Abstract

Solid state fermentation has been carried out using Bacillus halodurans KR-1 for the production of xylanase enzyme. Among different solid materials such as wheat straw, rice bran, wheat bran, soybean meal, wheat bran served as a very good inducer of xylanase. Maximum enzyme production of 33.3 U/gm was observed with wheat bran moisturized with water (1:1 ratio, w/v) and 10% size of inoculums (v/v) incubated at 40°C for 72h.

Keyword: Xylanase, Bacillus halodurans, Solid State Fermentation, Wheat bran

Introduction

Xylanase (Endo-1,4 – β-D xylan, xylano hydrolase; EC 3. 2.1.8) acts on α-1,4 xylan and cleaves β-1,4 glycosidic linkage randomly (1). The products are xylose, xylobiase and xylo-oligosaccharides. The enzyme belongs to the glycoside hydrolase family. Xylan degrading enzymes occur ubiquitously in wide diversity of sources viz. plants, animals and microorganisms, however not in mammals. Xylanase producing microbes include aerobic and anaerobic mesophiles and thermophiles. Multiple xylanases are reported in numerous microorganisms (2). The enzyme is of industrial importance and is used in paper manufacturing to degrade xylan, to bleach paper pulp increasing its brightness, improving the digestibility of animal feed and for clarification of fruit juices (3). Use of xylanase avoids the use of chemical processes that are very expensive and cause pollution (4, 5).

The relatively high cost of enzyme production has hindered the industrial applications of enzymatic process (6). The use of abundantly available cost-effective agricultural by-products viz. wheat bran and other lignocelluloses in solid state fermentation for xylanase production will definitely be economical. The technique of Solid-State Fermentation (SSF) involves the growth and metabolism of microbe on the moist solid in the absence of any free flowing water. The fermentation system that is closer to the natural habitat of microbes has been shown to be more efficient in producing certain enzymes and metabolites (7, 8). SSF offers distinct advantages over submerged fermentation including economy of space, simplicity of the medium, no complex machinery equipments and control systems, greater compactness of the fermentation vessel owing to a lower water volume, more product yield, reduced energy demand, lower capital and recurring expenditure in industry. The easier scale up processes, volume of solvent needed for product recovery, superior yields, absence of built up foam, and easier control of contamination due to the low moisture level in the system are other advantages (7,9,10,11,12,13).

Most of the reports for xylanase production using solid state fermentation are with fungi and
Actinomycetes. Comparatively, there are only few reports for xylanase production using bacteria in the solid state fermentation (1, 14, 15). Here we report production of xylanase by alkalo- thermophilic bacteria; Bacillus halodurans strain KR-1 using wheat bran under solid state fermentation conditions.

Materials and Methods

Organism and growth conditions: The bacterium used in present study was isolated from soil near riverbed of Indore. The bacteria were identified as Bacillus sp. by morphological studies. It was confirmed as Bacillus halodurans by the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh and has been registered as Bacillus halodurans strain KR-1, MTCC No. 9534. A medium (yeast extract, 0.5%; peptone, 0.5%; K$_2$HPO$_4$, 0.1%; MgSO$_4$.7H$_2$O, 0.02%; FeSO$_4$.7H$_2$O, 0.02% adjusted to 9.0 with 1% Na$_2$CO$_3$) supplemented with 1% xylan and 2.5% agar has been used for maintenance of the culture.

Xylanase production by SSF: To 5 gm of wheat bran in a 250 ml capacity Erlenmeyer flask, 5 ml of tap water was added and sterilized by autoclaving at 15 psi for 20 min. After cooling to room temperature (25°C), the flask was inoculated with 10% inoculum (freshly grown 24 h growth culture, w/v) and incubated in an Incubator at 40°C for 72 hours.

Xylanase extraction: The enzyme was squeezed from the semi-solid broth using two fold muslin cloths and collected in 50 mM glycine-NaOH buffer, pH 9.0 (50 ml of buffer was used for 5 g wheat bran). The squeezed enzyme collected in glycine-NaOH buffer was centrifuged at 10 x 000 g for 20 min in the cold condition (4°C) in a super speed cooling centrifuge model Sorvall RC-5B and the clear supernatant was used as enzyme extract.

Enzyme Assay: Xylanase enzyme was assayed by estimating the release of the reducing sugar from birch wood xylan using dinitrosalicylic acid (DNS) method (16). A 0.9ml sample of 1% birchwood xylan dissolved in 50 mM glycine-NaOH buffer, pH 9.0 was pre- incubated at 50°C for 5 min. To this, 0.1 ml of the enzyme was added and incubated at 50°C for 15 min. The reaction was stopped by adding 1.5 ml of DNS solution and the tubes were incubated in a boiling water bath for 15 min. A control was also run simultaneously where enzyme was added after the addition of DNS. A blank was also prepared where no enzyme was added and against the blank, zero was set in the colorimeter. D-Xylose was used as standard during the colorimetric estimation. One unit of the xylanase activity was taken as the amount of the enzyme required to release one ìmole of the reducing power as xylose equivalent per min under the conditions of the enzyme assay.

Effect of various agricultural by-products on xylanase production: During solid state fermentation, various carbon and nitrogen sources in place of wheat bran viz. rice bran, wheat straw, and soybean meal were tested for xylanase production.

Time course of enzyme production: Solid state fermentation was carried out upto 120 hours. An aliquot of the broth was taken out after every 24 hours for checking the amount of xylanase produced by the bacteria.

Effect of moisture level: It was checked by varying the ratio of the wheat bran and water from 1: 0.5 to 1: 4.

Effect of pH: It was checked by maintaining the pH of the broth varying from pH 5.0 to 12 before addition of the inoculum. The pH 5 and 6 was maintained by using citrate buffer, pH 7 and 8 by
using phosphate and pH 9 to 12 was maintained by using glycine-NaOH buffer.

**Effect of temperature:** It was checked by maintaining the temperature of the broth varying from 25°C to 60°C. The pH of the broth was maintained pH 9.0 and incubation period was 72 hours.

**Effect of various moisturizing agents:** Different moisturizing agents viz. MS1 (yeast extract, 0.5%; peptone, 0.5%; K<sub>2</sub>HPO<sub>4</sub>, 0.1%; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.02%; pH adjusted to 9.0 with 1% Na<sub>2</sub>CO<sub>3</sub>), MS2 (3% NaCl, 0.075% KCl, 0.7% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05% NH<sub>4</sub>Cl, 10% K<sub>2</sub>HPO<sub>4</sub>, 10% KH<sub>2</sub>P<sub>4</sub>, pH adjusted to 9.0 by using 1% Na<sub>2</sub>CO<sub>3</sub>), MS3 (0.2% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1% KH<sub>2</sub>P<sub>4</sub>, 1% K<sub>2</sub>HPO<sub>4</sub>, 1% NH<sub>4</sub>NO<sub>3</sub>, 0.02% CaCl<sub>2</sub>, 0.05% FeCl<sub>3</sub>, pH adjusted to 9.0 with 1% Na<sub>2</sub>CO<sub>3</sub>), MS4 (1.5% K<sub>2</sub>HPO<sub>4</sub>, and 0.5% MgSO<sub>4</sub>·7H<sub>2</sub>O, pH adjusted to 9.0 with 1% Na<sub>2</sub>CO<sub>3</sub>), double glass distilled water, and tap water were used for semi-solid broth taken for xylanase production.

**Effect of inoculum size:** Different inoculum sizes in the semi-solid fermentation broth viz. 2%, 5%, 10%, 20%, 30% and 40% were tested for maximum xylanase production. The fermentation was carried out at 40°C for 72 hours.

**Effect on different additives:** Different additives in 0.5% concentration viz. glucose, lactose, mannose and fructose as a carbon source, and KNO<sub>3</sub>, NaNO<sub>3</sub> and casein as a nitrogen source were tested for maximum production of xylanase. The fermentation was carried out at 40°C for 72 hours.

**Scaling up of enzyme production:** Scaling up of xylanase production was tested by growing the bacteria with different amount (up to 25 g) of wheat bran supplemented with 0.5% fructose as additive in a 2 l Erlenmeyer flask.

**Results and Discussion**

All the experiments were carried out at least thrice.

**Xylanase production on different agricultural by-products:** Different agricultural by-products viz. wheat bran, rice bran, wheat straw, and soybean meal were tested for xylanase production (Fig.1). The results showed maximum production of xylanase in the presence of wheat bran compared to other agricultural by-products. The maximum production of xylanase may be due to low lignin and silica contents in wheat bran compared to other agricultural by-products. Battan et al. (2006) also reported maximum xylanase production on various agricultural by-products.
production (5300 U/gm) with wheat bran by Bacillus pumillus. Khandeparkar et al. (1) also reported xylanase activity by using different agricultural by-products in solid state fermentation. They reported xylanase activity with wheat bran 35.70 U/gm; rice husk, 25.04 U/gm; rice bran, 18.64 U/gm; and Bagasse 17.29 U/gm by using Arthrobacter sp MTCC 5214. Muthezhilan et al. (17) studied xylanase production from Penicillium oxalicum by using wheat bran, rice bran, rice straw, sesame oil cake and wood husk and found maximum xylanase production with wheat bran followed by oil cake, rice bran, wood husk and rice straw, respectively. Gawande et al. (18) reported maximum xylanase production from Aspergillus terreus by using wheat bran. They reported xylanase production from Aspergillus terreus; 21.2 U/gm with wheat bran, 10.5 U/gm with rice straw, 10.2 U/gm with soybean hull, 3.5 U/gm with sugarcane bagasse. They also reported maximum xylanase production from Aspergillus niger with wheat bran, 26.7 U/gm. Antoine et al. (19) studied xylanase production by Penicillium canescens through solid-state fermentation with five different agro-industrial substrates viz. soya oil cake, soya meal, wheat bran, whole wheat bran and pulp beet, and found soya oil cake as best substrate in terms of enzyme production. Kavya and Padmavati (20) studied xylanase production by Aspergillus niger using different cheaper sources viz. wheat bran, rice bran, soya bran, ragi bran and dust, and found maximum xylanase production, 9.87 U/ml with wheat bran. Pal and Khanum (21) reported maximum xylanase production, 2596 U/gm by Aspergillus niger DFR-5 using wheat bran and soyabean cake in the ratio of 70:30. Sanghvi et al. (22) reported production of xylanase, 146 U/ml by Tricoderma harzianum through solid state fermentation using wheat straw. Murthy and Naidu (23) reported production of xylanase, 20388 U/gm by Penicillium sp. CFR 303 through solid state fermentation with coffee husk as substrate. Laxmi et al. (24) reported production of xylanase, 62480 U/l by Aspergillus sp. RSP-6 using palm fibres.

**Time course of enzyme production:** Solid state fermentation broth was incubated up to 120 hours. Aliquots were taken out at every 24 hours intervals for testing xylanase production. The results are shown in Fig. 2. The results indicated maximum production of xylanase after 72 hours incubation. Thereafter, a slight decrease in xylanase production was observed. The incubation time required depends on the growth rate of the

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**Fig.2.** Xylanase production from Bacillus halodurans at different incubation times using wheat bran.

Xylanase production using solid state fermentation
bacteria and its enzyme production pattern. Maximum xylanase production has been reported at 72 hours of incubation in solid state fermentation using *Bacillus pumilinus* (25) and *Arthrobacter*; (1). Muthezhilan et al. (17) reported xylanase production in *Penicillium oxalicum* at various time incubation and found maximum activity after 144 hours. Simoes et al. (26) reported maximum xylanase production from *Aspergillus japonicus* after 120 hours of incubation using wheat bran. Murthy and Naidu (23) studied production of xylanase by *Penicillium sp.* CFR 303 through solid state fermentation with coffee husk as substrate and found optimum fermentation time of 5 days. From these results, it is indicative that bacteria showed maximum xylanase production at 72 hours of incubation, whereas fungi showed at or after 120 hours of incubation.

**Effect of moisture level:** Initial moisture contents are considered to be one of the key factors influencing xylanase production. In the present study, maximum xylanase production was observed at the wheat bran and tap water ratio 1:1 (Fig. 3). The results indicated drastic decrease in xylanase production on increasing the ratio of tap water. There was about 80% xylanase production when wheat bran and tap water ratio was 1:0.5. It has been assumed that increase in the moisture level reduces the porosity of wheat bran and therefore limits oxygen transfer (27). On the other hand, Feniksova et al. (28) assumed that on decrease in the moisture level than the optimum ratio reduces the solubility of nutrients. Battan et al (25) reported maximum xylanase production from *Bacillus pumilinus* on keeping the ratio of wheat bran and moisture at 1:2.5. Khandeparker et al (1) reported maximum xylanase production from *Arthrobacter sp* MTCC 5214 on keeping the ratio of wheat bran and moisture at 3:1. Gessesse and Mamo (15) reported maximum xylanase production from *Bacillus sp*. AR-009 at wheat bran to moisture ratio of 1:1.

**Optimum pH for xylanase production:** In the present study, xylanase production was tested at pH of the broth ranging from pH 5.0 to 12. The results showed maximum xylanase production at pH 9.0 (Fig. 4). However, there was not drastic decrease in xylanase production at other pH tested. In this experiment, we found lesser activity (nearly 12 U/gm wheat bran) compared to while testing various agro-byproducts (nearly 16 U/gm of wheat bran). It may be due to enzyme handling. Khandeparker et.al (1) also reported maximum xylanase production from *Arthrobacter sp* MTCC 5214 at pH 9.0. Muthezhilan et al. (17) reported maximum xylanase production from *Penicillium oxalicum* at pH 8.0. Yang et al. (29)

![Fig. 3. Production of xylanase from Bacillus halodurans on different wheat bran to tap water ratio.](image-url)
reported maximum xylanase production from *Paecilomyces thermophila* J18 at pH 7.0 using wheat straw. Sanghvi et al. (22) reported maximum xylanase production was observed at pH 5.0 by *Tricoderma harzianum* through solid state fermentation. Murthy and Naidu (23) reported production of xylanase *Penicillium sp.* CFR 303 at pH 5 using solid state fermentation and coffee husk as substrate.

**Optimum temperature for xylanase production:** Optimum incubation temperature for maximum xylanase production under SSF was found to be 40°C (Fig.5). There was about 80% xylanase production at 55°C. Battan et al. (25) reported optimum temperature for xylanase production from *Bacillus pumillus* at 37°C. Khandeparker et al. (1) reported maximum xylanase production from *Arthrobacter sp* MTCC 5214 at 28°C. Muthezhilan et al. (17) reported xylanase production from *Penicillium oxalicum* at 45°C. Simoes et al. (26) reported maximum xylanase production from *Aspergillus japonicus* at 25°C using wheat bran. Yang et al. (29) reported maximum xylanase production from *Paecilomyces thermophila* J18 at 50°C using wheat straw. Antoine et al. (19) reported maximum xylanase production by *Penicillium*
canescens through solid-state fermentation at 30ºC. Kavya and Padmavati (20) reported maximum xylanase production by Aspergillus niger at 28ºC. Pal and Khanum (21) reported maximum xylanase production by Aspergillus niger DFR-5 at 37ºC. Sanghvi et al. (22) reported maximum xylanase production by Tricoderma harzianum through solid state fermentation at 28ºC. Murthy and Naidu (23) reported production of xylanase by Penicillium sp. CFR 303 through solid state fermentation at 30ºC using on coffee husk as substrate.

**Effect of different moisturing agents on xylanase production:** Among various moisturizing agents tested for xylanase production, tap water was found to be best in whose presence; xylanase production was 23.31U/gm wheat bran. There was 21.31 U of xylanase production per gm wheat bran in the presence of distilled water as moistureizing agent. There was decrease in xylanase production with other moisturizing agents, least being with MS4 (Table 1). Higher amount of xylanase production by tap water indicated that the present Bacillus halodurans does not require supplementation of mineral salts for xylanase production. Battan et al. (25) also reported maximum xylanase production with tap water (5984U/gm) compared to other mineral salt solutions. Yang et al. (29) reported maximum xylanase production from Paeclomyces themophila J18 in the presence of tap water with wheat straw. Therefore, our results coincided with other reported results. Here, we tested different combinations of mineral salts to check whether different combinations of mineral salts may be helpful in enhancing xylanase production. The results indicated that supplementation of any mineral salt is not required for enhancement in production of xylanase from Bacillus halodurans KR-1.

**Effect of inoculum size on xylanase production:** In the present study, xylanase production increased on increasing the inoculum size from 5 % to 10 % and thereafter decreased up to 40 % (Fig.6). Battan et al. (25) reported maximum xylanase at 15 % of inoculum size.

![Graph showing effect of inoculum size on xylanase production](image)

**Fig. 6.** Effect of inoculum size on xylanase production from Bacillus halodurans in solid state fermentation using wheat bran.

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Gessesse and Mamo (15) reported maximum xylanase production from *Bacillus sp.* AR-009 at 10% inoculum size. Murthy and Naidu (23) reported production of xylanase by *Penicillium sp.* CFR 303 through solid state fermentation at inoculum size 20% using coffee husk as substrate.

**Effect on different additives on xylanase production:** Different additives viz lactose, glucose, maltose, and fructose were tested as carbon source; and potassium nitrate, sodium nitrate, and casein were tested as a nitrogen source. The results are presented in Table 2. The results showed nearly 80% in xylanase production in the presence of fructose followed by lactose and glucose. However, there was no significant change in xylanase production in the presence of various nitrogen sources tested. Battan et al. (25) reported 20% increase in xylanase production in the presence of 4% peptone. Yang et al. (29) reported maximum xylanase production from *Paecilomyces thermophila* J18 in the presence of yeast extract and wheat straw. Murthy and Naidu (23) reported production of maximum xylanase by *Penicillium sp.* CFR 303 with peptone as nitrogen source in solid state fermentation and coffee husk as carbon source. Laxmi et al. (24) reported maximum production of xylanase by *Aspergillus sp.* RSP-6 using palm fibres as carbon source and beef extract as nitrogen source.

**Scaling up of enzyme production:** On five times scale up, increase in xylanase production was not observed linearly. During scale up, fructose was also used as additive. There was about two times increase in enzyme production upon five times scale up. Enzyme production with 25 gm of wheat bran was found to be 30.20 U/gm. There may be limitation of effective aeration upon scale up.

**Conclusion**

In the present study, conditions for xylanase production from *Bacillus halodurans* strain KR-1 under SSF have been optimized. The data showed economical production of xylanase. The process may be exploited for industrial applications.

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**Table 1.** Effect of various moisturizing agents on xylanase production in solid state fermentation using wheat bran

<table>
<thead>
<tr>
<th>Moistening agents</th>
<th>Xylanase production (U/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moistening agent 1</td>
<td>17.98 ± 0.017</td>
</tr>
<tr>
<td>Moistening agent 2</td>
<td>16.65 ± 0.024</td>
</tr>
<tr>
<td>Moistening agent 3</td>
<td>12.65 ± 0.022</td>
</tr>
<tr>
<td>Moistening agent 4</td>
<td>11.64 ± 0.011</td>
</tr>
<tr>
<td>Tap water</td>
<td>23.31 ± 0.038</td>
</tr>
<tr>
<td>Distilled water</td>
<td>21.31 ± 0.035</td>
</tr>
</tbody>
</table>

**Table 2.** Effect of different additives on production of xylanase

<table>
<thead>
<tr>
<th>0.5% Additives</th>
<th>Xylanase production (U/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21.97 ± 0.010</td>
</tr>
<tr>
<td>Glucose</td>
<td>29.97 ± 0.053</td>
</tr>
<tr>
<td>Fructose</td>
<td>33.3 ± 0.018</td>
</tr>
<tr>
<td>Lactose</td>
<td>27.30 ± 0.046</td>
</tr>
<tr>
<td>Maltose</td>
<td>25.97 ± 0.022</td>
</tr>
<tr>
<td>Casien</td>
<td>23.97 ± 0.030</td>
</tr>
<tr>
<td>KNO3</td>
<td>21.64 ± 0.030</td>
</tr>
<tr>
<td>NaNO3</td>
<td>24.64 ± 0.017</td>
</tr>
</tbody>
</table>

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DBT-TWAS post graduate fellowship awarded to him.

References


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