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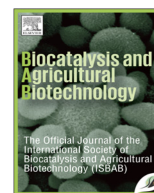

Original Research Paper

Investigation on lignocellulosic saccharification and characterization of haloalkaline solvent tolerant endo-1,4 β-D-xylanase from *Halomonas meridiana* APCMST-KS4

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ABSTRACT

The bacterial strain *Halomonas meridiana* isolated from marine sediment sample was used for the production of xylanase enzyme and it was also purified and characterized. Xylanase was purified to its homogenecity by using ammonium sulphate precipitation and DEAE Sepharose Fast Flow anion exchange chromatography with 17.23% total enzyme recovery and 15.83 fold purification. The molecular weight of the purified xylanase was 41 kDa and it was observed as an alkaline thermophilic. The optimum enzyme activity was found at pH 8 and also at 50 °C temperature. Further this xylanase was stable at wide pH range between 6 and 9 and temperature between 40 and 50 °C. This enzyme was active at 1.5 M NaCl. Metal ions such as manganese sulphate, zinc sulphate, zinc chloride, copper sulphate and magnesium chloride showed the highest influence on enhancing xylanase activity. The xylanase activity was also influenced by surfactants such as Tween 20, Tween 40 and Tween 60 and it was resistant to SDS. The enzyme metallo-xylanase was highly inhibited by EDTA. The other additives like beechwood xylan, birchwood xylan and all the tested organic solvents were positively influenced the enzyme activity when compared to the control. The enzyme registered maximum saccharification rate on pretreated rice bran, wheat straw and *Ulva* respectively.

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1. Introduction

Xylanases are 1,4-β-D-xylan xylanohydrolases which catalysers the hydrolysis of β-1,4 linkage of xylan, which is a major constituent of hemicellulose to release D-xylose as product. Xylanases have wide application in food and paper industry. In food industry, they are used to improve the physical and nutritional value of animal feeds by degrading xylan present in the agricultural wastes (Cheng et al., 2005). In baking industry, they are used to improve the dough properties of grains and cereal by degrading the xylan present in the cell wall materials of plants (Butt et al., 2008). In paper industry, they used to remove the lignin in Kraft pulp bleaching by changing the structure of pulp fibre and this is an eco friendly approach, hence xylanases replace toxic chlorinated compounds used for bleaching (Cheng et al., 2005).

Xylanases can be produced from bacteria, fungi, yeasts and actinomycetes. Among these xylanase from fungi and bacteria are extensively studied. Few examples for xylanase producing fungal species are *Aspergillus niger* DFR-5 (Pal and Khanum, 2011), *Penicillium sclerotiorum* (Knob and Carmona, 2010), *Rhizomucor miehei* NRRL 3169 (Fawzi, 2010), *Talaromyces thermophilus* (Maalej et al., 2009), *Paecilomyces themophila* (Li et al., 2006), *Aspergillus niveus* RS2 (Sudan and Bajaj, 2007) etc.

The existence of pollution problems associated with agroindustrial wastes, scarcity of places for its disposal, costlier treatment options and increased need to save valuable resources have forced to encourage the utilization and bioconversion of waste into high value industrially useful products. The recycling of resources is becoming valid and viable economic activity and is increasingly mentioned as a solution to some of the most serious problems of mankind. Enormous amounts of agricultural residues are being wasted in India. The huge amounts of residual plant biomass considered as “waste” can potentially be used to produce various value added products like biofuels, animal feeds, chemicals, enzymes etc. The demand for industrial enzymes, particularly of microbial origin is ever increasing owing to their applications in a wide variety of processes (Chapla et al., 2010).

Concerning the bacterial xylanases, more number of xylanases were purified and characterized and the important xylanase producing bacterial strains are *Flavobacterium johnsoniae* (Chen et al., 2013), *Bacillus* sp. MX47 (Chi et al., 2012), *Paenibacillus* sp. Hpl-002

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(Park et al., 2012), *Bacillus halodurans* (Kumar and Satyanarayana, 2011) *B. pumilus* GESF-1 (Menon et al., 2010), *B. subtilis* ASH (Sanghi et al., 2010), *Chromohalobacter* sp. TPSV (Prakash et al., 2009), *Bacillus* sp. GRE7 (Kiddinamoorthy et al., 2008), *Cellulomonas xavigena* (Santiago-Hernández et al., 2007), *Enterobacter* sp. MTCC 5112 (Khandeparkarand and Bhosle, 2006), *Pseudomonas* sp. WLUN024 (Xu et al., 2005) etc. Considering the facts discussed above, in the present study an attempt has been made to purify and characterize an organic solvent tolerant alkaline xylanase from a marine bacterium *Halomonas meridiana* strain APCMST-KS4 and to test its efficiency on licnocelluloytic saccharification.

2. Materials and methods

2.1. Microorganism used and xylanase production

H. meridiana APCMST-KS4 (GenBank: KF009686) used in this study was isolated from the coastal sediment collected from Kurumpanai, southwest coast, Kanyakumari District, Tamil Nadu, India. The strain was initially cultured in broth culture (tryptone-1%, yeast extract-1%, peptone-0.5%, magnesium sulphate-0.25%, trisodium citrate-0.3%, potassium chloride-0.2% and NaCl-8%) for 24 h. Then it was inoculated in the same halophilic broth with 1% xylose as production medium, and was incubated for 72 at 150 rpm in room temperature. Then the broth was centrifuged at 5000 rpm at 4 °C. Then the pellet was discarded and the supernatant (Xylanase) was used further.

2.2. Purification of xylanase

Xylanase was purified by using two steps namely ammonium sulphate precipitation and DEAE Sepharose Fast Flow anion exchange chromatography. The protein present in the culture supernatant (500 ml) obtained after centrifugation was precipitated with ammonium sulphate (75% saturation) at 4 °C through constant stirring. Then the precipitated protein was recovered through centrifugation (10,000 rpm) at 4 °C and mixed with minimal amount of 50 mM Tris–HCl buffer (pH 7.2). Then it was dialyzed with the same buffer for 12 h with continuous change in buffer for every 3 h intervals. Then the dialyzed sample was concentrated by using Amicon® Ultra centrifugal filters (10 kDa molecular weight cut off; Millepore Ireland Ltd, Ireland) and was then loaded on a DEAE-Sepharose Fast Flow® chromatography (1.2 cm × 20 cm) (Sigma Aldrich, USA), which was initially pre-equilibrated with 10 bed volumes of Tris–HCl buffer used as above. After that the xylanase was step wise eluted with gradients of NaCl (0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 M) in Tris–HCl (50 mM; pH 7.2) with a flow rate of 0.2 ml per minute and 2 ml fractions were collected. The eluted fractions were observed by taking optical density at 280 nm, and then the xylanase activity was analysed. Further the highly active elutions were pooled and concentrated by using Amicon® Ultra centrifugal filters.

The molecular weight of the purified xylanase was determined by using 12% SDS-PAGE on a vertical gel apparatus (V-GES, WEALTEC Corp., USA). After completion, the gel was stained with coomassie brilliant blue R-250 and the molecular weight was determined (Alphaimager mini system, Cell Biosciences, USA) by using standard molecular weight markers.

2.3. Characterization of purified xylanase

The purified xylanase from final chromatography step was suitably diluted for further characterization studies such as the effect of pH, temperature, metal ions (5 mM), inhibitors (5 mM), surfactants (5 mM), NaCl concentrations, substrate (5 mM) and different organic solvents (10%).

2.4. Analytical methods

Xylanase activity was assayed by using beechwood xylan as substrate. The assay mixture consisted of, 1.9 ml substrate [0.5% beechwood xylan mixed in Tris–HCl buffer (50 mM; pH 8.2)] and 0.1 ml enzyme source. Then it was incubated for 15 min in room temperature. After incubation, reaction was terminated by adding 3 ml DNS reagent and kept the same in water bath at 100 °C until the colour of the mixture turned into reddish brown. Then it was further incubated at 4 °C for 5 min. After that 3 ml distilled water was added to this mixture and the absorbance was taken at 575 nm (Miller, 1959). The enzyme activity was calculated by using xylose standard. The protein present in the samples was analysed by following the method of Bradford (1976).

2.5. Lignocellulosic sources and pretreatment

The substrates such as different seaweeds (*Sargassum wightii, Ulva lactuca, Gracilaria* sp., *Hypnea* sp.), ricebran, wheat bran, saw dust, orange peel and Hibiscus petals were used for the present work. They are cheap and readily available sources of lignocellulosis. Among them, seaweeds were collected from Chinnavilai and Muttom coasts of Kanyakumari District, Tamil Nadu. The rice bran and wheat bran, saw dust, orange peel and dried flowers were collected from Manavalakurichi, Kanyakumari District, Tamil Nadu. After collection, the substrates were cleaned in running tap water and sundried for two days to reduce the moisture content. After that, the dried materials were ball milled and pass through a 0.5 mm screen. Then it was treated with 1 M NaOH and incubated for 12 h in room temperature. After that the treated samples were thoroughly washed with distilled water until to neutralize. Finally the substrates were dried in oven at 80 °C. The treated substrates were further used for the enzymatic saccharification.

2.5.1. Saccharification

To determine the percentage of saccharification, 100 mg dry weight of equivalent lignocellulosic materials were mixed with 1 ml of buffer (Tris–HCl buffer-50 mM; pH 8.2) and then it was incubated with 1 ml crude xylanase obtained from *H. meridiana* APCMST-KS4 at 50 °C for different time intervals. The amount of reducing sugar produced was measured by DNS method. The percentage of Saccharification was calculated as:

$$\text{Saccharification}(\%) = \frac{\text{Reducing sugar}(\text{mg/ml})}{\text{Substrate}(\text{mg/ml})} \times 100$$

3. Results and discussion

Alkaline xylanae synthesized by *H. meridiana* APCMST-KS4 was purified through ammonium sulphate precipitation and DEAE-sepharose chromatography. In ammonium sulphate precipitation, 36.49% enzyme was recovered from the culture filtrate with 1.6 fold purity. Further purification by DEAE Sepharose anion exchange chromatography, the enzyme was eluted between 0.4 and 0.6 NaCl and here 17.23% enzyme was recovered with 15.83 fold purification (Table 1). On SDS page analysis, this purified xylanase exhibited single protein band with 41 kDa molecular weight (Fig. 1). Consistence with the present study, *B. pumilus* strain GESF-1 was reported to produce xylanase with the molecular mass of 39.6 kDa (Menon et al., 2010). Kiddinamoorthy et al. (2008) reported a 42 kDa xylanase from *Bacillus* sp. GRE7. Also recombinant

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