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EXTRACELLULAR ENZYME PRODUCTION BY INDIGENOUS THERMOPHILIC BACTERIA: PARTIAL PURIFICATION AND CHARACTERIZATION OF α-AMYLASE BY *BACILLUS* SP. WA21

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Abstract

Thermostability is a characteristic of most of the enzymes available for bulk industrial usage. Thermophilic microorganisms are of special interest as a source of novel thermostable enzymes. A total of 50 bacterial strains, isolated from local hot springs and ash samples were screened for the extracellular enzyme production including amylase, lipase, esterase, cellulase and β-galactosidase. As a follow up, studies on α-amylase were carried out with a bacterial strain identified as *Bacillus* sp. WA21 (from hot spring) on the basis of maximum zone of starch hydrolysis in agar plate medium. *Bacillus* WA21 showed growth over a wide range of temperature (35-55ºC) and pH (3-11) with optimum being 45ºC and pH 6. Maximum enzyme production was observed after 144 hours. The enzyme was found optimally active at 55ºC and pH 6. Temperature stability profile revealed that enzyme α-amylase retained more than half of its activity at 85ºC and between pH 5-9. Thus, *Bacillus* WA21 may be regarded as a promising source of α-amylase for biotechnological and industrial applications.

Introduction

For industrial applications, enzymes must be stable under process conditions. Generally, enzymes are preferred over chemical catalysts. Therefore, thermophilic microorganisms are believed to be potentially good alternative sources of thermostable enzymes (Egas *et al*., 1998). Hotsprings are a good source for the isolation of thermpohiles. Thermostable enzymes have been reported to have higher stability to organic solvents, acidic and alkaline pH and detergents (Vieille *et al.*, 1996). Other benefits include enhancement of reaction rate constant, increasing the diffusion rate as the medium viscosity decreases with an increasing temperature (Kumar & Swati, 2001). Amylases have wide spread applications in textile, paper, food and fermentation industries e.g., in manufacturing of bread, glucose and fructose syrups, fruit juices, sweeteners and alcoholic beverages (Haq *et al.,* 2010). Using thermostable amylases, thinning and liquefaction of starch is carried out at elevated temperature (Crabb and Mitchinson, 1997). To prevent the browning effect and to reduce the viscosity of the starch pastes for the production of sweeteners from the starch, the temperature should be 50˚C or more, for which thermostable α-amylase is required in order to sustain the process temperature (Castro *et al.*, 1999). Bacteria belonging to the genus Bacillus have been widely used for the commercial production of thermostable α-amylase (Kubrak *et al.*, 2010). We hereby report the production of thermostable α-amylase from a strain of *Bacillus* sp. WA21, isolated from the hotsprings of Karachi.

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Materials and Methods

Isolation of the organisms: Water samples from the Karachi hot springs and ash samples were mixed in 100ml of sterile water, agitated for 2 hours at 100 rpm, poured and spread onto BHI agar and LB agar plates respectively (Teodoro & Martins, 2000). The plates were incubated at 50°C for 24 hours and the colonies that appeared were purified by streaking on BHI agar plates. The purity was further checked by gram staining (Giffel *et al.,* 1995).

Temperature tolerance: Temperature tolerance profile of the isolates was checked on Luria-Bertani agar medium by incubating the plates for 24 hours at different temperatures ranging from 35-70°C (Narayan *et al.,* 2008).

Enzyme based screening: Potential of the isolates to produce enzymes of industrial importance like amylase, cellulase, β-galactosidase, lipase and esterase was determined on solid agar medium incorporated with starch (1%), cellulose (1%), lactose (1%), tween 20 (1%) and tween 80 (1%), respectively (Carrim *et al.,* 2006). Zone of hydrolysis was observed with 2% iodine solution (Nusrat & Rahman, 2007).

Considering the spectrum of industrial applications in different fields such as chemical, medical and analytical chemistry as well as in textile, paper, food, fermentation, brewing and distilling industries (Pandey *et al.,* 2000), the strain *Bacillus* WA21 which produced maximum zone of starch degradation (by producing amylase) was selected for further studies. Identification of the isolate was carried out on the basis of microscopic, cultural and biochemical characteristics (Holt *et al.,* 1994).

Optimization of growth conditions and enzyme production: To determine the effect of temperature and pH on the growth of *Bacillus* WA21, experiments were carried out at varying temperatures (35°C-60°C) and pH (3-11). The turbidity of the culture was determined at different time intervals by measuring the optical density at 600nm using spectrophotometer (Rasooli *et al.,* 2008). Enzyme assay was carried out based on dinitrosalicylic acid (Bernfeld, 1955).

Enzyme isolation and partial purification: Six days old broth culture, grown under optimized growth conditions was centrifuged at 6000 g at 4°C for 30 min. The cell free supernatant was precipitated with ammonium sulphate (80% saturation). Precipitates were resuspended in 20mM sodium phosphate buffer (pH 6.0) to get partially purified crude enzyme preparation which was then characterized (Konsoula & Laikopoulou-Kyriakides, 2007).

Enzyme assay: One ml of substrate solution containing 1% (w/v) soluble starch in 20mM sodium phosphate buffer (pH 6.0) and 1ml of enzyme solution (cell free supernatant) were incubated at 55°C for 3 min and the reaction was stopped by adding 96mM 3, 5-dinitrosalicylic reagent. After that tube was kept in a boiling water bath for exactly 15 min and then cooled on ice to room temperature. Absorbance was measured at 540nm using spectrophotometer (Bernfeld, 1955).

Characterization of α-amylase

Effect of temperature on the activity of enzyme: The enzyme activity was measured by incubating the partially purified enzyme preparation at various temperatures (35-95°C)

with starch as substrate prepared in 20mM sodium phosphate buffer at pH 6.0 (Behal *et al.,* 2006).

Effect of temperature on the stability of enzyme: In order to determine the temperature stability of the enzyme, enzyme solutions in different tubes were incubated at various temperatures from 35-95°C for 10 min (Carvalho *et al.,* 2008) and then assayed as mentioned earlier.

Effect of pH on activity of the enzyme: The activity was measured at different pH values from 3-11 with starch as the substrate. The different buffers used included: 20mM acetate buffer (pH 3.0-5.0); 20mM sodium phosphate buffer (pH 6.0-8.0) and 20mM Tris/HCl (pH 9.0-11.0) (Reyed, 2007).

Effect of pH on the stability of enzyme: To determine the enzyme stability at various pH values (3-11), enzyme solution was incubated in 20mM of each of the buffer at 55°C for 1 hour and then enzyme assay was performed (Carvalho *et al.,* 2008).

Results and Discussion

The present study reveals the potential of bacterial strains for extracellular enzyme production, isolated from ash samples and hotsprings. A total of 42 isolates from the hot springs and 8 isolates from the ash sample were identified on the basis of their microscopic characteristics. Microscopic characteristics of the isolates showed that 84% of the total isolates were gram-positive rods while the rest were gram-negative. This shows an increased presence of gram-positive bacteria in hot springs. Narayan *et al*., (2008) reported a total of 76.9% gram-positive organisms isolated from Savusavu hot spring in Fiji.

Temperature ranges for the growth of bacterial isolates were determined by incubating strains at temperatures ranging from 35-70°C for 24 hours. It was noted that most of the isolates could grow upto 65°C and were unable to grow beyond 65°C. The isolated strains meet the criteria of thermophilic organisms that grow at temperatures above 50°C (Perry & Staley, 1997). Adiguzel (2009) isolated bacterial strains from hot springs in Turkey which showed growth above 50°C. All the isolates (fifty in number) were screened for amylase, cellulase, β-galactosidase, lipase and esterase activities by employing zone clearing technique using respective substrates in agar medium. Ten *et al*., (2005) used plate assay for screening of polysaccharide and protein degrading microorganisms. Extracellular enzymatic profiles of the bacterial strains isolated from the hot spring of West Kameng District of Arunachal Pradesh, India were also studied by Bora & Kalita (2007).

In the present study, all of the strains have shown extracellular multi enzyme activity. However, majority of isolates were amylase (82%) and esterase (74%) producers while 66% of the isolates showed lipase and β-galactosidase production (Fig. 1). Percentage of cellulase producing isolates was less (50%) as compared to other enzymes. Low count of cellulase producers is most likely due to the low content of organic matter in the hotspring water (Kazue *et al.,* 2006). The production of extracellular lipases and esterases by *Bacillus* sp. was reported by Jung *et al*., (2003) while Batra *et al*., (2002) reported the production of β-galactosidase by *Bacillus* sp. isolated from hotspring.

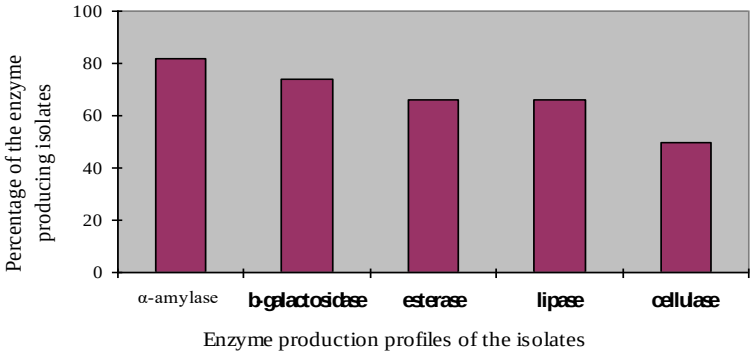

Fig. 1. Multi -enzyme production by the isolates.

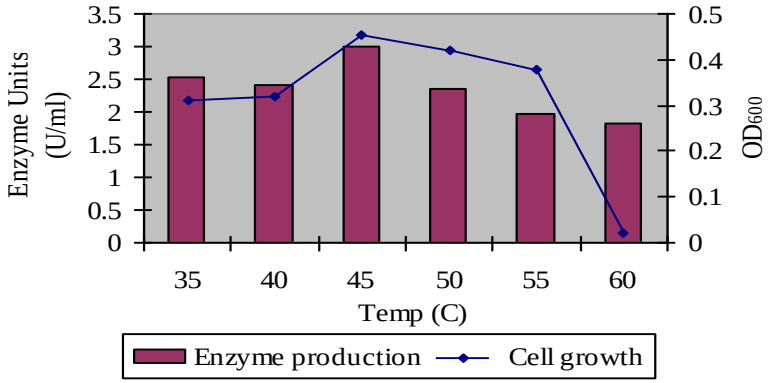

Fig. 2. Effect of temperature on the growth and α-amylase enzyme production.

The isolate showing the maximum zone of starch hydrolysis was selected and the strain was identified as gram-positive bacterium belonging to the genus *Bacillus* and designated as *Bacillus* sp. WA21. Reportedly, members of the genus *Bacillus* were found to be better producers of α-amylase (Adams & Kelly, 1998; Khajeh *et al*., 2001).

Optimization of cultural conditions such as medium, temperature and pH is crucial for the production of enzymes (Cherry *et al.*, 2004). Figure 2 indicates the effect of different temperatures on α-amylase production. Accordingly, the growth of *Bacillus* sp. WA21 and production of α-amylase was found optimum at 45°C thus, this temperature was selected for further enzyme production studies. Asif *et al*., (2009) reported 45°C as optimum temperature for the growth of *Bacillus* isolated from hot spring. Our findings are in agreement with Vieille *et al*., (1996) who reported that thermophiles produced optimally active enzyme at temperatures close to the producing organism's optimal growth temperature. Our results showed that the optimal conditions for the cell growth were equally adequate for the enzyme production.

The effect of pH is crucial in terms of growth of the producing organism and the biosynthesis of α-amylase. According to our studies, both the growth of *Bacillus* sp. WA21 and the α-amylase production continued over a wide range of pH (3-11) as shown in Fig. 3. However, optimum growth and enzyme production was achieved at pH 6. Utong *et al*., (2006) had described the ability of *Bacillus sphericus* to produce extracellular enzyme if grown at pH 6-9 range.

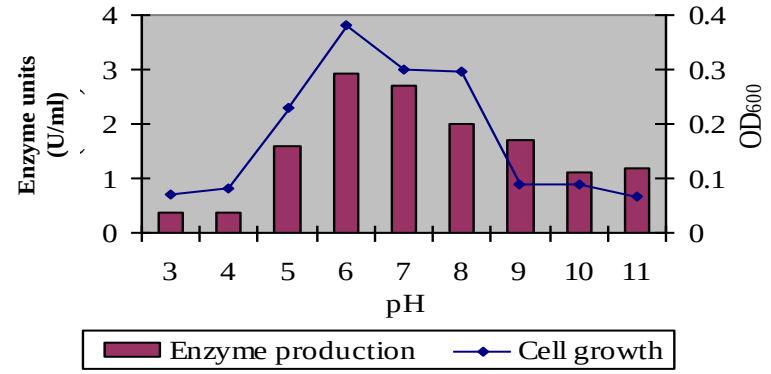

Fig. 3. Effect of pH on growth and α-amylase enzyme production.

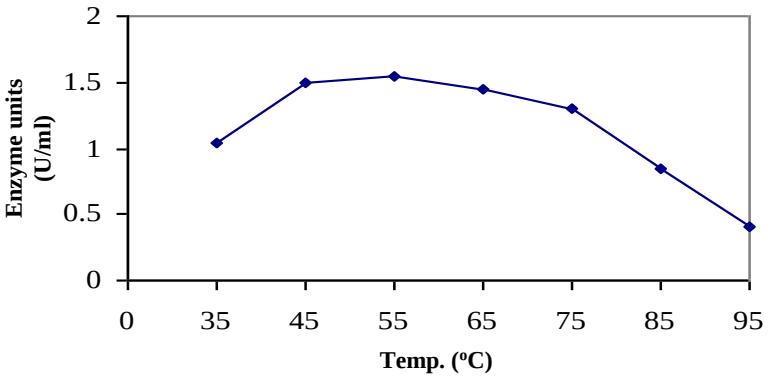

Fig. 4. Activity of partially purified α-amylase at different temperatures.

Optimal enzyme production was carried out under optimized cultural conditions by measuring the time when the yield of enzyme achieves maxima. Alpha amylase production according to our studies was increased with the increase in incubation time and a significant production was observed after reaching 144 hours of growth. Further increase in incubation period resulted in the decreased production of the enzyme. It might be due to the depletion of the nutrients, death phase of organism or due to the production of protease in the medium as suggested by Lealem & Gashe (1994).

Figure 4 shows the effect of temperature on the activity of α-amylase whereby, maximum enzyme activity was observed at 55°C. Chakraborty *et al*., (2000) found that maximum activity of α-amylase was observed at 55°C. Stability of α-amylase in our study was observed over a temperature range of 35-85°C (more than 60%) while 100% stability was observed at 65 °C (Fig. 5). Thermal stability of α-amylase was also reported by Al- Qodah *et al*., (2007). Accordingly, α-amylase remained stable at 64°C for 3.5 hours. However, Konsoula & Liakopoulo-Kyriakides (2007) found that α-amylase enzyme extract retained 96% activity when incubated at 60°C for 2 hours.

Hydrolytic action of α-amylase is greatly affected by pH. In the present study, maximum hydrolytic activity was achieved at slightly acidic pH (6) (Fig. 6) and the enzyme retained 100% stability at this pH as depicted in Fig. 7. Similar results were obtained by Khoo *et al*., (1994). However, Thippeswamy *et al*., (2006) reported 6.5 as optimum pH for α-amylase activity.

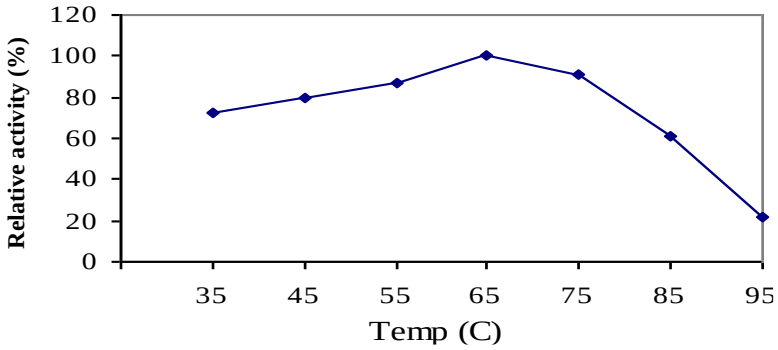

Fig. 5. Stability of partially purified amylase at different temperature.

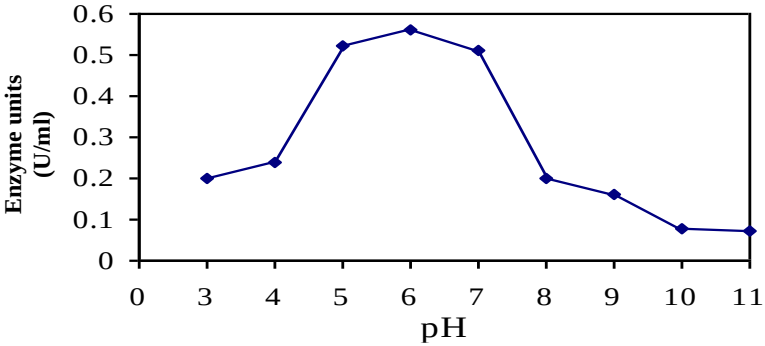

Fig. 6. Activity of partially purified α-amylase at different pH.

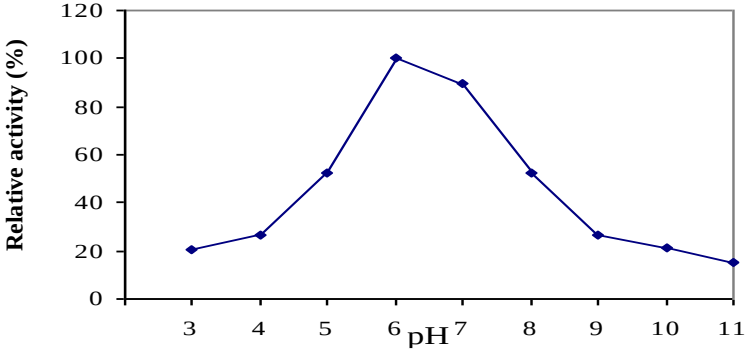

Fig. 7. Stability of partially purified α-amylase at different pH.

The usefulness of an enzyme from any organism for starch hydrolysis depends upon its potential to degrade native starch to oligosaccharides, glucose and other products at high temperatures and over a wide range of pH (Lealem & Gashe, 1994). The ability of *Bacillus* sp. WA21 to degrade native starch, at a wide range of pH and the thermal stability of α-amylase are the attractive attributes which make this bacterial strain to be a potential candidate in starch hydrolysis.

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