

Notes

Process optimization for hyperproduction of 1,4-alpha-D-glucan glucanohydrolase from locally isolated *Bacillus subtilis* BBT6 under solid state fermentation

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The extracellular enzyme 1,4-alpha-D-glucan glucanohydrolase (AMY) has wide application in clinical, medicinal and analytical chemistry and are used in industries such as food, textile, paper, etc. The α -amylase (AMY) produced from microbial sources, particularly bacterial is preferred over fungal, because of characteristic advantages, such as rapid growth rate and ability to release proteins into the extracellular medium. The current study deals with the isolation of novel bacterial strain and process optimization of AMY. Primary screening was performed on the basis of starch hydrolysis zone. Secondary screening was carried out using solid state fermentation (SSF). Molecular characterization using 16S r RNA gene sequencing technique was performed for the strain showing highest AMY production as compared to other isolates. The selected strain exhibited 91% similarity with the reference strain in the Gene Bank and identified as *Bacillus subtilis* on the basis of molecular characterization and analytical profile index testing. Different agricultural byproducts such as rice bran, rice husk, wheat bran, potato peel and coconut oil cake was tested. Among all, wheat bran proved to be the best for AMY production. Effect of other variables including incubation time, temperature, pH, inoculum size, carbon and nitrogen sources, surfactant and metal ion have also been investigated. Optimal production of AMY was obtained at 48 h of incubation period, 37°C, pH 7 and inoculum size 1 mL; 1 % ammonium sulphate and peptone as the best inorganic and organic nitrogen sources and 1.5 % starch, 0.2 % SDS found best for optimal production of enzyme.

Keywords: Amylase, AMY, SSF, Wheat bran;

The α -amylase, [1,4-alpha-D-glucan glucanohydrolase (AMY)] is an extracellular enzyme that randomly cleaves the α -1,4 glucosidic linkage in starch leading to the formation of maltose, maltotriose and glucose¹. It is one of the most versatile enzymes whose spectrum of application has widened in many sectors such as clinical, medicinal and analytical chemistry

with usage in industries such as food, textile, paper, etc. AMY is also used in starch liquefaction and sugar syrup production from starch^{2,4}. While it can be derived from a number of different sources viz. animals, plants, bacteria, and fungi⁵, generally, AMY from the microbial sources are reported to meet industrial demands. AMY produced by bacteria is preferred over fungal enzyme because of characteristic advantages, such as stability, rapid growth rates leading to shorter fermentation cycles and the ability to release proteins into the extracellular medium^{6,7}.

Molecular techniques such as 16S ribosomal DNA (16S rDNA) sequencing help in understanding the phylogenetic relationship between the living organisms and thereby useful in accurately assigning their taxonomical position⁸. In various cases, mere phenotypic recognition could not confirm the right taxonomic position, particularly in case of *Bacillus spp.* and in identifying novel species within the genera⁹.

Production of 1,4-alpha-D-glucan glucanohydrolase can be carried out by solid state fermentation (SSF) and submerged fermentation. SSF even though conventional, is extensively employed due to factor such as less energy requirements, high product yield, less catabolic repression and end-product inhibition, low capital investment and better product recovery^{10,11}. Increasing use and the subsequent demand of AMY has encouraged researchers to pay attention to produce 1,4-alpha-D-glucan glucanohydrolase locally and search for new and economical ways for its efficient production. The agricultural byproducts are attractive raw materials for the production of 1, 4-alpha-D-glucan glucanohydrolase. Majority of Asian countries has vast resources of the agricultural byproducts. These agricultural byproducts are economical because of their nutritional composition and easy availability at low cost. A large number of agricultural byproducts such as wheat bran, rice bran, rice husk, dried potato peel and banana peel have been used as substrates for SSF¹². In the present study, we have made an attempt to isolate a novel bacterial strain for economical production of 1,4-alpha-D-glucan glucanohydrolase using agricultural byproducts. Further, we have also carried out molecular characterization of highest 1,4-alpha-D-glucan glucanohydrolase producing strain.

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Materials and Methods

Isolation of organism

Fifteen bacterial strains were isolated from soil samples collected from different areas of Pakistan by serial dilution method¹³. One gram of soil sample was dissolved in 100 mL of sterilized distilled water. Suspension of soil was thus prepared and diluted up to 10^3 - 10^7 times. About 0.5 mL of each dilution was transferred in the starch agar plates. The plates were placed in an incubator at 37°C for 24 h. Young colonies showing a clear zone of starch hydrolysis were picked up and transferred to starch agar slants and incubated at 37°C for 48 h. Morphological and biochemical tests were performed (colony shape, gram staining, anaerobic test, motility test, starch hydrolysis catalase test, etc.) according to Berge's manual of determinative bacteriology¹⁴.

DNA isolation

Molecular characterization was carried out using 16s rRNA gene sequencing. For isolation of genomic DNA, freshly grown bacterial culture was used. The bacterial cells were centrifuged at 6500 rpm at 4°C for 5 min to obtain a pellet. The pellet was washed with 10 mL of TEN buffer and centrifuged at 6500 rpm (4°C) for 5 min. After this the pellet was incubated for 30 min at 37°C along with 5 mL of SET buffer and 1 mL of lysozyme. When specific time period was over 5 mL TEN buffer and 0.5 mL 25 % SDS was added and incubated again at 60°C for 45 min. The mixture was cooled prior to the addition of 1 mL 5M NaCl. DNA solution was treated with equal volume of choloform: phenol (1:1). Mixed and centrifuged (6500 rpm) at 4°C for 5 min. DNA was precipitated by adding double volume of ice cold absolute ethanol. Spool out the DNA and washed with 70% ethanol. Finally, dried and dissolved in TE buffer¹⁵. Isolated DNA was run in 1% agarose gel and visualized under the Gel doc imaging system. The 16s RNA region was amplified by using the universal primer (27F 5' to 3' AGAGTTTGATCCTGGCTCAG; 1541R 5' to 3' AAGGAGGTGATCCAGCCGCA) under optimized PCR conditions. The PCR product was purified and run in 1% agarose gel and visualized under the Gel doc system. The sequencing was performed commercially. The PCR product after gene clean was sent to 1st BASE Products & Services for sequencing. After the sequencing, the sequence was compared within GenBank using the BLAST search tool. Most closely related 16S rRNA genes of species were determined.

Preparation of inoculum

A loop full of bacterial culture was aseptically transferred to each flask containing 50 mL of sterilized nutrient broth. Flasks were placed in a rotary shaking incubator at 37°C for 24 h at 200 rpm¹⁶.

Solid state fermentation (SSF)

Ten gram of solid substrate was transferred to each of 250 mL Erlenmeyer flask. The substrate was moistened with phosphate buffer (0.02 M) in the ratio of 1:1. These flasks were autoclaved at 121°C for 15 min and allowed to cool down at room temperature (28°C) About 1 mL of vegetative inoculum was transferred aseptically to each flask and flasks were placed in the incubator at 37°C for 48 h. After a specific time period, 100 mL of water was added in each flask. These flasks were placed in an incubator shaker at 160 rpm for one hour, centrifuged and the supernatant was taken for estimation of 1,4-alpha-D-glucan glucanohydrolase. All the experiments were run in triplicate.

Fermentation media

Different culture media were tested for enzyme production. Details of the media were as follows: M1: Rice bran 5 g, 5 mL of Mineral salt solution g/L; KH_2PO_4 2, NH_4NO_3 10, NaCl 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1, distilled water 1000 mL; M2: Wheat bran 10 g, 10 mL of distilled water; M3: Rice husk 5 g, 5 mL of mineral salt solution g/L; NaCl 1, KH_2PO_4 2, NH_4NO_3 10, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1, distilled water 1000 mL; M4: Coconut oil cake 5 g, 5 mL of Mineral salt solution g/L; KH_2PO_4 2, NaCl 1, NH_4NO_3 10, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1, distilled water 1000 mL; M5: wheat bran 10 g, phosphate buffer 10 mL; M6: Wheat straw 10 g, 10 mL of phosphate buffer (0.02 M); and M7: Potato peel 10 g, 10 mL of mineral salt solution g/L; NH_4NO_3 10, KH_2PO_4 2, NaCl 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1, distilled water 1000 mL.

Optimization of cultural condition

Different cultural and nutritional parameters including incubation time, temperature, pH, inoculum size, carbon and nitrogen sources, surfactant and metal ions were optimized sequence wise

Enzyme assay

Estimation of 1,4-alpha-D-glucan glucanohydrolase was carried out according to the method of Rick and Stegbauer¹⁷ One unit of enzyme activity was that amount of enzyme, which liberates 1 mg of reducing group from 1% Lintner's soluble starch corresponding

to 1 mg of maltose hydrate in 10 min. Estimation of 1,4-alpha-D-glucan glucanohydrolase was carried out by incubating 1 mL of enzyme with 1% Litner's soluble starch at 40°C for 10 min. A blank was also run parallel by replacing enzyme solution with distilled water. Reducing sugar was measured at 546 nm by using the DNS method¹⁸.

Statistical analysis

All data was subjected to statistical analysis for determination of significance one way ANOVA and Duncan multiple range test was used to determine the significance. Significance has been presented in the form of probability ($P \leq 0.05$).

Results and Discussion

Choosing an appropriate bacterial strain is vital for the production of 1,4-alpha-D-glucan glucanohydrolase (AMY). All the selected isolates were screened for AMY production in solid state fermentation (Table 1). The bacterial isolates were identified on the basis of morphological characteristic and biochemical testing. For further confirmation, 16srRNA sequencing was performed for the strain showing the highest amylolytic potential. The selected strain was gram positive, rod shape, motile, aerobic and spore former. DNA was extracted from bacteria and the extraction result was visualized by Gel doc imaging system. The sequencing results were opened in the Chromas software and then BLAST. The similarity of 91% was found with *Bacillus subtilis* strain A405 (Accession no: AF058767.1). This showed the selected strain was

Bacillus subtilis. The strain was assigned the code *Bacillus subtilis* BBT-6 for further studies.

Screening of fermentation media

For the optimum AMY production, appropriate media as well as cultural conditions is mandatory. In the present study, different fermentation media (M1-M7) were tested for AMY production. Among all the fermentation media, the M5 media containing wheat bran and phosphate buffer gave the highest production (1010±0.3) U/mL/min) of 1, 4-alpha-D-glucan glucanohydrolase (Fig. 1A). This might be due to the presence of essential nutritional constituents in wheat bran i.e. 6.7% ash, 2.5% soluble dietary fibers, 17.1% protein, 43.6 % insoluble dietary fiber as well as different amino acids which are important for the production of 1,4-alpha-D-glucan glucanohydrolase¹⁹ and it contains a large portion of readily metabolizing carbohydrates as compared to other solid substrates²⁰. Presence of phosphate buffer enhances the production of the enzyme it might be due to the fact that phosphate acts as a stimulator of enzyme production as well as for the growth of bacteria²¹. Various concentrations of wheat bran were tested for the production of 1,4-alpha-D-glucan glucanohydrolase [Fig. A(i)]. Optimal production of the enzyme (1136.133±0.5 U/mL/min) was obtained at 5 g of wheat bran. The further increase resulted decrease in the AMY production.

Impact of time of incubation period

Incubation period plays an important role in the biosynthesis of AMY. In this study, fermentation media was incubated for different time intervals (0-72) and enzyme activity was calculated after every 8 h. It was found that maximum production of enzyme (1194 U/mL/min) was obtained after 48 h of incubation (Fig. 1B). The reason might be that bacteria entered into a stationary phase of growth after 48 h of incubation. After 48 h there was a gradual decrease in the enzyme production. This decline in the enzyme production was might be that important nutrient required for the growth exhausted or due to the accumulation of other byproducts such as proteases²² or it might be due to the denaturation of enzymes caused by interaction between other components in the medium.

Effect of incubation temperature:

Figure 1B(i) shows the effect of various incubation temperatures (17-57°C) on AMY production by *Bacillus subtilis* BBT 6. The optimal enzyme

Table 1—Screening of bacterial strains isolated from different soil samples for the production of 1,4-alpha-D-glucan glucanohydrolase

Isolates	Enzyme activity U/mL/min
<i>Bacillus</i> BBT 1	477±4.1 ^g
<i>Bacillus</i> BBT 2	157±4.2 ^l
<i>Bacillus</i> BBT 3	1199±5.6 ^b
<i>Bacillus</i> BBT 4	1084±8.0 ^c
<i>Bacillus</i> BBT 5	983±9.8 ^j
<i>Bacillus</i> BBT 6	1916±3.4 ^a
<i>Bacillus</i> BBT 7	747±1.6 ⁱ
<i>Bacillus</i> BBT 8	245±2.4 ^d
<i>Bacillus</i> BBT 9	243±1.7 ^d
<i>Bacillus</i> BBT 10	520±5.7 ^h
<i>Bacillus</i> BBT 11	442±1.3 ^f
<i>Bacillus</i> BBT 12	412±2.9 ^e
<i>Bacillus</i> BBT 13	344±2.5 ^d
<i>Bacillus</i> BBT 14	147±2.5 ^k
<i>Bacillus</i> BBT 15	49±3.5 ^m

[Each value shows the mean of three parallel replicates. ± indicates the standard deviation among the triplicates. Different superscripts indicates the mean difference is significant at $P \leq 0.05$]

production (1140 ± 1.32 U/ml/min) was obtained at 37°C . Beyond this level, a gradual decrease was observed. This might be due to the reason that at high temperature moisture contents reduced during solid state fermentation, thereby causing a decline in the growth of the organism. This leads to reduced production of AMY in the fermentation media²². Hence, optimum temperature in this case was 37°C .

Influence of pH

Influence of pH (3-10) on the production of 1,4-alpha-D-glucan glucanohydrolase by *Bacillus subtilis* BBT 6 was evaluated (Fig. 1C). The maximum production (1136 ± 2.13 U/mL/min) of AMY was obtained at pH 7. Any increase or decrease in the pH caused declined AMY production. Any change to pH caused deleterious impact on the enzyme production²³. So, optimum pH for the production of alpha amylase was found to be at pH 7.

Impact of inoculum concentration

Figure 1C(i) shows the impact of inoculum size (0.5-3 mL) on AMY production. The maximum yield of the enzyme (1136 ± 2.13 U/mL/min) was obtained at 1 mL. Further increase or decrease in this concentration resulted decrease in the production. Probably, it might be due to the reason that at lower inoculum concentrations the number of viable cells is lower in the medium²⁴. Whereas, low enzyme production at higher inoculum concentrations was might be due to the decline nutrient availability or accumulation of other toxic metabolites²⁵.

Influence of carbon sources

The choice of the carbon sources has a major influence on the production of AMY. In the present study, effect of different carbon sources such as glucose, lactose, xylose, sucrose, maltose, starch and CMC were studied (Fig. 1D). Out of all the carbon

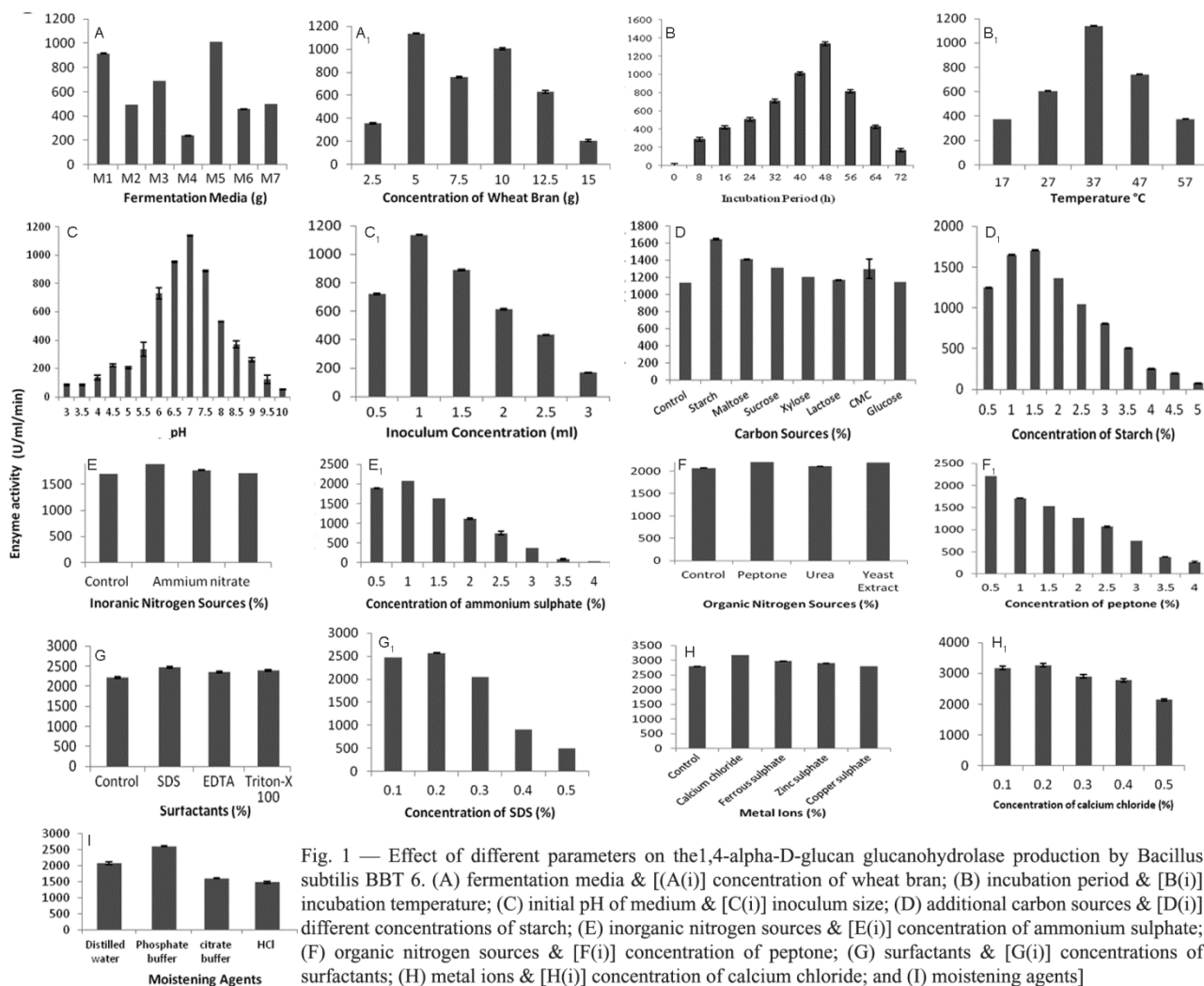


Fig. 1 — Effect of different parameters on the 1,4-alpha-D-glucan glucanohydrolase production by *Bacillus subtilis* BBT 6. (A) fermentation media & [(A_i) concentration of wheat bran; (B) incubation period & [(B_i) incubation temperature; (C) initial pH of medium & [(C_i) inoculum size; (D) additional carbon sources & [(D_i) different concentrations of starch; (E) inorganic nitrogen sources & [(E_i) concentration of ammonium sulphate; (F) organic nitrogen sources & [(F_i) concentration of peptone; (G) surfactants & [(G_i) concentrations of surfactants; (H) metal ions & [(H_i) concentration of calcium chloride; and (I) moistening agents]

sources tested starch gave the highest yield of enzyme (1648 \pm 5.8 U/mL/min) as compared to other carbon sources. It might be due to the fact that starch was slowly metabolized, so there was a significant accumulation of an enzyme in the media²⁶. Starch was further tested at different concentrations, i.e. (0.5-5%). The best production (1703 \pm 4.56 U/mL/min) was obtained at 1.5 %, further increasing the concentration of starch cause decrease in enzyme production [Fig. 1D(i)].

Effects of inorganic nitrogen sources

Effect of different inorganic nitrogen sources (ammonium sulphate, ammonium nitrate and potassium nitrate) was investigated on the AMY production by *Bacillus subtilis* BBT6 (Fig. E). The optimum production of the enzyme (1892 \pm 4.67 U/mL/min) was obtained by using 0.5% ammonium sulphate [Fig. E(i)]. This might be due to the reason that it provides both the ammonium and sulphate ion to the media.

Effect of organic nitrogen sources

Figure 1F shows effect of various organic nitrogen sources such as peptone, urea and yeast extract on AMY production. The maximum production (2213 \pm 2.3 U/ml/min) was obtained by peptone at the level of 1 %. [Fig. 1F(i)].

Effect of surfactants

Surfactants play an important role in the production of the enzyme by enhancing the permeability of cell membranes. Effect of different surfactants including SDS, EDTA and Triton-X 100 on 1,4-alpha-D-glucan glucanohydrolase production was studied (Fig. 1G). The maximum production (2568 \pm 2.1 U/mL/min) was obtained in the presence of 0.2% SDS [Fig. 1G(i)].

Influence of metal ions

Effect of different metal ions including ferrous sulphate, calcium chloride, zinc sulphate and copper sulphate was evaluated for enzyme production (Fig. 1H). The addition of CaCl₂ to the media increased enzyme production. The reason might be that Ca⁺² ions act as a better binder as well as stabilizer of an enzyme. Ca²⁺ had significant effects on the metabolism and physiology of bacteria as well as on the production of enzyme²⁸. Different concentrations of calcium chloride (0.1-0.5%) was further tested maximum production of 1, 4-alpha-D-glucan glucanohydrolase (3264 \pm 2.8) was obtained at 0.2% [Fig. 1H(i)].

Impact of moistening agents

Figure 1I depicted the impact of different moistening agents (distilled water, phosphate buffer, citrate buffer and HCl) on the production of 1, 4-alpha-D-glucan glucanohydrolase. Extremely less amount of the enzyme was produced with distilled water. It might be due to the fact that distilled water has no micro- or macronutrients. The maximum production of enzyme (2568 \pm 1.4) was obtained using phosphate buffer. It might be due to the enhancing effect of phosphate²⁷. Other moistening agents gave less production as compared to phosphate buffer.

Conclusion

The use of agricultural byproduct reduces the cost of fermentation media and leads to the reduction of final product cost. From the present study, it is concluded the wheat bran proved to be a good substrate for the production of 1,4-alpha-D-glucan glucanohydrolase (AMY) and helpful in making the medium economic. The isolated strain of *Bacillus subtilis* BBT6 has promising characteristics for converting the agricultural byproducts into the valuable products such as 1,4-alpha-D-glucan glucanohydrolase which are used in many industries such as textile, detergents, pharmaceutical, etc.

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