PRODUCTION OF CELLULASES FROM *PSEUDOMONAS STUTZERI* USING MANGO PEELS AS A SUBSTRATE IN SUBMERGED FERMENTATION

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Received April 8, 2024

Enzymes are biocatalysts produced by microorganisms using plant materials as a substrate. Green chemistry uses plant materials to produce enzymes, while fermentation technology produces enzymes on larger scales. These enzymes can be used in food, textile, paper industry and biofuel production. Cellulase is an industrial enzyme that breaks the *β*-1,4-glycosidic bond of polysaccharides in plant cells and can be produced by various microorganisms. Mango waste can be used to produce bioactive compounds, such as cellulase enzymes, from microorganisms in submerged fermentation (SmF). The production of endoglucanase and exoglucanase from *Pseudomonas stutzeri* using mango peels as a substrate in SmF was optimized using one factor at a time and response surface methodology. The optimum conditions for CMCase were 4.5% substrate concentration, 96 hours incubation time, and 2.5% inoculum size, while optimum conditions for FPase were 4.5% substrate concentration, 48 hours incubation time and 0.5% inoculum size. PBD was employed for the screening of various nutritional components, such as K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, NaCl, $MgSO_4$, $FeSO_4$ and $CaCl_2$, and it was found that significant nutritional parameters were $FeSO_4$, $MgSO_4$ and $(NH_4)_2SO_4$. Through the Central Composite Design, maximum production of endoglucanase, *i.e.* 120.112 IU/mL/min, was found at 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4$ and 0.45% $FeSO_4$, while maximum exoglucanase production, *i.e.* 161.38 IU/mL/min, was recorded at 0.1% $(NH_4)_2SO_4$, 0.5% $MgSO_4$ and 0.05% $FeSO_4$. The optimum temperature and pH for maximum CMCase and FPase activity were 50 °C and 7.0, respectively. Endoglucanases and exoglucanases were stable up to 50 °C and pH 7. Metal ions such as Mn^{2+} and Cu^{2+} activated the activity of CMCase and FPase, respectively, while Zn^{2+} and Na^{+} inhibited CMCase and FPase activity, respectively.

Keywords: *Pseudomonas stutzeri*, cellulases, submerged fermentation, lignocellulose biomass

INTRODUCTION

Enzymes are organic molecules that are also called biocatalysts. Enzymes produced by microorganisms in fermentation technology using plant substrates are less expensive and can be used in different industries, such as those of food, textile, paper and biofuel production.[1] The most widespread plant biomass on Earth is cellulose. Plant biomass is comprised of cellulose (40 to 60%), hemicelluloses (10 to 40%), and lignin (15 to 30%), which could meet 14% of the global energy demands.[2-4] When supplied with complete technology, this can account for 27% of the world's transport fuels by 2035. The maximum part of plant biomass is cellulose, which must be pretreated to liberate sugar molecules for biofuel synthesis.[5]

All cellulose-degrading enzymes or structures are referred to as cellulases generated by exogenous or cell-bound microorganisms, along with cellulases with multiple mechanisms of action. Cellulase can break cellulose into glucose at a minimum cost. It breaks the *β*-1,4-glycosidic bond of cellulose in plant cells. It is among the most widely used commercial enzymes secreted

by microorganisms like fungi and bacteria, which use plant cellulose as a substrate and secrete glucose to get energy from it.[6,7] There are three basic types of cellulases. CMCase or endoglucanase cleaves β-1,4-glycosidic bonds within the cellulose polymer randomly and produces new ends.[8,9] FPase or exoglucanases are cellobiohydrolases (CBH) and cellodextrinases, which cleave the cellulase at ends and cause cellobiose production.[10,11] β-Glucosidases effectively hydrolyze cellobiose, which is not degraded by CBHs and endoglucanases.[12] Cellulase enzyme is the 2nd most widely used industrial enzyme after protease. According to a research report on the Global Cellulase Market published in 2018, 32.84% of cellulase was consumed by Asia and the Pacific in 2016. According to that study, they are the top users of cellulase, having an estimated share in the market of roughly 32.84% by 2016. Additionally, the statistics revealed that, in 2016, 13.77% of cellulase was utilized in the textile sector, 26.37% was used for dietary supplements, and 29.71% was used as a feed ingredient. Additionally, it is foreseen that, by 2025, the need for cellulases will rise to 2300 million US dollars.[13]

Cellulases are produced by fermentation. Solid-state fermentation (SSF) is performed in the absence of water on a rigid support. The microbes need moisture for their growth.[14] In submerged fermentation (SmF), microorganisms are grown in a liquid broth medium. The nutrient media is optimized for better development of microorganisms. In this process, microbes are cultivated in nutrient media with oxygen and placed in a closed container. Bacteria are used mostly in SmF because they need large amounts of moisture for their growth. SmF is used to synthesise enzymes on a large scale because the purification and recovery of products can be controlled easily with this fermentation method. Different types of microbes and substrates, including sugarcane, rice husk, coconut coir pith, wheat bran and tea production waste, can be used in SmF.[15] *Pseudomonas stutzeri* is a bacterium with cellulolytic activities and de-nitrification ability, as it carries CAZymes genes in its genome.[16] In 1895, its initial description was presented by Burri and Stutzer.[17] Its unique property is its ability to endure an extensive range of pH and temperature. It mainly grows on starch and maltose, and can survive in a simple medium containing nitrogen and carbon sources.[18,19]

Mango (*Mangifera indica* L.) is from the family Anacardiaceae. It consists of edible parts, mainly pulp, and non-edible by-products, which constitute 35 to 55% of its total mass and can be utilised for various purposes.[20] Mango peel possesses a significant concentration of cellulose and lignin, *i.e.* 30 and 16%, respectively.[21] Mango peels contain considerable cellulase, so that they can be utilised as a substrate for microbial cellulase. In an earlier research, mango peels were utilised for fungal cellulase production.[22] In this research, the potential of mango peel to produce cellulase from *P. stutzeri* was assessed, and media were further optimized through one factor at a time methodology (OFAT) and response surface methodology (RSM) for maximum cellulase production.

EXPERIMENTAL

Substrate collection

Mango peels were used as substrate for cellulase production in this study. They were obtained by manually peeling fresh mangoes bought from a local market of Sargodha, a district of Punjab in Pakistan. The pulp was scraped using a sharp and clean knife. Mango peels were initially rinsed with distilled water (DW) to remove impurities. After that, mango peels were air-dried and ground to powdered form.

Inoculum preparation

The research study used the bacteria *Pseudomonas stutzeri*, which were obtained from the microbial culture collection of the Department of Biotechnology, University of Sargodha, Sargodha.

For inoculum preparation, a nutrient broth medium was prepared in a 250 mL Erlenmeyer flask containing 100 mL DW and autoclaved at 121 °C for 15 minutes to sterilize the nutrient broth medium, followed by cooling and then inoculation with the bacterial strain. After that, it was incubated at 35 °C for 24 h at an agitation speed of 120 rpm. The cell suspension obtained after growth was used as a source of inoculum for further experiments.

Enzyme production

Various nutrients were used for enzyme production, as per design, and 5% of the substrate was dissolved in DW in a 100 mL Erlenmeyer flask. The production medium was sterilized at 121 °C for 15 minutes. Then, it was allowed to cool at room temperature. After that, the production medium was inoculated with 1% bacterial strain. After inoculation, the production medium was placed in a rotary shaker and incubated at 35 °C and 120 rpm for 24 hours. After an incubation period of 24 hours, the fermentation broth was filtered through muslin cloth. In an Eppendorf tube, 2 mL of the filtrate was collected and centrifuged at 10,000 rpm for 10 minutes. While the

pellet was thrown away, the supernatant was employed as a source of crude enzyme.

Enzyme assay

Carboxymethyl cellulose (endoglucanase) assay

CMCase activity was performed as described in earlier research.[23] The CMCase assay was performed by taking 0.5 mL of crude enzymes in a test tube, and 0.5 mL of 1% (w/v) CMC solution (prepared in 0.05M citrate buffer of pH 5) and then incubated in a water bath at 50 °C for 30 min. After the termination of incubation time, each test tube received 1.5 mL of the DNS solution, which was then boiled for 10 min. After that, the reaction mixture was cooled at room temperature. After that, the optical density was measured at 540 nm using a spectrophotometer. One unit (U) of enzyme activity was the amount of enzyme, which released 1 µmol of glucose in a minute. CMCase activity was measured by using the following formula (Eq. 1):

$$\text{CMCase activity (IU/mL/min)} = \frac{\text{Optical density} \times \text{standard factor (SF)} \times \text{dilution factor (DF)}}{\text{Incubation time}} \quad (1)$$

Filter paper (exoglucanase) assay

Filter paper assay was performed using the method described by Irfan *et al.*[24] FPase activity was performed by taking 0.5 mL of crude enzymes in two test tubes and 0.5 ml distilled water in the third tube. After that, each test tube received 0.5 ml of 0.05M citrate buffer with pH 5, followed by adding a 1 × 6 cm strip of Whatman filter paper. These were then incubated in a water bath at 50 °C for 30 min. After the termination of incubation time, 1.5ml of DNS solution was added and incubated at 100 °C for 10 min. After that the test tubes were taken out from incubation and allowed to cool at room temperature and then measure optical density at 540 nm. FPase activity was measured using the formula below (Eq. 2).

$$\text{FPase activity (IU/mL/min)} = \frac{\text{Optical density} \times \text{SF} \times \text{DF}}{\text{Incubation time}} \quad (2)$$

Optimization of process conditions for maximum production of cellulase

One factor at a time (OFAT) methodology

Different physical parameters, including inoculum size, substrate concentration, and fermentation time, were adjusted by using the OFAT methodology. One factor was optimized to get a high quantity and yield quality, while others were kept constant. So, all factors were optimized one by one in the OFAT methodology.

Optimization of substrate concentration

To optimize substrate concentration, enzymes were produced using different concentrations of substrate. The concentrations tested were 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, and 5% (w/v). After enzyme production, the CMCase and FPase assays were performed according to the methods described above to find out the optimum concentration of substrate for maximum yield in SmF.

Optimization of incubation time

To optimize incubation time, production media containing the optimum concentration of substrate was then inoculated with a 1% (v/v) bacterial strain of *P. stutzeri*, followed by incubation for 24, 48, and 96 h. After enzyme production, the CMCase assay and FPase assay were performed according to the methods described above.

Optimization of the inoculum size

For inoculum size optimization, the production media containing the optimum substrate concentration were inoculated with 0.5%, 1%, 1.5%, 2%, 2.5%, and 3% (v/v) *P. stutzeri* inoculum, and then incubated in a rotary shaker for the optimum period of incubation. After enzyme production, CMCase and FPase assays were performed according to the methods described above.

Response surface methodology (RSM)

Significant independent variables were determined through the Plackett-Burman Design (PBD). Response surface methodology (RSM) was used to optimize the nutrients to reduce the number of nutrients and experiments required. Central Composite Design (CCD) was then used to examine the optimum amounts of key independent variables.

PBD

This design has two levels (Table 1) of factors and was employed for screening out and assessing the relative significance of different nutrients (independent variables) used in the production process. In PBD, 12 experimental runs were performed for CMCase and FPase at pH 5, 50 °C temperature, optimum substrate concentration, incubation time and inoculum size, as determined via OFAT. All experiments were performed twice according to the earlier method, and the average was taken as cellulase activity. Eight nutrients were tested in PBD to observe their influence on cellulase production. The tested nutrients were: yeast extract, K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, NaCl, $MgSO_4$, $FeSO_4$ and $CaCl_2$.

PBD depends upon the first-order polynomial model (Eq. 3):

$$Y = \beta_0 + \sum \beta_i X_i \quad (3)$$

where Y represents the predicted response (activity of cellulase), β_0 and β_i are the intercept and linear coefficient of the model, respectively, whereas X_i is the included independent variable.

CCD

Following PBD screening for key nutritional factors, they were substantially adjusted using the CCD of RSM. A 5-level design of CCD with 3

significant factors was employed. Each factor received all five of the stated levels. A 2^3 factorial design with 20 experimental runs was utilized, and the formula was $N = 2^k+2k+n_0$, where N is the number of experiments, k is the number of factors, which were included in the study, and n_0 is the number of experimental repetitions. It predicted the significant factors and interaction of one factor with the other (Table 2).

The coded values for factors were calculated by using the following formula (Eq. 4):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \text{where } i = 1, 2, 3,\ldots K \qquad (4)$$

where x_i and X_i represent the coded value and actual value of factors, respectively. X_0 is the value of the independent factor at the central point, and ΔX_i is the step change. The experimental data was analyzed using multiple regression analysis to calculate the response using the formula of the second-order polynomial model (Eq. 5):

$$Y = \beta_0 + \sum\beta_i X_i + \sum\beta_{ii} X_i^2 + \sum\beta_{ij} X_i X_j + \mathcal{E} \qquad (5)$$

where Y is the expected response, β_0 is intercepted, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the coefficient of interaction, while X_i and X_j are independent variables.

Table 1
Nutrients screened in PBD with their low and high values

Nutrients code	Nutrients (%)	Low value (-1)	High value (+1)
X1	Yeast extract (YE)	0.1	1.0
X2	K_2HPO_4	0.05	0.45
X3	KH_2PO_4	0.05	0.45
X4	$(NH_4)_2SO_4$	0.1	2.0
X5	NaCl	0.3	1.0
X6	$MgSO_4$	0.1	0.5
X7	$FeSO_4$	0.05	0.45
X8	$CaCl_2.2H_2O$	0.005	0.405

Table 2
Codes and levels of variables investigated in CCD

Variables (%)	Codes	Levels				
		-1	-α	0	+α	+1
$(NH_4)_2SO_4$	X4	-0.55	0.1	1.05	2.0	2.65
$MgSO_4$	X6	-0.04	0.1	0.3	0.5	0.64
$FeSO_4$	X7	-0.09	0.05	0.249	0.45	0.58

Characterization of cellulase produced from *P. stutzeri*

Effect of temperature on activity and stability of exoglucanase and endoglucanase

To investigate the effect of temperature on activity of CMCase and FPase, enzyme assay was performed by incubation at different temperatures (30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, 90 °C) for 30 min. The activity was then measured according to the method described above. To observe the effect of temperature on stability, 2 mL filtrate of the enzyme was taken in a test tube, which was pre-incubated at different temperatures (30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C) for one hour; after that, the CMCase and FPase assays were performed at optimum temperature to check the enzyme stability.

Effect of pH on activity and stability of exoglucanase and endoglucanase

To investigate the effect of pH on the activity of the CMCase enzyme, an assay was performed with 1% CMC prepared in buffers of different pH values (4, 5, 6, 7, 8, 9, 10, 11) and incubated at optimum temperature for 30 min. For FPase analysis, the enzyme assay was performed with buffers of pH 4-11. The activity was then measured according to the method described above. To observe the effect of pH on stability, 2 mL of filtrate of the enzyme was pre-treated with buffers of different pH values (4-11) for 1 h. After that, the enzyme assay was performed at optimum temperature for CMCase and FPase to optimize pH for enzyme stability.

Effect of metal ions on cellulase activity

The effect of different metal ions, including Na^+, K^+, Mn^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, on cellulase activity was investigated. Each metal ion was added to a test tube containing crude enzyme at optimum pH and then incubated at optimum temperature for 30 min. After that, enzyme activity was determined.

Statistical analysis

The importance of each factor and its influence on cellulase production was statistically analyzed using the ANOVA (analysis of variance). After finding the results of the 2nd order polynomial model, it was expressed in terms of 3D plots, which demonstrated the significance of each independent variable and the interaction among them. ANOVA was used to determine each term's value in the fitted equations and assess the goodness of fit. The fitted polynomial equation was subsequently displayed in 3D plots to illustrate the key and interacting impacts of the independent variables on the dependent variables.

RESULTS AND DISCUSSION

Biotechnologists are keenly interested in the use of cellulolytic enzymes due to their wide applications. Environmentally friendly methods of using low-cost substrates are gaining more popularity. This research study was carried out to produce exoglucanases and endoglucanases from *Pseudomonas stutzeri* by using mango peels as a substrate. For maximum production of exoglucanases and endoglucanases, various process parameters, including incubation time, inoculum size and substrate concentration, were optimized through one factor at a time methodology. In contrast, nutritional parameters were optimized through response surface methodology, and CMCase and FPase activities were evaluated. The results of the experiments were further analyzed through variance analysis. Furthermore, the effects of temperature, pH and metal ions on the activity and stability of the enzyme were analyzed.

The experiments described above found that maximum endoglucanase and exoglucanase production was observed at 4.5% substrate concentration. Maximum activity was 146.64 IU/mL/min and 70.58 IU/mL/min for CMCase and FPase, respectively. A slight decline in CMCase and FPase activity was observed at 5% substrate concentration, reaching 126.16 IU/mL/min and 69.68 IU/mL/min, respectively (Fig. 1). In an earlier study, peak production of CMCase from *Bacillus subtilis* K-18 occurred at 2% potato powder as a substrate.[25] By employing a 3% wheat bran concentration, Sharma *et al*. discovered that *Bacillus tequilensis* S28 produces the highest levels of cellulase.[26] The greatest cellulase yield from *Bacillus aerius* (MG597041) was obtained using 4% of *Bombax ceiba* at pH 5.5 with 1% inoculum size.[27]

Optimum incubation time for maximum endoglucanase and exoglucanase production was 96 h and 48 h, respectively. CMCase and FPase produced 43.716 IU/mL/min and 127.764 IU/mL/min reducing sugar, respectively (Fig. 2). A similar finding was reported by Abada *et al*., who observed maximum cellulase production at 96 h by *Bacillus albus*.[28] *Pseudomonas stutzeri* has been reported to hydrolyse various other substrates, such as corn cob, wheat straw, rice husk, eucalyptus leaves and *Bombax ceiba* seed pods, with the best cellulase activity of 170.9±4.1 IU/mL/min by using eucalyptus leaves at 37 °C after 24 h of incubation. Similarly, the optimum incubation time for maximum CMCase production by *Bacillus flexus* in media containing *Bombax ceiba* as a substrate was 96 h.[29] On the other hand, in other research studies, it was observed that *Bacillus subtilis* and *Paenibacillus* showed maximum cellulase activity at 24 h of incubation time.[25,30]

Inoculum size optimization resulted in the maximum endoglucanase production at 2.5% inoculum, while maximum exoglucanase production was observed at 0.5% inoculum. CMCase and FPase produced 77.99 IU/mL/min and 184.05 IU/mL/min of reducing sugar residues, respectively. Then, CMCase activity declined by 3%, *i.e.* to 49.286 IU/mL/min. A gradual decrease in FPase activity was observed from 1% to 3% inoculum size, *i.e.* from 177.84 IU/mL/min to 128.28 IU/mL/min, respectively (Fig. 3). Abada *et al*. noticed that cellulase production using *Bacillus albus* needed 3% inoculum (v/v) for peak production of cellulases.[28] In a closely related result, maximum cellulase activity was noticed at 2% inoculum size.[31] Fouda *et al.* reported that the maximum cellulase production occurred at a 1% (v/v) of inoculum size from *Bacillus amyloliquefaciens* M7.[32] Through PBD, significant variables having a substantial effect on cellulase production were determined. After performing 12 runs, it was observed that $MgSO_4$, $(NH_4)_2SO_4$ and $FeSO_4$ have prominent effects on cellulase production among the eight variables, while five were insignificant (Table 3). Contour plots for $MgSO_4$, $(NH_4)_2SO_4$ and $FeSO_4$ for maximum CMCase and FPase production were plotted (Fig. 4). At the same time, desirability charts for CMCase and FPase are illustrated in Figure 5. After this, CCD was employed to find optimum values of significant variables by performing an experiment with 20 runs.

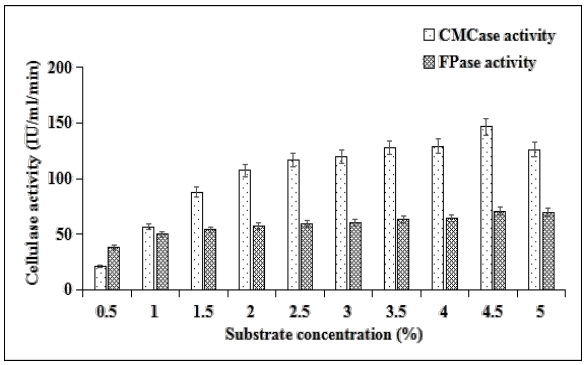

Figure 1: Effect of substrate concentration on cellulase production by *P. stutzeri* in submerged fermentation

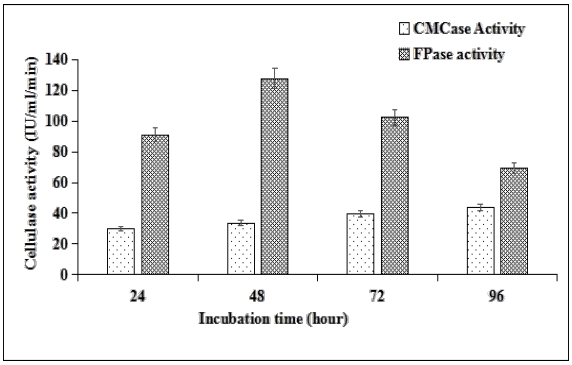

Figure 2: Effect of incubation time on cellulase production by *P. stutzeri* in submerged fermentation

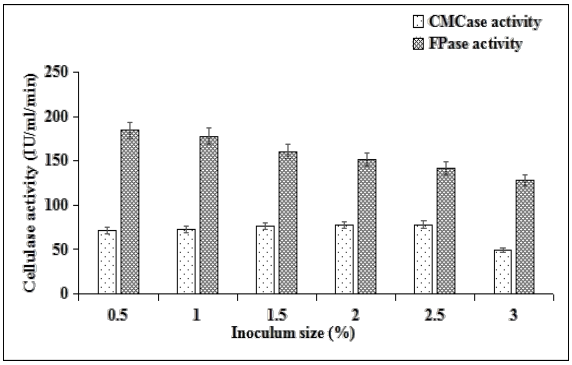

Figure 3: Effect of inoculum size on cellulase production by *P. stutzeri* in submerged fermentation

Maximum production of endoglucanase occurred at 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 0.45% $FeSO_4$ (Table 4). In a previous study by Ibrahim *et al.*, maximum production of CMCase by *Bacillus subtilis* (32.37 U/mL) occurred at 0.1% $FeSO_4.7H_2O$, 8% peptone, 18% CMC, 7% yeast extract (YE).[33] Nitrogen sources, such as NH_4NO_3, have been shown to decrease CMCase activity, while NH_4Cl stimulated the cellulase activity. Likewise, $MgSO_4$ also enhanced the cellulase activity, according to an earlier study.[34] Malik *et al*. also observed the positive effect of $MgSO_4$ and KH_2PO_4 on cellulase production.[35] Khalid *et al*. reported maximum endoglucanase at 2.5% sugarcane bagasse, with 0.01% of $MgSO_4$ and 0.5% of $(NH_4)_2SO_4$ after 24 h of incubation to optimize cellulase synthesis by *Bacillus aquimaris*.[27]

$MgSO_4$ has been found to be a significant variable in various previous research studies. In an earlier study by Tabssum *et al*., peptone, $MgSO_4$, and YE were considered important for endoglucanase production by utilizing a substrate of poplar biomass. The best yield required 0.09% $MgSO_4$, 0.5% yeast extract and 0.03% peptone.[36] Irfan *et al.* observed maximum CMCase production by using media composed of 3 g/mL of peanut shell, 0.45 g/mL of YE and 0.01 $MgSO_4$.[37] In this research, maximum production of exoglucanase by *P. stutzeri* occurred at 0.1% $(NH_4)_2SO_4$, 0.5% $MgSO_4$, 0.05% $FeSO_4$. Likewise, Ghazanfer *et al.* performed PBD, followed by CCD, and found that 0.5 g/L yeast extract, 0.2 g/L $FeSO_4$, 0.5 g/L, peptone and 0.02 g/L K_2HPO_4 were suitable for maximum FPase production with an F-value of 8.74 and a p-value of 0.00.[15] Maximum FPase production in the presence of peanut shell by *Bacillus paralichniformis* occurred by using 3 g/mL of peanut shell, 0.45 g/mL of YE and 0.3 g/L $MgSO_4$.[37] For cellulase production from *Bacillus tequilensis*, Sharma *et al.* used PBD and CCD to explore key nutritional factors. They found that 4.99% NH_4Cl, 4.94% peptone, 0.53% Tween-20, 2% yeast extract, 0.20% $CaCl_2$, and 0.60% $CoCl_2$ were key parameters.[26]

ANOVA results indicated that, for CMCase, the F-value was 4.13, and the P-value was 0.019. The interaction among different factors was analyzed (Table 5).

Table 3
Plackett-Burman design for screening of nutrients for CMCase and FPase production by *P. stutzeri* in submerged fermentation

Run No.	X1	X2	X3	X4	X5	X6	X7	X8	CMCase activity	FPase activity
1	1.0	0.45	0.45	2.0	1.0	0.5	0.45	0.405	127.592	28.721
2	0.1	0.45	0.05	2.0	1.0	0.5	0.05	0.005	128.494	30.653
3	0.1	0.05	0.45	0.1	1.0	0.5	0.45	0.005	126.690	26.918
4	1.0	0.05	0.05	2.0	0.3	0.5	0.45	0.405	125.364	26.145
5	0.1	0.45	0.05	0.1	1.0	0.1	0.45	0.405	114.913	81.656
6	0.1	0.05	0.45	0.1	0.3	0.5	0.05	0.405	118.414	83.717
7	0.1	0.05	0.05	2.0	0.3	0.1	0.45	0.005	128.07	79.853
8	1.0	0.05	0.05	0.1	1.0	0.1	0.05	0.405	125.311	78.951
9	1.0	0.45	0.05	0.1	0.3	0.5	0.05	0.005	123.136	76.633
10	1.0	0.45	0.45	0.1	0.3	0.1	0.45	0.005	120.536	141.288
11	0.1	0.45	0.45	2.0	0.3	0.1	0.05	0.405	121.226	141.55
12	1.0	0.05	0.45	2.0	1.0	0.1	0.05	0.005	116.875	140.386

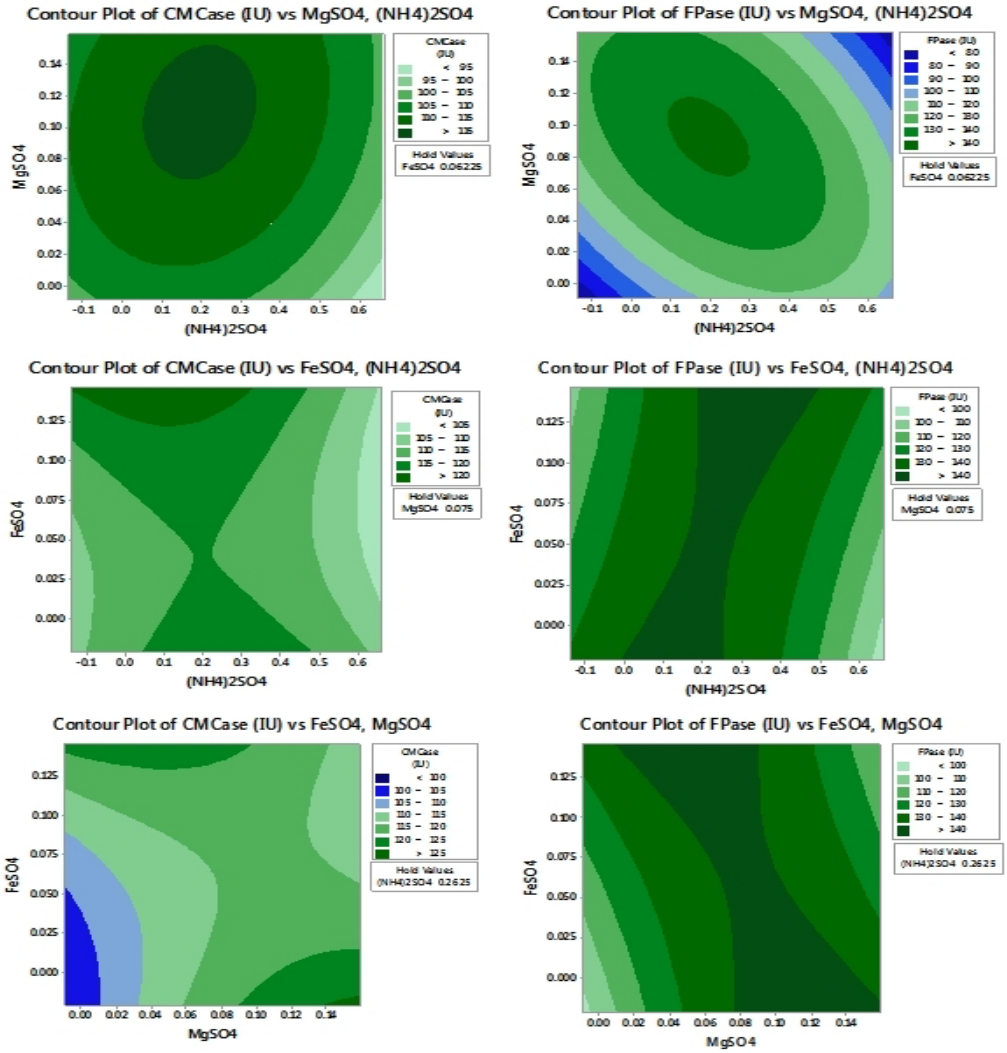

Figure 4: Contour plots showing the interaction of $(NH_4)_2SO_4$ $MgSO_4$ and $FeSO_4$ for FPase (right) and CMCase (left) production by *P. stutzeri* in submerged fermentation

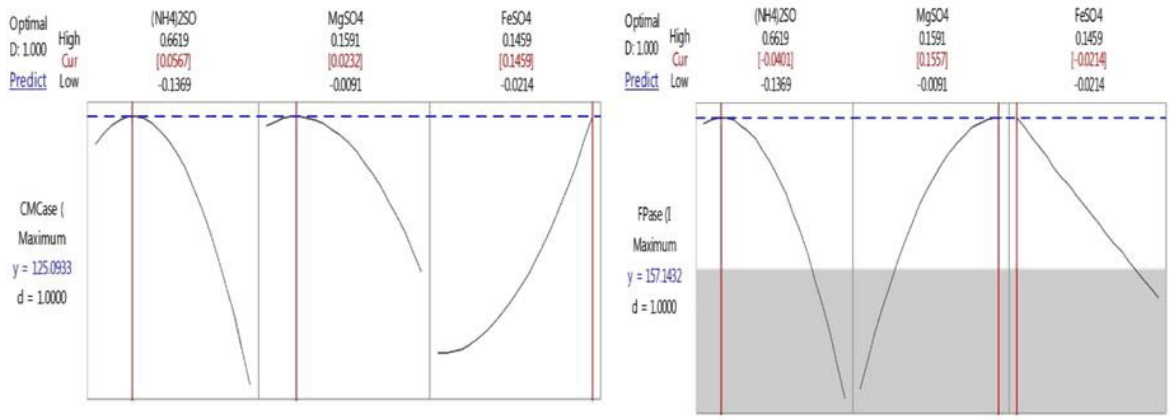

Figure 5: Desirability chart for FPase (right) and CMCase (left) production by *P. stutzeri* in submerged fermentation

Table 4
CCD design for optimization of significant variables for cellulase production by *P. stutzeri* in submerged fermentation

Run	X4	X6	X7	CMCase activity (IU/mL)	FPase activity (IU/mL)
1	1.05	0.64	0.249	116.876	122.226
2	1.05	0.3	0.249	111.570	120.294
3	2.0	0.5	0.05	115.974	123.128
4	0.1	0.1	0.45	120.112	119.264
5	1.05	0.3	-0.09	116.610	119.521
6	1.05	0.3	0.249	115.443	128.408
7	0.1	0.5	0.45	117.512	127.893
8	2.0	0.1	0.45	111.942	129.567
9	0.1	0.1	0.05	111.995	117.25
10	1.05	0.3	0.249	112.897	125.832
11	1.05	0.3	0.249	117.034	161.251
12	0.1	0.5	0.05	115.125	161.38
13	1.05	0.3	0.58	120.110	157.645
14	1.05	0.3	0.249	115.443	151.334
15	1.05	0.3	0.249	117.884	155.198
16	1.05	-0.04	0.249	100.80	113.597
17	-0.55	0.3	0.249	105.628	114.370
18	2.0	0.5	0.45	105.89	114.112
19	2.65	0.3	0.249	102.870	113.726
20	2.0	0.1	0.05	102.764	114.112

X4 = $(NH_4)_2SO_4$, X6 = $MgSO_4$, X7 = $FeSO_4$

The coefficient of determination (R^2) for CMCase activity was 78.82% and adjusted R^2 was 59.75%. The response of CMCase was calculated by using a regression equation (Eq. 6).

CMCase (IU) = 100.78 + 22.9 X4 + 211.2 X6 + 63.6 X7 - 58.5 X4*X4 - 670 X6*X6 + 683 X7*X7 + 70 X4*X6 - 121 X4*X7 - 1256 X6*X7

For FPase, the F-value was 50.81, and the P-value was 0.00. The interaction among different factors was analyzed (Table 6). The coefficient of determination (R2) for FPase activity was 97.86%, adjusted R^2 was 95.93 and predicted R^2 was 83.91%. The validity of results was described through a regression equation (Eq. 7). The graphs for predicted and observed values of CMCase and FPase are given in Figure 6.

FPase (IU) = 93.01 + 98.5 X4 + 817.4 X6 + 77.9 X7 - 164.7 X4*X4 -2886 X6*X6+ 109 X7*X7 - 654.7 X4*X6+ 432.8 X4*X7 - 2461 X6*X (7)

When an enzyme assay was conducted at 30 °C and 90 °C, a temperature-dependent rise in cellulase activity was observed at up to 50 °C. Maximum CMCase and FPase activity was observed at 50 °C of 149.13 IU/mL/min and 323.15 IU/mL/min, respectively. Afterwards, a decline in CMCase and FPase activity was observed, with the lowest activity at 90 °C, *i.e.* 117.78 IU/mL/min and 268.15 IU/mL/min (Fig.

7). Endoglucanases and exoglucanases were stable at 50 °C, after that, a gradual decrease in stability was observed (Fig. 8). Similarly, in a study by Goel *et al*., it was observed that the CMCase produced from *Pseudomonas* sp. showed a significant rise in activity at 50 °C, which above this temperature, declined; when incubated for 1 h, the cellulase displayed stability over a wide temperature range, which steadily declined above 60 °C.[38] Similarly, the optimum temperature for maximum stability and activity of cellulase from *Bacillus amyloliquefaciens* M7 was also 50 °C.[32] Narkthewan and Makkapan demonstrated that the FPase activity of *Bacillus velezensis* was maximum at 50 °C and was stable from 50 °C to 60 °C.[39]

Table 5
Analysis of variance (ANOVA) for CMCase production by *P. stutzeri* in submerged fermentation

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	9	534.963	59.44	4.13	0.019
Linear	3	184.702	61.567	4.28	0.035
X4	1	78.817	78.817	5.48	0.041
X6	1	88.312	88.312	6.14	0.033
X7	1	17.573	17.573	1.22	0.295
Square	3	250.451	83.484	5.81	0.015
X4*X4	1	156.763	156.763	10.9	0.008
X6*X6	1	40.466	40.466	2.81	0.124
X7*X7	1	41.219	41.219	2.87	0.121
2-Way interaction	3	99.81	33.27	2.31	0.138
X4*X6	1	5.498	5.498	0.38	0.55
X4*X7	1	16.262	16.262	1.13	0.313
X6*X7	1	78.05	78.05	5.43	0.042
Error	10	143.774	14.377		
Lack-of-fit	5	114.751	22.95	3.95	0.079
Pure error	5	29.022	5.804		
Total	19	678.736			

X4 = $(NH_4)_2SO_4$, X6 = $MgSO_4$, X7 = $FeSO_4$

Table 1
Analysis of variance (ANOVA) for FPase production by *P. stutzeri* in submerged culture fermentation

Source	DF	Adj SS	Adj MS	F-value	P-value
Model	9	3071.94	341.33	50.81	00
Linear	3	214.2	71.4	10.63	0.002
X4	1	79.5	79.5	11.83	0.006
X6	1	120.46	120.46	17.93	0.002
X7	1	14.24	14.24	2.12	0.176
Square	3	1865.15	621.72	92.55	0
X4*X4	1	1243.28	1243.28	185.07	0
X6*X6	1	750.23	750.23	111.67	0
X7*X7	1	1.05	1.05	0.16	0.701
2-Way interaction	3	992.58	330.86	49.25	0
X4*X6	1	483.57	483.57	71.98	0
X4*X7	1	209.22	209.22	31.14	0
X6*X7	1	299.78	299.78	44.62	0
Error	10	67.18	6.72		
Lack-of-fit	5	66.19	13.24	66.64	0
Pure error	5	0.99	0.2		
Total	19	3139.12			

X4 = $(NH_4)_2SO_4$, X6 = $MgSO_4$, X7 = $FeSO_4$

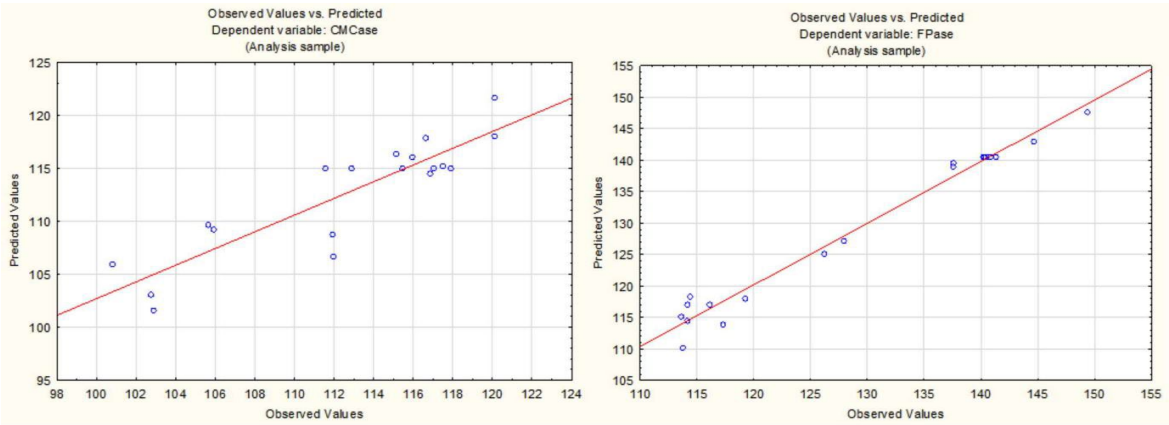

Figure 6: Predicted and observed values of FPase (right) and CMCase (left) production by *P. stutzeri* in submerged fermentation

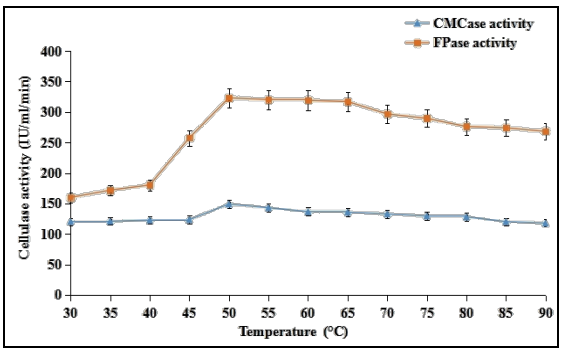

Figure 7: Effect of temperature on the activity of cellulase produced by *P. stutzeri* in submerged fermentation

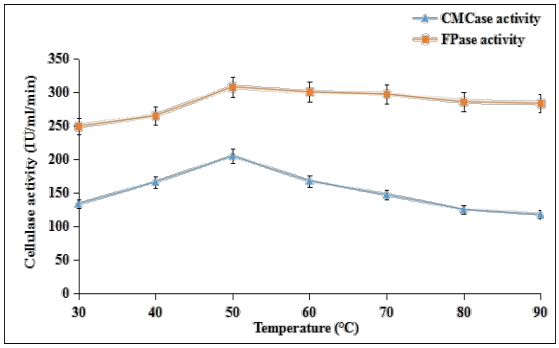

Figure 8: Effect of temperature on stability of cellulase produced by *P. stutzeri* in submerged fermentation

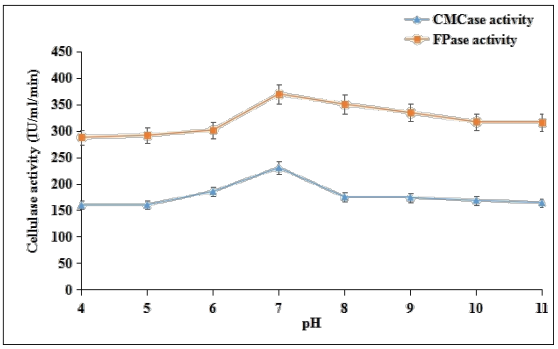

Figure 9: Effect of pH on the activity of cellulase produced by *P. stutzeri* in submerged fermentation

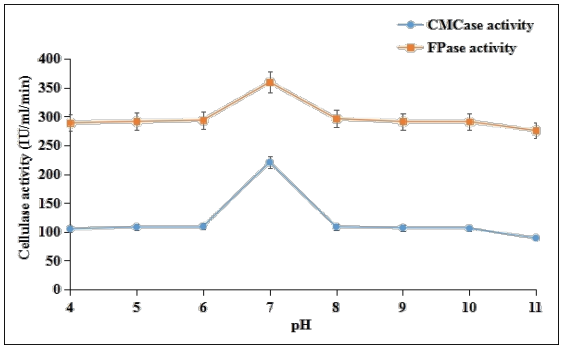

Figure 10: Effect of pH on stability of cellulase produced by *P. stutzeri* in submerged fermentation

In this study, a slight difference in cellulase activity in the range of pH 4-9 at 50 °C was observed, when using buffers with pH ranging from pH 4 to 11. However, maximum CMCase and FPase activity at pH 7 was 230.40 IU/mL/min and 369.90 IU/mL/min, respectively (Fig. 9). It was found that exoglucanases and endoglucanases remained stable up to pH 7, when treated with buffers having different pH for 1 hour. CMCase and FPase were unstable before and after pH 7 (Fig. 10). In a previous report, after evaluating the impact of pH on the synthesis of cellulase by 7 different isolates of *Bacillus*, it was noticed that the optimal pH for cellulase synthesis lies between 6 and 8, as S1-1, S7-2 and S7-6 showed peak production at pH 6, S1-4 showed maximum cellulase production at pH 7, while for S3-3 and S4-2, pH 8 was found to be the optimum for cellulase production.[40] Ibrahim *et al*. reported that the pH range for maximum cellulase activity from *Bacillus* sp. and *Pseudomonas* sp. was 7.5 to 8 at 50 °C, with the highest stability around pH 8.[41]

The endoglucanase produced by *Pseudomonas* sp. was functional between pH 6.5 and 8.0 at 50 °C, having the highest activity level at pH 7. It was stable over a wide pH range with maximum activity at pH 7.[38] According to Soeka *et al*., cellulase from strains of *Streptomyces macrosporeus* showed maximum activity after 120 h of incubation at pH 6 and 2% CMC.[42] In an earlier research study, it was found that *B. velezensis* had the maximum FPase activity at pH 7.0, and it declined in both acidic and alkaline pH.[39] On the other hand, a slightly alkaline environment, *i.e.* pH 7.5, was found suitable for CMCase production from *Pseudomonas aeruginosa* using sawdust as a substrate.[43]

The effect of different metal ions, including Na^+, K^+, Mn^{2+},Cu^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, on cellulase activity was investigated. It was observed that Na^+ inhibited the activity of CMCase and FPase up to 13.6% and 17.6%, and K^+ inhibited CMCase and FPase up to 3.12% and 8.6%, respectively. CMCase activity was also inhibited by Zn^{2+}. In the presence of Zn^{2+}, 84.7% of CMCase activity was retained. While other ions, *i.e.* Mg^{2+}, Cu^{2+}, Mn^{2+} and Ca^{2+}, significantly stimulated cellulase activity.

A slight inhibition of FPase activity was observed by Mg^{2+} ions (Table 7). In an earlier study, the CMCase activity from *Pseudomonas* sp. was minimized by adding K^+, Na^+, and Ca^{+2} ions at 5 mM and 10 mM concentrations, but had almost no effect at the concentration of 1 mM. Also, at concentrations of 5 mM and 10 mM, Cu^{+2}, Zn^{+2}, and Fe^{+2} ions reduced enzyme activity, while at the concentration of 1 mM, the activity was largely maintained. Mn^{+2} boosted the enzyme activity, elevating it three folds when the concentration of Mn^{+2} rose from 1 mM to 10 mM, compared to the control. Mg^{2+} ions were observed to exert a slight inhibition of FPase activity.[38] Another previous research has shown that the activity of CMCase produced from a subspecies of *B. subtilis* was boosted in the presence of Na^+, Fe^{2+} and Ca^{2+} at 50 °C and pH 5 by utilising rice straw as a substrate.[44] Lingouangou *et al*. reported that Zn^{2+}, Co^{2+} and Fe^{2+} were favourable for cellulase activity. It was observed that these metal ions have a positive impact on the activity of cellulase produced from *Pantoea dispersa* MLTBY6, *Pseudomonas monteilii* MLTBC10, *Pseudomonas aeruginosa* MLTBM2, *Lysinibacillus fusiformis* MLTBB7 and *Bacillus subtilis* MLTBC5 at pH 8 and 40 °C.[45]

Table 7
Effect of metal ions on the activity of cellulase produced by *P. stutzeri* in submerged fermentation

Metal ions	Residual activity (%)	
	CMCase	FPase
Control	100	100
Na^+	86.4	82.4
K^+	96.88	91.4
Mn^{2+}	132.59	119.42
Cu^{2+}	108.7	126.53
Zn^{2+}	84.7	123.33
Mg^{2+}	131.39	99.74
Ca^{2+}	124.19	108.39

CONCLUSION

After this research study, it was concluded that mango peels have the potential to produce cellulase enzyme from *Pseudomonas stutzeri* in SmF. Production media containing 4.5% substrate and 2.5% inoculum size were suitable for CMCase production, while 4.5% substrate and 0.5% inoculum size – for FPase production, at 48 h incubation time. Response surface methodology proved to be an effective and reliable tool for optimising nutritional parameters. Through RSM, it was found that 0.1% $MgSO_4$, 0.1% $(NH_4)_2SO_4$ and 0.45% $FeSO_4$ were suitable for maximum CMCase production, while 0.5% $MgSO_4$, 0.1% $(NH_4)_2SO_4$ and 0.05% $FeSO_4$ were suitable for maximum of FPase production. Maximum activities of CMCase and FPase were observed in the presence of Mn^{2+} and Cu^{2+}, respectively, at 50 °C and pH 7.

ACKNOWLEDGEMENT: The authors are thankful to the Deanship of Research and Graduate Studies, King Khalid University, Abha, Saudi Arabia, for financially supporting this work

through the Large Research Group Project under Grant no. R.G.P.2/506/45.

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