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PRODUCTION OF β-FRUCTOFURANOSIDASE FROM *Aspergillus flavus* IBK-02 USING ORANGE PEEL AS A LOW-COST SUBSTRATE: OPTIMIZATION THROUGH RESPONSE SURFACE METHODOLOGY

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ABSTRACT

β-fructofuranosidases have wide applications in diverse industrial sectors such as the food, confectionary, and pharmaceutical industries. This study was aimed at the optimization of β-fructofuranosidase production from a filamentous fungus, under submerged fermentation condition, using orange peel as a low-cost substrate. Fungi were isolated from decaying orange fruit and screened for β-fructofuranosidase production. The fungal strain with the most appreciable enzyme production was identified as *Aspergillus flavus* IBK-02 by the sequencing of the internal transcribed spacer region of the ribosomal DNA. β-fructofuranosidase production from the selected fungus was optimized using the conventional one-at-a-time approach by studying the effect of each of the medium parameters incubation period, carbon sources, nitrogen sources, pH, and temperature on the enzyme production. Also, the influence of four independent parameters (initial pH, temperature, inoculum concentration, and orange peel concentration) on β-fructofuranosidase production was studied using the Box Behnken Design (BBD) of response surface methodology (RSM). The coefficient of determination (R^2) obtained was 0.9600 showing that the quadratic model used for the prediction was significant ($P>0.05$). At the end of the experiment, the optimal levels of the four significant variables (initial pH, temperature, inoculum concentration, and orange peel concentration) were 6, 35 ^{o}C, 2.0% v/v, and 2% w/v, respectively, which produced 122.48 U/mL β-fructofuranosidase. The enzyme production increased by about 5-fold in the optimum fermentation medium condition by RSM compared with the medium optimized through the one-factor-at-a-time approach. This study has revealed that the statistical process development for the production of β-fructofuranosidase from *A. flavus* IBK-02, using orange peel as a low-cost substrate, could be used for industrial and biotechnological applications.

Keywords: *Aspergillus flavus*, β-fructofuranosidase**,** Optimization, Orange peel, Response surface methodology, Submerged fermentation .

INTRODUCTION

β-fructofuranosidases (EC 3.2.1.26), also known as invertases, catalyze the irreversible hydrolysis of the β-1,2-glycosidic linkage in sucrose molecule to obtain an equimolar mixture of D-glucose and D-fructose, known as invert sugar (Rustiguel *et al*., 2015). β-fructofuranosidases are produced by bacteria, fungi, higher plants and some animals (Aung *et al*., 2019). However, the majority of industrial applications of the enzyme are derived from particular fungi and bacteria sources because they are easier to manipulate, and produce enzymes that are more controllable and predictable (Awad *et al.,* 2013; Gracida-Rodriguez *et al.,* 2014).

β-D-fructofuranosidases are used in several sectors such as the food, beverage, bakery and pharmaceutical industries due to their wide biotechnological applications (Alves *et al.,* 2013). Invert syrup, obtained from sucrose hydrolysis is approximately 1.5 times sweeter than sucrose and possesses functionally more desirable properties like high solubility and hygroscopic nature (Keramat *et al*., 2017; Soares *et al*., 2019). Therefore, it is used in the confectionary industry as humectants in the manufacture of chocolate-coated soft-centered sweeteners, candy products, fondants, and after-dinner mints (Taskin *et al*., 2013). In the pharmaceutical industry, it is used as medication formulas or drugs in products such as cough syrups, digestive aid tablets, and powdered milk for infants' foods (Kulshrestha *et al*., 2013). Production of lactic acid and ethanol via fermentation of molasses, a by-product of the sugar cane industry has also been reported (Karandikar, 2007).

Globally, fruit and vegetable wastes generated from industrial processing account for 30 to 50% of the input materials (Di Donato *et al.,* 2011). Most of these wastes are generated from preliminary operations such as peelings and cuttings. The indiscriminate disposal of these wastes into the environment results in pollution hazards that are detrimental to its biological and physical components. However, agro-industrial wastes such as fruit peels are rich in moisture, carbohydrates, proteins, and other compounds which promote the growth of microorganisms leading to the production of a significant yield of enzymes and other value-added products (Ghosh *et al.,* 2014; Dapper *et al.,* 2016). Increasing demands, high nutrient costs, and environmental concerns have stimulated interest in the utilization of the agro-wastes as cost-effective substrates for the production of the enzyme. Several studies have evaluated different agro-wastes as cost-effective substrates for β-fructofuranosidase production (Rustiguel *et al.,* 2015: Qureshi *et al.,* 2017). As a result of the enormous size of the wastes generated globally and their biochemical characteristics, several studies have been carried out to transform the wastes to value-added products such as enzymes, organic acids, and bioethanol (Ghosh *et al.,* 2014).

The genus *Aspergillus* comprises several species useful in food fermentations and biotechnological applications (Varga and Samson, 2008). Some of these species have been implicated in β-D-fructofuranosidase production such as *A. versicolor* (Dapper *et al.,* 2016) *A. niger* (Oyedeji *et al.,* 2017; Tasar and Tasar, 2022), and *A. carbonarius* (Batista *et al.*, 2022).

The optimization of fermentation parameters such as pH, temperature, incubation period, and carbon and nitrogen sources, is one of the approaches employed for the achievement of high enzyme yield during the process (El-Hadi *et al.,* 2014). The conventional optimization procedure is to study the effect of these factors individually while keeping the others constant. However, the one-factor-at-a-time approach does not often result in the optimized yield of the enzyme or consider the possible interactions between the factors. On the other hand, statistical optimization methods offer a more reliable, effective, and economical technique of achieving high yield of products (Tasar, 2015). The response surface methodology (RSM) is a mathematical and statistical technique widely used to determine the effects of several variables and their interactions and to optimize different biotechnological processes (Zafar *et al.*, 2010). It has been extensively applied to optimize the culture medium and other process parameters for producing several enzymes (Ottoni *et al.*, 2012). On an industrial scale, the RSM technique is used to determine the feasibility of economic procedures meant to lower operational costs by using the optimized culture medium for the enzyme or by-product synthesis (Chang *et al.*, 2006).

This study aimed to statistically optimize the extracellular β-fructofuranosidase production from a fungus isolated from decaying orange fruit, using agro-waste as a low-cost substrate, under submerged fermentation conditions.

MATERIAL AND METHODS

Collection and preparation of agro-wastes

Orange (*Citrus sinensis*) and pineapple (*Ananas comosus*) peels were obtained locally from the fruit market. They were shredded and prepared by exhaustive washing with distilled water, dried at 60 °C for 48 h and thereafter milled and sieved to uniform 35 mesh sizes. They were stored in clean, dried, airtight containers and kept in the refrigerator at 4 °C and were subsequently used as substrate in enzyme fermentation media (Ahmed *et al.,* 2016).

Isolation of fungi and screening for β-fructofuranosidase production

Fungal strains were isolated from decaying orange fruits and characterized by morphological and microscopical examinations using lactophenol cotton blue solution mount (Benson, 1990). The isolates were screened for their relative β-fructofuranosidase production under submerged fermentation conditions. The fungal strain exhibiting the most appreciable enzyme production was then selected for further studies and maintained on potato dextrose agar (Fluka, St. Louis, Mo, USA) slants at 4 °C.

Molecular characterization and identification of selected fungus

Total genomic DNA from selected filamentous fungus was extracted from PDA growth medium using the Zymo Research (ZR) Fungal/Bacterial DNA Miniprep™ kit (ZYMO RESEARCH, USA). The internal transcribed spacer (ITS) sequence of fungal ribosomal DNA was amplified by polymerase chain reaction (PCR) using the primers ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White et al. 1990). The PCR product was resolved by electrophoresis on an agarose gel (1.5%) and observed under UV light. It was then isolated and sequenced using the same PCR primers. The fungal ITS sequence was deposited into GenBank Data Library with the accession number KX462776.1. A homology search was performed with GenBank database and the homologous sequences were selected for phylogenetic analysis by using the neighbor-joining (NJ) method. A phylogenetic tree showing an evolutionary relationship with other β-fructofuranosidase-producing fungal species in the GenBank was constructed using Molecular Evolutionary Genetics Analysis (MEGA 7.0.21).

Submerged fermentation for enzyme production

β-Fructofuranosidase was produced by submerged fermentation in Erlenmeyer flasks (250 mL) containing 100 mL of medium composed of KH_2PO_4 (1.0 g), K_2HPO_4 (6.27 g), $MgSO_4$ (0.25 g), peptone (5.0 g), biotin (0.0005 mg), thiamine (0.005 mg), $CaSO_4$ (0.005 mg), $FeSO_4$ (0.5 mg), $MnSO_4$ (0.26 mg), $ZnSO_4$ (0.1 mg), $CuSO_4$ (0.5 mg) all dissolved in 1L distilled water. Fruit peel (10.0 g) was added as the substrate (carbon source). The initial pH of the medium was adjusted to 6.0 and the culture medium was inoculated with 5×10^5 spores/mL and incubated at 30 ºC for 5 days. After incubation, the cultures were filtered through glass fibre filter paper (Whatman GF/A), and the cell-free supernatants were used to estimate β-fructofuranosidase activity. Fermentation was carried out in triplicates.

β-fructofuranosidase assay

β-Fructofuranosidase activity was determined by estimating the amount of reducing sugars released in a reaction mixture containing 0.02 mL of 1.0 %w/v sucrose in sodium acetate buffer (0.05 M, pH 4.5) and 0.01 mL of the enzyme extract (Bergmeyer and Bent, 1974). The reaction was for 60 min at 35 ºC. Reducing sugars were quantified by the addition of 3.0 mL of glucose oxidase-peroxidase reagent (Sigma-Aldrich, St. Louis, Mo, USA) and further incubation for 5 min at 35 ºC. The absorbance was read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to liberate 1.0 µmol glucose per milliliter per minute under the assay conditions.

Screening of agro-wastes (fruit peels) as substrates for enzyme production

The different agro-wastes orange peel, pineapple peel, and equal concentrations of orange and pineapple peels were each incorporated into the fermentation medium, as sole carbon source at $1.0\%^{w}/_{v}$ concentration. After inoculation with standardized spore suspension of fungus, the flasks were incubated at 30 °C for 96 h. At the end of incubation, the culture supernatant was assayed for enzyme activity.

Optimization of β-fructofuranosidase production using the one-factor-at-a-time approach

Effect of incubation period on β-fructofuranosidase production

The time course (24 to 196 h) of β-fructofuranosidase production in the fermentation medium was determined. At twenty-four hourly intervals, the β-fructofuranosidase activities were evaluated.

Effect of pH on production of β-fructofuranosidase

The effect of pH on the production of β-fructofuranosidase was determined by adjusting the pH of fermentation media to different levels 3.0 to 7.0. Each of the media, adjusted to different level, was inoculated with standardised spore suspension (5×10^5 spore/mL) and incubated for 120 h at 30 °C. Cultures were then filtered through glass fibre filter paper and the supernatant was

assayed for enzyme activity.

Effect of temperature on production of β-fructofuranosidase

The influence of temperature on production of β-fructofuranosidase was studied by varying the incubation temperature of fermentation culture from 25 to 60 °C. After incubation for 120 h, cell-free supernatant were obtained by filtration and enzyme production determined.

Effect of carbon sources on the production of β-fructofuranosidase

Various carbon sources (glucose, fructose, sucrose, rhamnose, maltose, starch, mannose, and orange peel), at 1.0% w/v, were investigated for their effect on β-fructofuranosidase production in the fermentation medium. Incubation was for 120 h at 30 °C.

Effect of different concentrations of orange peels on β-fructofuranosidase production

The production medium was supplemented with various concentrations of orange peels (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0%w/v). At the end of incubation, enzyme activity was determined.

Effect of nitrogen sources on the production of β-fructofuranosidase

Different nitrogen sources were investigated for their effects on β-fructofuranosidase production by supplementing the fermentation medium with each of the following: $(NH_4)_2SO_4$, NH_4Cl, KNO_3, $NaNO_3$, $Ca(NO_3)_2$, casein, peptone, and urea. They were added at 0.5%w/v concentrations.

Optimization of β-fructofuranosidase production using response surface methodology

The optimization of β-fructofuranosidase production from *A. flavus* IBK-02 was carried out by using the response surface methodology software. The four parameters of initial pH, incubation temperature, orange peel concentration, and inoculum concentration were chosen based on the results of the one-factor at a time approach. The Box-Behnken Design (BBD) was used for the optimization of β-fructofuranosidase production from the fungi and the study of interactions of the four variables. The software suggested a total of 29 runs of experiments in different combinations of the selected parameters. Each of the factors in the design was studied at three different levels (-1, 0, +1) and the coded and real values of the variables are presented in Table 1. A regression analysis was performed on the data obtained from the design experiments. Analysis of variance and multiple regression analysis were performed at $p < 0.05$ using Design Expert Version 7.1.5 statistical software (Stat-Ease Inc., Minneapolis, USA). Enzyme activity was taken as the dependent variable or response.

A second-order polynomial equation was used to predict the relationship between the independent variables and response as follows:

$$Y = \beta_o + \beta_a A + \beta_b B + \beta_c C + \beta_d D + \beta_{ab} AB + \beta_{ac} AC + \beta_{ad} AD + \beta_{bc} BC + \beta_{bd} BD + \beta_{cd} CD + \beta_{aa} A^2 + \beta_{bb} B^2 + \beta_{cc} C^2 + \beta_{dd} D^2$$

Where Y is the predicted β-fructofuranosidase response; β_o is the model constant; β_a, β_b, β_c, β_d are linear coefficients; β_{ab}, β_{ac}, β_{ad}, β_{bd} and β_{cd} are cross-product coefficients; β_{aa}, β_{bb}, β_{cc} and β_{dd} are quadratic coefficients and A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are the independent variables. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Triplicate determinations were carried out for each of the 29 experimental runs and the means were reported as response.

Statistical analysis

All data were subjected to statistical analysis for determination of mean and standard deviation using SPSS version 16. Experiments were carried out in triplicates.

Table 1: Range and levels of experimental variables selected for optimization of β-fructofuranosidase production from *Aspergillus flavus* IBK-02

Parameters		Level of factors		
	Symbol	-1	0	+1
Initial pH	A	4	5	6
Incubation temperature (°C)	B	25	30	35
Inoculum concentration (%)	C	1	2	3
Orange peel concentration (%)	D	1	2	3

RESULTS

Screening of fungi for β-fructofuranosidase production

Seven fungal strains isolated from decaying orange fruits were screened for β-fructofuranosidase production under submerged fermentation conditions. The fungal strain IBK-02 was found to exhibit the highest β-fructofuranosidase-production ability and was therefore selected for further studies.

Fungal strain identification

The study fungus, isolated from decaying orange (*Citrus sinensis*) fruit, was identified both by its morphological characteristics and the sequencing of the ITS region of the ribosomal DNA. The 600 bp fragment of the sequence showed 100% identity with other *A. flavus* strains. This sequence was deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under accession number KX462776.1. The study strain was therefore designated as *A. flavus* IBK-02. A phylogenetic tree was constructed based on the alignment of the sequences from the ribosomal genes from some *Aspergillus* species. Phylogenetic analysis revealed a close relationship between this species and other fungal species in the GenBank that have been implicated in β-fructofuranosidase production. There was a distant relationship with *Rhizopus oryzae*, a non-β-fructofuranosidase-producing fungus (Figure 1).

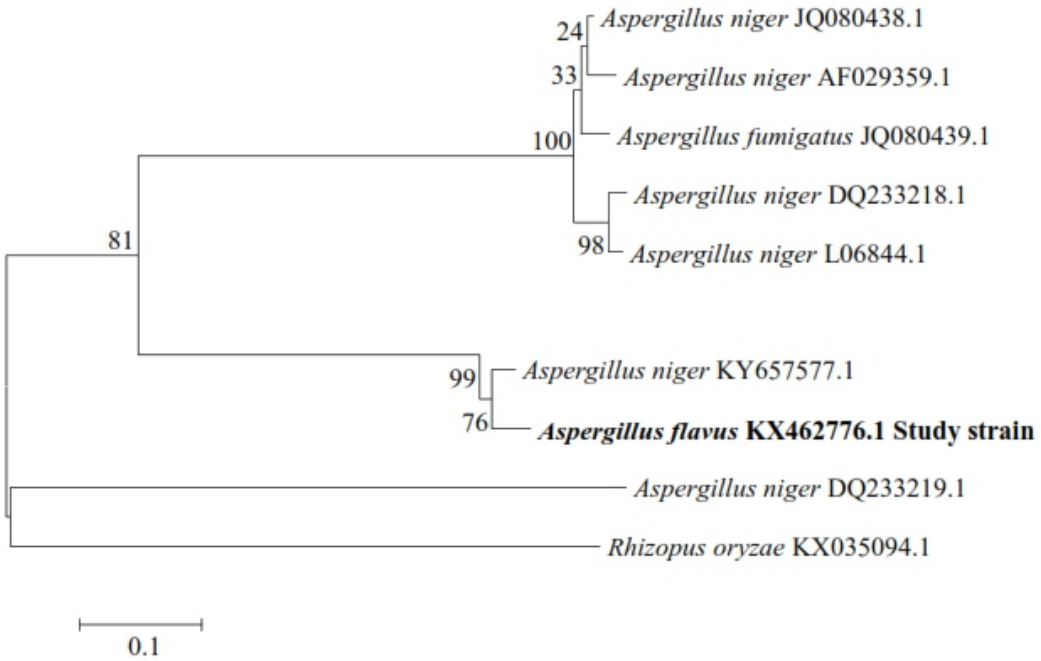


Figure 1: Phylogenetic relationship between *Aspergillus flavus* IBK-02 and other β-fructofuranosidase-producing *Aspergillus* strains in Genbank. GenBank accession numbers are included. *Rhizopus oryzae* was used as an outgroup. Bar, 20% sequence divergence.

Screening of fruit peels as substrates for β-fructofuranosidase production from fungus

β-Fructofuranosidase production was observed with the use of the two fruit peels, individually and in equal combination, as substrates for fermentation. Orange peel was observed to be the best substrate for β-fructofuranosidase production from *A. flavus* IBK-02, with β-fructofuranosidase activity of 27.47 ± 0.32 U/mL, followed by an equal combination of orange and pineapple peel and, pineapple peel (Figure 2).

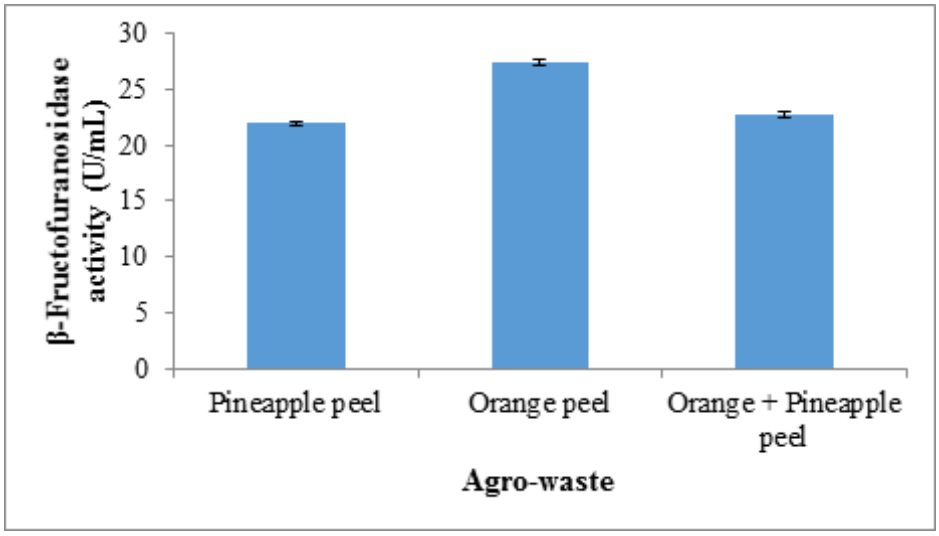


Figure 2 Screening of agro-wastes (fruit peels) as substrate for β-fructofuranosidase production from *A. flavus* IBK-02.

Optimization of β-fructofuranosidase production using one-factor-at-a-time approach

Effect of incubation period on β-fructofuranosidase production from fungus

Minimum enzyme activity of 12.77 ± 0.15 U/mL was observed at 24 h which increased with the incubation period and reached maximum at 120 h with enzyme activity of 25.44 ± 0.80 U/mL. The enzyme production gradually declined to 9.98 ± 0.10 U/mL at the end of the 216 h fermentation period (Figure 3).

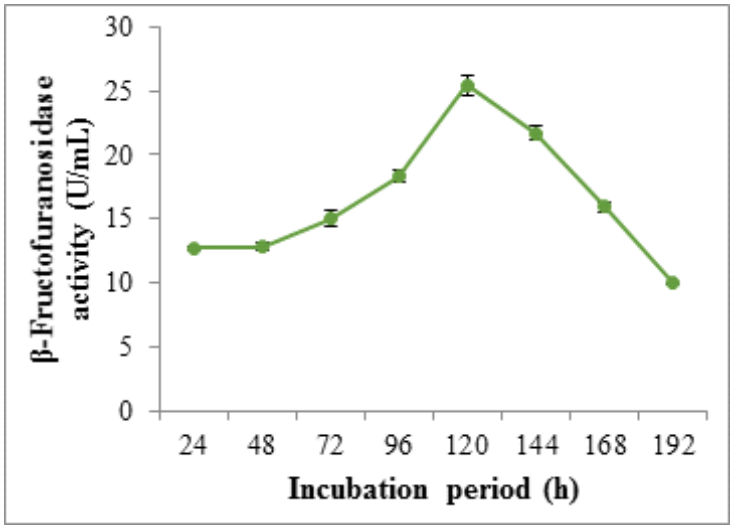


Figure 3. Effect of incubation period on β-fructofuranosidase production from *A. flavus* IBK-02. Each value represents the mean of triplicate determinations with ± SD

Effect of pH on β-fructofuranosidase production from fungus

Aspergillus flavus IBK-02 exhibited maximum β-fructofuranosidase production at pH 5.0 (24.99 ± 0.26 U/mL). Enzyme production then decreased gradually with increasing pH reaching the minimum value of 9.41 ± 0.19 U/mL at pH 7.0 (Figure 4).

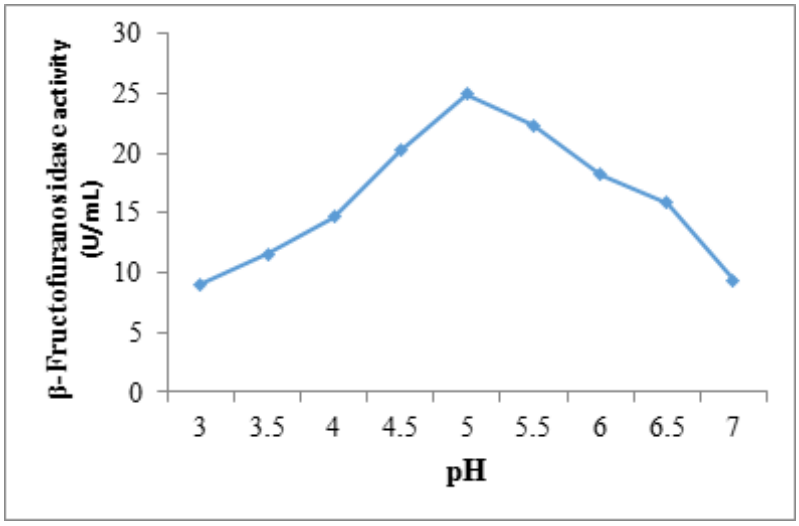


Figure 4: Effect of pH on β-fructofuranosidase production from *A. flavus* IBK-02 at 30 °C. Each value represents the mean of triplicate determinations with ± SD

Effect of temperature on β-fructofuranosidase production

Aspergillus flavus IBK-02 exhibited maximum β-fructofuranosidase production at 30 °C (23.45 ± 0.40 U/mL). Enzyme production then decreased, with the increase in temperature, down to a minimum value of 7.13 ± 0.45 U/mL at 60 °C (Figure 5).

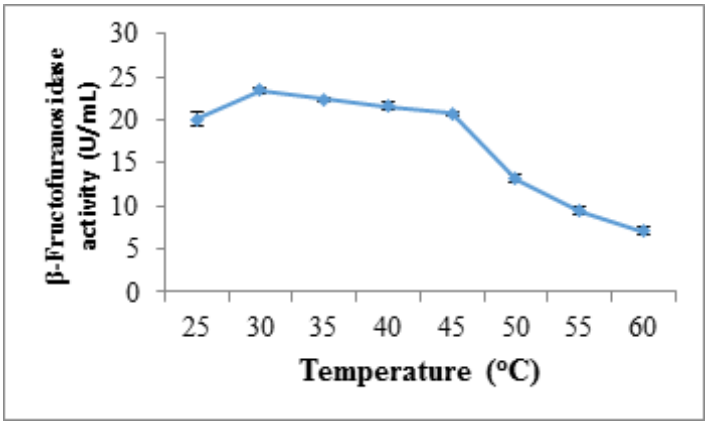


Figure 5: Effect of temperature on β-fructofuranosidase production from fungus using orange peel as substrate.

Effect of carbon sources on β-fructofuranosidase production

Aspergillus flavus IBK-02 exhibited maximum β-fructofuranosidase production with orange peel as carbon source in fermentation medium (24.93 ± 0.46 U/mL). This was followed by use of sucrose (21.74 ± 0.15 U/mL) and fructose (5.00 ± 0.41 U/mL) as carbon sources (Figure 6).

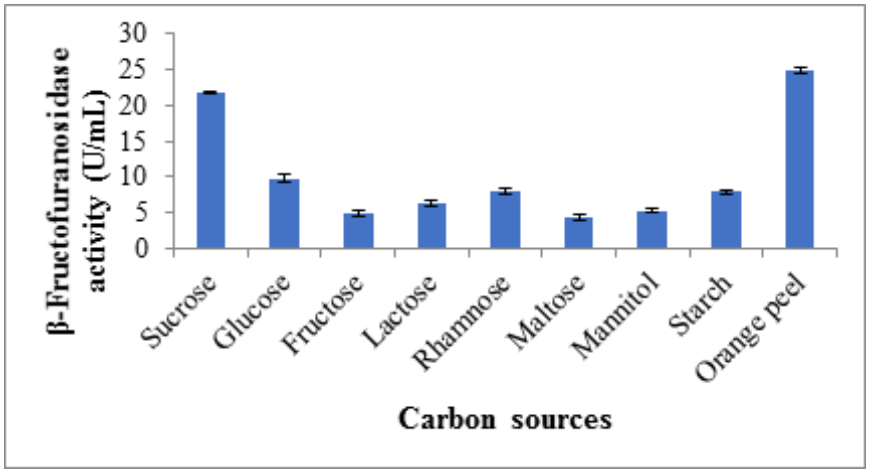


Figure 6: Effect of different carbon sources on β-fructofuranosidase production from *A. flavus* IBK-02.

Effect of orange fruit peel concentration on β-fructofuranosidase production from fungus

The fungus exhibited maximum β-fructofuranosidase production (25.62 ± 0.32 U/mL) with an orange peel concentration of 2.0% w/v. This was followed by a decrease in enzyme production as the orange peel concentration increased to 3.0% w/v (24.99 ± 0.26 U/mL) (Figure 7).

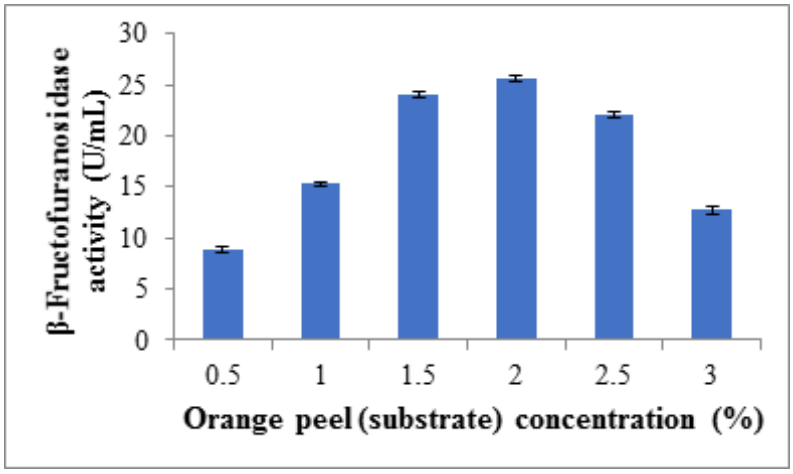


Figure 7: Effect of orange peel concentration on β-fructofuranosidase production from *A. flavus* IBK-02.

Effect of nitrogen sources on β-fructofuranosidase production from fungus

Peptone was observed to be the best nitrogen source for β-fructofuranosidase production from *A. flavus* IBK-02 (22.42 ± 0.44 U/mL), followed by urea (19.27 ± 0.59 U/mL). The minimum enzyme production was observed using ammonium sulphate as a nitrogen source (Figure 8).

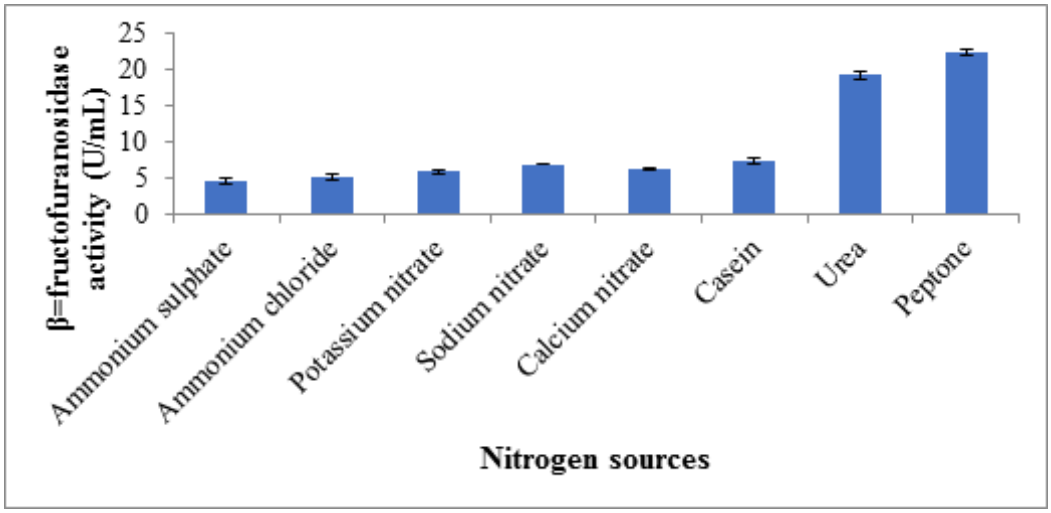


Figure 8: Effect of nitrogen sources on β-fructofuranosidase production from fungus.

Effect of inoculum concentration on β-fructofuranosidase production from fungus

β-Fructofuranosidase production from *A. flavus* IBK-02 was maximum when fungal spore inoculum concentration of 2.0% v/v (25.06 ± 0.26 U/mL) was used in the fermentation medium. Enzyme production then decreased as inoculum concentration increased (Figure 9).

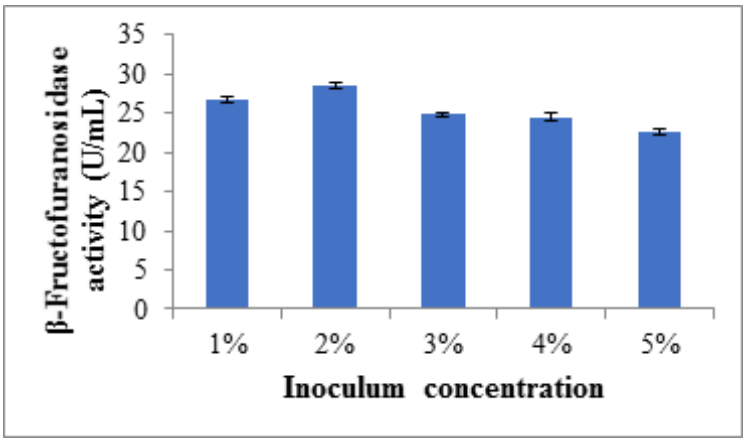


Figure 9: Effect of inoculum concentration on β-fructofuranosidase production from *A. flavus* IBK-02.

Optimization of β-fructofuranosidase production using experimental design

Optimization of β-fructofuranosidase production from *A. flavus* IBK-02, using the Box-Behnken experimental plan with the observed and predicted responses for the experimental runs is presented in Table 2.

The effect of the factors on β-fructofuranosidase production was expressed in a second-order polynomial regression equation of the form:

β-fructofuranosidase activity (U/mL/min) = 843.89200 – 112.39917 x Initial pH – 22.0703 x Temperature – 112.59400 x Inoculum concentration – 57.13233 x Orange peel concentration + 0.35700 x Initial pH x Temperature + 9.50250 x Initial pH x Inoculum concentration + 6.83500 x Initial pH x Orange peel concentration + 1.05000 x Temperature x Inoculum concentration + 7.00000E-003 x Temperature x Orange peel concentration + 6.018000 x Inoculum concentration x Orange peel concentration + 7.17350 x Initial pH^2 + 0.32299 x $Temperature^2$ + 4.96600 x $Inoculum\ concentration^2$ + 3.13725 x $Orange\ peel\ concentration^2$.

This regression equation provided the level of β-fructofuranosidase production as a function of initial pH, incubation temperature, inoculum

concentration, and orange peel concentration.

The statistical significance of each of these factors was evaluated using the analysis of variance (ANOVA). From the ANOVA table (Table 3), the model was found to have an F-value of 24.03 implying that the model was significant and there was only a 0.01% chance that the model F-value could occur due to error. Probability values of "Prob < *F* < 0.05" indicated that such model terms are significant. Thus, A, B, D, AC, AD, BC, CD, A^2, B^2, C^2, and D^2 are significant model terms for maximizing β-fructofuranosidase production from *A. flavus* (Table 3). The "Lack of fit *F*-value" of 1.81 implies the Lack of fit is not significant, relative to the pure error. The non-significant lack of fit is good for the model to fit. Prob < *F* value of lack of fit indicated that the quadratic model was valid and adequate for the optimization of the factors for optimum production of the enzyme. The summary of the ANOVA for β-fructofuranosidase production from *A. flavus* is presented in Table 4. The multiple correlation coefficient (R^2) value obtained was 0.9600. The closeness of the multiple correlation coefficient (R^2) value to 1 indicated a better correlation between the observed and predicted values. The coefficient of variation (C.V.%) in this study was 2.57% while the ratio obtained for β-fructofuranosidase adequate precision was 15.170.

Three-dimensional (3D) response surface plots were used to analyze the optimal levels and interaction effects of the factors or variables in maximizing β-fructofuranosidase production from *A. flavus*. The possible combinations of variables in maximizing enzyme production are presented in Figures 10 and 11. From the analysis of the 3D response plot between pH and inoculum concentration in Figure 10a, it could be observed that an increase in pH with a decrease in inoculum concentration led to an increase in β-fructofuranosidase production. The 3D plot between pH and orange peel concentration in Figure 10b showed that an increase in pH with a decrease in orange peel concentration led to an increase in β-fructofuranosidase production. Analysis of the 3D plot between inoculum concentration and orange peel concentration in Figure 10c showed that an increase in orange peel concentration with an increase in inoculum concentration led to an increase in enzyme production. Figure 10d shows the 3D response plot between temperature and inoculum concentration. From the plot, it could be observed that an increase in temperature correlated with an increase in inoculum concentration for increased enzyme production. Figure 11a shows that an increase in temperature with a simultaneous increase in orange peel concentration led to an increase in the β-fructofuranosidase concentration.

The response surface plot between pH and temperature in Figure 11b showed that a slight decrease in pH with an increase in temperature led to an increase in β-fructofuranosidase production. Maximal β-fructofuranosidase production of 122.48 U/mL was achieved from *A. flavus* IBK-02 at pH 6.0, temperature 35 °C, 2.0% v/v inoculum concentration and orange peel concentration 2.0%v/v (Table 2). This corresponds to an approximately 5.0-fold increase in *A. flavus* β-fructofuranosidase production due to RSM optimization.

The validity of the proposed model was estimated by prediction of *A. flavus* β-fructofuranosidase production for each trail of the matrix. The experimental results obtained showed that the maximum observed β-fructofuranosidase production (122.48 U/mL) was in excellent agreement with the predicted value (120.2 U/mL) in run 22.

Table 2: Box Behnken experimental design matrix and results of optimization of β-fructofuranosidase production from *A. flavus* IBK-02.

Run	pH	Incubation temperature (°C)	Inoculum concentration (%)	Orange peel concentration (%)	Observed β-fructofuranosidase activity (U/ml)	Predicted β-fructofuranosidase activity (U/ml)
1	5	30	2	2	97.66	90.13
2	5	35	2	1	104.84	105.8
3	5	35	1	2	100.68	103
4	5	30	2	2	95.17	94.03
5	5	25	2	3	96.58	99.67
6	5	30	3	1	86.08	88.39
7	6	30	2	1	96.18	90.94
8	6	25	2	2	94.88	99.46
9	6	30	3	2	118.89	115.6
10	5	35	2	3	106.86	109.4
11	4	30	2	1	100.48	99.1
12	6	30	2	3	118.68	115.6
13	4	35	2	2	104.01	109.3
14	5	30	1	3	98.44	95.69
15	5	30	2	2	94.06	89.96
16	5	35	3	2	116.80	113.7
17	4	30	3	2	96.94	92.3
18	5	30	3	3	98.02	110.4
19	5	30	2	2	94.44	88.86
20	6	30	1	2	98.94	96.09
21	5	25	2	1	93.38	96.21
22	6	35	2	2	122.48	120.2
23	5	25	3	2`	91.74	93.8
24	4	25	2	2	95.46	95.7
25	5	25	1	2	102.84	104.1
26	4	30	2	3	97.46	97.23
27	5	30	1	1	106.60	101.6
28	5	30	2	2	97.02	92.16
29	4	30	1	2	114.90	110.8

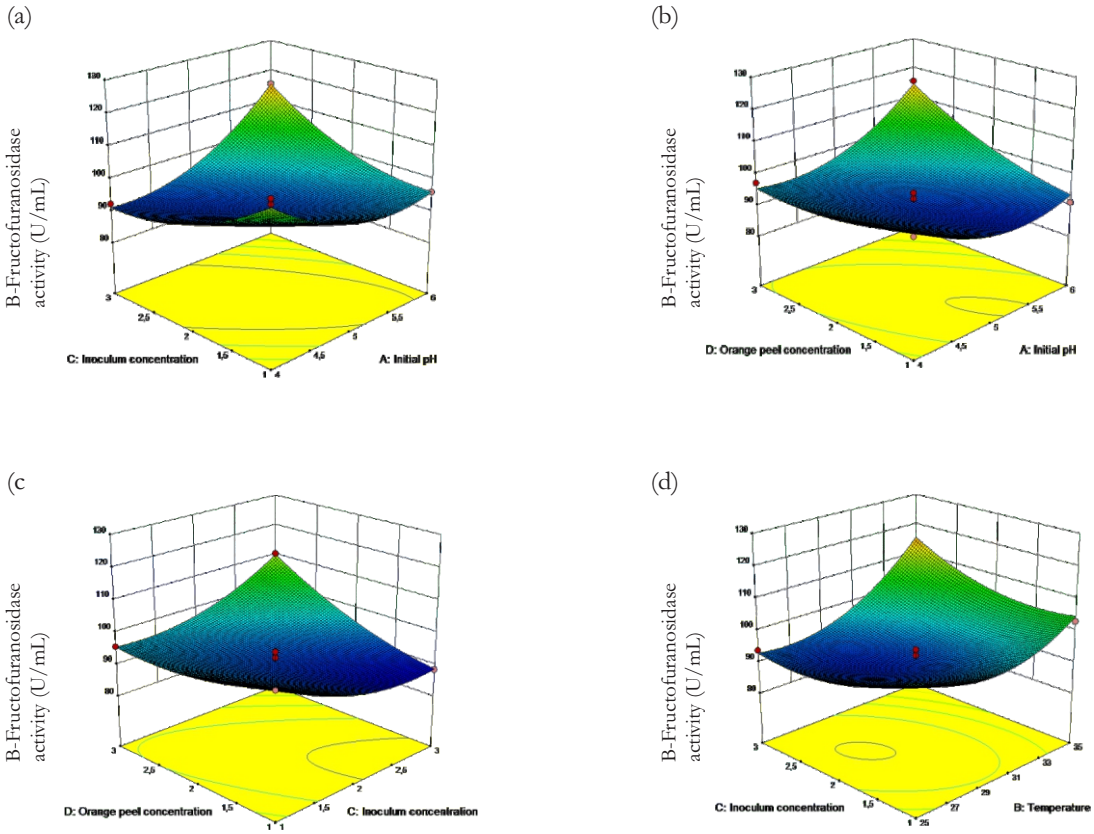
Table 3: Analysis of variance (ANOVA) for the Box Behnken quadratic model for β-fructofuranosidase production from *A. flavus* IBK-02

Source	Sum of Squares	df	Mean square	*F*-Value	*p*-value Prob > *F*
Model	2245.64	14	160.40	24.03	< 0.0001*
A-Initial pH	88.84	1	88.84	13.31	0.0026*
B-Temperature	437.54	1	437.54	65.54	< 0.0001*
C-Inoculum concentration	0.71	1	0.71	0.11	0.7499
D-Orange peel concentration	169.80	1	169.80	25.44	0.0002*
AB	12.74	1	12.74	1.91	0.1887
AC	361.19	1	361.19	54.10	< 0.0001*
AD	186.87	1	186.87	27.99	0.0001*
BC	110.25	1	110.25	16.51	0.0012*
BD	4.900E-003	1	4.900E-003	7.340E-004	0.9788
CD	194.88	1	194.88	29.19	< 0.0001*
A^2	333.79	1	333.79	50.00	< 0.0001*
B^2	422.93	1	422.93	63.35	< 0.0001*
C^2	159.96	1	159.96	23.96	0.0002*
D^2	63.84	1	63.84	9.56	0.0080*
Residual	93.46	14	6.68		
Lack of Fit	76.52	10	7.65	1.81	0.2987
Pure Error	16.94	4	4.24		
Cor Total	2339.11	28			

A-pH; B-Incubation temperature; C-Inoculum concentration; D-Orange peel concentration; R^2= 0.9600; Adj R^2= 0.9201; Predicted R^2= 0.8003; Adeq Precision 15.170; df- degree of freedom; C.V = 2.57%; * Statistically significant ($P < 0.05$)

Table 4: Summary of the ANOVA for β-fructofuranosidase production from *A. flavus* IBK-02

Variance parameter	Value
Standard deviation (SD)	2.58
Mean	100.69
Coefficient of variation (C.V.%)	2.57
PRESS	467.23
Multiple correlation coefficient (R^2)	0.9600
Adjusted R-squared	0.9201
Predicted R-Squared	0.8003
Adequate Precision	15.170



Ultrafilteration of crude enzyme

The crude enzyme was passed through ultrafilteration process using amicon Ultr

Figure 10: Three-dimensional (3D) response surface plots showing the effect of (a) pH and inoculum concentration, (b) pH and orange peel concentration, (c) inoculum concentration and orange peel concentration, and (d) temperature and inoculum concentration, on β-fructofuranosidase prduction from *A. flavus* IBK-02

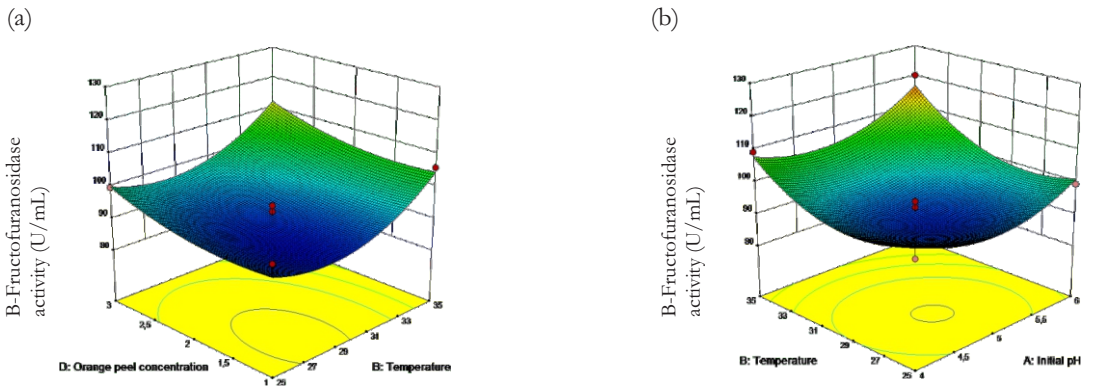


Figure 11: Three-dimensional (3D) response surface plots showing the effects of (a) temperature and orange peel concentration and (b) pH and temperature, on β-fructofuranosidase production from *A. flavus* IBK-02

DISCUSSION

Fungal strains isolated from decaying orange fruit were screened for their relative β-fructofuranosidase production abilities, under submerged fermentation conditions. The strain IBK-02 exhibiting the most appreciable enzyme production was selected for further study. It was presumptively identified by phenotypic method as *Aspergillus* sp. IBK-02. Several species of fungi have been implicated in the decay of orange fruits (Tournas and Katsoudas, 2005; Akinmusire, 2011). Fruits generally contain high levels of sugars and nutrient molecules and their low pH values make them particularly susceptible to fungal decay (Singh and Sharma, 2007).

The identity of the fungus was confirmed by molecular identification method, based on the fungal ITS region (gene) sequencing and analysis, as *Aspergillus flavus* IBK-02 with a maximum identity of 100% to other similar species. Several strains of *A. flavus* have been implicated in β-fructofuranosidase production (Uma *et al.,* 2010; Matei *et al.,* 2017). Phylogenetic comparative analysis revealed a close relationship between the fungus and other fungal species in the GENBANK implicated in β-fructofuranosidase production.

Orange peel was the best substrate for β-fructofuranosidase production out of pineapple peel and, an equal combination of the two fruit peels. These fruit peels are rich in moisture, carbohydrates, protein, fat, and other compounds (Wijngaard *et al.,* 2009). The peels are rich sources of nutrients such as carbohydrates, and the sufficient sugars and protein they contain will promote the growth of microorganisms, producing a significant yield of useful enzymes (Bennet *et al.,* 2002). Studies on the utilization of different agro-wastes as cost-effective substrates for β-fructofuranosidase production from *Aspergillus* and other fungal species include the use of wheat bran (Giraldo *et al.,* 2011), pineapple peel (Oyedeji *et al.*, 2017), date syrup (Qureshi *et al.,* 2017), pineapple crown waste (Batista *et al.*, 2022), and cassava-soybean (Osiebe *et al.*, 2023). The difference in the suitability of the fruit peels as substrates for β-fructofuranosidase production could be attributed to the differences in the concentrations of the nutrient molecules in the different peels leading to differences in microbial growth and enzyme production.

The optimization of β-fructofuranosidase production from the test fungus, using the one-factor-at-a-time approach, revealed the following parameters for optimum enzyme production: incubation period 120 h, initial pH 5.0, temperature 30 °C, orange peel as the best carbon source, nitrogen source peptone and fungal spore inoculum concentration 2.0%v/v. The application of response surface methodology (RSM) for optimization of β-fructofuranosidase production from fungi allows the simultaneous determination of the main and interaction effects of important factors on enzyme production. Response surface methodology is a collection of statistical and mathematical techniques convenient for developing, improving, and optimizing processes in which several variables influence the response of interest (Myers *et al.,* 2009). The optimization of processes involving varying one factor while the other factors were kept at their constant levels, that is, the one-factor-at-a-time approach is unable to consider the interactive effect among all the factors on the outcome (Bas and Boyaci, 2007). Moreover, the approach is time-wasting, laborious, and wasteful of reagents. Response surface methodology can be used to overcome these limitations since its application can identify and quantify the various interactions among several parameters with fewer experimental runs (Shankar *et al.,* 2015). The optimum values of the process variables were obtained from the quadratic regressions. The adequacies of models were justified through analysis of variance (ANOVA). Prob > F-value less than 0.05 indicates that the model terms are significant while values greater than this indicate the model terms are not significant. Therefore, it can be assumed that the developed statistical model is reasonably accurate. In this study, fitting the data to various models (linear, two factorial, quadratic, and cubic) and their subsequent ANOVA revealed that β-fructofuranosidase production was most suitably defined with the quadratic polynomial model. The parameter combination eliciting the maximum response of 122.48 U/mL for *A. flavus* IBK-02 β-fructofuranosidase production was initial pH (6.0), temperature (35 °C), inoculum

concentration (2.0%) and orange peel concentration (2.0%v/v). This corresponds to an approximately 5.0-fold increase in *A. flavus* β-fructofuranosidase production due to RSM optimization. Results from this study have demonstrated a significant increase in the enzyme production from *A. flavus* IBK-02, through RSM compared to the non-optimized condition. A good correlation between the experimentally obtained data and predicted data specified by the model indicates that the Box Behnken design could be effectively applied to optimize the process for β-fructofuranosidase production from the study fungus. Statistical experimental design had also been used to improve the yield of extracellular β-fructofuranosidase from strains of *A. carbonarius* (Batista *et al.,* 2021) and *A. niger* (Tasar and Tasar, 2022).

CONCLUSION

β-fructofuranosidase is utilized in several biotechnological applications in diverse industries. In this study, *Aspergillus flavus* IBK-02 isolated from decaying orange fruit was efficient in β-fructofuranosidase production. Orange peel was used as a low-cost substrate for production of the industrially-important enzyme from the fungus, under submerged fermentation conditions. Optimized enzyme production was successfully achieved using the statistical optimization tool response surface methodology (RSM), leading to a five-fold increase in β-fructofuranosidase yield. The parameter combination eliciting optimum *A. flavus* IBK-02 β-fructofuranosidase production was initial pH (6.0), temperature (35 °C), inoculum concentration (2.0% w/v), and orange peel concentration (2.0% w/v). This corresponds to an approximately 5.0-fold increase in *A. flavus* β-fructofuranosidase production due to RSM optimization. This study has demonstrated an enhanced β-fructofuranosidase production from *A. flavus* IBK-02 using orange peel as a low-cost substrate, and the RSM as a statistical optimization tool.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Oyedeji O.: Conceptualization; investigation; methodology; data curation; formal analysis; manuscript preparation. Oluduro, O. A.: Conceptualization; supervision; validation; manuscript preparation. Mokoena, M. P.: Supervision; investigation; methodology; resources; visualization. Olaniran, A. O.: Supervision; data curation; formal analysis; resources; visualization; manuscript preparation.

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REFERENCES

Ahmed, I., Zia, M. A., Azhar, M., Akram, Z., Naveed, T. and Nowrouzi, A. (2015). Bioprocessing of citrus waste peel for induced pectinase production by *Aspergillus niger*, its purification and characterization. *Journal of Radiation Research and Applied Science*, 9(2):148-154.

Akinmusire, O. O. (2011). Fungal species associated with the spoilage of some edible fruits in Maiduguri, North Eastern Nigeria. *Advanced Environmental Biology*, 5(1):157-161.

Alves, J. N. O., Jorge, J. A. and Guimaraes, L. H. S. (2013). Production of invertases by Anamorphic (*Aspergillus nidulans*) and teleomorphic (*Emericela nidulans*) fungi under submerged fermentation using rye flour as carbon source. *Advanced Microbiology*, 3:421-429.

Aung, T., Jiang, H., Liu, G. L., Chi, Z., Hu, Z and Chi, Z. M. (2019). Overproduction of a β-fructo-furanosidase1 with a high FOS synthesis activity for efficient biosynthesis of fructooligo-saccharides. *International Journal of Biological Macromolecules,* 130:988-996.
doi: 10.1016/j.ijbiomac.2019.03.039

Awad, G. E. A., Amera, H., El-Gammal, E. W., Helmy, W. A., Esawy, M. A. and Elnashar, M. M. M. (2013). Production optimization of invertase by *Lactobacillus brevis* Mm-6 and its immobilization on alginate beads. *Carbohydrate Polymer*, 93:740-746.

Bas, D. and Boyaci, I. H. (2007). Modeling and optimization II: comparison of estimation capabili-ties of response surface methodology with artificial neural networks in a biochemical reaction. *Journal of Food Engineering*, 78(3):846-854.

Batista, R. D., Melo, F. G., Do Amaral Santos, C. C. A., De Paula-Elias, F. C., Perna, R. F., Xavier, M. C. A., Morales, S. A. V. and De Almeida, A. F. (2021). Optimization of β-fructofura-nosidase production from agrowaste by *Aspergillus carbonarius* and its application in the production of inverted sugar. *Food Technology and Biotechnology*, 59(3):306-313. doi: 10.17113/ftb.59.03.21.6934

Bennet, J. W., Wunch, K. G., Faison, B. O. (2002). Use of Fungi in Bioremediation. In: Manual of Environmental Microbiology, 2nd Edition, Hurst, C. J., Crawford, R. L., Garland, J. L., Lipson, D. A., Mills, A. L., Stetzenbach, L. D. (Eds.). ASM Press, Washington DC. pp. 960-971.

Benson, H. J. (1990). Microbiological Applications. A Laboratory Manual in General Microbiology. 5th edition. USA. Wm C. Brown Publishers. pp. 38-43.

Bergmeyer, H. O. and Bent, E. (1974). Methods of Enzymatic Analysis. Bergmeyer, H. O. (Ed.) 2nd Edition. Academic Press. New York. pp. 1205-1212.

Chang, C. Y., Lee, C. L, Pan, T. M. (2006). Statistical optimization of medium components for the production of *Antrodia cinnamomea* AC0623 in submerged cultures. *Applied Microbiology and Biotechnology*, 72:654-61.

Dapper, T. B., Arfelli, V. C., Henn, C., Simoes, M. R., Dos Santos, M. F., Della Tore, C. L., Da Conceicao Silva, J. L., De Cassia Garcia Simao, R., Kadowaki, M. K. (2016). β-fructofu-ranosidase production by *Aspergillus versicolor* isolated from atlantic forest and grown on apple pomace. *African Journal of Microbiology Research*, 10(25):938-948.

Di Donato, P., Fiorentino, G., Anzelmo, G., Tommonaro, G., Nicolaus, B. and Poli, A. (2011). Re-use of vegetable wastes as cheap substrates for extremophile biomass production. *Waste Biomass Valorization*, 2:103-111.

El-Hadi, A. A., El-Nour, S. A., Hammad, A., Kamel, Z. and Anwar, M. (2014). Optimization of cultural and nutritional conditions for carboxymethylcellulase production by *Aspergillus hortai*. *Journal of Radiation Research and Applied Sciences*, 7(2014):23-28.

Ghosh, K., Dhar, A. and Samanta, T. B. (2014). Purification and characterization of an invertase Produced by *Aspergillus ochraceus* TS. *Indian Journal of Biochemistry and Biophysics*, 38(3):180-185.

Giraldo, M. A., da Silva, T. M., Fernanda, S., Terenzi, H. F., Jorge, J. A. and Guimaraes, L. H. S. (2011). Thermostable invertases from *Paecylomyces variotii* produced under submerged and solid-state fermentation using agro-industrial residues. *World Journal of Microbiology and Biotechnology*, 28:463-472.

Gracida-Rodriguez, M. A., Goncalves, H. B., Furriel, R. D., Jorge, J. A. and Guimaraes, L. H. S. (2014). Characterization of the co-purified invertase and beta-glucosidase of a multifunctional extract from *Aspergillus terreus*. *World Journal of Microbiology and Biotechnology*, 30:1501-1510.

Karandikar, S. (2007). Microbial Enzymes: Studies on β fructofuranosidase from a thermotolerant strain of *Kluyveromyces marxianus*. Ph.D thesis submitted to Pune University, Pune, India.

Keramat, A., Kargari, A., Sohrabi, M., Mirshekar, H. and Sanaeepur, H. (2017). Kinetic model for invertase-induced sucrose hydrolysis: Initial time lag. *Chemical Engineering and Technology*. 40(3):529-36. doi: 10.1002/ceat.201400389

Kulshrestha, S., Tyagi, P., Sindhi, V. and Yadavilli, K.S. (2013). Invertase and its applications – A brief review. *Journal of Pharmacy Research* 7(9):792-797.

Linde, D., Macias, I., Fernandez-Arrojo, L., Plou F. J., Jimenez, A., Fernandez-Lobato, M. (2009). Molecular and biochemical characterization of a β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. *Applied and Environmental Microbiology*, 75(4):1065-1073.

Matei, G. M., Matei, S., Pele, M., Dumitrescu, F. and Matei, A. (2017). Invertase production by fungi, characterization of enzyme activity and kinetic parameters. *Revista Chimie*, 68(10):2205-2208.

Myers, R. H., Montgomery, D. C., Anderson-Cook, C. M. (2009). Response Surface Methodology: Process and product optimization using designed experiments. Third edition. Wiley, New York, U.S.A. pp. 112.

Nehad, E. A. and Atalla, S. M. M. (2020). Production and immobilization of invertase from *Penicillium* sp. using orange peel waste as substrate. *Egyptian Pharmaceutical Journal*, 19:103-112. doi: 10.4103/epj_41_19

Osiebe, O., Adewale, I. O. and Omafuvbe, B. O. (2023). Production and characterization of intra-cellular invertase from *Saccharomyces cerevisiae* (OL629078.1), using cassava-soybean as a cost-effective substrate. *Scientific Reports*, 13:16295. doi: 10.1038/s41598-023-43502-2

Ottoni, C. A., Cuervo-Fernandez, R., Piccoli, R. M., Moreira, R., Guilarte-Mareoma, B., Sabino, E., Rodrigues, MFA. and Maiorano, A. E. (2012). Medium optimization for β-fructofu-ranosidase production by *Aspergillus oryzae*. *Brazilian Journal of Chemical Engineering*, 29(01):49-59.

Oyedeji, O., Bakare, M. K., Adewale, I. O., Olutiola, P. O. and Omoboye, O. (2017). Optimized production and characterization of thermostable invertase from *Aspergillus niger* IBK1, using pineapple peel as alternate substrate. *Biocatalysis and Agricultural Biotechnology*, 9(2017):218-223.

Qureshi, A. S., Khusk, I., Ali, C. H., Majeed, H. and Ahmad, A. (2017). Production of invertase from *Saccharomyces cerevisiae* Angel using date syrup as a cost-effective carbon source. *African Journal of Biotechnology*, 16(45):777-781.

Rustiguel, C. B., Jorge, J. A. and Guimaraes, L. H. S. (2015). Characterization of a thermotolerant mycelial β-fructofuranosidase from *Aspergillus phoenicis* under submerged fermentation using wheat bran as a carbon source. *Biocatalysis and Agricultural Biotechnology*, 4(3):362-369.

Salim, R. G., Abo-Sereh, N. A. and Khalil, B. E. (2020). Optimization and molecular characterization of novel *Aspergillus* spp. Producing invertase enzyme. *Egyptian Pharmaceutical Journal*, 19:321-329. doi: 10.4103/epj_34_20

Shankar, T., Sathees, R. and Anandapandian, K. T. K. (2015). Statistical optimization for ethanol production by *Saccharomyces cerevisiae* (MTCC 170) using Response Surface Methodology. *Journal of Advances in Medical Life Sciences*, 2(3):6-10.

Singh, D. and Sharma, R. R. (2007). Postharvest diseases of fruits and vegetables and their management. In: Sustainable Pest Management. Prasad, D., (Ed.). Daya Publishing House, New Delhi, India. 2007.

Soares, A. S., Augusto, P. E. D., Leite, B. R. C., Jr, Nogueira, C. A., Vieira, E. N. R., Barros, F. A. R., Stringheta, P. C. And Ramos, A. M. (2019). Ultrasound-assisted enzymatic hydrolysis of sucrose catalyzed by invertase: Investigation on substrate, enzyme and kinetics parameters. *Lebensm Wiss Technology*. 107:164-170. doi: 10.1016/j.lwt.2019.02.083

Tasar, O. C., Erdal, S. and Algur, O. F. (2015). Utilization of leek (*Allium ampeloprasum* var. Porrum) for inulinase production. *Preparative Biochemistry and Biotechnology*, 45:596-604.

Tasar, O. C. and Tasar, G. E. (2022). Effects of different nitrogen sources on invertase production by *Aspergillus niger*. *Eurasian Journal of Biological and Chemical Sciences*, 5(2):95-96. doi: 10.46239.ejbcs.1138487and

Taskin, M., Esim, N., Genisel, M. Ortucu, S., Hasenekoglu, I. Canli, O. and Erdal, S. (2013). Enhancement of invertase production by *Aspergillus niger* OZ-3 using low-intensity static magnetic fields. *Preparative Biochemistry and Biotechnology*. 43(2):177-188.

Tournas, V. H. and Katsoudas, E. (2005). Mould and yeast flora in fresh berries, grapes and citrus fruits. *International Journal of Food Microbiology*, 105:11-17.

Uma, C., Gomathi, D. and Muthulaksmhmi, C. (2010). Production, purification, and characterization of invertase by *Aspergillus flavus* using fruit peel waste as substrate. *Advances in Biological Research*, 4(1):31-36.

Varga, J. and Samson, R. A. (eds). (2008). *Aspergillus* in the Genomic Era. Wageningen: Wageningen Academic Publishers. 2008.

White, T. J., Bruns, T. D., Lee, S. B., Taylor, J. W., Innis, M. A., Gelfand, D. H. and Sninsky, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenets. In: PCR Protocols: A Guide to Methods and Applications, (Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. eds). pp. 315-322.

Wijngaard, H. H., Roble, C. and Brunton, N. (2009). A survey of Irish fruit and vegetable waste and by-products as a source of polyphenolic antioxidants. *Food Chemistry*, 116:202-207.

Zafar, M., Kumar, S. and Kumar, S. (2010). Optimization of naphthalene biodegradation by a Genetic algorithm-based response surface methodology. *Brazilian Journal of Chemical Engineering*, 27(1):89-99.