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High level expression of a recombinant xylanase by *Pichia pastoris* cultured in a bioreactor with methanol as the sole carbon source: Purification and biochemical characterization of the enzyme

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ABSTRACT

The xylanase gene *xyn11A* from *Cellulomonas uda* was expressed in *Pichia pastoris* under the control of an inducible promoter AOX1. The recombinant xylanase was named Xyn11A$_{AOX1}$. The *P. pastoris* clone (C9) showing the highest xylanase activity was selected to evaluate the production of Xyn11A$_{AOX1}$ in liquid cultures in a bioreactor. The culture was carried out by fed-batch fermentation using two strategies, one-stage method using methanol, and two-stage method using glucose and methanol as carbon sources. Interestingly, after 48 h of fermentation using one-stage method, a dry cell weight of 34 g/L and total protein concentration of 1.16 g/L were obtained, where Xyn11A$_{AOX1}$ was the major enzyme secreted into the culture medium. Xyn11A$_{AOX1}$ was purified from the culture supernatant of *P. pastoris*/pPICZαB – *xyn11A* and showed an estimated molecular mass of 45 kDa. The optimal temperature and pH were 50 °C and 6.5, respectively. The K_M and V_{max} values were 4.5 mg/mL and 5000 U/mg protein, respectively. This is the first report on cultivating *P. pastoris* with methanol as the sole carbon source in a minimal salt medium in which the recombinant enzyme was obtained as the major enzyme secreted into the culture supernatant within a short fermentation time.

1. Introduction

Xylan is one of the major components of hemicellulose and is the second most abundant renewable resource after cellulose. It is a complex polysaccharide composed of a main chain consisting of xylose residues connected via β-1,4-glycosidic linkages [1]. Given the complexity of this compound, its degradation requires the synergistic action of several xylanolytic enzymes including *endo*-xylanases, β-xylosidases, α-glucuronidases, α-arabinofuranosidases, and esterases. Endo-β-1,4-xylanase is the most important enzyme in this process [2].

Endoxylanases act on the main chain of xylan by hydrolyzing the internal β(1 → 4) linkages between molecules of xylose, leading to a mixture of different sizes of xylooligosaccharides [3]. Endoxylanases have important applications in industry because of their enormous potential to transform lignocellulosic materials and they are widely used as raw materials in a large number of industrial processes. Currently, the major industrial application of xylanases is in the paper industry [4]. The treatment of pulp with xylanases prior to chemical bleaching provides significant economic and environmental benefits [5].

Although numerous studies have examined xylanases from various sources, it is still important to develop new methods for improving the production of xylanase because of its potential for industrial applications, such as in biobleaching and the animal feed industry [1]. Heterologous expression systems may be useful for obtaining increased amounts of xylanase. In eukaryotic systems, *Pichia pastoris* has been successfully used to produce large amounts of different enzymes. This organism is a methylotrophic yeast capable of producing significant quantities of recombinant protein [6]. In the absence of glucose, this yeast can use methanol as the sole carbon source because of its highly regulated methanol metabolism pathway [7]. The alcohol oxidase enzyme (AOX1) is responsible for the first methanol oxidation reaction in *P. pastoris*; its synthesis is regulated by the AOX1 promoter, which has been broadly used in the expression of recombinant proteins in this microorganism [8,9].

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In bioreactor cultures, *P. pastoris* can grow to high cell densities (100 g/L) in minimum salt medium [10,11]. A common strategy for achieving high cell densities involves three stages in fed batch cultures. The first stage corresponds to a batch culture with glucose or glycerol as the carbon source. Secondly, fed-batch control is initiated by feeding concentrated glucose and glycerol until a certain cell density is reached. The third stage consists of an induction step during which methanol is added to induce heterologous protein production. The duration of the induction period is longer than that of biomass generation, resulting in a total fermentation time of approximately 100 h [12,13].

We previously reported the cloning and expression of the *Cfl xyn11A* xylanase gene from *Cellulomonas flavigena* in *Escherichia coli*; however, most of the protein was found in the insoluble fraction of the bacterial cell lysate in inclusion bodies [14]. The aim of this work was to extracellularly overexpress Xyn11A xylanase from *C. uda* CDBB-1960 in *P. pastoris* under fed-batch fermentation in minimal salt medium using methanol as the sole carbon source. Further, the recombinant enzyme was purified and characterized.

2. Materials and methods

2.1. Chemicals

The DNA gel extraction kit and restriction enzymes were obtained from Qiagen (Hilden, Germany). Birchwood xylan, 3,5-dinitrosalysilic acid, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). SDS-PAGE markers were obtained from Bio-Rad (Hercules, CA, USA). All other chemicals were of analytical grade. Xylo- oligosaccharide standards (xylose, xylobiose, xylotetrose and xylohexose) were purchased from Megazyme (Wicklow, Ireland).

2.2. Molecular techniques

DNA manipulations were performed using standard methods [15]. *P. pastoris* was manipulated as described in the manual of the EasySelect™ Pichia Expression Kit (Invitrogen, Carlsbad, CA, USA).

2.3. Strains, plasmids, and mediums

C. flavigena CDBB-531 strain was identified as *Cellulomonas uda* CDBB-1960 by taxonomic analysis based on 16S rDNA, and this strain was deposited in the National Collection of Microbial Cultures, CINVESTAV (Mexico City, México). *E. coli* JM109 (Invitrogen) was used for high-level production of plasmid DNA, and *P. pastoris* X-33 (Invitrogen) was used for xylanase expression. The vector pPICZαB was supplied by Invitrogen. *E. coli* JM109 was grown at 37 °C and 150 rpm on Luria-Bertani (LB) medium containing 25 μg Zeocin/mL to select clones transformed with the pPICZαB vector. *P. pastoris* was grown in a shaking flask at 28 °C in rich medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin, and 1% (w/v) glucose or 0.5% (v/v) methanol (BMMY) for induction. For strain maintenance, YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose was used.

2.4. Subcloning of xylanase gene

The *Cfl xyn11A* gene was subcloned into pPICZaB vector as follow. Plasmid DNA from *E. coli* XL1-Blue MRF carrying the *Cfl xyn11A* gene [14] was used as a template to amplify the coding region. The coding region of the *Cfl xyn11A* gene (GenBank accession number AM182259.2) was amplified by PCR using forward primer (5′-CTGCAGCGGTGACGTCCAACCAG-3′) and reverse primer (5′-TCTAGACCGCAGAACGCGCTGGGCGTCCA-3′) into the restriction sites XbaI and PstI (underlined), respectively. PCR was performed using HotStar HiFidelity DNA Polymerase (Qiagen) under the following conditions: one cycle for 5 min at 95 °C, followed by 30 cycles for 1 min at 94 °C, 1 min at 59 °C, 1.2 min at 72 °C; and one cycle for 10 min at 72 °C in a Gene-Cycler (Bio-Rad). The PCR products were purified and directionally cloned in the pPICZαB vector in the XbaI and PstI sites, generating the construct pPICZaB-*xyn11A*, which was transferred into *E. coli* JM109 by heat shock. *E. coli* JM109 cells harboring the construct pPICZaB-*xyn11A* were cultivated in LB medium at 37 °C supplemented with 25 μg Zeocin/mL. A transformant *E. coli* JM109/pPICZαB-*xyn11A* clone was selected on an LB agar plate containing Zeocin (100 μg/mL) and analyzed by restriction profiling and DNA sequencing.

2.5. Expression in pastoris

For expression in *P. pastoris*, 10 μg of the pPICZαB-*xyn11A* construct was linearized with SacI and transferred into *P. pastoris* X-33 by electroporation (4.5 kV/cm, 25 mF and 400 W for 11 ms) using (EasyjecT Optima, Equibio Ltd., New York, NY, USA). pPICZαB without the insert was used as a negative control. Transformed clones were selected based on their ability to grow on YPD agar plates containing 100, 200, 500, or 1000 μg Zeocin/mL.

2.6. Selection of recombinant clones

Candidate clones were cultured at 30 °C for 8 h on BMMY medium agar plates containing 0.5% (v/v) methanol and 0.2% (w/v) xylan. Positive clones were selected for expression of the xylanase activity, which was visualized using Congo Red staining [16].

2.7. Xylanase and protein assay

A 20 μL supernatant aliquot was added to 980 μL citrate-phosphate buffer (50 mM, pH 6.5) containing 0.2% (w/v) birchwood xylan. The xylanase activity was determined from the amount of reducing sugars released during incubation at 50 °C. The reducing sugar was measured by the dinitrosalicylic acid method using xylose as a standard [17]. One unit of activity (U) is defined as 1 μmol of xylose released per minute under the assay conditions. The protein concentration was measured using the Lowry method [18], with bovine serum albumin as a standard. All tests were carried out in triplicate and the average values were recorded.

2.8. Fed-batch culture for enzyme production in bioreactor

Fermentations were carried out in a 7 L bioreactor (BIOSTAT Aplus, Sartorius, Goettingen, Germany). For propagation, *P. pastoris* was cultured in YPD medium. The propagation culture for the reactor was carried out in 200 mL of YPD medium inoculated with 2% inoculum in a 1 L Erlenmeyer flask incubated at 28 °C with shaking at 200 rpm for 24 h. The medium was then centrifuged at 8000 rpm for 10 min and the pellet was resuspended in 300 mL of minimum salt medium (MSM) to inoculate the bioreactor.

For fermentation, the basal media MSM [19] containing the trace element solution PTM1 (Amresco, Solon, OH, USA) was used. The MSM contained the following: 4 g/L KH_2PO_4, 4 g/L $(NH_4)_2SO_4$, 0.38 g/L $CaCl_2$, 18.2 g/L K_2SO_4, 9.4 g/L $MgSO_4 \cdot 7H_2O$, 1 mL PTM1, and 2.5 g/L methanol or 40 g/L glucose. The trace element solution PTM1 contained the following: 5.99 g/L $CuSO_4 \cdot 5H_2O$, 8 mL 10× NaI stock solution, 3 g/L $MnSO_4 \cdot H_2O$, 0.20 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.50 g/L $CoCl_2 \cdot 6H_2O$, 20.04 g/L $ZnCl_2 \cdot 5H_2O$, 65.05 g/L $FeSO_4 \cdot 7H_2O$,800 μL 100× H_3BO_3 stock solution,19.2 mL 96.2% H_2SO_4, and 0.40 g/L biotin.

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