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Priyanka L. P, Subramaniyan S, Sandhia G. S and Sathish D. K.

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Effect of Medium Components and Culture Conditions on Xylanase Production by *Bacillus mojavensis* PSS1

Priyanka L. P[1], Subramaniyan S*[2], Sandhia G. S[3] and Sathish D. K.[4]

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ABSTRACT

In this study, an attempt was made to evaluate the cultural and nutritional conditions of *Bacillus mojavensis* PSS1 for xylanase production under submerged fermentation conditions. Several factors influence enzyme production by microorganisms. Selection of substrate, different media components and physical parameters significantly influences xylanase production. Different carbon sources, nitrogen sources, initial medium pH, inoculum size, incubation period, and agitation speed were studied to understand their role in the enhancement of xylanase production. PSS1 favored maximum xylanase production in the presence of 0.5% beechwood xylan, yeast extract and peptone at a concentration of 0.25% each, an initial medium pH of 8, inoculum size of 10%, 72 hours of incubation period, and rotation speed of 150 rpm. Characterization of crude xylanase was also carried out. Crude xylanase shows an optimum pH of 7 and optimum temperature at 50ºC. The enzyme retained maximum activity in the presence of 0.5 M NaCl (99.5%). It shows resistance to all the metals tested except the presence of Hg2+. The enzyme activity was enhanced in the presence of organic solvents like Hexane (164%), Methanol (141%), ethanol (140.8%), and ethyl acetate (128.2%). Surfactants such as Triton X 100 (174%), SDS (168%), and inhibitors like PMSF (162.92) and EDTA (140%) also enhanced enzyme activity. So that the properties of xylanase from *Bacillus mojavensis* PSS1 confirmed its potential application in industrial field.

***Key words*:** Xylan, Submerged fermentation, Characterization, Crude xylanase

Xylanases are hydrolytic enzymes which gained immense interest in various biotechnological applications recently. Xylan the major hemi cellulosic component comprises more than half of plant biomass and represents one of the topmost renewable organic molecules in the terrestrial system. Bioconversion of this lignocellulosic biomass into fermentable products can be efficiently utilized for various purposes. Even though this noncellulosic polymer is found in almost all plant species it is found abundantly in woody and annual plants [1]. Xylan degradation occurs naturally by a diverse group of soil microflora. Xylanases, the xylan hydrolyzing enzymes are produced mainly by bacteria, fungi, and actinomycetes [2]. For industrial purposes, bacteria are more preferred due to their high growth rate and production rate [3]. Pre-treatment of forage crops by xylanase along with other cell wall hydrolyzing enzymes such as cellulase help to improve the nutritional efficiency and digestibility of animal feed [4]. It also facilitates the process of composting. For isolating potent xylanase producing bacteria, soil samples were collected from different forest regions of Kerala and carried out screening procedures and staining techniques.85 bacterial strains were obtained from various soils collected and the maximum enzyme producing strain was obtained from them by a three-step screening procedure. The bacterial strain PSS1 was obtained from the soil collected in the forest regions of Bonacaud, Western Ghats of Kerala. It was identified as *Bacillus mojavensis* by 16SrRNA sequencing followed by morphological and biochemical analysis. The present study aims to analyze the effect of various parameters influencing xylanase production such as different carbon sources, nitrogen sources, initial medium pH, inoculum size, incubation time, and Rotation speed. Effect of pH, temperature, metal ions, NaCl concentrations, organic solvents and additives on crude xylanase activity was also carried out.

* **Subramaniyan S.**
✉ subr44@gmail.com

1-5 Post Graduate Department of Botany and Research Centre, University College, Thiruvananthapuram - 695 034, University of Kerala, Kerala, India

MATERIALS AND METHODS

Organism

Initially a basic minimal media containing (g/l): xylan, 5.0; yeast extract, 0.6; peptone, 0.6; K_2HPO_4, 1; and $MgSO_4$, 0.2.was used for the isolation of PSS1and maintained in the xylan agar medium in an incubator. After examining the pattern of xylanase production in the minimal

medium a study was carried out by regulating various chemical and physical growth parameters. The preinoculum for the enzymatic studies was produced by inoculating a loop of freshly grown bacterial strain into 100 ml sterilized basic xylan medium in 250 ml Erlenmeyer flask and incubated in a rotary shaker maintained at 120 rpm for 24 hours. Sterilized media were inoculated with 24hour old-grown pre-inoculum and incubated in a shaking incubator. Samples were collected at successive intervals and centrifuged for enzyme extraction and xylanase assay was carried out.

Xylanase assay

The xylanase activity was determined by using 0.5% beechwood xylan as the substrate by the DNS method [5]. Xylan substrate was prepared in 0.2 M Sodium phosphate buffer (pH 7). To the 0.9ml of pre-incubated substrate 0.1ml of the suitably diluted enzyme was added. After 10 minutes of incubation, the reaction was terminated by adding a 1.5ml DNS reagent. Like that, an enzyme blank and reaction blank was also prepared. After boiling and sudden cool the absorbancy was measured at 540nm. One unit of xylanase activity was defined as the amount of xylanase required to release 1µmol of xylose residues per minute under controlled assay conditions [6].

Effects of medium components and culture conditions on xylanase production

After examining the pattern of xylanase production by *Bacillus mojavensis* in a selected medium, investigations were carried out by using different carbon and nitrogen sources. Along with that, the influence of various physical parameters such as initial medium pH, inoculum size, incubation period, and rotation speed was also analyzed.

Effect of carbon source on xylanase production

Carbon serves as an essential element for the growth and metabolism of microorganisms. Both simple and complex forms of carbon were analyzed for their ability to induce maximum levels of xylanase production. Various monosaccharides, disaccharides, polysaccharides, and lignocellulosic substances were supplemented in the growth medium to study whether they improve or impair the yield of xylanase. Glucose, fructose, and xylose are simple sugars selected which are the monomeric constituents of the plant cell wall. Disaccharides used were maltose, sucrose, and cellobiose. The complex polysaccharides employed were Carboxymethylcellulose, pectin, and Beechwood xylan. Lignocellulosic substances are agricultural wastes that contain starch, protein, and cellulose in addition to hemicellulosic substances. Wheat bran, rice straw, and sugarcane bagasse are the different lignocellulosic substances utilized as carbon sources in the fermentation process. 12 different sources (0.5%) were tested as the sole carbon source in the growth media and the influence of different concentrations of the best carbon source at 0.2%, 0.4%, 0.6%, 0.8%, and 1% was also studied.

Effect of different sources of nitrogen

To determine the best nitrogen source which stimulates maximum enzyme production different organic and inorganic sources were screened. 13 different nitrogen sources (0.5%) in the presence of selected carbon sources were investigated for xylanase production. Casein, gelatine, urea, thiourea, yeast extract, beef extract, peptone, and tryptone are the organic nitrogen sources supplemented in the fermentation medium. The inorganic sources used were KNO_3, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, NH_4CH_3COOH. Different combinations of yeast extract and peptone (0.25% + 0.25%, 0.5% + 0.5%, 0.75% + 0.75%, 1% + 1%) were also studied for xylanase production by *Bacillus* PSS1.

Effect of initial medium pH on xylanase production

The pH of the production medium has a huge influence on the secretion of microbial xylanase. To study the effect, fermentation mediums with different pH (4-11) were inoculated with the bacterial strain PSS1 and the impact of medium pH for xylanase production was evaluated.

Effect of inoculum size on xylanase production

The impact of inoculum size on xylanase production by PSS1 was analyzed by using various concentrations of 24 hour's old grown pre-inoculum. Different concentrations such as 5%, 10%, 15%, 20%, and 25% were studied.

Effect of incubation period on xylanase production

The culture medium was inoculated with 24-hour old pre-inoculum and samples were taken from the fermented medium from zero hours onwards up to 144 at an interval of 24 hours. Samples were centrifuged and the supernatant obtained was taken as crude xylanase and standard assay for xylanase was carried out.

Effect of rotation speed on xylanase production

The effect of rotation speed on xylanase production was studied from 50 to 200 rpm in a shaking incubator at 37°C.

Characterization of crude xylanase from Bacillus mojavensis PSS1

Samples were taken from the fermented medium at the peak hour (72h) of enzyme production and they were subjected to centrifugation at 8000 rpm for 20 minutes. The supernatant obtained was taken as the source of crude xylanase.

Effect of pH on activity and stability of xylanase

The effect of pH on enzyme production was evaluated by preparing xylan substrate using buffers of different pH. Xylanase activity was analysed within the pH range of 4-11 using the following buffer solutions of 0.2 M Sodium acetate buffer (pH4-5), 0.2M Sodium phosphate buffer (pH 6-8), 0.2 M Carbonate buffer (pH 9-11). pH stability was checked by incubating xylanase with respective buffers and assay was done every 30 minutes up to 2 hours.

Effect of temperature on activity and stability of xylanase

The effect of temperature on xylanase activity was analysed by following the standard procedure mentioned earlier for xylanase assay within a temperature range of 30°C - 70°C. The temperature stability was evaluated by incubating the enzyme with the corresponding temperature and enzyme activity was checked every 30 minutes up to 2 hours.

Effect of metal ions on xylanase activity

The influence of metal ions on xylanase activity was tested by using various metal salts such as Ba2+, Ca2+, Cu2+, Fe2+, Hg2+, Mg2+, Mn2+, K2+, Na+ and Zn2+ at a

concentration of 10mM and incubated at room temperature for 30 minutes and xylanase activity was evaluated and expressed as residual activity (%).

Effect of NaCl concentrations

Xylanase activity was analysed by incubating xylanase with different concentrations of NaCl (0.5-2.5M) and a standard assay was carried out.

Effect of organic solvents on xylanase activity

Solvent stability of xylanase was analysed by incubating the crude xylanase with different organic solvents at a concentration of 30% for 30 minutes and xylanase activity was assayed. Organic solvents with different logP values represent different hydrophobicity. Lower the log P value lower will be the hydrophobicity.

Effects of various additives

The enzyme activity was checked out by incubating it with surfactants (1%) like Sodium Dodecyl sulphate (SDS), Triton X-100, Cetyl Trimethyl Ammonium Bromide (CTAB), and inhibitors like Ethylene Diamine Tetra Acetic acid (EDTA) and Phenylmethyl sulfonyl fluoride (PMSF).

RESULTS AND DISCUSSION

Effects of medium components and culture conditions on xylanase production

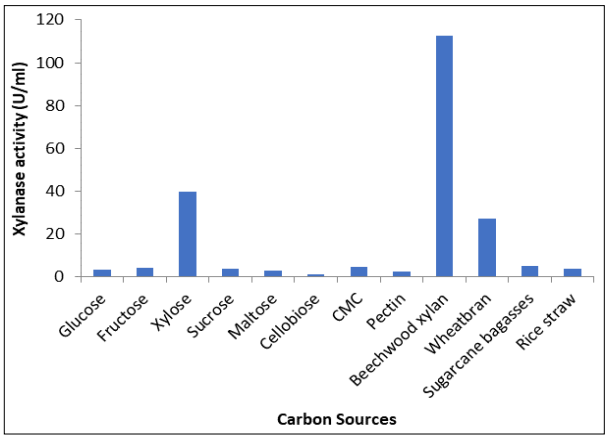

Fig 1 Effect of different carbon sources on xylanase production

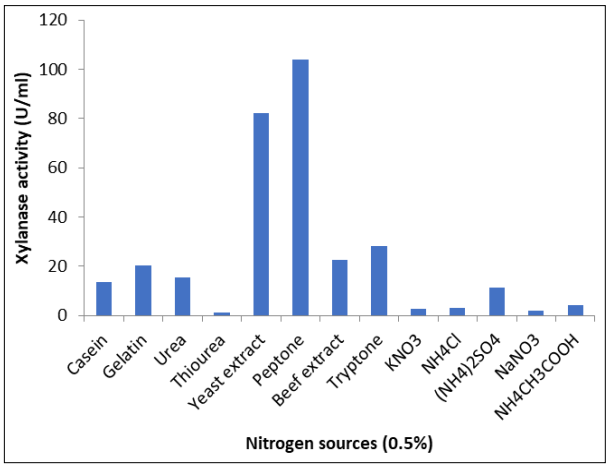

Fig 3 Effect of different nitrogen sources on xylanase production

Xylanase production by the strain PSS1 in the minimal medium was 112.38 U/ml. Here xylanase production was increased when the nutritional and physical conditions altered.

Effect of different carbon sources on xylanase production

The carbon sources supplemented in the fermentation medium play a crucial role in xylanase production. Among the different carbon sources screened, maximum xylanase production was induced by Beechwood xylan (112.38U/ml) followed by xylose and wheat bran (Fig 1). On varying the concentration of beechwood xylan from 0.2 to 1% the highest enzyme activity of 120.27U/ml was obtained at a concentration of 0.4% (Fig 2). Similarly, there are many reports of pure forms of xylan supporting maximum xylanase production [7-9]. On the other hand, the complex lignocellulosic material wheat bran induced significant levels of xylanase (27.02 U/ml). This finding was following the earlier reports of Nagar *et al.* [10] and Bajaj *et al.* [11]. When the production medium was supplemented with glucose and fructose xylanase production was negligible which could be due to catabolic repression of monosaccharides on enzyme secretion [12]. In contrast, xylose a monosaccharide contradicted the above finding by producing a significant amount of xylanase (39.63 U/ml). Similar findings have been documented by Shanti and Roymon that xylose is a good inducer for xylanase production [13].

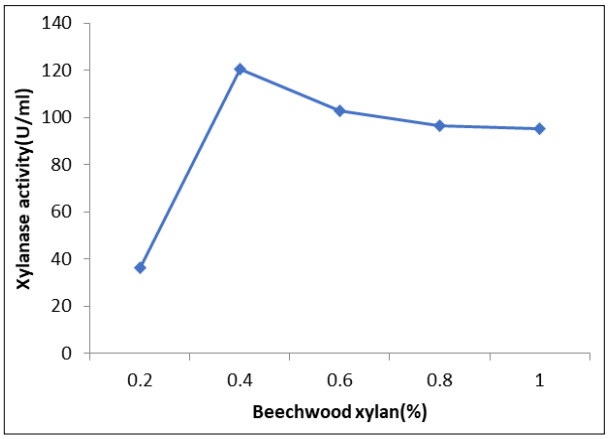

Fig 2 Different concentrations of beechwood xylan on xylanase activity

Effect of nitrogen sources on xylanase production

Different organic and inorganic nitrogen sources were screened to determine which of them influences maximum xylanase production (Fig 3). The highest xylanase secretion was observed in the presence of peptone (104 U/ml) and yeast extract (82 U/ml). There are several reports of good xylanase production in presence of yeast extract and peptone. Since these two sources were observed as better sources of nitrogen, a study was carried out by combining different combinations of both (Table 1). Yeast extract and peptone at 0.25% each were founded as the best concentration (150 U/ml). Yeast extract plays an important role in cell division by providing growth factors and essential elements [14]. Like that yeast extract, a complex organic nitrogen source favours bacterial growth by providing NH4+ and nitrogen assimilating enzymes [15]. So that they complement each other and induce maximum xylanase production. *Bacillus* SSP 34 gave 380 IU/ml of

xylanase production at 0.25% of both yeast extract and peptone [16]. Similarly, *Bacillus* sp. MCC2728 showed a significant increase in xylanase production by the combined effect of yeast extract and peptone [13]. Sepahy *et al*. [12] reported that a combination of yeast extract and tryptone enhanced xylanase production in *Bacillus mojavensis* AG137.

Table 1 Different combinations of yeast extract and peptone on xylanase production

Different combinations of yeast extracts (%) + peptone (%)	Xylanase activity (U/ml)
Yeast extract (0.25%) + Peptone (0.25%)	150
Yeast extract (0.5%) + Peptone (0.5%)	135.2
Yeast extract (0.75%) + Peptone (0.75%)	85.7
Yeast extract (1%) + Peptone (1%)	76.5

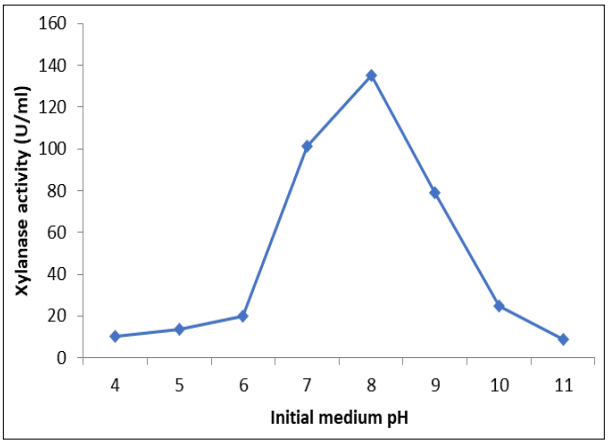

Fig 4 Effect of initial medium pH for xylanase production

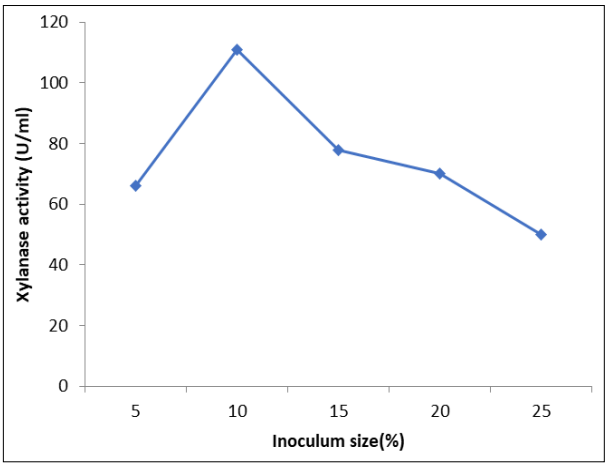

Fig 5 Effect of inoculum size for xylanase production

Effect of incubation period on xylanase production

Bacillus sp PSS1 showed the highest enzyme production at 72 hours of incubation (112.38U/ml). After 96 hours of incubation, a drastic reduction was observed in the enzyme titer (Fig 6). This may be due to the secretion of toxic metabolites by the microbes which inhibit the enzyme synthesis. The optimum incubation time required for the highest xylanase production is different for different Bacillus strains. *Bacillus* SSP34 produced maximum enzyme secretion after 96 hours of incubation [21]. Like that *Bacillus subtilis* ASH after 48 hours [20].

Effect of rotation speed on xylanase production

Effect of initial medium pH on xylanase production

The pH of the fermentation medium significantly influences the production of enzymes. Every organism has an optimum pH for fermentation medium at which maximum enzyme production occurs. In the presence of favourable medium pH transport of nutrients across the cell membrane occurs and promotes cell growth and enzyme production [17]. Xylanase production pattern of *Bacillus* sp PSS1 was studied within a pH range of 4-11 and maximum production of 135.13 U/ml occurs at a pH of 8 (Fig 4). So that it can be confirmed that the bacteria can tolerate alkaline conditions. Two bacterial strains *B. subtilis* BS04 and *B. megaterium* BM07 exhibited their peak activity at a pH of 8 [18]. Similarly, Sepahy *et al*. [12] reported that *B. mojavensis* AG137 produces maximum xylanase at a pH of 8.

Effect of inoculum size on xylanase production

Various inoculum size ranging from 5-25% were analysed for enhanced production of xylanase by *Bacillus mojavensis* PSS1. The highest production was observed at 10% inoculum size (110.81U/ml). Enzyme production declines drastically when the inoculum size increases (Fig 5). This may be due to the faster nutrient consumption of proliferating bacteria in the fermentation medium [19]. The results of this study are in accordance with that of Sanghi *et al*. [20] who observed that lower inoculum size is suitable for hyperproduction of xylanase. Similarly, Shanti and Roymon reported that 5% inoculum is suitable for maximum xylanase production in both *Bacillus sp*. MCC2727 and MCC2728 [13]. To obtain maximum enzyme yield a balance should be maintained between growing bacteria and available nutrients in the fermentation medium.

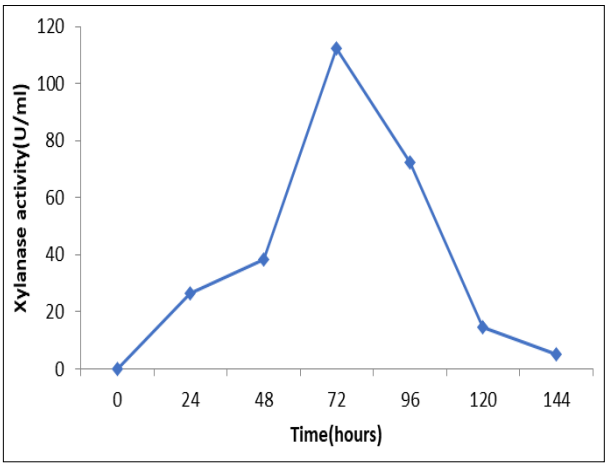

Fig 6 Effect of incubation period on xynanase production

For the uniform distribution of dissolved oxygen throughout the fermentation medium agitation plays a very vital role. In the present study maximum enzyme activity (144.14U/ml) was obtained at 150 rpm (Fig 7). Similar findings were reported in the case of *Bacillus* sp. MCC2727 and MCC2728 where a rotation speed of 150 was optimum for maximum enzyme production [13]. The lower yield of xylanase was observed at a very low and high agitation speed of 50 and 200 rpm. The lower enzyme yield during lower agitation speed may be due to the limited dissolved oxygen supply, improper mixing of media components, and cell clumping. Like that higher agitation speed of 200 rpm may cause shear stress in bacterial cells and lower yield.

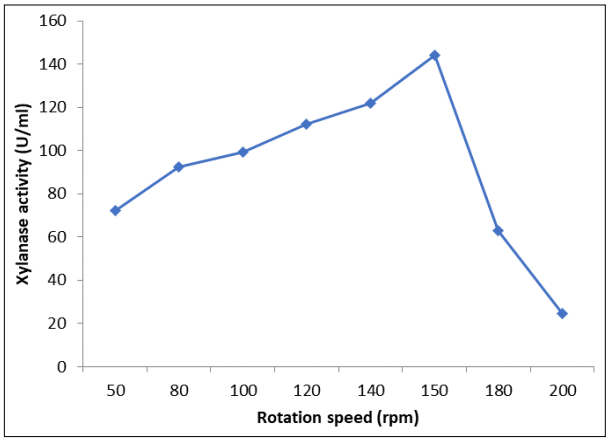

Fig 7 Effect of rotation speed on xylanase production

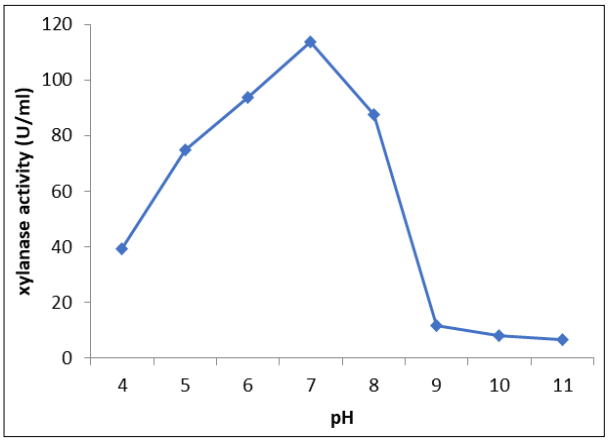

Fig 8 Effect of pH on enzyme activity

Characterization of crude xylanase from Bacillus mojavensis PSS1

Effect of pH on activity and stability of xylanase

The optimum pH of xylanase was tested by incubating crude xylanase in different buffers. A pH of 7 was revealed as optimum for maximum xylanase activity (113.58 U/ml) by *Bacillus* sp PSS1 (Fig 8). Further increase in pH resulted in a gradual decrease in enzyme activity. An optimum pH of 7 for xylanase activity from *Bacillus subtilis* ASH was previously reported by Sanghi *et al*. [20]. Similarly, for *Bacillus amyloliquefaciens* a pH range of 6.8-7 was optimum for maximum xylanase activity [22]. Stability of enzyme at pH 4, 7 and 11 were analysed. At pH 7 the enzyme was stable up to two hours while at pH 4 and 11there was a drastic loss of activity after 30 minutes (Fig 9).

Effect of temperature on activity and stability of xylanase

For every enzyme, there will be an optimum temperature during which the activity will be higher. Xylanase activity was observed at temperatures ranging from 30°C to 60°C (Fig 10) and a maximum activity of 111.71 U/ml was observed at a temperature of 50°C. In several *Bacillus* sp. the optimum temperature has been reported in the range of 50-55°C [6], [23]. A decline in enzyme activity occurred above and below the optimum temperature. In the case of 50°C which is the most favourable one, the enzyme was stable for up to one hour. 87% of activity retained up to 90 minutes at 50°C after that it decreases gradually (Fig 11).

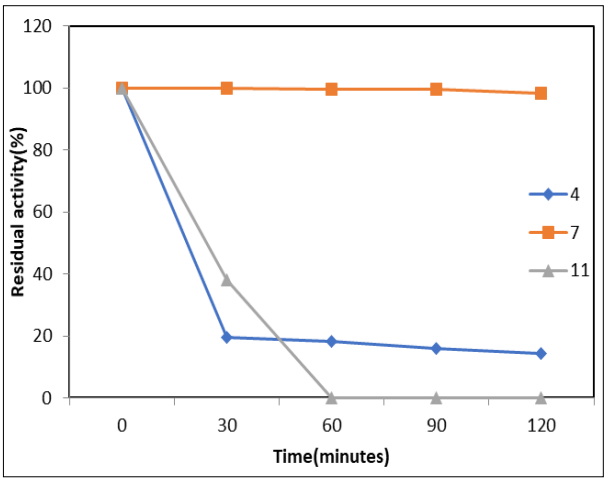

Fig 9 Stability of xylanase at pH 4, 7 and 11

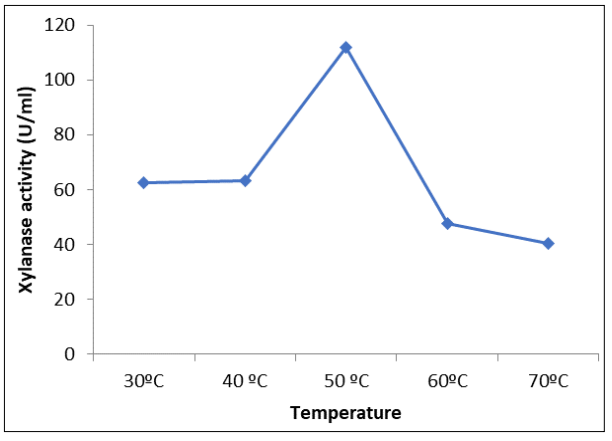

Fig 10 Effect of temperature on activity

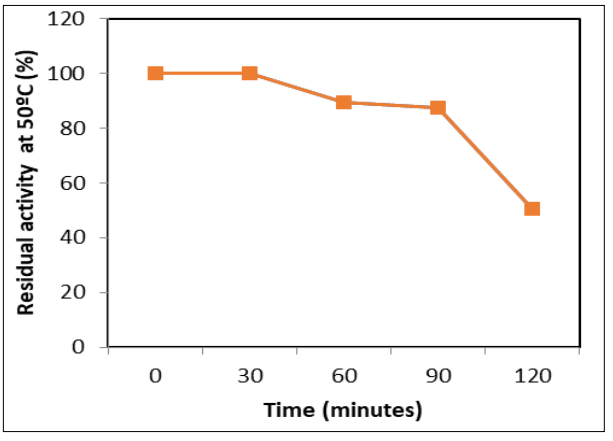

Fig 11 Stability of xylanase at 50°C

Effect of metal ions on xylanase activity

Xylanase assay was carried out under standard assay conditions in the presence of various metal salts at 10mM concentration. Only Hg2+ causes an inhibitory effect on xylanase activity. The enzyme loses 97% of enzyme activity in the presence of Hg2+. This inhibitory effect may be due to its interaction with sulfhydryl groups on the enzyme. Several researchers have reported similar findings of less activity in the presence of Hg^{2+} [23]. The other metal ions such as Cu^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, K^{+} and Zn^{2+} stimulated the xylanase activity (Table 2).

Effect of different concentrations of NaCl on xylanase activity

Various salt concentrations ranging from 0.5 to 2.5 M were monitored for xylanase activity. Maximum enzyme activity was observed in the presence of 0.5 M NaCl (Table 3). More than 99% of original activity was retained in the presence of 0.5M NaCl. Even though there occurs a slight decrease in enzyme activity in the presence of increasing salt concentration more than 90% of xylanase activity was retaining. So that it can be confirmed as salt-tolerant xylanase from *Bacillus mojavensis* PSS1. Salt tolerant xylanases have significant applications in the processing of marine food. Usually, halotolerant xylanases have been reported from the marine environment [24].

Table 2 Effect of metal ions on xylanase activity

Metal ions	Residual activity (%)
Ba^{2+}	115.67
Ca^{2+}	124.2
Cu^{2+}	141.96
Fe^{2+}	136.2
Hg^{2+}	3
Mg^{2+}	138.4
Mn^{2+}	120.2
K^{2+}	142.2
Na^{+}	140.28
Zn^{2+}	133.9

Table 3 Effect of NaCl on xylanase activity

NaCl(M)	Residual activity (%)
0.5	99.5
1	94.3
1.5	92.56
2	92.18
2.5	91.54

Effect of various organic solvents on xylanase activity

In this study, xylanase activity was checked in the presence of different organic solvents with different log P values. The findings of the study revealed that xylanase from PSS1 had good tolerance in the presence of a 30% concentration of hexane, methanol, ethanol, ethyl acetate, and acetone (Table 4). Arunachalam and Beena have reported halophilic organic solvent tolerant xylanase from *Bacillus subtilis* [24]. Like that *Provencia* sp exhibited maximum xylanase activity in the presence of 25% hydrophilic solvents [25].

Table 4 Solvent stability of xylanase from PSS1

Organic solvent	log P value	Residual activity (%)
Methanol	-0.69	141
Acetone	-0.61	118
Ethanol	-0.18	140.8
Ethyl acetate	0.71	128.2
Butanol	0.839	93.4
Choloroform	1.67	80.96
Hexane	3.769	164

Effect of various additives on xylanase activity

In the presence of various additives, xylanase activity was assayed and it was observed that the enzyme showed maximum activity in the presence of Triton X- 100, SDS, PMSF, and EDTA (Fig 12). Kumar and Satyanarayana have also reported Triton X-100 and SDS tolerant xylanase from *B. halodurans* [26]. On the other hand, *B. mojavensis* strain AG137 exhibited a severe reduction of enzyme yield in the presence of SDS [12]. In this study, EDTA and PMSF do not inhibit enzyme activity. Unlike that, there are reports that about 90% of inhibition by EDTA and partial inhibition by PMSF [24].

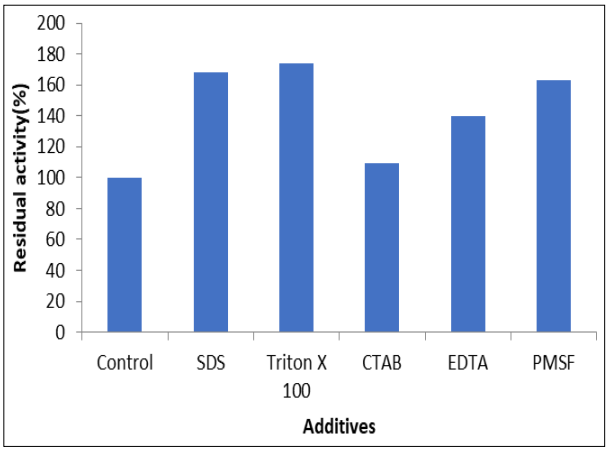

Fig 12 Effect of additives on xylanase activity

CONCLUSION

Xylanase can be considered as an emerging biomolecule in the present and future perspective. This study aimed to evaluate enzyme production by altering medium components and fermentation process parameters. It was observed that original activity was enhanced by these treatments. Characterization of crude xylanase revealed that the enzyme shows maximum activity at neutral pH and is thermophilic. Metal ions like Cu2+, Ca2+, Fe2+, Mg2+, Mn2+, K+ and Zn2+ stimulated the xylanase activity. Since it is resistant to various concentrations of NaCl and Organic solvents it can be considered as halophilic and organic solvent resistant xylanase. Surfactants like SDS, CTAB, and Triton X 100 and inhibitors like PMSF, EDTA also stimulated xylanase activity. The Bacillus strain PSS1 in this study were found to be a potential tool in various industrial applications.

Acknowledgement

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Conflict of interest

Authors have declared that no competing interests exist.

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