Asian Journal of Chemistry; Vol. 28, No. 3 (2016), 669-674

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2016.19462

Optimization of Various Parameters for Maximum Production of Glucoamylaze by *Neurospora sitophila*

FATIMA SYED[1,*], M. JAVAID ASAD[2], KHURSHID ALI[1], MUHAMMAD GULFRAZ[2], AFTAB AHMAD[3], ZAHID KHAN[1], MUHAMMAD IMRAN[1], RAHEELA TAJ[1] and SHAFIULAH KHAN[4,*]

[1]Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan
[2]Department of Biochemistry, PMAS, Arid Agriculture University, Rawalpindi, Pakistan
[3]Beijing University of Chemical Technology, Beijing, P.R. China
[4]Institute of Chemical Sciences, Gomal University, D.I. Khan, Pakistan

*Corresponding authors: E-mail: fatimasyed_chem81@yahoo.com; s.khan@gu.edu.pk

Received: 11 July 2015; *Accepted*: 6 September 2015; *Published online*: 5 December 2015; AJC-17673

Glucoamylaze production by *Neurospora sitophila* was investigated under solid state fermentation. A number of process parameters including moisture content (%), pH, incubation temperature (°C), inoculum size (%), nutrients and duration of fermentation (h) were optimized using response surface methodology. The statistical results showed that the best culture conditions for glucoamylaze production were pH 5, temperature 37.5 °C and 72 h of incubation period while the optimum medium conditions were 15 g wheat bran, moisture content 65 %, inoculum level 20 %, starch 1.17 %, NH_4NO_3 0.66 % and SDS 0.3 % of the substrate weight. Under these optimized conditions, the enzyme production was enhanced to 335.2 ± 12 U/gds which is approximately 2.6-fold increase in the enzyme production when compared with the initial fermentation conditions. To our best of knowledge, this is the first report on optimization of process variables using response surface methodology for glucoamylaze production under solid state fermentation from *Neurospora sitophila*.

Keywords: Glucoamylaze, *Neurospora sitophila*, Response surface methodology, Optimization.

INTRODUCTION

The prominent role of enzymes in various industrial processes mainly depends on the efficiency of enzyme extraction, purification and characterization. Therefore, optimization of culture and medium conditions for the production and purification of enzyme is highly imperative for its industrial viability. Various industrial processes exploit the vital role of biological catalysts in the manufacturing of commercially important products [1]. The industrial applications of amylolytic (α-amylase, EC 3.2.1.1 and glucoamylaze, EC 3.2.1.3) enzymes have been extensively exploited in the fields of food processing, brewing, pharmaceuticals, paper, sugar, detergents, textiles and environmental waste decomposition, *etc*. [2]. Glucoamylaze hydrolyzes both starch and glycogen in a step wise mechanism releasing a single glucose unit from the non reducing end [3]. Nowadays, the overall global glucoamylaze production is around 26 % of the total enzymes production after proteases [2].

Conventionally, the production of glucoamylaze has been carried out under submerged fermentation process. Recently, the process of submerged fermentation for enzyme production has been replaced by a more economical and favourable technique called solid state fermentation (SSF). In solid state fermentation, the micro-organisms are grown on the surface of a solid mass with limited amount of water content. Solid state fermentation provides more favourable environmental conditions for microbial growth and product formation [4] and its applicability is of great importance when the crude fermented mass is directly used as enzyme source. Enzyme production under solid state fermentation requires low-cost and abundantly available agro-industrial wastes. However, the selection of suitable microorganism for production of industrially important enzymes under solid state fermentation is a time consuming and complicated process.

A number of microorganisms like bacteria, fungi and yeast have been employed for the large scale production of glucoamylazes [5]. *Aspergillus* is among the most abundant fungi which comprises hundreds of species distributed worldwide [6].

Neurospora, is a model of model microbes for biochemical genetics and molecular biology [7] and is therefore a valuable organism from biotechnological point of view. *Neurospora* has the ability to grow on a large number of carbon sources

[8] and could be a good microorganism for producing secondary metabolites under solid state fermentation system. The great cellulose hydrolyzing potential of *N. sitophila* makes it one of the fastest growing fungi [9]. The above mentioned properties are beneficial to glucane industry which is of great importance in glucoamylaze research.

In this study, response surface methodology (RSM) was employed to optimize various process parameters for glucoamylaze production on low cost substrate under solid state fermentation using *Neurospora sitophila*. To the best of our knowledge, this is the first report on optimization of process variables using response surface methodology for glucoamylaze production under solid state fermentation by *Neurospora sitophila* fungal strain.

EXPERIMENTAL

Screening of solid substrates: Commercial quality wheat bran, rice straw, corn cobs, tea waste, orange peels, turnip peels and corn cobs as solid substrates were screened for the production of glucoamylaze. The substrate with maximum enzyme production was selected for subsequent experiments.

Inoculum preparation: A 7 days old petri plate culture of the organism was used for inoculum preparation using 100 mL sterile inoculum medium: [glucose 50, $MgSO_4 \cdot 7H_2O$ 0.5, urea 3, KCl 0.15, KH_2PO_4 0.08, $ZnSO_4 \cdot 7H_2O$ 0.01(g/L) with a pH of 5.5]. The flasks were incubated on a rotary shaker at 220 rpm (35 °C) for 72 h.

Solid state fermentation: The static experiments for glucoamylaze production under solid state fermentation were conducted in 250 mL Erlenmeyer flasks containing 15 g of the solid substrates. The initial substrate moisture content was kept 65 % with distilled water. The contents were sterilized, cooled and inoculated with a 20 % inoculum and incubated at 37 °C for 72 h. During the initial screening process, the fermentation was carried out for 4 days and maximum enzyme production was observed at 72 h. All the experiments were performed in duplicate and the average values are represented as mean ± SD using graphpad prism-5.

Enzyme extraction: At the end of fermentation, the glucoamylaze was extracted with 100 mL of 0.1 M sodium acetate buffer pH 5. The fermented biomass was thoroughly agitated on rotary shaker (150 rpm) for 30 min at room temperature. The whole contents were filtered through Whatman No. 1 filter paper and centrifuged for 10 min at 10,000 rpm. The clear supernatant was used as the enzyme source for further analysis.

Enzyme activity measurement: Glucoamylaze activity was assayed by the method described previously [10]. Briefly, 0.1 mL of the crude enzyme solution was mixed with 0.1 mL 1 % starch solution in 0.1 M sodium acetate buffer (pH 5). The mixture was incubated at 37 ºC for 10 min. The reaction was stopped by addition of 1 mL of glucose oxidase reagent. The amount of glucose released was measured at 500 nm using spectrophotometer. One unit of enzymatic activity (U) was defined as the amount of enzyme that releases one µmol of glucose per minute per mL of reaction.

Optimization of factor using central composite design (CCD): In order to optimize and evaluate the mutual interaction of the most important parameters that influence the production of glucoamylaze, response surface methodology using central composite design (CCD) was employed. The factors selected for optimization using central composite design (CCD) include; incubation time-substrate weight, pH temperature, inoculum size-moisture and carbon-nitrogen source. Each factor was studied at five-levels; $\pm \alpha$- 0- $\pm$ 1 (-1 for low level, +1 for high level and 0 center point).

Response surface methodology is a useful statistical tool for analyzing few process variables (less than 6) and is usually not employed for large number of process parameters to avoid high number of experimental runs. According to the design, the total number of treatment combinations is $2^k + 2k + no$, where k is the number of independent variables and no is the number of repetition of experiments at the central point [11]. The mathematical relationship of response (enzyme production) and proposed variables were analyzed by a quadratic model equation. Each run of the experimental design was performed in duplicate.

RESULTS AND DISCUSSION

Effect of substrate on glucoamylaze production: The selection of suitable substrate that could act as solid support and carbon source for microbial growth is very important parameter in solid state fermentation. In order to select the most suitable substrate, various agricultural by-products (as substrate) were screened for maximum glucoamylaze production under solid state fermentation. The growth of microorganism and enzyme production was observed with all the substrates used, however, maximum glucoamylaze activity (170 U/gds) was obtained with wheat bran at 72 h of incubation. This high titre of enzyme production may be attributed to the presence of various essential nutrients in the wheat bran and the ease of microbial colonization and anchorage to this solid support. Moreover, wheat bran remains loose even under moist conditions, providing a large surface area for fungal growth. The effect of wheat bran on glucoamylaze production was significant ($P \leq 0.05$) as compared to other tested substrates. The order of substrate suitability was wheat bran > corn cob > orange peel > tea waste > rice straw. Furthermore, wheat bran is an excellent source of carbohydrates (27 %), proteins (14 %), fat (6 %), minerals (5 %) and B-vitamin [12] and is therefore a suitable source for promoting microbial growth and enzyme production [13]. Wheat bran has been widely reported as excellent substrate for various enzymes production under solid state fermentation [13,14]. It has been previously published that the highest glucoamylaze production was obtained with lingo cellulosic substrates that release relatively small amount of glucose compared to those that produce the highest amount of reducing sugars [15]. Excessive glucose in the fermentation media causes enzyme repression while its scarcity induces enzyme production. The different behaviour of substrates in the present study may be due to the different physiochemical characteristics of the substrates that could lead to differential release of glucose in to the fermentation media.

Effect of carbon source supplementation: The growth of microorganisms may not be thoroughly supported by the solid substrate alone and therefore, the exogenous addition of

carbon and nitrogen supplements improve both microbial growth and product synthesis. The potential of *N. sitophila* for glucoamylaze production was investigated on media containing different carbon sources (1 % of the substrate weight) and slightly different response of *N. sitophila* for glucoamylaze production was observed (Fig. 1). In comparison to control, the production of glucoamylaze was significantly ($P < 0.05$) induced by the tested carbon sources except glucose which suppressed the enzyme production. However, the highest enzyme titre was obtained with starch as polysaccharide (312. ± 12 U/gds) followed by fructose (286 ± 11 U/gds), lactose (272 ± 12 U/gds) and maltose (263 ± 7 U/gds) respectively. Starch, maltose and lactose have been reported to have similar inducing effect on glucoamylaze production from *Aspergillus terreus* [15]. The inducing effect of starch on glucoamylaze production from *Aspergillus flavus* A 1.1 has also been previously established, whereby glucoamylaze was differently induced by starch from different sources [16]. The chemical composition of starch (with respect to amylose amylopectin composition) varies with different sources and could therefore, have different inducing effect on amylase production. Furthermore, it was also observed that amylase production from *Rhizopus* sp was strongly induced in the presence of amylose rather than amylopectin [17]. In another study, the production of glucoamylaze from *Aspergillus niger* was considerably induced when maltose was used as exogenous source of carbon [18]. Lactose has also been shown as an inducer of amylase production when used in combination with wheat bran in other microbes [19]. Singh and Soni [20] also published that lactose was a strong inducer for amyloglucosidase production by *Aspergillus oryzae* HS-3 under solid state fermentation. It has been previously reported that glucose enhances the yield of fungal cell mass, but suppresses glucoamylaze production and the rate of substrate utilization [14]. Catabolic repression plays an important role in the regulation and secretion of inducible enzymes. The different response of microbes to different carbon sources for enzymes synthesis has been attributed to repression of mRNA formation [21,22].

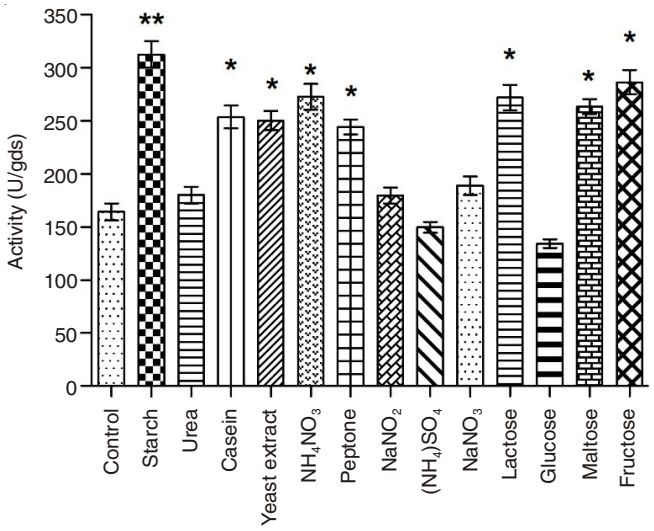

Fig. 1. Effect of carbon and nitrogen sources on glucoamylaze production (where **p = < 0.0001 *vs.* control, *p = 0.0002 *vs.* control)

Effect of nitrogen supplementation: Nitrogen is an essential component of protein and enzymes and therefore, plays a vital role in microbial growth for the synthesis of a desired product. *Neurospora sitophila* was grown on both organic and inorganic nitrogen supplements (1 % of the substrate weight) for glucoamylaze production (Fig. 1). Both organic and inorganic nitrogen sources (except ammonium sulphate) had positive effects on glucoamylaze production compared to control. Most of the tested nitrogen supplements that include, casein, yeast extract and peptone had significant effect ($P < 0.05$) on enzyme production, while $NaNO_2$, $NaNO_3$ and urea revealed a slight positive effect when compared with the experimental control.

However, maximum enzyme activity was obtained with NH_4NO_3 (272 ± 12) followed by casein (253 ± 10) (Fig. 1). Ammonium nitrate has been reported to have inducing effect on α-amylase production by *Bacillus sp*. KR-8104 [19]. Maximum β-galactosidase production has been shown with nitrogen supplements that release ammonium ions [23]. The different behaviour of microbes towards different nitrogen sources for product synthesis has been attributed to a regulatory mechanism. This regulatory mechanism lowers the enzyme synthesis when the microorganism is provided with a medium containing different nitrogen sources and a substrate that is easy to be metabolized [14].

Optimization of parameters using central composite design (CCD): Conventionally, one factor at a time approach was applied to optimize different variables that influence the product synthesis under the study design. We also screened various carbon-nitrogen sources, initial pH and temperature, moisture content and inoculum size employing one factor a time approach to find the most suitable candidates that significantly affect the enzyme production. However, this approach is laborious and time consuming. Afterwards, those parameters were optimized using a statistical tool called response surface methodology. In the present study, central composite design was employed to optimize major significant factors and to evaluate their mutual interaction for glucoamylaze production under solid state fermentation. The design plan included 13 experiments (two factors at a time) and two levels (low and high) of concentrations for each factor. The responses of the central composite design were fitted into a second-order polynomial equation in terms of coded factors and the four proposed models as shown below:

$$\text{Sqrt (enzyme activity U/gds)} = +5.22 - 0.29\,(A) + 0.61(B) - 0.32\,(AB) - 1.12\,(A^2) - 0.67\,(B^2) \quad (1)$$

$$\text{Sqrt (enzyme activity U/gds)} = +5.84 - 0.039\,(A_1) - 0.23\,(B_1) + 0.016\,(A_1B_1) - 0.15\,(A_1^2) - 0.76\,(B_2^2) \quad (2)$$

$$\text{Sqrt (enzyme activity U/gds)} = +3.85 - 0.21\,(A_2) - 0.073\,(B_2) + 0.094\,(A_2B_2) + 0.68\,(A_2^2) + 0.64\,(B_2^2) \quad (3)$$

$$\text{Sqrt (enzyme activity-U/gds)} = +5.66 - 0.044\,A_3 + 0.13\,B_3 + 0.18\,A_3\,B_3 - 0.53\,A_3^2 - 0.73\,B_3^2 \quad (4)$$

where, A is incubation time (h), B is substrate weight (g), A_1 is pH, B_1 is temperature (C), A_2 is inoculum size (%), B_2 is moisture level (%), A_3 is starch (%) and B_3 is NH_4NO_3 (%).

Adequacy of the model: The analysis of variance (ANOVA) was used to validate the proposed models; AB, A_1B_1, A_2B_2 and A_3B_3. The observed data significantly fit with the

proposed models, as shown by the values of the parameters F (24.26, 12.53, 65.09 and 50.99) and p (< 0.0003, 0.0022, 0.0001 and 0.0001) for the models AB, A_1B_1, A_2B_2 and A_3B_3 respectively, suggesting that there is only 0.03, 0.22. 0.01 and 0.01 % chance, respectively that a model F-value this large could occur due to noise. The higher values of R^2 for four models (0.9454, 0.8994, 0.9789 and 0.9444, respectively) also indicated the efficiency of the model, indicating that 94.54, 89.94, 97.89 and 94.44 %, respectively; of the responses variability could be explained by them. Adequate precision measures the signal to noise ratio and a value > 4 is considered appropriate for desirable models. The adequate precision value of 10.77 for glucoamylaze production indicates that the model can be used to navigate the design space. The significance of each variable and their mutual interaction for enhanced glucoamylaze production was also evaluated from the p-value.

The responses indicated that four (B; substrate weight, B_2; temperature, A_3, B_3; starch and NH_4NO_3 respectively), out of the eight coefficients and one interaction term A_3B_3 (starch and NH_4NO_3) have significant ($p < 0.0001$) effect on glucoamylaze production.

The response surface plots were obtained to calculate the optimal levels for different test variables. Therefore, four response surfaces were obtained from the combination of eight experimental variables. Based on the models equations, three dimensional plots were plotted in order to investigate the mutual interactions among the independent variables and to find out their optimum concentrations for glucoamylaze production.

Effect of substrate weight and incubation time: The objective of response surface optimization is to indicate a desirable location in the design space. The use of response surface was to find out the optimum values of the influential variables for which the response was maximized. The 3D plots shown in Fig. 2a-d were obtained from the correlation of concentrations of two variables keeping other variables at their optimum levels. To investigate the interaction between incubation time and substrate weight and to determine their optimum concentrations for maximum glucoamylaze production, the fermentation was carried out for 139.88 h at different levels of substrate concentration. A 3D plot of the incubation time and substrate weights (Fig. 2a) showed that maximum glucoamylaze activity (183.41 U/gds) was obtained at 72 h and with 15 g of the substrate weight. However, a decline in enzyme production was observed with further increase in incubation time. The experimental decline in glucoamylaze activity after 72 h of incubation could be a result of protease degradation, decrease in nutrient availability in the medium and catabolic repression of the enzyme [24].

Effect of pH and temperature combination: pH and temperature are two important parameters that strongly influence the growth of microorganisms and therefore, synthesis of the desired product.

Statistical tool was used to optimize these parameters and to determine their optimum levels for maximum enzyme production. A 3D plot of the pH and temperature (Fig. 2b) showed that maximum enzyme activity (213.77U/gds) was obtained at pH 5 and 37.5 °C which were in agreement with the predicted value. Further increase in reaction temperature

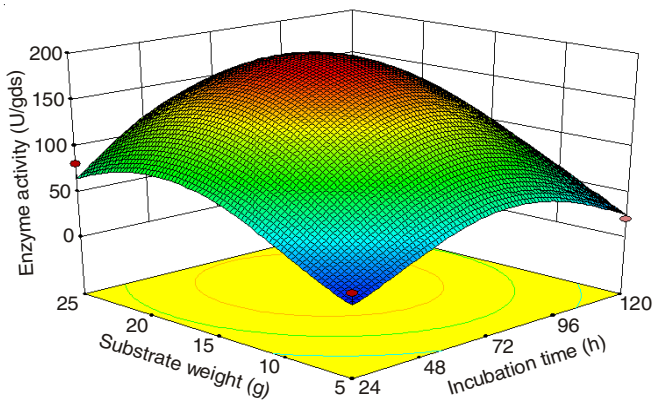

Fig. 2a. (3D plot) Response surface plot for substrate weight (g) and incubation time (h)

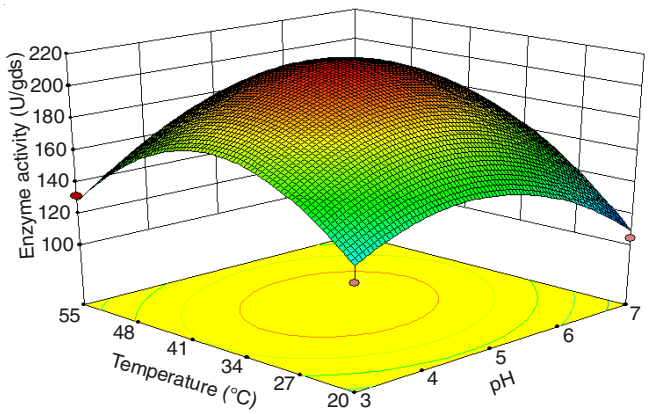

Fig. 2b. (3D) Contour plot of pH and temperature (°C) combination for glucoamylaze production

greatly reduced the enzyme activity. At higher temperature the organism consumes a lot of energy in fighting the unfavourable temperature conditions and therefore negatively affects its growth and enzyme production [25]. The metabolic activities of microorganisms are highly sensitive to pH variation and therefore glucoamylaze production by *Neurospora sitophila* showed significant reduction above or below the optimum pH. Fungal glucoamylazes are usually active at wide range of pH varying from 3.5 to 7 [13,26].

Effect of inoculum level and moisture content combination: The combined effect of inoculum level and moisture content is depicted in Fig. 2c. Maximum activity was shown at 20 % of the inoculum size and 65 % moisture content. The enzyme production at this combination was (227.2 U/gds) which was almost equal to the predicted value (227.6 U/gds). Adjustment of inoculum size for enzyme production under solid state fermentation is an important factor. Higher inoculum level not only increases the spore count, but also adding water content to the growing media, thereby affecting the fungal growth and enzyme synthesis. Lower inoculum size, on the other hand, introduces lesser number of the fungal cells to the growing medium. This will take relatively longer time to attain optimal growth for the product formation [27]. Water content plays a critical role in solid state fermentation influencing the physical state of the solid mass, nutrients solubility and exchange of gases [28]. Higher level of moisture content results in the reduction of substrate porosity, development of stickiness, distortion of the particle structure and disturbing the oxygen

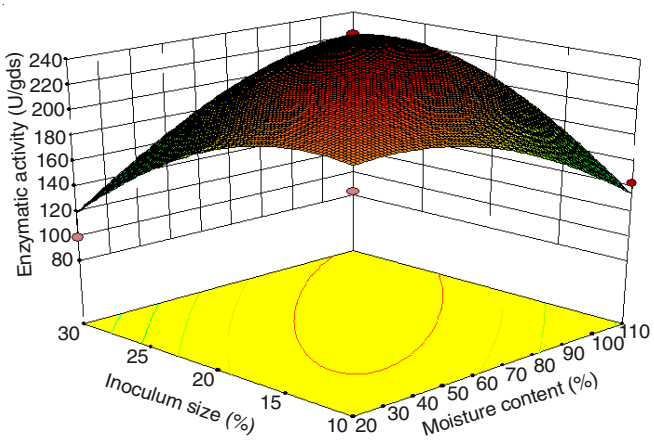

Fig. 2c. (3D plot) Contour plot of moisture level (%) and inoculum size (%) combination

transfer by reducing the gas volume [29]. On the other hand, lower level of water content causes poor substrate swelling and reduces the nutrients solubility of the substrate [27].

Effect of carbon- nitrogen combination on glucoamylaze production: The interactive effect of starch and NH_4NO_3 on glucoamylaze production was investigated between the low and high levels and the result is plotted in Fig. 2d. Based on the response surface plot, a concentration of 1.17 % of starch and 0.6 % of NH_4NO_3 was found to be the optimum combination for maximum glucoamylaze production. This combination of carbon and nitrogen exhibited the highest enzyme production (323.5 U/gds) which was higher than the predicted value (319.6 U/gds). Thus, it is evident from the data that the optimized combinations of the selected carbon and nitrogen sources revealed strong synergistic effects on glucoamylaze production.

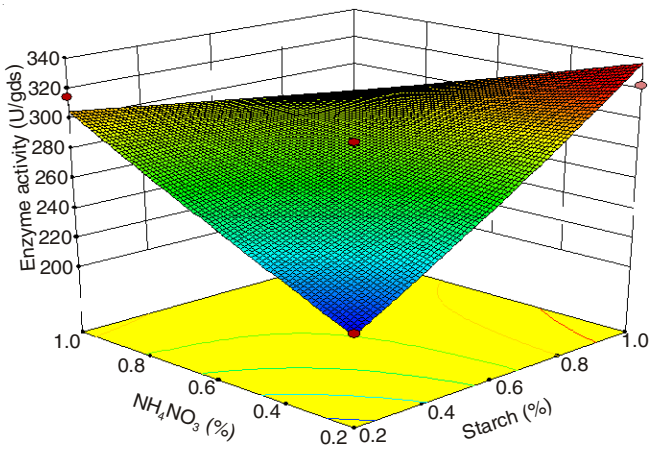

Fig. 2d. (3D plot) Contour plot of starch (%) and NH_4NO_3 (%) combination

Effect of surfactants: To evaluate the effect of surfactants on glucoamylaze production, the media components optimized by response surface methodology, were supplemented with different concentrations of the sodium dodecyl sulfate (SDS), Tween-20 and Tween-80.

Maximum enzyme production was obtained with SDS (335.2 ± 12 U/gds) at 0.3 % of the substrate weight and was found as a better surfactant than both Tween-20 and Tween-80 (Fig. 3). A decline in glucoamylaze production was observed at higher concentration of the SDS (beyond 0.3 %).

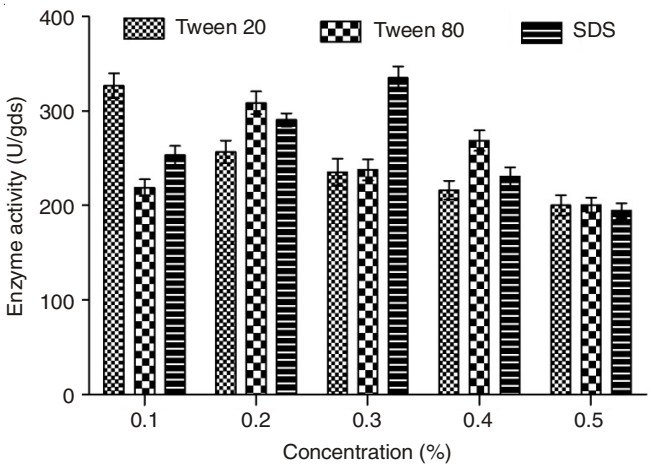

Fig. 3. Effect of surfactants on the glucoamylaze production under the optimized conditions

At higher surfactant concentration, the cells form aggregates in flasks, which affects the excretion of the polymer from the cells and its yield [30]. Surfactants promote the enzyme production by increasing the cell membrane and substrate permeability and also facilitating the enzyme-substrate interaction [31]. Previous reports also indicated that addition of surfactant to the fermentation media significantly increased the enzymes production [32,33].

Conclusion

In the present study, the glucoamylaze was produced under solid state fermentation on low cost agricultural substrates using *Neurospora sitophila*. Wheat bran was proved to be the most suitable substrate for fungal growth and enzyme production. Preliminary screening of exogenous carbon and nitrogen sources revealed that the effect of starch and ammonium nitrate on enzyme production was statistically significant. Statistical tool was employed to optimize different culture and media parameters that significantly influence the enzyme production. Among the screened factors, the interactive effect of starch and ammonium nitrate on glucoamylaze production was highly significant. The results indicated that statistical approach was significantly useful for optimization of process parameters and maximum glucoamylaze yield (335.2 ± 12 U/gds) was obtained under these optimized conditions. However, purification and characterization studies may provide some unexplored information about this enzyme.

ACKNOWLEDGEMENTS

This study was supported by Higher Education Commission of Pakistan (PIN: 117-8864-PS7-022) for providing financial support. The authors are highly grateful to the Department of Biochemistry, PMAS, Arid Agriculture University, Rawalpindi, Pakistan for providing lab facility.

REFERENCES

1. G.D. Najafpour, *Bioresour. Technol.*, **86**, 91 (2003).
2. A. Pandey, P. Nigam, C. Soccol, V. Soccol, D. Singh and R. Mohan, *Biotechnol. Appl. Biochem.*, **31**, 135 (2000).
3. H. Anto, U. Trivedi and K. Patel, *Bioresour. Technol.*, **97**, 1161 (2006).
4. G.S. Dhillon, H.S. Oberoi, S. Kaur, S. Bansal and S.K. Brar, *Ind. Crops Prod.*, **34**, 1160 (2011).

5. C.T. Slivinski, A.V.L. Machado, J. Iulek, R.A. Ayub and M.M. Almeida, *Braz. Arch. Biol. Technol.*, **54**, 559 (2011).
6. Hawksworth, *Med. Mycol.*, **49**, 70 (2011).
7. R.H. Davis and D.D. Perkins, *Nat. Genet.*, **3**, 397 (2002).
8. N. Mahanta, A. Gupta and S. Khare, *Bioresour. Technol.*, **99**, 1729 (2008).
9. D.M. Updegraff, *Anal. Biochem.*, **32**, 420 (1969).
10. A. Gupta, N. Gautam and D.R. Modi, *Biotechnol. Pharm. Res.*, **1**, 1 (2010).
11. V.S. Prajapati, U.B. Trivedi and K.C. Patel, *Food Sci. Biotechnol.*, **22**, 31 (2013).
12. M. Haque, M. ShamsUdDin and A. Haque, *Int. J. Food Sci. Technol.*, **37**, 453 (2002).
13. P. Ellaiah, K. Adinarayana, Y. Bhavani, P. Padmaja and B. Srinivasulu, *Process Biochem.*, **38**, 615 (2002).
14. M.I. Rajoka and A. Yasmeen, *World J. Microb. Biot.*, **21**, 179 (2005).
15. S. Ali, S. Mahmood, R. Alam and Z. Hossain, *World J. Microb. Biot.*, **5**, 525 (1989).
16. E. Gomes, S.R. Souza, R.P. Grandi and R.D. Silva, *Braz. J. Microbiol.*, **36**, 75 (2005).
17. R. Cruz, E.L. Souza, E.H.E. Hoffmann, M.Z. Bellini, V.A. Cruz and C.R. Vieira, *Rev. Microbiol.*, **28**, 101 (1997).
18. L.L. Barton, C.E. Georgi and D.R. Lineback, *J. Bacteriol.*, **111**, 771 (1972).
19. M. Hashemi, S.H. Razavi, S.A. Shojaosadati, S.M. Mousavi, K. Khajeh and M. Safari, *J. Biosci. Bioeng.*, **110**, 333 (2010).
20. H. Singh and S.K. Soni, *Process Biochem.*, **37**, 453 (2001).
21. T. Fowler, R. Berka and M. Ward, *Curr. Genet.*, **18**, 537 (1990).
22. X. Li and P. Gao, *Appl. Environ. Microbiol.*, **22**, 209 (1996).
23. G.G. Rao and B.V. Prajapati, *Biocatal. Agric. Biotechnol.*, **3**, 181 (2014).
24. N. Kango, *J. Food Eng.*, **85**, 473 (2008).
25. H.N. Bhatti, R. Hamid, M. Nawaz, R. Asgher, M.R. Perveen and A. Jabbar, *Food Technol. Biotechnol.*, **45**, 11 (2007).
26. W.C. Lam, D. Pleissner and C.S.K. Lin, *Biomolecules*, **3**, 651 (2013).
27. F. Zadra•il and H. Brunnert, *Eur. J. Appl. Microbiol. Biotechnol.*, **11**, 183 (1981).
28. A.M. Torrado, S. Cortés, J.M. Salgado, B. Max, N. Rodríguez, B.P. Bibbins, A. Converti and J.M. Domínguez, *Braz. J. Microbiol.*, **42**, 394 (2011).
29. S. Martins, S.I. Mussatto, G. Martínez-Avila, J. Montañez-Saenz, C.N. Aguilar and J.A. Teixeira, *Biotechnol. Adv.*, **29**, 365 (2011).
30. S. Arockiasamy and R.M. Banik, *J. Biosci. Bioeng.*, **105**, 204 (2008).
31. J. Gao, H. Weng, D. Zhu, M. Yuan, F. Guan and Y. Xi, *Bioresour. Technol.*, **99**, 7623 (2008).
32. A.P. Goes and J.D. Sheppard, *J. Chem. Technol. Biotechnol.*, **74**, 709 (1999).
33. Y.R. Liu, R. Yuan, Y. Chai, D. Tang, J. Dai and X. Zhong, *Sens. Actuators B*, **115**, 109 (2006).