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ScienceDirect
Enzyme and Microbial Technology 41 (2007) 733–739
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry

Bindu Battan [a], Jitender Sharma [a,*], Saurabh Sudha Dhiman [a], Ramesh Chander Kuhad [b]

[a] *Department of Biotechnology, Kurukshetra University, Kurukshetra 136119, India*
[b] *Department of Microbiology, University of Delhi South Campus, New Delhi 110021, India*

Received 21 February 2007; received in revised form 15 June 2007; accepted 15 June 2007

Abstract

A very high level of cellulase-free, thermostable xylanase has been produced from newly isolated strain of *Bacillus pumilus* under submerged fermentation in a basal medium supplemented with wheat bran (2%, w/v) pH 8.0 and at 37 °C. After optimization of various production parameters, an increase of nearly 13-fold in xylanase production (5407 IU/ml) was achieved. The produced xylanase is stable in neutral to alkaline pH region at 70 °C. The suitability of this xylanase for use in the bioleaching of eucalyptus Kraft pulp was investigated. A xylanase dose of 5 IU/g of oven dried pulp of 10% consistency exhibited the optimum bleach boosting of the pulp at pH 7.0 and 60 °C after 180 min of treatment. An increase of 5% in brightness along with an increase of 21% and 28% in whiteness and fluorescence respectively, whereas 18% decrease in the yellowness of the biotreated pulp was observed. Enzyme treated pulp when subjected to chemical bleaching, resulted in 20% reduction in chlorine consumption and up to 10% reduction in consumption of chlorine dioxide. Also a reduction of about 16% in kappa number and 83% in permanganate number, along with a reduction in COD value and significant improvement in various pulp properties, viz. viscosity, tensile strength, breaking length, burst factor, burstness, tear factor and tearness were observed in comparison to the conventional chemical bleaching.

Keywords: Thermostable; Xylanase; Paper and pulp; Biobleaching

1. Introduction

Xylanases (endo-1,4-β-D-xylanohydrolase; EC 3.2.1.8), the xylan degrading enzymes, have been reported mainly from bacteria [1–3], fungi [2], actinomycetes [4] and yeast [5,6]. The main reason for investigating its production by bacteria and fungi is its wide variety of biotechnological applications such as bleaching of paper pulp, increasing the brightness of pulp, in food processing, poultry feeds, degumming of plant fibers, etc. [7]. Xylanases have been widely reported to improve the effectiveness of conventional bleaching chemicals in removing lignin from hardwood and softwood kraft pulps [8–10]. Since the last two decades the bleaching of pulp has become an issue of great concern primarily because of the environmental hazards caused by the release of the adsorbable organic halogens and due to increasing public awareness. This increased environmental awareness has become a significant driving force for imposing strict regulations.

In the process of paper production, pulping is a step in which fibres are broken apart and most of the lignin is removed. The residual lignin is then removed by a multistep bleaching process [11]. Chemical pulping which is frequently used around the world is mainly achieved by sulphate (kraft) process. The kraft process consists of cooking the wood chips at high temperatures and at high alkaline pH, in which part of the xylan is dissolved in the pulping liquor; while short-chain xylan precipitates in a more or less crystalline form on the surface of cellulose microfibrils. This process removes around 95% of the total lignin but the 5% residual lignin gives paper a brownish colour and so the paper needs to be bleached [12]. The chemicals used for bleaching process have resulted in the release of large amounts of chlorinated organic compounds that are known to have toxic, mutagenic and carcinogenic effects. In response to government and environmental protection groups, paper industries are currently changing practices to reduce organic pollutant loads on the environment [13–15]. Thus, the use of enzymes for biobleaching has provided an alternative and cost-effective method for process change.

The use of microbial enzymes for bleaching of paper and pulp is now gaining momentum. The microbial enzymes have

* Corresponding author. Tel.: +91 1744 239239.
E-mail address: jksharmakuk@rediffmail.com (J. Sharma).

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.06.006

high specificity for their substrates, the reaction conditions are mild and there is no substrate loss due to chemical modifications. Several criteria are essential for choosing the suitable xylanase producing microorganism for biobleaching of pulp. It is essential that the enzyme should be active at neutral and alkaline pH and should be thermostable. Interest in cellulase-free xylanase has been of more recent origin following the concept of utilizing xylanase in pulp and paper industry to produce rayon grade paper pulps or superior quality dissolving pulps [16]. Any cellulase activity will have serious economic implications in terms of cellulose loss, degraded pulp quality, and increased effluent treatment cost.

Not much literature has been reported on the high-level production and process economy of utilizing xylanolytic enzymes. Wide scale industrial applications of xylanase require their cost-effective production to make the process economically viable. Thus, the aim of our studies was to reduce the cost of xylanase production by optimizing the fermentation medium and to study its application in the pulp and paper industry. In the present investigation, we report very high level production of a thermostable and cellulase-free xylanase using agro-residues in submerged fermentation from a newly isolated strain of *Bacillus pumilus* that has been potentially applied in the selective hydrolysis of the hemicellulose component in the pulp and paper industry.

2. Materials and methods

2.1. Microorganism

B. pumilus was isolated from a soil sample collected from sanitary landfill using xylan agar medium (pH 7.0) at a temperature of 37 °C. Its ability to produce xylanase was qualitatively confirmed when it formed transparent digestion halos on birchwood xylan agar plates on treatment with Congo red followed by washing with 1 M NaCl. The organism was identified as *B. pumilus* ASH from the Institute of Microbial Technology (IMTECH), Chandigarh, India on the basis of its morphological, physiological and biochemical characteristics. It has been deposited at MTCC (Microbial Type Culture Collection), Chandigarh, India and has been given accession number 7411. The culture was maintained and stored at 4 °C on nutrient agar medium

2.2. Xylanase production under submerged fermentation

The enzyme production was studied in Erlenmeyer flask (250 ml) containing 50 ml of basal medium having (g/l): yeast extract, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 1.0 supplemented with wheat bran, 5.0 and peptone, 5.0; pH 8.0. The flasks were inoculated with 2% (O.D. ~0.5) of the overnight grown inoculum and incubated at 37 °C under shaking conditions (200 rpm). The enzyme was harvested by centrifuging at 10,000 × *g* for 15 min. The supernatant was treated as enzyme and was assayed for xylanase activity. The optimization studies were also performed by altering the fermentation conditions and composition of this basal medium under optimum shaking conditions (200 rpm).

2.3. Xylanase assay

Birchwood xylan and carboxymethyl cellulose were used as the assay substrates for xylanase and cellulase, respectively. The reaction mixture for each enzyme assay contained 490 μl of 2% of respective substrates prepared in phosphate buffer, pH 7.0 and 10 μl of appropriately diluted enzyme and was incubated at 60 °C for 10 min. Both undiluted enzyme and a series of dilutions were assayed against carboxymethyl cellulose for cellulase activity at different pH values ranging from pH 6 to 9. The enzyme activity was determined by measuring the release of reducing sugars during the enzyme substrate reaction using Miller's method [17]. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of reducing sugars from the respective substrates in one min under the standard assay conditions.

All the experiments were carried out independently in triplicates and the results presented here are the mean of the three.

2.4. Chemicals and pulp samples

Birchwood xylan, carboxymethyl cellulose, 3,5-dinitrosalicylic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of highest purity available commercially. Pulp used for biobleaching was hardwood pulp obtained from Ballarpur Industries Limited (BILT), Yamunanagar, Haryana, India. Agro residues were procured locally.

2.5. Parametric optimization of xylanase production

The optimization for xylanase production by *B. pumilus* was carried out for the following parameters:

1. Incubation period: submerged fermentation (SmF) was carried out for 18–30 h.
2. Inoculum age and size: a 12–24-old culture at a level of 1–4% was used as inoculum.
3. Temperature: xylanase production at temperature ranging from 30 to 55 °C was studied.
4. pH: basal medium of pH ranging from 5 to 10 was used for xylanase production.
5. Carbon source and the concentration of the selected carbon source: various carbohydrates and agricultural residues/byproducts were tested as sole carbon source for xylanase production and the effect of different concentrations of the selected carbon source on xylanase production was studied.
6. Effect of nitrogen source and the concentration of the selected nitrogen source on xylanase production: production medium devoid of any nitrogen source was supplemented with different inorganic and organic nitrogen sources like peptone and yeast extract and the effect of various concentrations of the selected nitrogen source on xylanase production was studied.
7. Effect of various surfactants, detergents and other additives: different additives like EDTA and SDS at a concentration of 0.2 (w/v) and Tween 80, Tween 20, glycerol, Triton X, olive oil and oleic acid at a concentration of 0.2 (v/v) were supplemented in the medium and the effect of various concentrations of the selected additive was studied.

After optimization of these parameters, xylanase production was carried out under optimized nutritional and fermentation conditions for maximum yield of the enzyme to be applied in pulp and paper industry.

2.6. Optimization of enzyme dose and other reaction parameters for biobleaching

The optimization of enzyme dose and retention time for biobleaching was carried out by treating moistened unbleached pulp with varying doses of xylanase, ranging between 0 and 12.5 IU/g for variable time intervals starting from 30 min to 3 h. The optimum pH and temperature for efficient biobleaching were determined by carrying out the enzymatic treatment at 10% (w/v) pulp consistency in transparent plastic bags under different pH values ranging from 6 to 10 and at different temperatures varying from 55 to 70 °C.

2.7. Biobleaching of kraft pulps ($ECDED_1D_2$)

Pulp used for bleaching was a mixture obtained from woods of six different trees, namely eucalyptus, poplar, eucalyptus rulla, small vaneer, bamboo, and debarka bamboo hardwood (DBH). Fifty grams extensively washed oven dried kraft pulp of 10% consistency was treated with xylanase (5 IU/g) in plastic bags under optimum conditions. Then xylanase treated pulp was exposed to $CDED_1D_2$ bleaching sequence to obtain pulp with different properties. Thereafter, various experiments were conducted to measure the reduction in chlorine