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# GLUCOAMYLASE FROM A THERMOPHILIC STRAIN OF *BACILLUS LICHENIFORMIS* RT-17: PRODUCTION AND CHARACTERIZATION

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**Abstract**

Glucoamylase is an industrially important enzyme that finds many applications including in the production of glucose and fructose syrups. Commercially it is obtained from the species of *Aspergillus* and *Bacillus*. *Bacillus* being source of thermostable enzymes has been studied widely for the prospects of getting proteins having industrially relevant properties. In the present study, a thermophilic strain of *Bacillus licheniformis* RT-17 was investigated for its ability to produce glucoamylase. After initial experiments, the factors affecting glucoamylase production were screened using Plackett-Burman design (PBD). The data indicated that out of six factors, one factor i.e. incubation period was found having significant effect on the response and hence was studied separately. The strain produced the maximum titers (0.017 IU $mL^{-1}$) after 24 h of incubation, the production dropped significantly if cultivation is extended to 48 or 72 h that could be linked with the proteolytic potential of this strain. The enzyme in cell-free culture supernatant was partially purified by 1.3 fold with 65% yield using 80% ammonium sulphate. The partially purified enzyme was characterized for its temperature and pH optima. The results showed that glucoamylase from RT-17 performed optimally at 50°C under alkaline conditions (pH 8.2) in presence of 1.8% starch. Thermophilic nature of the strain, heat- and alkali-stability of the glucoamylase indicate possible candidature of the strain for future biotechnological applications.

**Key words:** Glucoamylase, *Bacillus licheniformis*, Plackett-Burman design, Alkalozyme.

## Introduction

Glucoamylase is a glycoside hydrolase that acts preferably on α-1,4- than α-1,6 glycosidic linkages present in starch and other related polysaccharides, thereby releases β-glucose (Sauer *et al*., 2000). The enzyme is applied in many industries, including for the production of glucose and fructose syrups. On industrial scale, the enzyme is obtained from the members of the genus *Aspergillus, Rhizopus* (Bennett, 1998) and *Bacillus*. Although, filamentous fungi are preferred choice for the production of industrial enzymes owing to the good yields, however, bacilli bear an edge for being house of thermostable proteins (Bajpai, 1989) and hence can produce enzymes with desirable properties. Furthermore, the higher growth rate and ease with which genetic manipulation can be carried out in bacteria also render them suitable organism for industrial production.

Application of thermostable glucoamylase in starch industry would render the process more cost effective as a higher loading of starch could be made with sustained enzymatic catalysis. Therefore, various approaches have been listed to engineer this enzymes (Sauer *et al*., 2000). However, the isolation and screening of native organism with novel properties remain at the core of industrial microbiology. Various thermophilic bacteria from indigenous sources have been isolated with the ability to produce one or more industrially important enzymes. The strain RT-17 of *B. licheniformis* was originally isolated from a soil sample and was studied for its ability to produce endoglucanase (Tariq *et al*., 2018). The strain was found to produce amylase, however, detailed studies were required to evaluate its potential to be applied on industrial scale. Since, thermostable and alkali-stable amylases are widely applied in detergent and in some other industries therefore, it was worthwhile to optimize the production of amylase from this strain.

Traditionally, optimization studies were carried out by applying one-factor-at-a-time approach however, this approach does not provide any insights about the interactive effect between the factors. Furthermore, it needs to conduct a large number of experiments in a sequential manner that requires more time (Box *et al*., 1978). As an alternative approach, statistical designs have been developed to study a large number of factors affecting a response simultaneously and quantifying the effect of individual variable on a process. The designs are based on matrices that investigate the interactive effect of the factors with minimum number of experiments, without compromising the statistical relevance of the model. Plackett-Burman design (PBD) is a popular design that screens a large number of factors for their significant effect on a process (Plackett & Burman, 1946). As a follow-up, the significant factors can be optimized to yield an optimum response by adopting methodologies available within Response Surface (Sen & Swaminathan, 1997). PBD was adopted by Yahya *et al*., (2016) to screen factors affecting alpha amylase of the *A. tubengiensis* by manipulating production parameters. In this study, PBD was employed to evaluate the factors for their significant effect on glucoamylase production by *B. licheniformis* RT-17 and the enzyme was characterized for its optimum activity.

## Materials and Methods

**Bacterial strain, screening and inoculum preparation:** Bacterial strain, *B. licheniformis* RT-17 was obtained from the Department of Microbiology, University of Karachi. The strain was revived on nutrient agar (Oxoid, UK) at 50°C for 24 h. Amylolytic potential was observed by spot inoculating the strain on nutrient broth containing 0.5% soluble starch (NB-starch) as a sole source of

carbon. After incubation for 48 h at 50°C, the plates were stained with iodine solution and clear zone around the colonies were observed. Inoculum of the strain to study amylase production was prepared by transferring a single colony from nutrient agar to nutrient broth and cultivating at 50°C until $OD_{600}$ reached to 1.0.

**Optimization of factors affecting glucoamylase production by adopting one variable at a time (OVAT) approach:** Various factors affecting glucoamylase production by the strain RT-17 were studied initially by adopting OVAT. The effect of incubation time was studied by transferring 5% inoculum of the strain in NB-starch and cultivating at 50°C for 96 h in an orbital shaker. Aliquots were withdrawn periodically and centrifuged to obtain cell-free culture supernatant (CFCS). CFCS was assayed for glucoamylase based on estimation of glucose by GOD-PAP (Farrance, 1987).

The effect of pH on glucoamylase production was studied by transferring the inoculum (5%) to NB-starch with pH adjusted to 6, 7, 8 and 9 and incubated at 50°C for 48 h (the optimum incubation period as investigated in the previous experiment). Post incubation CFCS was obtained and assayed for glucoamylase activity.

Likewise, effect of cultivation temperature on glucoamylase production was studied by keeping incubation period and pH of the medium constant and the production medium was incubated at variable temperature 37-70°C. CFCS were analyzed for the enzyme activity. Similarly, effect of nitrogen source (Yeast extract, tryptone, peptone, ammonium sulphate and urea) and effect of carbon sources (rice starch, potato starch, cereal starch, corn starch) were studied by incorporating the compound in NB and cultivating the strain by keeping other factors constant. Post incubation CFCS were subjected to glucoamylase

**Optimization of factors affecting glucoamylase production by using statistical design:** Six factors were screened by employing Plackett-Burman design (PBD) using Minitab 17 to determine their significant effect on glucoamylase production by the strain RT-17. The factors included temperature (37 and 45°C), pH (7 and 9), Inoculum size (5 and 10 mL), Medium (Nutrient broth or Mineral salt medium, MSM, both with 2% starch), Incubation period (24, 48 h) and agitation (with shaking or without shaking). A design of 12 runs was generated using Minitab 17 and experiments were conducted in triplicate. Inoculum was transferred to the production medium and all the conditions were adjusted as suggested by the PBD. Post incubation CFCS was obtained and glucoamylase assay was performed. IU $mL^{-1}$ was taken as response and data was analysed using Minitab 17.

**Glucoamylase assay and protein estimation:** The activity of glucoamylase was assayed by GOD-PAP Method (Randox England). Reagent (1 mL) was mixed with 50 µL of partially purified enzyme preparation and incubated at 37°C for 15 min. Amount of glucose released was estimated by taking absorbance at 540 nm ($OD_{540}$) and compared with standard curve obtained by standard glucose solutions. One unit of glucoamylase was defined as the amount of the enzyme required to release one µmole of glucose in one minute under standard set of assay conditions.

Total proteins present in the enzyme preparation and CFCS were estimated by Lowry's method (Lowry *et al*., 1951) by taking Bovine serum albumin as standard.

**Characterization of glucoamylase activity:** The strain RT-17 was cultivated under optimum conditions as predicted by the PBD, and CFCS obtained was subjected to ammonium sulphate precipitation (80% saturation level) maintained at 4°C. Pellets were obtained after centrifugation at 12000 ×*g* for 15 min and re dissolved in the minimum amount of buffer. The aliquots were kept in freezer (at -20°C) until used. The glucoamylase activity present in partially purified enzyme was characterized for its optimum activity by varying one factor at a time. Assay was performed and optimum temperature was determined by incubating the reaction mixture at different temperatures. Similarly, the effect of pH was studied by changing the pH of the reaction buffer and effect of substrate concentration was determined by performing the assay in presence of different amount of starch.

## Results and Discussion

The strain RT-17 of *B. licheniformis* was previously studied for the production of endoglucanase (Tariq *et al*., 2018), and it was found amylase producer upon plate screening. The thermophily of the organism was known as it was able to grow at 50°C. The proteins from thermophiles are expectedly thermostable and hence can find numerous biotechnological applications (Fitter *et al*., 2001). Therefore, it was worthwhile to evaluate amylolytic potential of the strain. *B. licheniformis* amylase (BLA) has been widely studied for its desirable properties and industrial application (Bajpai, 1989), however, native strains are yet to be isolated and screened to obtain enzymes with novel properties (Sohail *et al*., 2005).

**Pre-screening of factors affecting glucoamylase production by RT-17:** Large scale production of microbial enzymes is influenced by various nutritional (Djekrif-Dakhmouche *et al*., 2006), chemical and environmental factors. The variables are set at the level to obtain optimal yield with the consideration of economic viability of the process. In this study, the parameters affecting production of glucoamylase were optimized. Initially, the parameters were studied by adopting one-variable-at-a-time approach. Such experiments indicated the robustness of the process that can tolerate the level of individual factors and aid to design further experiments by adopting statistical tools.

The data obtained showed that the glucoamylase titers were comparable when the strain was cultivated either for 24 or 48 h (Table 1), however, the titers dropped significantly when the incubation period was extended to 72 or 96 h. This observation can be attributed to the fact that the production of amylase and other hydrolases is growth-linked and it reaches to its peak levels during log phase of the growth (Sachslehner *et al*., 1998).

**Table 1. Effect of temperature, pH and incubation period on glucamylase production by *B. licheniformis*RT-17 as studied by varying one factor at a time.**

| Factors | Levels | Glucoamylase production (IU $mL^{-1}$) |
|---|---|---|
| Temperature ($^oC$) | 40 | 0.75 ± 0.007 |
| | 50 | 1.08 ± 0.026 |
| | 60 | 0.240 ± 0.026 |
| | 70 | 0.141 ± 0.0023 |
| pH | 6 | 1.66 ± 0.049 |
| | 7 | 0.091 ± 0.011 |
| | 8 | 0.054 ± 0.002 |
| | 9 | 0.0503 ± 0.002 |
| Incubation period (h) | 24 | 0.258 ± 0.004 |
| | 48 | 0.261 ± 0.0017 |
| | 72 | 0.244 ± 0.0009 |
| | 96 | 0.244 ± 0.013 |

The results related to the effect of pH showed that the neutral pH was more favorable to produce glucoamylase, whereas, acidic pH exerted drastic effect than that of slightly alkaline pH. pH exerts its influence on the growth of the organism and it can modify extracellular proteins by ionization or by triggering the process of glycosylation (Stals *et al*., 2004), thereby, induction of the enzyme is affected.

When the effect of cultivation temperature was studied, it was found that the glucoamylase production has increased with the increase in temperature from 40 to 50°C; considerable production was not detected at higher temperatures (60-70°C). Decreased production of cellulase at temperature higher than the optimum growth temperature has already been reported (Sohail *et al*., 2009).

**Studies on factors affecting glucoamylase production by RT-17 using PBD:** The results of pre-screen experiments were, hence, utilized to generate PBD where six factors (temperature, pH, incubation period, medium type, inoculum size and shaking) were studied. A design of 12 runs was generated to evaluate the effect of these factors at high (+) and low (-) levels. All the experiments were conducted randomly and in triplicate. The average IU $mL^{-1}$ of glucoamylase was taken as response. The data showed that the response varied from 1.46-10.9 IU $mL^{-1}$ (Table 2). The variation highlighted that the factors had profound effect (Soni *et al*., 2006; Yahya *et al*., 2016) on glucoamylase production by RT-17 and these parameters need to be optimized. The data indicated that the maximum glucoamylase activity was obtained in run number 10, where 5% inoculum was transferred to MSM containing 2% starch with a pH of 9, incubated at 48°C for 48 h without shaking. While the minimum glucoamylase activity was observed in run number 12, that differed with run 10 on the basis of pH (7) and incubation time (24 h). Statistical analysis of run 12 for residual effect showed negative value (-6.5983) hence confirming the obtained results.

The data was subjected to multiple linear regression analysis to estimate t- and p- values for each variable, that showed positive effect on glucoamylase production by all the factors except for X3 and X4 (as inferred by negative values). It revealed that only incubation period (X5) had a significant effect on glucoamylase production by RT-17 as indicated by smaller than 0.05 p-value (Table 3). Pareto chart (Fig. 1) also confirmed about the significant effect of incubation period, though, the effect of shaking, pH, inoculum size and temperature also had pronounced positive effect, but the value was beyond the threshold p-value, that was in agreement with the findings of Qunhui *et al*., (2008) where none of the studied factors was found having significant effect on the response. The composition of media had negligible effect on the production of glucoamylase by RT-17.

As incubation period appeared as a significant factor affecting glucoamylase production by RT-17; in another experiment, it was varied from 24-72 h while keeping other factors constant as were maintained in run 10. The results showed that the highest titers (0.017 IU $mL^{-1}$) were obtained after 24 h of cultivation and the production diminished when the strain was cultivated for 72 h. The ability of the strain to yield higher titers in shorter incubation period can be considered as a desirable attribute as more products can be obtained in a shorter period of time. Decline in glucoamylase production under extended incubation may be linked with the ensuing stationary phase of the bacterial growth or it can be attributed to the production of protease that digests extracellular proteins, as the strain was reportedly protease producer (Tariq *et al*., 2018). Denaturation of the enzyme during the period beyond 24 h may also be a contributing factor in this regard (Table 4).

**Table 2. Production of glucoamylase by *Bacillus licheniformis* strain RT-17 as studied by using Plackett-Burman design (PBD).**

| Run No. | Temperature ($^oC$) | pH | Inoculum size (%) | Medium | Incubation period (h) | Shaking | Response (IU $mL^{-1}$) |
|---|---|---|---|---|---|---|---|
| 1. | 37 | 9 | 5 | MSM | 48 | Yes | 5.67 ± 0.019 |
| 2. | 45 | 7 | 5 | NB | 48 | Yes | 4.92 ± 0.018 |
| 3. | 37 | 9 | 10 | NB | 48 | No | 5.92 ± 0.012 |
| 4. | 45 | 7 | 10 | NB | 48 | No | 6.25 ± 0.0062 |
| 5. | 37 | 7 | 10 | MSM | 48 | Yes | 1.54 ± 0.0014 |
| 6. | 45 | 9 | 10 | NB | 24 | Yes | 1.54 ± 0.0055 |
| 7. | 37 | 7 | 5 | NB | 24 | Yes | 2.37 ± 0.0017 |
| 8. | 45 | 9 | 10 | MSM | 24 | Yes | 1.54 ± 0.0014 |
| 9. | 37 | 7 | 10 | MSM | 24 | No | 4.19 ± 0.0026 |
| 10. | 45 | 9 | 5 | MSM | 48 | No | 10.9 ± 0.0083 |
| 11. | 37 | 9 | 5 | NB | 24 | No | 4.75 ± 0.011 |
| 12. | 45 | 7 | 5 | MSM | 24 | No | 1.46 ± 0.00029 |

**Table 3. Estimated effect of factors affecting glucoamylase production by *B. licheniformis* RT-17. The coded values are temperature, pH, inoculum size, medium, incubation period and shaking from X1 to X6.**

| Constant | Main effect | coefficient | St dev coefficient | T-value | P-value |
|---|---|---|---|---|---|
| | - | **11.575** | **1.521** | **7.61** | **0.00f*** |
| X1 | 0.807 | 0.403 | 1.521 | 0.27 | 0.801 |
| X2 | 4.147 | 2.073 | 1.521 | 1.36 | 0.231 |
| X3 | -3.920 | -1.960 | 1.521 | -1.29 | 0.254 |
| X4 | -0.037 | -0.018 | 1.521 | -0.01 | 0.991 |
| X5 | 8.880 | 4.440 | 1.521 | 2.92 | 0.033 |
| X6 | 6.983 | 3.492 | 1.521 | 2.30 | 0.070 |

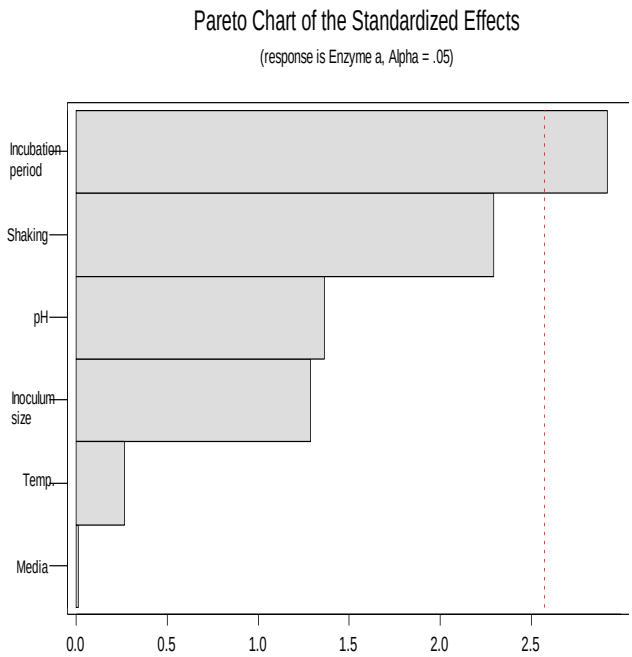


Fig. 1. Pareto chart showing effect of various factors on glucoamylase production by *B. licheniformis* RT-17.

**Table 4. Analysis of variance (ANOVA) for Plackett-Burman design generated for glucoamylase production by *B. licheniformis* RT-17.**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Main effects | 6 | 482.5 | 80.42 | 2.90 | 0.132 |
| Residual Errors | 5 | 138.7 | 27.75 | | |
| Total | 11 | 621.2 | | | |

**Characterization of partially purified glucoamylase:** The studies on stability of the enzymes are necessarily required prior to evaluate its possible application on commercial scale (Haki & Rakshit, 2003). In order to characterize glucoamylase of *B. licheniformis* RT-17, the crude glucoamylase preparation was partially purified with 80% ammonium sulphate saturation. The result showed that protein concentration was decreased from 0.4 mg to 0.2 mg and the specific activity was improved from 1110.5 IU $mg^{-1}$ to 1446.26 IU $mg^{-1}$, hence a purification level of 1.3 fold with a yield of 65% was achieved.

The partially purified enzyme was used to investigate the effect of various factors on the activity by adopting one-variable-at-a-time approach. Initially, the effect of temperature was investigated. The enzyme showed its maximum activity at 50°C, while it exhibited 85% of its activity when temperature was increased to 55°C, however, any further increase in temperature resulted in drastic decline in the activity of the enzyme (data not shown). A temperature optima in the range of 50-60°C has been reported for amylase from various organisms (Norouzian *et al*., 2000).

The effect of pH on glucoamylase activity by RT-17 was observed by incubating the partially purified enzyme preparation in different buffers. The results showed that the enzyme performed optimally when it is exposed to neutral or alkaline pH and the highest activity (1.87 IU $mL^{-1}$) at pH 8.2. Production of alkali-stable amylases has distinctly been reported from bacterial origin (Highara *et al*., 2001) as normally fungal amylases work better under acidic conditions (Kandra, 2003).

Consequently, the effect of amount of starch was investigated by assaying the enzyme in presence of varying concentration of starch from 0.2-2%. The data showed that there was an increasing trend of the glucoamylase activity with increasing concentration of starch until 1.8% (1.7 IU $mL^{-1}$). A slight decrease in the activity was observed when the concentration was further increased.

## Conclusion

The strain RT-17 of *B. licheniformis* and its glucoamylase offer tremendous potential for ultimate application on industrial scale such as for the production of glucose syrups from corn starch and in detergent industry due to thermophily and stability at higher temperature and alkaline pH.

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