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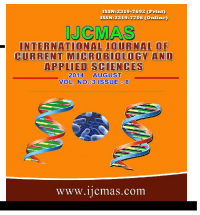

Original Research Article

Isolation and Screening of Alkaline Thermostable Xylanase Producing Bacteria from Soil in Bhilai – Durg Region of Chhattisgarh, India

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Keywords

Xylanase, Screening, Wheat bran, Congo red assay, Alkaline-thermostable

A B S T R A C T

Sixteen different soil samples in and around Bhilai- Durg region of Chhattisgarh were screened for alkaline thermostable xylanolytic bacteria. Isolation and screening of potent indigenous isolates were done in three distinct stages. All the sixty five bacterial isolates obtained through primary screening by enrichment technique were subjected to plate assay. Twenty four isolates showed clear zone around them thus proving to be xylanolytic in nature. The maximum zone diameter was measured to be around 2.1cm. Ten isolates which showed clear zone diameter greater than 1.0cm were subjected to final screening procedures wherein isolate ISL 3 proved to be the most potent and efficient xylanase producer with an enzyme activity of 23.33 IU/ml. The optimum pH and temperature for maximum xylanase production by ISL 3 was 9.0 and 50ºC respectively. Xylanase production was substantially enhanced when a combination of wheat bran (1%) and xylan (0.5%) were provided in the medium. Yeast extract and peptone combined together at a concentration of 0.5% each proved to be efficient nitrogen sources.

Introduction

Xylan is one of the most abundant polysaccharide in nature. About one third of the dry weight is comprised of xylan in higher plants. It is a complex heteropolysaccharide consisting of a linear backbone made of β- 1, 4 linked xylose residues with side branches comprising of various substituent groups. Xylan is present in the secondary cell wall and forms an interface between lignin and other polysaccharides (Dhiman et al. 2008). Xylanases are hydrolytic enzymes which catalyze the breakdown of xylan with significant industrial applications. The industrial uses of xylanases have increased significantly in recent years (Techapun et al. 2003). They are being used in waste treatment, textile processing, for recovery of hexoses and pentoses, animal feed processing etc. and the most important application being their use for pulp bleaching in paper - pulp industries (Beg et al. 2000; Collins et al. 2005; Azeri et al. 2010). The process of pulping and bleaching both during paper manufacturing are carried out at high temperatures and alkaline pH

(Srinivasan and Rele, 1999) and therefore the need for alkaline thermostable xylanase enzymes. Moreover the enzyme should be free of cellulases which otherwise may destroy the cellulose microfibres and thus deteriote the paper quality. Microorganisms are the most preferred source of industrially important enzymes for commercial applications. There are many microbes which have been reported to produce xylanases like fungi (Taneja et al. 2002; Sudan and Bajaj, 2007), actinomycetes (Ball and Mccarth, 1989) and bacteria (Nakamura et al., 1993; Gupta et al., 2001; Azeri et al., 2010) but among all; bacteria are more preferred in having high pH and temperature optima thereby facilitating their use for industrial purposes. Moreover the production of xylanases from bacteria can be advantageous as the enzyme production rate is normally higher due to the high growth rate of bacteria. The state of Chhattisgarh (India) comprises of 44% forest area with a fairly rich biodiversity. Such rich forest resource should be able to sustain diverse group of microorganisms and thus, owing to the industrial applications of xylanases; the main objective of the present study was to isolate and screen indigenous alkaline, thermostable xylanase producing bacterial strains from the rich soil of Bhilai – Durg region of Chhattisgarh and also to evaluate the effects of various physical and cultural characteristics on xylanase production by the most potent isolate.

Materials and Methods

Isolation and Screening: A total of sixteen soil samples from different areas in and around Bhilai – Durg region were collected which included decomposed lignocellulosic rich forest soil, dung, animal feed etc. all of which were suspended in sterile distilled water. Isolation and screening of potent indigenous isolates were done in three distinct stages using wheat bran agar medium for primary screening and xylan agar medium and liquid production medium for secondary and final screenings respectively. In the primary screening, the sample suspensions were serially diluted and 0.1 ml of the highest dilution were spread to enrich xylanase producing organisms on wheat bran agar medium with the following composition in g/l: wheat bran, 5.0; yeast extract, 3.0; peptone, 5.0; NaCl, 5.0 and agar, 20.0, pH-9.0

The bacterial colonies obtained from the first screening procedure were then subjected to secondary screening wherein the cultures were plated on xylan agar medium containing birch wood xylan (0.5 %) from HiMedia which was substituted for wheat bran. Following four days of incubation, the xylanolytic property of the colonies obtained was assessed by Congo red assay method which was done by flooding the plates with 1.0 % (w/v) Congo red for 15 minutes (Kumar et al., 2010) followed by destaining with 1M NaCl. The colonies showing clear zone with a minimum diameter of 1cm around them were selected and maintained in nutrient broth. The final screening was performed in liquid production medium (Anuradha et al., 2007) containing (g/l): xylan, 5.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 1.0 and $MgSO_4.7H_2O$, 0.2. 10% (v/v) fresh 18 hour old culture was used to inoculate 50 ml medium in 250 ml Erlenmeyer flask. The inoculated flasks were incubated for 96 hours on a rotary shaker (200 rpm) at 45 ºC ± 2 ºC.

Crude enzyme extraction: The selected isolates were inoculated individually into production medium already mentioned. After proper incubation period, cells were separated by centrifugation at 10,000 rpm for 10 minutes at 4ºC (REMI Cooling

Centrifuge). The supernatant obtained served as the source of extracellular crude enzyme (Subramaniyan et al., 1997).

Xylanase assay: 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 1.8 ml of 1% birch wood xylan in 50mM Glycine – NaOH buffer (pH 9.0) and 0.2 ml of crude enzyme and incubating at 55 ^{o}C for 10 minutes. The reaction was terminated by adding 3 ml 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 estimated by colorimeter at 540 nm against blank. All the experiments were set up in triplicates and the results are the mean of the three sets. One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose units per minute under the specified assay conditions.

Protein estimation: The method of Lowry et al. (1951) was used to estimate the protein concentration using bovine serum albumin as the standard.

Cellulase assay: The cellulase activity was determined by measuring the amount of reduced sugars released from 1 % (w/v) CMC as substrate under same conditions described as for xylanase assay.

Optimization of factors affecting enzyme production: Various factors affecting the xylanase enzyme production by the selected isolate were evaluated. The effect of carbon sources on enzyme production was studied by replacing xylan in the production medium with different synthetic and natural agriculture based carbon sources (1 %w/v). The ability of different organic and inorganic nitrogen sources (0.5% w/v) to induce optimum xylanase production was also studied. The effect of pH and temperature on enzyme production was evaluated by adjusting the pH of the production medium using different buffers and by incubating the inoculated flasks at various temperatures. All the optimization studies were carried out by cultivating the selected bacterial isolate in 100 ml Erlenmeyer flask containing 20 ml of production medium and 10% w/v inoculum followed by incubation at conditions already specified. Enzyme activity was determined under specified reaction conditions.

Results and Discussion

Screening of potent xylanase producers

The primary screening of sixteen different soil samples in and around Bhilai - Durg region produced 65 bacterial isolates by enrichment method by plating on wheat bran agar medium (Table-1). To screen only xylanase producing organisms, secondary screening was performed to eliminate non xylanase producers as wheat bran, in addition to hemicelluloses also contains starch, protein and cellulose (Subramaniyan et al., 1997). Screening results prove that soil supports a number of diverse organisms as it is a rich resource of nutrients.

Secondary screening by Congo red plate assay method was performed using xylan agar medium containing xylan as the sole carbon source. Out of sixty five, 24 isolates were detected to be xylanolytic in nature by the clear zone around them and among these 10 isolates which showed zone diameter greater than 1.0 cm were selected for further screening In the final screening, the 10 isolates were evaluated by measuring the enzyme activity and the bacterial strain; ISL

3 showed maximum activity (23.33 IU/ml). The summary of the results also show that ISL 3 produces considerably low cellulase activity which is significant for industrial use (Table-2).

Preliminary morphological characterization showed that the isolate was gram positive, rod shaped, aerobic and endospore former. There are reports on isolation of xylanase producing organisms from soil (Poorna, 2011; Porsuk et al., 2013).

Optimization of factors affecting enzyme production

Effect of carbon sources: The essential element for microorganisms during growth and metabolism is carbon. Therefore the ability of different synthetic and natural agriculture based carbon sources to stimulate maximum xylanase production by the selected strain was studied. The results depicted in figure - 2 indicate that the inexpensive and abundantly available substrate wheat bran resulted in good yield of xylanase followed by xylose. Enzyme production was negligible on sugars like glucose, mannose and sucrose. However a combination of wheat bran and xylan supported maximum xylanase production

Wheat bran serves as a good source of carbon as determined by the enzyme activity. It may be due the fact that it contains sufficient nutrients with 40% xylan which is a substrate for xylanase (Thiago and Kellaway, 1982). The enhancement of xylanase production by xylan in the presence of wheat bran might be an indication that xylan would be acting as an inducer. These results can be attributed to gene expression and that xylanase production takes place in the presence of inducer suggests that xylanase could be an inducible enzyme (Parachin et al., 2009; Hiremath and Patil, 2011). These results prove that agriculture based wastes can serve as inexpensive and reliable carbon sources for xylanase production.

Effect of nitrogen sources: Various nitrogen sources including both organic and inorganic were tested to determine the best nitrogen source supporting maximum xylanase production. Nine different nitrogen sources were examined of which highest xylanase production was observed in medium containing yeast extract and peptone (0.5 % w/v each). Different enzyme activities were obtained with yeast extract (2.33 U/ml) and peptone (10.9 U/ml) when added separately but when combined produced maximum activity (Figure 3). This may be an indication that both these sources might be complementing each other. Moreover yeast extract has been reported to play an important role in xylanase production because of presence of essential elements and growth factors necessary for the growth of organisms (Porsuk *et a*l., 2013). Peptone, like yeast extract is a complex organic nitrogen source which might be stimulating growth by releasing NH_4^+ and improving the expression of nitrogen assimilating enzymes (Wang et al., 2009).

Effect of temperature/ pH: Each organism has its own pH range for growth and activity with an optimum value in this range. One of the factors which play a crucial role in transportation of nutrients across the membrane and the functioning of enzyme systems with in an organism is pH (Gupta and Kar, 2008). The influence of pH on enzyme production as depicted in Figure – 4 indicates that the optimum pH for maximum xylanase production by the selected strain was found to be 9.0, although significant amounts of xylanase was produced at pH 10

also. 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).

The effect of incubation temperatures on xylanase enzyme production was evaluated and the results (Figure 5) show that xylanase was produced at temperatures ranging from 20ºC to 80ºC but maximum enzyme production by the isolated strain was found to be at 50ºC and a drastic decrease in xylanase production was observed at temperatures lower than and higher than the optimum temperature.

Table.1 Primary Screening of xylanase producers

Sample type	**No. of bacterial isolates**
Soil	33
Cow feed	10
Dung	15
Rotting Wood	07

Table.2 Screening results of 10 potent isolates

Isolate No.	Zone Diameter (cm)	xylanase activity(IU/ml)	Cellulase activity (IU/ml)
ISL 3	2.1	23.33	0.053
ISL 5	1.3	3.0	1.33
ISL 6	1.7	13.66	0.082
ISL 17	1.8	14.0	3.33
ISL 19	2.0	17.3	2.0
ISL 36	1.4	2.66	4.33
ISL 43	1.5	4.50	2.23
ISL 52	1.6	3.22	4.33
ISL 56	1.8	5.0	1.52
ISL 58	1.8	21.0	0.15

Figure.1 Strain ISL 3 showing Clear zone in Congo red plate assay method

Figure.2 Effect of carbon sources on xylanase production

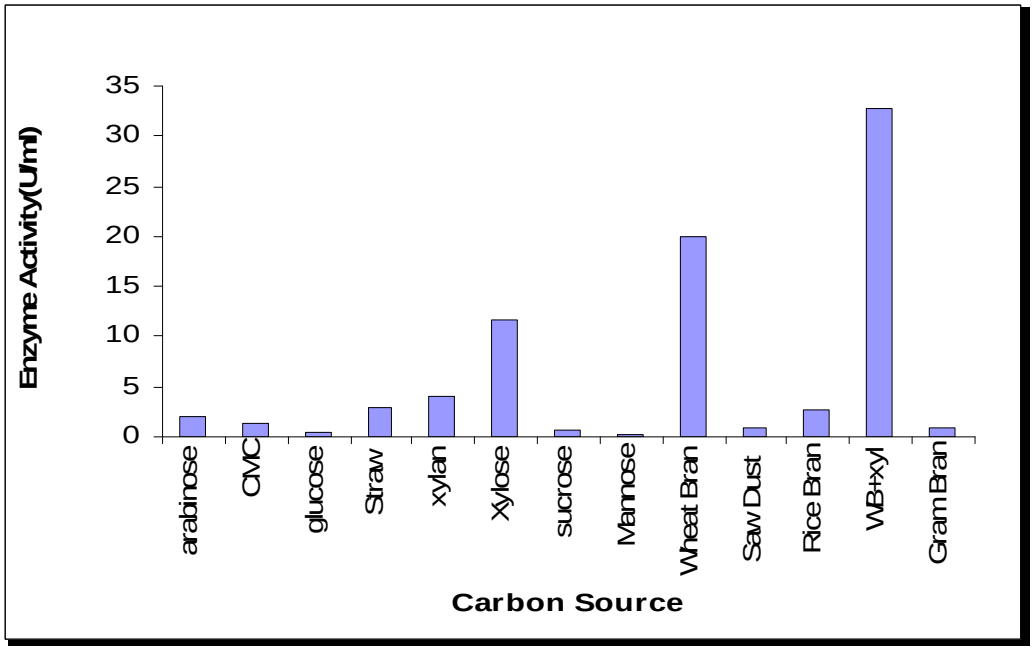


Figure.3 Effect of nitrogen sources on xylanase production

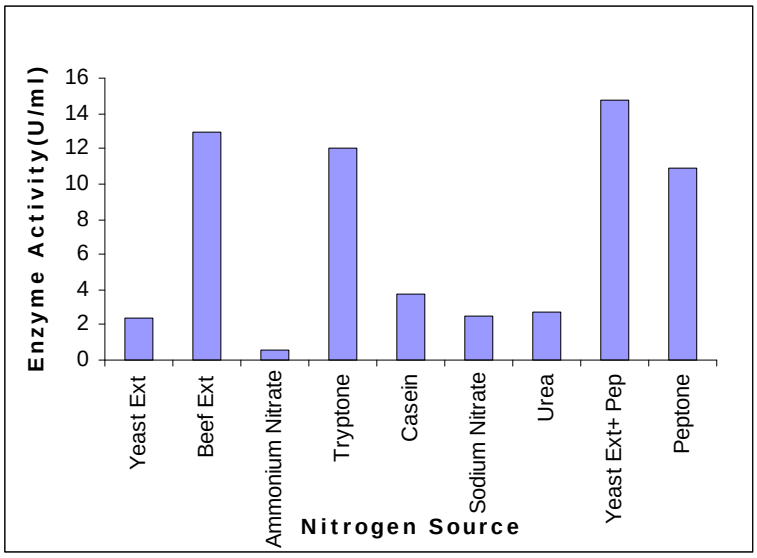


Figure- 4: Effect of Temperature

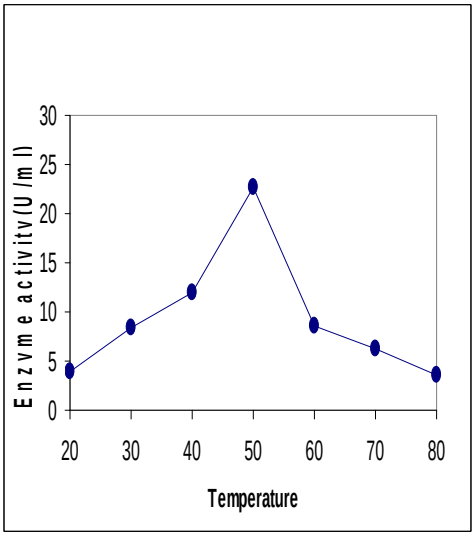
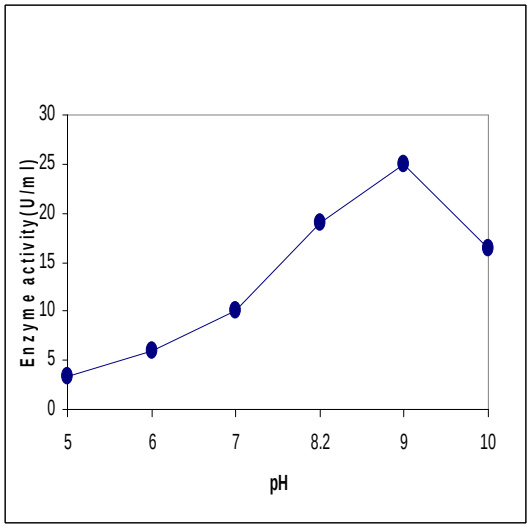


Figure- 5: Effect of pH



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