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Co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates

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Abstract

Various nutrients belonging to three categories, carbon, organic nitrogen and complex organic sources, were investigated for the first time in terms of their effect on the co-production of extracellular thermostable α-amylase and β-galactosidase by *Bacillus subtilis*, a bacterium isolated from fresh sheep's milk. Among the organic nitrogen sources tested, tryptone and corn steep liquor favored their production. Substitution of soluble starch by various starchy substrates, such as corn flour, had a positive effect on both enzyme yields. Furthermore, a two-fold higher production of both enzymes was achieved when corn steep liquor or tryptone was used in combination with the different flours. Among the divalent cations examined, calcium ions appeared to be vital for α-amylase production. The crude α-amylase and β-galactosidase produced by this *B. subtilis* strain exhibited maximal activities at 135 °C and 65 °C, respectively, and were also found to be significantly stable at elevated temperatures.

Keywords: *Bacillus subtilis*; α-Amylase; β-Galactosidase; Thermostable; Co-production; Organic substrates

1. Introduction

Hydrolases are among the enzymes of increasing industrial application. Within these, α-amylase and β-galactosidase receive special attention. α-Amylase, which catalyzes the hydrolysis of starch to low molecular weight products, is produced by a wide variety of microorganisms, but for commercial applications α-amylase is mainly derived from the genus *Bacillus* (Pandey and Nigam, 2000; Violet and Meunier, 1989). α-Amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries (Machius and Wiegand, 1995; Pandey and Nigam, 2000).

β-Galactosidase (or lactase) hydrolyzes the milk sugar, lactose, to its components glucose and galactose and thus it is used for the treatment of milk and its derivatives for consumption by people who have lactose intolerance, for prevention of lactose crystallization in frozen and condensed milk products, for the reduction of water pollution caused by whey and also for increasing the sweetening properties of lactose (Furlan and Schneider, 2000; Patel and Mackenzie, 1985). Although many microbial β-galactosidases have been investigated, very few studies have been reported on thermostable β-galactosidase (Batra and Singh, 2002; Brady and Marchant, 1995; Chang and Mahoney, 1994; Fischer and Scheckermann, 1995; Lind and Daniel, 1989; Pandey and Nigam, 2000; Vasiljevic and Jelen, 2001).

In view of the advantages offered by the application of thermostable hydrolases, such as reduction of reaction time and contamination risk which provide considerable energy saving (Grueniger and Sonnleitner, 1984; Pandey and Nigam, 2000), there is always a requirement for hydrolases capable of functioning at elevated temperatures. Furthermore, in order to meet this demand the development of a low cost fermentation medium is necessary.

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In a previous work, the authors reported the production of an extracellular thermostable α-amylase from *Bacillus subtilis* and its employment for the hydrolysis of various starches (Konsoula and Liakopoulou-Kyriakides, 2004). This paper describes the screening of various organic substrates and the development of a suitable low cost fermentation medium for the co-production of α-amylase and β-galactosidase from *B. subtilis*, and some characteristic properties of β-galactosidase are presented as well.

2. Methods

2.1. Chemicals

Soluble starch and D-(+)-glucose were purchased from J.T. Baker. Peptone was obtained from Sigma, while tryptone and yeast extract were purchased from Scharlau. Potato, rice, corn, wheat, oat and chestnut flour were purchased from the local market and corn steep liquor was obtained from local industries (Thessaloniki, Greece). Bovine serum albumin was obtained from Acros Organics.

2.2. Microorganism

The *B. subtilis* strain was obtained from the Laboratory of Microbiology and Hygiene, Faculty of Agriculture, Aristotle University of Thessaloniki. The microorganism was isolated from fresh sheep's milk and identified according to the criteria described in Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986) by Prof. E. Litopoulou-Tzanetakis.

2.3. Enzyme production

Unless stated otherwise the bacterium was grown in shake flasks in a basal medium of the following composition: (% w/v) 0.25 KH_2PO_4; 0.25 Na_2HPO_4; 0.1 NaCl; 0.2 $(NH_4)_2SO_4$; 0.005 $MgSO_4 \cdot 7H_2O$ and 0.0075 $CaCl_2$. The basal medium was supplemented with 0.2% (w/v) appropriate organic substrate. The concentration of the liquid substrate, corn steep liquor (CSL), was kept at 0.2% (v/v). The pH of the medium was adjusted to 7.0. Erlenmeyer flasks (500 ml) containing 100 ml medium were inoculated at 10% (v/v) level and incubated at 40 °C on a rotary shaker (120 rpm) for 48 h. Samples were removed at intervals for the determination of biomass and enzymic activities.

The effect of the carbon source concentration was studied by the addition of various concentrations of tryptone (0–2% w/v) or corn steep liquor (0–2% v/v) in the basal medium. In the case of starch the basal medium containing corn steep liquor (0.2% v/v) was supplemented with various concentrations of corn flour (0–0.2% w/v).

The basal medium containing 0.2% (w/v) tryptone was supplemented with 0.2% (w/v) glucose after 24 h of cultivation in order to study the effect of glucose on α-amylase production.

The effect of calcium ions on α-amylase production was investigated by addition of various concentrations of $CaCl_2$ (0.00125–0.015% w/v) in the basal medium containing 0.2% (w/v) tryptone.

2.4. Enzyme isolation

Cells were removed from the culture broth by centrifugation at 4500*g* for 20 min at 4 °C. The cell-free culture supernatant was precipitated with $(NH_4)_2SO_4$ (80% saturation). After centrifugation (at 4500*g* for 45 min), the precipitate was collected and resuspended in 0.1 M sodium phosphate buffer (pH 7.0) to obtain the partially purified crude enzyme preparation. Protein was estimated according to the method of Bradford (1976) using crystalline bovine serum albumin as standard.

2.5. Enzyme assay

The characterization of the α-amylase was performed according to the methods of Jensen and Olsen (2003) and Ramesh and Lonsane (1989). The reduction in the color of the blue-colored starch–iodine complex was determined according to the method of Ramesh and Lonsane (1990) and the release of reducing sugar equivalents was estimated by the method of Miller (1959).

2.5.1. α-Amylase

The crude enzyme solution was diluted to a final volume of 0.5 ml with 0.1 M sodium phosphate buffer (pH 6.5), and was added to 0.5 ml of 1% (w/v) soluble starch in the same buffer. The reaction mixture was incubated at 50 °C for 10 min. α-Amylase activity was determined by the rate of absorbance decrease of the colored starch–iodine complex (Ramesh and Lonsane, 1990). The enzyme activity was expressed as specific activity (U mg^{-1} soluble protein) and one unit of enzyme activity (U) was defined as the amount of enzyme that causes 1% decrease in the absorbance of the colored starch–iodine complex per minute.

2.5.2. β-Galactosidase

The crude enzyme solution was diluted to a final volume of 0.5 ml with 0.1 M sodium phosphate buffer (pH 6.0), and was added to 0.5 ml of 6 mM *o*-nitrophenol-galactopyranoside (ONPG) in the same buffer. The reaction mixture was incubated at 40 °C for 30 min. The reaction was ended by adding 0.5 ml of 1 M Na_2CO_3 and the concentration of *o*-nitrophenol (ONP) released from ONPG was determined by measuring the absorbance at 420 nm, using a standard calibration curve. The enzyme activity was expressed as specific activity (U mg^{-1} soluble protein) and one unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 nmol ONP per minute (Vasiljevic and Jelen, 2001).