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Original Article

Screening, optimization and characterization of xylanase by locally isolated bacteria

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Abstract
Xylanase enzyme is of great importance commercially because of its high stability at elevated temperatures and in alkaline conditions. The present study was aimed to isolate and screen xylanase producing bacteria and to optimize cultural conditions for maximum production of enzyme from locally isolated bacteria and to optimize the assay conditions under which enzyme possessed maximum activity. Two bacterial isolates XPB-GS02 and XPB-CW01 with maximum activity ratio showed 18.3 and 12.2 U/ml xylanase activity, respectively. Maximum xylanase was produced in medium M1 containing xylan after 48 hours of incubation. Maximum amount of enzyme was produced by XPB-GS02 at 45° C, pH 9 using 1% xylan concentration, while XPB-CW01 produced maximum amount of xylanase at 50° C, pH 7 with 1.5% xylan concentration. Maximum xylanase activity was achieved at temperature 50° C, pH9 with 2% xylan concentration. Xylanase produced by XPB-GS02 was active from 35-80°C, while XPB-CW01 was active from 30 ‒70°C with optimum temperature 50°C.
Key words: Xylanase, *Bacillus,* optimization, characterization

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INTRODUCTION

Enzymes are biological catalyst which are being extensively used in various biotechnological processes in the industry. Xylanases (EC 3.2.1.8) are a group of hydrolytic enzymes that facilitates the complete hydrolysis of xylan which contains a backbone of 1,4-linked-β-D-xylopyranose residues substituted with arabinose, acetyl groups and 4-O-methylglucuronic acid residues. Xylanase catalyzes the depolymerization of xylan backbone by hydrolyzing β-1, 4-glycosidic bonds resulting in the formation of xylose and xylo-oligosaccharides. It has a wide range of industrial applications which include textile industry, texture improvement of bakery products, bleaching of pulp, xylo-oligosaccharide production, and improvement in animal feed digestibility, extraction and clarification of fruit juices, conversion of lignocellulosic biomass into valuable products and wastewater treatment (Wong *et al*., 1988; Subramaniyan and Prema, 2002; Polizeli *et al*., 2005). Xylanases are also used to make material for scientific research, besides being used as research material themselves. Xylanases have been reported from fungi (Ishihara *et al*., 1997; Puchart *et al*., 1999) and bacteria (Gessesse and Mamo, 1998; Inagaki *et al*., 1998). Due to high growth rate of bacteria, the xylanase production from bacteria is more advantageous than other microorganisms (Nagar *et al*., 2010). The present study was aimed to screen xylanase producing bacteria from soil and water and to optimize the culture conditions for maximum enzyme production and to optimize the enzyme activity under laboratory conditions.

MATERIALS AND METHODS

Screening and isolation of bacterial isolates

Xylanase producing bacterial isolates were isolated from water and soil samples by serial dilution method followed by spreading on two agar medium A and B. The agar medium A plates contained yeast extract 0.3%,NaCl 0.5%, peptone 0.5%, agar 2.0% and wheat bran 0.5% while in agar medium B only wheat bran was replaced by xylan 0.5%. The pH of the medium

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was adjusted at 9 and it was autoclaved at 15 lbs pressure for 15 minutes. The plates were inoculated by streaking straight in the middle with inoculating loop followed by incubation for 24 h at 45±1ºC. Xylanase activity was detected on culture plates by staining the plates with iodine for 15 minutes (Fig. 1). The enzyme activity was determined as the ratio of hydrolysis zones (clear areas) to that of the colony.

Quantitative estimation of xylanase production

Two bacterial isolates (XPB-DS01 and XPB-GS02) was used for further studies. Fermentation medium: xylan 0.5%, peptone 0.5%, K_2HPO_4 0.1%, yeast extract 0.5% and $MgSO_4.7H_2O$ 0.02% were inoculated with 1% inoculum and were incubated in shaking water bath at 120rpm for 24 hours at 45°C. Culture medium was centrifuged at 3000rpm for 20 minutes and supernatants was used as crude enzyme. The xylanase enzyme activity was assayed by determining the concentration of reducing sugars liberated by the activity of the enzyme on its substrate xylan using DNS reagent (Miller, 1959).The reaction mixture was prepared by adding 1.8ml of 1% birch wood xylan in 50mM Glycine NaOH buffer (pH 9.0) and crude enzyme(0.2ml). This reaction mixture was incubated at 45ºC for 10 minutes and reaction was terminated by adding 2ml DNS reagent followed by incubation in boiling water for 10 minutes to release the reducing sugars. A control was also set up where crude was added after adding DNS. All the tubes were cooled to room temperature and the absorbance was estimated by spectrophotometer at 540nm against blank. The xylanase activity was measured from the standard curve prepared by using xylose as a standard. The enzyme activity has been expressed as units of enzyme activity/ml of the bacterial medium. All the experiments were performed in triplicates.

Protein Estimation

Soluble protein concentration was estimated by Lowery method (Lowry *et al.*, 1951) from the supernatant after pelleting out the bacteria. The soluble proteins were estimated by preparing the protein standard curve using BSA.

Optimization of carbon sources for xylanase Production

The effect of two carbon sources *i.e.*, wheat bran and brich wood xylan on enzyme production was studied by using media M1 and M2 at pH 9. Fermentation medium M1 contained xylan 0.5%, peptone 0.5%, K_2HPO_4 0.1%, yeast extract 0.5% and $MgSO_4.7H_2O$ 0.02% while fermentation medium M2 contained 0.5% wheat bran instead of xylan and rest of the composition was same as M1. Both media were inoculated with respective bacterial isolates and incubated at 45°C for 24 and 48 hours, in shaking water bath with gentle shaking at 120rpm.To check the effect of concentration of carbon sources on enzyme production various concentrations 3, 0.5, 1, 1.5, 2, and 2.5% of xylan were used in fermentation medium and incubated at 45°C for 24 and 48 hours, in shaking water bath with gentle shaking at 120rpm.

Incubation Time for xylanase Production

Inoculated fermentation media were incubated at 45°C for 24, 48, 72, 96, 120 and 140hours in shaking water bath at 120rpm. After each incubation period the bacterial broth was centrifuged at 3,000 rpm for 20 minutes. The supernatants were collected in separate test tubes and were processed for the estimation of protein contents and enzyme activity.

Determination of temperature optima for xylanase Production

Inoculated media (M1) was incubated at 37, 40, 45, 50, 55, 60 °C for 48 hours in shaking water bath with gentle shaking at 120rpm. After 48 hours incubation xylanase production assay was performed for every temperatures and effect of temperature on xylanase production was recorded.

Determination of optimum medium pH for xylanase Production

Inoculated fermentation media (M1) was incubated at 45°C for 48 hours at a wide range of pH from 5-13. Xylanase production assay was performed for every pH and the effect of pH on enzyme production was recorded.

Characterization of xylanase activity

The xylanase activity was characterized by the optimization of xylanase assay conditions for maximum units/ml of xylanase.

Optimization of temperature, pH and substrate concentration for xylanase activity

Optimum temperature for activity of xylanse was determined by carrying out at selected temperatures from 30-100°C by incubating crude enzyme substrate mixture at

selected temperature for 10 minutes. The optimum pH was found by monitoring xylanase activity at pH values between 5-12using50mM Glycine-NaOH buffer solution. Substrate concentration for maximum xylanse production was optimized by using 0.25-3.5% of xylan substrate.

Identification of bacterial isolates

Single colony of the selected isolates grown on xylan nutrient agar plates were observed for morphological characteristics in terms of margin colour, surface, opacity and shape. Selected isolates were subjected to gram staining and motility test by hanging drop method. Biochemical tests *i.e.,* oxygen requirement, catalase test, oxidase test, citrate utilization test and production of urease were carriedout (Theivendrarajah, 1990).

RESULTS

Screening of Isolates for xylanase Activity

Out of eight isolates, four (XPB-GS01, XPB-CW01, XPB-GS and XPB-DS03) showed the xylanase activity (clear areas) on xylan containing agar plates after 24 hrs incubation. The activity ratio was determined by measuring areas of hydrolysis around the streaked areas (Fig. 1). The XPB-GS01 and XPB-CW01with highest hydrolysis zone (best enzyme producers) was selected for further study (Table I).

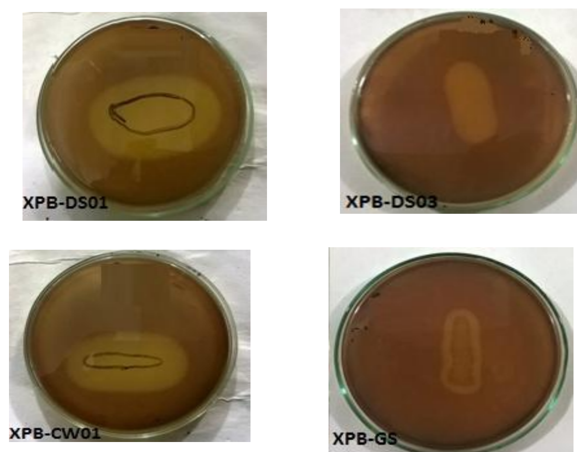

Figure 1: Zone of Hydrolysis of xylanase activity of bacterial isolates XPB-DS01, XPB-DSO3, XPB-CW01 and XPB-GS02

Effect of culture medium for xylanase Production

Among the used carbon sources, xylan exhibited a significant effect on the production of the β-xylanase activity (Fig. 2) and high level of production was achieved when the cells ofXPB-CW01 and XPB-GS02 were grown in a medium containing 1.5 and 1% xylan, respectively (Fig.3).

Table I: Activity ratio, xylanase activity and protein estimation of bacterial isolates

Bacterial isolates	Activity ratio	Xylanase activity (U/ml)	Total Protein (mg/dl)
XPB-GS02	19.5	18.3	0.543
XPB-CW01	9.67	12.2	0.371

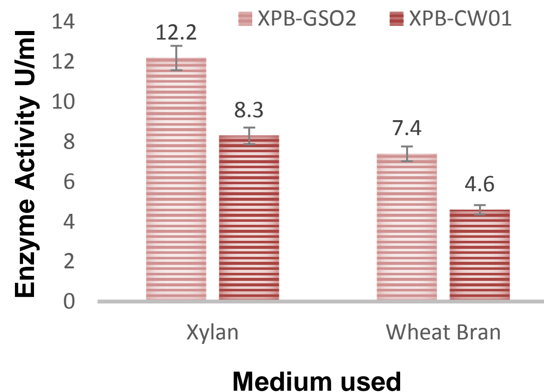

Figure 2: Effect of carbon sources on enzyme production

Optimal temperature and pH for xylanase production

Maximum xylanase production by XPB-GS02 (18.95U/ml) and XPB-CW01 (14.5U/ml) was recorded at 45 and 50°C, respectively (Fig. 4). The XPB-GS02 produced maximum xylanase (21.08U/ml) at pH 9 whileXPB-CW01 produced maximum xylanase (10.70U/ml) at pH 7 (Fig. 5).

Incubation Time for xylanase Production

The production of xylanase was recorded at various incubation time period for 24, 48, 72, 96 and 120 hrs., respectively. The optimum incubation time was 48hrs. and production of enzyme was decreased after 48 hours (Fig. 6).

Characterization of xylanase enzyme

Xylanase enzyme activity is affected by

various factors such as temperature, pH and substrate concentration,

Effect of temperature and pH on enzyme activity

Xylanase produced by XPB-GS02 was active from 35-80°C,while XPB-CW01 was active from 30 ⁻70°C with optimum temperature 50°C (Fig. 7).The enzyme produced by XPB-GS02 and XPB-CW01 were active between pH 4-12. Xylanase produced by XPB-GS02 exhibited maximum activity at pH 9 while xylanase produced by XPB-CW01 exhibited maximum activity (11.427U/ml) at pH 8 (Fig. 8).

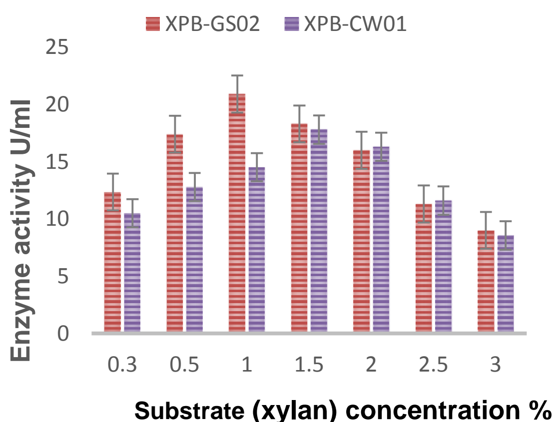

Figure 3: Effect of xylan concentration on xylanase production

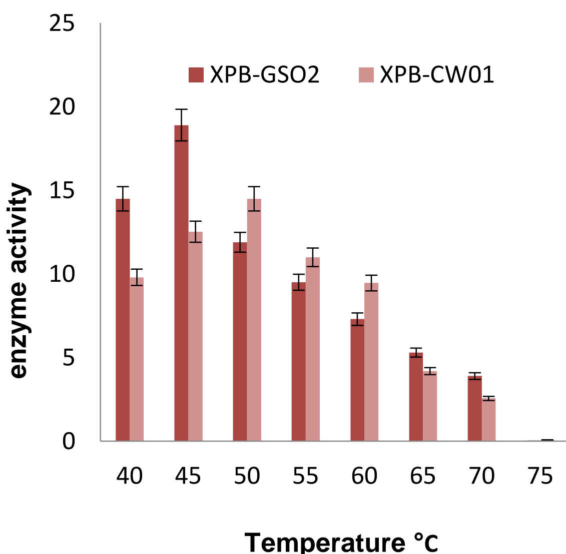

Figure 4: Effect of temperature on xylanase production

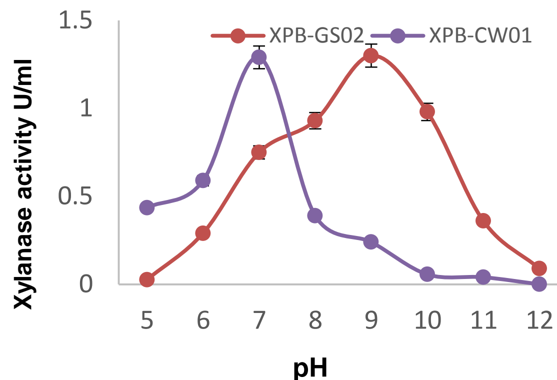

Figure 5: Effect of pH on xylanase production

Effect of substrate concentration on xylanase activity

Xylanase produced byXPB-GS02 andXPB-CW01, exhibited activity from 0.5-4.0%. Enzyme produced by XPB-CW01 exhibited maximum activity at 2% substrate concentration and then remained almost constant till 4.0% substrate concentration. Xylanase produced by XPB-GS02 produced maximum amount of sugars (U/ml) at 2.5% substrate concentration, respectively (Fig. 9).

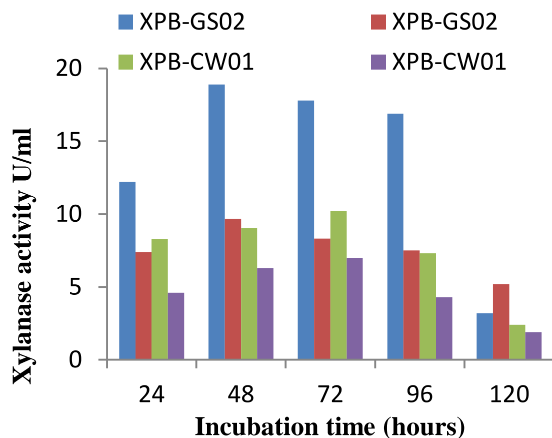

Figure 6: Effect of incubation time on xylanase production.

Identification of bacterial isolates

Among the selected two isolates, the XPB-CW01 isolate showed circular, convex, entire yellow color, moist and shiny colony. The colony of theXPB-GS02 isolate was circular, flat, irregular, white color, dry and rough. Both the isolates were stained as blue- violet rods with spores showing that they are Gram-positive

rods. These isolates moved swiftly across the field of microscope with twisting and this indicated the motility. The isolates were non-branching and spore forming rods belonging to the Family Bacillaceae.

Table II: Morphological and Biochemical Identification of Bacterial Isolates

Characters	Strain	
	XPB-GS02	**XPB-CW01**
Colony color	creamy white	white
Shape of cell	rod	rod
Margin	Entire	Irregular
Elevation	flat	convex
Gram staining	+ve	+ve
Motility	+ve	+ve
Surface	moist shiny	dry rough
Oxidase test	-ve	-ve
Catalase test	+ve	+ve
Spore formation	+ve	+ve

DISCUSSION

Xylanases are hydrolytic enzymes which catalyze the breakdown of xylan with significant industrial applications (Techapun *et al.*, 2003). The enzyme xylanases having high activities at alkaline pH are most preferable for industrial applications. In present study, four bacterial isolates were capable of producing xylanase enzyme isolated from soil and water sample. Bacteria produce a large number of extracellular enzymes which play an important role in the biodegradation of low cost plant biomass and toxic organic pollutants. Many efforts are going to evaluate the hidden potentials of bacteria (Peixoto *et al.*, 2011). For the qualitative screening of bacterial isolates Iodine test was applied, in which Gram's iodine solution was used for staining xylan agar plates (Fig. 1).However, Congo red solution can also be used for this purpose as reported by Rattanasuk and Ketudat-Carins (2009). But Iodine test is reported to be better for this purpose because it gives sharp zone of hydrolysis making the process better and efficient. Moreover, it also avoids the use of toxic chemicals (Kanasa *et al.*, 2008).

The bacterial cultivation media, composed of different nutrients, minerals carbon sources play important role in bacterial enzyme production. Two culture media were used for the production of xylanase and birch wood xylan was found best carbon source. The xylanase production on Birchwood xylan was maximum after 48 hours but the time for maximum xylanase production on wheat bran was 72 hours as also reported by Khandeparkar and Bhosle (2006).When grown on Birchwood xylan the bacterial isolates gave maximum xylanase activity in the early stationary growth phase (48h), while in case of wheat bran the maximum enzyme activity was obtained in the late stationary phase from *Bacillus*as reported by Subramaniyan *et al.* (1997).

The effect of incubation temperatures on xylanase enzyme production was evaluated and results showed maximum enzyme production at 45 and 50ºCby the XPB-GS02 and XPB-CW01, respectively. Shanthi and Roymon (2014) also reported maximum enzyme production at 50ºC and a drastic decrease in xylanase production was observed at temperatures lower and higher than the optimum temperature.

One of the factors which play a crucial role in transportation of nutrients across the membrane and the functioning of enzyme systems within an organism is pH (Gupta and Kar, 2008). In this study, optimum pH 9 was recorded, although significant amounts of xylanase was produced at pH 10. The observations that xylanase production was seen at different pH may be an indication that the organism may have the ability to induce multiple xylanases at different pH (Sharma and Bajaj, 2005).Several alkaline tolerant xylanase producing bacterial strains have also been characterized recently by Mamo *et al.*(2006).

The optimal temperature for xylanase activity was 50°C for XPB-GS02 and XPB-CW01. In accordance to the present results, *Bacillus* activity was found to be gradually increased with increasing temperature and found significantly declined at 80°C. Kulkarni and Rao (1996) reported 50°C as optimum temperature for xylanase activity from *Bacillus sp.* and Liu *et al.*(1998) reported similar temperature optima for enzyme activity from *Trichosporon cutaneum*

Xylanase produced by XPB-GS02 exhibited maximum activity at pH 9 while xylanase produced by XPB-CW01 exhibited maximum activity at pH 7.0.Khandeparkar and Bhosle (2006) also reported the optimum pH 9

for xylanase activity by *Arthrobacter* sp. Xylanase isolated from *Bacillus subtilis* exhibited maximum activity at pH 6 (Pereira *et al*., 2002). Xylanase isolated from *B.licheniformis*A99and *B.coagulans* BL69 had the optimum pH of 7 (Archana and Satyanarayana, 1997; Wang *et al.,* 2003). Gupta *et al*., (2001) reported that purified xylanase was active between pH 6.0-10.5 and retained more than 70% of its activity. It possessed a unique property of having dual pH optima at 7.5 and 9.2, respectively.

Conclusion

The xylanase produced by bacterial isolates XPB-GS02 and XPB-CW01 were highly stable at alkaline pH (7-9) and can withstand high temperature 80ºC.

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