such as corncob (266 U/mL) and their precursor, xylose, induced maximum level of xylanase free of cellulase, while the easily metabolisable sugars (fructose, glucose and maltose) appeared to repress xylanase synthesis (Damaso et. al., 2000). A. foetidus MTCC 4898 maximum xylanase yield (210 U/mL) was obtained in SmF with negligible cellulase activity at 30 °C with 1 % birch wood xylan as substrate in 3 days (Shah and Madamwar, 2005). Bocchini et. al., (2005) reported that grass and sugarcane hydrolysates, which contain sugars, mainly xylose, mannose, arabinose and galactose, have an inducing effect on xylanase production in SmF by B. circulans D1.

2.6.0. SOLID-STATE FERMENTATION

In Western countries, important problems have originated recently, such as pollution of soils, the potential use of bioremediation, as well as the necessity to find alternatives for animal feeding (Durand, 2003) which has directed the search for new technologies to produces by product utilizing these sources. SmF is advantageous as it is well characterized, and homogeneous conditions are maintained throughout and it is easier to scale up. However, SmF being an energy intensive process, SSF is gaining more importance. Economic analysis has indicated that SSF technology can considerably reduce the capital investment and total production cost as well as increases profitability, thereby making it an ideal technology in several industrial sectors (Castilho, 2000). It is
gaining more and more attention in recent years, due to the possibility of using cheap and abundant agro industrial waste as substrates, higher productivity, simplicity, low energy requirement, better recovery of product, lesser waste water output, and there is no catabolic repression (Pandey et al., 2000, Krishna, 2005). Nevertheless, its use is limited by the fact that not all organisms are able to grow in SSF, and the process cannot be well characterized. Fungi produce higher levels of xylanase than bacteria or yeasts. However, fungal xylanases are generally associated with cellulase (Steiner et al., 1987) and more active in acidic range (Haltrich et al., 1996). Utilization of oat spelt xylan is very costly in the production of xylanase enzyme (Bocchini et al., 2005), as well as SmF has limitations, so SSF was carried out.

2.6.1. Xylanase production using agro industrial residues by SSF

The selection of a suitable substrate for SSF process depends on several factors mainly related with cost and availability, and the heterogeneous nature of the substrates makes the problem difficult. Fig. 2. 10. illustrate xylanase production from B. pumilus on various agro-industrial residues by solid-state fermentation. Wheat bran (5582 U/gds) was found to be the best substrate followed by soya meal untoasted (4215 U/gds) and rice straw (1876 U/gds). The results also indicated that these substrates promoted high biomass, which would have been the reason for better production; however, in rice bran biomass was high but production was comparatively low. Production was very low with sawdust, rice husk and coir pith; the presence of polyphenol in high quantity in these substrates would have inhibited the growth and enzyme production. Xylanase was produced by T. lanuginosus ATCC 46882 (5098 U/g) with bagasse pulp by SSF (Christopher et al., 2005). Substrates for SSF are heterogeneous products from agriculture or by-products of agro-industries. This basic macro-molecular structure confers the properties of a solid to the substrate. The structural macromolecules may provide an inert matrix within which the carbon and energy sources are adsorbed. Preparation and pretreatment are the necessary steps to convert the raw substrate into a form suitable for use (Krishna, 2005). Wheat bran is produced worldwide in enormous quantities as an important by-product of the cereal industry. The outer tissues of the
wheat kernel imply that the wheat bran consists mainly of cell wall polysaccharides. Arabinoxylan is the main polysaccharide in wheat bran, but only a minor fraction of the flour. It is an integral part of the cell walls of endosperm material in the flour and of aleuronic layer cells present in bran.

Fig. 2. Xylanase production by *Bacillus pumilus* on various agro industrial residues (BG- sugarcane bagasse, RS- rice straw, RH- rice husk, RB- rice bran, WB- wheat bran, CP- coconut pith, TDH- thur dal husk, SD- sawdust, and SM- Soy meal flakes at 30°C under SSF.

It has been shown that wheat aleurone cell walls contain about 65 % arabinoxylan, with an arabinose to xylose ratio of 0: 35 (Beldman, 1996). Xylans consist of a linear backbone of β -(1, 4) linked D-xylopyranosyl residues containing individual α -L-arabinofuranosyl residues attached through O-2 and/or O-3 (Izydorczyk *et. al.*, 1993). A
feature of some xylans is the existence of feruloyl residues that are esterified with L-arabinofuranose. Feruloyl groups can form cross-linkages between polysaccharide chains by peroxidase-catalysed dimerization or between lignin and polysaccharides via ether-ester bonds (Perez et al., 2002). Due to their structural complexity several specific enzymes are required for the complete depolymerization of wheat bran xylans.

2.6.2. Effect of pretreatment on enzyme production

Different agro industrial residues, all pretreated with acid and alkali and untreated were used for endoxylanase production by Bacillus pumilus by SSF. The results were given in Fig. 2.11. Of different agro industrial residues used, untreated wheat bran was more effective for enhanced production of endoxylanase; next to this was rice straw and then rice bran, while alkali and acid treatment has inhibited enzyme production in wheat bran by ~16 to 20 fold. Rice straw alkali treatment has not effected very much whereas in rice bran acid and alkali treatment has decreased enzyme production. The biomass was also very low when the production has decreased. On the other hand, alkali treated saw dust, as well as acid and alkali treated sugar cane bagasse were found to be effective to enhance enzyme titer, but the overall production was very less compared to rice straw and wheat bran. In order to increase the accessibility of the cellulose and hemicellulose present in the substrates, mild alkali (1N NaOH) and/or acid treatment (1N HCl) is a widely accepted method for solubilizing lignin from lignocellulose. In this study, xylanase activity did not increased even after mild alkali or acid treatment relatively it was reduced. Such negative effects of alkali treatment on xylanase production were also reported by studies on A. foetidus. A possible explanation for this observation has been suggested that rapid consumption of carbon sources and concurrent release of monomeric sugars would lead to a repression in enzyme synthesis (Shah and Madamwar, 2005). There were similar reports on endoxylanase production by Bacillus sp. on SSF using wheat bran (Archana and Sathyanarayana, 1997, Gessesse and Mamo, 1999, Virupakshi et al., 2005) wheat straw by Thermoascus aurantiacus (Kalogeris et al., 1998).
Fig. 2. 11. Xylanase production by *Bacillus pumilus* on various agro industrial residues (production with acid and alkali treated substrate compared with untreated substrates) at 30 °C under SSF.

Pretreatment with acid and alkali did not enhance xylanase production in wheat bran. The removal of major part of hemicelluloses from wheat bran during pretreatment (Koullas *et al.*, 1993, Kalogeris *et al.*, 1998) was the reason for this reduction in enzyme production. The α- (1-6) linkages of galactose are very sensitive to acid and alkali and may be cleaved during alkaline extraction (Timell, 1965, Jeffries, 1994). It was reported that agro industrial residues from graminaceous plants contain arabinoxylan, which supports high xylanase activity (Singh *et al.*, 2000). The biochemical composition of wheat bran
(Lequart et al., 1999) indicated that wheat bran contained considerable amount of soluble sugar like glucose, xylose, arabinose and galactose required for the initiation of growth and replication of the microorganism.

Arabinoxylan is the main polysaccharide in wheat bran; aleurone cell walls of it contain about 65% arabinoxylan, the degree of substitution of the main chains by arabinose is higher it is approximately 1 in 5 xylose residues (Lequart et al., 1999). This might be the reason for enhanced production of endoxylanase on wheat bran, where the substrate itself is highly nutritive sources for Bacillus pumilus. This study highlighted that choosing an appropriate source of carbon could improve enzyme production markedly. Developing a process for the production of xylanase using wheat bran as a potential substrate is very attractive as it is readily available and inexpensive source of carbon. Ninawe and Kuhad (2005) reported wheat bran and corncob as an enhancer for xylanase production by Streptomyces cyaneus SN32. Thermomyces lanuginosus (D2W3) was reported to give high xylanase activity on sorghum straw by SSF (Sonia et al., 2005), A. fischeri Fxn by wheat bran (Senthilkumar et al., 2005) and Bacillus JB-99- (3644 U/g) (Virupakashi et al., 2005) on rice bran. But these reports on xylanase activities were lower compared to that obtained by B. pumilus on wheat bran. The universal suitability of wheat bran as substrate is that it contains sufficient nutrients and able to remain free even in high moist condition providing large surface area (Archana and Sathynarayana, 1997).

2.7.0. Enzyme profile in SSF and SmF

Related to plate assay the crude enzyme extract was subjected to enzyme profile studies. Related to xylanase activity, protease, β-xylosidase, CMC ase and FP ase activity were estimated as explained in the procedure. Enzyme profiles of the culture in agro industrial residue like wheat bran, rice straw and soybean flakes are recorded and related with that of submerged fermentation using Oat spelt xylan and well explained in Table. 2.4. The results indicate that wheat bran was the best source for endoxylanase production by SSF. The production of proteases and cellulase was low in the case of wheat bran. In agro
industrial residues, generally protease synthesis is inhibited by C-sources indicating the presence of catabolic repression of protease biosynthesis (Pandey et. al., 2000). Heck et. al., (2002) reported soybean industrial residue as suitable sources for cellulase and xylanase production by \textit{Bacillus} strains.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>SSF (IU gds^{-1})</th>
<th>SmF (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RS</td>
<td>WB</td>
</tr>
<tr>
<td>CMCCase</td>
<td>3.2</td>
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</tr>
<tr>
<td>Filter paper</td>
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</tr>
<tr>
<td>Xylanases</td>
<td>1873</td>
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</tr>
<tr>
<td>(\beta)-xylosidases</td>
<td>0.11</td>
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</tr>
<tr>
<td>Protease</td>
<td>124.1</td>
<td>121.1</td>
</tr>
</tbody>
</table>

\textbf{Table. 2.4. Enzyme profile of \textit{Bacillus pumilus}}

Ferreira and coworkers have reported high proteolytic activity with wheat bran as substrate in SSF by \textit{Aspergillus tamari} (Ferreira, 1999). Soya meal has the highest level of protease. This can be explained based on the chemical composition of these agro industrial materials, compared to wheat bran (11.5-18 %) and rice straw (4.5 %), soya meal (44 - 47 %) is having high protein content. The production of protease affects the storage of enzyme, which can be rectified by using proteolytic inhibitors or by protein purification methods (Walsh and Headon, 1994). In this study it was found that the production of protease was comparatively lower in SSF when compared to SmF. Protease production was very low in all agro-industrial residues. The \(\beta\)-xylosidase production was found to be very low and about 2 IU/ gds in wheat bran. There was a report of high secretion of \(\beta\)-xylosidase (45 U/mL) by \textit{Aspergillus fumigatus} with 3 % corncob as carbon sources in SmF (Lenartovicz et. al., 2003). Minor cellulosic activity was found in the crude filtrate. The results point out that, the culture was a poor producer of cellulase
related to xylanase, indicting that this enzyme was a persuasive source for biobleaching process.

2.8.0. Conclusion

From these data we can conclude that the isolate *Bacillus pumilus* was highly alkalophilic and thermo tolerant. Xylanase production was investigated using different carbon sources in different mode of fermentation, where SSF has shown 58-fold increase in production related to SmF. It was possible to verify that a lignocellulosic waste, wheat bran, was an important substrate for the production of xylanase. Use of wheat bran as substitute, was an efficient alternative to reduce the costs of xylanase production by *B. pumilus* in SmF as well as in SSF, since these materials are often available in tropical countries like India. It is as an inexpensive source of components that propitiate the bacterial growth and the enzyme production. The thermo tolerances of the microbe and enzyme production as well as its high stability at alkaline pH values are superiors to the mesophilic counterparts used for prebleaching of pulp. These properties together with the production of extremely high levels of xylanase, with poor cellulolytic activity making this strain a promising entrant for industrial applications.