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OPTIMIZATION OF ALKALI-THERMOSTABLE AND CELLULASE-FREE XYLANASE PRODUCTION FROM *BACILLUS* SP.

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Abstract

Context: To analyze the nutritional and physicochemical parameters for the production of alkali-thermostable and cellulase free xylanase from bacteria.

Objectives: The aim of this study was to isolation and identification and of alkali-thermostable and cellulase free xylanase producing bacteria from soil as well as optimization of process parameters for xylanase production.

Materials and Methods: The bacterium *Bacillus* sp. was isolated from soil by serial dilution technique on xylan agar medium and identified by morphological and biochemical studies. The production of xylanase was carried out on xylan broth medium and xylanase activity was assayed by dinitrosalicylic acid (DNS) method. The effect of cultural parameters on the production of xylanase was determined by measuring the activity of xylanase. The effect of temperature and pH on the activity of partially purified xylanase as well as substrate specificity of xylanase were examined.

Results: The maximum xylanase production (4000 U/L) by a *Bacillus* sp. was attained when the medium containing 0.5% wheat bran xylan and peptone at pH 8.0 and 50-55°C within 48-60 h. The partially purified xylanase was optimally active at pH 9.0 and 55°C. The xylanase showed high substrate activity towards wheat bran xylan but no activity towards cellulose, carboxymethyl cellulose and starch. Thus the enzyme was alkali-thermostable and cellulase free xylanase.

Conclusion: The results obtained in this study suggest that the *Bacillus* sp. used is highly potential and useful for the production of cellulase free xylanase.

Key words: Alkali-thermostable, cellulase-free xylanase, optimization, *Bacillus* sp.

Introduction

Xylanase, an extracellular enzyme, is the key enzyme for the breakdown of xylan. Xylan, the major hemicellulose component in plant cell walls, is a polysaccharide composed of β-1, 4-linked xylo-pyranose units (Saha 2003). Xylanase degrades xylan to short chain xylooligo-saccharides of varying lengths (Krengel *et al.*1996). To date xylanases have increased their importance due to their potential applications in industrial processes such as in biomass fuel, bio bleaching, wine industry, improving animal feed and production of ethanol (Tucker *et al.* 1989, Xu *et al.* 2000, Nunez *et al.* 2001, Sudha *et al.* 2003, Moers *et al.* 2003), modification of cereal-based food stuffs (Tuohy *et al.* 1989), improving the digestibility of animal feed stocks (Yin *et al.* 2001), bioconversion of lingo cellulosic material and agro-wastes to fermentable products (Prema 2005) and pre-bleaching of paper pulps (Gubitz *et al.* 1997, Viikkari *et al.* 1986). These uses have increased a great demand for xylanase production (Bocchini *et al.* 2003). Haltrich *et al* (1996) gave an overview of fungal xylanase and showed that the enzyme can be produced by a number of microorganisms including bacteria, yeast and filamentous fungi.

Chlorinated organic compounds produced from chemical bleaching technologies are harmful to the environment and need to be substituted by eco-friendly procedures. Xylanases cleave and solubilize re-precipitated xylan and lignin located on the surface of the micro fibrils. This facilitates pulp bleaching and lowers chlorine consumption thereby reducing the discharge of toxic-organochlorine compounds in the environment (Senior *et al.* 1992, Tolan and Canovas 1992). Pre-bleaching of paper pulp also requires the

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use of cellulase free xylanases, since cellulases may adversely affect the quality of the paper pulp by destroying the structure of cellulose and thus diminish the quality of the pulp (Techapun *et al.* 2003). For the development of suitable xylanase as a pre-bleaching agent, the stability of enzyme at higher optimum pH and temperature is desirable (Beg *et al.* 2000). The major current application of xylanases is in the pulp and paper industries where the high temperature and alkaline pH of the pulp substrate requires thermo-alkaliphilic enzymes for efficient biobleaching (Kulkarni *et al.* 1999). A thermostable xylanase would also be beneficial in animal's feeds if added to the feeds before the pelleting process. To search commercial feasibility, enzyme production must be increased by introducing a more potent strain and by optimizing nutritional and cultural conditions such as carbon and nitrogen sources, time course, incubation period and initial pH. So the main aim of the present investigation reported here was to optimize the production of an alkali-thermostable and cellulase free endo-xylanase by an extreme thermophile *Bacillus* sp.

Materials and Methods

Isolation and identification of Bacillus sp.: For the isolation of the bacterium *Bacillus* sp. the waste soil samples were collected using pre-sterilized sample bottles and sterile spatula from different areas of sugar-cane industry. The soil samples were diluted by serial dilution and cultured on xylan agar media. The isolate producing clear transparent zone around the colony on xylan agar plate was selected as xylanolytic bacteria and purified by sub cultured. The identification of *Bacillus* sp. was confirmed on the basis of morphological and biochemical characteristics using the criteria of Bergey's Manual of Systematic Bacteriology (Holt *et al.* 1993). The culture was maintained on wheat bran xylan agar slants by weekly transfers onto fresh slants and was stored at 4^{o}C in refrigerator.

Xylanase production and assay: The production of xylanase was carried out using *Bacillus* sp. cultured in 250 ml Erlenmeyer flasks containing 100 ml of medium [0.5% wheat bran xylan, 0.5% yeast extract, 1% peptone, 0.5% NaCl] at 50◦C for 2 days in an incubator on a rotary shaker. After this period of time the culture was centrifuged at 8000 rpm for 15 min at 4◦C and the cell free supernatant was used as the crude enzyme preparation. Xylanase was assayed by DNS method (Miller 1959) using wheat bran xylan (1%) as substrate and phosphate buffer at pH 7 and 50◦C. One unit of xylanase is defined as the concentration of enzyme that catalyzes the formation of 1 μmole of product (xylose) $ml^{-1}min^{-1}$ under the assay conditions. The concentration of enzyme in crude biological preparation is expressed as unit/ml (U/ml) or unit/litter (U/L). Cellulase was assayed according to Mandels and Sternburg (1976) using sodium citrate buffer. One unit of cellulase is defined as the amount of the enzyme that liberates 1 μmol reducing sugar as glucose ml^{-1} min^{-1} under the assay conditions. The soluble protein content in the crude extract was determined according to Lowry *et al* (1951) using bovine serum albumin as the standard.

Optimization of cultural parameters: In order to get xylanase, the bacterium was grown in the liquid cultural medium. Various physical and nutritional parameters for xylanase production were optimized by maintaining all factors at a constant level except the one being studied. The parameters studied in our laboratory were pH, temperature, carbon and nitrogen sources, % of xylan, incubation period and metal salts. The effect of pH on xylanase production was assessed by cultivating the strain *Bacillus* sp. in media of varied pH ranging from 5.0-11.0. The influence of temperature on xylanase production was studied by cultivating the strain *Bacillus* sp. at different temperatures such as 30◦C, 40◦C, 50◦C, 60◦C and 65◦C. Xylanase production by *Bacillus* sp. was studied by cultivating the strain in a set of media having different carbon sources such as wheat bran xylan, birch wood xylan, oat spelt xylan, rice bran xylan, glucose, xylose, lactose, cellobiose, cellulose, carboxymethyl cellulose and starch. Xylanase production from *Bacillus* sp. was also examined in a set of media containing wheat bran xylan with various percentages (0.5%, 1.0%, 2.0%, 3.0% and 4.0%).

To select the best nitrogen sources for xylanase production by *Bacillus* sp., both organic and inorganic nitrogen sources were incorporated separately into the production media. Different nitrogen sources used in

media were peptone, yeast extract, tryptone, casein, urea, asparagine, ammonium phosphate and potassium nitrate. Incubation period is also one important factor for the production of enzyme in highest amount. In this case the strain *Bacillus* sp. was cultivated with various incubation periods such as 24 h, 36 h, 48 h, 60 h, 72 h and 96 h. A variety of metal salts that have different effect on the production of enzyme from microorganism due to their stimulatory and inhibitory effect on xylanase production was assessed by cultivating the strain in media keeping all other constituents constant except salt.

Effect of pH and temperature on xylanase activity: The xylanase partially purified from *Bacillus* sp by ammonium sulfate saturation and heat treatment was assessed for its activity in different pH and temperature. The effect of pH on xylanase activity over wheat bran xylan was studied in 50mM phosphate buffer with various desired pH ranging from 5.0-11.0. The effect of temperature on xylanase activity was examined by incubating the reaction mixtures at the desired temperatures ranging from 30-80◦C.

Substrate specificity of xylanase: To determine the substrate specificity of xylanase towards various substrates including wheat bran xylan, rice bran xylan, oat spelt xylan, birch wood xylan, cellulose, carboxymethyl cellulose and starch were used as substrate. The activity of xylanase towards substrates was determined by DNS method..

Results

The strain *Bacillus* sp. isolated from soil was identified according to the Bergey's Manual of Systematic Bacteriology. The isolate was an aerobic, endospore forming, gram positive, rod-shaped bacterium producing a clear zone on xylan agar plate.

In our laboratory, the influence of various cultural factors on the production of xylanase was studied. The production of xylanase from *Bacillus* sp. was better in alkaline pH (Fig. 1a) but the highest (3800 U/L) being observed at pH 8.0 and declined sharply at pH values on either side of this optimum pH. The production of xylanase by *Bacillus* sp was excellent at a temperature rang from 30-65°C, but the optimum temperature for the production of xylanase (3800 U/L) was found to be 50-55°C (Fig. 1b). The carbon sources in the media exerted a profound effect on the enzyme production behavior of the bacterium. Some carbon sources supported good growth with low enzyme production, while others supported good growth as well as enzyme secretion. In the case of *Bacillus* sp.*,* wheat bran xylan (1%) supported a high enzyme secretion (Fig. 2), followed by oat spelt xylan, rice bran xylan and birch wood xylan; but mono- and di- saccharides failed to support high xylanase titres. The production of xylanase at different percentage of wheat bran xylan was assessed and it was observed that the medium containing 0.5 % of wheat bran xylan was suitable for maximum xylanase production (4000 U/L) by *Bacillus* sp. (Fig. 3). The best nitrogen source for xylanase production by *Bacillus* sp. was peptone, followed by tryptone and yeast extract, conversely xylanase production was not supported by ammonium nitrate, potassium nitrate, asparagine and casein (Fig. 4). The production of xylanase was found to be (3400 U/L) when peptone was used as nitrogen source but in case of tryptone and yeast extract the enzyme activity were (3100 U/L) and (2800 U/L) respectively. The time course of xylanase production from *Bacillus* sp. was investigated and maximum production (4000 U/L) was observed between 48-60 hours (Fig. 5). Further incubation after this period did not show any increment in the level of enzyme production. Metal salts had a profound effect on the production of xylanase. Among the different metal salts (2mM) tested, Ca^{2+} and Mg^{2+} enhanced xylanase production from *Bacillus* sp. but Cu^{2+}, Cd^{2+} and Hg^{2+} strongly inhibited xylanase production (Fig. 6).

The effect of pH on xylanase activity was assessed at various pH values ranging from 5.0-11. The xylanase of the *Bacillus* sp. exhibited good activity around the pH range 7-10, but the optimum pH of the xylanase activity was 9.0 (Fig. 7a). The effect of temperature on the activity of xylanase obtained from *Bacillus* sp. was examined in the temperature range of 30-80°C. The xylanase showed best activity around 50 - 60°C with

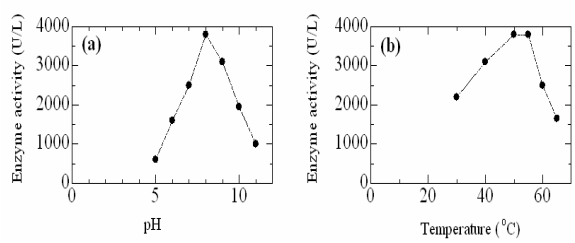

Fig. 1. Effect of pH (a) and temperature (b) on xylanase production by *Bacillus* sp.

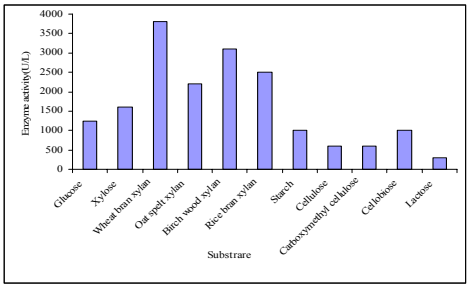

Fig. 2. Effect of different carbon sources on xylanase production by *Bacillus* sp.

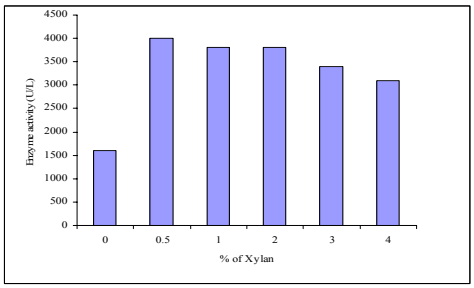

Fig. 3. Effect of % of xylan on xylanase production by *Bacillus* sp.

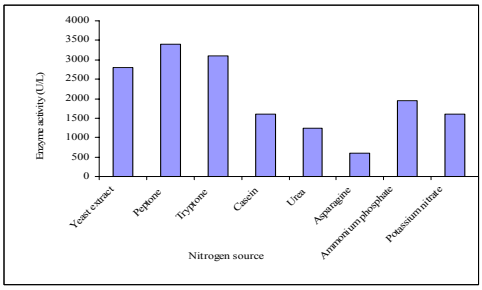

Fig. 4. Effect of various nitrogen sources on xylanase production by *Bacillus* sp.

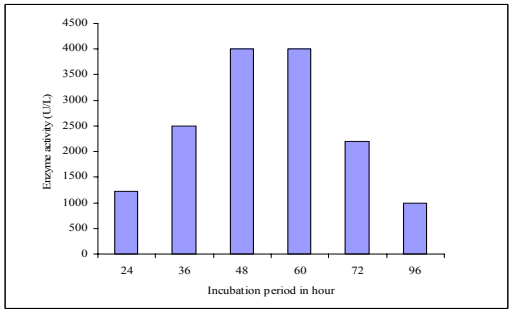

Fig. 5. Effect of incubation period on xylanase production by *Bacillus* sp.

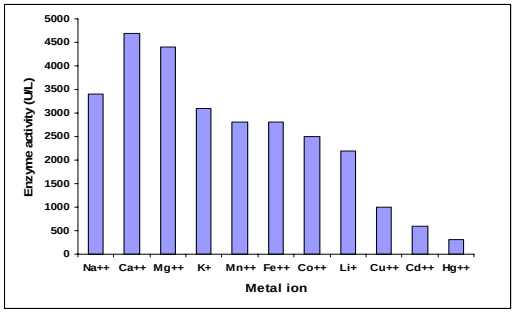

Fig. 6. Effect of different metal ions on xylanase production by *Bacillus* sp.

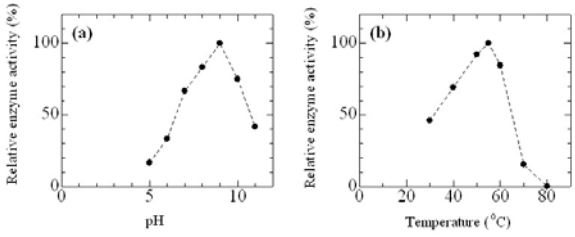

Fig. 7. Effect of pH (a) and temperature (b) on xylanase activity

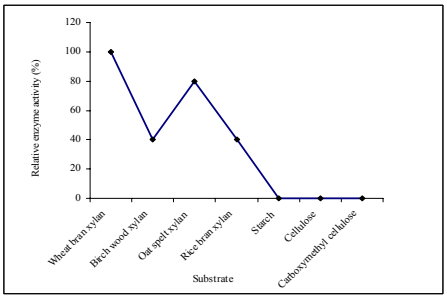

Fig. 8. Substrate specificity of xylanase

optimum activity at 55°C (Fig. 7b). With further rise of the temperature above optimum value, the activity of xylanase gradually decreased and 15 % of the activity was only retained at 70°C but destroyed at 80°C. The substrate specificity of xylanase was studied using various polysaccharides as the substrates. The xylanase of *Bacillus* sp. was able to strongly hydrolyze wheat bran xylan, oat spelt xylan, rice bran xylan and birch wood xylan, but the xylanase showed highest substrate activity towards wheat bran xylan (Fig. 8) and no activity towards cellulose, carboxymethyl cellulose and starch, and it was a true xylanase.

Discussion

Xylanases are widely used enzymes in paper and pulp industries as well as in food, animal feed and pharmaceutical industries. Members of the genus *Bacillus* produce a great variety of extracellular enzymes, of which xylanases are of significant industrial importance. In general cellulase free xylanases produced by thermophilic microorganisms are usually more thermostable than their mesophilic counterparts (Techapun *et al.* 2003) and the cellulase free xylanases have also been reported from other microbial strains, such as *B. amyloliquefaciens* (Breccia *et al.*1998) and *B. licheniformis* A99 (Arehana and Satyanarayana 1998), but most are not alkali stable. The xylanase of *Bacillus* sp. studied in our laboratory was cellulase free and active at elevated temperatures and in an alkaline pH environment.

The cultural conditions were found to have profound influence on xylanase production. The initial pH of the medium has been reported to strongly influence many enzymatic systems by affecting the transport of a number of chemical products and enzymes across the cell membrane (Moon and Parulekar 1991). In the present study, the optimum pH for the production of xylanase of *Bacillus* sp. was 8.0. However, the optima for xylanases production from many bacteria was reported in the neutral pH range such as *B. circulans* WL-12 (Esteban *et al.* 1982) and *B. thermoleovorans* strain K-3d (Sunna *et al.*1997). The maximum production of xylanase by *Bacillus* sp was between 50°- 55°C that is similar to the optimum temperature (55°C) recorded for the release of xylanase from *Neocallimastix frontalis* (Mountfort *et al.*1989).

In our laboratory, it was seen that wheat bran xylan supported a high xylanase production from *Bacillus* sp than other substrate. The cost effective hemicellulosic substrates such as corncobs, wheat straw, rice bran, rice straw, corn stalks and sugar cane bagasse have been found to support xylanase production (Kulkarni *et al.* 1999). A similar finding also reported for *Streptomyces* sp. (Vyas *et al.* 1990), *Trichoderma reesei* (Bailey *et al.* 1993) and *Melano carpus albomyces* II-68 (Saraswat and Bisaria 1997). It was found that the xylanase production by *Bacillus* sp became maximum when the medium containing 0.5 % wheat bran xylan. The induction of xylanase by xylan has been reported in various xylanase-producing microorganisms like *Trametes trogii* (Levin and Forschiassin 1998), *Streptomyces* sp. QG-II-3 (Beg *et al.* 2000) and *Humicola lanuginose* (Kamra and Satynarayana 2004). The effect of different nitrogen sources on xylanase production was also examined and it was found that the maximum xylanase production was attained when peptone is used as nitrogen source. A high xylanase titre was obtained from *B. circulans* AB16 (Dhillon *et al.* 2000), when tryptone was used as a nitrogen source, while high xylanase production was observed in yeast extract (Bakri *et al.* 2003, Purkarthofer *et al.*1993). Incubation period is a significant parameter in xylanase production.The time course for highest xylanase production was 48-60 h. This finding was in accordance with the work reported by Irwin and Wilron (1993). Xylanase production was increased in the presence of Ca^{2+} and Mg^{2+} in the medium, while decreased by Cd^{2+} and Hg^{2+} due to their positive and negative modulating ability on xylanase secretion.

In the present study, the optimum pH for xylanase of *Bacillus* sp. was 9.0. However the pH optima for xylanases isolated from many bacteria was reported in the neutral pH range, for example, xylanase of *Dictyoglomus thermolacticum* exhibited optimum activity at pH 7 (Mathrani and Ahring 1992). Xylanase from *Bacillus* sp. was thermostable. The enzyme was stable up to 70°C, but thermally inactivated at 80°C. The

enzyme showed optimum activity at 55°C. Xylanase of *Streptomyces livindas* 1326 was optimally active at 60°C and pH 6 (Morosoli *et al.* 1986). Xylanase that are active at high temperature and pH values are required in the pulp and paper industry (Karlsson *et al*. 1998). Xylanase of *Bacillus* sp. showed the highest substrate activity towards wheat bran xylan, but no activity towards cellulose, carboxymethyl cellulose and starch. Thus it was a true cellulase free xylanase. Xylanase produced by *Aeromonas caviae* ME-1 did not hydrolyze starch, cellulose and carboxymethyl cellulose (Kubata *et al.*1995) and xylanase from *Streptomyces lividans*-1326 showed no activity towards carboxymethylcellulose (Morosoli *et al.* 1986). The cellulase free xylanase will be more applicable and beneficial in pre-bleaching of paper pulp because cellulase may adversely affect the quality of the paper pulp by destroying the structure of cellulose.

Conclusions

From the study it can be concluded that the production of xylanase by *Bacillus* sp. was markedly increased through optimization of process parameters. This observation is interesting due to the low cost of carbon source since wheat bran xylan is a less expensive substrate for xylanase production in commercial scale than oat spelt xylan. The maximum production of xylanase was achieved after 48 h of inoculation at pH 8.0 and 50-55°C. The xylanase of *Bacillus* sp. was optimally active at pH 9.0 and 55°C as well as showed high substrate activity to wheat bran xylan, but no activity towards cellulose, carboxymethyl cellulose and starch. Thus the xylanase of *Bacillus* sp. was alkali-thermostable and cellulase free, that will be significantly useful in pre-bleaching pulp in the paper industry and improving nutritive value of animal feed.

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