ISOLATION AND MOLECULAR CHARACTERISATION XYLANASE PRODUCED BY *SPOROLACTOBACILLI*

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Raed Kadhim JASIM

The Iraqi Ministry of Education, Educational Directorate of Basra, Alackramen Preparatory school for boys, Basra, Alackramen, IRAQI

*Corresponding author: Mobile: +9647702254154, Email: radkazim@yahoo.com

**Abstract.** The purpose of this study was to determine the effect of some cultural conditions on the Xylanase enzyme production by *Sporolactobacilli* isolated from the industrial soil and to investigate their potential to produce xylanase utilizing grass and other materials as a substrate. Xylanase activity was detected using the Dinitrosalicylic acid assay method. It was isolated from the industrial soil, secrets extra cellular xylanases when grown in liquid media supplemented with rice bran, grass, corncob, or sugar baggage as a carbon sources (which were treated with 2N NaOH for removing the cellulose from these substrates). The maximal enzyme production was obtained when rice bran xylan was used as a carbon source. It was shows the high enzyme activity at high temperatures 55ºC and high enzyme activity was found at pH 8. The extra cellular enzyme has an apparent molecular weight of 68 KD, as determined by SDS–PAGE. The purified enzyme was confirmed by Zymogram analysis. The *Sporolactobacilli* show high enzyme activity of 4.7 U/mL. The newly isolated bacterial species in this experiment are having alkalophilic and thermophilic enzymatic properties, which is advantageous to maintain the conditions during industrial processing.

**Keyword:** Sporolactobacilli, industrial soil, xylanase.

**Introduction**

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall [TIMELL, 1967, BUTNARIU and CAUNII, 2013]. It is composed of a linear backbone of 1,4–linked–D–xylopyranosyl units that often has side chains of O–acetyl, arabinosyl and methylglucuronosyl substituents [MAHESHWARI, et al., 2000; BLANCO, et al., 1997, RASHED and BUTNARIU, 2014, BUTNARIU and GIUCHICI, 2011]. Endo–1,4–xylanase (1,4–D–xylan xylanohydrolase: E.C. 3.2.1.8) is the main enzyme responsible for the cleavage of the linkages within the xylan backbone [SELFAGHI, et al., 2002, BUTNARIU et al., 2012]. Bacterial, fungal and actinomycete xylanases have attracted considerable research interest [BASTAWDE, 1992] because of their potential applications in recovery of fermentable sugars from hemicellulose, biobleaching of pulp and paper industry and to other industrial applications [KANG, et al., 1996, KUHAD and SINGH, 1999].

It has a great application in the pulp and paper industry [DIIKER and RKHARDS, 1976; LEE, et al., 1998, PUTNOKY et al., 2013].

The wood used for the production of the pulp is treated at high temperature and basic pH, which implies that the enzymatic procedures require proteins exhibiting a high thermostability and activity in a broad pH range [JACQUES, 2000, BUTNARIU, 2012].

Treatment with xylanase at elevated temperatures disrupts the cell wall structure. This, as a result, facilitates lignin removal in the various stages of bleaching.

This study deals with strain *Sporolactobacilli* was isolated from various agricultural fields in the Shendra Industrial Area, Aurangabad, (India) and showed the highest activity among the other xylanase producing *Sporolactobacilli* strains. In this laboratory study, the effect of different cultural conditions on the xylanase.

**Material and methods**

**Soil samples and isolation and characterization of bacteria**

Composite surface soil samples from the first 15 cm were collected from
various agricultural fields in the Shendra Industrial Area, Aurangabad (India), which had been irrigated with wastewater from factories for more than two decades.

Soil sample (10 g) was suspended in 90 mL normal saline solution, serially diluted and plated on nutrient agar.

The isolates were then identified based on the morphological, cultural and biochemical characteristics following Bergey’s Manual of Determinative Bacteriology (1994) [BOSTAN et al., 2013, CAUNII et al., 2015, BUTNARIU et al., 2006].

Morphological characterization
Among 35 colonies obtained on 10⁻⁵ plate were selected and pure cultures of these isolates were obtained by repeated streaking on nutrient agar slants.

Finally, pure cultures were stored on nutrient agar slants at 4°C in a refrigerator. These Cultures were grown in the xylanase production media by using lignocellulosic materials as a carbon source [MATSUO, et al., 1997].

The selected substrate material (grass, corn cob) was treated for xylan. Then the material was finely chopped to small pieces of (2–3) cms and treated using (2M) NaOH for about 6 hours. This treatment degrades the cellulose present in the material. The material was thoroughly washed with distilled water until the pH reaches up to 7.0. Finally, it was dried on a hot plate at 60°C for 24–32 hours and then powdered using a mortar and pestle. The powder was then sieved using a mesh and stored at 32 hours and then powdered using a mortar and pestle. The powder was then sieved using a mesh and stored at

Xylanasae production media
The media were prepared and their pH was adjusted to 8.0–8.5. The cultures were transferred to (250 mL) Erlenmeyer flasks, containing (100 mL) of the xylanase production media and fermentation was carried out on a rotary shaker at 250 rpm under the same inoculum conditions [GOMES, et al., 1993].

The fermented media carrying was centrifuged for 20 min at 5000 rpm and at 24 hours and 48 hours time interval for the assay activity. DNS was used for the determination of the amount of xyllose released due to the action of xylanase.

Xylose standard was prepared by mixing 100 mg of xylose in 100 mL of distilled water. Grams, endospore, Capsule staining catalase test and Oxidase test were performed [YOSHIOKA, et al., 1981; KHANDKE, et al., 1989].

Preparation of culture media and reagents for biochemical characterization
The cultivation of microorganisms in the laboratory requires that the needed nutrients and suitable environmental conditions be provided. Nutrients are those raw materials that are used to sustain living organisms, promote growth, replace cellular constituents, and provide energy for metabolic reactions and cell movement. Enriched medium is any basal medium, which has been supplemented (enriched) with serum, blood or extracts of plant or animal tissues etc. in order to be able to support or enhance the growth of particular microorganisms. LB—broth, LB—agar, citrate agar, tryptone broth, indole test, methyl red test (MR—test), voges—proskauer test (VP—test) and triple sugar iron agar medias were prepared [ANAND and VITHAYATHIL, 1990; TONG, et al., 1980].

Rapid Biochemical Assay
The API—20E employs a plastic strip composed of 13 individual micro tubes, each containing a dehydrated medium in the bottom and an upper cupule.

The media become hydrated during inoculation of a suspension of the test organism, and the strip is then incubated in a plastic covered tray to prevent evaporation. In these manner 13 carbohydrates tests are performed. Following incubation, identification of the organism is made by using differential charts supplied by the manufacture or by means of a computer—assigned system called PRS [GANJU, et al., 1989].

Xylanase activity assay
Assays for crude xylanase were performed using 0.5 % soluble oat spelt xylan in (50 mM) sodium phosphate buffer, pH 7.0. The reaction mixture was composed of 1.8 mL substrate and 0.2 mL crude enzyme. The mixture was incubated in a water bath at 60°C for 15
The released reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNSA) method Miller, [MILLER, 1959] in which the reaction was stopped by adding (3 mL) of DNSA acid reagent.

A reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 min. After cooling the reaction tubes to room temperature, the O.D. was measured at (575 nm) with xylose as the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 mol xylose / min / mL under the above mentioned conditions [PURKARTHOFER, 1993].

**Effect of various Carbon and Nitrogen source on xylanase Activity**

Effect of various nitrogen sources on the xylanase production was assessed by culturing the bacteria in the production medium (pH 7.0–7.5) at room temperature (28±2ºC). Either of corncob, sugarcane and plant material was used as carbon source individually in the xylanase production media. After 24 h of the culture growth, xylanase activity was estimated [MATSUO and YASUI, 1985].

**Gel electrophoresis and zymogram of crude xylanase**

Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in a discontinuous buffer system where in the buffer used to cast the gel.

The SDS–Polypeptide complexes in the sample applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes.

After migrating through the stacking gel of high porosity complexes get deposited in a very thin zone on the surface of the resolving gel. On further electrophoresis, polypeptides get resolved based on their size in the resolving gel. In this experiment the PAGE was performed on 12.5 % denaturing polyacrylamide gels by the method of LaemmLi (1970).

A modified protocol of the procedure reported earlier was used incubation of the protein samples with sample buffer at 50ºC for 10 min instead of 100ºC for 2–5 min. The samples were electrophorized on the gel using a mini protein II system, a broad range protein marker (Promega) being used for molecular weight determination. Proteins were stained with 0.1 % (w/v) Coomassie blue R (Fluka) in 50 % (v/v) methanol, 10 % (v/v) acetic acid. Protocol for preparation of laemmlı gels: Assemble the gel mould, mix the solutions below in order and pour gel immediately after adding APS (MONTI, et al., 1991).

**Zymogram analysis for xylanases**

For the zymogram analysis, the crude enzyme samples were electrophoresed as above on SDS–PAGE containing xylan (0.1 %).

After running, the gel was washed four times for 30 min in 100 Mm phosphate buffer (pH 7.0), the first two washes containing 25 % (v/v) isopropyl alcohol, to remove SDS and renature protein in the gel. The gel was then incubated for 20 min at 37ºC before soaking in Congo red solution for 5 min at room temperature and washing with 1 M NaCl until excess dye was removed from the active band. The zymogram was prepared after soaking the gel in 0.5 % acetic acid solution. The background turned dark blue, and clear zones were observed in the areas exposed to xylanase activity [NAKAMURA, et al., 1993].

**Immobilization of enzyme using sodium alginate**

Sodium alginate (0.6 g) was dissolved in 10 mL–sterilized water and mixed thoroughly by placing ion magnetic stirrer for 30 minutes until a homogenous solution is obtained.

To this, 10 mL of phosphate buffer (pH 7.2) containing the enzyme source was mixed and placed on a shaker for 30 minutes at 150 rpm.

This alginate–enzyme mixture was added drop wise from a height of 50 mm with sterilized 10–mL glass syringe (diameter, 2.0 mm) into an autoclaved solution of calcium chloride (0.1 M, pH 7.0), where calcium alginate beads of uniform diameter (2 mm) are formed instantaneously.

The beads are left in this gelling solution for 5 min before being harvested, rinsed with sterilized 10 % (wt/vol) sucrose solution, and used directly.
Using immobilized beads xylanase activity was determined as mentioned earlier in the methods section.

**Polymerase chain reaction**  
Test Primer xyn gene (endo β 1.4 D–xylanase) from bacillus sp. to detect xylanase gene in *Sporolactobacilli*.

**FORWARD PRIMER:**  
5′–CTGCCGGGAATTACAGTTGTT–3′

**REVERSE PRIMER:**  
5′–TGGTGATTCAATGGGTACT–3′

**PCR Mixture:** The PCR mixtures were prepared with sterile H₂O, 2 μL of 20 μmol of both forward and reverse primers, 1 μL of 10 mM dNTP, 5 μL of 1U Taq DNA polymerase, 4.0 μL of 25 mM MgCl₂, 1μL DNA Sample. Water was added to adjust the final reaction volume to 50 μL.

**PCR Products** were analyzed with 2% agarose gel electrophoresis [TUOHY, et al., 1993].

**Results and discussion**

**Screening of soil samples for xylanase producing bacteria**

Screening of soil samples resulted in 15 well–isolated colony from 10⁻⁵ dilution plate. One colony from 10⁻⁵ dilution plates were selected for further study. This colony are inoculated into nutrient broth and incubated at 37ºC on an incubator shaker at 200 rpm for 24 hrs.

Growth obtained from these flasks was streaked onto agar media plates and after confirming purity; it was maintained as stocks on agar slants. This isolate was further characterized by morphological and biochemical studies [BAGIU et al., 2012].

**Morphological characteristics**

Grams staining, capsule and spore staining, motility and colony morphology of *Sporo lactobacilli* was studied for identification. The results of this observations are listed in Table 1.

### Table 1.  
**Morphological, physiological of *Sporolactobacilli***

<table>
<thead>
<tr>
<th>Property</th>
<th><em>Sporo lactobacilli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology of the colonies on nutrient agar</td>
<td></td>
</tr>
<tr>
<td>1. Form</td>
<td>Circular</td>
</tr>
<tr>
<td>2. Elevation</td>
<td>Raised</td>
</tr>
<tr>
<td>3. Colour</td>
<td>White</td>
</tr>
<tr>
<td>4. Size</td>
<td>1.5–2.0 mm</td>
</tr>
<tr>
<td>Morphology of the cells in nutrient broth</td>
<td></td>
</tr>
<tr>
<td>1. Gram stain</td>
<td>Gram–positive bacilli</td>
</tr>
<tr>
<td>2. Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>3. Spores</td>
<td>Sporulate</td>
</tr>
<tr>
<td>4. growth</td>
<td>Turbid, with sediment</td>
</tr>
</tbody>
</table>

**Biochemical tests**

Species *Sporolactobacilli* are identified by using biochemical criteria.

### Table 2.  
**Biochemical characterization of *Sporolactobacilli***

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>Reaction</th>
<th><em>Sporolactobacilli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>Bubbles were produced</td>
<td>Positive</td>
</tr>
<tr>
<td>ONPG</td>
<td>Purple</td>
<td>Negative</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>Purple</td>
<td>Positive</td>
</tr>
<tr>
<td>Ornithine</td>
<td>Purple</td>
<td>Positive</td>
</tr>
<tr>
<td>Urease</td>
<td>Orangeish Yellow</td>
<td>Negative</td>
</tr>
<tr>
<td>Phenyl alanine deamination</td>
<td>Colourless</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Colourless</td>
<td>Negative</td>
</tr>
<tr>
<td>H₂S production</td>
<td>Orangeish yellow</td>
<td>Positive</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>Blue</td>
<td>Positive</td>
</tr>
<tr>
<td>Voges proskauers</td>
<td>Colourless</td>
<td>Negative</td>
</tr>
<tr>
<td>Methyl red</td>
<td>Methyl red</td>
<td>Negative</td>
</tr>
<tr>
<td>Indole</td>
<td>Colourless</td>
<td>Negative</td>
</tr>
<tr>
<td>Malonate</td>
<td>Light green</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Assay of enzyme activity**

Enzyme activity was estimated by using the formula as it represented in methods and methodology. One unit (U) of enzyme activity equals to one mole of
xyloses released per mL of enzyme per minute generally represented in units (U).

**Enzyme activity in different carbon sources**

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Enzyme activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>0.08</td>
</tr>
<tr>
<td>Corn cob</td>
<td>0.13</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>0.505</td>
</tr>
<tr>
<td>Rice bran</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Effect of different carbon sources on xylanase activity

In the table 3. Shows maximum activity of 0.7 units when rice bran was used as a carbon source.

**Enzyme activity in different nitrogen sources**

Different Nitrogen sources like ammonia (inorganic), urea (organic), Soya protein and maximum enzyme activity was observed in these sources.

No activity was observed when ammonia was used as a Nitrogen source.

Maximum activity was observed in urea and ammonia nearly of (2.5–3.0) units. Enzyme activity was more in buffer pH 8 for *Sporolactobacilli* and releases (4.7 U/mL) of xyloses.

**Poly acryl amide gel electrophoresis (PAGE)**

Using PAGE with 12.5 % gel strength isolated crude extra cellular enzyme xylanase. SDS PAGE for this resulted in a single band of (68 KDa).

**Zymogram analysis**

The single band exhibiting a xylanolytic activity revealed by zymogram analysis indicates that this enzyme (68 KDa) is relatively large compared with others.

**Figure 1.** 12.5 % SDS–PAGE and zymogram analysis of crude xylanase from *Sporolactobacilli*. M=broad range protein marker (kda); CE=crude enzyme; Z=zymogram.

**Immobilization of crude enzyme**

The immobilized enzyme in calcium alginate beads showed highest activity than normal crude enzyme.

The activity of the immobilized enzyme was found to be (4.7 U/mL)

**Identification of xylanase gene by rapid molecular techniques (PCR)**

DNA was isolated by the procedure mentioned in materials and methods, and observed under UV–Tran illuminator.
The isolated genomic DNA and plasmid DNA samples were amplified with suitable primers mentioned in materials and methods.

Conclusions

The present experiment was aimed to isolate the xylanase producing bacteria from soil which have their potential to degrade the xylan which is the complex polymer and second to cellulose in its abundance, represents a major reserve of fixed carbon in the environment.

Xylanase pre bleaching technology is now in use at several mills worldwide. The newly isolated bacterial species in this experiment are having alkalophilic and thermophilic enzymatic properties, which is advantageous to maintain the conditions during industrial processing.

The xylanase enzyme produced by the Sporo lactobacilli is thermostable and alkalophilic which has wide industrial applications.

The enzyme activity was detected at different pH ranges and Temperatures. The maximum activity was obtained at pH 8 for Sporo–lactobacillus sp. (4.7 U).

References


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