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α-Amylase Production by *Bacillus licheniformis* under Solid State Fermentation Conditions and its Cross Linking with Metalosalts to Confer Thermostability

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ABSTARCT

The production potential of *Bacillus licheniformis* strain RT_7YC for extracellular α-amylase was analyzed through solid state fermentation. Enzyme concentration produced under varying concentrations of substrate and ecological variables was calculated by using DNS and starch-iodine methods. The maximum production of α-amylase was obtained at 20% wheat bran. It was observed that corn steep liquor was found as best nitrogen source for α-amylase production. It was also found that enzyme was stable at 60°C when treated for 10 min. Moreover, it was also much stable at 10 mM concentration of $CaCl_2$. © 2010 Friends Science Publishers

Key Words: α-Amylase; Solid state fermentation; *Bacillus licheniformis*; Stability

INTRODUCTION

Enzymes are among the most important products obtained for human needs through microbial sources. Among these biomolecules, α-amylase (α-1,4 glucan glucanohydrolase) is an exo-acting enzyme that yield maltose upon hydrolysis of various starches. The enzyme has been placed in family 13 of glycosyl hydrolases category in IUPAC classification. The α-amylase is being isolated from a number of microbial sources and every enzyme moiety isolated from various microbial sources is unique in their characteristics. Thus, commercial production of any thermostable α-amylase from some unexplored microbe is, therefore, imperative. Various attempts have been made so far on studying the yield potential of α-amylase from different microbial strains. *Bacillus* spp., have been found to be the best candidate for commercial production of this enzyme that is a soil-borne thermophilic bacterial genus (Reilly, 1991). Baysal *et al.* (2003) studied the production potential of α-amylase from *B. subtilis* on wheat bran and rice husk. Studies show that, *B. lichenifomis* is the best studied bacterial strain. Most thermostable α-amylases used in industry are produced from *B. licheniformis.* It has an optimal temperature of 90°C and required additional Ca^{2+} for its thermostability (Volkin & Middaugh, 1992; Arikan, 2008; Haq *et al.,* 2010).

Solid substrate fermentation has attracted renewed interest and attention from researchers due to recent development in the field of biotechnology. Solid state fermentation (SSF) has tremendous potential for the production of enzymes not only for laboratory but also for commercial production (Goes *et al.,* 1999). The present study has been designed for the selection of new medium such as metallosalt for the production of α-amylase by *Bacillus* for conferring thermostability to the enzyme.

MATERIALS AND METHODS

Inoculum preparation: *B. licheniformis*-RT_7YC strain was obtained from the culture collection of Industrial Biotechnology Division, NIBGE, Faisalabad, Pakistan. Inoculum was prepared in conical flasks after following Castro *et al.* (1999) in aqueous milieu with pH adjusted at 7. The medium was autoclaved for 15 min at 121°C. Spores of the *B. licheniformis* RT_7YC strain were transferred into medium by following aseptic streaking technique. The inoculum was allowed to grow at 37°C on rotatory shaker at 180 rpm for 24 h. The spore suspension was used for inoculation of fermentation medium.

Solid state fermentation: Enzyme was produced in conical flasks by *B. licheniformis* strain RT_7YC, using wheat bran and maize bran as inducing substrates. Corn steep liquor

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used as an additional nitrogen source at different levels (%) and the culture was grown for 8 days at 37°C in an incubator. After centrifugation at 12,000 rpm for 15 min the supernatant was carefully collected for analysis (Miller, 1959).

Analytical: Enzyme assay methodology was performed by following starch-iodine method and DNS method as described by Miller (1959). Enzyme suspension samples at various steps were subjected to protein estimation spectrophotometrically, while protein contents were measured at 610 nm along with running the substrate blank (Bradford, 1976).

Thermostability of enzyme towards metalosalts: The effect of NaCl and $CaCl_2$ were studied by subjecting the enzyme to varying concentrations of aforesaid salts at temperature regimes of 60, 70, 80 and 90˚C to seek the stability of *Bacillus licheniformis* α-amylase.

RESULTS AND DISCUSSION

Hamilton *et al.* (1999) analyzed that growth and enzyme production by microorganisms are greatly influenced by both environmental conditions and nutrients available with growth medium. Extensive work has been going on all over the world to select the suitable organisms and efficient inducers for production of concentrated α-amylases using biomass wastes in solid state fermentation.

Effect of carbon/nitrogen sources on enzyme production: The effect of different concentrations of wheat and maize bran on biosynthesis of α-amylase from *B. licheniformis* RT_7YC strain was studied. It was found that maximum growth and enzyme production occurred in fermentation medium with wheat bran 10 % and CSL 8% (Table I). The maximum enzyme activity i.e., 1338 U mL^{-1} obtained at 20% wheat bran medium by DNS method after 192 h of incubation. The protein estimation was 10.13 mg mL^{-1} and the specific activity observed by DNS method was 132 U mg^{-1}. Maximum enzyme activity of 3163 U mL^{-1} at 20% wheat bran level was observed by starch decolourization method after 192 h of incubation with 312 mg mL^{-1} specific activity. By utilizing maize bran as carbon source (20%; 192 h incubation), enzyme activity and specific activity obtained to be 1500 U mL^{-1} and 208 U mg^{-1}, respectively with DNS method.

In present investigation, wheat bran obtained the highest level of α-amylase activity (5433 U mL^{-1}) as compared to maize bran. These studies indicate that raw starches have the significant potential to be used in industrial biotechnology, whereas wheat bran at 20% level is the better carbon source for α-amylase production and this conclusion is also supported by Hamilton *et al.* (1999). It was also suggested from the results mentioned in Table I, that DNS method of enzyme assay is preferred over starch-iodine.

Thermostability of enzyme: We studied the effect of temperature at various levels with varying time periods of

Fig. 1: Effect on the thermostability of native and cross linked α-amylase of *Bacillus licheniformis* RT_7YC at 60°C

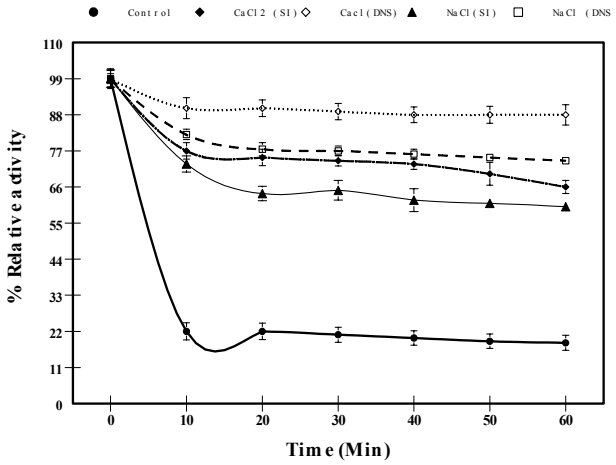
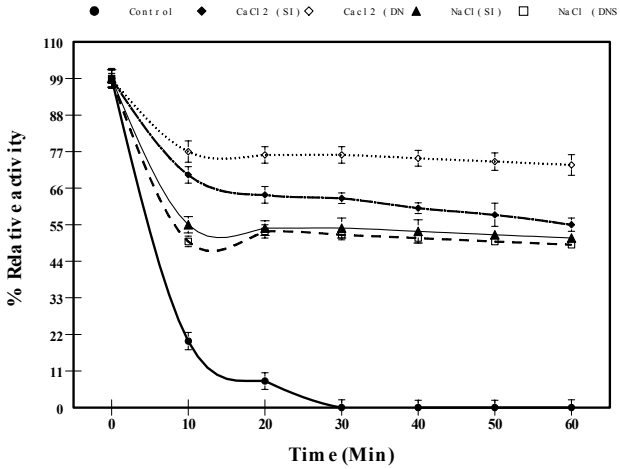


Fig. 2: Effect on the thermostability of native and cross linked α-amylase of *Bacillus licheniformis* RT_7YC at 70°C



10, 20, 30, 40, 50 and 60 min. It was observed that maximum loss of enzyme activity was occurred during first 10 min of exposure and afterwards enzyme was stable up to 60°C. The activity was lowered significantly at 70°C that further showed a decline at 80°C beyond that enzyme became inactive.

Effect of enzyme stability with addition of metalosalts (NaCl & $CaCl_2$; 10 mM each) was observed at 60, 70, 80 and 90°C temperatures under optimal substrate conditions of 20% wheat bran at pH 7 (Figs. 1-4). Interaction between thermostability verses time of exposure of enzyme was found to be maximum at 60°C up to 10 min. Goyal *et al.* (1995) demonstrated that α-amylase from a hyperproducing strain of *Bacillus sp*. E2 was stable at 70°C for 30 min but quickly inactivated at higher temperature than 70°C. Elhawary (1991) studied that a partially purified α-amylase

Table I: Effect of substrate concentration on α-amylase production under solid state fermentation by *Bacillus licheniformis* RT_7YC

Substrate concentration (% wheat bran)	Enzyme Activity (U mL^{-1})		Protein Estimation (mg mL^{-1})	Specific activity (U mg^{-1})		Substrate utilization (%age)
	DNS Method	Starch Iodine method		DNS method	Starch Iodine method	
20	1338 ±0.02	3163 ± 0.1	10.13 ± 0.02	132 ± 0.1	312 ± 0.01	40.50
15	1528 ± 0.01	1631 ± 0.2	7.17 ± 0.1	213 ± 0.2	224 ± 0.03	46.50
10	2344 ± 0.02	1482 ± 0.3	8.17 ± 0.1	287 ±0.04	181 ± 0.04	41.0

was active over a wide range of temperature and retained 44% of its activity at 90°C for 2 h.

It was observed that enzyme is more stable in the presence of $CaCl_2$ as compared to NaCl that showed 66% of activity with $CaCl_2$ and 44% activity with NaCl at 60°C. Furthermore, linking with $CaCl_2$ conferred extreme stability towards enzyme molecule that showed an appreciable activity at 90°C. This result was also supported by Goyal *et al.* (1995) who studied α-amylase in the presence of 10 mM Ca^{2+}. However, the enzyme was stable at 90°C for 10 min and after 30 min at 100°C still retained 26% of its initial activity. Kichakova (1991) observed that *Bacillus* sp. 86 being periodically cultivated in the optimized medium containing 4% soluble starch and 0.5% $CaCl_2$ showed an 8 fold increase in amylolytic activity as compared to the enzyme activity in primary medium.

In relation to the studies made on α-amylase characterization obtained from *B. licheniformis*, we found this bacterial strain to be the potential alternative microbe for the production of α-amylase as it possesses an active species of the enzyme that is a bit thermolabile. The handicap can be overcome by linking it with metal ion and $CaCl_2$ is better moiety as compared to NaCl for conferring thermostability to α-amylase of *B. licheniformis* RT_7YC. As a large amount of foreign exchange is involved to import such enzymes and meet local consumption in our country, serious attention and efforts are required to produce the indigenous amylases by using locally improved strains. The enzyme produced by *B. licheniformis* RT_7YC retained its activity at higher temperatures.

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