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Characterization and stability studies on surfactant, detergent and oxidant stable α-amylase from marine haloalkaliphilic *Saccharopolyspora* sp. A9

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ABSTRACT

A halopalkaliphilic marine *Saccharopolyspora* sp. stain A9 with an ability to produce surfactants, oxidant and detergent stable α-amylase was isolated from marine sediments collected from west coast of India. The α-amylase from strain A9 was purified to homogeneity with the aid of ammonium sulfate precipitation and gel filtration chromatography by using Sephadex G-75, insoluble corn starch and sephacryl S-100 column, with a 39.01-fold increase in specific activity. SDS-PAGE and zymogram activity staining showed a single band equal to molecular mass of 66 kDa. Enzyme was found to be stable in presence of wide range of NaCl concentration with maximum activity found at 11% (w/v) of NaCl. Enzyme showed remarkable stability towards laboratory surfactants, detergents and oxidants. Glucose, maltose and maltotriose were the main end product of starch hydrolysis, indicating it is α-amylase.

1. Introduction

Amylases (endo-1,4-α-D-glucanohydrolases EC 3.2.1.1) are extracellular endoenzymes that randomly hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units [1]. This class of industrial enzyme constitutes 25% of the sales in the enzyme market [2]. Amylase has a great significance with extensive biotechnological applications in many industrial processes such as sugar, textile, paper, brewing, baking and in distilling industries. It is also used in preparation of digestive juices, cakes, fruit juices, starch syrups and in pharmaceutical industry as a digestive aid. The demand for amylase is increasing day by day because of its extensive applications in all above mentioned industrial sectors [3].

α-Amylases are universally distributed throughout the animal, plant and microbial kingdoms. Over the past few decades, considerable researches have been undertaken with the extracellular α-amylase being produced by a wide variety of microorganisms. The major advantages of using microorganisms for the production of amylases are the economical bulk production capacity of microbes and their easier manipulation to obtain enzymes of desired characteristics. Since many of the commercially available amylases do not withstand industrial reaction conditions and also they do not meet a large industrial demand of this enzyme, therefore, isolation and characterization of novel amylases with desirable properties such as thermostability, alkaline stability and halophilicity are very important to meet the industrial demands [4]. These may be the reasons why researchers all over the globe are now trying to exploit extremophiles which are the valuable source of novel enzymes [4–6]. Among the extremophiles, halophiles are microorganisms that live, grow, and multiply in highly saline environments. Moderately halophilic bacteria are able to grow over a wide range of salt concentrations from 0.4 to 3.5 M with optimum growth at 0.5–2.0 M [7]. Exoenzymes from these organisms with polymer-degrading ability at low water activity are of interest in many harsh industrial processes where concentrated salt solutions would inhibit enzymatic conversions [3].

Marine microorganisms are capable of catalyzing various biochemical reactions with novel enzymes such as amylase, lipase, deoxyribonuclease and protease [8]. There are many reports on thermostable α-amylase production from bacteria belonging to genus *Bacillus* such as *Bacillus coagulans*, *Bacillus stearothermophilus* and *Bacillus candolyticus* [9,10]. Amylase production also has been

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reported in eubacterial moderate halophiles e.g. *Acinetobacter* [11], *Micrococcus halobius* [12], yeast such as *Cryptococcus flavus* [13] and *Halomonas meridiana* [14]. But very limited research has been directed towards α-amylase production from marine actinomycetes [15]. The present study was carried out to select a haloalkaliphilic *Saccharopolyspora* strain that produces detergent, oxidant and thermostable amylase and to investigate its properties and stabilities.

2. Materials and methods

2.1. Source of marine actinomycetes strain

Sediment samples were collected from Goa, Alibagh and Mumbai coastal region of India at the time of low tide. Heat pretreatment at 40 °C for 30–60 days was employed for isolation of marine actinomycetes [16]. Marine soil samples were suspended in sterile water and thoroughly mixed on rotary shaker at 150 rpm for 20 min. Different marine actinomycetes were isolated by using different selective media such as glycerol yeast extract agar, starch casein agar, maltose yeast extract agar and glucose aspargine agar. The isolated strains were screened for amylase production by using starch agar medium prepared in artificial sea water. The artificial sea water (ASW) contains (g/l) NaCl, 23.37; Na_2SO_4, 3.91; $NaHCO_3$, 0.19; KCl, 0.66; KBr, 0.09; $MgCl_2$, 4.98; $CaCl_2$, 1.10; $SrCl_2$, 0.02 and H_3BO_3 0.02. Maximum amylase producing marine *Saccharoplospora* sp. A9 was maintained on glycerol yeast extract agar medium.

2.2. Identification of strain A9

Identification of strain was done by scanning electron microscopy (SEM), 16S r-DNA sequencing, biochemical and cultural characterization. The method adopted for preparation of slide culture for SEM analysis was as described by Williams and Davies [17].

Cell extract (5 μl), standard amino acids [DL-DAP (5 μl)] and glycine mixture (1 μg/ml)] were added to the baseline of the Whatmann filter paper No. 1 to analyse the amino acids present in the cell wall. An ascending chromatography was performed using methanol:distilled water:6N HCl:pyridine (80:26:4:10 v/v) respectively for 4 h. Chromatographic paper was air dried and developed with 0.2% (w/v) of ninhydrin. Finally, paper was kept at 100 °C for 3 min.

Cell hydrolysate and standard sugar solution were applied to the baseline of the Whatman filter paper No. 1 to determine the sugar content of cell wall. Finally ascending chromatography was performed in solvent system [*n*-butanol:distilled water:pyridine:toluene (10:6:6:1 v/v)] and sugar spots were developed by spraying aniline phthalate and by keeping at 100 °C for 4 min [18–20].

2.3. Inoculum preparation and culture condition

The glycerol yeast extract medium containing starch (Hi-Media) 1% (w/v) prepared in artificial sea water (ASW) was used for development of inoculum. The seed culture was prepared in 100 ml of conical flask containing 50 ml of medium by inoculating 2.0 ml of spore suspension containing 2.5–3.0 × 10^6 CFU ml^{-1} and cultivated under agitation at 200 rpm at 55 °C for 4 days. Then 50 ml of seed culture was inoculated in the 500 ml of fermentation medium containing 2% starch, 0.2% beef extract, 0.2% tryptone and 11% (w/v) NaCl prepared in artificial sea water. The pH of the medium was adjusted to 11 and fermentation was carried out 14 days under agitation at 200 rpm at 55 °C. After removal of cells by centrifugation at 3000 × *g* for 20 min, cell free supernatant was subjected to purification and characterization.

2.4. Purification of α-amylase enzyme

The enzyme was precipitated by bringing the culture filtrate to 90% saturation with solid ammonium sulfate and kept at 4 °C for overnight. The precipitate was centrifuged at 12,000 × *g* for 30 min. The precipitate was dissolved in 50 mM glycine–NaOH buffer of pH 11 and dialyzed for 48 h against the same buffer.

Enzyme obtained (2 ml) from the above step was loaded onto a Sephadex G-50 column (1.2 × 135 cm), pre-equilibrated with 50 mM glycine–NaOH buffer, pH 11, at flow rate of 10 ml/h. Fractions (3.5 ml) were collected and those having specific activities more than 200 U/mg in the void volume were pooled and used for the next step. The enzyme obtained after Sephadex G-50 was loaded onto an equilibrated DEAE-Cellulose column (2 cm × 10 cm). The fractions with specific activities more than 300 U/mg were pooled and loaded onto insoluble corn starch column [21].

The enzyme obtained from the above step was loaded onto an insoluble corn starch column (2.5 cm × 10 cm), pre-equilibrated with 50 mM glycine–NaOH buffer, pH 11. The column was washed with cold water and the bound enzyme was eluted by incubation in 50 mM of glycine–NaOH buffer of pH 11 at 55 °C. Fractions with high amylase activities (above 1200 U/mg) were pooled and dialyzed against 50 mM of glycine–NaOH buffer of pH 11. Enzyme (2 ml) from starch column was loaded onto a Sephacryl S-400 column (1.2 cm × 130 cm). The fractions (3.5 ml) were collected and checked for the enzyme activities and protein concentrations [22].

2.5. Assay of amylase

The activity of α-amylase was estimated by determining the amount of reducing sugar released from starch. 1 ml of enzyme solution was added to 1 ml of starch solution (1%, w/v) in 50 mM of glycine–NaOH buffer (pH 11) and in presence of 10 mM of Ca^{2+}. The mixture was incubated at 55 °C for 60 min. The reaction was stopped by the addition 2 ml of 3,5-dinitrosalicylic acid (Himedia) and $A_{540\,nm}$ was measured in Jasco V-530 spectrophotometer. One unit (U) of enzyme activity is defined as the amount of enzyme required for the liberation of 1 μmol of reducing sugar as glucose per minute under assay condition [23].

2.6. Effect of metal ions, EDTA and various chemicals on enzyme activity

The effect of metal ions and EDTA on amylolytic activity was determined by the addition of different concentrations of each ion (5 mM and 10 mM) to the standard assay. All metal ions were used in salt form such as $CaCl_2$, $MgCl_2$, $HgCl_2$, $FeCl_3$, $CoCl_2$ $CuSO_4$, KCl, urea (8 mM), glycerin (1%, v/v) olive oil (1%, v/v) and EDTA (5 mM and 10 mM).

The effects of metal ions, various chemicals and chelating agent on amylolytic activity were determined by pre-incubating the enzyme in the presence of additives for 30 min at 55 °C and then performing the assay in the presence of the same concentrations of additives at optimum temperature for 60 min. Enzyme activity was determined as percentage relative activity as compared to control (without additives), considered to have 100% of relative activity.

2.7. Stability of amylase

The pH stability of amylase was determined by incubating the enzyme in presence of different buffer systems in presence of 11% NaCl and 10 mM Ca^{2+} at 55 °C. Residual amylase activity was expressed as % relative activity as compared to control which was considered as 100% of relative activity. Enzyme activity after pre-incubation at 55 °C for 60 min in glycine–NaOH buffer (pH 11)