STATISTICAL OPTIMIZATION OF CELLULASE PRODUCTION BY *TRICHODERMA LONGIBRACHIATUM* ON WHEAT BRAN UNDER SOLID-STATE FERMENTATION UTILIZING PLACKETT-BURMAN DESIGN

HIND LEGHLIMI,[*,**] FATIMA-ZOHRA KENZA LABBANI,[**,***,****] AICHA BELMESSIKH,[*****] NAIMA BOUTAGHANE[**,******] and ZAHIA KABOUCHE[**]

[*]*Department of Microbiology, Faculty of Natural and Life Sciences, University of Frères Mentouri-Constantine 1, Algeria*
[**]*Laboratory of Therapeutic Substances (LOST), Department of Chemistry, Faculty of Exact Sciences, University of Frères Mentouri-Constantine 1, Algeria*
[***]*Department of Natural Sciences, Teacher Training College El Katiba Assia Djebar of Constantine, University Town Ali Mendjeli, Constantine 25000, Algeria*
[****]*Laboratory of Biotechnology, Biodiversity Conservation and Didactics, Teacher Training College El Katiba Assia Djebar of Constantine, University Town Ali Mendjeli, Constantine 25000, Algeria*
[*****]*Laboratory of Biologie and Environnement, Department of Microbiology, Faculty of Natural and Life Sciences, University of Frères Mentouri Constantine 1, Constantine, Algeria*
[******]*Department of Biochemistry, Faculty of Natural and Life Sciences, University of Frères Mentouri-Constantine 1, Algeria*
✉ *Corresponding author: H. Leghlimi, hind.leghlimi@umc.edu.dz*

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Cellulases are essential industrial enzymes extensively utilized in biofuel production, textile processing, and waste management. However, high production costs limit their widespread commercial application. This study aimed to optimize cellulase production by *Trichoderma longibrachiatum* isolate under solid-state fermentation (SSF) using wheat bran, an abundant and inexpensive substrate. Fermentation parameters were first optimized using a one-factor-at-a-time (OFAT) approach, identifying 30 °C and 2×10^7 spores per gram of dry substrate (gds) as optimal conditions. Subsequently, medium components affecting enzyme production were statistically evaluated using a Plackett-Burman design comprising eight experimental runs with five variables: carboxymethyl cellulose (CMC), ammonium sulfate ($(NH_4)_2SO_4$), $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$. Statistical analysis demonstrated that CMC, $(NH_4)_2SO_4$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$ significantly enhanced filter paper activity (FPA) ($p < 0.001$). Endoglucanase activity (EGA) showed a significant positive correlation with CMC and $(NH_4)_2SO_4$ ($p < 0.001$). Pareto charts confirmed that CMC and $(NH_4)_2SO_4$ were the most influential factors for cellulase production. Under optimized conditions, wheat bran was found to be an effective substrate for producing high cellulase titers with low operational cost. These findings highlight the potential of combining agricultural residues with statistical optimization to advance cost-effective enzyme production and support sustainable bioconversion technologies.

Keywords: cellulase, *Trichoderma longibrachiatum*, solid-state fermentation, wheat bran, Plackett-Burman design, enzyme optimization

INTRODUCTION

Cellulose is the most abundant polymer in lignocellulosic biomass and represents a renewable feedstock for chemicals and energy, making its biological degradation a subject of intensive research. Cellulases are a group of enzymes that hydrolyze the β-1,4-glycosidic bonds in cellulose and comprise three main types: endoglucanases (EC 3.2.1.4), which randomly cleave internal bonds to create new chain ends, exoglucanases (EC 3.2.1.91), which cleave cellulose chain ends into oligosaccharides, and β-glucosidases (EC 3.2.1.21), which finally act to hydrolyze cello-oligosaccharides into glucose molecules.[1] These

enzymes synergistically convert cellulose into glucose monomers.[2,3]

Cellulases are naturally produced by diverse microorganisms, including fungi, bacteria, and protozoa.[4,5] Among them, fungi are the primary source of commercial cellulases due to their high secretion capacity and extracellular enzyme characteristics, which facilitate enzyme recovery.[6,7] Several fungal species from the phyla Chytridiomycota, Ascomycota, and Basidiomycota efficiently degrade cellulose, with ascomycetes such as *Trichoderma reesei* and *Aspergillus niger* widely utilized for industrial cellulase production.[6-8] Cellulase is produced by a large number of microorganisms. They are either cell bound or extracellular. Although a large number of microorganisms can degrade cellulose, only a few of them produce significant quantities of free enzymes capable of completely hydrolysing crystalline cellulose.[9]

Due to their broad industrial applications, including biofuel production, textile softening, detergent formulation, food processing, pulp and paper industry, and pharmaceutical uses, cellulases are regarded as the third most important industrial enzymes after proteases and amylases.[5-12] The growing demand for highly active, stable, and specific cellulases necessitates continued research to improve production cost-efficiency, substrate specificity, and enzyme activity.[3-13]

Microbial cellulase production depends on multiple nutritional and physicochemical parameters such as medium composition, pH, temperature, incubation time, agitation, moisture, inoculum size, and additives. Optimizing these parameters is essential to maximize enzyme yield and develop economically feasible fermentation processes.[14] A major limiting factor in cellulase commercialization is the cost of fermentation media, which motivates the use of low-cost agricultural residues as substrates. Utilizing agro-industrial wastes not only reduces production expenses, but also addresses environmental pollution by valorizing lignocellulosic residues.[15-18] Numerous studies have demonstrated cellulase production using agricultural organic wastes, such as corn stover, wheat straw, rice straw, and bagasse.[19,20]

Filamentous fungi, especially *Trichoderma* species, are considered preferred cellulase producers and solid-state fermentation (SSF) is regarded as an efficient and economical method for producing concentrated enzyme preparations.[21] However, the complex nature of lignocellulosic substrates and multivariate fermentation parameters necessitate the application of statistical experimental designs, such as Plackett-Burman design, to identify significant factors affecting enzyme production rapidly and efficiently.

Despite previous work on cellulase production optimization, few studies have systematically investigated the combined effects of medium components and fermentation conditions on cellulase production by *Trichoderma longibrachiatum* using wheat bran in SSF. Wheat bran is an attractive substrate due to its abundance, nutrient content, and low cost, making it promising for industrial enzyme production.

Therefore, this study aimed to optimize cellulase production by *T. longibrachiatum* isolate under SSF using wheat bran as substrate. Initial optimization of critical biological parameters (incubation temperature and inoculum size) was conducted using a one-factor-at-a-time approach, followed by Plackett-Burman design for the screening of key medium components. The overall goal was to maximize cellulase yield economically and contribute to the advancement of sustainable bioconversion technologies.

EXPERIMENTAL

Microorganism, inoculum preparation and spore preservation

The cellulase-producing microorganism used in this study was a previously characterized *Trichoderma longibrachiatum* isolate. This fungus was isolated from soil samples collected near a thermal spring in Hammam Debbagh, Guelma, Northeastern Algeria. The species-level taxonomic identification of the isolate, based on colony habit and morphological characteristics, was carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig Germany), as reported by Leghlimi.[22]

For routine maintenance, the fungus was grown on Potato Dextrose Agar (PDA) plates at 30 °C until abundant sporulation was observed. Spores were then harvested by adding 10 mL of sterile distilled water to the culture surface. The resulting spore suspension was used for the inoculation of culture media and enzyme production. Spore concentration was determined by using a Thomas cell counting chamber. For short-term storage, spore suspensions were maintained at 4 °C. For long-term preservation, spores were suspended in 10 mL of sterile distilled water containing 0.1% Tween 80 supplemented with 20% (v/v) glycerol as a cryoprotectant. The suspensions were then stored as glycerol stocks at −20 °C to maintain spore viability and minimize strain variation until further use.[23]

Substrate preparation and solid-state fermentation medium

Wheat bran was assessed as a substrate for cellulolytic enzymes production under solid-state fermentation. Industrial wheat bran was kindly provided by Hamma Bouziane's Group at Unit 314 located in Constantine province, Northeastern Algeria. The substrate was subsequently stored in airtight containers at room temperature until use.

Enzyme production by *T. longibrachiatum* isolate was carried out in 250 mL Erlenmeyer flasks, each containing 5 g of untreated wheat bran. The solid substrate was moistened to 70% level (v/w),[24] (based on dry substrate weight) by adding an appropriate volume of distilled water supplemented with 1% glucose as moistening agent, for the approach of incubation temperature and inoculum size optimization. Regarding the Placket and Burman design, the composition of the wetting agent varies according to the particular matrix test. The content mixture was thoroughly homogenised to ensure uniform moisture distribution. The flasks were plugged with sterile cotton wool and covered with aluminium foil to allow gas exchange and maintain adequate aeration for fungal growth, while minimizing contamination. All materials were sterilized by autoclaving at 120 °C for 20 minutes prior to inoculation, and cooled thereafter at room temperature. All subsequent handling was performed under aseptic conditions. Flasks were inoculated with a spore suspension at a density of 2×10^7spores per gram of substrate. The inoculum was mixed thoroughly into the substrate using a sterile glass rod to ensure uniform distribution. Cultures were then incubated at 30 °C for 4 days. All experiments were performed in duplicate.

Optimization of fermentation conditions

SSF production of cellulase is influenced by various factors, including media components and environmental parameters.[25] A combination of experimental strategies and statistical tools was applied to evaluate the most significant variables affecting cellulase production.

One-factor-at-a-time (OFAT) approach

Initial optimization of two biological parameters, incubation temperature and inoculum size, was performed using an OFAT strategy. A preliminary temperature screening was conducted to evaluate the thermal response of the studied *Trichoderma longibrachiatum* isolate. Based on previously reported optimal growth and cellulase production conditions for this species, the analysis was subsequently focused on the biologically relevant temperature range of 28-35 °C. Inoculum concentrations were evaluated at three levels: 1.5×10^7, 2×10^7, and 2.5×10^7 spores/gram of dry substrate (gds). The substrate moisture content was adjusted to 70% (v/w) by adding a calculated amount of moistening agent prior to sterilization. During incubation, moisture was maitained by minimizing evaporation using cotton-plugged flasks.

Plackett-Burman experimental design (PBD)

The Plackett–Burman Design (PBD) is an efficient statistical method used to identify the most influential factors among numerous variables in experimental processes. In this study, a two-level fractional factorial PBD,[26] which assumes no interactions between factors, was employed to detect significant fermentation parameters affecting cellulase production under SSF conditions. The main effect of each parameter was calculated as the difference between the average response values at the high level (+1) and the low level (–1) for that factor.

The Plackett-Burman design was employed to evaluate the significance of selected medium components on cellulase production. Five independent medium component variables were selected for evaluation. To accurately estimate experimental error and improve model reliability, two dummy variables were also included, resulting in a total of seven variables studied. Each variable was evaluated at two levels: low (-1) and high (+1), corresponding to the concentrations presented in Table 1. The factors included: carboxymethyl cellulose (CMC) as a soluble cellulose analogue and inducer (carbon source), ammonium sulfate as an inorganic nitrogen source, and mineral salts ($MgSO_4\cdot7H_2O$, $FeSO_4\cdot7H_2O$, $ZnSO_4\cdot7H_2O$) as essential cofactors for microbial metabolism and enzyme synthesis. Wheat bran was used as the basal substrate due to its lignocellulosic composition, cost-effectiveness in supporting fungal growth under solid-state fermentation conditions and its reported effectiveness in supporting cellulase production. The total number of experimental runs was calculated as n +1, where n is the number of variables; hence, eight trials were conducted. All experiments were performed in triplicate to ensure reproducibility. The average values of filter paper cellulase activity (FPA) and endoglucanase activity (EGA) were measured and used as responses (dependent variables) for further statistical analysis.

Table 2 presents the PBD matrix, with each column representing an independent variable and each row corresponding to a specific experimental run. Fungal spore suspensions were inoculated into the solid medium for each run and incubated at 30 °C for 72 hours, in accordance with the design matrix.

Statistical analysis

Experimental design and data analysis were performed using Minitab software (version 22). The Plackett-Burman (PB) experimental design followed a first-order linear regression model represented by the equation:

$$Y = \beta_0 + \Sigma\beta i\ Xi \quad (1)$$

where Y is the dependent variable representing enzyme activity (FPA or EGA), β_0 is the model intercept, βi are the estimated coefficients for each independent variable Xi, and i denotes the variable

index. The analysis of variance (ANOVA) using the F-test was employed to assess the overall significance of the model and each individual term. The statistical significance of the regression coefficients was further evaluated using Student's t-test. Model adequacy and goodness of fit were determined by the coefficient of multiple determination (R^2) and the lack-of-fit test. The statistical significance was determined using the test of probability (p). Variables with p-values less than 0.05 were considered statistically significant and retained as influential factors affecting cellulase production.

Table 1
Variables studied at different levels in Plackett-Burman design for cellulase production under solid state conditions

Variables (%)	Variable code	Low level (-)	High level (+)
CMC	X_1	0	1
$(NH_4)\ SO_4$	X_2	0	0.5
Error	X_3	-	-
$MgSO_4\ 7H_2O$	X_4	0	0,05
Error	X_5	-	-
$FeSO_4\ 7H_2O$	X_6	0	0.02
$ZnSO_4\ 7H_2O$	X_7	0	0.02

Table 2
Plackett and Burman design matrix for screening variables influencing cellulase production (FPA and EGA) of each treatment

Run N°	X_1	X_2	X_3	X_4	X_5	X_6	X_7	FPA activity (U/gds)	EGA activity (U/gds)
1	+	+	+	-	+	-	-	27.21±1.21	296.70±8.53
2	+	+	-	+	-	-	+	24.42±1.10	262.71±10.97
3	+	-	+	-	-	+	+	23.22±0.54	218.49±3.19
4	-	+	-	-	+	+	+	23.06±0.21	212.33±1.22
5	+	-	-	+	+	+	-	22.25±0.38	206.59±7.26
6	-	-	+	+	+	-	+	15.11±1.89	172.17±10.73
7	-	+	+	+	-	+	-	17.30±1.81	208.97±10.42
8	-	-	-	-	-	-	-	6.66±1.39	147.03±10.27

Note: Variables were tested at two levels: the higher level designated as +1 and the lower level designated as −1

Analytical methods

Enzyme extraction

Crude enzyme extracts were obtained by mixing the fermented substrate with extraction buffer (0.1 M citrate buffer, pH 4.8) at a ratio of 1:20 (w/v) for 2 minutes. Insoluble solids were removed by filtration through Whatman No. 1 filter paper, followed by centrifugation at 20,000 × g for 10 minutes at 4 °C. The clarified supernatant was collected and stored at 4 °C for no longer than 48 hours prior to analysis to preserve enzymatic activity; for extended storage, samples were kept at -20 °C. The dry weight of the moldy wheat bran was determined by drying 5 g of the fermented substrate to constant weight at 105 °C.

Enzymatic assays

The filter paper activity FPA and endoglucanase EGA were assayed in the crude extracts following the protocols described by Ghose,[27] using filter paper and carboxymethyl cellulose (CMC) as substrates, respectively. The release of reducing sugars was quantified using the dinitrosalicylic acid (DNS) method.[28] Enzyme activities were expressed in international units per gram of dry substrate (IU/gds), where one international unit is defined as the amount of enzyme releasing one µmol of reducing sugars (expressed as glucose equivalents) per minute from filter paper or CMC under assay conditions.

RESULTS AND DISCUSSION

Optimization of incubation temperature

The temperature of the growth medium is considered one of the most important variables in enzyme production in SSF. Temperature is a critical factor, it plays a major role, significantly affecting fungal growth, germination, sporulation, and generally metabolic activities, thus affecting enzyme biosynthesis.[31] The temperature can change the physical properties of the cell membrane and thus influence extracellular enzyme secretion.[32]

Incubation temperature significantly affected cellulase production by *T. longibrachiatum* isolate under SSF using wheat bran as substrate. The highest FPA of 32.85 ± 1.59 U/gds and EGA of 261.48 ± 1.80 U/gds were obtained at 30 °C (Fig. 1). Temperatures above 30 °C resulted in a decrease in both enzyme activities, with the lowest values observed at 28 °C. Thus, 30 °C was identified as the optimum incubation temperature for further experiments.

The influence of temperature on FPA and EGA was statistically assessed using one-way analysis of variance (ANOVA), revealing a highly significant effect of incubation temperature on both enzyme activities ($p < 0.001$). For EGA, the highest mean activity was observed at 30 °C (261.48 ± 1.80 U/gds), while lower activities were found at 35 °C (209.52 ± 1.13 U/gds) and 28 °C (137.15 ± 0.26 U/gds). Post-hoc Tukey tests demonstrated that all pairwise comparisons between temperature groups were statistically significant ($p < 0.05$). Similarly, incubation temperature significantly affected FPA activity ($p < 0.001$). The highest mean FPA was detected at 30 °C (32.85 ± 1.59 U/gds), followed by lower activities at 35 °C (22.78 ± 1.27 U/gds) and 28 °C (22.09 ± 0.23 U/gds). Tukey's post-hoc analysis confirmed that all temperature groups differed significantly from each other ($p < 0.05$).

Based on these results, 30 °C was identified as the optimum incubation temperature for maximizing cellulase (FPA: 32.85 ± 1.59 U/gds; EGA: 261.48 ± 1.80 U/gds) production by *T. longibrachiatum* isolate in SSF using wheat bran. This is consistent with previous studies indicating that optimal cellulase production by *Trichoderma* species generally occurs at temperatures ranging from 28 to 32 °C.[33,34,35] These temperatures balance fungal metabolic activity and enzyme stability. Below the optimum, reduced nutrient transport and slower fungal growth are likely to limit enzyme synthesis, while temperatures above 30 °C may induce thermal denaturation of enzymes and disrupt cellular metabolism.[32-36]

Furthermore, elevated temperatures accelerate moisture loss in the SSF substrate, impairing fungal growth and enzyme secretion by creating dry microenvironments unfavorable for fungal metabolism, consistent with the findings of Raimbault,[31] and Sun *et al*.[35] The impact of cultivation temperature on both the growth rate and cellulase production is significant.[37] According to Das *et al*.,[38] the fungal strains exhibited their highest level of activity at a temperature of 30 °C, however this activity dropped when the incubation temperature exceeded 37 °C. Furthermore, incubation at higher temperature affected the fungus harmfully, which reflected on the enzyme synthesis. Since enzyme is a primary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield.[35]

The observed statistically significant differences in enzyme activities at various temperatures ($p < 0.001$) confirm the robustness of this parameter's impact on cellulase yields.

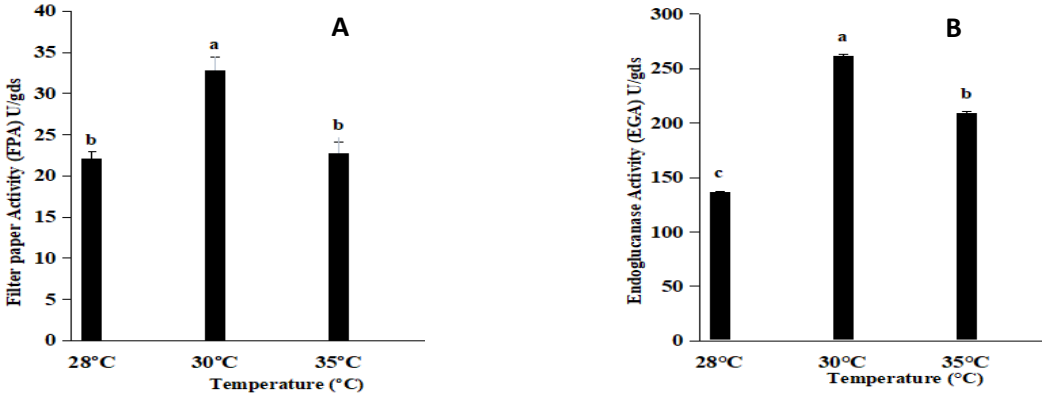

Figure 1: Effect of incubation temperature on cellulase production by *T. longibrachiatum* isolate under SSF using wheat bran as substrate; (A) FPA and (B) EGA were measured after incubation at 28 °C, 30 °C and 35 °C. Bars represent mean enzyme activities (U/gds) ± standard deviation (SD) (n = 3) of three independent experiments. Different letters above bars indicate statistically significant differences between temperature groups (Tukey's test, $p < 0.05$)

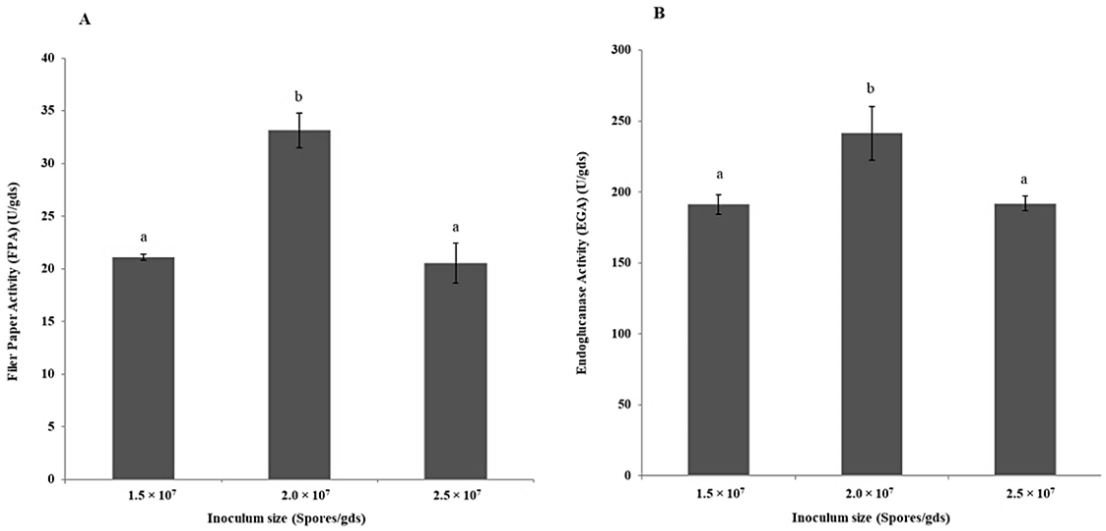

Figure 2: Effect of inoculum size on cellulase production by *T. longibrachiatum* isolate under SSF using wheat bran as substrate; (A) FPA and (B) EGA measured at inoculum sizes of 1.5×10^7, 2×10^7, and 2.5×10^7 spores per gds. Bars represent means ± SD from three independent experiments (n = 3). Different letters above bars indicate statistically significant differences between inoculum sizes according to Tukey's test ($p < 0.05$)

Optimization of inoculum size

The effect of inoculum size on cellulase production by *T. longibrachiatum* isolate was assessed using three different spore concentrations: 1.5×10^7, 2×10^7, and 2.5×10^7 spores/gds. The highest FPA and EGA values were obtained at an inoculum size of 2×10^7 spores/gds, yielding 33.91 ± 1.43 U/gds and 252.90 ± 11.28 U/gds, respectively (Fig. 2). Further increases in inoculum size beyond this optimal value resulted in a decrease in enzyme activities.

Statistical analysis using one-way ANOVA revealed that the effect of inoculum size on both FPA and EGA was highly significant ($p < 0.001$). Post-hoc Tukey's test confirmed that, for both enzyme activities, the values at 2×10^7 spores/gds were significantly higher than at the other inoculum sizes ($p < 0.001$). No significant difference was found between the lowest (1.5×10^7 spores/gds) and highest (2.5×10^7 spores/gds) inoculum treatments.

These findings demonstrate that an inoculum size of 2×10^7 spores/gds is optimal for maximizing cellulase production (FPA (33.91 ± 1.43 U/gds) and EGA (252.90 ± 11.28 U/gds)) by *T. longibrachiatum* isolate under SSF using wheat bran as substrate. Both lower and higher inoculum densities significantly decreased enzyme production ($p < 0.001$), indicating that an optimal fungal biomass concentration is essential. This finding corroborates previous studies where moderate inoculum sizes favored substrate colonization and prevented adverse effects of overcrowding.[39,40] Excessive inoculum sizes can lead to nutrient depletion, reduced oxygen transfer, and anaerobic conditions, all of which are detrimental to cellulase biosynthesis. Conversely, insufficient inoculum can result in delayed substrate utilization, slower micelial growth and enzyme accumulation.[35-42]

However, the maximum cellulase activity (19.13 ± 1.5 FPU/g) was obtained by *Trichoderma reesei* in solid fermentations on municipal solid waste, using 0.5×10^6 spores/g.[41] Acording to Alaam *et al*.,[37] a 5% inoculum size of *Aspergillus niger* potentially resulted in an upregulation of cellulase yield. Prior research has indicated that smaller inoculum sizes (0.5%, 1%, and 2%) result in reduced cellulase synthesis.[38]

Screening of significant medium components using PBD

The PBD was employed to identify the most influential medium components for cellulase production by *T. longibrachiatum* isolate under SSF. However, this model is unable to explain the interaction between different variables. Eight experimental runs, comprising seven variables at two levels each, demonstrated a wide range of enzyme activities (Table 2). The FPA ranged from 6.66 ± 1.39 U/gds to 27.21 ± 1.21 U/gds, while EGA varied from 147.03 ± 10.27 U/gds to 296.70 ± 8.53 U/gds. The highest enzymatic activities were obtained in run 1, and the lowest in run 8.

Regression analysis yielded statistically robust first-order linear models for both FPA (Eq. 2) and EGA (Eq. 3), with high coefficients of determination (R^2 = 96.23% for FPA, R^2 = 96.36% for EGA). The mathematical models representing the FPA and EGA as a function of the independent variables were expressed by the following equations:

$$\text{FPA activity (U gds}^{-1}) = 19.904 + 4.371\ X_1 + 3.093\ X_2 + 0.807\ X_3 - 0.135\ X_4 + 2.006\ X_5 + 1.553\ X_6 + 1.549\ X_7 \quad (2)$$

$$\text{EGA activity (U gds}^{-1}) = 215.62 + 30.50\ X_1 + 29.55\ X_2 + 8.46\ X_3 - 3.01\ X_4 + 6.32\ X_5 - 4.03\ X_6 + 0.80\ X_7 \quad (3)$$

where X_i are the independent variables as defined in Tables 1 and 2.

The adequacy of the goodness of fit model was assessed using the coefficient of determination (R^2). The coefficient (R^2) of determination is defined as the ratio of the explained variation to the total variation, and is a measurement of the degree of fitness.[29] A small value of R^2 indicates poor relevance of the dependent variables in the model. The model can fit well with the actual data when R^2 approaches unity. Joglekar and May[30] demonstrated that the R^2 should be at least 80% to indicate the suitability of the used model. As shown in Tables 3 and 4, the R^2 value of this model was determined to be higher than 0.96 in both models, which showed that the regression model defined well the true behaviour of the system and demonstrated that the model had the capacity to account for a maximum of 96.0% of the responses variability, FPA or EGA, respectively. Also, the high values of adjusted R^2 (94.58%for FPA-94.77% for EGA) and predicted R^2 (91.52% for FPA-91.81% for EGA) indicated the high correlation between predicted and experimental values for both enzymes.

Accordingly, the main effects of the individual parameters on enzyme production were calculated as described in Experimental, and the significance levels (p values) identified by means of Fisher's test on the ANOVA obtained for each variable are shown in Tables 3 and 4, respectively. ANOVA revealed that nearly all tested factors significantly influenced FPA activity ($p < 0.05$), except for $MgSO_4 \cdot 7H_2O$ ($p = 0.665$), which showed no significant effect (Table 3A). For EGA, only carboxymethyl cellulose (CMC; $p = 0.000$) and ammonium sulfate ($p = 0.000$) had significant positive effects, while the mineral salts exhibited either negligible or negative impacts (Table 3B).

The models' significance was further confirmed by high F-values (58.35 for FPA; 60.52 for EGA) and very low probability values ($p < 0.0001$ at 95% confidence level), indicating that most of the variation in the response could be explained by the included variables (Table 4).

Table 3
Statistical analysis applied on Plackett-Burman design for cellulase production from *T. longibrachiatum* isolate

A: FPA activity						
Term	Main effect	Coefficient	SE Coef	T-Value	P-Value	VIF
Constant		19.904	0.306	65.10	0.000	
X1	8.742	4.371	0.306	14.30	0.000	1.00
X2	6.187	3.093	0.306	10.12	0.000	1.00
X3	1.613	0.807	0.306	2.64	0.018	1.00
X4	-0.270	-0.135	0.306	-0.44	0.665	1.00
X5	4.012	2.006	0.306	6.56	0.000	1.00
X6	3.107	1.553	0.306	5.08	0.000	1.00
S= 1.49778 R-Sq = 96.23% R-sq (adj) = 94,58% R-sq (pred) = 91.52%						
B: EGA activity						
Term	Main effect	Coefficient	SE Coef	T-Value	P-Value	VIF
Constant		215.62	2.14	100.73	0.000	
X1	61.00	30.50	2.14	14.25	0.000	1.00
X2	59.11	29.55	2.14	13.81	0.000	1.00
X3	16.92	8.46	2.14	3.95	0.001	1.00
X4	-6.03	-3.01	2.14	-1.41	0.178	1.00
X5	12.64	6.32	2.14	2.95	0.009	1.00
X6	-8.06	-4.03	2.14	-1.88	0.078	1.00
X7	1.60	0.80	2.14	0.37	0.713	1.00
S= 10.4869 R-Sq = 96.36% R-sq (adj) = 94.77% R-sq (pred) = 91.81%						

T –value = student t-test; p – value = corresponding level of significance

Table 4
Analysis of variance for the experiment on optimization of cellulase production by *T. longibrachiatum* isolate

A: FPA activity					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	916.271	130.896	58.35	0.000
Linear	7	916.271	130.896	58.35	0.000
X1	1	458.500	458.500	204.38	0.000
X2	1	229.649	229.649	102.37	0.000
X3	1	15.617	15.617	6.96	0.018
X4	1	0.437	0.437	0.19	0.665
X5	1	96.561	96.561	43.04	0.000
X6	1	57.908	57.908	25.81	0.000
X7	1	57,598	57.598	25.68	0.000
Error	16	35.894	2.243		
Total	23	952.165			
B: EGA activity					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	46587.3	6655.3	60.52	0.000
Linear	7	46587.3	6655.3	60.52	0.000
X1	1	22326.0	22326.0	203.01	0.000
X2	1	20961.6	20961.6	190.60	0.000
X3	1	1717.4	1717.4	15.62	0.001
X4	1	218.0	218.0	1.98	0.178
X5	1	959.4	959.4	8.72	0.009
X6	1	389.5	389.5	3.54	0.078
X7	1	15.5	15.5	0.14	0.713
Error	16	1759.6	110,0		
Total	23	48346.9			

F = Degree of freedom; Adj SS = Adjusted sum of squares; Adj MS = Adjusted Mean of squares; F = Fishers's function; P = Corresponding level of significance

Further, the higher F values revealed that the maximum variation in the responses (FPA and EGA activities) could be explained by the model equation. From Table 4, the F-values are high as well as their probability p-values are less than 0.05 for the variables, which implies that each variable was highly significant, and indicating that the models can be used for prediction of the responses.

The Plackett-Burman statistical design efficiently screened multiple medium components, revealing significant variables influencing cellulase production. CMC and ammonium sulfate were identified as the most significant factors favorably influencing both FPA and EGA. The regression models exhibited excellent goodness-of-fit ($R^2 > 0.96$), indicating strong predictive validity.

CMC has been shown to act as both an inducer and an efficient additional carbon source, thereby stimulating the transcription and secretion of cellulolytic enzymes, particularly endoglucanases, as supported by Thakkar and Saraf[43] and Shajahan *et al*.,[44] while wheat bran served as a substrate providing additional carbon and structural support for solid-state fermentation.

There are reports indicating 1% CMC in the medium was optimum for secretion of cellulose.[45] Additionally, it is worth mentioning that the ideal CMC concentrations may vary based on factors such as type of microorganism, cellulase enzyme, and experimental settings, resulting in potential changes compared to the published literature.[46] Among five carbon sources, CMC showed significant improvement in cellulase production. In general, the cellulase production was induced by CMC.[43] This is expected and, for instance, Lucas *et al*.[47] showed that CMC was the desired substrate for endoglucanase production. The highest cellulase activity was observed when CMC was used as a carbon source and the lowest cellulase activity was observed when sucrose was used as a carbon source.[48] These findings are in line with Abu-Gharbia *et al*.,[49] who said that CMC-supplemented medium saw the highest levels of enzyme synthesis. According to Dina *et al*.,[21] the best carbon source of all the native substrates

studied (bagasse, cotton, green gram hull, wheat bran and rice straw) was wheat bran, which produced higher cellulase activities. Enzyme activities measuring 5.6 IU, 17 IU and 14 IU per gram dry weight of the carbon source for FP, pNPG and CMC were obtained, respectively. Supplementation of wheat bran with either lactose or carboxymethyl cellulose (CMC) enhanced cellulase production. Among them, lactose improved the cellulase production the most.[50]

Pareto chart analysis

A Pareto chart of effects was constructed to identify and rank the main effects of medium components on cellulase production by *T. longibrachiatum* isolate. The horizontal axis represents the main effect estimates for each variable, with factors ordered by their relative importance to the response. As shown in Figure 3, the Pareto chart indicates that CMC, $(NH_4)_2SO_4$, ferrous sulfate, and zinc sulfate were the most influential variables for FPA production by *T. longibrachiatum.*

For EGA activity, the Pareto chart demonstrates that only CMC and $(NH_4)_2SO_4$ were significant contributors to enzyme production (Fig. 4). These results identified the most influential medium components for enhancing FPA and EGA activities under the conditions tested.

The estimated effect obtained for each variable, illustrated by the Pareto charts (Figs. 3 and 4), indicated that an increase in the concentrations of CMC and ammonium sulfate resulted in an enhancement of both activities. Based on these results, the factors CMC and ammonium sulfate were selected for further optimization to improve cellulase production. Further optimization studies were carried out, considering the most significant variables, such as CMC and ammonium sulfate. However, mineral salts tested showed very little positive result on FPA activity, and they exhibited a negative effect on EGA activity, hense they were kept at a lower level in the medium for further studies.

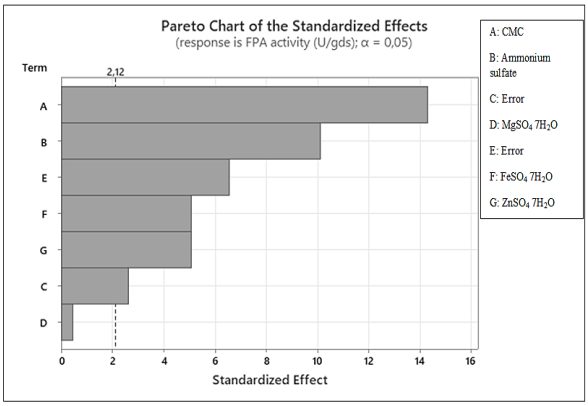

Figure 3: Pareto chart for nutrients as significant variables for FPA produced from *T. longibrachiatum* isolate

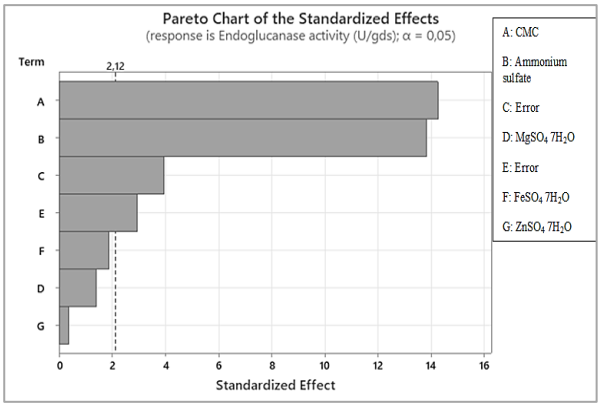

Figure 4: Pareto chart for nutrients as significant variables for EGA produced from *T. longibrachiatum* isolate

Many papers have reported that ammonium compounds are the most favorable nitrogen sources for protein and cellulase synthesis.[34] The significance of ammonium sulfate highlights the importance of readily assimilable inorganic nitrogen in supporting fungal growth and enzyme biosynthesis, in agreement with Rajoka[51] and Mandels and Reese.[52] The ammonium salt of $(NH_4)_2SO_4$ can be directly decomposed to produce ammonia and used by microorganisms, which are also known as a quick-acting nitrogen source.[53] It has been shown that ammonium sulfate as a nitrogen source is more reasonable and balanced for *Penicillium* to produce cellulases,[54] which is similar to the results of this study.

Some microbes produce higher enzyme in presence of some metal ions. Most divalent cations as Cu and Zn induce and contribute to an increase in enzyme activity, hence acting as a cofactor in inducing the activity of the enzyme. Meanwhile, the least noted for this is the Mg ion. Interestingly, while $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ positively influenced FPA levels, they showed negligible or even negative impacts on EGA activity. This disparity reflects the complex role of metal ions in enzyme regulation, potentially acting as cofactors

or inhibitors depending on concentration, enzyme subtype, and fungal physiology.[3-55] $MgSO_4 \cdot 7H_2O$ showed no significant effect on FPA and a negative, though statistically non-significant effect on EGA. The addition of the divalent metal cations $HgCl_2$, $BaCl_2$, $MgCl_2$ and $FeCl_2$ (2 mM) did not enhance cellulase production by *A. flavus*, whereas it was marginally increased by $CaCl_2$ and $ZnCl_2$ (2 mM).[56] A study by Imran *et al.*[57] revealed that cellulase activity is enhanced by the metal ions K^+, Mg^{2+}, Ca^{2+} and Mn^{2+}, while it is inhibited by Zn^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Ag^+ and Fe^{3+} using *Aspergillus* and *Humicola* species.

Results suggest that Fe^{2+} apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active form of the enzyme at high temperature (100 °C).[58] The major action of these metal ions is to work as cofactor of the enzyme. The effect of balance between different metal ion concentrations could be more important than their individual effects. For example, magnesium is needed for cellulase production, but it is also inhibitory at high concentrations and the inhibition is counteracted by calcium.[52] It has been hypothesized that the metal may prevent some components necessary for induction from leaking out of the cells.[58] The results underscore the relevance of balancing trace mineral salts carefully during medium formulation.

Implications for industrial application and future research

The suitability of wheat bran as lignocellulosic agro-industrial by-product, combined with statistically identifined key medium components, highlights its potential application as a substrate for cellulase production under solid-state fermentation conditions. The use of agro-industrial by-products as fermentation substrates may contribute to the valorization of renewable biomass resources and support more sustainable bioprocessing approaches.

The high significance and predictive power of the Plackett-Burman derived models indicate these factors' suitability for further optimization using response surface methodology (RSM) or other multivariate strategies.[44-59] Additionally, scale-up studies should evaluate the robustness of these conditions and the economic feasibility of industrial production.

Future studies should focus on improving the reproducibility and scalability of cellulase production under solid-state fermentation conditions using wheat bran-based substrates. It is important to pay particular attention to strain-specific responses to substrate composition and the interaction between physicochemical parameters affecting enzyme yield. Further clarification regarding the industrial applicability of the proposed system may be obtained through comparative studies involving alternative lignocellulosic by-products and process validation at pilot scale.

CONCLUSION

This study presents a systematic and effective optimization strategy for enhancing cellulase production by *Trichoderma longibrachiatum* isolate under SSF using wheat bran as a sustainable and cost-effective substrate, combining classical OFAT approach with statistical optimization via PBD. The findings contribute valuable insights into fermentation parameter control and highlight the critical influence of medium components on enzyme yield. Precise regulation of critical parameters, including incubation temperature, inoculum size, and key medium components such as CMC and ammonium sulfate, substantially improved both filter paper activity and endoglucanase yields. These optimized conditions contribute significantly to the development of sustainable, cost-efficient bioconversion processes for lignocellulosic biomass, thereby bolstering the industrial applicability of fungal cellulase production. The insights gained herein provide a robust foundation for future scale-up and commercial exploitation of cellulase-producing microbial systems.

REFERENCES

[1] R. K. Sukumaran, M. Christopher, P. Kooloth-Valappil, A. R. Sreeja-Raju, R. M. Mathew *et al*., *Bioresour. Technol*., **329**, 124746 (2021), http://dx.doi.org/ 10.1016/j.biortech.2021.124746

[2] G. Jini and R. K. Singh, *J. Bioresour*., **11**, 71 (2024), https://doi.org/10.5281/zenodo.11318516

[3] N. Fatima, A. Irfan, H. A Shakir, M. Khan, M. Franco *et al*., *Cellulose Chem. Technol*., **58**, 705 (2024), https://doi.org/10.35812/CelluloseChemTechnol.2024.58.64

[4] S. D. Wijayanti, K. Oliviani, J. Kusnadi and R. A. C. Putri, *Conf. Ser. Earth Environ. Sci*., **475**, 1 (2020), https://doi.org/10.1088/1755-1315/475/1/012085

[5] F. Z. K. Labbani, S. Dakhmouche, L. Bennamoun, A. Ait-Kaki and T. Nouadri, *Int. J. Ecosyst. Ecol. Sci*., **12**, 7 (2022), https://doi.org/10.31407/ijees12.102

[6] J. K. Saini, R. Saini and L. Tewari, *Biotech*, **3**, 337 (2015), https://doi.org/10.1007/s13205-014-0246-5

[7] D. Troiano, V. Orsat and M. J. Dumont, *Renew. Sust. Energ. Rev.*, **117**, 10947 (2020)
[8] C. Payne, B. C. Knott, H. B. Mayes, H. Hansson, M. E. Himmel *et al.*, *Chem. Rev.*, **115**, 1308 (2015), https://doi.org/10.1021/cr500351c
[9] C. Koomnok, in *Procs. 31st Congress on Science and Technology of Tailand of Technology*, Suranaree University, 18–20 October, 2005
[10] J. R. Cherry and A. L. Fidants, *Curr. Opin. Biotechnol.*, **14**, 438 (2003), https://doi.org/10.1016/S0958-1669(03)00099-5
[11] S. Sajith, P. Priji, S. Sreedevi and S. Benjamin, *J. Nutr. Food Sci.*, **6**, 1 (2016), https://doi.org/10.4172/2155-9600.1000461
[12] P. Bajaj and R. Mahajan, *Appl. Microbiol. Biotechnol.*, **103**, 8711 (2019), http://dx.doi/10.1007/s00253-019-10146-0
[13] S. Sethi, A. Datta, B. L. Gupta and S. Gupta, *ISRN Biotechnol.*, **19**, 985685 (2013), http://dx.doi.org/10.5402/2013/985685
[14] S. Sarsan and R. Merugu, "Role of Bioprocess Parameters to Improve Cellulase Production: Part II. New and Future Developments in Microbial Biotechnology and Bioengineering", 2019, pp. 77-97
[15] S. Nighojkar, Y. Phanse, D. Sinha, A. Nighojkar and A. Kumar, *Process Biochem.*, **41**, 1136 (2006), https://doi.org/10.1016/j.procbio.2005.12.009
[16] R. Liguori, A. Amore and V. Faraco, *Appl. Microbiol. Biotechnol.*, **97**, 6129 (2013), https://doi.org/10.1007/s00253-013-5073-2
[17] S. Uzuner and D. Cekmecelioglu, *J. Mol. Catal. B Enzym.*, **113**, 62 (2015), https://doi.org/10.1016/j.molcatb.2015.01.003
[18] V. Poondla, S. K. Yannam, S. N. Gummadi, R. Subramanyam and V. S. Reddy Obulam, *Biocatal. Agric. Biotechnol.*, **6**, 40 (2016), https://doi.org/10.1016/j.bcab.2016.02.007
[19] M. D. Romero, J. Aguado, L. Gonzalez and M. Ladero, *Enzyme Microb. Technol.*, **25**, 244 (1999), https://doi.org/10.1016/S0141-0229(99)00035-6
[20] A. Pandey, P. Selvakumar, C. R. Soccol and P. Nigam, *Curr. Sci.*, **77**, 149 (1999)
[21] S. Dina, V. T. Thankamani and T. E. Abraham, *J. Microbiol. Biotech. Food Sci.*, **12**, e4754 (2023), https://doi.org/10.55251/jmbfs.4754
[22] H. Leghlimi, PhD Thesis, Department of Microbiology, Faculty of Natural and Life Sciences, University of Frères Mentouri-Constantine 1, Algeria, 2013
[23] B. Botton, A. Breton, M. Fevre, S. Gauthier, P. H. Guy *et al.*, "Moisissures utiles et nuisibles importance industrielle", 2nd ed., Masson, Collection Biotechnologies, 1990, pp. 34-428
[24] H. Leghlimi, I. Djezzar-Mihoubi, H. BoukhalfaLezzar, S. Dakhmouche, L. Bennamoun *et al.*, *Int. J. Sci.*, **6**, 46 (2017), https://doi.org/10.18483/ijSci.1457
[25] N. Darabzadeh, Z. Hamidi-Esfahani and P. Hejazi, *Food Sci. Nutr.*, **7**, 572 (2019), https://doi.org/10.1002/fsn3.852
[26] R. L. Plackett and J. P. Burman, *Biometrica*, **33**, 305 (1946), https://doi.org/10.1093/biomet/33.4.305
[27] T. K. Ghose, *Pure Appl. Chem.*, **59**, 257 (1987)
[28] G. L. Miller, *Anal. Chem.*, **31**, 426 (1959)
[29] A. Nath and P. K. Chattopadhyay, *J. Food Eng.*, **80**, 1282 (2007), https://doi:10.1016/j.jfoodeng.2006.09.023
[30] A. M. Joglekar and A. T. May, *Cereal Foods World*, **32**, 857 (1987)
[31] M. Raimbault, "Fermentation en milieu solide, croissance de champignons filamenteux sur substrat amylacé", ORSTOM, Paris, 1983, pp. 1-42
[32] S. K. Pramanik, S. Mahmud, G. K. Paul, T. Jabin, K. Naher *et al.*, *Curr. Res. Microb. Sci.*, **2**, 100013 (2021), https://doi.org/10.1016/j.crmicr.2020.100013
[33] S. Ahmed, N. Aslam, F. Latif, M. Rajoka and A. Jamil, *Front. Nat. Prod. Chem.*, **1**, 73 (2005)
[34] S. Sethi and S. Gupta, *Biolife*, **2**, 989 (2014)
[35] H. Sun, X. Ge, Z. Hao and M. Peng, *Afr. J. Biotechnol.*, **9**, 163 (2010), http://www.academicjournals.org/AJB ISSN 1684–5315
[36] D. Dutt and A. Kumar, *Cellulose Chem. Technol.*, **48**, 285 (2014), https://www.cellulosechemtechnol.ro/pdf/CCT3-4(2014)/p.285-298.pdf
[37] H. Alaam, I. E. Mousa, N. M. H. Rizk, H. A. Hamza and A. S. Eldourghamy, *J. Curr. Vet. Res.*, **6**, 72 (2024), https://doi.org/10.21608/jcvr.2024.384945
[38] A. Das, S. Bhattacharya, K. S. Roopa and S. S. Yashoda, *Dyn. Biochem. Process Biotech. Mol. Biol.*, **5**, 18 (2011)
[39] A. Aygan, B. Arikan, H. Korkmaz, S. Dincer and O. Colak, *Braz. J. Microbiol.*, **39**, 547 (2008), https://doi.org/10.1590/S1517-83822008000300027
[40] P. Saravanan, R. Muthuvelayudham, R. R. Kannan and T. Viruthagiri, *Front. Chem. Sci. Eng.*, **6**, 443 (2012), https://doi.org/10.1007/s11705-012-1225-1
[41] J. J. Abdullah, D. Greetham, N. Pensupa, G. A. Tucker and C. Du, *J. Fundam. Renew. Energ. Appl.*, **6**, 206 (2016), https://doi.org/10.4172/2090-4541.1000206
[42] V. H. Vu, T. A. Pham and K. Kim, *Mycobiology*, **39**, 20 (2011), https://doi.org/10.4489/MYCO.2011.39.1.020
[43] A. Thakkar and M. Saraf, *Nat. Prod. Bioprospect.*, **4**, 341 (2014), https://doi.org/10.1007/s13659-014-0046-y
[44] S. Shajahan, I. Ganesh Moorthy, N. Sivakumar and G. Selvakumar, *India J. King Saud Univ. Sci.*, **29**, 302 (2017), https://doi.org/10.1016/j.jksus.2016.08.001
[45] Y. Lugani, R. Singla and B. S. Sooch, *J. Bioproc. Biotech.*, **5**, 1 (2015), https://doi.org/10.4172/2155-9821.1000264

[46] A. Fouda, K. S. Alshallash, H. M. Atta, M. S. El Gamal, M. M. Bakry *et al*., *J. Microbiol. Biotechnol*., **34**, 207 (2024), https://doi.org/10.4014/jmb.2309.09023
[47] R. Lucas, A. Robles, M. T. Garcia, G. A. De Cienfuegos and A. Galvez, *J. Agric. Food Chem*., **49**, 79 (2001), https://doi.org/10.1021/jf000916p
[48] M. S. Demissie, N. H. Legesse and A. A. Tesema, *Plos One*, **19**, e0301607 (2024), https://doi.org/10.1371/journal. pone.0301607
[49] M. A. Abu-Gharbia, N. W. El-Sawy, A. M. Nasr and L. A. Zedan, *J. Pharm. Appl. Chem*., **4**, 1 (2018), https://doi.org/10.18576/jpac/040201
[50] J. Singh and P. Kaur, *Braz. Arch. Biol. Technol*., **55**, 505 (2012), https://doi.org/10.1590/S1516-89132012000400004
[51] M. I. Rajoka, *Electr. J. Biotechnol*., **7**, 256 (2004), http://www.ejbiotechnology.info/content/vol.7/issues/full/2/
[52] M. Mandels and E. T. Reese, *J. Ind. Microbiol. Biotechnol*., **22**, 225 (1999), https://doi.org/10.1038/sj.jim.2900635
[53] H. Li, M. Dou, X. Wang, N. Guo, P. Kou *et al*., *Sustainability*, **13**, 6006 (2021), https://doi.org/10.3390/su13116006
[54] R. Saini, J. K. Saini, M. Adsul, A. K. Patel, A. Mathur *et al*., *Bioresour. Technol*., **188**, 240 (2015), https://doi.org/10.1016/j.biortech.2015.01.048
[55] Y. S. Soeka, N. Suharna, E. Triana and T. Yulinery, *Makara J. Sci*., **23**, 65 (2019), https://doi/10.7454/mss.v23i2.11043
[56] A. Bano, X. Chen, S. Prasongsuk, A. Akbar, P. Lotrakul *et al*., *Appl. Biochem. Biotechnol*., **189**, 1327 (2019), http://dx.doi.org/10.1007/s12010-019-03086-y
[57] M. Imran, Z. Anwar, M. Irshad, M. J. Asad and H. Ashfaq, *Adv. Enzyme Res*., **4**, 44 (2016), https://doi.org/10.4236/aer.2016.42005
[58] I. F. Okonkwo, *Int. J. Environ. Agric. Biotechnol*., **4**, 727 (2019), http://dx.doi.org/10.22161/ijeab/4.3.20 ISSN: 2456-1878
[59] S. Yahya, S. Jahangir, S. S. Shaukat, M. Sohail and S. Ahmed Khan, *Pak. J. Bot*., **48**, 2557 (2016)