OPTIMIZATION OF MINERAL SUPPLEMENTS FOR THE PRODUCTION OF
ALPHA AMYLASE FROM RICE BRAN USING Aspergillus Oryzae
THROUGH SUBMERGED FERMENTATION

Muralikandhan Kamaraj1*, Dhanasekaran Subramaniam2

1Bioprocess Laboratory, Department of Chemical Engineering, Faculty of Engineering and Technology, Annamalai University, Annamalai Nagar – 608 002, Tamil Nadu, India
2Mass Transfer laboratory, Department of Chemical Engineering, Faculty of Engineering and Technology, Annamalai University, Annamalai Nagar – 608 002, Tamil Nadu, India

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ABSTRACT

This study is aimed to investigate the optimum level of mineral supplements for the maximum production of fermentative α-amylase by using cheap substrate rice bran. The fungal strain Aspergillus oryzae MTCC-8624 is used to investigate the α-amylase production capability. The culture is maintained on potato dextrose agar (PDA) and sub-cultured at an interval of three months. The culture is initially screened for amylase production by starch agar plate assay on standard media. The report concludes that the optimization of mineral supplements for the fermentative α-amylase production and its suitability for a large-scale production using cheap and easily available substrate rice bran. The significant media components identified by Plackett-Burman design are KH2PO4 = 2.69 g/L; MgSO4 = 1.70 g/L; CaCl2 = 0.53 g/L; FeSO4 = 0.5 g/L and (NH4)2SO4 = 4.95 g/L for Rice Bran

KEYWORDS
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Rice Bran
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* Corresponding author
E-mail: muralikandhan1976@gmail.com (Muralikandhan Kamaraj)

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1 Introduction

Application of amylase enzyme is versatile and its demand in the various industries such as food, fermentation, textile, paper, detergent, pharmaceutical, and sugar are paramount (Asrat & Girma, 2018). Further, the economic and technological significance forced to pay great attention to the production of amylase enzyme. Its role in the hydrolysis of α-D-(1,4) glycosidic linkage in starch components and related polysaccharides to release maltose and disaccharide is the main reason for the continuous intensive research (Shah et al., 2014; Avwioroko & Tonukari, 2015; Bharathiraja et al., 2016; Subash et al., 2017; Asrat & Girma, 2018). Selection of substrate, the potential of microorganism, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, incubation period and thermostability are the main factors which directly influence the rate of amylase production (Pankaj et al., 2015). The utilization of agriculture waste is a promising one for the sustainable production of amylase. Cheaper cost and abundant availability are the supporting thoughts for the above statement (Bharathiraja et al., 2016). Due to the increasing demand for alpha-amylase in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and there cost-effective production techniques (Konsula & Liakopoulou-Kyriakides, 2004; Shivaramakrishnam et al., 2006). The selection of appropriate carbon and nitrogen sources or other nutrients is one of the most critical stages in the development of an efficient and economic process (Jiby et al., 2016). Further, Asrat & Girma (2018) investigated the potential of newly isolated strain Aspergillus niger FAB-21 using fruit peel wastes for the production of amylase. The report infers the maximum amylase activity of 1.241 U/ml at a pH of 6.0 and temperature at 45°C. Further, Elmansy et al. (2018) studied the effect of various fermentation conditions on α-amylase production through shake-flask culture Bacillus sp. NRC22017 and the maximum yield of α-amylase is inferred to be 15.15±0.47 U/ml at a pH of 6.0 with an inoculum size of 500 μl at 45°C and anaerobic incubation period of 72 h. Physical factors that are affecting the production of the α-amylase from a newly isolated Bacillus sp. M10 strain. lus sp. M-10 namely temperature, pH, aeration, inoculum size, and inoculum age are optimized by Demirkiran et al. (2017). Among the important factors, 37°C temperature, pH 7.0, 150 rpm for aeration, 2.5% (v/v) inoculation size and 2 days for inoculation age are found to be the optimum rate for maximum α-amylase production of 30 U/mL at the hour of 48. Pathania et al., (2017) optimized the process variables included pH, temperature, inoculum size, incubation days and substrate concentration for production of amylase by B. amyloliquefaciens SH8 using response surface methodology and achieved the maximum amylase activity of 16.07 IU/ml at the optimum condition of pH 5, temperature 45°C, inoculum size 5%, incubation day of 5, and substrate concentration of 0.60%. Similarly, Jiby et al. (2016) tested the activity of α- amylase from A. niger utilizing coconut water, tapioca water, rice water, and white yam. The maximum activity of α-amylase is recorded as 0.29 x 10^3 μmoles/sec after 7 days of submerged fermentation on white Yam water at pH 7.0 and 28°C. Among the three medium, rice water recorded as second (0.09 x 10^3 μmoles/sec) and tapioca water (0.06 x 10^3 μmoles/sec) as third position. Vimal et al. (2015) identified as B.amyloliquefaciens KCP2 using 16S rDNA gene sequencing data yielded the maximum amylase activity of 63.12 U/ml. Similarly, Ahmed et al. (2015) optimized the experimental parameters for alpha-amylase production by A. fumigatus in submerged fermentation, these researchers used sunflower waste, cotton stalk, rice husk, date syrup, and molasses are tested as carbon source. The maximum production of α-amylase is found to be 7.01 U/ml by A. fumigatus are 72 h of incubation period at initial pH 5.5, temperature 35°C, inoculum size of 6x10^6 conidia in 50 ml of culture medium and agitation rate of 150 rev/min. Shah et al. (2014) isolates a total of 17 fungal cultures from soil samples. Out of which IP31, A.oryzae, exhibited good amylolytic enzymes production. After parametric optimization maximum amylolytic activity was observed, when pH of the mineral salt medium was 7.0, incubation temperature of 45°C, after incubation of 72 hrs by using 50 ml of starchy wastewater as sole carbon source with 5 discs of A. oryzae. After studying the kinetic properties of α-amylase, its maximum activity was found at pH 6, the temperature of 50°C, with 1.5% of substrate concentration. The Vmax and Km value was 37.037 IU/mL and 1.4 mg/mL respectively for α-endoglucanase. Submerged fermentation has been defined as fermentation in the presence of excess water. Almost all the large-scale enzyme-producing facilities are using the proven technology of submerged fermentation due to better monitoring and ease of handling (Singhania, 2011). It is the preferred technology for industrial enzyme production due to ease of handling at large-scale when compared to SSF. Conventional fermenters for submerged fermentation technology are properly advanced and offer online manage over several parameters together with pH, temperature, dissolved oxygen, and froth formation with easy mass transfer and heat removal. These benefits make submerged fermentation technology superior to solid-state. It is widely conformist for industrial metabolites production. The choice of an appropriate condition for the substrate is a key aspect of submerged fermentation. It depends on various factors, including cost, availability, particle size, and moisture content. This screening process involves, avoiding the formation of several by-products and the substrate of choice being the one that not only serves as the best nutrient source but also acts as the best support for cell growth (Rajagopalan & Krishnan, 2008). The medium in submerged fermentation is liquid, which remains in contact with the microorganisms (Singhania et al., 2015). With this few introductions, this investigation is aimed to optimize the mineral supplements for the production of alpha-amylase from A.oryzae using rice bran through submerged fermentation.
2 Materials and Methods

2.1 Microorganism and its Maintenance

The strain of *A. oryzae* MTCC-8624 used in this investigation was obtained from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture is maintained on potato dextrose agar (PDA) and sub-cultured at an interval of three months (Ellaiah et al., 2002). The culture is initially screened for amylase production by starch agar plate assay on standard media. The inoculated plates, containing media, supplemented with starch is stained with Gram's iodine reagent, after 72 hr of incubation. The plates are then flooded with iodine solution for 15 minutes and washed with warm water to remove the excess colour (Mabel et al., 2006).

2.2 Inoculum Preparation

The strain is sub-cultured on PDA slants and incubated for 72 hr at a temperature of 25°C. After the incubation period, the spore suspensions are prepared by adding 10 ml of sterile water to the PDA slant containing sporulated slant cultures. The spores on the surface of the medium were dislodged using inoculation needle under aseptic conditions. The spore suspensions are filtered using sterile muslin cloth into sterile flasks. The filtered spore suspension is transferred into 250 ml Erlenmeyer flask containing 100 ml of potato dextrose broth and incubated for three days at 25°C. Appropriate volumes of inoculums (% v/v) are used to inoculate the production medium (Tanyildizi et al., 2005).

2.3 Fermentation Medium

In this investigation, rice bran is utilized as a substrate. It is collected from nearby areas of Chidambaram, Tamil Nadu, India. Since this agricultural by-product is not available in completely dried form, it is necessary to dry this substrate prior to use them in the fermentation process. In the present study, the substrate is dried by keeping them in the oven at 80°C for 12 hr. After drying, the substrates are powdered in a laboratory grinder and sieved using a 40mm sieve. An adequate amount of the powdered substrate is mixed with 100ml of the corresponding mineral salt media in a 250ml Erlenmeyer flask. After adjusting the pH, the contents of the flask were sterilized in an autoclave at 121°C and 15 psi pressure for 15 minutes. Appropriate volumes of inoculums are added to the flasks after cooling it down to room temperature. All the experiments for media optimization were carried out with a substrate concentration of 20g/L, inoculum size of 5% (v/v), and fermentation time of 72 hr. The pH and temperature are maintained at 5 and 25°C respectively (Sarra et al., 1993).

2.4 Extraction of amylase from the fermentation medium

At the end of the fermentation period, contents of the flask are filtered using a Whatman No.44 filter paper followed by filtration through a muslin cloth. The filtrate is then centrifuged at 10,000 rpm for 10 min and the supernatant was used as the source of enzyme for assay (Ahuja et al., 2004).

2.5 Assay of amylase

Assay system for amylase activity is carried out by measuring the amount of reducing sugar according to DNS method (Fogarty, 1983). Amylase activity is determined by incubating a mixture of 1 ml of the aliquot of each enzyme source and 1% soluble starch dissolved in 0.1 M phosphate buffer, at pH 7, at 55°C for 15 min. The reaction is arrested by adding 1 ml of 3, 5 DNS Acid followed by10 minutes boiling. The final volume is made up of 12 ml with distilled water and the reducing sugar released was measured at 540 nm. Reducing sugar (Glucose or maltose) concentration is determined from a standard curve under the same condition using glucose. Figure 1 is used to represent the calibration curve for glucose concentration using Bio-spectrophotometer (Ellaiah et al., 2002; Mabel et al., 2006).

![Figure 1 Calibration chart for glucose concentration using Bio-spectrophotometer](http://www.jebas.org)

3 Results and Discussion

3.1 Screening of media components for *Aspergillus oryzae*

Fifteen variables were screened in twenty trials, each variable being a media constituent. The medium constituents used for *A. oryzae* MTCC-8624 are KH₂PO₄(A), (NH₄)₂SO₄(B), KCl(C), MgSO₄(D), CaCl₂(E), Urea(F), FeSO₄(G), ZnCl₂(H), NH₄NO₃(J), NaCl(K), MnSO₄(L), K₂HPO₄(M), CoCl₂(N), NaNO₃(O) and FeCl₃(P). Two level design (-1 indicates the lower level and +1 indicates the higher level) are generated for fifteen variables with one replicate. The coded and actual values of the variables are shown in Table 1. Table 2 shows Plackett-Burman design for conducting 20 trials and the corresponding amylase production was noted. The effect of the variables and their significance on...
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Table 1 Variables screened in Plackett–Burman design for amylase activity using *A. oryzae* MTCC-8624

| Variables   | Low level (-) values (g/L) | High level (+) values (g/L) |
|-------------|-----------------------------|-----------------------------|
| KH₂PO₄      | 1                           | 5                           |
| MgSO₄       | 1                           | 3                           |
| Peptone     | 1                           | 10                          |
| Yeast extract | 2                         | 8                           |
| FeSO₄       | 2                           | 5                           |
| CaCl₂       | 0.2                         | 2                           |
| NaCl        | 1                           | 3                           |
| NaNO₃       | 0.5                         | 1.5                         |
| Urea        | 0.5                         | 5                           |
| MnSO₄       | 0.1                         | 0.9                         |
| (NH₄)₂SO₄   | 1                           | 5                           |
| CoCl        | 0.005                       | 0.5                         |
| ZnSO₄       | 1                           | 5                           |
| ZnCl₂       | 0.1                         | 1                           |
| KCl         | 1                           | 7                           |

Table 2 Plackett–Burman experimental design matrix for screening of important variables for *A. oryzae* MTCC-8624 utilizing rice bran

| Runs | A  | B  | C  | D  | E  | F  | G  | H  | J  | K  | L  | M  | N  | O  | P  | Amylase Activity (U/ml) (mol H₂/mol glucose) | Exp. | Pred. |
|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-------------------------------------------------|------|-------|
| 1    | -1 | 1  | 1  | -1 | 1  | 1  | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1    | 9.60                                          | 9.647 |       |
| 2    | 1  | -1 | 1  | -1 | -1 | -1 | -1 | -1 | 1  | 1  | 1  | 1  | 1  | 1  | -1   | 9.87                                          | 9.837 |       |
| 3    | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | -1   | 8.93                                          | 8.877 |       |
| 4    | 1  | -1 | 1  | 1  | 1  | 1  | -1 | -1 | 1  | -1 | 1  | 1  | 1  | -1 | -1   | 4.62                                          | 4.487 |       |
| 5    | -1 | 1  | -1 | 1  | 1  | 1  | 1  | -1 | 1  | -1 | 1  | 1  | -1 | 1  | 1    | 9.15                                          | 9.103 |       |
| 6    | 1  | -1 | -1 | 1  | 1  | 1  | 1  | -1 | 1  | -1 | -1 | 1  | -1 | -1 | 1    | 7.98                                          | 7.669 |       |
| 7    | 1  | 1  | 1  | 1  | 1  | -1 | 1  | 1  | 1  | 1  | -1 | 1  | -1 | 1  | 1    | 4.62                                          | 5.011 |       |
| 8    | -1 | 1  | 1  | -1 | 1  | 1  | 1  | 1  | -1 | 1  | -1 | 1  | 1  | -1 | 1    | 9.65                                          | 10.041|       |
| 9    | 1  | -1 | -1 | 1  | 1  | 1  | 1  | 1  | -1 | -1 | -1 | -1 | -1 | 1  | 1    | 4.63                                          | 4.339 |       |
| 10   | -1 | -1 | 1  | 1  | -1 | 1  | 1  | 1  | -1 | -1 | 1  | 1  | -1 | -1 | 1    | 7.54                                          | 7.507 |       |
| 11   | 1  | -1 | 1  | -1 | -1 | 1  | 1  | 1  | 1  | -1 | -1 | -1 | -1 | 1  | 1    | 5.97                                          | 6.003 |       |
| 12   | -1 | -1 | -1 | -1 | -1 | 1  | 1  | 1  | -1 | -1 | -1 | -1 | -1 | 1  | -1   | 9.03                                          | 9.241 |       |
| 13   | -1 | 1  | 1  | -1 | 1  | -1 | 1  | 1  | 1  | -1 | -1 | 1  | 1  | -1 | 1    | 7.98                                          | 8.271 |       |
| 14   | -1 | -1 | 1  | -1 | 1  | -1 | -1 | 1  | 1  | -1 | -1 | -1 | -1 | 1  | 1    | 9.80                                          | 9.489 |       |
| 15   | -1 | 1  | 1  | -1 | -1 | 1  | 1  | -1 | 1  | 1  | -1 | -1 | -1 | 1  | 1    | 10.17                                         | 9.779 |       |
| 16   | -1 | 1  | 1  | 1  | 1  | -1 | 1  | 1  | 1  | 1  | -1 | -1 | 1  | -1 | -1   | 8.73                                          | 8.783 |       |
| 17   | -1 | -1 | 1  | 1  | 1  | 1  | -1 | 1  | -1 | -1 | -1 | -1 | 1  | 1  | -1   | 8.43                                          | 8.219 |       |
| 18   | 1  | 1  | 1  | -1 | 1  | -1 | 1  | -1 | 1  | -1 | -1 | 1  | 1  | -1 | 1    | 9.79                                          | 9.843 |       |
| 19   | 1  | -1 | 1  | 1  | 1  | 1  | 1  | 1  | -1 | 1  | 1  | -1 | 1  | 1  | 1    | 6.72                                          | 6.931 |       |
| 20   | 1  | -1 | -1 | 1  | 1  | 1  | 1  | -1 | 1  | 1  | 1  | 1  | -1 | -1 | 1    | 6.49                                          | 6.623 |       |

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Table 3 Estimated effects and coefficients of the Plackett–Burman design for *A. oryzae* MTCC-8624 utilizing rice bran

| Terms | Effect | Coeffi. | SE Coeffi. | T   | P    |
|-------|--------|---------|------------|-----|------|
| Constant | 7.985 | 0.1122 | 0.000 |
| A      | -2.046 | -1.023 | 0.1122 | -9.12 | 0.001 |
| B      | 1.394 | 0.697 | 0.1122 | 6.21 | 0.003 |
| C      | 0.552 | 0.276 | 0.1122 | 2.46 | 0.070 |
| D      | -1.850 | -0.925 | 0.1122 | -8.25 | 0.001 |
| E      | -0.870 | -0.435 | 0.1122 | -3.88 | 0.018 |
| F      | -0.310 | -0.155 | 0.1122 | -1.38 | 0.239 |
| G      | 1.020 | 0.510 | 0.1122 | 4.55 | 0.010 |
| H      | -0.206 | -0.103 | 0.1122 | -0.92 | 0.410 |
| J      | 0.416 | 0.208 | 0.1122 | 1.85 | 0.137 |
| K      | 0.182 | 0.091 | 0.1122 | 0.81 | 0.463 |
| L      | 0.590 | 0.295 | 0.1122 | 2.63 | 0.058 |
| M      | -0.484 | -0.242 | 0.1122 | -2.16 | 0.097 |
| N      | -0.344 | -0.172 | 0.1122 | -1.53 | 0.200 |
| O      | 0.050 | 0.025 | 0.1122 | 0.22 | 0.835 |
| P      | -0.606 | -0.303 | 0.1122 | -2.70 | 0.054 |

Figure 2 Parity plot between the experimental and predicted values of important variables for *A. oryzae* MTCC-8624 utilizing rice bran

the enzyme activity is found by analyzing the responses statistically. Variables with P value < 0.05 are significant. The effect estimates of amylase activity from the result of Plackett-Burman design for rice bran utilizing *A. oryzae* MTCC-8624 was represented in Table 3. The Parity plot between the experimental and predicted values is shown in Figure 2. Figure 3 shows the Pareto chart for rice bran. From the Pareto chart *KH₂PO₄*, *(NH₄)₂SO₄, MgSO₄, CaCl₂* and FeSO₄ were found to be significant. It is reached based on the p values noted in Table 3.
3.2 Optimization of media composition of *Aspergillus oryzae* MTCC-8624 using CCD for rice bran

The optimum levels of significant variables obtained from the Plackett-Burman design for *A. oryzae* MTCC-8624 is determined by the central composite design of RSM. Table 1 gives the detail of the actual and coded values employed in the design for the substrate rice bran. The 52 run design matrices using the five independent variables with the experimental and predicted responses are shown in Table 2. The second-order polynomial Equation 2 for amylase production (Y) for *A. oryzae* MTCC-8624 utilizing rice bran as substrate was found as follows:

\[
Y = 13.5532 + 0.0420755A - 0.00609608B + 0.248739C
  + 0.273624D - 0.166398E
  - 0.648658A^2 + 0.241188B^2
  - 0.596509C^2 + 0.341067D^2
  - 0.500166E^2
  - 0.199062AC - 0.212813AD
  + 0.300938AE + 0.369063BC
  + 0.134063BD - 0.154063BE
  - 0.0209375CD - 0.170313CE
  + 0.252188DE \rightarrow \text{(2)}
\]

Where A - KH₂PO₄; B - (NH₄)₂SO₄; C - MgSO₄; D - CaCl₂ and E - FeSO₄

The parameters estimated and the corresponding P-values are shown in Table 3. From the data the terms A, D, E, B², C², D², E², AB, AC, AE, BC, BE, and DE were found to be significant for rice bran. Here the $R^2$ values of 0.9833 indicate a good agreement between the experimental and predicted values for rice bran. The $R^2$ predicted values of 0.9353 were also in good agreement with $R^2$ adjusted values of 0.9725 correspondingly. The parity rice bran is represented in Figure 4.

For rice bran, the minimum and maximum production was 10.41 U/ml and 14.71 U/ml respectively for Run No. 25 and Run No. 49. The results obtained by CCD are analyzed by standard analysis of variance (ANOVA) are shown in Table 4. The central composite experimental design with five independent variables for media optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate is shown in Table 5. The three-dimensional response surface curves constructed by the regression model are shown in Figure 5.1 to 5.10 for rice bran. The
Figure 4 Parity plot between the experimental and predicted values of important variables for *A. oryzae* MTCC-8624 utilizing rice bran

Table 5 The Central Composite experimental design with five independent variables for media optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate

| Run No | A   | B   | C   | D   | E   | Amylase Activity (U/ml) | Exp. | Pred. |
|--------|-----|-----|-----|-----|-----|-------------------------|------|-------|
| 1      | 0   | 0   | 0   | 0   | 0   | 14.71                   | 14.701|
| 2      | 1   | 1   | 1   | 1   | -1  | 12.09                   | 12.296|
| 3      | -1  | -1  | -1  | 1   | -1  | 13.31                   | 13.232|
| 4      | 0   | 0   | 0   | 0   | -2.38| 11.23                   | 11.004|
| 5      | 0   | 0   | 0   | 0   | 2.38 | 11.01                   | 11.243|
| 6      | -1  | 1   | -1  | 1   | 1   | 12.98                   | 12.832|
| 7      | 0   | 0   | 0   | 2.38| 0   | 13.26                   | 13.221|
| 8      | -1  | -1  | -1  | 1   | 1   | 13.08                   | 12.972|
| 9      | 1   | -1  | -1  | 1   | -1  | 12.92                   | 12.916|
| 10     | 2.38| 0   | 0   | 0   | 0   | 12.97                   | 12.788|
| 11     | -1  | -1  | 1   | 1   | 1   | 11.21                   | 11.286|
| 12     | 0   | 0   | 0   | 0   | 0   | 14.65                   | 14.701|
| 13     | 0   | 0   | 0   | 0   | -2.38| 12.69                   | 12.736|
| 14     | 0   | 0   | 0   | 0   | 0   | 14.68                   | 14.701|
| 15     | 1   | 1   | 1   | -1  | -1  | 11.81                   | 11.970|
| 16     | -1  | 1   | 1   | 1   | -1  | 12.59                   | 12.605|
| 17     | 1   | 1   | -1  | 1   | -1  | 11.71                   | 11.833|
| 18     | 1   | -1  | 1   | 1   | 1   | 12.92                   | 12.780|
| 19     | 1   | 1   | -1  | 1   | 1   | 12.02                   | 11.959|
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| Run No | A  | B  | C  | D  | E  | Exp.  | Pred.  |
|--------|----|----|----|----|----|-------|--------|
| 20     | -1 | 1  | 1  | -1 | 1  | 11.61 | 11.624 |
| 21     | -1 | -1 | 1  | -1 | 1  | 13.13 | 12.891 |
| 22     | 1  | 1  | 1  | -1 | 1  | 12.64 | 12.430 |
| 23     | -1 | -1 | 1  | -1 | -1 | 11.25 | 11.134 |
| 24     | 1  | 1  | -1 | -1 | -1 | 11.55 | 11.502 |
| 25     | 0  | 0  | 2.38 | 0 | 0 | 10.41 | 10.659 |
| 26     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 27     | 0  | -2.38 | 0  | 0  | 0  | 11.58 | 11.763 |
| 28     | 0  | 0  | -2.38 | 0  | 0  | 12.35 | 12.108 |
| 29     | 1  | -1 | -1 | 1  | 1  | 12.72 | 12.999 |
| 30     | 1  | -1 | -1 | 1  | -1 | 12.28 | 12.356 |
| 31     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 32     | -1 | 1  | -1 | 1  | -1 | 13.36 | 13.609 |
| 33     | -1 | 1  | 1  | 1  | 1  | 12.01 | 11.818 |
| 34     | -1 | 1  | 1  | -1 | -1 | 12.21 | 12.066 |
| 35     | 1  | -1 | 1  | -1 | -1 | 11.81 | 11.938 |
| 36     | -1 | -1 | 1  | 1  | -1 | 11.55 | 11.555 |
| 37     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 38     | 1  | 1  | 1  | 1  | 1  | 12.35 | 12.412 |
| 39     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 40     | 1  | -1 | -1 | -1 | -1 | 12.02 | 12.142 |
| 41     | 0  | 2.38 | 0  | 0  | 0  | 11.81 | 11.634 |
| 42     | 1  | -1 | 1  | 1  | -1 | 12.43 | 12.147 |
| 43     | 1  | -1 | -1 | -1 | 1  | 13.31 | 13.130 |
| 44     | -1 | 1  | -1 | -1 | -1 | 13.15 | 13.065 |
| 45     | -1 | -1 | 1  | -1 | 1  | 11.39 | 11.209 |
| 46     | -1 | -1 | -1 | -1 | -1 | 12.61 | 12.805 |
| 47     | -1 | 1  | -1 | -1 | 1  | 12.36 | 12.633 |
| 48     | -2.38 | 0  | 0  | 0  | 0  | 12.68 | 12.870 |
| 49     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 50     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 51     | 0  | 0  | 0  | 0  | 0  | 14.71 | 14.701 |
| 52     | 1  | 1  | -1 | -1 | 1  | 11.78 | 11.972 |
Figure 5.1 3D Plot shows the interaction between the medium components (NH4)2SO4 and KH2PO4 for *Aspergillus oryzae* using rice bran

Figure 5.2 3D Plot shows the interaction between the medium components MgSO4 and KH2PO4 for *Aspergillus oryzae* using rice bran

Figure 5.3 3D Plot shows the interaction between the medium components CaCl2 and KH2PO4 for *Aspergillus oryzae* using rice bran

Figure 5.4 3D Plot shows the interaction between the medium components FeSO4 and KH2PO4 for *Aspergillus oryzae* using rice bran

Figure 5.5 3D Plot shows the interaction between the medium components (NH4)2SO4 and MgSO4 for *Aspergillus oryzae* using rice bran

Figure 5.6 3D Plot shows the interaction between the medium components (NH4)2SO4 and CaCl2 for *Aspergillus oryzae* using rice bran
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Optimum values of the variables were found from the equations derived by the differentiation of the obtained second-order polynomial equations. The optimum values were found to be: $\text{KH}_2\text{PO}_4$ - 2.69 (g/L); $(\text{NH}_4)_2\text{SO}_4$ - 4.95 (g/L); MgSO$_4$ - 1.70 (g/L); CaCl$_2$ - 0.53 (g/L); and FeSO$_4$ - 0.50 (g/L) for rice bran shown in Table 5. Table 6 shows the results of the regression analysis of the second-order polynomial model for media optimization of *A. oryzae* MTCC-8624 utilizing rice bran as a substrate. The significant media components identified by Plackett-Burman design are $\text{KH}_2\text{PO}_4$ = 2.69 g/L; $(\text{NH}_4)_2\text{SO}_4$ = 1.70 g/L; CaCl$_2$ = 0.53 g/L; and FeSO$_4$ = 0.5 g/L. These results are in agreement with the findings of previous researchers such as Konsula & Liakopoulou-Kyriakides (2004), Shivaramakrishnam et al. (2006), Bharathiraja et al. (2016), Subash et al. (2017) and Asrat & Girma (2018).

**Conclusion**

This investigation concludes that the optimization of mineral supplements for the fermentative α-amylase production and its suitability for a large-scale production using cheap and easily available substrate rice. The fungal strain *A. oryzae* MTCC-8624 is used in this study to investigate the α-amylase production capability. The significant media components identified by Plackett-Burman design are $\text{KH}_2\text{PO}_4$ = 2.69 g/L; MgSO$_4$ = 1.70 g/L; CaCl$_2$ = 0.53 g/L; FeSO$_4$ = 0.5 g/L; and $(\text{NH}_4)_2\text{SO}_4$ = 4.95 g/L for rice bran.
### Table 6 Results of the regression analysis of second order polynomial model for media optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate

| Term constant | Regression coefficient | T-statistics | P-value |
|---------------|------------------------|--------------|---------|
| Intercept     | 14.7015                | 240.049      | 0.000   |
| A             | -0.0173                | -0.585       | 0.000   |
| B             | -0.0271                | -0.915       | 0.563   |
| C             | -0.3046                | -10.288      | 0.367   |
| D             | 0.1019                 | 3.443        | 0.000   |
| E             | 0.0503                 | 1.697        | 0.002   |
| A^2           | -0.3311                | -12.997      | 0.100   |
| B^2           | -0.5308                | -20.84       | 0.000   |
| C^2           | -0.5865                | -23.026      | 0.000   |
| D^2           | -0.3045                | -11.956      | 0.000   |
| E^2           | -0.6325                | -24.83       | 0.000   |
| A.B           | -0.225                 | -6.532       | 0.000   |
| A.C           | 0.3669                 | 10.651       | 0.000   |
| A.D           | -0.0531                | -1.542       | 0.133   |
| A.E           | 0.2256                 | 6.55         | 0.000   |
| B.C           | 0.1681                 | 4.881        | 0.000   |
| B.D           | 0.0294                 | 0.853        | 0.400   |
| B.E           | -0.1294                | -3.756       | 0.001   |
| C.D           | -0.0013                | -0.036       | 0.971   |
| C.E           | -0.0025                | -0.073       | 0.943   |
| D.E           | -0.0863                | -2.504       | 0.018   |

R-Sq = 98.33%  R-Sq(pred) = 93.53%  R-Sq(adj) = 97.25%

### Table 7 ANOVA for the fitted polynomial model for media optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate

| Sources of variation | Sum of squares | Degrees of freedom (DF) | Mean square (MS) | F-value | P-value |
|----------------------|----------------|-------------------------|------------------|---------|---------|
| Regression           | 69.3666        | 20                      | 3.4683           | 91.34   | 0.000   |
| Linear               | 4.6235         | 5                       | 0.9247           | 24.35   | 0.000   |
| Square               | 55.3906        | 5                       | 11.0781          | 291.76  | 0.000   |
| Interaction          | 9.3525         | 10                      | 0.9352           | 24.63   | 0.000   |
| Residual Error       | 1.1771         | 31                      | 0.038            | -       | -       |
| Lack-of-Fit          | 1.1734         | 22                      | 0.0533           | 130.09  | 0.000   |
| Pure Error           | 0.0037         | 9                       | 0.0004           | -       | -       |
| Total                | 70.5437        | 51                      | -                | -       | -       |

### Table 8 Optimum values of the media components obtained from regression equation for *A. oryzae* MTCC-8624 utilizing rice bran as substrate

| Independent variables | Optimum value (coded) | Optimum value (real) (g/L) |
|-----------------------|-----------------------|----------------------------|
| KH2PO4 (g/L)          | -0.31231              | 2.69                       |
| (NH4)2SO4 (g/L)       | -0.02402              | 4.95                       |
| MgSO4 (g/L)           | 0.40841               | 1.70                       |
| CaCl2 (g/L)           | 0.16817               | 0.53                       |
| FeSO4 (g/L)           | 0.02402               | 0.50                       |
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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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