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Isolation and Culture Medium Optimization for Thermostable Extracellular α-Amylase Production by Thermophilic *Alicyclobacillus acidocaldarius*

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A thermophilic *Alicyclobacillus acidocaldarius*, which produces thermostable α-amylase, was isolated from the hot water effluent of a boiled rice mill near Tirupati, Andhra Pradesh, India. The effect of different culture conditions on the growth and production of extracellular α-amylase by thermophilic *A. acidocaldarius* was investigated in laboratory scale. The results showed that the optimum conditions for the production of α-amylase are a temperature of 60°C, pH of 6.0, and medium starch concentration of 1.0%, and yeast extract and tryptone of 0.2%. Surfactants, like Tween-20 and SDS, up to 0.02%, were found to increase the bacterial growth and enzymes. Further increase in their concentration resulted in significantly decreased enzyme production.

Key words : Isolation, *Alyclobacillus acidocaldarius*, α-amylase, cultural conditions, optimization

Introduction

Historically, the selection of microorganisms that produce enzymes has been empirical, starting with samples from very diverse natural sources. Cultures enriched by growth on substrates were used to inoculate fermentation media. Amylases, (α-amylase EC 3.2.1.1) hydrolyze starch, glycogen, and related polysaccharides by cleaving internal α -1,4-glucosidic bonds at random. The reports on the industrial use of bacterial amylases go back to the early 1920s, with a product trade-name "Rapidase," marketed by a European company with the same name [33]. The thermostable α-amylase from *Bacillus* sp. is perfectly compatible with detergent conditions, and now a day's small amount of this enzyme is widely added to detergent powder formulations for the removal of starch stains [33]. With the advent of new frontiers in biotechnology, the spectrum of amylase application has extended in many other fields, such as clinical, medicinal and analytical chemistry, textile, food, brewing and distilling industries, as well as starch saccharification [4,26]. Apart from the use of α-amylases for the production of sweeteners, the enzyme has also been applied in fuel ethanol production from liquefied starch [6]. In all these processes thinning and liquefaction of starch is prerequisite and is carried out at elevated temperatures using thermostable amylases [14]. The requirement for thermostable enzymes with improved properties has initiated a continuous search for thermophilic microorganisms producing novel amylases for industrial applications. Optimization of cultivation conditions is expected to improve the enzyme production. In the present study, the effect of different cultivation conditions on growth and amylase production by thermophilic bacterium isolated from hot water effluents of local boiled rice making mill was investigated.

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Materials and Methods

Screening and selection of high amylase producing bacterial isolate

Thermophilic *Bacillus* species were isolated from different environmental samples like wheat flour mill solid waste, spoiled potatoes, cattle farmyard manure and hot water effluent from boiled rice making mill around Tirupati, India. Initially the isolates were identified as *Bacillus* sp. by gram staining and spore staining. The ability to produce extracellular α-amylase was determined by culturing at 60°C and the starch-iodine plate assay method [2]. A total of 10 colonies were selected and tested for enzyme production in flask scale culture. The SYT broth contained (in g/l) potato

soluble starch 10, yeast extract 3, tryptone 3, K_2HPO_4 1.2, KH_2PO_4 0.2, $MgSO_4$ 0.02 and $CaCl_2$ 0.01 and the final pH adjusted to 6.0. The 50 ml SYT broth was prepared in 250 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. The 5 h old cultures (0.2 $OD_{600\ nm}$) were inoculated at 5% (v/v) to the SYT broth and the flasks were incubated at 60°C for 24 h with 120 rpm in an orbital shaking incubator.

Identification of bacterial isolate

Microbial type culture collection centre, Chandigarh identified the selected bacterial culture isolated from the effluent from boiled rice making mill as *Alyclobacillus acidocaldarius* MTCC 8766. This culture was used for further studies.

Culture medium

The selected *Bacillus* sp. isolates were cultured in SYT broth. The 5 h old bacterial culture (0.2 $OD_{600\ nm}$) at 5%(v/v) level was inoculated into 50 ml broth in 250 ml Erlenmeyer flask to study the effects of cultural conditions and optimization of medium composition. The enzyme activity in the fermented broth medium was measured by centrifugation of bacterial culture at 10,000 rpm for 15 min at 4°C and the crude centrifugal supernatant was used as an enzyme source.

Assay of α-amylase

α-Amylase activity was routinely measured at 70°C in a 2 ml reaction mixture that contained 0.9 ml of a 1.0% (w/v) of soluble starch (from potato; SD fine) in potassium phosphate buffer (pH 6.0) and 0.1 ml of a suitably diluted solution of enzyme. The reducing sugar formed was measured by the dinitrosalicylic acid method [22] at 540 nm using a spectrophotometer. One unit of enzymatic activity was defined as the amount of enzyme that produced 1 mM of reducing sugar as glucose per min under the standard assay conditions.

Estimation of bacterial growth

The bacterial growth in the fermented broth medium was determined by measuring the turbidity in a spectrophotometer at 600 nm.

Optimization of cultural conditions

The effect of different factors including temperature (35-70°C), initial medium pH (4-9) and various concentration of medium constituents like starch (0, 0.5, 1.0, 1.5, and 2.0%), tryptone (0, 0.1, 0.2, 0.3, 0.4 and 0.5%), yeast extract (0.1, 0.2, 0.3, 0.4 and 0.5%), Tween-20 and SDS (0, 0.01, 0.02, 0.03 and 0.04%) on bacterial growth and amylase production were determined.

Statistical analysis

Data presented are the averages of three replicates. Significance of the test variables were analyzed by the Experimental Design Module of Duncun's Multiple Range test using the SPSS Statistical software package (SPSS 10.0 for windows).

Results and Discussion

Screening and selection of potent isolate

Ten thermophilic bacterial cultures were isolated from local environmental samples and screened for α-amylase production. The isolate from hot water effluent of boiled rice making mill around Tirupati was found promising and used for further studies (Fig. 1). The selected bacterial isolate was identified by Microbial Type Culture Collection Centre, Chandigarh, as *Alyclobacillus acidocaldarius* MTCC 8766.

Effect of incubation temperature

The bacterial growth and enzyme production were not observed at 35-40°C and further increase in temperature from 50°C to 60°C resulted increased bacterial growth and enzyme production and further increase in temperature led

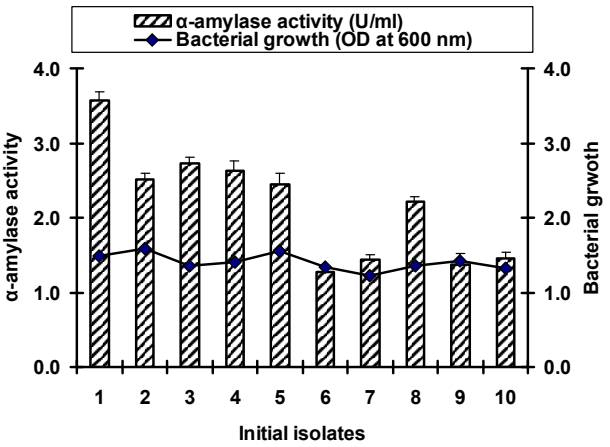

Fig. 1. Screening and selection of promising isolate for α-amylase production. 1.*Alicyclobacillus acidocaldariurs* 2.*Bacillus* sp.I 3. *Bacillus* sp. II 4. *Bacillus* sp III. 5. *Bacillus* sp. IV 6. *Bacillus* sp. V 7. *Bacillus* sp. VI 8. *Bacillus* sp. VII 9. *Bacillus* sp.VIII 10. *Bacillus* sp. IX

to decreased bacterial growth and enzyme production. Maximum bacterial growth and enzyme production (3.89 U/ml) were noticed at 60°C (Fig. 2).

Amylases have been mainly reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, such as α-amylase and glucoamylase [27]. α-Amylase may be obtained from several bacteria, yeasts and fungi. Bacterial amylase, is generally preferred than fungal amylase due to several characteristic advantages that it offers [1]. Strains of *Aspergillus* sp. and *Bacillus* sp. like *Bacillus amyloliquefaciens* and *B. licheniformis*, were mainly used for commercial applications [8]. Thermostable α-amylases are generally preferred as their application reduces contamination risk and minimize reaction time, thus providing significant energy saving. Hydrolysis carried out at higher temperature also minimizes polymerization of D-glucose to isomaltose.

Recent research on thermostable α-amylase production has concentrated on the enzymes of thermophiles and extreme thermophiles. α-Amylase production at optimum level has been reported between 50-55°C for the thermophilic fungal culture *Thermomyces lanuginosus* [15]. Bacterial α-amylases are produced at a much wider range of temperature. *Alicyclobacillus acidocaldarious*, *Bacillus amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce α-amylase at temperatures 37-60°C [7,17,28,29]. In the present study, 60°C temperature was found to be ideal for both growth and amylase production by *A.acidocaldarius* (Fig. 2) Certain hyperthermophiles such as *Thermococcus profundus* and *Theermotoga maritima have been reported to produce α-amylase even at 80°C* [3,19]. *Rhodothermus marinus*, a marine thermophilic bacterium was reported to give maximum yields of thermostable α-amylase at 61°C [12]. A cold active α-amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum α-amylase production at 4°C [10].

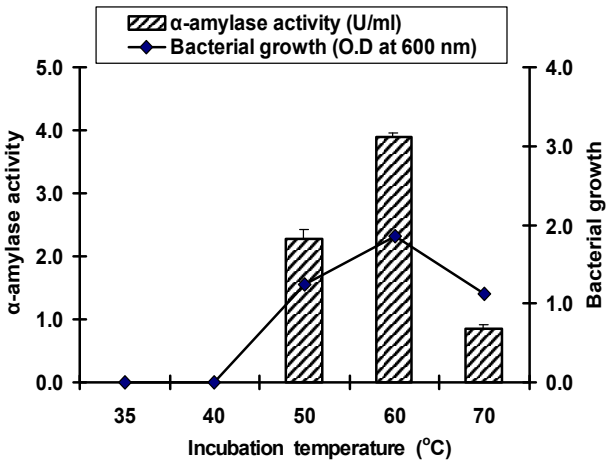

Fig. 2. Effect of incubation temperature on bacterial growth and α-amylase production.

Effect of medium pH

The higher bacterial growth and enzyme activity (3.7 U/ml) were noticed at pH 6.0 and further increase in pH resulted in decreased bacterial growth and enzyme production. The pH 6.0 appeared to be ideal for maximal bacterial growth as well as enzyme production (Fig. 3).

The enzyme is very sensitive to pH, hence, the selection of optimum pH is very essential for the production of amylase [21,23]. Different sources of α-amylases exhibiting considerable pH stability were reported [6]. The pH change observed during the growth of microbes also affects product stability in the medium. Earlier studies have revealed that fungi require slightly acidic pH and bacteria require neutral pH for optimum growth. pH is known to affect the synthesis and secretion of α-amylase just like its stability [11]. pH of 6.0 was found to be ideal for maximum bacterial growth and amylase production by *A.acidocaldarius* (Fig. 3).

Effect of medium constituents (starch, tryptone and yeast extract)

The concentration of starch as substrate had influence on the production of the enzyme (Fig. 4). It was observed that increase in the concentration of starch up to 1% in the culture

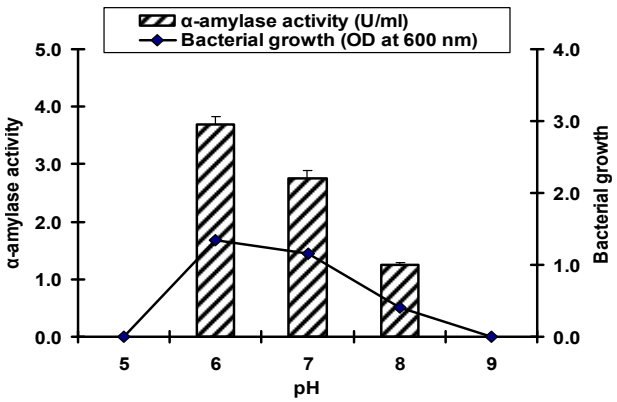

Fig. 3. Effect of initial medium pH on bacterial growth and α-amylase production.

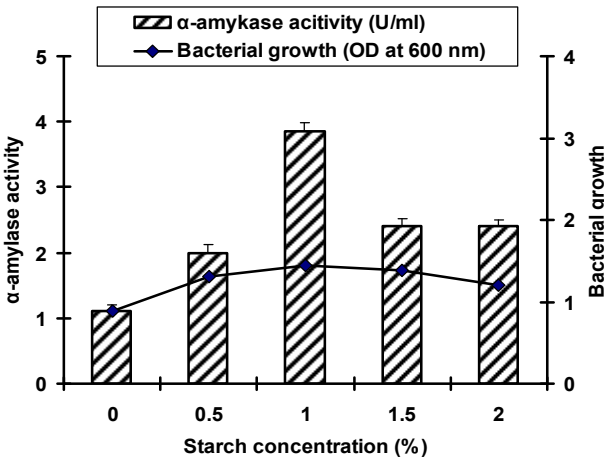

Fig. 4. Effect of starch concentration on bacterial growth and α-amylase production.

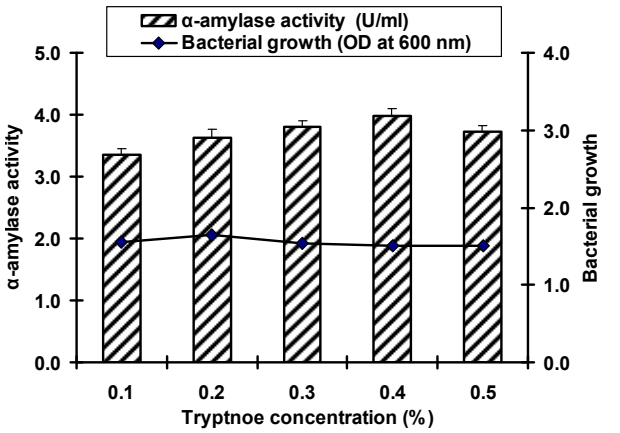

Fig. 5. Effect of tryptone concentration on bacterial growth and α-amylase production.

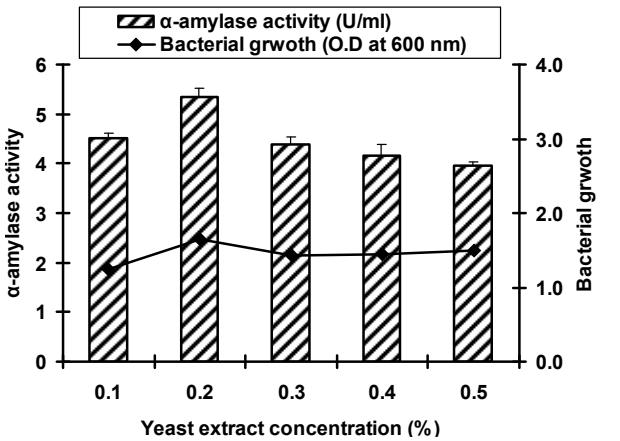

Fig. 6. Effect of yeast extract concentration on bacterial growth and α-amylase production.

medium enhanced bacterial growth and enzyme production with 3.8 U/ml and decreased thereafter. The bacterial growth was slightly increased when tryptone concentration increased from 0.1 to 0.2% but further increase in its concentration has no significant effect on bacterial growth. However, the enzyme production increased (3.98 U/ml) with the increase of tryptone concentrations up to 0.4% and decreased with further increase in its concentration (Fig. 5). Both bacterial growth and enzyme production were higher at 0.2% yeast extract concentration (Fig. 6). The maximal enzyme activity of 5.34 U/ml was obtained with 0.2% yeast extract. Further increase in its concentration resulted in decreased bacterial growth as well as enzyme production.

Effect of surfactants on bacterial growth and enzyme production

Bacterial growth and enzyme production were increased with the increase of concentration of surfactants like Tween-20 and SDS from 0.01 to 0.02% and decreased thereafter (Fig. 7, 8).

α-Amylase is an inducible enzyme and is usually induced in the presence of carbon sources such as starch, its hydrolytic product, or maltose [23]. Carbon sources such as starch and glycerol were known to increase enzyme production in *Bacillus* sp. PS-7 and *Bacillus* sp. I-3 [13,28]. Soluble starch has been found to be the best substrate for production of α-amylase by *B. stearothermophilus* [29]. Narayana and Vijayalakshmi [24] reported that, among the studied carbon sources viz., dextrose, lactose, maltose, mannitol, sucrose and trehalose used for amylase production, starch was found to be the best substrate. Kuo and Hartman reported that *Thermoactinomyces vulgaris* produces maximal activity of α-amylase when starch or maltose used as a carbon source [18]. In this study, starch at 1.0% concentration in the medium yielded maximum bacterial growth and amylase (Fig. 4). Dettori *et al.* reported that strains of *B. stearothermophilus* and *B. amylolyticus* secreted maximum α-amylase in a medium supplemented with 1% peptone, 0.5% yeast extract and 0.5% maltose under vigorous shaking conditions [5]. Dharani Aiyer compared the influence of organic and inorganic nitrogen sources and reported peptone to be a better nitrogen source for enzyme production by *B. licheniformis* SPT278 than ammonium hydrogen phosphate, the best among inorganic nitrogen sources [7]. Nguyen *et al.* reported for *Thermomyces lanuginosus*, the yeast extract showed a significant effect on α-amylase production [25,32]. The addition of the surfactant to the enzyme-substrate system increased the amount of reducing sugars during the hydrolysis

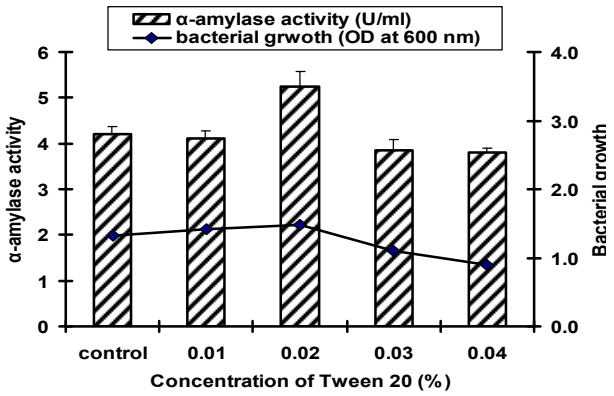

Fig. 7. Effect of Tween-20 on bacterial growth and α-amylase production.

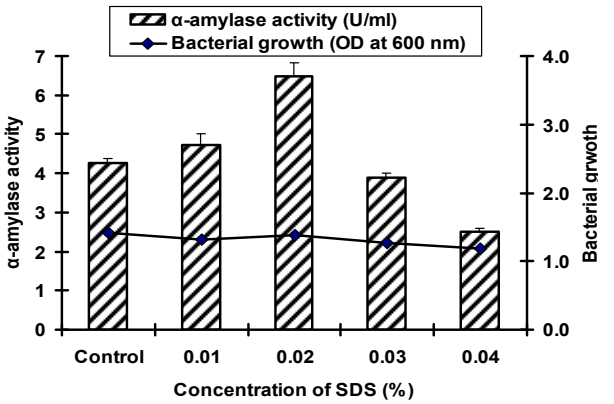

Fig. 8. Effect of SDS on bacterial growth and α-amylase production.

for *Bacillus amyloliquefaciens* [9]. Moreover, some nonionic surfactants are reported to increase the catalytic activity of enzymes [20]. An increasing usage of bacterial α-amylases in biotechnology has meant that a wide variety of conditions such as high temperature, extreme pH, and the presence of surfactants and organic solvents have to be applied to conventional production techniques [31].

Conclusion

In this investigation, medium carbon source i.e. starch at 1.0 % and nitrogen sources i.e. yeast extract, tryptone at 0.2% and surfactants i.e. SDS, Tween-20 at 0.02% concentrations have resulted in the better bacterial growth and enzyme production. The nature of culture conditions and composition of media for optimal production of α-amylase by *Alyclobacillus acidocaldarius* MTCC 8766 has been improved in this investigation.

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초록 : 세포외 고온성 α-아밀라제를 생산하는 *Alicyclobacillus acidocaldarius*의 분리 및 효소생산용 최적 배양 조건

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고온성 α-아밀라제를 생산하는 내열성 *Alicyclobacillus acidocaldarius* 균을 인도 Tirupati, Andhra Pradesh 지역의 가열한 미강 열수 추출물에서 분리하였다. 분리균인 내열성 *Alicyclobacillus acidocaldarius*가 생산하는 세포 외 α-아밀라제의 생산과 성장에 미치는 배양조건을 실험실 규모로 조사하였다. 그 결과 α-amylase의 고생산 최적 조건은 온도 60°C, pH 6.0 및 배지의 전분농도 1.0%, yeast extract와 tryptone은 0.2%를 나타냈다. Surfactants like Tween-20과 SDS 같은 계면활성제는 0.02%까지 균주의 성장과 효소 생산을 증가 시켰으나, 그 이상의 농도에서는 α-amylase 효소의 생산이 현저하게 감소하였다.