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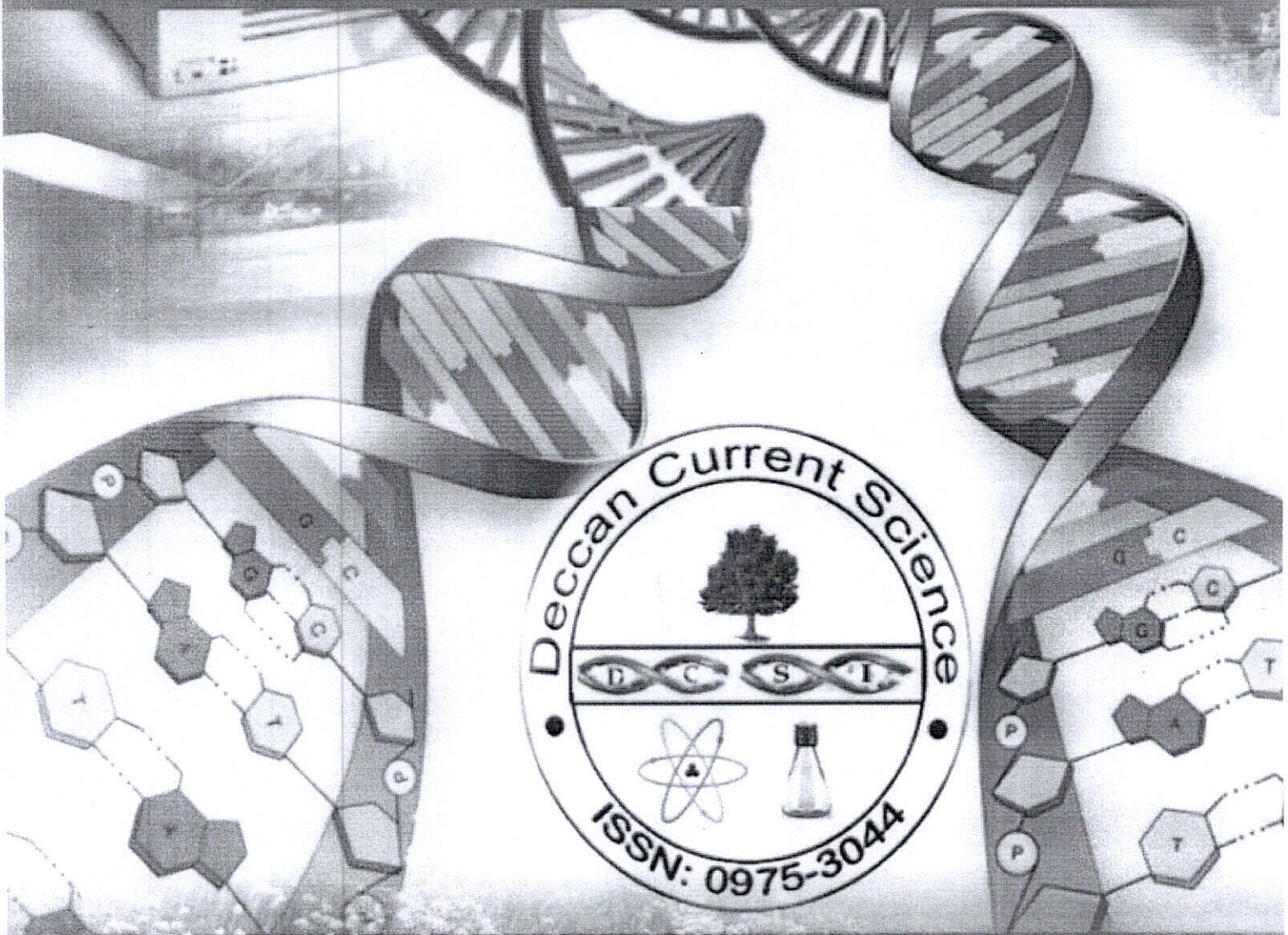

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Isolation and Optimization of Xylanase Production from Newly Isolated *Bacillus* sp.

V. Shanthi* and M G. Roymon
Department of Microbiology & Biotechnology, St. Thomas College, Bhilai (Chhattisgarh)
*Corresponding author: shanti_162@rediffmail.com

Abstract:
Xylanase is an industrially important enzyme which serves multipurpose uses in food and other industries. The objective of the present study was to isolate an indigenous and potential xylanase producing bacteria from soil of Bhilai-Durg region. The isolation and screening was followed up in three stages and on the basis of clear zone formation on xylan agar medium and enzyme activities in liquid xylan medium, a potent xylanase producing bacterial strain was isolated. The isolate was identified as *Bacillus sp.* on the basis of morphological, biochemical and molecular characterization. Experimental reports reveal that the isolate is thermotolerant and alkali stable xylanase producer. Optimization studies of various nutritional parameters for optimum xylanase production were carried out. It was observed that the novel isolate showed highest activities with wheat bran and yeast extract as carbon and nitrogen source respectively. Xylan proved to be an efficient supplement for wheat bran in enhancing xylanase production. These results conclude that *Bacillus sp.* is a novel and unique thermoalkalophilic xylanase producing bacterial isolate capable of enhanced xylanase production which makes it suitable for use at industrial scale.

Keywords: Xylanase, *Bacillus sp.*, Alkalophilic, thermotolerant, optimization

Introduction:

Hemicellulose is the second most abundant polymer present in nature after cellulose. Hemicellulose consists of xylan as the major component. Xylan is a heteropolymer with the backbone structure consisting of β-1,4 linked D-xylose units. The branching pattern varies with the plant species mainly consisting of arabinose, glucuronic acid, mannose or acetyl residues (Virupakshi *et al.*, 2005). Xylan is attacked by a class of hydrolytic enzyme; Xylanases (EC 3.2.1.8) which randomly hydrolyze the backbone to produce xylooligosaccharides of different lengths (Vikarii *et al.*, 2007). This ability of xylanase to act on xylan renders it to be used as a potential biotechnological tool for using the abundantly present xylan in nature as biomass. Although xylanases are produced by plants, insects and microorganisms (Anuradha *et al.*, 2007) but microbial xylanases have found a unique position for industrial purposes. Among

the various microorganisms which produce xylanases, fungal and bacterial sources are predominantly used for industrial applications (Khandeparkar and Bhosle, 2006). Considering the fact that fungal xylanases are associated with a plethora of problems, the present study aims at isolation and optimization of xylanase produced from newly isolated *Bacillus sp.* from soil.

Materials and methods:

Isolation of xylanolytic bacteria

Several soil samples were collected from different places of Bhilai- Durg region of Chhattisgarh state and were suspended in sterile distilled water followed by serial dilution and the highest dilutions were plated out for screening of xylanolytic bacteria.

Screening

Screening of xylanolytic bacteria was done in three stages to give emphasis on isolation of the most potent bacteria. The primary screening was done on the medium containing hemicellulose rich wheat bran agar medium with the following composition in g/L: Wheat Bran (50.0), Peptone (5.0), Yeast extract (3.0), NaCl (5.0), Agar (15.0), pH 9.0. 0.1 ml of the highest serially diluted soil samples were spread plated on wheat bran agar medium and incubated for 2-3 days at 50ºC. Unless and otherwise specified the sterilization steps were performed at 121ºC or 15 lb/inch^2 for 20 minutes. Well isolated bacterial colonies on wheat bran agar plates were selected and subjected to Secondary screening by spot inoculation on xylan agar medium (Nakamura *et al.*, 1983) where Wheat bran in preliminary screening procedure was replaced with Birch wood xylan as carbon source. The plates after incubation were subjected to qualitative screening for by Congo red plate assay method in which the plates were flooded with 1% Congo red solution for 10-15 minutes followed by destaining with 1M NaCl solution for the visibility of clear zone around the colony. Bacterial isolates showing a clear zone of at least 1 cm were selected for tertiary screening. The final screening procedure involved the inoculation of isolates into liquid xylan medium for quantitative screening of potent xylanase producing bacteria.

Xylanase production

The bacterial isolates selected through screening procedures were used for production of xylanase enzyme. Cell free extract was obtained by centrifugation at 10,000 rpm for 15 minutes at 4ºC and was used as a source of crude extracellular enzyme.

Xylanase Assay

Xylanase assay with some modifications was performed following the method of Bailey *et al.* (1992). The reaction mixture prepared by mixing 1.8 ml pre incubated substrate (0.5% xylan) and 0.2 ml crude enzyme was incubated at 55ºC for 10 minutes. The reaction was terminated by addition of 3.0 ml of 3, 5 dinitrosallicylic acid DNS and the concentration of reducing sugars released was quantified at 540nm against blank using xylose standard. One unit of xylanase activity was defined as 1µmole of xylose liberated per minute per ml of enzyme preparation under standard assay conditions.

Total soluble protein estimation

Total soluble protein was estimated following Lowry's method (Lowry *et al.* 1951) using Bovine serum albumin as standard.

Cellulase Assay

The cellulase assay was performed by determining the amount of reducing sugars liberated from 1% Carboxy methyl cellulose CMC prepared in 0.2 M sodium phosphate buffer (pH 7.0). The reaction mixture consisted of 0.5 ml of crude and 1.0 ml of substrate followed by incubation at 50□C for 15 minutes. 3.0 ml DNS was added to stop the reaction and the liberated reducing sugars were estimated.

Enzyme activity was calculated using glucose standard and expressed as µmoles of glucose released per ml per minute of crude under standard reaction conditions.

Optiization of xylanase production

The potent bacterial isolate obtained from three stages of screening process was subjected to optimization of various physical and nutritional factors for maximum xylanase production. Xylanase production was studied at different pH (7.0-12.0), and temperatures (40□C-80□C). The effect of shaking and agitation speed (50-250 rpm) was studied. The effect of various carbon and nitrogen sources was studied by substituting xylan in the basal medium with different simple and complex forms of carbon. Different organic and inorganic forms of nitrogen were substituted for peptone and yeast extract in the basal medium. Various supplements were added to the medium to study their effect on enhancing the xylanase production in association with the optimized carbon source. Effect of various metal ions on xylanase production was assessed by estimating the enzyme activity of the crude obtained after appropriate incubation.

Results and discussion:

Isolation and screening

A total of 27 soil samples were collected for isolation and screening purpose which produced 68 bacterial isolates on xylan agar medium (secondary screening medium) indicating their potential to hydrolyze xylan and thus proved to be xylanolytic in nature. Further screening of these isolates by Congo red plate assay method produced 24 isolates. This screening process helped to weed out those isolates which produced negligible amounts of xylanase. The final screening based on xylanolytic and cellulolytic activities, the isolate designated as ISL-58 produced the highest amount of xylanase with negligible amounts of cellulase. The results indicate the ability of soil to render a rich ecological habitat to several diverse microorganisms including xylan degrading microbes. This ability of soil to encourage the growth and survival of xylanase producing organisms was supported by Cordeiro *et al.* (2002) who successfully isolated a thermophillic bacterial isolate from local soil. Also Roy and Habib (2009) isolated and screened out *Bacillus cereus* from soil. The potent bacterial isolate was identified and the identification included morphological, phenotypic and biochemical characterization according to Bergey's Manual of Systematic Bacteriology. The isolate after molecular characterization was suggested to belong to *Bacillus sp.*

Optimization

Both physical and nutritional parameters were studied for their ability to produce maximum xylanase production. Xylanase production was studied after adjusting the initial medium pH at different pH values ranging from 7-12. Detectable amounts of xylanase activity were seen at all medium pH evaluated (Figure 1a), however, maximum titers of xylanase production were observed at pH 9.0 and the lowest activity was observed at pH 12.0. It is clear that medium pH affects the production, stability and activity of extracellular enzymes. These results are in accordance with that of *Staphylococcus* sp. (Gupta *et al.*, 2001) and *Enterobacter* sp. MTCC 5112 (Khandeparkar and Bhosle, 2006) which have been reported to produce maximum xylanases at pH 9.0. The effect of temperature on xylanase production by newly isolated *Bacillus sp.* was evaluated at varying temperatures in the range of 40°C-80°C with 5°C increment. The data obtained (Figure 1b) revealed that xylanase enzyme was produced in the temperature range of 40°C to 70°C. Elevated xylanase activity was recorded at 50°C. The results are supported by those of

Subramaniam and Prema, (2000) who reported maximum xylanase production by *Bacillus sp.* at 50°C. However, Literature survey reveals that varying optimum temperatures for xylanase production by various bacterial isolates were obtained; 30°C for *Bacillus circulans* (Ratto *et al.*, 1992), 37°C for *Bacillus sp.* (Nagar *et al.*, 2012). It can be argued that fermentation temperature largely mediates extracellular enzyme production in microorganisms (Smits *et al.*, 1998).

The influence of shaking of production medium on yields of xylanase enzyme by *Bacillus sp.* was investigated by incubating the inoculated cultures at different rotating speeds (Figure 1c). The enzyme yields were compared with those kept at static condition. Results indicate that shaking of media indeed caused an increase in enzyme production. Shaking conditions proved beneficial for xylanase production by *Bacillus sp.* as manifested by the increased xylanase titers when compared to static conditions. The results are convincing pertaining to the fact that shaking causes uniform mixing and balanced mass transfer reactions during the fermentation process (Purwanto *et al.*, 2009). The optimum agitation speed was found to be 150 rpm. In contrast to these results, maximum xylanase production was recorded at 200 rpm by *Bacillus subtilis* ASH (Sanghi *et al.*, 2009) and *Bacillus pumilus* VLK-1 (Kumar *et al.*, 2014).

Carbon is an essential element for growth and metabolism of all living organisms. So, optimization of carbon source is critical for any process development. Among the different carbon sources evaluated for maximum xylanase production, wheat bran was found to be the best and optimum carbon source (Figure 2). Similar findings by Yang *et al.* (1995), Sa-Pereria *et al.*, (2002) indicate that suitability of inexpensive wheat bran as optimum carbon source may be due to the presence of 20-30 % xylan.

The influence of nitrogen sources on xylanase production by *Bacillus sp.* was investigated and the results indicate that yeast extract caused maximum xylanase yield (Figure 3). Among the inorganic nitrogen sources investigated, ammonium nitrate comparatively was better in producing significant levels of xylanase. This finding is in accordance with those of Dhillon and Khanna, (2000) and Subramaniyan *et al.*, (2001) who reported highest levels of xylanase yields by *Bacillus sp.* B16 and *Bacillus circulans* AB16 respectively. The ability of yeast extract to render maximum xylanase production can be attributed to its rich micronutrient composition.

The effect of supplements in inducing maximum xylanase production revealed the ability of xylan in optimum xylanase production. The ability of xylan to act as an additive to wheat bran in inducing maximum xylanase production may be because xylan might be acting as an inducer for xylanase production which might be increasing the production manifold later. In contrast to these results, findings of Giridhar and Chandra, (2010) and Kumar *et al.*, (2014) have revealed Tween 80 was the best additive for *Bacillus pumilus* VLK-1

Conclusions:

A newly isolated potent, high yielding xylanolytic bacteria was sucessfully isolated through three screening stages from forest soils of Bhilai-Durg region and was identified to belong to *Bacillus sp.* The optimum pH and temperature for optimum xylanase production was found to be 9.0 and 50C respectively. Shaking conditions induced high xylanase titers than static conditions. Shaking speed of 150rpm was found to be effective in inducing optimum xylanase levels. Wheat bran and yeast extract were the best carbon and

nitrogen source respectively for Bacillus sp. Tween 80 proved to be an effective supplement for maximum xylanase production. It can be thus concluded that the new isolate is considerably alkalophilic and thermotolerant and also its unique ability to use inexpensive, agricultural waste; wheat bran as carbon source enables it to be exploited for various industrial applications.

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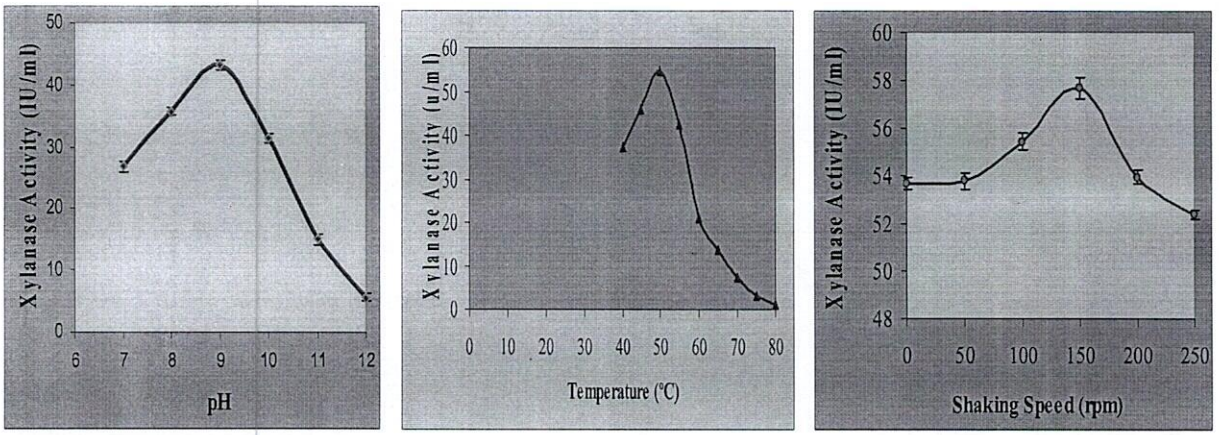
Figure 1: Effect of physical parameters on xylanase production



a) Effect of pH b) Effect of Temperature c) Effect of shaking

Figure 2: Effect of carbon source on xylanase production

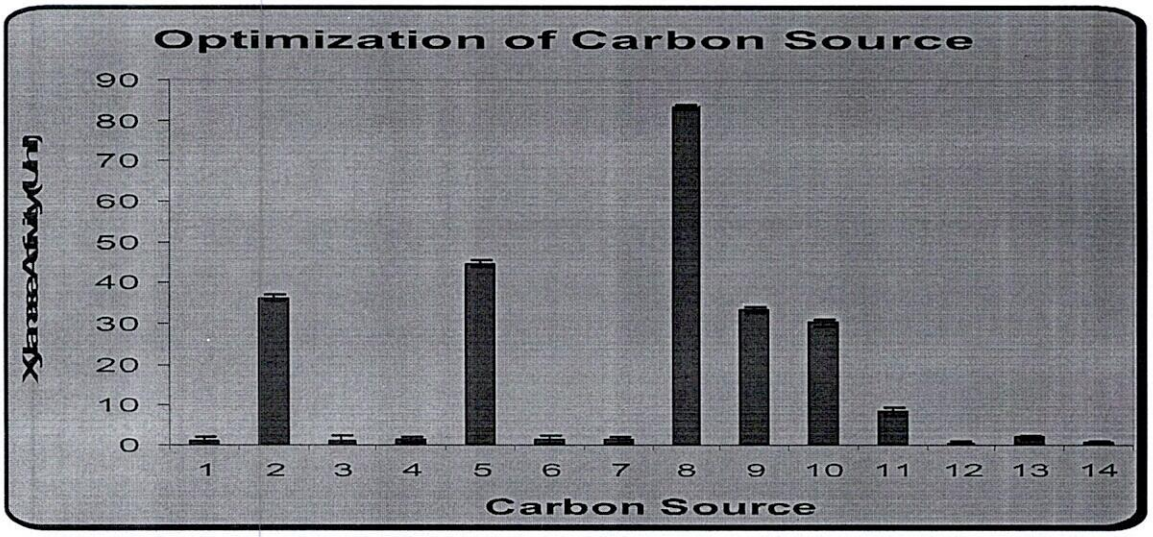


Figure 3: Effect of Nitrogen source

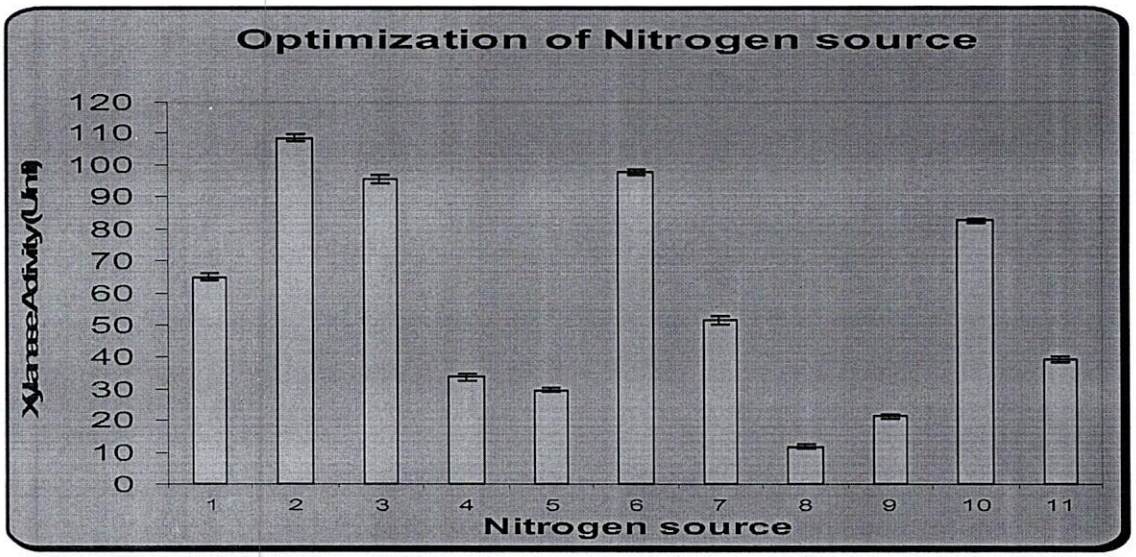


Figure 4: Effect of supplements