

Parthenium hysterophorus Weed as a Novel Substrate for β -Glucosidase Production by *Penicillium citrinum* NAF5: Application of the Crude Extract to Biomass Saccharification

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Abstract: Fungal cellulases are well-studied enzymes with various industrial applications. The key goal of this work was to evaluate the possibility of non-conventional *P. hysterophorus* weed biomass valorization by using it to produce β -glucosidases using the fungus *P. citrinum* NAF5. For a comparative analysis, three independent variables selected from the 'one variable at a time' approach were subjected to response surface methodology. To assess the interacting outcome of the significant factors, the central composite design (CCD) was used. The CCD was applied by considering carbon source (lactose % w/v), nitrogen source (peptone % w/v), and pH as the model factors for the present analysis. The maximum β -glucosidase production (2.48 U/ml) was obtained with lactose 1.50% (w/v), peptone 1.50% (w/v), and pH 6. The crude enzyme preparations from *P. citrinum* NAF5 were used to saccharify sodium carbonate (1% w/v) pretreated *P. hysterophorus* biomass. After 7 h enzymatic hydrolysis of sodium carbonate pretreated *P. hysterophorus* biomass, a maximum release of reducing sugars of 92.48 mg/g substrate was observed, which is 3.3-fold higher than that of untreated biomass (28.02 mg/g substrate), indicating that *P. citrinum* NAF5 has the potential to produce the crude enzyme for lignocellulosic biomass hydrolysis.

Keywords: β -Glucosidases; OVAT; RSM; *P. citrinum* NAF5; saccharification.

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1. Introduction

One of the world's most harmful invasive weeds, *P. hysterophorus*, can be found very commonly in Asia, Africa, America, and Australia [1]. Common physical, chemical, and biological treatments and integrative techniques have struggled to manage this weed. Various microorganisms can efficiently use the lignocellulosic materials bound in their cell walls for cellulase production. This will also give an effective strategy for controlling this weed through its utilization. Recently, researchers have begun to take a novel approach to weed control by utilizing weeds as a bioresource for numerous uses [2,3]. *P. hysterophorus* has been recognized as a potential choice for producing α -cellulose because it is an annual plant with high cellulose [1]. *P. hysterophorus* has the potential to be a low-cost feedstock for the production of endoglucanase [4], cellulase [5], xylanase [6], and laccase [7].

The use of weed biomass for the production of β -glucosidase could be a useful way to increase the value of biomass. Furthermore, the plenty of weed biomass may provide a solution to the issue of the high expenses of β -glucosidase production. Fungal cellulases are among the most important enzymes for converting lignocellulosic substrate into easily fermentable sugars for bioethanol production. Cellulase is made up of three types of enzyme complexes: cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and β -glucosidases (EC 3.2.1.21), which work together to convert complex carbohydrates present in lignocellulosic biomass into sugar [8]. Cellulose is a high-molecular-weight linear polymer of D-glucopyranose units joined by β (1-4) glycosidic linkages [9]. These enzymes work together to cleave β (1-4) glycosidic bonds between glucose units in the cellulose polymer at distinct sites, resulting in total cellulose hydrolysis. Filamentous fungi are capable of producing large amounts of extracellular cellulases. Exo-type glycosyl hydrolase known as β -glucosidases break β -glycosidic bonds in disaccharide or glucose-substituted compounds. β -glucosidases are grouped into glycosyl hydrolase families 1 and 3 by the Carbohydrate active enzymes classification system [10,11]. Glycosyl hydrolase family 1 enzyme folds into an (a/b)₈ barrel structure and acts as a β -retaining system with Glu as the catalytic nucleophile; however, glycosyl hydrolase family 3 β -glucosidases use an Asp residue in nucleophile attacks, even though only a few structures have been solved [12,13]. The β -glucosidase synthesized has a wide range of industrial applications, as evidenced by its features and high yield in low-value culture media. It can be used to produce 2G ethanol and in the juice and alcohol industries [14]. Food, animal feed, beverages, biofuel, and many other commercial domains have all used β -glucosidase to their full potential [15]. It can also be used as a flavoring enzyme to enhance the flavor of wine, tea, and fruit juices, as well as other foods [16]. The fungal genera *Aspergillus*, *Penicillium*, and *Trichoderma* are responsible for most commercial cellulase production [17–19]. *Penicillium* has been studied as a potential source of β -glucosidase in recent years. It produces all three main cellulase components with adequate β -glucosidase, a significant advantage over *Trichoderma* and the fungus with cellulolytic potential [20]. In addition to producing high-activity of β -glucosidases, fungi of the genus *Penicillium* also have profitable possibilities for the industrial production of cellulases [21]. This study aimed to assess the production of β -glucosidase by the fungal strain *P. citrinum* NAF5 utilizing *P. hysterophorus* as a substrate under static conditions, as well as optimize the enzymatic activity. RSM approach was used to conduct statistical optimization of β -glucosidase production for this fungus. Enzymatic saccharification of microwave pretreated *P. hysterophorus* was also used to determine the efficacy of crude cellulase.

2. Materials and Methods

2.1. Microorganism.

This study was carried out with a fungal strain, *Penicillium citrinum* NAF5 (accession no. KX858345), obtained from the collection of cultures of the Fermentation laboratory, Department of Microbiology, Kurukshetra University, Kurukshetra, isolated from garden soil, purified, grown, and stored at 25°C on potato dextrose agar plates.

2.2. Substrate.

P. hysterophorus, an annual herbaceous weed, was procured from the university campus and used as a lignocellulosic substrate for the β -glucosidase in the present study.

2.3. Medium.

The modified Mandel's medium was (g/l) KH_2PO_4 , 2.0; urea, 0.3; $(\text{NH}_4)_2\text{SO}_4$, 1.4; and trace elements (mgL^{-1}): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5; $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$, 2.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 16; and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2.0 used for production of β -glucosidase under stationary conditions of growth.

2.4. Inoculum Preparation and β -glucosidase production.

The isolate was cultivated on the PDA slant at 25°C for 7 days until complete sporulation for inoculum development. For the production of β -glucosidase, the flasks were inoculated with 2×10^7 spores/ml and incubated at 25°C for 5 days under stationary conditions. To extract the enzyme, the contents were filtered through muslin cloth to separate the solid mycelial portion from the culture filtrate liquid portion, then centrifuged at 4°C for 20 minutes at 10,000 rpm. After filtering the supernatant via Whatman filter paper no. 1, the clear supernatant was employed as a crude enzyme source in subsequent assays.

2.5. Determination of β -glucosidase activity.

The β -glucosidase activity was assayed by Ng *et al.* [22]. At 50°C for 30 minutes in the dark, we incubated the reaction mixture 1 μl with 200 μl of enzyme solution that had been appropriately diluted with 800 μl of substrate solution (2 mM pNPG or 4-nitrophenyl- β -D-glucopyranoside). To stop the reaction, 1 ml of 1 M Na_2CO_3 solution was added. The color generated due to the liberation of pNP was assessed using pNP as a standard at 405 nm throughout the experiment. For β -glucosidase, the enzyme activity was measured in U/ml.

2.6. Optimization of β -glucosidase production conditions.

By altering the fermentation time from 48 to 288 h, the influence of the incubation period was examined. *P. citrinum* NAF5 was cultured in a 150 ml flask containing a 20 ml optimized medium with several pH ranges from 3.0-8.0 to find the optimal pH. Hydrochloric acid (1 N) or sodium hydroxide (1 N) was used to alter the pH of the medium. The effect of temperature was studied by producing β -glucosidase from *P. citrinum* NAF5 at different temperature conditions, i.e., 20, 25, 30, and 35°C . Different carbon sources 1% (w/v) sugar concentration (glucose, fructose, CMC, lactose, mannitol, maltose, galactose, cellobiose, sucrose, and dextrose) were used to study their effects on enzyme production. Ammonium nitrate, ammonium sulfate, peptone, beef extract, yeast extract, and urea were used to substitute nitrogenous components in the medium to examine the influence of nitrogen supplementation. The effects of different surfactants (Triton X-100, SDS, Tween-20, and Tween-80) on β -glucosidase production were investigated. The 'one variable at a time' strategy was used under static conditions to maximize the synthesis of β -glucosidase by *P. citrinum* NAF5.

2.7. Response surface methodology (RSM).

RSM model central composite design optimized the effect of three independent variables on β -glucosidase production: lactose (A, %), peptone (B, %), and pH (C) [23]. The low level (-1) and high level (+1) of each independent variable are specified in Table 1. The entire design consisted of 17 possibilities, each with three replicates at a central point, and was carried out randomly (Table 2). To fit a 2-order polynomial model to experimental data, multiple regression equations (1) were used.

$$Y = \beta_0 + \sum_{i=0}^n \beta_i X_i + \sum_{i=0}^n \beta_{ii} X_i^2 + \sum_{i \neq j=1}^n \beta_{ij} X_i X_j \quad (1)$$

Where Y is the expected response, β_0 is the intercept; n is the number of components to be considered, β_i , β_{ii} , and β_{ij} are the linear (main effect), quadratic, and interaction model coefficients, respectively. Accordingly, X_i and X_j indicate the levels of the independent parameters. For the RSM model, the statistical software Design Expert 8.0.7.1 was used for analysis.

Table 1. Factor independent variables used in experimental design.

Symbol	Independent variables	Coded Levels	
		-1	+1
A	Lactose (%)	0.75	2.5
B	Peptone (%)	0.25	2.75
C	pH	5	7

2.8. Pretreatment of biomass with sodium carbonate.

P. hysterothorus was obtained from various locations on the University campus. A consistent weight was achieved by drying the chopped biomass in an oven at 60°C. It was then placed in plastic bags for later use. 2g of weed biomass were suspended in 20 ml of 1% sodium carbonate, with a solid to liquid ratio of 1:10 w/v. These mixtures were autoclaved at 121°C for 30 minutes to hydrolyze the biomass under 15psi pressure. Filtration was used to collect the solid residue, thoroughly rinsed with double distilled water until the pH was neutral. The pretreated biomass was dried in the oven at 60°C and utilized as the substrate for saccharification studies to maintain a steady weight.

2.9. Enzymatic hydrolysis.

A crude cellulase enzyme from *P. citrinum* NAF5 (10 U/g) was used to hydrolyze pretreated biomass enriched with 0.1 M sodium citrate buffer (pH 5.0) at 40°C and 100 rpm for 7 hours. The samples were centrifuged at 10,000 rpm for 10 minutes [24,25], and the supernatant was used to estimate reducing sugars [26] using Miller’s dinitrosalicylic acid (DNS) method.

3. Results and Discussion

3.1. OVAT.

Using the ‘one variable at a time’ approach, different culture conditions were optimized for maximal β -glucosidase production.

3.1.1. Effect of the incubation period.

The effect of the incubation period on the generation of β -Glucosidase by *P. citrinum* NAF5 was investigated by incubating for varied periods, ranging from 2 to 12 days at a 2-day interval. However, the other fermentation conditions include pH 6.0, temperature 25°C with no extra carbon and nitrogen source as Mandel’s media provided. Under stationary conditions, maximum β -Glucosidase (0.473±0.01 U/ml) production was achieved on the 6th day of incubation (Figure 1a). An increase in the incubation time beyond the optimum period leads to decreased enzyme activity. With increasing time, the fermentation medium also becomes rich in inhibitory metabolites. These factors collectively affect the fungal physiology and

consequent production of extracellular enzymes [27]. Enzyme activity is reduced when incubated for more than 8 days, owing to pH changes, the buildup of inhibitory metabolites like cellobiose [28], or a decrease in nutrients in the fermentation medium [29]. Gautam *et al.* [27] reported maximal cellulase productivity from *A. niger* and *Trichoderma* sp. on the 4 and 5 days of incubation, respectively, under stationary conditions.

3.1.2. Effect of pH.

The effect of initial pH on β -glucosidase synthesis by *P. citrinum* NAF5 was investigated in this work, which is influenced not only by other environmental factors but also by the kind of substrate used for the production of the enzyme. The fungus exhibited the highest β -glucosidase production when the initial pH of the medium was set as 6.0, under the stationary condition (Figure 1b). Under stationary conditions, the maximum β -glucosidase activity was recorded (0.474 ± 0.07 U/ml). However, the other fermentation conditions include the 6th day incubation period, temperature 25°C with no extra carbon and nitrogen source as Mandel's media provided them. *P. miczynskii* highest productivity of β -glucosidases was shown to be optimal at pH 5.5, according to Beitel and Knob [30]. The pH influences because of changes in cell morphology and also by influencing enzyme transport [31]. The highest β -glucosidase production by *P. roqueforti* ATCC 10,110 occurs at pH 5.5 [32].

3.1.3. Effect of temperature.

The influence of temperature on the production of β -glucosidase by *P. citrinum* NAF5 was investigated by increasing the incubation temperature from 20 to 35°C at 5°C intervals. The fungus produced a maximum titer of β -glucosidase (0.437 ± 0.09 U/ml) at 25°C under stationary conditions (Figure 1c). However, the other fermentation conditions include the 6th day incubation period, pH 6.0, with no extra carbon and nitrogen source as Mandel's media provided them. In a study similar to ours, Nathan *et al.* [33] found that a temperature of 25°C is optimum for producing high titers of cellulases from *T. viride* VKF3. Enzyme production was drastically reduced under stationary conditions at 20°C. This could be due to microorganisms' metabolisms slowing down due to their slower growth at low temperatures. The results showed that fungal cellulase activity peaked at 25°C and declined as the temperature was raised. Membranes and enzymes are both harmed by high temperatures [34]. According to Gautam *et al.* [27], *A. niger* and *Trichoderma* sp. produce the highest levels of cellulases at 40°C and 45°C, respectively. However, a decrease in enzyme activity at high temperatures is mostly caused by the denaturation of the enzyme [29,35].

3.1.4. Effect of carbon source.

When the stimulating impact of different carbon sources on the production of β -glucosidase by *P. citrinum* NAF5, was discovered that the fungus generated the most β -glucosidase when lactose was added to the medium as an additional carbon source under stationary conditions. Several other carbon sources such as sucrose, CMC, and cellobiose have also enhanced enzyme production. The results revealed that the fungus showed the highest β -glucosidase activity (0.929 ± 0.08 U/ml) when the medium was supplemented with 1% lactose under stationary conditions (Figure 1d). The activity increased to 0.929 ± 0.08 U/ml from 0.413 ± 0.01 U/ml in control under stationary conditions. However, the other fermentation conditions include the 6th day incubation period, pH 6.0, temperature 25°C with no extra

nitrogen source as Mandel's media provided. The carbon source significantly impacts extracellular enzyme production [36,37]. While studying the production of cellulases in the model fungus *T. reesei*, research showed that lactose has a stimulatory impact [38]. Prassana *et al.* [39] generated the greatest titers of cellulases from *Penicillium* sp. using 0.5% lactose in a fermentation medium, including cellulose and sawdust as substrate.

3.1.5. Effect of nitrogen source.

Figure 1e shows the results of optimization experiments that looked at the influence of various nitrogen sources on β -glucosidase production by *P. citrinum* NAF5 under static growth conditions. Under stationary conditions of fermentation, peptone was the best nitrogen source for improved β -glucosidase production by the fungus. While determining the optimal peptone concentration for maximizing β -glucosidase production, it was discovered that peptone was most efficient at 1.0% (w/v) in inducing the maximum amounts of β -glucosidase production under static (1.109 \pm 0.03 U/ml) conditions. The activity increased to 1.109 \pm 0.03 U/ml from 0.265 \pm 0.04 U/ml in control under stationary conditions. However, the other fermentation conditions include the 6th day incubation period, pH 6.0, temperature 25°C with an extra carbon source (1% lactose). According to Gautam *et al.* [27], *A. niger* produced more cellulases in a medium containing 1% peptone. *P. waksmanii* F10-2 produced more cellulases in the presence of 2% peptone in a wheat straw-containing medium, according to Han *et al.* [40].

3.1.6. Effects of surfactant.

The different studies have shown different, *i.e.*, stimulatory and inhibitory, effects of various surfactants on β -glucosidase production by microorganisms.

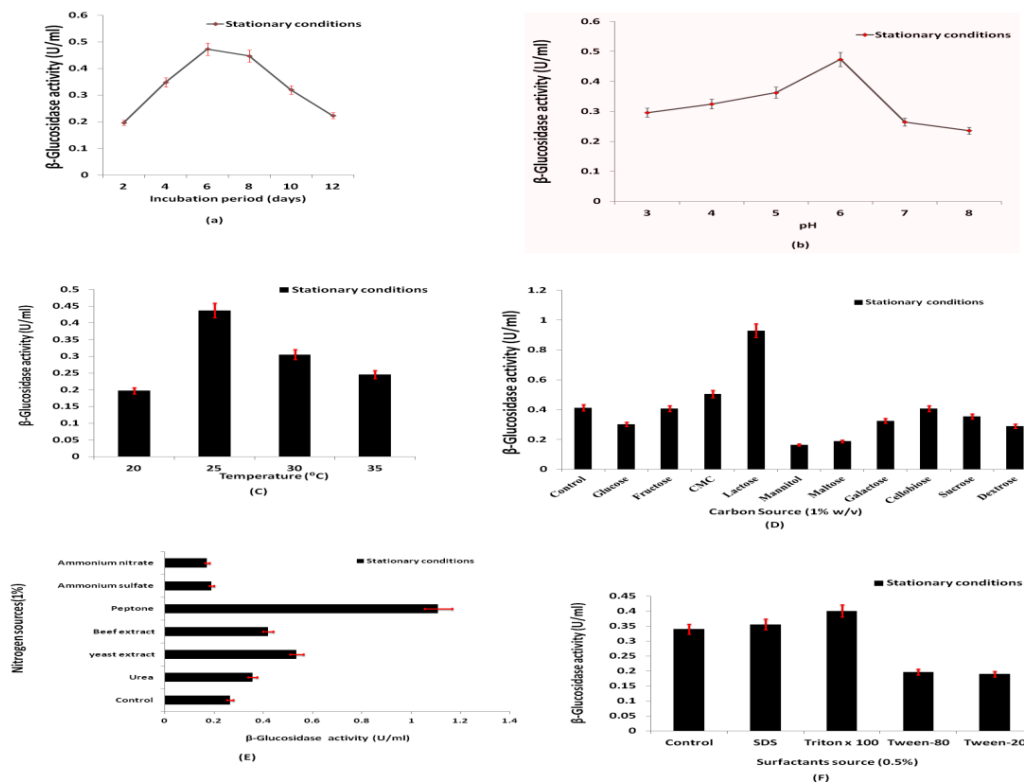


Figure 1. Effect of different cultural conditions on β -Glucosidase production by *P. citrinum* NAF5: (a) Incubation period; (b) pH; (c) Temperature; (d) Carbon source; (e) Nitrogen sources; (f) Surfactants source.

The present work was conducted to know the effect of Tween-80, Tween-20, Triton X-100, and SDS on the enzyme manufacture by *P. citrinum* NAF5, under static conditions. Figure 1f shows the effect of surfactants on *P. citrinum* NAF5 β -glucosidase production after 6 days of incubation. The results revealed that the fungus showed the highest β -glucosidase activity (0.401 ± 0.01 U/ml) when the medium was supplemented with 0.5% Triton X-100 under stationary conditions (Figure 1f). In the presence of SDS, Tween 20, and Tween 80, Prasanna *et al.* [39], found that *Penicillium* sp. produced less cellulase than in control. Triton X-100 seemed to be the optimum surfactant for the synthesis of β -glucosidase, according to Prasanna *et al.* [39]. Figure 1 shows the effect of different cultural conditions on β -glucosidase production.

Under optimized conditions, three ingredients (lactose, peptone, and pH) selected from the OVAT approaches were subjected to RSM for further β -glucosidase-production statically optimization. It appeared as though lactose (1% w/v), peptone (1% w/v), and pH were the best combinations of the independent variables for β -glucosidase production. These three independent variables were further investigated to find their effect on the production of β -glucosidase.

3.2. Statistical analysis of β -glucosidase production by RSM.

RSM is an accurate statistical optimization technique that employs statistical and analytical tools to create a multifactorial model that can be applied to various areas, including biology, agriculture, and food processing, to determine the optimum response. Faster optimization methods evaluate the interactions of several factors with fewer trials [41].

Table 2. Results of central composite design of β -Glucosidase activity (U/ml).

Run no	A (%)	B (%)	C	Activity (U/ml)
1	1.50	0.60	6.00	1.49
2	1.50	1.50	6.00	2.48
3	0.75	2.75	5.00	1.42
4	2.76	1.50	6.00	1.39
5	1.50	1.50	6.00	2.46
6	2.25	0.25	7.00	1.19
7	0.75	0.25	5.00	1.38
8	0.75	2.75	7.00	1.24
9	2.25	2.75	5.00	1.28
10	2.25	2.75	7.00	1.36
11	0.24	1.50	6.00	1.56
12	1.50	1.50	7.68	1.47
13	1.50	3.60	6.00	1.24
14	0.75	0.25	7.00	1.40
15	2.25	0.25	5.00	1.35
16	1.50	1.50	4.32	1.69
17	1.50	1.50	6.00	2.42

A, Lactose (%); B, Peptone (%); C, pH; BGL, β -Glucosidase activity (U/ml).

Table 3. ANOVA for the quadratic model of central composite design.

Source	Sum of squares	df	Mean square	F value	p-value prob> F
Model	0.40	9	0.045	22.97	0.0002 Significant
A-Lactose	3.893E-003	1	3.893E-003	2.01	0.1996
B-Peptone	2.590E-003	1	2.590E-003	1.33	0.2859
C-pH	4.690E-003	1	4.690E-003	2.42	0.1640
AB	1.190E-003	1	1.190E-003	0.61	0.4593
AC	1.394E-004	1	1.394E-004	0.072	0.7964
BC	4.645E-005	1	4.645E-005	0.024	0.8814
A2	0.20	1	0.20	103.19	< 0.0001
B2	0.25	1	0.25	130.45	< 0.0001
C2	0.16	1	0.16	81.37	< 0.0001

Source	Sum of squares	df	Mean square	F value	p-value prob> F
Residual	0.014	7	1.941E-003		
Lack of Fit	0.013	5	2.679E-003	28.11	0.0347
Pure Error	1.906E-004	2	9.530E-005		
Cor Total	0.41	16			

Based on the results of the OVAT approach, lactose (A), peptone (B), and pH (C) were chosen to be optimized by response surface methodology (RSM) to achieve the maximum β -glucosidase production. The current research optimized RSM for cultural conditions to increase β -glucosidase production. Under selected experimental conditions produced β -Glucosidase ranges from 1.19 U/ml to 2.48 U/ml is shown in Table 2. ANOVA was used to examine the results obtained after cultural conditions optimization shown in Table 3.

The final response equation for highest β -glucosidase production after excluding the non-significant terms:

$$Y_1 = 3.18 + 0.63A + 0.24B + 1.39C - 0.013AB - 5.56AC + 1.92BC - 0.2369A^2 - 0.0959B^2 - 0.1183C^2 \quad (2)$$

Figures 2 and 3 show 3-D interaction and contour plot graphs for the production of β -glucosidase. According to Figure 4, a comparison of the actual and predicted values can be seen [23].

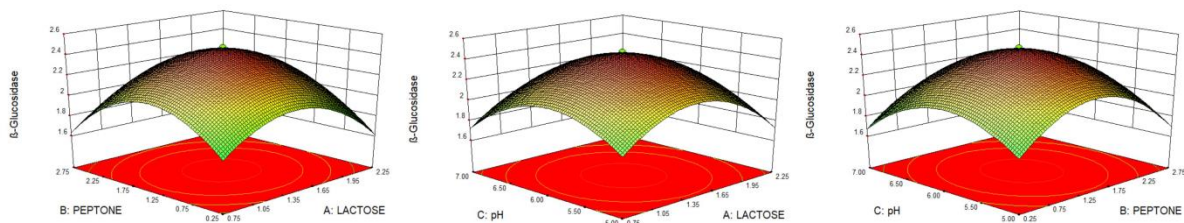


Figure 2. 3-D plot graphs showing the effect and interaction of: (a) lactose vs. peptone; (b) lactose vs. pH; (c) peptone vs. pH concentration on β -glucosidase production

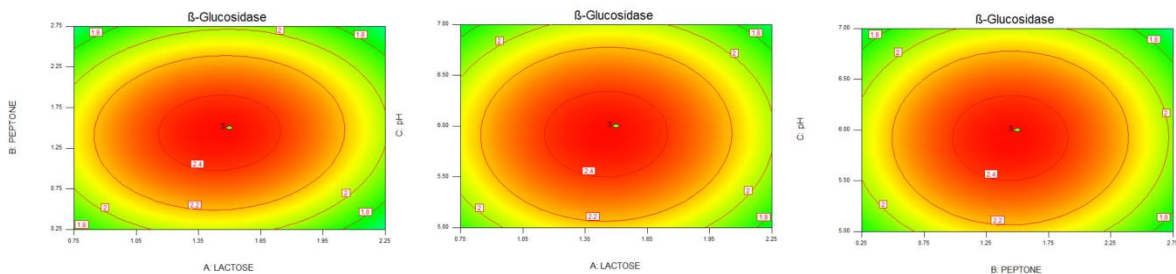


Figure 3. Contour plot graphs showing the effect and interaction of: (a) lactose vs. peptone; (b) lactose vs. pH; (c) peptone vs. pH concentration on β -glucosidase production.

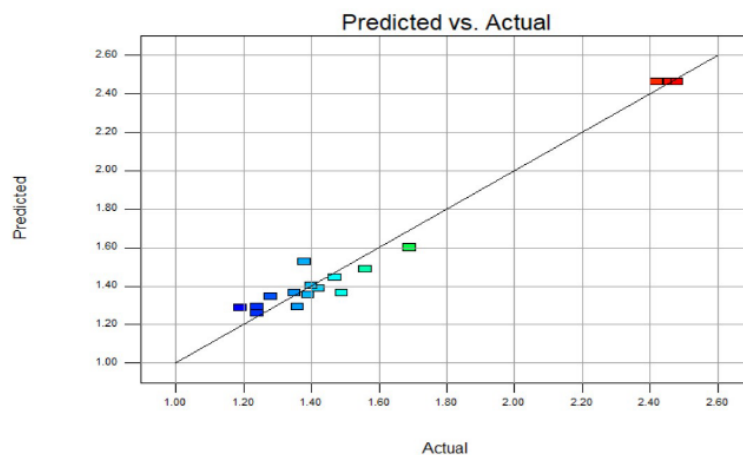


Figure 4. The least squares fit graph for actual and predicted.

The significance of the model is indicated by the model F-value of 22.97. Model terms are significant when “Prob > F” is less than 0.0500. A², B², and C² are suitable model variables in this case. The “Pred R-Squared” of 0.7367 is in fair agreement with the “Adj R-Squared” of 0.9252. As a result, the optimal concentrations for lactose, peptone, and pH are 1.50% (w/v), 1.50% (w/v), and 6, respectively. The maximum β -glucosidase production under these culture conditions is 2.48 U/ml. The model developed 2.2-fold improved β -glucosidase production. *P. miczynskii* produced 1.99 U/ml of β -glucosidase when grown in pineapple peel [30], whereas *P. funiculosum* produced 1.83 U/ml when grown in sugarcane bagasse [42]. According to Marina *et al.* [43], the response surface approach revealed the highest β -glucosidase (6.5 U/ml) activity in the presence of lower quantities of wheat bran 1% (w/w), an inoculum concentration of 10⁵ spores/ml, and temperatures of 33°C. *Penicillium* sp. FSDE15 produced the highest 8.72 + 0.42 U/g β -glucosidase when grown in wheat bran [44]. *Talaromyces amestolkiae* was found to produce around 11 U/ml β -glucosidase [45].

3.3. Enzymatic hydrolysis.

The liberation of reducing sugars (92.48 mg/g substrate) from sodium carbonate (1% w/v) pretreated *P. hysterothorus* biomass was 3.3-fold higher as compared to untreated *P. hysterothorus* biomass (28.02 mg/g substrate). β -glucosidase derived from *P. citrinum* NAF5, therefore, played a vital role in the liberation of reducing sugar from *P. hysterothorus* biomass. The alkaline and deacetylating characteristics of sodium carbonate break down the lignocellulosic biomass’s recalcitrant structural design, resulting in delignification and enhanced the sugar yields from enzymatic hydrolysis biomass [46,47]. According to Yang *et al.* [48], sodium carbonate is a potential pretreatment for increasing sugar output from agricultural residues like rice straw. All fermentable sugars are recovered in a single enzymatic hydrolysis stage during the sodium carbonate pretreatment procedure, producing no fermentation inhibitors. According to Yongcan Jin *et al.* [49], most sugars are retained in the solid fractions after sodium carbonate treatment. Salehi *et al.* [50] and Shen *et al.* [51] reported increased saccharification of sodium carbonate and steam pretreated lignocellulosic biomass. Wenhai *et al.* [52] reported glucose concentration reached 110.47 g/L after 48 hours of hydrolysis. Enzymatic saccharification of *P. hysterothorus* biomass prepared with choline chloride and sorbitol (CCS, molar ratio 1:5) yielded the highest sugar (148.54 mg/g biomass) [53].

4. Conclusions

β -glucosidase production from *P. citrinum* NAF5 was enhanced 2.2-fold by optimizing the cultural conditions using the RSM approach. Furthermore, RSM was a better experimental technique than OVAT since it decreases the number of experiments and assumes random mistakes. In contrast, OVAT is regarded as a time-consuming and difficult technique. The crude enzyme produced from *P. citrinum* NAF5 was able to saccharify the lignocellulosic biomass. The capability of our crude enzyme for lignocellulosic biomass conversion systems is highlighted in this investigation.

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Conflicts of Interest

The authors declare no conflict of interest.

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