SQU Journal for Science, 2022, 27(2),77-83 DOI:10.53539/squjs.vol27iss2pp77-83
Sultan Qaboos University

Optimization of Independent Variables for the Production of Extracellular Alpha Amylase by *Bacillus subtilis* IMD34 Using Plackett-Burman Design

Henshaw Effiom* and Josiah Lennox

Antimicrobials and Bioactive Molecules Research Group, Microbiology Department, Faculty of Biological Sciences, University of Calabar, Nigeria.
Email: henzefilo@yahoo.co.uk.

ABSTRACT: *Bacillus* species are widely used for thermostable α-amylase production for various applications such as in the detergent and food industries. This research aimed to study the production of α-amylase by *B. subtilis* IMD34 in submerged fermentation conditions using Plackett-Burman (PB) design, along with the optimization of certain environmental factors for optimal growth of amylolytic bacteria. Plackett-Burman design was used to evaluate the effect of ten independent variables on α-amylase production. Maximum enzyme activity was recorded in a medium containing (g/l); starch (6.0), peptone (12.0), K_2HPO_4 (1.0), Na_2HPO_4 (2.0), NaCl (0.6), Na_2SO_4 (0.1), $MgSO_4.7H_20$ (1.0), $FeCl_3.6H_2O$ (0.02), $CaCl_2.2H_2O$ (0.02) and with an agitation speed (200 rpm). From the pareto chart it was observed that the most important independent variables for amylase production were starch, peptone, NaCl, Mg_2SO_4, $CaCl_2.2H_2O$ and agitation. Optimization of environmental parameters to assess their effect on the growth of bacteria showed that optimal pH, incubation time and temperature were recorded at 6, 48h and 37˚C. Evaluating independent variables statistically using PB design revealed that some of the medium's components had a positive impact on α-amylase production by *B. subtilis* IMD34.

Keywords: Optimization; Independent variables; Alpha amylase; *Bacillus subtilis* IMD34; Plackett-Burman Design.

تحسين المتغيرات المستقلة لإنتاج أميلز ألفا خارج الخلية بواسطة *BACILLUS SUBTILIS* IMD34 بإستخدام تصميم Burman-Plackett

هينشو إفيوم و جوسيا لينوكس

الملخص: من بين البكتيريا ، من المعروف أن البكتيريا العصوية Bacillus تستخدم على نطاق واسع في إنتاج إنزيم ألفا amylase-α المستقر حراريا لتطبيقات مختلفة مثل المنظفات والصناعات الغذائية. كان الهدف من هذا البحث دراسة إنتاج إنزيم ألفا بواسطة بكتريا عصوية IMD34 في حالة التخمير المغمور باستخدام تصميم بلاكيت-بورمان مع تحسين بعض العوامل البيئية للنمو الأمثل للبكتيريا. تم استخدام تصميم بلاكيت بورمان لتقييم تأثير عشرة متغيرات مستقلة على إنتاج أنزيم ألفا. تم تسجيل أقصى نشاط للإنزيم في وسط يحتوي على (جم / لتر) ؛ النشا (6.0) ، البيتون (12.0) ، K2HPO4 (1.0) ، Na2HPO4 (2.0 ، كلوريد الصوديوم (0.6) ، ((0.1) 2SO4 (Na4)، MgSO4.7H20 (1.0)، FeCl3.6H2O (0.02)، CaCl2.2H2O (0.02) ، سرعة التحريك (200 دورة في الدقيقة). من مخطط باريتو لوحظ أن أهم المتغيرات المستقلة لإنتاج الإنزيم كانت النشا ، البيتون ، كلوريد الصوديوم ، Mg2SO4، CaCl2.2H2O التحريض. أظهر تحسين المعلمات البيئية لتقييم تأثيرها على نمو البكتيريا أنه تم تسجيل درجة الحموضة المثلى ووقت الحضانة ودرجة الحرارة عند 6 و 48 ساعة و 370 درجة مئوية. كشف تقييم المتغيرات المستقلة إحصائيًا باستخدام تصميم PB أن بعض مكونات الوسيط كان لها تأثير إيجابي على إنتاج أنزيم ألفا بواسطة B. subtilis IMD34.

الكلمات المفتاحية: التحسين، المتغيرات المستقلة ، ألفا أميليز، Bacillus subtilis IMD34 ، تصميم بلاكيت بورمان.

1. Introduction

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. They are a class of industrial enzymes having an approximately 25% share of the market for enzymes [1]. These enzymes are very useful in industrial processes such as making starch, paper and detergents, and in the brewing, textile and pharmaceutical industries. Although there are large number of microorganisms capable of producing useful enzymes, bacterial amylase is generally preferred for starch liquefaction due to its high stability [2] and neutral to alkaline pH. In the food industry, amylases are used in the production of glucose syrups, crystalline glucose, high fructose corn syrups, maltose syrups, and for the reduction of viscosity of sugar syrup and the reduction of haze formation in juices, as well as for solubilization and saccharification of starch for alcohol fermentation in the brewing industry and retardation of staling in the baking industry [2]. In the detergent industry amylases are used as an additive to remove starch based dirt, as well as forin warp sizing of textile fibers in the textile industry. Conventionally the production of alpha amylase has been carried out by submerged fermentation [3,4] of a number of bacterial species such as *Bacillus subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. acidocaldarius* and *B. marcesens* [3,5,6]. Designing an appropriate fermentation medium for highly efficient enzyme production is imperative and necessary as the medium's constituents and incubation condition can significantly affect the yield of this product [7-9]. Screening of independent variables in the medium used can be carried out by PB experimental design [10]. It is a partial factorial design where a large number of independent variables (N) are studied in small number of experiments (N+1) [11-12].

There are reports on the use of PB design for medium composition for enzyme production by bacteria [3,4,5,7,8,13-17]; however information on the use of PB design to study the influences of independent variables on the production of α-amylase by *B. subtilis* IMD34 along with the optimization of environmental process parameters for the growth of this bacterial strain is not available. In this research, ten independent variables, namely starch, peptone, $MgSO_4$, K_2HPO_4, $NaHPO_4$, NaCl, Na_2SO_4, $FeCl_3$.6HO, $CaCl_2.2H_2O$, and agitation speed were screened for their influences on α-amylase production by *B. subtilis* IMD34 under submerged fermentation using PB design. In addition, the optimization of certain environmental factors including pH, temperature and incubation time on the growth of this bacterial strain was studied.

2. Materials and method

2.1. Microorganism and cultivation

Bacillus subtilis IMD34 was obtained from Department of the Microbiology, University of Ibadan, Ibadan-Nigeria. All chemicals used in the experiment were of analytical grade. The organism was maintained on a nutrient agar (**Oxoid, Difco, USA**) slant and preserved at 4˚C.

2.2. Plackett-Burman experimental design

The importance of each independent variable including starch, peptone, MgSO4, K_2HPO_4, Na_2HPO_4, NaCl, Na_2SO_4, $FeCl_3.6H_2O$, $CaCl_2.2H_2O$, and agitation speed in the fermentation medium required for enzyme production in a shake flask culture using *B. subtilis* IMD34 was screened for. The PB experimental design of twelve runs (Table 2) for ten variables, nine nutritional factors and one environmental factor (Table 1), were used to evaluate the influence of each on α-amylase production. In Table 2, each row represents an experiment and each column represents an independent variable. For each variable two different concentrations, high and low, were tested (Table 1).

2.3. Submerged fermentation

All experiments were carried out in triplicate using 250 ml Erlenmeyer flasks containing 30 ml fermentation media as per experimental design, with pH adjusted to 7.0. Flasks were autoclaved at 15psi and 121˚C for 15 minutes, after which they were inoculated with an 18h old culture with a cell concentration of $1.6x10^5$ using aseptic procedures, and then incubated on an orbital incubator shaker (Model 10X400.XX2.C, Sanyo Gallenkamp PLC, UK) at 37˚C and 100 rpm for 24h [3,18].

2.4. Enzyme extraction assay

The culture fluids were centrifuged using a refrigerated high-speed ultra-centrifuge at 10,000xg (4˚C) for 5 minutes, after which the filtrates (supernatants) were assayed for extracellular 2-amylase using the DNSA reagent method [19]. Boiled culture served as a control. A standard curve was prepared with glucose, and the calibration curve so established was used to convert the spectrophotometer values to glucose equivalent, and activities were calculated [18, 20].

2.5. Optimization of environmental process parameters

Environmental process variables such as incubation time, temperature, and pH were varied to evaluate their effects on enzyme production and growth of *B. subtilis* IMB34. The fermentation medium as defined by PB design above was employed for the optimization process. The effect of incubation time on growth and amylase production

was evaluated at 12h, 24h, 36h, 48h, 60h, 72h, and at each 12 hourly interval growth (optical density) was measured at 610 nm using a Perkin-Elmer lambda UV/VIS spectrophotometer, and the enzyme activity was assayed as stated above. The incubation temperature was studied at (25˚C, 37˚C, 45˚C and 55˚C) and the pH of the fermentation medium was also varied (pH:4, pH:6, pH:7, pH:8 and pH:10). The procedure for measuring the growth (optical density) and enzyme assay as described above was adopted.

The data were statistically evaluated adhering to the following formula

a) The effect of each independent variable was evaluated with the equation below

Exi = 2(∑Hxi-∑Lxi)/N [13], where, Exi is the concentration effect of the independent variable, Hxi and Lxi are the concentrations of amylase at high and low levels of the same variables, and N is the number of trials (12). When the sign is positive, the influence of the variable on enzyme production is greater at high concentrations, and when negative the influence is greater at low concentrations.

b) The mean square of each variable was calculated as follows

Vxi = (∑Hxi-∑Lxi)/N [13] where Vxi is the mean square of the variable. Factors showing larger effects were identified using F-test [13]. Percentage concentration was calculated from F-test.

3. Results and Discussion

Amylases are ubiquitous enzymes produced by plants, animals and microorganisms, in which they play a dominant role in carbohydrate metabolism. Amongst the sources of amylase, microbes have been paid a great deal of attention because of their greater stability and safe use [21]. Among bacteria *Bacillus* species are known to be widely used for thermostable amylase production for various applications such as fermentation, detergents, food etc. [1, 22]. To enhance the production of amylase by *B. subtilis* IMD34 using PB design, ten independent variables were screened to evaluate their effects on enzyme production, and in addition, the optimization of certain environmental factors for the growth of *B. subtilis* IMD34 was investigated. The amylase activities from the twelve runs are presented in Table 2, where the results indicate a variation in amylase production with a range from 0.134-0.547Uml^{-1} by *B. subtilis* IMD34. These results have shown the need to screen the medium's components for efficient amylase yield [7, 14, 15, 23, 24, 25]. Figure 1 shows that the most important independent variables for amylase production were starch, peptone, NaCl, Mg_2SO_4, $CaCl_2.2H_2O$ and agitation.The highest amylase activity was recorded in trial number 9 containing (g/l); Starch (6.0), Peptone (12), K_2HPO_4 (1.0), $NaHPO_4$ (2.0), NaCl (0.6), Na_2SO_4 (0.2), $MgSO_4.7H_2O$ (1.0), $FeCl_3.6H_2O$ (0.02), and $CaCl_2.2H_2O$ (0.02), with an agitation speed of 200rpm. The data in Table 2 was statistically evaluated to ascertain the effect of each independent variable and its contribution to amylase production, as presented in Table 3.A positive effect indicates a direct relationship and the contribution of independent variables to the considered response (amylase production), and a negative effect indicates an inverse relationship between the variable and the response (Table 3). A positive sign for the effect means that by increasing the independent variable from low to high level leads to an increase in the response. An effect with a negative sign means that by changing the level of the independent variable from low to high, the response decreases.

Results from Table 3 indicate that starch, peptone, NaCl, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and increased agitation speed each showed a positive effect on amylase production while K_2HPO_4, Na_2HPO_4, Na_2SO_4 and $FeCl_3.6H_2O$ had negative effects. In this study, the positive effect shown by starch and peptone, $CaCl_2$ and $2H_2O$ is consistent with the research of Bansode [8], who reported the positive effect of the media components, starch, tryptone and $CaCl_2.2H_2O$, on amylase production using bacteria. A similar report by [7] revealed that peptone, agitation and $MgSO_4.7H_2O$ directly affected the rate of amylase production from the fungus *Aspergillus tubingensis* 1. Agitation intensity influences the mixing and oxygen transfer rate during submerged fermentation, and thus product formation [25]. Starch has been found to be the best carbon source for the production of amylase by *B. stearothermophilus* [26].

The commercial production of amylase is carried out in stages, essentially because the environmental factors required for the optimum growth of bacteria being employed for production may differ from those required for the maximum production of enzymes. In this study three environmental factors including pH, temperature and incubation time were optimized to ascertain the optimal conditions for bacterial growth. Optimum temperature, pH and incubation time were determined by varying incubation temperature (25˚C, 37˚C, 45˚C, 55˚C) (Figure 3), adjusting the fermentation medium pH (4, 6, 7, 8, 10) (Figure 4), and incubating the medium for various periods of time (12h, 24h, 36h, 48h, 60h, 72h) (Figure 2). Results obtained showed the optimum temperature for growth of *B. subtilis* IMD34 to be 37˚C (OD_{600nm}; 2.12) (Figure 3). Optimal growth of amylolytic *B. amyloliquefaciens* at 36˚C has been reported [27] and *B. subtilis* at a range of 35-37˚C [18]. The optimum pH for optimal bacterial growth (OD_{600nm}; 1.89) was observed at pH 6. The pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. It has been suggested that the metabolic activity of bacteria is very sensitive to the pH level of their media [16]. The result obtained in this study is similar to the observation of [27] who reported pH 6.5 to be the most favourable for growth of *B. marcescen*. The pH dependent changes in the growth might have been due to pH control of metabolic gene expression [17]. The optimum incubation time for growth (OD_{600nm}; 2.64) was observed at 48h (Figure 2). This result agrees with the earlier report by [18] who found that the optimum incubation time for optimal growth of *Bacillus* sp. was in the range 36-48h. The effect of incubation time on optimal growth decreases as its duration increases because the growth of the cells may reach their peak, move through the stationary phase without numerical increase and enter the decline phase [29].

Table 1. Plackett-Burman design for studying the influences of ten variables on α-amylase production by *B. subtilis* IMD 34.

Independent variables	Units	Low Level(-)	High Level(+)
Z_1: Starch	g/l	3.0	6.0
Z_2: Peptone	g/l	6.0	12.0
Z_3: K_2HPO_4	g/l	1.0	2.0
Z_4: Na_2HPO_4	g/l	2.0	4.0
Z_5: Nacl	g/l	0.6	1.2
Z_6: Na_2SO_4	g/l	0.1	0.2
Z_7: $MgSO_4.7H_2O$	g/l	0.5	1.0
Z_8: $Fecl_3.6H_2O$	g/l	0.02	0.04
Z_9: $Cacl_2.2H_2O$	g/l	0.01	0.02
Z_{10}: Agitation speed	rpm	100	200

Table 2. Plackett-Burman experimental design in 12 runs for studying the influences of independent variables on α-amylase production in shake culture and amylase activity.

Trials No	Z_1	Z_2	Z_3	Z_4	Z_5	Z_6	Z_7	Z_8	Z_9	Z_{10}	Amylase activity(Uml^{-1})
1	+	+	-	+	+	+	-	-	-	+	0.513
2	-	+	+	-	+	+	+	-	-	-	0.352
3	+	-	+	+	-	+	+	+	-	-	0.284
4	-	+	-	+	+	-	+	+	+	-	0.243
5	-	-	+	-	+	+	-	+	+	+	0.501
6	-	-	-	+	-	+	+	-	+	+	0.314
7	+	-	-	-	+	-	+	+	-	+	0.223
8	+	+	-	-	-	+	-	+	+	-	0.134
9	+	+	+	-	-	-	+	-	+	+	0.547
10	-	+	+	+	-	-	-	+	-	+	0.172
11	+	-	+	+	+	-	-	-	+	-	0.412
12	-	-	-	-	-	-	-	-	-	-	0.182

Table 3. Effects of independent variables on α-amylase production by *B. subtilis* IMD34 in shake culture.

Independent variables	ΔH	ΔL	MS	Effect	F-value	% contribution
Starch	3.243	1.124	0.212	0.216	989.12	65.23
Peptone	2.127	1.520	0.141	0.084	201.41	13.28
K_2HPO_4	1.587	1.810	0.012	-0.043	62.03	4.09
Na_2HPO_4	1.702	1.791	0.131	-0.024	44.01	2.90
NaCl	1.423	1.645	0.045	0.052	32.41	2.14
Na_2SO_4	1.413	1.522	0.061	-0.023	95.38	6.29
$MgSO_4.7H_2O$	1.782	1.325	0.064	0.018	55.26	3.64
$Fecl_3.6H_2O$	1.524	1.487	0.071	-0.017	22.41	1.48
$Cacl_2.2H_2O$	1.674	1.534	0.052	0.041	8.49	0.56
Agitation speed	1.824	1.642	0.021	0.032	5.92	0.39

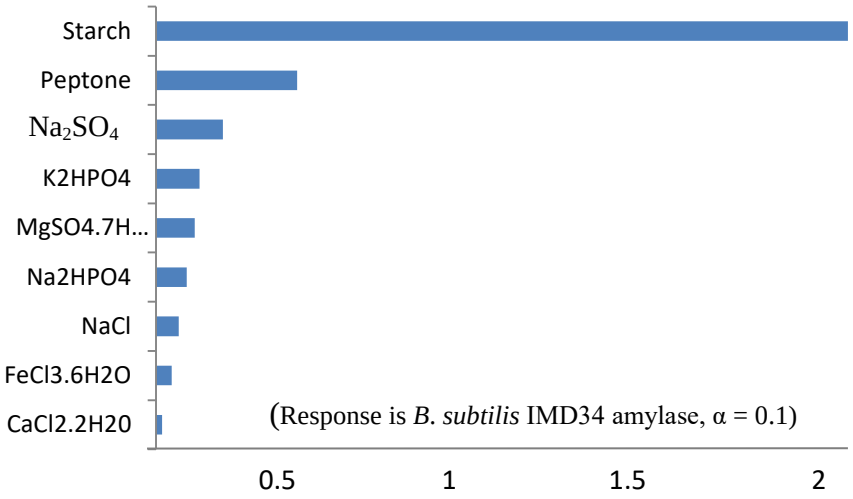

Figure 1. Pareto chart of the influences of independent variables on the response variable by *B. subtilis* IMD34

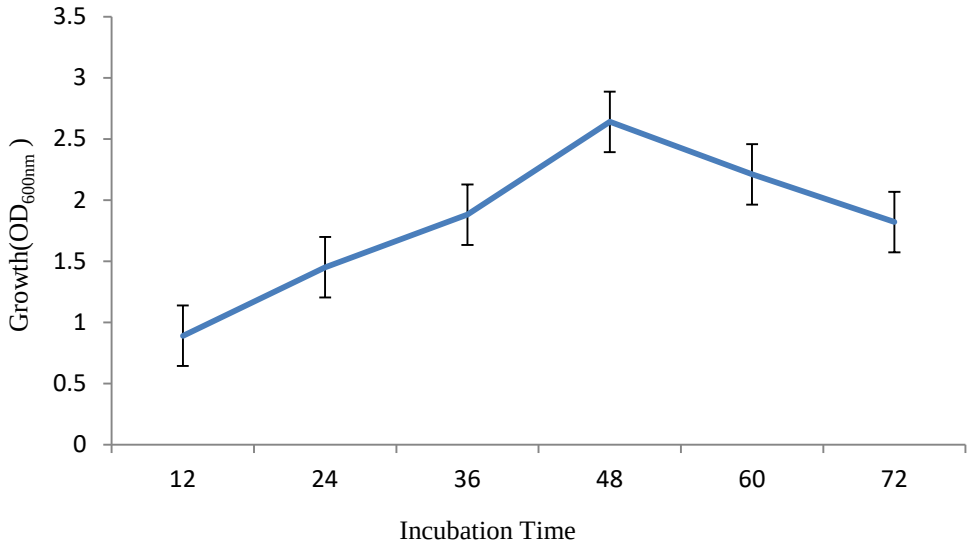

Figure 2. Growth pattern of *B. subtilis* IMD34 for various incubation times.

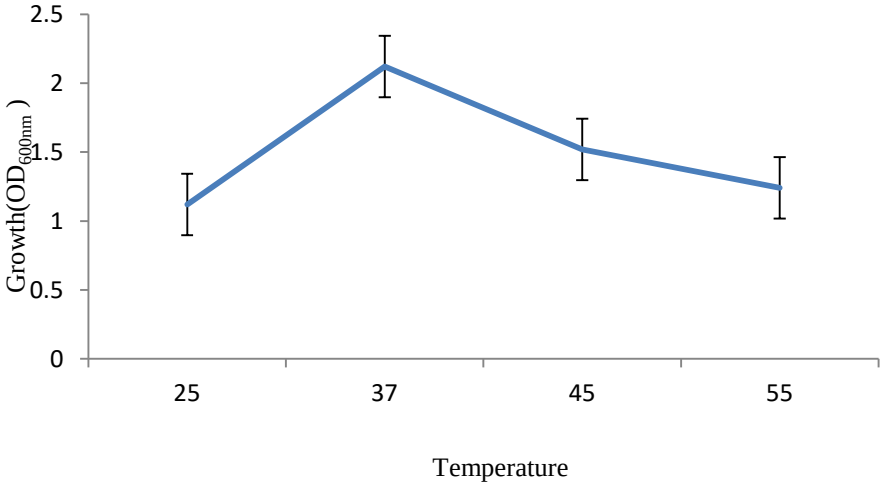

Figure 3. Growth pattern of *B. subtilis* IMD34 at different incubation temperatures.

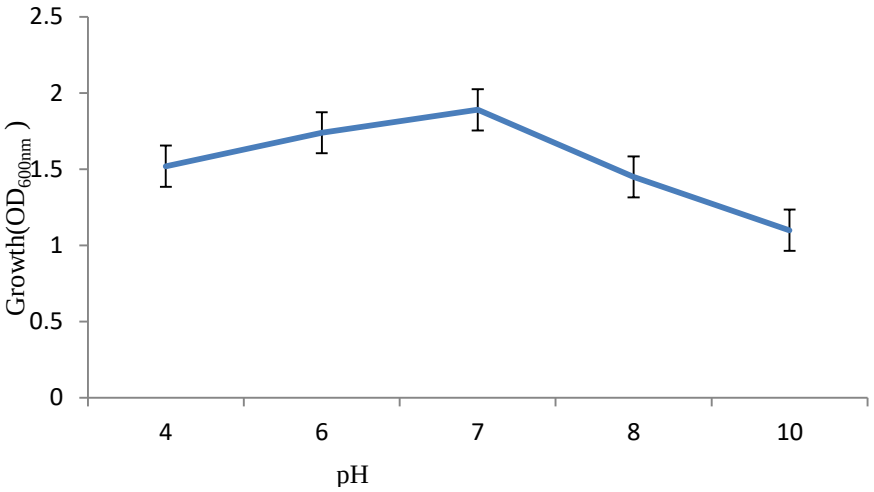

Figure 4. Growth pattern of *B. subtilis*IMD34 at various pH.

Conclusion

This study revealed, using PB design, that certain independent variables in the composition of the growth medium used had a positive impact on α-amylase production by *B. subtilis* IMD34.

Conflict of interest

The authors declare no conflict of interest.

Ethical Approval

Not applicable

Acknowledgment

The corresponding author acknowledges the valuable contributions of Professor Josiah Asime Lennox to this study. This research was not funded by any funding agency.

References

1. Das, S., Singh, S., Sharma, V. and Soriz, M.L. Biotechnological applications of industrially importance amylase enzymes. *International Journal of Pharmaceutical and Biological Science* 2011, **2**, 486-496.
2. Pandey, A., Soccol, C.R., Rodriquez-Leon, J.A. and Nigam, P. *Solid state fermentation in biotechnology; fundamental and applications*. New Delhi, Asia Technology 2001, pp. 3-7.
3. Enhasy, H.A.E. Bioprocess development for the production of α-amylase by *Bacillus amyloliquefaciens* in batch and fed-batch culture. *Research Journal ofMicrobiology* 2007, **2(7)**, 560-568.
4. Kunamneni, A., Permaul, K. and Singh, S. Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces funginosus*. *Journal of Bioscience and Bioengineering* 2005, **100(2)**, 168-171.
5. Konsoula, Z. and Liakopoulou-Kyrikides, M. Thermostable α-amylase production by *Bacillus subtilis* entrapped in calcium alginate gel capsules. *Enzyme Microbial Technology* 2006, **39(4)**, 690-696.
6. Aiyer, P.V. Amylase and their applications. *African Journal of Biotechnology* 2005, **4(13)**, 1525-1529.
7. Yahya, S., Jahanair, S., Shaukat, S.S., Sohail, M. and Khan, S.A. Production optimization by using Plackett-Burman design and partial characterization of amylase from *Aspergillus tubingensis*1. *Pakistan Journal of Biology* 2016, **48(6)**, 2557-2561.
8. Bansode, S. Screening of nutritional component for α-amylase production in submerged fermentation by bacteria isolated from soil using Plackett-Burman design. *International Journal of Pharmaceutical Science* 2010, **2(1)**, 93-98.

9. Khosravi, D.K., Zogli. A., Alavi, S.A. and Fatemi, S.S.A. Application of Plackett-Burman design for citric acid production from pretreated and untreated wheat straw. *Iran Journal of ChemicalEngineering*. 2008, **27(1)**, 91-104.
10. Plackett, R. I. and Burman, J. P. The design of optimum multifactorial experiments. *Biometrika* 1946, **33,** 305-325.
11. Naveena, B.J., Altaf, M., Bhadriah, K. and Reddy, G. Selection of medium component by Plackett-Burman design for the production of L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. *Bioresource Technology* 2005, **96**, 485-490.
12. Sayyad, S.A., Panda, B.P., Javed, S. and Ali, M. Screening of nutrient parameters for lovastatin production by *Monascus purpureus* MTCC 369 under submerged fermentation using Plackett-Burman design. *Research Journal* of *Microbiology* 2007, **2(7)**, 601-605.
13. Fossi, B.T., Tavea, F. and Ndjouenkeu, R. Production and partial characterization of a thermostable amylase from ascomycetes yeast strain isolated from starchy soil. *African Journal of Biotechnology* 2005; **4(1)**, 14-18.
14. Kumar, P. and Satyanarayana, T. Optimization of culture variables for improving glucoamylase production by alginate-entrapped *Thermomucorindicaeseudaticae* using statistical method. *Bioresource Technology* 2007, **98(6)**, 1252-1259.
15. Soni, P., Prasad, G.S. and Baneryee, U.C. Optimization of physicochemical parameters for the enhancement of carbonyl reductase production by *Candida viswanathii*. *Bioprocess and Biosystem Engineering* 2006, **20(3)**, 149-156.
16. Prakash, B., Vidyasagar, M., Madhukumar, M.S., Muralikrishna, G. and Sreeramulu, K. Production, purification and characterization of two extremely halotolerant, thermostable and alkali stable α-amylase from *Chromohalobacter* sp. TVSP.101. *Process Biochemistry* 2009, **44**, 10-15.
17. Yang, H., Liu, L., Li, J., Du, G. and Chen, J. Heterologous expression, biochemical characterization and overproduction of alkaline alpha amylase from *Bacillus alcalophilus*. *Microbiological Cell Factor* 2012, **10**, 71-77.
18. Henshaw, E. and Wakil, S.M. Effect of agitation speed and incubation time on amylase production by *Bacillus* species isolated from malted and fermented maize (*Zeamays*). *Microbiology Research Journal International* 2019, **27(3)**, 1-7.
19. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 1959, **31**, 426-428.
    Haq, I., Ali, S., Javed, M.M., Hammed, U., Saleem, A., Adrain, F. and Qadeer, A. Production of α-amylase from randomely induced mutant strain of *B. amyloliquefaciens* and its applications as a desizer in textile industry, *Pakistan Journal of Biology* 2010, **42(1)**, 473-484.
20. Sodhi, H. K., Sharma, K. and Soni, J. K. Production of a thermostable α-amylase from *Bacillus* sp PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Process Biochemistry* 2005, **40**, 525-534.
21. Rao, O.M., Swamy, A.N. and Sivarmakrishna, G. Biological process technology strategies, production and purification of amylases: an overview. *International Journal of Genomics and Proteomics* 2009, **2**, 27-29.
22. *Process Biochemistry* 2005, **40**, 525-534.
23. Bertrand, T.F., Fredric, T. and Robert, N. Production and partial characterization of a thermostable amylase from ascomycetes yeast strain isolated from starchy soil 2004, New York; McGraw Hill 53-85.
24. Gupta, R., Gigras, P., Mohapatra, H. and Goswani, V.K, Chauhan B. Microbial alpha amylase. *Process Biochemistry* 2003, **2**, 1-18.
25. Qader, S.B., Aman, A., Norman, S. and Abid, A. Enhanced production and extracellular activity of commercially important amylolytic enzymes by a new isolated strain of *Bacillus* sp AS-1. *Turkish Journal of Biochemistry* 2006, **31, 135**-140.
26. Alrumman, S.A., Mostafa, Y.S., Efan, S.A., Alamri, S.A. and Abd El-latif, H. Amylase and its industrial applications. *Advances In Life Sciences and Technology* 2014, **12**, 35-40.
27. Unakal, C., Kallur, R.I. and Kalural, B.B. Amylase production and application. *European Journal of Experimental Biology* 2012, **4**, 1044-1055.

Received 21 November 2021
Accepted 24 July 2022