Isolation and Production of Novel β -galactosidase from a Newly Isolated, Moderate Thermophile, *Bacillus* sp. Strain B1.1

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Abstract: The enzyme β -galactosidase (lactase; EC 3.2.1.23) is a commercially important enzyme due to its various applications in dairy and food industries, which are based on the β -galactosidase-catalysed hydrolysis of lactose into glucose and galactose. The objectives of this work were to identify novel and attractive sources of this industrially relevant enzyme, and to study the effect of selected growth parameters (carbon source, lactose concentration, nitrogen source, peptone concentration, initial pH and temperature) on the formation of β -galactosidase. Based on a screening of isolates from Tha Pai hot spring, Mae Hong Son Province, Thailand, strain B1.1 was selected for further studies. Strain B1.1 is a Gram-positive, rod-shaped, catalase-positive bacterium that forms endospores. Based on the sequence of the 16S rDNA determined, this isolate is most closely related to *Anoxybacillus* sp. and *Bacillus* sp., and hence the strain is designated as *Bacillus* sp. B1.1. β -Galactosidase was produced by this strain with lactose and peptone as carbon and nitrogen sources, respectively. Optimal enzyme production occurred at an initial culture pH of 8.5 and at 45 °C. Under these optimum culture conditions, maximal volumetric and specific β -galactosidase activity of 0.478 U mL⁻¹ and 0.338 U mg⁻¹ protein, respectively, were obtained after 13 h of cultivation in a medium contain 2.5% lactose, 2.0% peptone, 0.3% K₂HPO₄, 0.1% KH₂PO₄ and 0.05% MgSO₄·7H₂O.

Key words: β -Galactosidase, isolation, production, thermophile, prebiotic.

1. Introduction

β -Galactosidase or β -D-galactoside galactohydrolase (EC 3.2.1.23), commonly known as lactase, catalyzes the hydrolysis of the disaccharide lactose or structurally related galactosides, with the former cleaved to its monosaccharide constituents glucose and galactose. It is a commercially important enzyme due to its various applications in food and pharmaceutical industries [1]. The production of lactose-reduced or lactose-free dairy products is one

example of an important commercial application, since these products can be used by lactose-intolerant consumers who are unable to digest the milk sugar lactose. β -Galactosidase also offers a solution for the problem of whey surplus and disposal by increasing the value and possibilities of use of whey. In addition, galacto-oligosaccharides (GOS), prebiotic lactose derivatives, are commercially produced from lactose using the transferase activity of β -galactosidases [2, 3].

Over the last decades, numerous biological systems, plants, animals, microorganisms including yeast, molds, bacteria and actinomyces, have been reported to produce β -galactosidase, and a variety of culture conditions can affect the production of β -galactosidase.

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More recently, enzymes from thermophilic micro-organisms, which show a higher degree of thermostability, are preferred because of several advantages that are encountered when performing the reactions at elevated temperatures, namely eliminated undesired microbial contamination during their application, increased substrate (lactose) solubility, higher product concentrations, and increased reaction rates. These results improve the economy of the lactose hydrolysis process [4]. Although numerous studies on β -galactosidase have been performed, suitable enzyme activities and culture condition for the production of β -galactosidase are still major factors limiting the application of this enzyme. Consequently, both the screening for new strains of thermophilic β -galactosidase producer and the effect of culture conditions on the production of β -galactosidase activity are the primary objectives in this study.

Our experiments were aimed at screening for a suitable novel thermophilic β -galactosidase producer with beneficial properties and the identification of this strain. Culture conditions that affect enzyme production by the selected strain were further studied.

2. Materials and Methods

2.1 Microorganisms

Environmental samples were collected at Ta Pai hot spring (75 °C), Mae Hong Son Province, Thailand and Nong Ya Plong hot spring (45 °C), Petchaburi Province, Thailand.

2.2 Screening of Microorganisms

Lactose medium (0.5% lactose, 0.5% peptone, 0.3% beef extract, and 1.5% agar) was used in a plate-based screening of microorganisms aiming at the identification of strains producing thermophilic β -galactosidase activity. The ability of the different isolates to produce β -galactosidase was examined on lactose medium plates containing 50 $\mu\text{g mL}^{-1}$ of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) as a chromogenic substrate and 1 mM of isopropyl

β -D-1-thiogalactopyranoside (IPTG) as an additional inducer for the synthesis of β -galactosidase [5].

2.3 Characterization of Selected Bacterial Isolates

The morphological and physiological properties of the selected isolate B1.1 were investigated according to Bergey's manual of determinative bacteriology [6].

For the determination of the 16S rDNA sequence, strain B1.1 was cultivated in lactose medium for 24 h at 50 °C. Cells were subsequently lysed after their separation from the medium by centrifugation, and DNA was extracted using the phenol/chloroform/isoamyl alcohol method. The 16S rDNA gene was then amplified using the universal primers SFD (5' GAC GGG TGA GTA ACA CGT G 3') and SRD (5' GCT TTC TGG TTA GGT ACC GT 3'). The PCR conditions were initial denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C (30 sec), 50 °C (30 sec), 72 °C (1 min), and final extension at 72 °C for 7 min. The amplification products were purified using the Hi Yield Gel/PCR DNA Fragment Extraction kit (Real Biotech Corp., Taipei, Taiwan). The sequencing of the purified products was performed by a commercial provider (Bioservice Unit, Bangkok, Thailand). Sequence data were complied with the BLAST program and compared with those available from the GenBank database of the National Center for Biotechnology Information. The phylogenetic relationship of isolate B1.1 was determined by using ClustalW2 (<http://www.ebi.ac.uk>).

2.4 Culture Condition

Inoculum was prepared in 250 mL Erlenmeyer flasks containing 100 mL of cultivation medium, which consisted of 0.5% lactose, 0.5% peptone, 0.3% yeast extract, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 0.3% K_2HPO_4 , 0.1% KH_2PO_4 , and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The selected strain was incubated at 50 °C for 24 h. Cells were harvested by centrifugation (4,000 rpm for 10 min at 4 °C), washed twice with saline solution, diluted to a dry cell weight of 0.1 g L^{-1} and used as the inoculum. The

production of β -galactosidase was performed by transferring an aliquot (1.0 mL) of the inoculum to a 250 mL Erlenmeyer flask containing 100 mL of the same culture medium. After 13 h incubation at 50 °C, cell growth and β -galactosidase activity were examined. To determine the effect of various carbon sources on β -galactosidase production, lactose, galactose or glucose were used as the main carbon source in the medium. The nitrogen sources studied included $(\text{NH}_4)_2\text{HPO}_4$, $(\text{NH}_4)_2\text{SO}_4$, peptone and yeast extract. To investigate the effect of pH, the initial pH of the medium was adjusted to various values (6.0-10.0) with sterile 1.0 N NaOH or 1.0 N HCl solutions. Various culture temperatures (40-60 °C) were also tested with respect to growth and β -galactosidase yields.

2.5 Determination of β -galactosidase Activity

Cells were first harvested from the culture broth by centrifugation (4,000 rpm for 10 min at 4 °C). After washing twice with 50 mM sodium phosphate buffer (pH 6.5), cells were suspended in 50 mM sodium phosphate buffer pH 6.5 to obtain a cell suspension, and were then broken by three passages through a French Press (Aminco, USA) at 1,200 bar and 4 °C. The cell extract was separated from cell debris by centrifugation at 4,000 rpm and 4 °C for 30 min. This crude supernatant served as the enzyme source.

β -Galactosidase activity was quantitatively assayed at 40 °C by incubating 20 μL of suitably diluted enzyme solution with 480 μL of 22 mM *o*-nitrophenyl- β -D-galactopyranoside (oNPG) in 50 mM phosphate buffer (pH 6.5) as the substrate for 15 min. The reaction was stopped by adding 750 μL of 0.4 M Na_2CO_3 and the amount of *o*-nitrophenol (oNP) released was determined by reading the absorbance at 420 nm [7].

One unit of β -galactosidase activity (U) was defined as the amount of enzyme that releases 1 μmol of oNP from oNPG per minute under the experimental conditions described above.

2.6 Determination of Cell Dry Weight

Cell dry weight (CDW) was estimated by centrifuging 3 mL of the culture samples at 4,500 rpm for 15 min, washing the pellet twice with distilled water, and drying the sample in an oven at 70 °C to constant weight.

3. Results and Discussions

3.1 Screening and Identification of a Selected Isolate

In a screening of moderately thermophilic microorganisms from hot spring water samples, aiming at identifying suitable thermophilic producers of β -galactosidase activity, an approach based on lactose agar plates with an incorporated chromogenic substrate was used. By using this approach, more than 30 phenotypically different colonies were isolated after incubation of the agar plates at 50 °C, and 5 colonies were identified as potential β -galactosidase producers since they showed blue colonies on lactose agar containing X-gal. Strain B1.1 (from Ta Pai hot spring) was found to exhibit the highest of β -galactosidase activity (data not shown). This strain has the ability to produce an intracellular β -galactosidase, and extracellular β -galactosidase activity was not detected during the cultivation. Isolate B1.1 is a gram-positive, rod-shaped bacterium that is catalase-positive. In addition, it also forms endospores under appropriate conditions. Its colonies were found to be circular, milk white, smooth and humid.

In order to identify this isolate, the 16S rDNA of strain B1.1 was sequenced directly following PCR amplification. The 16S rDNA sequence of strain B1.1 was determined to be 406 bp long. An alignment of the 16S rDNA gene sequence of isolate B1.1 with other sequences available in the GenBank databases at NCBI demonstrated that the highest degrees of identity is with the type strain *Anoxybacillus* sp. and *Bacillus* sp., sharing 99% 16S rDNA similarity. Fig. 1 shows the phylogenetic tree of isolate B1.1. Based on the 16S rDNA sequence and the biochemical and physiological characteristics, we determined B1.1 to be a *Bacillus* species and designated it *Bacillus* sp. B1.1.

Isolation and Production of Novel β -galactosidase from a Newly Isolated, Moderate Thermophile, *Bacillus* sp. Strain B1.1

Fig. 1 The phylogenetic tree showing the position of isolate B1.1 to a selected number of members of *Bacillus* Gram-positive rod bacteria. (FN666244.1: *Anoxybacillus pushchinoensis*, JF830129.1: Uncultured *Anoxybacillus* sp. clone Bac-A12-1, FJ808715.1: *Anoxybacillus* sp. G18, GQ153549.1: *Anoxybacillus eryuanensis* strain E-112, FJ842661.1: *Anoxybacillus* sp. SW, HM032715.1: *Anoxybacillus* sp. X2, AY198416.2: *Bacillus* sp. YMY1010, FJ527830.1: *Anoxybacillus* sp. PGDN13, HM016869.1: *Anoxybacillus flavithermus* subsp. *yunnanensis* strain E13, FJ527829.1: *Anoxybacillus* sp. PGDY12).

3.2 Factors Affecting β -galactosidase Production

In general, β -galactosidase production is affected by numerous factors, such as the components of the culture medium and the cultivation conditions [8-10]. The effects of some of the key factors, like carbon source, nitrogen source, amount of carbon, amount of nitrogen, initial pH and temperature, on the formation of β -galactosidase activity by *Bacillus* sp. B1.1 were determined.

3.2.1 Effect of Carbon Source on the Production of β -galactosidase

To investigate the effect of different carbon sources on the production of β -galactosidase by the selected organism *Bacillus* sp. B1.1, lactose, galactose and glucose were used as the main carbon sources and compared with respect to the yields of β -galactosidase attained. β -Galactosidase activity was determined after growth of the organism for 13 h. As shown in Fig. 2, *Bacillus* sp. B1.1 can utilize a variety of different carbon for the synthesis of β -galactosidase. The highest β -galactosidase activity was detected after growth on lactose followed by galactose and glucose. The stimulatory effect of lactose has been similarly observed with *Geobacillus stearothermophilus*

SAB-40 [11] and *Lactococcus lactis* A2 [12]. Several researchers have described that various carbon sources control the production of β -galactosidase in different microorganisms. Nagy et al. [8] reported that lactose is the only sugar used by *Penicillium chrysogenum*, whereas glucose is not used. Shaikh et al. [13] reported on the influence of some carbon sources on the production of β -galactosidase by *Rhizomucor* sp.. They indicated that lactose was the best carbon source while glucose was the poorest carbon source.

Different lactose levels (10-35 g L⁻¹) were added to the medium and the activities of β -galactosidase as well as growth of B1.1 were estimated after 13 h of incubation. The results presented in Fig. 3 indicate that the concentration of lactose in the medium affected both enzyme activity and growth of the organism. The β -galactosidase activity increased as the lactose concentration in the medium was raised up to 25 g L⁻¹. Under these growth conditions, a maximum β -galactosidase activity of 0.166 U per mg dry cell weight was observed. A further increase in the initial lactose concentrations led to a decrease in β -galactosidase activity and cell growth. Increased lactose concentrations in the culture medium may repress the biosynthesis of β -galactosidase [14, 15].

Fig. 2 Effect of carbon source on the production of β -galactosidase by strain B1.1. Determinations were made after 13 h cultivation at 50 °C. Symbols: dry cell weight; β -galactosidase activity; consumed sugar.

Fig. 3 Effect of lactose concentration on the production of β -galactosidase by strain B1.1. Determinations were made after 13-h cultivation at 50 °C. Symbols: dry cell weight; β -galactosidase activity; specific activity.

3.2.2 Effect of Nitrogen Source on the Production of β -galactosidase

A variety of different nitrogen sources were added to the medium containing 25 g L⁻¹ lactose and the activity of β -galactosidase was determined after 15 h of fermentation at 50 °C (Fig. 4). Peptone was the best

nitrogen source for the production of β -galactosidase by strain B1.1. Our results agreed with those observed for *Streptococcus thermophilus* and *Kluyveromyces lactis* by Rao and Dutta [16] and Barreto et al. [5], respectively, who demonstrated that peptone can be a good nitrogen source which that a maximum synthesis

Isolation and Production of Novel β -galactosidase from a Newly Isolated, Moderate Thermophile, *Bacillus* sp. Strain B1.1

Fig. 4 Effect of nitrogen source on the production of β -galactosidase by strain B1.1. Determinations were made after 13 h cultivation at 50 °C. Symbols: dry cell weight; β -galactosidase activity; specific activity.

Fig. 5 Effect of peptone concentration on the growth and β -galactosidase production by strain B1.1. Determinations were made after 13 h cultivation at 50 °C. Symbols: dry cell weight; β -galactosidase activity; specific activity.

of β -galactosidase. On the other hand, these results were different from the report of Domingues et al. [17] and Hsu et al. [9], which indicated that the highest activity of this enzyme was obtained by *Aspergillus niger* and *Bifidobacterium longum* CCRC 15708, respectively, when using yeast extract. A further increase in β -galactosidase activity was observed with an increase in the peptone concentration in the medium,

and a maximum activity was obtained in the presence of 20 g L⁻¹ peptone. A further increase in the concentration of peptone did not enhance the enzyme activity any further (Fig. 5).

3.2.3 Effect of Initial pH and Temperature on Enzyme Production

The effect of the initial pH value of the medium on enzyme production was studied over a pH range of 6.0

Fig. 6 Effect of initial pH on the production of β -galactosidase by strain B1.1. Determinations were made after 13 h cultivation at 50 °C. Symbols: dry cell weight; β -galactosidase activity; specific activity.

Fig. 7 Effect of temperature on the production of β -galactosidase by strain B1.1. Determinations were made after 13 h cultivation at 50 °C. Symbols: dry cell weight; β -galactosidase activity; specific activity.

to 10.0. The maximum enzyme production was observed between pH 8.5 and 9.0 (Fig. 6), while higher initial pH values resulted in a sharp decrease in β -galactosidase activity. There have been few reports on β -galactosidase production at this high range of pH.

As presented in Fig. 7, various culture temperatures

(40-60 °C) were tested in order to investigate their effect on β -galactosidase production. At temperatures between 40 °C and 50 °C, the strain grew well, and the highest levels of β -galactosidase activity were obtained. The maximum enzyme activity value was found when the cultivation was performed at 45 °C.

4. Conclusions

A newly isolated, moderate thermophile, *Bacillus* sp. strain B1.1 is able to synthesis β -galactosidase with lactose and peptone as carbon and nitrogen sources, respectively. Optimal enzyme production occurred at an initial culture pH of 8.5 and at 45 °C. After optimizing the culture condition for the production of β -galactosidase, authors obtained maximal volumetric and specific β -galactosidase activity of 0.478 U mL⁻¹ and 0.338 U mg⁻¹ protein, respectively. Thus purification, characterization and application of this enzyme for prebiotic GOS are in progress.

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