

Optimization of Solid State Fermentation Conditions and Characterization of Thermostable Alpha Amylase from *Bacillus subtilis* (ATCC 6633)

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Abstract

Alpha amylase production using microbial source and solid state fermentation has been conducted for past few years in search of thermostable enzyme. Owing to the prolific use of thermostable alpha amylase in various industries like paper, food, detergent, brewing and starch liquefaction process, the production of alpha amylase is still going on. In the present work, Bacillus subtilis (ATCC 6633) has been utilized for generation of alpha amylase followed by optimization of the fermentation media. Change in fermentation conditions like fermentation hour, temperature, inoculums size, nitrogen and sugar sources have pivotal role to enhance alpha amylase yield. The thermal, pH and detergent stability of partially purified alpha amylase have been tested and compared with purified porcine pancreatic amylase. The result is encouraging with approximate 80% retention of alpha amylase activity comparable to purified porcine pancreatic amylase in presence of drastic condition of temperature (60°C), pH (6-11) and detergents. This makes it apt for use in various industries like detergent, food and paper industries.

Keywords: Fermentation; SSF; Thermostability; Activity; PPA

Abbreviations: WB: Wheat Bran; PPA: Porcine Pancreatic Amylase; SSF: Solid State Fermentation; DNS: Dinitrosalicylate; SDS: Sodium Dodecyl Sulphate; PB: Phosphate Buffer

Introduction

The ubiquitous starch splitting enzyme amylase [EC 3.2.1.1] has been a centre of research since decades. It splits starch liberating simple sugars like glucose, maltose and maltotriose [1]. Owing to its prolific use in industry, large scale production of amylase has been carried out using submerged and solid state fermentation with the help of microorganism like bacteria and fungi. However solid state fermentation is superior to submerged fermentation because of its number of additional advantages like simplicity, cost effectivity, easy availability, better productivity and lesser water output [2]. Wealth of information exists in support of wheat bran as the best sources among all the agro sources for extracellular amylase production for its higher starch content [3-6]. Bacillus species have been exploited by a number of researchers for the production of amylase using SSF [7-11]. They are growing increasingly important subject of study because of their thermostable enzyme productivity that has got myriad applications in number of industries. Bacillus species has become utilized due to their rapid growth rates giving rise to short fermentation cycles, known gene sequences, handling safety and capability to secrete extracellular enzymes in the fermentation media. Presently amylase encompasses considerable area of global enzyme market making starch liquefaction easier than former. In these respect members of the Bacillus family namely Bacillus licheniformis, Bacillus stearothermophilus etc. are being best utilized for thermostable alpha amylase production [12,13]. Thermostable amylase production would become an active area of research as it is being utilized in starch liquefaction, food, brewing, paper, pulp and detergent industry [14-17].

Keeping in mind the growing importance of alpha amylase, in the present work alpha amylase production has been optimized from *Bacillus subtilis* (ATCC 6633) using wheat bran as source of nutrient in SSF. Effect of fermentation hour, temperature, inoculum size, nitrogen and sugar source and extraction solvent have been tested for improved alpha amylase yield. Alpha amylase from fermented extract is characterized with a view point of pH, thermal and detergent stability in order to evaluate it for the use in detergent industry [18,19]. The extracted partially purified amylase manifests moderate thermal and pH stability with an optimum activity at 60°C and at pH 7. It withstands alkaline pH with residual 81.39% of activity after three hours incubation at pH 11 at 60°C. Amylase preserves up to 50% of its initial activity even in presence of 5% SDS over three hours of incubation that qualifies it for the use of various industries.

Materials and Methods

Organism used growth and solid state fermentation

25 ml of nutrient broth is inoculated with a loop full of *Bacillus subtilis* (ATCC 6633) cells from a 24 hour old slant and kept at 37°C in a shaker. After 16-18 hours of growth, 1 ml inoculum (around $1.5-2 \times 10^8$ cfu/ml) from this broth culture has been added to the WB. Solid state fermentation has been carried out with 4 gm dry wheat bran collected from local market in a 100 ml Erlenmeyer flask. The moisture level of the wheat bran is kept at 50% (w/w) with autoclaved distilled water. The contents of the flask are autoclaved prior to solid state fermentation [20].

Optimization of solid state fermentation parameters like fermentation hour, temperature and inoculum size for maximum amylase production

With handful of existing information regarding the inoculum size used for amylase production from SSF, the present study has been conducted with varying inoculums size from 2.5 to 20% (v/w).

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With optimum inoculums size, SSF has been carried out for various fermentation periods (24, 48 and 72 hours) at different temperatures (30° C, 40° C, 50° C and 60° C).

Amylase extraction after solid state fermentation

Following fermentation the media comprising of wheat bran is mixed with 25 ml of ice cold phosphate buffer (20 mM, pH=7.0) for 30 minutes at 4°C in a rotary shaker at 150-200 rpm. The supernatant has been collected followed by centrifugation at 8000 rpm for 15 min at 4°C and used for amylase assay. For optimization of appropriate extraction solvent, phosphate buffer (20 mM, pH=7.0) has been substituted with phosphate buffer (20 mM pH=7.0) that has incorporated 1% Triton-X 100 and Tween 20. After extraction the supernatant has been assayed for enzyme activity.

Effect of supplements in wheat bran for optimum amylase production

4 gram of wheat bran is supplemented with various inorganic nitrogenous salt (0.125 M) like NH_4Cl , $(NH_4)_2SO_4$ and NH_4NO_3 followed by incubation for 48 hours at 40°C. The extraction of the enzyme is carried out following the same procedure as described earlier. Effect of organic nitrogen sources are also checked by addition of (1% by weight) peptone, tryptone and yeast extract in WB and extracted after 48 hours of fermentation as mentioned above. Simultaneously another set of fermentation experiments has been carried out in presence of sugars like glucose, starch and maltose (each with 1% by weight) in WB. The alpha amylase activity is determined according to DNS method [21].

Amylase assay

Alpha amylase activity of the extract is measured by DNS method [21]. In brief the reaction mixture containing 1% soluble starch, 20 mM phosphate buffer (pH=7) and fermented extract is taken and incubates at 37°C for 20 minutes followed by the addition of 3,5-Dinitrosalicylic Acid (DNS). The amount of the reducing sugar liberated during assay is estimated by measuring color development at 540 nm by UV-VIS spectrophotometer (Hitachi). 1 U of amylase activity is defined as the amount of enzyme that liberates 1 micromole of maltose per minute under standard assay condition. The experiments are carried out in triplicates and standard error is calculated.

Protein estimation

The protein content of the extract is determined following Lowry's method [22].

Purification of amylase of *Bacillus subtilis* (ATCC 6633) from fermented extract

Fermented extract has been subjected to ammonium sulfate precipitation at 30-80% saturation. The precipitate is collected by centrifugation at 12000×g for 20 min at 4°C. The precipitate is suspended in 20 mM ice cold phosphate buffer (pH 7) and dialyzed against the same buffer for 10 hours with three changes. The dialyzed solution is applied onto DEAE cellulose ion exchange chromatography pre equilibrated with the same 20 mM phosphate buffer (pH 7). All the bound fractions with high OD₂₈₀ have been collected after elution with 100 mM NaCl. The flow through as well as elute have been estimated for protein content and amylase activity after desalting by dialysis. The entire procedure has been carried out at 4°C.

Effect of temperature on amylase activity

The optimum temperature of the amylase is determined in 20 mM phosphate buffer (pH 7) over a range of temperature from 35-80°C. Thermal stability of the partially purified amylase is tested after incubation of the purified enzyme in the same buffer at 60°C for 2 hours. In order to assess the thermal stability of purified amylase it has been compared with PPA incubated under identical experimental conditions. In both the cases, the residual amylase activity has been measured as described earlier.

Sensitivity of amylase in various pHs and detergents

The influence of pH and various detergents on the stability as well as activity of extracted amylase from *Bacillus subtilis* (ATCC 6633) has been checked to find its suitability in industry. Amylase activity has been measured in presence of buffers of different pHs using 20 mM citrate (pH=6.2), phosphate (pH=7.0), Tris-HCl (pH=8.0) and Glycine-NaOH (pH 9-11) by standard amylase assay. pH stability of the amylase has been estimated at 40°C after incubation of partially purified amylase for 0-3 hours in buffers of different pHs (6.2-11). The same assay procedure has been repeated accompanying incubation of extracted amylase in presence of detergents (0-5%) namely Triton-X 100, Tween 20 and SDS for a period of 1 hour at 60°C.

Statistical analysis

Effect of each parameter was studied in triplicate and graphically represented as the mean \pm SD (n=3) using Origin 5.

Results and Discussion

Effect of fermentation hours on amylase production during solid state fermentation

With prior knowhow regarding the preferred use of WB among all agro wastes for improved alpha amylase production through SSF, the present work has been conducted with Bacillus subtilis ATCC 6633 and WB [2-5]. Success of SSF for enzyme production utilizing microbial resources largely depends on incubation period. The yield of alpha amylase from SSF varies with incubation period (24, 48 and 72 hours). The yield is maximum with 1160 ± 9.4 U/g around 48 hours of fermentation. The alpha amylase activity declines on the both side at 24 hours and at 72 hours with activity 970 \pm 7.3 U/g and 1050 \pm 9.8 U/g respectively (Figure 1). The value can be correlated with the results of other workers supporting highest amylase production after 48 hours fermentation period [20]. Earlier study depicts that a clear correlation exists between bacterial growth and alpha amylase production [23]. The activity or production of amylase rises linearly with time up to 42 hours that merges with the sporulation pattern of Bacillus subtilis [23]. The fall in activity of amylase beyond 48 hours may be due to the denaturation or degradation of alpha amylase in presence of other components in the fermentation medium like protease etc. as noticed earlier [24].

Effect of temperature and inoculum size on the production of amylase in solid state fermentation

Temperature has profound effect on amylase yield as noticed from the result of SSF carried out at different temperatures for 48 hours. The activity of alpha amylase in the extracted media is highest at 40°C (1160±2.8 U/g) that decays both at the higher and lower temperatures (Figure 2). Optimization of SSF for amylase production is largely dependent on inoculum size. Lower inoculum size implies Citation: Maity S, Mallik S, Basuthakur R, Gupta S, et al.(2015) Optimization of Solid State Fermentation Conditions and Characterization of Thermostable Alpha Amylase from Bacillus subtilis (ATCC 6633). J Bioprocess Biotech 5: 218 doi: 10.4172/2155-9821.1000218

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Figure 1: Optimization of fermentation hour for maximum amylase production by SSF from *Bacillus subtilis* (ATCC 6633).



lesser number of cells in the production medium. This eventually increases fermentation time for enzyme synthesis as longer time is required to attain an optimum number of cells to utilize the substrate forming the desired product. Inoculum size regulates alpha amylase yield as observed from the variation of enzyme activity with change in inoculums' size (Figure 3). With 2.5% (V/W) inoculum, the activity in the fermented extract is 900 ± 16.2 U/g increasing there by with the rise in inoculum size. The alpha amylase activity is highest when 10% inoculum has been used that is around 1150 ± 6.7 U/g. Decay in the amylase activity is evident thereafter with 20% inoculum 1020 ± 9.6 U/g. Comparison with earlier reports from Anto et al. is in agreement with the present result which shows that increase in inoculum size adversely affects the enzyme production [25]. Although work by Baysal et al. reports 20% inoculum size as optimum for SSF with wheat bran in amylase production [26]. However this may be an outcome of species variations that has been used for carrying out fermentation by different workers. However a likely explanation can be put forward in support of the above observation. Lower inoculum size accounts for less number of bacterial cells and lower nutrient consumption leading to the formation of less biomass that eventually manifests low amylase yield during SSF. However the productivity of amylase rises as inoculum size increases up to 10% due to optimum growth of *Bacillus* under specified medium environment with maximum available nutrient. With further hike in inoculum size, there is again a fall in alpha amylase activity at 20% inoculum size. It can be related with the inhibition of bacterial growth due to limiting nutrient supply in the fermentation media with concomitant poor amylase yield after 48 hours. Similar observation has also been reported by Gangadharan et al. [27].

Effect of extraction solvent and nitrogen sources on the amylase yield from *Bacillus subtilis* (ATCC 6633):

The nature of solvent used for extraction of crude enzyme may improve the yield of alpha amylase. Figure 4 shows highest activity of amylase when extracted with 20 mM phosphate buffer (pH 7) compared to the solvents with nonionic detergent like Triton-X 100 and Tween 20 used for extraction (activity of amylase varies from 1030 to 1080 U/g in presence of detergents in extraction solvent). This suppression in alpha amylase yield in presence of detergents like Triton-X 100 and Tween 20 can be attributed to the increased biomass formation. Similar observation regarding detergent effect on alpha amylase yield has also been mentioned by Benjamin et al. [28].

Presence of nitrogenous supplement in WB may improve the nutritional quality of WB that can influence amylase synthesis by producer microorganism. Alpha amylase yield has been augmented from solid state fermented production media with proper supplementation of C and N sources as they are absolutely necessary for microbial growth [29,30]. From earlier literature it is known that the presence of (NH₄)₂SO₄, NH₄Cl and NH₄ HPO₄ in SSF media along with WB stimulates enzyme production [31]. However in our studies, highest amylase yield has been observed in presence of 0.125M $NH_{1}NO_{2}$ (1410±16.9 U/g) compared to the others (Figure 5). Organic nitrogen sources also modulates alpha amylase yield when present with WB in the SSF media. Among organic nitrogen sources 1% tryptone is proved to be a better candidate (1730±26.4 U/g) followed by peptone $(1360 \pm 35.6 \text{ U/g})$ when present in the SSF media. In contrary, yeast extract supplementation in WB is not promising enough in terms of alpha amylase activity ($880 \pm 13.2 \text{ U/g}$). Earlier results also corroborates with our observation albeit a different school of thought exists [32,33]. Work done by Pandey et al. [17] reveals contradictory results indicating no differences in the amylase yield through SSF conducted in WB aided with different N sources [27]. According to them, WB apart from being a C source also serves as a source of N that abrogates the requirements of additional N sources in the fermentation media [34].

Effect of sugar additives on the amylase yield from *Bacillus* subtilis (ATCC 6633) during solid state fermentation

Being inducible enzyme, alpha amylase production is influenced by the presence of starch or its hydrolyzed product maltose in the fermentation media. Table 1 presents the activity of alpha amylase in presence of various C sources in SSF media along with WB. The amylase yield is highest in presence of maltose and soluble starch (1260 \pm 8.2 U/g and 1230 \pm 24.6 U/g) respectively compared to fermented WB (1170 \pm 16.5 U/g). This is in agreement with the observation reported in case of *Bacillus thermoleovorans* preferring starch, glucose, lactose, maltose and maltodextrin as carbon sources for amylase production [31]. Wealth of information is also available in this context regarding improved yield of amylase from *Bacillus licheniformis*,







Figure 4: Effect of extraction solvent on amylase production after 48 hour SSF using *B. subtilis* (ATCC 6633).



Figure 5: Effect of nitrogen sources (0.125 M for inorganic salts and 1% for organic nitrogenous sources) on amylase yield from SSF using *Bacillus subtilis* (ATCC 6633).

Bacillus species I3 and *Bacillus Stearothermophilus* in presence of starch in the fermentation media [10,34]. However in the present study, glucose in wheat bran represses alpha amylase yield $(1110 \pm 14.3 \text{ U/g} \text{ compared to } 1170 \pm 16.5 \text{ U/g})$ because of its rapid uptake by bacteria in preference to other complex sugars like maltose, dextrin etc. due to catabolite repression. This promotes better bacterial growth reducing the necessity of alpha amylase production. This is in agreement with the observation of Pandey et al. with similar trend of decrease in alpha amylase yield in the presence of glucose compared to WB alone [17,27].

Optimum pH and temperature of amylase

Earlier reports depict a temperature range with optimum alpha amylase activity oscillating from 40°C for an alkalophilic *Bacillus* species to 95°C for amylase from *Bacillus* subtilis and *Bacillus* amyloliquefaciens [35]. However majority of alpha amylase have their optimum activity of pH 7.0 at approximate 65°C [36-38]. From literature review it is known that alkalophilic amylase is mostly thermolabile [18]. On the other hand acidophilic alpha amylase as it is related to the use of enzyme in various sectors of industry. Alpha amylase extracted from SSF has optimum activity at pH 7.0 (1230±21.23 U/g). In the present case alpha amylase is showing maximum activity around 60°C (1650±9.81 U/g) with 50% decline in activity (800 ± 9.23 U/g) at 80°C (Figure 6a and Figure 6b).

Purification of amylase of *Bacillus subtilis* (ATCC 6633) from solid state fermented extract

Amylase from the fermented extract has been purified after ammonium sulphate fractionation at 30-80% saturation. The activity and specific activity of amylase in the fermented extract is 1160 (U/ml) and 446 (U/mg) respectively. Table 2 presents the gradual purification of amylase as observed from the improvement of specific activity values of the amylase in comparison with the amylase from fermented extract. For the estimation of the structure and molecular weight of amylase, the SDS-PAGE analysis has been conducted (Figure 7). SDS gel electrophoresis analysis manifests the presence of a single band near 66 kDa. This molecular weight matches with the previous reports (54 to 68 kDa) regarding the molecular weight of purified amylase from other sources [39].

Estimation of thermal stability of amylase

During thermal stability the activity of purified amylase has been compared with the activity of PPA incubated under identical conditions. The amylase activity estimated under 0 hour of incubation has been considered as 100% for both the purified as well as for PPA. Activity of the incubated amylases at various hours has been calculated as the residual activity and plotted against period of incubation. In the present case, purified alpha amylase from Bacillus subtilis keeps back approximate 80% activity upon incubation for 2 hour at 60°C. Its thermal stability has been compared with purified PPA incubated under identical condition that retains 87% of the activity (Figure 8). Thermal stability of alpha amylase has been disseminated from the constituent amino acids as revealed from work by Suzuki et al. [13]. They identified a region (Gln 178) and region II (255 to 270 residues) of Bacillus licheniformis amylase to be essential for imparting thermostability [13]. Deamidation of Asn/Gln residue for Bacillus licheniformis has been evolved as a cause of thermal inactivation. However extra thermal stability has been induced due to additional salt bridge formation among lys 385, lys 88 and /or lys 253 [12]. Amylases

Inducer	Enzyme Activity (U/g)		
WB	1170 ± 16.5		
WB + Glucose (0.01gm)	1110 ± 14.3		
WB + Maltose (0.01gm)	1260 ± 8.2		
WB + Starch (0.01gm)	1230 ± 24.6		

Table 1: Effect of sugars on amylase yield from SSF.

Sample	Activity (U/ml)	Protein content (mg/ ml)	Specific activity (U/mg)	Purification fold	
Crude fermented Extract	1160	2.6	446	1	
30-80% (NH ₄) ₂ SO ₄ saturation	6820	4.1	1664	3.7	
DEAE cellulose chromatography (Flow	4259	1.6	2662	6	

 Table 2: Partial Purification of amylase from Bacillus subtilis (ATCC 6633) fermented extract .







Figure 7: 12% SDS PAGE of 48 hour fermented extract of *B. subtilis* (ATCC 6633) Lane 1, 48 hours fermented extract (4 µg), Lane 2, 0-30% fraction of ammonium sulphate precipitated fermented extract, Iane 3, 30-80% fraction of ammonium sulphate precipitated fermented extract, Lane 4, Purified amylase, Lane M, Standard molecular weight markers (Sigma).



vary in their thermostability as evident from the series from *Bacillus licheniformis* CUMC305 to *Bacillus stearothermophilus*. In the former the maximum activity of amylase is retained at pH 9.0 with 91% residual activity at 100°C [40]. Owing to the thermal stability at 60°C this alpha amylase from *Bacillus subtilis* is able to preserve its activity against thermal inactivation. Thermostable alpha-amylases are used for starch liquefaction process at high temperature whereas thermolabile amylases are used for the saccharification of starch in baking. This can be an effective addition to industries for long lasting stability and activity.

pH and detergent stability of amylase

Owing to the rising demands of amylase in detergent industry, it is essential to check the stability of amylase in presence of detergents. In addition it is worthwhile to determine the pH stability of alpha amylase. Amylase stability has been derived from the estimation of amylase activity at varying conditions of buffer pH and detergents. Activity of amylase has been presented as % of residual activity as stated before. Amylase is able to retain about around 80% of its activity after 3 hours of incubation at 40°C. This trend is maintained even at pH 11

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although in the acidic pH zone, the loss in alpha amylase activity is more pronounced (Figure 9). When incubated in the presence of 1% SDS the amylase activity is preserved up to an extent of 77% albeit it decreases thereafter in the presence 5% SDS (with 52% remaining activity) at 60°C after 1 hour. Nonionic detergents like (0.1%) Tween-20 or Triton X 100 also stabilizes alpha amylase. In presence of 5% Tween-20, the activity of alpha amylase increases from 1400 U/g to 1624 U/g. Triton X 100 also offers protection and protects alpha amylase activity as manifested from (Table 3). Compared to the purified PPA, subjected under similar experimental conditions, alpha amylase of *Bacillus subtilis* (ATCC 6633) has retained its activity up to a significant extent in presence of various detergents. This substantiates its direct use in a number of industries.

Conclusion

Alpha amylase produced after solid state fermentation from *Bacillus subtilis* (ATCC 6633) appears to have potential in industries due to its thermal, pH and detergent stability. Further studies are to be continued with the goal of performing its production in pilot scale. Data originated from this study will help to design experimental set up for large scale production of amylase. Evaluation of other biochemical and biophysical parameters like sensitivity towards ions, inhibitors, reaction kinetics and structural studies are to be performed to validate its use in industries. Divalent cations like calcium and magnesium ions increase the thermal stability of amylase so it will be relevant to check the effects of these ions in the fermentation media for the improvement

	SDS		Tween 20		Triton X 100	
Concentration of detergents (%)	Amylase activity (U/g)					
	from fermented extract	(PPA)	from fermented extract	(PPA)	from fermented extract	(PPA)
0	1140 ± 17.5	1390 ± 9.82	1150 ± 11.3	1400 ± 8.62	1150 ± 7.29	1400 ± 9.64
1	878 ± 6.37	1126 ± 12.4	1173 ± 8.73	1442 ± 5.46	1311 ± 11.5	1638 ± 13.9
3	730 ± 9.89	1036 ± 14.3	1208 ± 4.27	1512 ± 12.2	1414 ± 13.8	1764 ± 7.51
5	593 ±10.48	897 ± 7.84	1277 ± 9.57	1624 ± 9.17	1495 ± 5.38	1918 ± 8.32

Table 3: Effect of detergents on alpha amylase activity.



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of thermal stability of amylase. This may pave the pathway to justify its commercial utility.

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