Bioresource Technology 102 (2011) 9723–9729

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

High level expression of a recombinant xylanase by *Pichia pastoris* NC38 in a 5 L fermenter and its efficiency in biobleaching of bagasse pulp

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ARTICLE INFO

Article history:
Received 18 May 2011
Received in revised form 15 July 2011
Accepted 16 July 2011
Available online 27 July 2011

Keywords:
Pichia pastoris
Xylanase
Glycerol aldehyde 3-phosphate promoter (GAP)
Bagasse pulp
Biobleaching

ABSTRACT

A genetically modified XynA gene from *Thermomyces lanuginosus* was expressed in *Pichia pastoris* under the control of GAP promoter. *P. pastoris* expressed greater levels of xylanase (160 IU ml^{-1}) on BMGY medium without zeocin after 56 h. The xylanase production by recombinant *P. pastoris* was scaled up in a 5 L fermenter containing 1% glycerol and the highest xylanase production of 139 IU ml^{-1} was observed after 72 h. Further studies carried out in fermenter under controlled pH (5.5) yielded a maximum xylanase production of 177 IU ml^{-1} after 72 h. The biobleaching efficacy of crude xylanase was also evaluated on bagasse pulp and a brightness of 47.4% was observed with 50 IU of crude xylanase used per gram of pulp, which was 2.1 points higher in brightness than the untreated samples. Reducing sugars (24.8 mg g^{-1}) and UV absorbing lignin-derived compounds values were considerably higher with xylanase treated samples.

1. Introduction

An increasing awareness on environmental pollution has enforced the pulp and paper industries to strive for an alternate greener technology which will replace the use of harsh chemicals in their processes with microbial enzymes. The application of biocatalysts not only makes the process less toxic but also decreases costs associated with the production and consumption of resources (water, electricity, fuels). Pulp bleaching with microbial xylanases has already been demonstrated as an environmentally friendly technology for the pulp and paper industries (Shirkolaee et al., 2008). Xylanases aid in enhancing the brightness of pulp, diminish impurities, and retention period of pulp (Viikari et al., 1994; Bajpai, 1999; Singh et al., 2003). Cellulase-free, alkali and thermo-stable microbial xylanases are mostly ideal for biopulping and bleaching processes.

Xylanases are produced by numerous microorganisms, *viz.*, bacteria, actinomycetes and fungi. Xylanases hydrolyse xylan, which is one of the major components of hemicellulose found in the plant cell wall (Jeffries, 1996). Xylan is considered to be the second most abundant polysaccharide in nature; it is composed of a linear backbone of 1,4-β-linked D-xylose units and often contains side chains of other sugar residues such as arabinose and glucuronic acid (Biely, 1985). Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic and ρ-coumaric acid esterases. All these enzymes act co-operatively to convert xylan into its constituent sugars (Collins et al., 2005).

Potential application of xylanase in pulp and paper industries has urged considerable research efforts towards producing more thermophilic and alkalophilic xylanases by screening for naturally-occurring xylanases. Alternatively, xylanases from extremophiles have been genetically modified for enhanced stability at higher temperature and pH conditions (Stephens et al., 2009). We previously reported on the expression of an alkalo-tolerant fungal recombinant xylanase (able to retain 84% activity at pH 10 for 90 min at 60 °C) developed by directed evolution in *Pichia pastoris* GS511 and *Escherichia coli* BL21. *P. pastoris* exhibited 545-fold higher levels of recombinant xylanase expression (extracellular) than the levels observed intracellularly with *E. coli* (Mchunu et al., 2009). However, the usage of multifaceted undefined medium and addition of antibiotics for the growth and xylanase production by *P. pastoris* will not be economically viable for any industrial applications. Thus the present study was focused on optimizing the growth conditions of *P. pastoris* using buffered medium containing zeocin or devoid of zeocin for obtaining the maximum expression of recombinant xylanase in shake flasks and then

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doi:10.1016/j.biortech.2011.07.059

scaling up further in a lab scale fermentor. The biobleaching efficiency of crude recombinant xylanase was also assessed on bagasse pulp. To the best of our knowledge, this is the first report on biobleaching of bagasse pulp using a recombinant xylanase expressed in *P. pastoris*.

2. Methods

2.1. Strain maintenance

P. pastoris NC38 was used in this study (Mchunu et al., 2009). Yeast cultures were maintained on YPD medium (g l^{-1}: 10 yeast extract, 20 peptone and 20 dextrose) containing zeocin (100 μg ml^{-1}) for plasmid maintenance.

2.2. Growth and recombinant xylanase production by P. pastoris on buffered minimal glycerol yeast (BMGY) medium incorporated with or without zeocin

Two hundred millilitres of BMGY containing glycerol (1%), yeast nitrogen base with ammonium sulphate (13.4%), biotin (0.02%), 1 M potassium phosphate buffer (20 ml, pH 6.0) and zeocin (100 μg ml^{-1}) was dispensed into 500 ml Erlenmeyer flasks. An additional set of experiment was conducted using BMGY medium deprived of zeocin. A 10% (v/v) of 16-h-old *P. pastoris* cultures grown on BMGY medium was used as inoculum. Culture flasks were incubated at 30 °C with shaking at 200 rpm for 72 h. Five millilitres of sample was withdrawn every 8 h to determine the optical density (600 nm) and pH. For xylanase activity, the culture broths were withdrawn every 8 h and centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was used for the assay.

2.3. Effect of glycerol concentration on the growth and xylanase production by P. pastoris

Two hundred millilitres of BMGY medium devoid of carbon sources was dispensed into 500 ml Erlenmeyer flasks. Glycerol concentrations of 0.1%, 1%, 2.5% or 5% were added. A 10% (v/v) of 16-h-old *P. pastoris* culture grown on the BMGY medium was used as an inoculum. Culture flasks were incubated at 30 °C with shaking at 200 rpm for 72 h. Samples (5 ml) were withdrawn every 8 h to determine optical density (600 nm), pH and xylanase activity.

2.4. Laboratory scale production of recombinant xylanase by P. pastoris

This study was carried out in a 5-L glass fermenter (Minifors, Infors HT, Switzerland) with a working volume of 3 L BMGY medium containing 1% glycerol as a carbon and 13.4% yeast nitrogen base with ammonium sulphate as a nitrogen source. A seed culture of *P. pastoris* (16-h-old) was prepared in BMGY broth and inoculated at 10% (v/v). The agitation, aeration rate and temperature were maintained at 200 rpm, 1.5 vvm (gas volume per liquid reactor volume per minute) using the cascade mode and 30 °C, respectively, for 72 h. Using the similar growth conditions, a separate batch run was carried out by maintaining the pH at 5.5. Fifty millilitre samples were withdrawn through the sampling port and centrifuged (10,000*g* for 10 min) and the clear supernatant was used to determine the xylanase activity.

2.5. Determination of β-xylanase activity

The xylanase activity was determined according to the method described by Bailey et al. (1992). The reaction mixture consisted of 1.8 ml of 1% birchwood xylan (Roth, Karlsruhe, Germany) solution in sodium citrate buffer (50 mM, pH 6.5) and 0.2 ml of appropriately diluted enzyme. After 5 min incubation at 50 °C, the liberated reducing sugars were determined as xylose equivalents. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 μmol reducing sugars (xylose) per minute.

2.6. Biobleaching of bagasse pulp with a recombinant xylanase

Unbleached sugarcane bagasse pulp was obtained from Sappi Fine Paper, Stanger, South Africa. The pulp was carefully washed using tap water to remove the fine and water soluble contaminants. For biobleaching, bagasse pulp sample (10 g) was pre-treated with 500 IU of crude xylanase obtained from *P. pastoris* for 3 h at 10% pulp consistency (Manimaran et al., 2009). The reaction was terminated by heating the enzyme pre-treated pulp samples at 100 °C for 10 min. Ten millilitres of biobleached test and control samples were centrifuged at 10,000*g* for 15 min and the supernatant was analysed for reducing sugars (Bailey et al., 1992). Lignin-derived compounds in the supernatant were also observed by measuring the absorption spectrum between 200 and 465 nm using UV–Vis Spectrophotometer (Varian Cary 100, USA) against the control supernatant from sample pre-treated with denatured enzyme. The pulp was subjected to standard handsheet making processes and the final paper product was analysed (TAPPI, 1996). Biobleaching efficiency of crude xylanase (50 IU g^{-1}pulp) on bagasse pulp was also examined at various temperatures (30–70 °C) and pH (5–9) conditions for 3 h.

2.7. Analytical methods

Standard paper sheets were made from enzyme pre-treated and untreated pulps using the handsheet former (PTI Lab equipment, Austria) and air dried according to TAPPI methods (TAPPI, 1996). The brightness and opacity of the final paper product was measured using a reflectance meter (Technidyne, USA). The ceramic reference (TAPPI) with a brightness of 47.5% was used as a standard. The paper was also tested for tensile index, burst factor and tear index. For scanning electron microscopy, xylanase pre-treated and control pulp samples were mounted on stubs coated with gold palladium and examined under a scanning electron microscope (Philips, USA) at 10 kV.

3. Results and discussion

3.1. Xylanase production in shake flask cultures

Xylanases are secreted by a number of microorganisms and their productivity diverges significantly with respect to cultivation conditions (Collins et al., 2005). In this study, the thermo and alkalo-tolerant recombinant fungal xylanase was expressed in *P. pastoris* and its growth conditions were optimized for maximum xylanase production. *P. pastoris* expressed a high level of xylanase (165 IU ml^{-1}) on BMGY medium incorporated with zeocin after 56 h and there was no considerable improvement in xylanase production after 48 h. Comparatively, the xylanase production level was slightly lower (160 IU ml^{-1}) in the yeast cells grown on BMGY medium devoid of zeocin. Although, there was a negligible difference in enzyme production levels, the xylanase expression trend was comparable on both the media.

The growth pattern of yeast cells was relatively similar between the cells grown on these two media. The cell OD was steadily increased and maximum growth was observed after 48 h in both the yeast cultures. The shake flask experiments indicated that there was no difference in yeast cell growth pattern. However, a marginal diminution in xylanase expression level was noticed in