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Production of New Thermostable and Acidstable Alpha-Amylase from Bacillus sp.B1 and B2 under Solid State Fermentation

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Abstract
This study was conducted to identify and screen native *Bacillus* strains from hot spring samples for alpha amylase activity. Positive strains for the enzyme activities were *Bacillus, SP.B1 and Bacillus, SP.B2.*The isolated cultures screened by solid state fermentation using wheat bran as its substrate. A crude extract enzyme applied to amylase assay to determine the enzyme's activity against standard curve of maltose. As a result, wheat bran was shown to be the normal solid organic substrate for the highest alpha amylase production with 244.10 and 187.15 U/ml/min with pH 5 and 5.5in *Bacillus.Sp*.B2 and *Bacillus.Sp*.B1, respectively in 48 h. The incubation temperature 50°C was found to be optimum for the production of alpha amylase. The enzymes were stable for 30 min between 65°C-70°C.

Keywords: alpha-amylase, *Bacillus*, Enzyme activity, wheat bran, solid state fermentation

Introduction
Microbial enzymes are widely used in several industries, notably in detergent, food processing, brewing, and pharmaceuticals [1]. Bacterial amylases have longer shelf life and can be stored for weeks without significant loss of activity [2]. The production alpha amylase by fermentation has been a worth praising achievement in the field of Industrial microbiology. Enzymes production was carried out by solid state fermentation and it had been established as a superior technique for the production of enzymes [3]. Solid state fermentation (SSF) has numerous advantages such as: superior productivity, simple technique, low capital investment, low energy requirement and less waste water output, better product recovery and lack of foam build-up [4]. The present study, reports on the purification, characterization of an acidophilic thermostable alpha-amylase produced by *Bacillus sp*. B1 and B2 which were newly isolated from the Jowshan hot spring in Kerman, Iran. The properties of the enzyme, including theirs pH profiles and thermostability, revealed that they can be considered new alpha amylase.

2. Material and Methods
2.1. Isolation of Bacteria
Two strains were isolated from Jowshan hot spring in Kerman, Iran. The identification of bacterial strains were based on morphological, biochemical and 16s rRNA gene sequencing [5].

2.2. Screening of bacterial isolates
Primary screening of bacterial isolates for production of alpha amylase was done by the starch agar plate containing (g l^{-1}) starch (Merck, Germany), 10.0; peptone, 2.0; yeast extract, 5.0; NaCl, 1.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; $CaCl_2$, 0.1; agar, 20.0 [6]. After 16 hour growth at 50°C isolates that showed the biggest zone of clearance in starch hydrolysis were selected for production in Solid State Fermentation.

2.3. Enzyme production in Solid State Fermentation
2.3.1. Solid state fermentation
tests were conducted in 250ml Erlenmeyer flasks containing 10g of wheat bran impregnated with 10ml of sterile liquid nutrient medium containing(%): (KH2PO4 - 0.1, NaCl-0.25, MgSO4.7H2O- 0.01, CaCl2- 0.01). The flasks were autoclaved and inoculated with 1ml of the prepared inoculums, thoroughly mixed and followed by incubation at 37°C for 5 days. The organism was grown at 37°C for 5 days in medium with shaking on a shaker (140 rpm). Samples were taken from 12 to 120 h. after 5 days 100 ml of the phosphate buffer (pH 6.8) was added in each flask. Flasks were rotated on the rotary shaker for one hour and then capacity was filtered through muslin cloth. Filtrate was centrifugation. The supernatant of the

culture after centrifugation (10.000 rpm, 10 min) at 4°C was used to determine extracellular amylase activity.

2.4. Enzyme assay
Alpha-amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate [7]. The reaction mixture contained 0.5 ml of crude enzyme and 1 ml of sodium phosphate buffer (pH 7.0) containing 1% soluble starch and incubated at 37°C for 10 min, the amount of reducing sugar released in the mixture was determined by the addition of 2 ml of 3, 5dinitrosalicylic acid method [8] followed by boiling for 10 min and to develop color. The absorbance of the mixture was measured at 550 nm, in a UV-spectrophotometer and glucose was used to create a standard curve. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1 µmol glucose per minute under the assay condition. All analytical measurements were performed at least in triplicates.

2.5. Assay of protein concentration
The protein concentration was determined by the Lowry's method using bovine serum albumin used as Standard [9].

2.6. Optimization of fermentation conditions for enzyme production
The optimization of medium components and fermentation process is of primary importance in any fermentation process. Combinations of the best substrates were employed for further optimization of process parameters, namely initial moisture content (20, 30, 40, 50, and 60 %), incubation time (12, 24, 48, 72, 96, 120 h), incubation temperature (37-100°C), initial pH of the medium (pH 3.0–10.0), organic nitrogen sources (casein, peptone, yeast extract and urea). To study the efficacy of various inducers, the medium was supplemented independently with 1 % Glucose, Maltose, Starch and Sucrose [10].

2.7. Purification of alpha- amylase and SDS-PAGE
Amylase purification and SDS–PAGE the crude amylase was subjected to ultra filtration in a stirred cell using a 30 kDa Millipore membrane. The retentate was desalted by PD 10 column and loaded on DEAE cellulose column (1.6 cm ×45 cm). [DE52, What-man] Unbound proteins were removed by washing with Tris–HCl buffer (50 mM; pH 8.0). Elution was carried out by using 0–1.0 M NaCl gradient at a flow rate of 20 mL/h. Active fractions were pooled and loaded onto Sephadex G-100 column(1.6 cm ×50 cm)(Merck Germany) [11] equilibrated with citrate phosphate buffer pH 6.8. The active fractions were pooled and subjected to polyacrylamide gel electrophoresis (PAGE).SDS–PAGE was carried according to the method of [12] using 12% cross-linked polyacrylamide gel. For zymography, SDS gel was washed with 20% (v/v) isopropanol (to remove SDS) for 30 min and then with citrate phosphate buffer (50 mM; pH 7.0) for 30 min. Further, the gel was soaked in 1% (w/v) starch solution in citrate phosphate buffer for 2 h at 40°C.The molecular mass was determined by using a low molecular weight marker (Color Brust Marker SIGMA.USA). After electrophoresis, protein bands were visualized by staining with comassie blue G 250. It was then washed with distilled water and stained with 0.1 N iodine solutions. A white zone of hydrolysis on gel indicated amylase activity.

3. Result
In the present studies wheat bran as the substrate were used for growth and α-amylase production by the *Bacillus Sp.*B1 and B2. The results were shown high titer of α-amylase activity (244.10 and 187.15 U/ml/min in *Bacillus.Sp*.B2 and *Bacillus.Sp*.B1).

3.1. Effect of fermentation conditions for enzyme production
The results of the present study showed that amylase production increased with increase in incubation time linearly till 48 h and on further incubation till 120 h, there was a decrease in the amylase production. Maximum amylase production was obtained at 48h. Maximum enzymes production were obtained with an initial moisture content of 60% and the maximum enzymes production obtained were 244.10 and 187.15 U/ml for B2 and B1. Up to 60%, there were a linear increase in enzyme production and upon further increase; there were a decrease in enzymes production. The effect of additional nitrogen sources like, casein, urea, yeast extract, peptone at 1% supplementation on the medium was tested and the results were further analyzed "Fig.1".

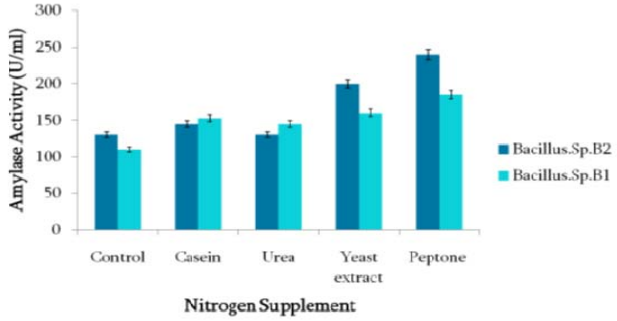

Fig 1: Amylase activity with additional supplementation of various nitrogen substrates

A control flask was maintained without additional nitrogen supplementation. Several carbon substrates like glucose, starch, maltose, lactose and sucrose were tested along with control to evaluate the enzyme production by SSF "Fig. 2". On supplementation of various carbon substrates, maximum enzyme production was exhibited by starch (1% w/w). Results showed different impact on enzyme production with different supplement substrates. The maximum enzyme production obtained was 235.11 and 163.2 U/ml for B2 and B1 with 1% w/w starch. Supplementation of glucose also enhanced enzyme.

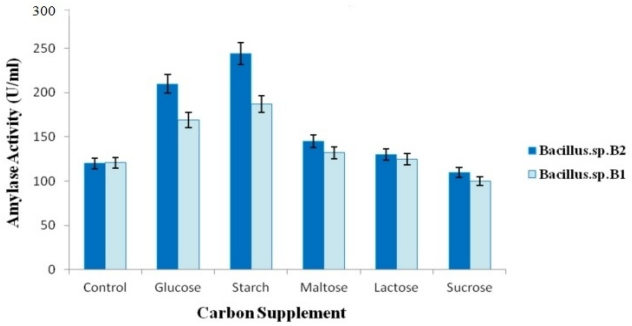

Fig 2: Amylase activity with additional supplementation of various carbon substrates

3.2. Effect of pH on the enzyme activity and stability

The amylase activity was measured at various values of pH for two strains and the maximum activity was observed at pH 5 and 5.5 for B2 and B1. The pH stability was tested by 60 min of pre-incubation of the purified enzyme in phosphate buffers. Our enzymes were more acidophil. These result as shown in "Fig. 3" and the enzymes were stable from pH 4.0-6.5 "Fig. 4".

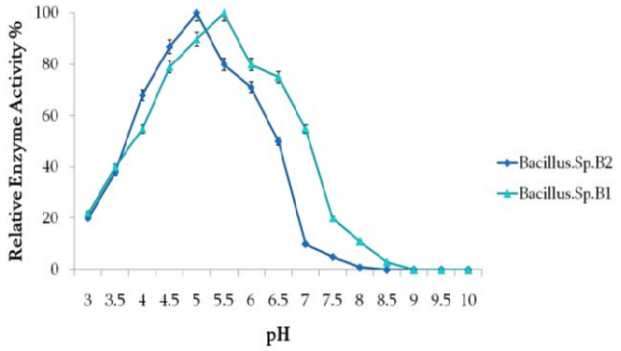

Fig 3: Effect of pH on activity of the purified amylase

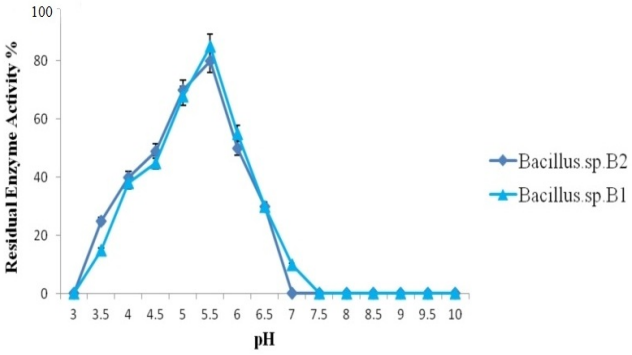

Fig 4: Stability alpha-amylase for two isolates Bacillus. Sp. B1and B2 in 60 min

3.3. Effect of temperature on the enzyme activity

The isolates Bacillus strain were tested in a wide range of temperatures ranging from 37 to 100°C. Optimum amylase production was obtained at a temperature of 50°C as shown in "Fig. 5". In the present experiment with increase in temperature, enzyme production increased up to a certain level and upon further increase of temperature, production decreased. The enzymes were stable for 30 min between 65°C-70°C that were shown in "Fig. 6".

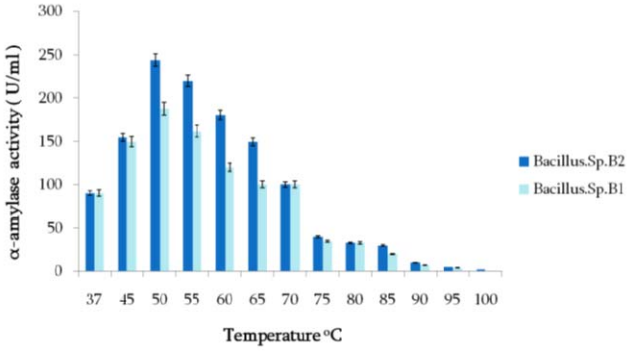

Fig 5: Effect of temperature on activity of the purified amylase in 48 h

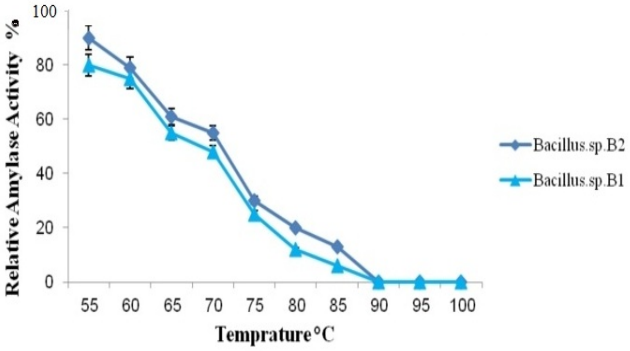

Fig 6: Thermostability of the purified amylase at 30 min

3.4. Purification of the enzyme

The purification of the enzyme was performed as described in methods section. the purified enzyme demonstrated an apparent single protein band on SDS-PAGE. The molecular weight of the single protein band was estimated to be 36 kDa and 28 kDa for B2 and B1.The result was shown in "Fig.7".

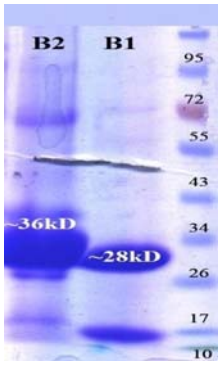

Fig 7: Polyacrylamide gel electrophoresis of the purified amylase from Bacillus sp. B1 and B2 SDS-PAGE

4. Discussion

Solid-state fermentation (SSF) is fermentation of solid substrates at low moisture levels or water Activities; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism [13]. The selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation [14]. In the present studies wheat bran as the substrate were used for growth and α-amylase production by the *Bacillus Sp.*B1and B2. The results were shown high titer of α-amylase activity (244.10 and 187.15 U/ml/min in *Bacillus.Sp.*B2 and *Bacillus.Sp.*B1 in 48h).But it was reported that wheat bran was found to be the best substrate and suitable for necessary manipulation [15, 16].

The effect of moisture level on enzyme production for wheat brarn.60% moisture content enhanced maximum enzyme production when compared to 20, 30, 40 and 50% of wheat bran. The effect of different nitrogen sources on the production of α-amylase. When different nitrogen sources was supplemented in the production medium, peptone gave the highest α- amylase activity, although slight reduction in amylolytic activity was observed when urea was used as supplements. Addition of nitrogen sources has been reported to have an inducing effect on the production of various enzymes including α-amylase. Similar observations were

noticed in case of amylase production by other reporter [17, 18]. The supplementation of wheat bran with the different carbon sources; glucose, starch, sucrose, lactose, maltose at 1 % (w/w) concentration showed increased production of enzyme with starch (235.11 and 163.2 U/ml for B2 and B1).Supplementation of glucose also enhanced enzyme. The stability of α-amylase was observed to best able over a temperature range of 65ºC to 70ºC. A gradual increase in stability was observed as the temperature was increased gradually from 50ºC to70ºC, amylase stability slightly decreased as the temperature was further increased to 70ºC This agrees with the report of [19] who reported that α-amylase was highly stable for 1 hour at 60˚C and 70˚C, while at 80˚C and 90˚C, 12%and 48% of the original activities were lost, respectively. [20] Also reported reduction in enzyme activity at temperatures above70ºC.The pH optima of the enzyme were found to be 5 and 5.5 for B2 and B1 with stability in range 4-6.5. Some are only stable within a low pH range [21]. The purified enzyme demonstrated an apparent single protein band on SDS-PAGE. The molecular weight of the single protein band was estimated to be 36 and 28 KDa for B2 and B1. The mass of α-amylases from various microbial sources vary From 22.5 to 184 kDa [22].

5. Conclusion

Commercial α -amylase is usually produced by submerged fermentation; however, SSF appears promising due to the natural potential and advantages it offers. Based on the present study, it appears that cotton stalk, which is inexpensive and readily available agricultural substance, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields of α-amylase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF.

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