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SCREENING AND OPTIMIZATION OF β-GLUCOSIDASE PRODUCTION BY *Candida* sp. JK9/1

Atsadaporn Pakdeelun and Jantaporn Thongekkaew*

Department of Biological Science, Faculty of Science, Ubon-Ratchathani University, Ubon-Ratchathani, 34190 Thailand

*Corresponding Author: jantaporn.t@ubu.ac.th

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Abstract

A total of 73 yeast strains were screened for an extracellular β-glucosidase producing ability by using substrate *p*-nitrophenyl-β-D-glucoside (*p*NPG). Among them strain JK9/1 showed the maximum β-glucosidase activity which was further identified as *Candida* sp. strain JK9/1 based on ITS1-5.8S-ITS2 and D1/D2 domains of the subunit (26S) ribosomal DNA. Optimum conditions for β-glucosidase production were determined using different biophysical and biochemical parameters. The maximum β-glucosidase production was obtained in the optimum medium containing 1.5% (w/v) of xylose as a carbon source and 0.02 % (w/v) KNO_3 as additional inorganic nitrogen, with an initial medium pH of 4.0 and at 25ºC for 6 days of cultivation. Under optimal conditions, *Candida* sp. strain JK9/1 produced the enzyme of 7.21 ± 0.19 $U.mL^{-1}$, which was 4.2 times higher than the amount before optimization (1.71±0.03 $U.mL^{-1}$). This can contribute its β-glucosidase producing ability for a great potential to be used in the biotechnological application.

INTRODUCTION

β-Glucosidases (E.C 3.2.1.21) are a group of enzymes that hydrolyses a variety of glycosides, including aryl- and alkyl-β-glucoside and *p*-nitrophenyl-β-D-glucoside, and disaccharides such as cellobiose. These enzymes have broad applications such as release of aroma from wine grapes [1-3] and hydrolysis of bitter compounds during juice extraction, formation of alkyl- and aryl-glycosides by trans-glycosylation from natural polysaccharides or their derivatives, leading to products with useful in pharmaceutical, cosmetic, and detergent industries [4] and also used in cellulose-degrading systems for fuel ethanol production [5-8].

β-Glucosidase could be isolated from plants, animals and microorganisms. Even β-glucosidase has been reported in several studies in order to figure out their characteristics for industrial application [9-13]. In view of the potential application of this enzyme, β-glucosidase-producing strains screening and enzyme production from various sources are desirable. Therefore, the present investigation aims at screening potential yeast capable of producing considerable amounts of β-glucosidase and optimizing the parameters for maximum enzyme production.

MATERIALS AND METHODS

Materials

Candida sp. strain JK9/1 was isolated from soil in forest in Ubon-Ratchathani, Thailand. Sugars used as a carbon source and substrates for hydrolytic activity such as xylose, glucose, fructose, maltose, sucrose, cellobiose, carboxymethyl cellulose sodium salt, xylan and *p*-nitrophenyl-β-D-glucopyranoside were products of Acros (Acros organic,Belgium).

Methods

Selection of potential strain

Seventy three yeast isolates were inoculated onto media containing 0.5% yeast extract, 0.5% peptone and 1% cellobiose. After incubated at 25ºC for 48 h at 140 rpm, the culture supernatants were assayed for β-glucosidase activity. The highest level of β-glucosidase strain was molecularly identified by sequencing the ITS1-5.8S-ITS2 and D1/D2 domains of the subunit (26S) ribosomal DNA and selected in the present study.

Molecular identification of selected yeast

Pure isolated yeast cells from 3 mL of 24-h culture were harvested by centrifugation and DNA extraction as described by Sambrook et al. 1989 [14]. Identification was carried out by sequencing the

gene that encodes the 5.8S regions of ribosomal ribonucleic acid (rRNA) and the spacer regions ITS-1 and ITS-2 after PCR amplification. To do so, the universal primers ITS4 (5′ TCCTCCGCTTA-TTGATATGC 3′) and ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) were used. The D1/D2 domains of the 26S subunit were also sequenced by using the primers NL-1 (5′ GCATATCAATAAGCGGAGGAAAAG 3′) and NL-4 (5′ GGTCCGTGTTTCAAGACGG3’), according to the methodology described by Kurtzman and Robnett 1997 [15]; Esteve- Zarzoso et al. 1999 [16] and Leaw et al. 2006. [17]. The PCR product was purified by using QIAquick PCR Purification Kit and analyzed with 1.5% agarose gel electrophoresis. The PCR products were sequenced using the ITS4, ITS5, NL-1 and NL-4 primers using either Amersham Pharmacia ALF Express II or ABI 310 (capillary) automated DNA sequencer, following the manufacturer’s instructions. For identification, the obtained sequences were compared with those of all known yeast species, available at the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) [18].

Enzyme Assay

The standard β-glucosidase activity was determined by incubating the enzyme with *p*-nitrophenyl-β-D-glucopyranoside (*p*-β-NPG) as the substrate by a modification of Yeoh method [19]. The enzymatic reaction mixtures (500 µL) containing 450 µL of 3 mM *p*-β-NPG (final concentration) in 50 mM citrate buffer (pH 4.5) and 50 µL of the culture supernatant were incubated at 50 °C for 15 min. After incubation, the reaction was stopped by adding 2 mL of 200 mM sodium carbonate buffer (pH 10). The activity of β-glucosidase was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 410 nm. One unit of β-glucosidase activity was expressed as the amount of enzyme required to release 1 µmole of *p*-nitrophenol per minute under the assay conditions. All assays were performed in duplicate.

Optimization of enzyme production of the selected strain

Carbon source

To determine the best carbon source for enzyme production, *Candida* sp. JK9/1 was grown in sterile YM medium (1% yeast extract, 2% peptone and 2% glucose) for 16 h at 25ºC as a preculture. For β-glucosidase production, a medium containing 1% yeast extract, 0.5% peptone and 1% of various carbon sources (glucose, xylose, fructose, maltose, sucrose, cellobiose, xylan and carboxymethylcellulose sodium salt) was inoculated with 1% (v/v) of preculture. The cultures were grown under shaking (140 rpm) at 25ºC. After 4 days, the cultured broth was centrifuged at 7,500 rpm (4ºC, 15 min). The resulting supernatant solution was used for β -glucosidase activity assay.

Concentration of the carbon source

Concentration of the selected carbon source was varied from 0.5% to 2.0% for maximum enzyme production.

Inorganic nitrogen source

Effect of inorganic nitrogen sources such as KNO_3, NH_4NO_3, NH_4Cl and $(NH_4)_2SO_4$ were evaluated by incorporating each as the sole nitrogen source at 0.02% level.

Temperature and pH

To determine the optimum temperature for enzyme production, the inoculated medium was incubated at a temperature of 25 and 30ºC. Initial pH optimum for enzyme production was determined by adjusting the pH of production media from 4.0 to 6.0.

RESULTS

Screening of the isolated strain

For screening of β-glucosidase producing yeast, a total of 73 yeast strains were grown in production medium. After incubation, the culture supernatants were assayed for β-glucosidase activity by spectrophotometrically using *p*NPG as the substrate. The result of β-glucosidase producing yeast screening showed that 22 strains performed positive for enzyme activity. Among them, strain namely JK9/1 showed the highest level of enzyme activity (**Figure 1**). Therefore, yeast strain JK9/1 was selected and further investigated the optimization of enzyme production.

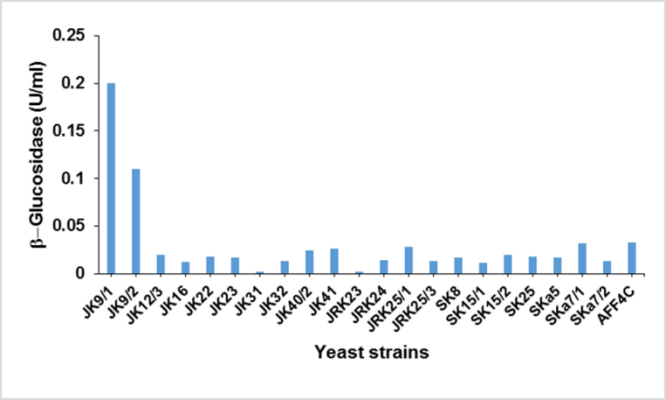

Figure 1. Enzyme activity for 22 β-glucosidase producing yeasts after 48 h at 25ºC

Identification of isolated strain

Sequencing of the ITS1-5.8S-ITS2 and D1/D2 of 26S rDNA region of the isolated yeast was performed. A comparison of the ITS1-5.8S-ITS2 and D1/D2 of 26S rDNA gene sequences, the isolated strain showed the highest identity (99%) with *Candida* sp. ST-390. Thus, it was named *Candida* sp. JK9/1.

Effect of carbon and inorganic nitrogen sources

Among the carbon sources tested (glucose, xylose, fructose, maltose, sucrose, cellobiose, xylan and carboxymethylcellulose sodium salt), maximum enzyme production (1.70 U/ml) was noticed when xylose was used as the carbon source (**Figure 2**). An increase in xylose concentration resulted in an increased production; the optimum concentration was determined to be 1.5-2% (**Figure 3**). Among the inorganic nitrogen sources, KNO_3 was found to be the best for enzyme production, while ammonium salts resulted in least production of the enzyme (**Figure 4**).

Effects of pH and temperature

The influence of culture medium pH on β-glucosidase production was studied for pH values in the range from 4.0 to 6.0 at 25ºC. The yeast was found to have maximum enzyme production at pH 4.0; an increase in pH thereafter caused decrease in enzyme production (**Figure 5**). In order to investigate the effect of

temperature on production, yeast strain was grown at 25 or 30ºC in optimum media and pH. A temperature of 25ºC was found to be the optimum temperature for enzyme production. Therefore, 25°C was considered as optimum temperature for the production of β-glucosidase by *Candida* sp. JK9/1 (**Figure 6**).

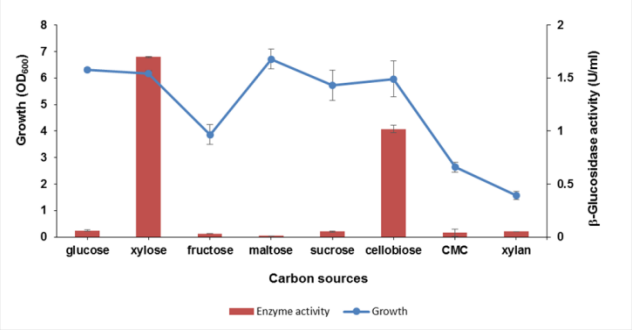

Figure 2. Effect of different carbon sources (1%) on β-glucosidase production by *Candida* sp. JK9/1

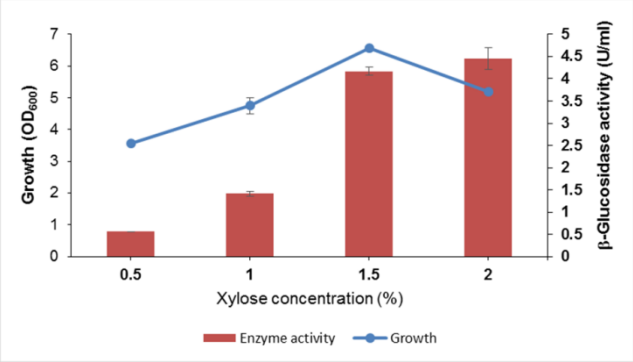

Figure 3. Effect of xylose concentration on β-glucosidase production by *Candida* sp. JK9/1

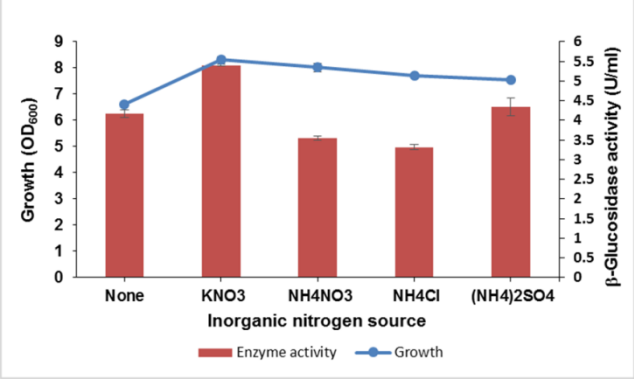

Figure 4. Effect of different inorganic nitrogen sources on β-glucosidase production by *Candida* sp. JK9/1

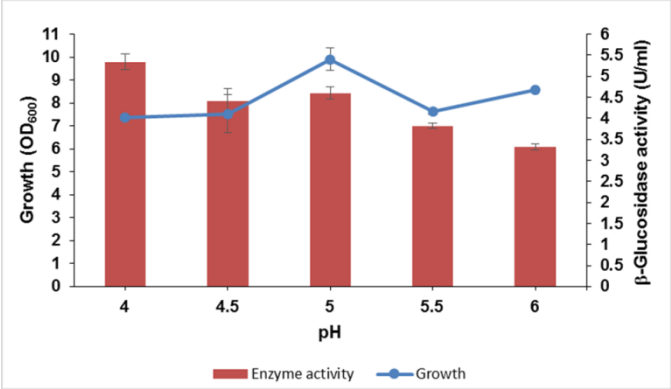

Figure 5. Effect of pH on β-glucosidase production by *Candida* sp. JK9/1

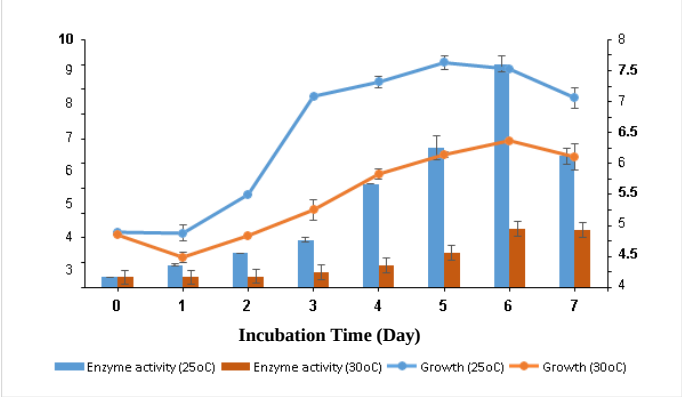

Figure 6. Time course of β-glucosidase production by *Candida* sp. JK9/1 at cultivation temperature of 25 and 30ºC

Production under Optimal Conditions

β-glucosidase production by *Candida* sp. JK9/1 in the optimum medium containing 1% (w/v) yeast extract, 0.5% (w/v) peptone, 1.5 % (w/v) xylose and 0.02 % (w/v) KNO_3, at 25 °C and initial pH of 4.0 for 6 days was 7.21 ± 0.19 $U.mL^{-1}$, which was 4.2 times higher than the amount in basal medium containing 1% (w/v) yeast extract, 0.5% (w/v) peptone and 1%(w/v) xylose and initial pH of 4.5 (1.71±0.03 $U.mL^{-1}$) (**Figure 7**).

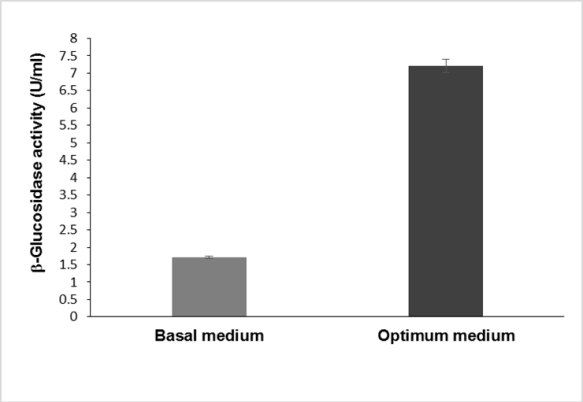

Figure 7. β-glucosidase production by *Candida* sp. JK9/1 in the basal and optimum medium

DISCUSSION

For optimization of β-glucosidase production from Candida sp. JK9/1, effect of biophysical and biochemical factors on β-glucosidase production were investigated. Firstly, for effect of different carbon sources on β-glucosidase production, Candida sp. JK9/1 was grown on carbon sources of concentration at 1%. Among the carbon sources tested, xylose exhibited the maximum β-glucosidase activity whereas when glucose was used, minimum activity was reported. Moreover, an increase in xylose concentration resulted in an increased production. This result was similar to screening of carbon sources for β-glucosidase production by Aspergillus saccharolyticus [20] indicated that xylose was considered as good carbon source for enzyme production. However, the presence of glucose or fructose reduces the activities [21]. Correspondingly, some researchers previously stated that sugar monomers like glucose are often inhibitors of enzyme production based on the CreA repression mechanism [22]. Subsequently, different inorganic nitrogen sources were assessed for β-glucosidase production. It was revealed that the use of KNO3 showed the highest value of enzymatic production by Candida sp. JK9/1 when compared to other inorganic nitrogen sources. Similarly, Elyas et al. 2010 reported that KNO3 was found to be the best for β-glucosidase production by Aspergillus-SA 58. Finally, the influence of culture medium pH and temperature on β-glucosidase production were studied. The yeast was found to have maximum enzyme production at pH 4.0, an increase in pH thereafter caused decrease in enzyme production, suggesting that at low pH range was advantageous not only maintains asepsis [23] but also enhances enzyme production in this strain. In order to investigate the effect of temperature on β-glucosidase production, yeast strain was grown in optimum media and pH. A temperature of 25oC was found to be the optimum temperature for enzyme production while at higher temperature (30oC) was found to reduce production, indicating that temperature is a critical factor for production in this strain. Under the optimal conditions, the amount of β-glucosidase produced by Candida sp. JK9/1 (7.21 ± 0.19 U.mL-1) was 4.2 times higher than the amount before optimization (1.71±0.03 U.mL-1). This can contribute its β-glucosidase producing ability for industrial processes such as liberating flavors, aromas and isoflavone aglycons for the synthesis of oligosaccharides and alkylglycosides as well as a great potential to be used in the biotechnological application.

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