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Thermostable amylase production from hot spring isolate *Exiguobacterium sp*: A promising agent for natural detergents

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ABSTRACT

The purpose of the study is isolation and application of novel Hot spring bacterial enzymes. It also reports on purification and characterization of thermostable α-amylase from a newly hot spring isolate, *Exiguobacterium* sp. This thermostable amylase is Ca2+-independent an added improvement in starch saccharification process at a higher temperature because it removes the addition of Ca2+ for improving the stability of amylases. Maximum enzyme activity was obtained at 45 °C at pH 8.5 and stability at concentration of 3.0% NaCl. Thermostable α-amylase from *Exiguobacterium sp* was purified by 3.9 fold with 54.6% recovery and specific activity was 1083 U/ml. The molecular weight of α-amylase was 54 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Apparent K_m and V_{max} value was 5.88 mg/ml and 250μmol/min/ml, respectively for the hydrolysis of soluble starch. An initial analysis of the circular dichroism (CD) spectrum in the ultraviolet range revealed that the amylase is predominantly turn structure and a detailed structural composition showed alpha helix 10.8%, Beta sheet 27.1%, Turn 29.8% and Randomness 32.3% respectively. The amylase combined with soap-nut extract was able to de stain blood stained cloth within 30 min

1. Introduction

Investigations of the enzymes from thermophilic organisms are of global concern because they are capable of functioning at high temperatures and researchers to investigate enzymes as replacement of chemical catalysts in various biochemical processes. The continual exploration of enzymes and their utilities have expanded their industrial market with the growth of 7.6% per year (David et al., 2009). As the technique is easy, cost effective, fast and can be modified to obtain enzymes of desired characteristics, microbial enzyme is more effective than that of other sources (Sen et al., 2014). In the above scenario, amylases are among the most important industrial enzymes having great significance in biotechnological studies and represent about 25–33% of the world enzyme market (Nguyen et al., 2002; Ashwini et al., 2011). Amylases are the most versatile enzyme with a long history of catalytic applications in food and pharmaceutical industries. Thermostable enzymes, derived mainly from thermophilic microorganisms have found a number of commercial applications because of less reaction time and contamination risk (Grueninger et al., 1984; Demirijan et al., 2001). Alpha-amylases are the widely used thermostable enzymes in various industries like starch processing (Leveque et al., 2000; Sarikaya et al., 2000), textile (Pandey et al., 2000), food process (Satosi et al., 2001), detergent (Lin et al., 1998) and pulp and paper industries (Bahrami et al., 2001). Recently, their roles in synthesis of bioactive peptides and as additive in commercial detergents are gaining attention. The stability of the enzyme is greatly manifested due to the composition and nature of the surfactants in detergents. In addition α-amylases with pH values higher than 8.0 have a potential application as an ingredient in automatic dishwasher and laundry detergent formulations (Kim et al., 1995). The use of enzymes in detergents formulations known as “green chemicals in detergents” enhances the ability of the detergents to remove tough stains, making the detergent environmentally safe (Hmidet et al., 2009). The alkaline α-amylases are used in detergents to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate, etc. (Souza and Magalhaes, 2010). Detergent enzymes account for about 40% of the total worldwide enzyme production and thus they represent one of the largest and most successful applications of modern industrial biotechnology (Igarashi et al., 2003). Growth of microorganism and its metabolite synthesis mainly depends on

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Table1
Characterization of the SSB11 strains.

	Test	SSB11
Colony characters	Color	Pale yellow
	Shape	Circular
	Elevation	Flat
Morphology of bacteria	Gram character	+
	Shape	Rod
	Arrangement	Single
	Motility	+
Biochemical characterization	Lecithinase	+
	Catalase	+
	Oxidase	–
	Indole	–
	Protease	+
	Lipase	+
	Amylase	+
Physiochemical characterization	Temp. tolerance range	20–80 °C
	Optimum temperature	50 °C
	pH tolerance range	2–12
	Optimum pH	8
Sugar fermentation	Glucose	+
	Arabinose	–
	Galactose	+
	Fructose	+
	Raffinose	–

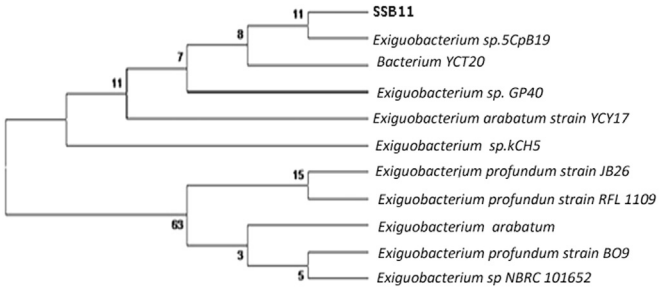

Fig. 1. Neighbour- joining phylogenetic tree from analysis of 16 S r DNA gene sequence of bacterial isolates.

the medium nutrients and the growth conditions (Prescott et al., 2002). For maximizing the microbial metabolites production and minimizing the production cost, optimization of media components and process conditions plays a vital role (Bezbaruah et al., 1994). The drupes (soapnuts) contain saponins which are a natural surfactant. They have been used for washing for thousands of years by native peoples in Asia as well as Native Americans (Austin, 2004). Sapindaceae is one of the important family of plant kingdom consisting about 150 genera and 2000 species (Evans, 1989). The soapberry family comprises nearly 2000 species, which are primarily tropical in nature. The soapnut tree commonly known as Reetha by Indians and found in most of the hilly regions. The genus *Sapindus* includes two major species *Sapindus mukorossi* and *Sapindus emarginatus* in north and south India. *S. mukorossi* is one of the most important sources for saponins. Literature review shows that no work has been done on mixing reetha extract with amylase from hot spring isolates which has been used as a detergent.

In the present study, we report the taxonomic identification of a high-titer and a natural detergent stable alkaline amylase producing bacterial strain isolated from Taptapani Hot Spring of Odisha in India and optimize the medium for production of thermostable alpha-amylase from *Exiguobacterium* sp. along with enzyme purification and characterization.

2. Materials and methods

2.1. Microorganism

Water samples were collected from Taptapani (84°40′E and 19°50′N) hot water spring (Odisha), India and carried in sterile plastic containers to the laboratory at ambient temperature for further analysis. The hot spring which was studied here is located on the eastern slope of the Eastern ghat in India, Odisha, which is a hot water sulfur spring set at the greenery of a lush forest. One ml of water sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-6} dilution. The isolation of bacteria was performed in starch-agar plate containing flucanazole (antifungal agent). Plates were incubated in inverted position at different temperatures (40 °C, 50 °C and 60 °C), pH 7.5 for 24 h. The bacterial isolates were further sub-cultured on the respective media in order to obtain pure culture and among them the new isolate *Exiguobacterium* sp. SSB11 (GenBank accession number- KC923292) was found to be highest alkaline amylase producer and employed in this study. The preliminary characterization (morphological, biochemical, physiological and molecular) of the pure isolates were carried out using existing method (Sarkar et al., 2008). The details of the morphological, biochemical and physiological characterization are presented in Table 1 and the neighbour-joining phylogenetic tree from analysis of 16 S rDNA gene sequence of bacterial isolate is represented in Fig. 1.

2.2. Effect of nutritional parameters on bacterial growth and amylase production

The effect of various natural carbon sources such as seed of Tamarind, Jackfruit, Mango, Red ber and Jambolina at a concentration of 0.5% (w/v) were evaluated for bacterial growth. For evaluating nitrogen sources, basal medium was supplemented with yeast extract(YE), tryptone (TRP), urea(UR), peptone(PEP), sodium nitrate(SN) and control(CL) at a final concentration of 0.5% (w/v). For evaluating phosphate source KH_2PO_4, K_2HPO_4, Na_2HPO_4, NaH_2PO_4, $(NH_4)HPO_4$, $KH_2PO_4+K_2HPO_4$ and substrate: moisture ratio was also studied on bacterial growth and amylase production.

2.3. Amylase production

Amylase activity was determined by its saccharolytic properties following the method of Bernfield (1955). The reaction was carried out by stirring the free or immobilized enzyme with 1 ml of starch solution (0.5%, w/v, in 0.1 M phosphate buffer, pH 6.5) for 5 min at 90 °C. The reaction was terminated by the addition of 1 ml of 3, 5-dinitrosalicylic acid (3%, w/v). The reaction mixture was then boiled for 15 min for color development and the absorbance measured at 540 nm. One unit of enzyme activity is defined as the amount of enzyme which produces 1 mmol of reducing sugar (sucrose) per minute under the specified conditions.

2.4. Amylase purification

Crude enzyme extract was used for purification of amylase. 80% ammonium sulfate was added slowly to the crude enzyme till saturation level. By centrifugation at $10{,}000 \times g$, the precipitated products were separated and re-suspended in a minimum amount of acetate buffer (0.2 M, pH 5.0). By thorough dialysis residual ammonium sulfate was removed using the same buffer. Concentrated 5 ml sample was then loaded onto Sephadex G-75 column with a bed size of 2.5 cm × 70 cm. The column was equilibrated with 0.2 M acetate buffer (pH 5.0) and was eluted with the same equilibrating buffer at a flow rate of 1 ml/min. All the purification steps were carried out at 4 °C. In each sample, the protein content was estimated at 280 nm using a spectrophotometer. The fractions matching to high amylase activity were pooled, lyophilized and stored at −20 °C for further characterization.

2.5. Molecular weight determination

The molecular weight of the purified amylase was determined by SDS-PAGE (Laemmli, 1970) using a 12.5% resolving gel. Electrophoresis was performed at 200 V and the protein bands were visualized with Coomassie Brilliant Blue R-250 staining. The molecular weight of the amylase was determined by comparison with standard molecular weight markers of 97.4, 66, 43, 29, 20.1 and 14.3 kDa (Bangalore Genei Pvt. Ltd., Bangalore, Karnataka, India). The molecular weight was determined by software built into the Lark gel documentation system (Bangalore, India). Zymogram analysis was performed for the purified amylase (Maity et al., 2009).

2.6. Enzyme kinetics

Km and Vmax values of the enzyme were determined by measuring its activity with various concentrations of the substrate urea (0.1–1 g/100 ml). Kinetic constants were calculated from Lineweaver–Burke plot.