Partial purification and characterization of alpha amylase from *Bacillus amyloliquefaciens* by solid state fermentation

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Abstract

Among different types of enzymes obtained from microbial sources, amylases are the most widely used in industries. In the present study, different agro products were used for production of alpha amylase by using *Bacillus amyloliquefaciens.* The present study showed that wheat flour and wheat bran has higher efficiency in α- amylase production using *Bacillus amyloliquefaciens.* The partial purification of the enzyme was done by fractionation of raw extract with ammonium sulphate salt in a variety of saturated degree to get the partial purified enzyme. The partial characterization of partially purified enzyme was done by determination of the optimum pH and temperature at which the enzyme activity is maximum. The purified alpha amylase obtained from *Bacillus amyloliquefaciens* make it good candidate for wide application as additives and starch modification.

Keywords: alpha amylase, agro products, *Bacillus amyloliquefaciens.*

Introduction

Alpha (α)-amylases are the enzymes that are extra-cellular and hydrolyze internal 1, 4-glycosidic cordons in starch to produce low molecular weight products, such glucose, and maltose as well as maltotriose units. These are the most important class of industrial enzymes which are of excellent significance within biotechnology and also occupy around 25% on the world enzyme market. Amylases can be obtained through plant, animal and microbial sources. Presently, majority of microbial amylases tend to be commercially available and the starch processing industrial sectors, they have nearly changed chemical hydrolysis of starch. The broad applications of microbial amylases within the industries are usually endorsed for their superior balance in comparison to amylases of plant and animal origin. The production of amylases utilizing microorganisms features a major benefit of economic industrial production and simple manipulation associated with microbes with regard to obtaining the nutrients of preferred characteristics. The actual fungal along with bacterial α-amylases have large applications inside the brewing, food, fermentation, textile, paper, detergent, and pharmaceutical industries along with many areas such as medical, medicinal in addition to analytical biochemistry. Partly purified

amylase, has been utilized in digestives, in the present work, we have partially purified the alpha amylase extracted through *Bacillus amyloliquefaciens*. Knowing that solid state fermentation (SSF) is a much cost effective as well as efficient compared to submerged fermentation (SmF), we certainly used wheat bran as substrates for production associated with α-amylase.

Materials and methods

Procurement and Maintenance of Culture

Bacillus amyloliquefaciens (MTCC 610) used in the present study was obtained from Microbial Technology Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh (Punjab), India. The strains were grown on nutrient agar slants and maintained at 4°C.

Substrates and its pre-treatment

Various agro by-products and their residues viz. Wheat flour, Barley flour, Corn flour, Gram flour, Coconut oil cake, Banana peel, Potato peel, Sweet Potato peel, Wheat bran, and Rice bran were used as substrate for solid state fermentation (SSF). These were obtained from local market Agro residues were chopped and dried (70°C) for 16 h. The dried residues were then ground to powder form (40 mm mesh) and stored in polythene bags at room temperature (30±2°C) till use as substrate for alpha amylase production (Asghar *et al*., 2002).

Preparation of Inoculum

For the preparation of inoculum, a volume of 50 ml of nutrient broth was inoculated with a loopfull of cells from a 24h old culture and kept at 37°C in a rotary shaker (100 rpm). After 18 h of incubation, 2 ml of this nutrient broth (Appendix 1.2) culture of *B. amyloliquefaciens* (MTCC 610) was used as the inoculum for solid state fermentation (Gangadharan *et al*., 2006).

Production and isolation of Alpha amylase
Preparation of medium for SSF

Five grams of each of the dried substrate were placed in 250 ml Erlenmeyer flasks and then moistened with mineral salt medium (Appendix 1.3). Distilled water was added to the mineral salt solution in order to maintain the concentration of mineral elements in the medium and to adjust the required moisture level (Gangadharan *et al*., 2006)

Solid state fermentation

The fermentation media in the flasks were autoclaved at 121°C for 20 minutes and cooled to about 30°C. The flasks were inoculated with 1% inoculum of *B.amyloliquefaciens* (MTCC 610) and the contents of the flask

were mixed thoroughly to ensure uniform distribution of the inoculum. The flasks were incubated at 37°C for 24 h in a shaking incubator operated at speed of 100 rpm. All the experiments were run parallel in triplicates (Gangadharan *et al*., 2006).

Isolation of enzyme

After fermentation, the fermented matter in each flask was extracted by the addition of different extraction medium like distilled water, 0.1M Phosphate buffer (pH 7±0.2) 0.1% Tween-80 and Triton-X-100 to a total extract volume of 200 ml. The entire content was mixed thoroughly at 30°C for 1 h in rotary shaker at 180 rpm and filtered using a Whatman filter paper no.1. The suspensions were then centrifuged at 8000 rpm at 4°C for 10 minutes. The supernatant was carefully collected and used as crude enzyme for the estimation of total protein content and alpha amaylase activity.

Partial purification of alpha amylase produced by *Bacillus amyloliquefaciens* (MTCC 610)

The fermented broth was filtered and subsequently centrifuged at 8000 rpm at 4°C for 10 min to remove the cells. The cell free broth (crude enzyme) was then used for further purification.

Ammonium sulphate precipitation

Proteins were precipitated from cell free broth using ammonium sulphate. The calculated amount of ammonium sulphate was added to the supernatant to obtain 50% (w/v) saturation. The suspension was stirred for 20 h at 4°C in an ice bath. After sufficient shaking the precipitate was collected by centrifugation at 8000 rpm for 10 min at 4°C.The pelleted precipitate was resuspended in a minimum volume (2-4ml) of phosphate buffer (0.1M, pH 6±0.2).

Dialysis

For the removal of salts from enzyme solution, the precipitated solution so obtained was introduced into 12 kD molecular weight cut off dialysis bag (membrane tied at both end) and dialyzed overnight against the same phosphate buffer (0.1M, pH 6) at 4°C with continuous stirring.

Characterization of partially purified alpha amylase produced by *Bacillus amyloliquefaciens* (MTCC 610)

Thermostability of alpha amylase

The thermal stability of the alpha amylase enzyme was determined by incubating enzyme fraction at various temperatures viz. 55, 60, 65, 70 and 75°C without substrate for 30 min and aliquots of incubated enzyme were assayed for activity by Okolo *et al*. (1995).

Effect of pH on the stability of alpha amylase

The effect of pH on the stability of alpha amylase was measured by incubating enzyme fractions at different pH values from 4 – 7. Enzyme solution was incubated in different buffers at 37±1°C for 30 minutes and then enzyme assay was performed. The different buffers used included - sodium acetate buffer (pH 4.0), citrate phosphate buffer (pH 5.0), sodium phosphate buffer (pH 6.0 – 8.0).

Effect of metal ions on the stability of alpha amylase

The effect of different ions such as $MnSO_4$, $CaCl_2$, $FeCl_2$ and $CuSO_4$ (5mM) on α- amylase stability was determined by the addition of the corresponding ion in purified enzyme followed by incubation at 37±1^{o}C for 30 min. Aliquots of incubated enzyme were assayed for activity Okolo *et al*. (1995).

Effect of substrate on alpha amylase kinetics

In the present study the effect of different substrate concentration on enzyme kinetics was investigated. The enzyme was kept constant whereas the concentration of starch was taken in increasing order. Different concentration of starch soluble (0.1mg/ml, 0.2 mg/ml, 0.3 mg/ml,0.4mg/ml,0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml and 1.0 mg/ml) were taken in 11 clean and dry test tubes. Label the tubes as control "C" and Test "T" for each concentration. 1 ml of distilled water was added to all the tubes. 0.5 ml of alpha amylase enzyme was added to the tubes labeled T of respective concentration. All the tubes were incubated at 37°C for 10 minutes. After incubation, immediately 2 ml DNS reagent was added to all tubes. The solutions in the test tubes were mixed properly. All the tubes were kept in boiling water bath for 5 minutes at 100°C and cooled it. All the tubes were diluted by adding 9 ml of distilled water. The solutions in each test tube were mixed by using vortex mixer. The absorbance of test tube solutions was read at 540 nm against the control. The K_m and V_{max} values were determined by using graphical representation (Lineweaver and Burk, 1934). The reciprocal values of substrate concentration (1/[S]) and reaction velocity (1/V) were plotted to obtain a straight line graph. The graph allowed for the calculation of both the V_{max} and Michaelis Menten constant (K_m) through linear regression of the data points.

Determination of molecular weight of partially purified alpha amylase protein by SDS PAGE

The homogeneity of the purified enzyme was confirmed by sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). Standard protein markers (Sigma-Aldrich, USA). viz., β-Galactosidase (116 kDa) was used to determine the molecular weight of the partially purified α-amylase from *Bacillus amyloliquefaciens* from the gel.

Statistical Analysis

The data obtained during the course of study were statistically analyzed by Analysis of variance, correlation coefficient, regression analysis and the result was interpreted (Cox, 2006).

Results and discussion

Evaluation of various agrobased substrates for production of alpha amylase by *Bacillus amyloliquefaciens* (MTCC 610)

Fermentative production of alpha amylase using *Bacillus amyloliquefaciens* from different agrobased substrates was investigated. Fifteen different substrates were screened for the alpha amylase production. From the set of experiments, the highest α- amylase enzyme yield (145.56 IU/ml) was observed from wheat flour when extracted with phosphate buffer. Barley flour gave high amylase enzyme yield (138.64 IU/ml) under Triton X-100 extraction solvent followed by Tween80 medium (134.59 IU). Wheat flour also gave significant amylase enzyme production (131.48 IU/ml) in distilled water as an extracting medium. Among all the substrates, soybean husk was found to give lowest enzyme yield (40.44 IU/ml) when extracted with distilled water (Table 1.1). Overall Wheat flour was found to show maximum enzyme production and the differences among the other substrates being statistically significant ($P< 0.05$).

Table 1.1: Production of α-amylase by *Bacillus amyloliquefaciens* (MTCC 610) in different agro byproducts and extraction medium

S.No	Substrate (Agrobyproducts)	Enzyme Activity(IU/ml) in different extraction medium			
		Distilled water	Phosphate Buffer	Tween 80	Triton X-100
1	**Wheat flour**	**134.64**	**145.56**	114.36	112.66
2	**Barley flour**	72.45	128.10	**131.48**	**138.64**
3	**Corn flour**	60.39	92.44	62.22	52.58
4	**Gram flour**	95.72	83.47	53.74	59.64
5	**Coconut oil cake**	51.26	96.71	50.25	75.50
6	**Rice bran**	76.76	93.62	98.35	98.44

7	**Wheat bran**	92.07	108.05	109.13	116.67
8	**Potato peel**	53.73	86.60	87.23	91.87
9	**Sweet potato peel**	60.95	95.92	89.45	90.15
10	**Banana peel**	75.40	133.44	95.56	99.17
Due to Substrate: $F_{(cal)} = 9.88 > F_{(tab)} = 1.93$ (S) at 5%; Due to Extraction medium: $F_{(cal)} = 8.04 > F_{(tab)} = 2.82$ (S) at 5%					

The investigation revealed that alpha amylase was produced by the *Bacillus amyloliquefaciens* (MTCC 610) in all the agrobased substrates. Different workers (Losane and Ramesh 1990; Haq *et al*., 2003; Gangadharan *et al*., 2006) conducted on amylase production using different microbes and reported wheat bran as best substrate for enzyme synthesis. Irfan *et al*. (2011) reported wheat bran as best substrate among other substrates such as rice bran and cottonseed meal. In the present study wheat bran, rice bran gave higher amylase production in comparison to mustard oil cake, soybean husk. Similar findings were also observed in another study conducted by Saxena and Singh (2011) for alpha amylase production.

Partial purification of α-amylase enzyme produced by *Bacillus amyloliquefaciens* (MTCC 610)

From optimized conditions of different process as well as nutrient parameters, the α- amylase was produced under solid state fermentation (SSF) by *Bacillus amyloliquefaciens*. Alpha amylase was purified by ammonium suphate precipitation at 50% saturation and subjected to dialysis against 0.1 M phosphate buffer (6±0.2). The crude extract contained 0.42 mg/ml protein and showed a specific activity of 403.5 (IU/mg). The enzyme was further subjected to ion exchange column and the specific activity increased to 970.84 IU/mg with purification of 2.40 fold (Table 1.11).

Table 1.11: Partial purification summary of alpha amylase produced by *Bacillus amyloliquefaciens* (MTCC 610)

Steps	**Volume (ml)**	**Total enzyme (IU)**	**Enzyme activity (IU/ml)**	**Total protein (mg/ml)**	**Sp. activity (IU/mg)**	**Purification fold**	**Recovery or % yield**
Crude enzyme	78	13218.66	169.47	0.42	403.5	1	100
Purified enzyme	17	3135.82	184.46	0.19	970.84	2.40	23.72

Swain *et al*. (2006) reported that alpha amylase was partially purified using ammonium sulphate fractionation. The crude extract contained 327.23 mg/ml protein and showed that specific activity increased to 39.61 units/mg yield protein and 3 fold purification. Uyar *et al*. (2003) reported that the purification of α-amylase by ion exchange and gel filtration resulted in 73.1 fold purification with specific activity of 170.4 U/mg.

Characterization of partially purified α-amylase from *Bacillus amyloliquefaciens* (MTCC 610)

Effect of temperature on the stability of purified alpha amylase

As starch liquefaction is generally carried out at higher temperature ranging from 65-80°C, the thermostability of α-amylases has great significance in starch processing industries. Thermal activity of alpha amylase produced by *Bacillus amyloliquefaciens* (MTCC 610) was tested by incubating the enzyme at various temperatures such as 55°C, 60°C, 65°C, 70°C and 75°C for 30 minutes and then assay of enzyme was performed. The investigation indicated that enzyme produced by the organism was stable in temperature range of 55°C to 70°C for period of 30 minutes, with maximum stability (219.44 IU/ml) at 65°C and least (196.43 IU/ml) at 55°C (Table 1.12). On analyzing the data by correlation the effect of temperature on alpha amylase activity was found non-significant (P< 0.5).

Table 1.12: Stability of partially purified alpha amylase from *Bacillus amyloliquefaciens* (MTCC 610) under different temperature

S. no	Temperature (^{0}C)	Enzyme activity (IU/ml)
1.	55	196.43
2.	60	205.74
3.	65	**219.44**
4.	70	214.16
5.	75	209.95
r= 0.642, t_{cal} =1.45 < t_{tab} = 3.18 at 5% (NS) , Y = 0.709 X + 163.04		

The high temperature inactivation could be due to incorrect conformation by the hydrolysis of the peptide chain, destruction and aggregation of amino acids (Schokker and Boekel, 1999). Irfan *et al*. (2011) reported that α-amylase produced by *Bacillus* sp. showed optimum activity at 60°C for 20 minutes. Sodhi *et al*. (2005) studied α-amylase reaction at different temperatures in the range of 40-80°C and found that α-amylase from *Bacillus* sp. PS-7 was optimally active at 60°C. It could be a good candidate for the efficient liquefaction of starch.

Stability of alpha amylase to different hydrogen ion concentration (pH)

To determine the pH stability, the purified α-amylase from *B. amyloliquefaciens* was dissolved in different buffers systems viz., Sodium acetate buffer (pH 4.0), Citrate phosphate buffer (pH 5.0), and sodium phosphate buffer (pH 6.0-7.0). The purified α- amylase was incubated in buffers of different pH for 30 minutes at 37°C. After mixture was kept at 37°C for 30 minutes, the residual activity was measured under standard assay conditions. The effect of pH on α- amylase activity is shown in Table 1.13. The alpha amylase activity of *Bacillus amyloliquefaciens* was found to be active in pH range from 4-7 with optimum activity (212.76 IU/ml) at pH 6.0. The enzyme activities at pH 5.0 and 7.0 were 146.49 IU/ml and 197.48 IU/ml respectively. Since there was constant change in amylase activity with increase or decrease of pH the data was found to be statistically non-significant (P< 0.5).

Table 4.13: Effect of pH on the stability of partially purified alpha amylase

S. No	pH	Enzyme activity (IU/ml)
1.	4	129.03
2.	5	146.49
3.	6	**212.76**
4.	7	197.48
r= 0.876, t_{cal} =2.56 < t_{tab} = 4.30 at 5% (NS) , y = 27.16 X + 22.03		

Stability of partially purified alpha amylase to different metal ions

The activity of α-amylase was measured at pH 6.0 in the presence of various metal ions each at the concentration of 5mM. This was done by incubating the enzyme with metal ions solution for 30 minutes at 37°C. All the metal ions were added as sulphate and chloride salts. After incubation the residual activity of amylase was measured by standard assay procedure. The observation obtained in the present investigation revealed that the enzyme was found to be inhibited by Cu^{2+} whereas addition of Ca^{2+}, Mn^{2+}, Fe^{3+} ions had significant effect on α-amylase activity. The maximum activity (227.40 IU/ml) of α-amylase was recorded in Ca^{2+} whereas minimum activity (103.68 IU/ml) was noted when enzyme was incubated in Cu^{2+} (Table 1.14). The effect of metal ions on the amylase activity was found to be statistically non significant (P< 0.5)

Table 1.14: Effect of metal ions on the stability of partially purified alpha amylase

S. No	Metal ions (5mM)	Enzyme activity (IU/ml)
1.	$CuSO_4$	103.68
2.	$FeCl_3$	223.86
3.	$MnSO_4$	218.49
4.	$CaCl_2$	**227.40**
F_{cal} =39.59 >F_{tab} = 5.98 at 5% (S)		

The present research showed the effect of metal ions on the stability of α-amylase and the observations presented in the table and graph are in agreement with the reports of Hassan *et al*. (2011) in which the amylase enzyme was inhibited by Cu^{2+}. Irfan *et al*. (2011) also evaluated metal affinity profile of the amylase enzyme and reported enhanced metal affinity by Ca^{2+} followed by Mn^{2+}. Asghar *et al*. (2007) reported that α- amylase from *Bacillus subtilis* JS 2004 did not require any ions for catalytic activity except Ca^{2+} and also observed stronger inhibitory effect in case of Cu^{2+}. Hayashida *et al*. (1988) reported that the Cu^{2+} at concentration 2mM inhibited the enzyme activity while Ca^{2+} did not inhibit the enzyme which is in agreement with the present investigation.

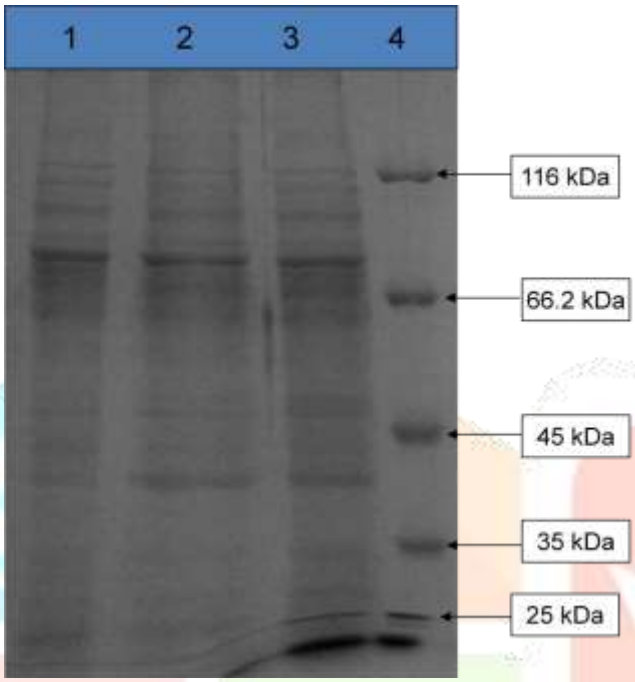
Determination of molecular weight by SDS PAGE

After anion exchange, the pattern of elution was used to determine the molecular weight of alpha amylase on SDS-PAGE. Electrophoretic mobility of purified alpha-amylase obtained from *Bacillus licheniformis* with reference to mobilities of protein marker fractions was analyzed. The mobility of the purified a-amylase from *Bacillus licheniformis* was determined by calculating relative migration distance from different bands appeared on the PAGE gel. The standard curve of molecular mass was drawn between relative molecular masses and Logarithm of molecular weights. The regression line Y (log mol.wt) = -2.081 X (Relative migration distance) + 12.13 was obtained from standard curve of molecular mass. The molecular weight of alpha amylase protein band corresponding to relative migration distance (0.457) was estimated as 71kDa (Table 1.15 and Fig 1.1).

Table 1.15: Variation in relative migration distance (Rf) with molecular weights

S. No.	Relative migration distance (Rf)	Log (Molecular weight)
1.	0.254	11.6
2.	0.494	11.1

3.	0.682	10.71
4.	0.802	10.46
5.	0.965	10.12
Y = -2.081 X + 12.13		



The findings presented in this study outline the characterization of α-amylase from *Bacillus amyloliquefaciens* (MTCC 610) by SDS-PAGE. The results showed that the α- amylase from *Bacillus amyloliquefaciens* consisted of a single polypeptide. The results obtained in this study agree closely with those reported in other studies where α- amylase was purified from other strains of *Bacillus* sp. (Ahmadi *et al*., 2010; Khan and Priya, 2011; Khodayari *et al*., 2014; Roy *et al*., 2014). The molecular mass of alpha amylase is usually between 40 and 72 kDa (Liu and Xu, 2008; Hmidet *et al*., Rai and Solanki, 2014). However several high molecular weight amylases (above 100kDa) produced by various *Bacillus* sp. including *Bacillus licheniformis* have also been reported (Tabassum *et al*., 2014). Demirkan (2011) and Gangadharan *et al*. (2009) reported apparent molecular weight of 56 and 58 kDa of purified alpha amylase produced from *Bacillus amyloliquefaciens* and *Bacillus* sp. Molecular weights of α-amylases were usually between 50-66 kDa but variations in molecular weights ranging from 40 KDa-150 kDa were reported in the literature (Gupta *et al*., 2003).

The present study addressed the significant factors affecting extracellular alpha amylase enzyme production. The produced enzyme was partially purified and characterized where it showed high thermal, pH and metal stability. The purified alpha amylase obtained from *Bacillus amyloliquefaciens* make it good candidate for

wide application as additives and starch modification. Further, all fermentation should be carried out in reactor system and the time required for maximum enzyme production should be optimized so that enzyme production could be taken at large scale.

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