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Characterization of a thermostable, $CaCl_2$-activated and raw-starch hydrolyzing alpha-amylase from *Bacillus licheniformis* AT70: Production under solid state fermentation by utilizing agricultural wastes

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ABSTRACT

B. licheniformis AT70 which produced a thermophilic, raw-starch degrading alpha-amylase was isolated from Gorooh hot springs in Kerman province. Maximum production of AT70 alpha-amylase was obtained in the presence of starch (as a carbon source) and ammonium chloride (as a nitrogen source) with 388 and 329 U/ml enzyme yield, respectively. SSF was also carried out using various agricultural and kitchen wastes and results showed that the maximum yield of AT70 alpha-amylase production was obtained by date waste and wheat bran, respectively (10%, w/v). The thermal stability of the AT70 alpha-amylase was increased about 2.5 folds at 60 °C. AT70 alpha-amylase showed the maximum activity at 1.5 M NaCl by 42% and local detergent Shooma enhanced the alpha-amylase activity about 34%, compared to control. Furthermore, AT70 alpha-amylase exhibited remarkable hydrolytic activity in a range of 14–20% (w/v) of raw corn starch at 55 °C. These results indicated that AT70 alpha-amylase has great potential applications for the raw-starch degrading.

1. Introduction

Starch is a major constituent of agriculture and domestic wastes. These cheap agricultural wastes could become appreciated resources to be converted into high value compounds. In recent years, a global interest has been focused on the raw-starch hydrolyzing amylases to simplify the procedure of starch digestion [1,2]. Alpha-amylase is a key enzyme in the industrial processes such as starch saccharification, textile, paper, brewing, food, distilling industries and pharmaceuticals [3]. Alpha-amylase (E.C 3.2.1.1 1,4-α-glucan-glucanohydrolase) catalysis the hydrolysis of the internal α-D-(1,4) glycosidic linkages in starch at random locations. This enzyme constitutes a class of industrial enzymes that account for ~25% of the enzyme market [4]. Alpha-amylase can be derived from several sources, such as animals, plants and microorganisms. However, enzymes from microbial sources generally meet industrial demands [4]. Whereas many of the commercial alpha-amylases do not accordance industrial process conditions, isolation and characterization of alpha-amylases with favorite features such as thermostability, alkaline stability and halophilicity is of special importance [5]. Activity of alpha-amylases at higher temperatures is desirable for gelatinization and liquefaction of starch to economize the processes; as the need to continually search for more thermophile and thermostable alpha-amylases is increasing [4]. Using thermostable alpha-amylase in the industrial processes has advantages, including the decreased risk of contamination, cost of external cooling, increased diffusion rate, a better solubility of substrates, a lesser viscosity permitting accelerated mixing and pumping. Moreover, they showed a resistance in the presence of denaturing agents, solvents and proteolytic enzymes [3]. Among the genus *Bacillus, B. licheniformis* and *B. amyloliquifaciens* are identified as good producers of thermostable alpha-amylase. Due to significant thermal resistance, the alpha-amylase produced by *B. licheniformis* widely used in the starch liquefaction process [6]. Bacterial organisms are now being progressively considered for the production of enzymes by solid-state fermentation (SSF) [7]. SSF has several advantages over submerged fermentation (SmF) because of the low capital investment, the simple technique, low energy requirement, end-product inhibition, low waste water output and better product recovery [8]. SSF has been engaged in the

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production of thermophilic alpha-amylase by *Bacillus subtilis* and *B. cereus* MTCC 1305 [9]. In the present research, we report the biochemical characterization of a Ca^{2+}-activated, thermophilc and detergent-stable alpha-amylase from *B. licheniformis* AT70 and its potential to degrade raw starch granules was also investigated. The production of the alpha-amylase was also performed by using different kinds of agro-residues and the kitchen wastes, which some of them were not reported yet i.e. Hajibadam sweets, Sehen Komath sweets in SSF manner. The properties of this enzyme, including its pH and temperature profiles, kinetic parameters, irreversible thermo-inactivation and raw starch digestibility revealed that it has significant potential for the starch industry.

2. Materials and methods

2.1. Identification of the microbial strain and culture conditions

Bacillus licheniformis AT70 strain which used in this research was isolated from Gorooh hot spring, located in Jiroft city, Iran. For identification of the isolated strain, a number of morphological and biochemical tests described in Bergey and especially 16 S rRNA gene sequence analysis were performed [10,11]. The isolate was detected to be an alpha-amylase producer on the starch-agar medium composed of (%) soluble starch 1.0, yeast extract 0.2, peptone 0.5, NaCl 0.1, $MgSO_4{\cdot}7H_2O$ 0.1, $CaCl_2{\cdot}2H_2O$ 0.02, after incubating at 55 °C for 72 h. Alpha-amylase production was indicated by flooding the plates with 1% (w/v) I_2 and 2% (w/v) KI solution.

2.2. Production of alpha-amylase

Alpha-amylase production was performed in the culture medium containing (g/l): KH_2PO_4 1.0, $Na_2HPO_4{\cdot}2H_2O$ 3.13, tryptone 2.0, $(NH_4)_2SO_4$ 2.0, $MgSO_4{\cdot}7H_2O$ 0.05, $CaCl_2{\cdot}2H_2O$ 0.05, soluble starch 1.0 (pH 7.0) [12]. The medium seeded with 10% (v/v) of the pre-culture composed of nutrient broth medium and incubation was carried out at 55 °C with 150 rpm for 72 h. The cells were harvested after centrifugation at 10,000*g* for 10 min at 4 °C and cell-free supernatants were collected as crude enzymes.

2.3. Alpha amylase assay

Alpha amylase activity was measured using 3,5-dinitrosalicylic acid (DNS) reagent according to Bernfeld [13]. A mixture of 0.5 ml of enzyme solution and 0.5 ml of soluble starch (1.0%) in phosphate buffer (50 mM, pH 7.0), as substrate, was incubated at 55 °C for 20 min. The reaction was stopped by adding 1 ml of DNS reagent. The mixture was boiled for 5 min and after cooling in room temperature, the mixture was diluted with distilled water. The amount of reducing sugars released during the starch hydrolyzing was measured by recording absorbance at 540 nm and using the glucose standard curve. A unit of enzyme activity was defined as the amount of enzyme required to release of 1 μmol of sugar reducing per 1 min under enzyme assay conditions.

2.4. Optimization of enzyme production conditions

The alpha-amylase production was optimized in the presence of the different carbon sources by supplementing the basal medium (without carbon source) with 0.1% (w/v) of starch, glucose, galactose, maltose, fructose [14]. For this order, the mediums were inoculated with 10% of an overnight culture of the AT70 isolate. After 48 h of incubation at 55 °C, the culture mediums were harvested by centrifugation with 11,000*g* and 4 °C for 10 min and the obtained supernatants were used for the investigation of the alpha-amylase production. The organic nitrogen sources, including yeast extract, gelatin, peptone and inorganic nitrogen sources, including ammonium chloride and sodium nitrate replaced by ammonium sulphate in the basal medium at a concentration of 2.0% [14]. In each case, the culture medium was inoculated with 10% of the overnight grown bacterial culture. After 48 h, the culture medium was centrifuged at 11,000*g* and 4 °C for 10 min and the alpha- amylase activity was assayed for the resulting supernatants. The effect of different metal ions on the enzyme production was also investigated by adding a concentration of 1.0% of KCl, $MgSO_4{\cdot}7H_2O$, NaCl and $CaCl_2$ in the basal medium (without metal ion) [14]. Alpha-amylase activity was measured after 48 h of incubation. The effect of pH on the alpha-amylase production was studied by growing the bacterium in the culture media with various pH 5.0, 6.0, 7.0, 8.0 and 9.0. After 48 h of incubation, the alpha-amylase activity of the resulting supernatants was measured under the standard conditions.

2.5. Solid state fermentation

The production of alpha-amylase was investigated under solid state fermentation method on the eight different types of local agricultural wastes as substrate including banana peel, potato peel, orange peel, Hajibadam sweets, Sehen Komath sweets; a kind of local cake, wheat bran [9], rice bran and a kind of date waste. First of all, the AT70 isolate was inoculated into 100 ml of the pre-culture medium containing (%) glucose 1.0, peptone 0.25, yeast extract 0.2, NaCl 0.15, KH_2PO_4 0.05, $MgSO_4$ 0.05, $CaCl_2$ 0.01 and was incubated at 55 °C for 16 h. After this period, 3 ml of pre-culture was added within the culture medium composed of 10 gr of agricultural waste and 25 ml of optimized salt solution containing ammonium chloride 2.0 gr/l, $CaCl_2$ 1.0 gr/l and KH_2PO_4 1.0. After 7 days, 30 ml of sodium phosphate buffer (50 mM, pH 7.0) was added to the culture mediums and the obtained mixture was passed from a textile. Then, centrifugation was performed in 11,000*g* for 10 min at 4 °C. The gathered extract was used as enzyme and its activity was assayed under the standard conditions by using the DNS method.

2.6. Partial purification of the alpha-amylase

A Q-Sepharose column pre-equilibrated with 50 mM phosphate buffer pH 7.6 was used for the partial purification of the alpha-amylase produced by *B. licheniformis* AT70. The supernatant was passed through the pre-equilibrated column. The attached proteins were removed from the column using a linear gradient of NaCl (0.0–5.0 M) in the same buffer at a flow rate of 1.0 ml/min [15,16]. The fractions were used to determine the alpha-amylase activity and protein content. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was also performed according to the Laemmli method to determine the purity and the molecular mass of alpha-amylase [17]. Proteins were observed by silver staining method [18]. Zymography of alpha-amylase was also performed in non-denatured gel containing 10% polyacrylamide and 1% soluble starch for the confirmation of the amylolytic activity [12]. Enzyme activity was detected using staining with KI/I_2 solution as a white zone in dark background.

2.7. Biochemical characterization

2.7.1. Effect of pH on the enzyme activity and stability

The effect of pH on the enzyme activity was studied under standard assay conditions in the following buffer systems at a concentration of 50 mM: glycine (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris (pH 8.0–10.0), glycine (pH 11.0 and 12.0). pH stability was also measured by pre-incubation of the enzyme solution at room temperature for 1 h in