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Production and immobilization of a novel thermoalkalophilic extracellular amylase from bacilli isolate

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ABSTRACT

A Thermoalkalophilic amylase was produced from an environmental bacterial isolate. The enzyme was then immobilized through its amino groups onto the epoxy rings of magnetic poly glycidyl methacrylate [m-poly (GMA)] beads. The free enzyme was active within a large pH range, between 7 and 12 and displayed the optimum activity at 95 °C and pH 10. The immobilization appeared to increase the stability of the enzyme as its bound form showed optimum activity at 105 °C and pH 11.0. Kinetic studies demonstrated that immobilized enzyme had higher K_m and lower V_{max} values. The activity of the free and bound enzyme was determined, at 37 °C and pH 10.0 and pH 11.0, respectively, in the presence of various organic solvents and detergents (5%, v/v). Results obtained indicated that detergents, sodium dodecyl sulfate (SDS) and TritonX-100, caused six fold increase and that various organic solvents also increased the activity of the amylase.

1. Introduction

Amylases, 1-4-D glucan glucanohydrolase, for example, are industrial enzymes which break down starch or glycogen into oligosaccharide differing in size. The members of amylase family are classified according to their type of starch hydrolysis molecules. Alpha-(EC 3.2.1.1), beta-(EC 3.2.1.2), and glucoamylase -(EC 3.2.1.3), have been widely used in detergent, food, paper, and textile industry [1–4].

Microbial amylases are extracellular enzymes especially produced by *Bacillus* species and they have often been preferred for a number of reasons: easy isolation, cost-effective production [5], and high stability in harsh environmental conditions such as elevated levels of temperature and pH that have often been required in food, textile or paper industry [6–8].

Immobilized enzymes onto insoluble supports could often display better physiological and biochemical properties such as increased stability and activity in nonaqueous media, enzyme reuse [9–12]. It has been reported that different support materials have been used for different types of amylases: for α-amylases, for example, nitrocellulose membrane [13], calcium alginate beads [3], chitin and chitosan [14], poly (HEMA-GMA) membranes [15] have been preferred, while chitosan microbeads [16] or poly(acrylamide/acrylic acid) resins [17] have been used for β-amylases.

In this study, an α-like amylase produced from bacilli isolate was immobilized on 1.6 μm monosize [m-poly (GMA)] microbeads. The amylase characterized both in free and immobilized form appeared to be a thermoalkalophilic enzyme.

2. Materials and methods

Soil samples were aseptically collected into sterile plastic bags from the ground soil of cherry orchards of Tokat, Turkey.

2.1. Isolation of bacteria

Ten grams of soil sample was diluted in 90 mL sterile 0.85% NaCl solution. Suspensions were shaken for 6 h at room temperature. One milliliter aliquots were transferred into 9 mL 0.85% NaCl. Serial dilutions, up to 10^{-6} were made and pour-plated in Horikoshi-I agar [1% glucose, 0.5% polypeptone, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% Na_2CO_3], pH 10.2 medium and incubated for 72 h at 37 °C.

2.2. Extracellular amylase screening and production

Amylase activity was screened on mineral medium supplemented with 0.5% starch (0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.5% NaCl, and 1% Na_2CO_3) (pH 10.0). Individual colonies were transferred onto mineral medium agar plates containing 0.5% starch and incubated at 37 °C overnight.

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Colonies with amylase activity were identified by iodine staining [18]. Bacterial colonies displaying amylase activity were then inoculated in 250 mL mineral medium broth and grown for 3 days at 37 °C in a shaker incubator (Shellab, USA). Cells were harvested by centrifugation for 15 min at 10,000 rpm at 4 °C. Supernatant was transferred into glass bottles and two volumes of 95% ethanol were added. Proteins were precipitated at −20 °C overnight. The precipitate was air-dried and stored at −20 °C. Protein content of the precipitate was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [19]. The amount of protein was determined at 280 nm in a spectrophotometer (Optima, Tokyo, Japan).

2.3. Synthesis of M-poly(GMA) beads

M-Poly(GMA) microbeads were synthesized as described by Akkaya et al. [20].

2.4. Immobilization studies

For the optimization of immobilization conditions four different buffers, 0.1 M Na-acetate, pH 3, 4 and 5; 0.1 M Na-carbonate, pH 9.0 and 10.0; 0.1 M K-phosphate, pH 6.0, 7.4, and 8.0; 0.1 M Tris–NaOH, pH 11.0 and 12.0, and a range of protein concentrations, 0.5–5 mg/mL, were used. Therefore, immobilization of the amylase onto m-poly(GMA) beads was conducted in each buffer for 2 h at 25 °C, using 3 mg/mL enzyme, with continuous stirring. The enzyme–polymer conjugates were precipitated by centrifugation for 5 min at 5000 rpm and the precipitate was washed with the same buffer for three times, and dried. Amylase concentration was determined using absorbance at 280 nm before and after the immobilization process. The amount of immobilized amylase was calculated using mass balance equation [21].

2.5. Assessment of the free and immobilized amylase activity

The activities of free and immobilized amylase were determined by measuring the amount of glucose formed by the amylase-catalyzed hydrolysis of soluble starch. For the free enzyme activity, the reaction medium was NaOH–glycine buffer (2 mL 50×10^{-3} M, pH 10.0) including 2 mL 2.0% starch. After 5 min preincubation at 37 °C, the assay was started by the addition of amylase solution (1.0 mL, 0.1 mg/mL) and incubation was continued for 15 min. The amount of glucose was determined by DNS (Dinitrosalicylic acid) method [22].

The activity of the immobilized enzyme was determined in the same assay medium. The assay was started by the addition of 0.5 g m-poly(GMA)-enzyme conjugate into 10 mL assay medium and was incubated for 15 min at 37 °C with continuous stirring. The reaction was terminated by the removal of polymer–enzyme conjugate by centrifugation. One unit of thermostable amylase activity was taken as 1 μmole of glucose per minute per mg solid enzyme. The activity of the immobilized amylase was presented as a percentage of the activity of free enzyme of same quantity.

Thermostability profiles of free and immobilized enzymes were determined within 25–125 °C temperature range. To determine the optimum pH, the same assay conditions were used with different buffers: phosphate buffer, pH 6.0–8.0; glycine–NaOH buffer, pH 8.5–10.5; and Tris–NaOH, pH 11.0–12.0. The results of temperature dependence, the effects of pH, metal ions, organic solvents, substrate specifity, and storage and thermal stability were presented in a normalized form with the highest value of each set being assigned the value of 100% activity. All the experiments were carried out in triplicate.

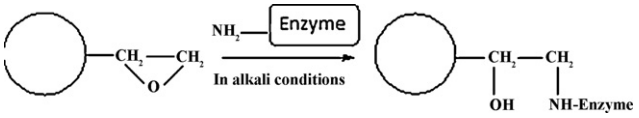

Fig. 1. A schematic representation of covalent immobilization of amylase onto m-poly(GMA).

2.5.1. Determination of kinetic constants

Various starch concentrations (0.1–4.0%, w/v) were used for the kinetic analysis. The enzyme kinetic parameters, K_m and V_{max} were determined by using Lineweaver–Burk plot [23] and the experimental conditions described in Section 2.4.

2.5.2. Substrate specifity

Substrate specifity was investigated by incubating the free and immobilized enzyme with 2% corn starch, wheat starch, amylose, or maltose.

2.5.3. Effects of metal ions, organic solvents, and detergents

Both free and immobilized amylase were incubated in aqueous media including each of the following metal salts NaCl, KCl, $ZnSO_4$, $CaCl_2$, $MgCl_2$, $FeCl_3$, $HgCl_2$, $CuSO_4$, $MnCl_2$ at 5 mM final concentration.

The amylase activity was also determined in the presence of various organic solvents (5% final concentration) acetone, aniline, benzene, chloroform, ethanol, ethyl acetate, methanol, 1-propanol, toluene, and detergents SDS and Triton X-100, under the reaction conditions described in Section 2.4.

2.5.4. Thermal and storage stability

Thermal stability of free and immobilized amylase were assayed for 2 h by measuring the activity at different temperatures, 95, 105, and 125 °C, in the buffer medium described in Section 2.4. Aliquots were taken with 15 min intervals and the activity was determined.

The activity of free and immobilized amylase was measured in NaOH–glycine buffer (0.05 M, pH 10.0) and NaOH–glycine buffer (0.05 M, pH 11.0), respectively.

The enzyme samples were stored for 40 days for free enzyme and 80 days at 4 °C and were then assayed under the experimental conditions described above.

3. Results and discussion

3.1. Bacterial isolation and amylase screening and production

Iodine staining indicated clear zones around individual colonies possessing amylase activity. The isolate displaying the highest amylase activity was used for amylase production. Extracellular enzymes are released into the culture media. Thus extracellular protein content was readily recovered by precipitating the cell free supernatant with two volumes of 95% ethanol at −20 °C for 24 h. A fraction of the precipitate was resolved in 12% SDS–PAGE gel (supplemented data, Fig. 1). A single protein band with ~50 kDA MW was obtained after the Coomassie-Brilliant Blue staining. This size appeared to be within the range of amylase 45-105 kDa [24,25].

3.2. Immobilization of amylase

A covalent immobilization were expected to occur between hydroxyl groups on the epoxy ring of m-poly(GMA) and the amino groups of amylase (Fig. 1) [26–28]. Immobilization of amylase onto m-poly(GMA) beads was carried out at pH range between of 4.0 and 12.0, and its effect on the immobilization was depicted. As can be seen the best binding results were obtained with Tris–NaOH