JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ENHANCING CELLULASE PRODUCTION VIA RESPONSE SURFACE METHODOLOGY FROM *CLADOSPORIUM* SP.: FRUIT WASTE ADDITION IMPACT

Rama Mohan Poludasu [1, 2 *]

Address(es):
[* 1] SV University, Department of Biochemitry, Tirupati, India-517502.
[2] Biomass conversion technology group, Dalian institute of chemical physics, CAS 457, Zhongshan road, Dalian, China- 116023.

*Corresponding author: bioraam@gmail.com

https://doi.org/10.55251/jmbfs.11117

ARTICLE INFO

Received 10. 3. 2024
Revised 8. 4. 2024
Accepted 17. 4. 2024
Published 1. 6. 2024

Regular article

OPEN ACCESS

ABSTRACT

Accumulating organic waste stands as a significant challenge in developing nations. Researchers have extensively studied and utilized sugarcane bagasse fiber residues to produce various compounds. *Cladosporium* sp. NCIM 901 employs peracetic acid (PAA) treated bagasse in solid substrate fermentation (SSF) to generate cellulase enzyme. We utilized a Box-Behnken Design (BBD) to investigate how the fermentation medium components - watermelon juice, lemon peel powder, KH_2PO_4, $(NH_4)_2SO_4$, and $MgSO_4$ - affect the synthesis of Filter Paperase (FPase), Carboxymethyl cellulase (CMCase), and β-glucosidase enzymes. Under the enhanced conditions, the trial exhibited β-glucosidase, CMCase, and FPase activities of 18.6, 19.4, and 17.6 U/gds, respectively. The model calculated R^2 values ranging between 91.2–91.4%, suggesting their suitability and potentially usable for estimating the level of culture medium variables needed to achieve optimal enzyme generation by the fungal strain *Cladosporium* sp. Observations confirm that supplementing lemon peel powder and watermelon juice with $MgSO_4$ enhances enzyme production. The findings of model validation revealed high consistency between the observed experimental and anticipated outcomes. In the simultaneous saccharification and fermentation (SsF) process, the processed bagasse yielded more ethanol (26 g/L) compared to the untreated substrate. Based on the result, it can be conclude that, fruit processing wastes are useful for increasing cellulase enzyme production by fungi under solid state fermentation.

Keywords: Watermelon juice, Lemon peel powder, *Cladosporium* sp., Box-Behnken design, Cellulase production, Solid state fermentation

INTRODUCTION

The quest for renewable energy, functioning as a feasible alternative to non-renewable energy sources, has arisen as an urgent requirement in modern society (**Passoth and Sandgren, 2019**). Second-generation biofuels derive from forestry and agricultural residues of lignocellulosic biomass, which are abundant resources. Crucially, these sources of energy do not interfere with food requirements and do not cause any negative impacts on the markets for food and animal feed (**Rastogi et al., 2017**). Unlike traditional energy sources, lignocellulosic residues provide not just carbon for producing energy, but also a broad variety of sustainable products (**Straathof, 2014**). Significantly, India is a leading global producer of sugarcane (*Saccharum* spp.), with an approximate harvest of 419.25 million tonnes during the 2021-22 fiscal year (**Konde et al., 2021**).

According to **Ellila et al. (2017)**, the carbon source might result to over 50% of the overall enzyme production costs, even when employing pure glucose. As a result, a range of techniques have been implemented to decrease the expenses related to producing enzymes. For example, scientists have employed solid state fermentation (SSF) and used lignocellulosic biomass as both carbon sources and enzyme inducers. Researchers (**Irfan et al., 2017; Jampala et al., 2017**) have examined various lignocellulosic residues, such as fruit pomace, waste paper, corn cob, wheat and soy bran, sugarcane bagasse, wheat and rice straw for their potential in producing cellulases. In its composition, generally, bagasse typically contains approximately cellulose (47-52%), hemicellulose (25-28%), and lignin (20-21%) (**Dotaniya et al., 2016**). Given its widespread availability, it holds the capability to be utilized as a material for the microbial synthesis of value-enhanced items, for instance pharmaceutically important substances, organic acids, aminoacids, enzymes and protein-rich animal feed (**Parameswaran, 2009**). Additionally, it serves as a carbon source supporting the filamentous fungi growth (**Ferreira et al., 2018**).

With a substantial influence on industrial progress, filamentous fungi stand as versatile organisms. They find application in producing industrially significant enzymes like pectinase, cellulase, laccase, xylanase, and amylase (**Ferreira et al., 2018**). Cellulases comprise an ensemble of enzymes collaborating to thoroughly hydrolyze intricate carbohydrates. This enzymatic ensemble consists of three complex types: endoglucanases or CMCases, cellobiohydrolases, and β-glucosidases. The combined effect of their actions helps convert cellulose into glucose (**Prasanna et al., 2016; Satyamurthy et al., 2016**). Recently, there's been increasing attention on *Cladosporium* for its role in producing vital industrial enzymes. Prior research has demonstrated the fungus's utility in pectinase production (**Moharram et al., 2022**) and endoglucanase, exoglucanase, and xylanase (**Moharram et al., 2021**) through solid-state fermentation (SSF).

Furthermore influencing costs, the substrate functions as a catalyst inducer. Moreover, its origin can result in the formation of enzymatic blends with varying catalytic capabilities for cellulose degradation (**Li et al., 2017; Pandey et al., 2016**). Opting for lignocellulosic materials over commercial alternatives can help attain specific enzyme pools, which has the potential to increase cellulose hydrolysis yields (**Cunha et al., 2012**). Inducers are crucial for generating cellulase. Sophorose and lactose are the main soluble agents frequently employed for induction. Nonetheless, utilising these methods of induction leads to elevated expenses (**Huang et al., 2016**). **Ferreira et al. (2018)** emphasized that the synthesis of fungal cellulase relies significantly on media composition and culture conditions. Hence, developing the right fermentation strategy is vital to maximize the potential of involved microorganisms in the process. In their study, **Ozcelik and Yaruz-Duzgum (2016)** pointed out that fruit and vegetable waste serve as substantial reservoirs of carbohydrates, proteins, vitamins, and minerals. Researchers **(Neelima et al., 2021)** demonstrate that these resources find application in generating value-added items like phytochemicals, biofuels and enzymes. By employing low-cost or surplus substrates such as waste from fruit processing, it becomes plausible to lower enzyme production expenses. This process not only confronts fruit industry waste challenges but also contributes to pollution reduction. **Ozcelik and Yaruz-Duzgum (2016)** reported watermelon juice's content: protein at 0.44 g/L, dietary fiber at 0.07 g/L and reducing sugars at 4.5 g/L. It also contains diverse minerals (Ca, Mg, P, Na, and K) and vitamins (Niacin, riboflavin, vitamins A, B, and C). Meanwhile, Lemon peel powder, as per **Raajeswari and Nischala (2017)**, comprises 163.3 mg Calcium, 1.66 g protein, 18.67 g carbohydrates, and 10 mg fiber. Moreover, lemon peel's dry weight composition, as studied by **Pham et al. (2020)**, exhibits ash at 1.7%, protein at 3.59%, total fiber at 4.78%, and carbohydrate at 30.74%.

Experimental design holds significance across various scientific and industrial domains, facilitating thorough process observation and system operation understanding. Effective planning and design of experiments are imperative for deriving conclusive outcomes. Among the widely utilized optimization methodologies, the response surface methodology (RSM) stands out, aiding in the systematic enhancement of results. Because of its capability to assess the impacts

of many variables and their associations on various response variables, the RSM proves valuable **(Aydar, 2018)**. Typically, for statistical optimization, two main design types are employed: the box-behnken design (BBD) and central composite design (CCD). BBD designs typically employ fewer points than CCDs, reducing costs for the same factors. They effectively estimate first and second-order coefficients, utilising 3 levels per factor—distinct from CCDs which can have up to 5 levels. Unlike CCDs, BBDs omit extreme factor combinations, such as all low settings **(Minitab, 2021)**. Researchers have applied BBD experimental designs to generate cellulase enzymes through SSF **(Verma and Kumar, 2019; Gares et al., 2023)**.

Enhancing pretreatment effectiveness involves hemicellulose removal or delignification. This process is essential for breaking the interaction between lignin and hemicellulose and disrupting cellulose's crystalline structure. Methods include and hydrothermal treatment, steam explosion, alkali, ionic liquids, and organic acids **(Kumar et al., 2009)**. Peracetic acid, produced by combining acetic acid and hydrogen peroxide with a sulfuric acid catalyst, is a potent oxidant. It serves as an efficient pretreatment for biomass delignification, minimizing lignin content and increasing the accessibility of cellulose. This selective delignification enhances the cell wall's surface region, pore diameter, and porosity, thereby improving cellulose conversion in saccharification and hydrolysis (**Gill et al., 2021; Wi et al., 2015; Zhao et al., 2007)**.

The simultaneous saccharification and fermentation (SsF) method enables enzyme-assisted hydrolysis and fermentation, while this method combines biomass pretreatment saccharification with simultaneous fermentation of the liberating reducing sugars. SsF enhances substrate utilization, boosts product yield, and reduces cellulase inhibition and deactivation. This occurs by preventing high concentrations of released sugars in a single reactor **(Wang and Lu, 2021)**. Considerable research has focused on optimizing selective media for microbial cellulase synthesis via lignocellulose, utilising diverse fungal strains. Nonetheless, the use of waste from vegetable or fruit industry for cellulase synthesis by *Cladosporium* sp. NCIM 901 via SSF in a nutrient-rich medium is scarcely studied. This research investigates enzyme synthesis through SSF utilising pretreated bagasse, supplemented with lemon peel powder and watermelon juice. The study also delves into ethanol production utilising crude cellulase through SsF.

MATERIALS AND METHODS

Microorganisms

We used *Cladosporium* sp. NCIM 901 from India's National Collection of Industrial Microorganisms for enzyme synthesis through SSF. The microorganism was cultivated, maintained, and stored on potato dextrose agar (PDA) medium slants at 4 ºC. For ethanol fermentation, the flocculating and ethanol-tolerant *Saccharomyces bayanus* strain given by Roberto Ambrosoli of the Univerita degli Studi di Torino, Italy was used. The culture was kept alive in a medium called MGYP that included (g/L): 3 g of malt extract, 5 g of bacteriological peptone, 5 g of yeast extract, and 10 g of glucose.

Substrate preparation

S.V. Sugar Factory in Tirupati, India, supplied the sugarcane bagasse substrate, which underwent thorough rinsing before being dried using a laboratory hot air oven at 65–70 ºC for 8 h. The bagasse was then powdered, dried, and sieved through a 1 mm mesh before being stored in polythene bags for subsequent experiments. The finely ground bagasse powder underwent pretreatment with 5% peracetic acid (PAA) for 90 min at 90 ºC, as conducted by **Mohan et al. (2013)**. The resulting transparent liquid and solid forms were separated. Later SSF experiments were done using the solid biomass fraction.

Preparation of watermelon juice

We purchased overripe watermelon fruits affected by soft rot from the local farmers market in Tirupati. Subsequently, we macerated the fruits using a juice mixer grinder. The extracted liquid juice underwent filtration by whatman cellulose filter paper No.1 (1-11µm), and we used the resulting clear and clarified juice for the experiments.

Lemon peel powder preparation

The lemon peels were collected, allowed to dry in laboratory oven at 65 to 75 ºC for 8 h, milled, and filtered through a 0.5 mm mesh. The resulting oven-dried powdered lemon peel was stored in polythene bags for subsequent experiments.

Fungal inoculum preparation

Cladosporium sp. (NCIM 901) spores were attained by cultivating the culture for 4-5 days at ambient temperature on PDA (Himedia, Mumbai, India) slants. The culture was then suspended in sterile, clean water with Tween 80 (0.1%, w/v). To remove mycelia, the *Cladosporium* sp. spore suspension was passed through glass wool. The resulting spore count was verified through direct microscopic counting using a hemocytometer. The inoculum, containing 1×10^8 spores/mL, was permitted to grow for 24 h in a preculture medium consisting of (w/v) 0.1% $MgSO_4.7H_2O$, 0.4% yeast extract, 0.2% K_2HPO_4, and 1.5% glucose, with a pH of 7.0, to acquire a mycelial suspension.

Enzyme production via solid substrate fermentation (SSF)

SSF was conducted in 250 mL graduated conical flasks with 5 g of prepared bagasse (carbon source) and Mary Mandel's mineral medium (MM) (15 mL) with the subsequent composition (g/L): $CaCl_2$ 1 g, Peptone 5 g, NaCl 5 g, NH_4NO_3 5.0 g, Urea 2 g, Tween-80 0.5 mL, and trace element mixture: $ZnSO_4.7H_2O$ 0.001 g, $FeSO_4.7H_2O$ 0.005 g, $MnSO_4.7H_2O$ 0.001 g, and $CoCl_2$ 0.002 g **(Jeffries, 1996)**. The pH was adjusted to 5.5 before autoclave sterilization. After being sterilized for 15 min at 121ºC under 15 lbs pressure in an autoclave, the trial flasks were cooled to room temperature. Following this, a 2 mL mycelial solution from a 24 h old culture cultivated on glucose pre-culture medium was introduced into the experimental media. The components of the flask were homogeneously stirred and incubated under prescribed conditions of humidity and temperature. In each flask, the total available moisture content was adjusted and maintained at 75-80% by incubating the experimental flask in a water-saturated atmosphere in an incubator at 37 ºC for 5 days. Subsequently, the flasks were held at 20 °C for an h while being gently shaken (at a speed of around 120 rpm), and 50 mL of freshly made trisodium-citrate buffer (pH 6.0, 50mM) was mixed for obtaining the enzyme. The resulting solid and liquid combination was separated by being strained using a fresh muslin cloth with a pore size of 2 mm. Afterwards, a 10-min centrifugation at 10,000 g was done, and the resultant clear supernatant was utilized as the crude extracted enzyme (10 mL).The creation of the crude enzyme cellulase was conducted under aseptic conditions.

Development of yeast inoculum

An inoculum of flocculating and ethanol-tolerant *Saccharomyces bayanus* was created by cultivating the yeast developed on MGYP media, along with basal medium (BM) (100 mL) containing 36 g/L of glucose, in a 250 mL flask. The combination was placed in incubation at 33 °C on a laboratory incubator shaker for 12 h. Subsequently, 10% (v/v) of the yeast, with a density of 1.5×10^8 cells per mL, was utilized as an inoculum for the fermentation medium.

Simultaneous saccharification and fermentation (SsF)

The SsF method included the incorporation of a crude cellulase preparation from SSF, 5% (w/v) PAA-pretreated bagasse substrate, 1% yeast inoculum, and MM mineral medium into the reaction solution. The SsF process pH was adjusted to 5.5 utilising a citrate buffer (0.05 M). The SsF procedure was conducted in 250 mL flasks with a functional volume of 100 mL for duration of 72 h on an orbital incubator shaker operating at 120 rpm. Aseptic conditions (maintained through laminar air flow) were upheld, and samples were collected regularly to analyze their ethanol content.

Assay of components of bagasse, fruit biomass and cellulases

The cellulose, hemicelluloses, and lignin percentage of both untreated and pretreated bagasse substrates were examined utilising the **Goering and Van Soest (1971)** procedure. To evaluate various components, the **AOAC (1990)** techniques were employed, including measurements of total moisture, dry matter, ash percentage, crude protein, fat, and fiber. For carbohydrate content determination, the method described by **Dubois et al. (1956)** was followed. Enzymatic actions such as Filter Paperase (FPase) and Carboxymethyl cellulase (CMCase) were determined using the **Ghose (1987)** procedure. Exactly, FPase and CMCase activities were evaluated using 50 mg of Whatman No.1 filter paper and a 2% (w/v) carboxymethyl cellulose solution in sodium-acetate buffer (pH 4.8, 50mM), respectively. Following a 60 min incubation at 50 ºC (30 min for CMCase samples), the liberated reducing sugar in the reaction solution was quantified at 540 nm with the 3, 5-dinitrosalicylic acid (DNS) technique **(Miller 1959)** and a UV-spectrophotometer. For β-glucosidase activity assessment, a reaction mixture containing 0.9 mL of 1 mM p-nitrophenyl-β-D-glucopyranoside (pNPG) and 0.1 mL of well-mixed enzyme was incubated at 50 ºC for 30 min. The ensuing p-nitrophenol was quantified at 410 nm by introducing 2 mL of 2 M sodium carbonate (Na2CO3) to enhance color intensity **(Gokhale et al. 1988)**.The standard enzyme activity unit is often represented as the enzyme quantity needed to liberate 1 mole of glucose per min at 50 ºC during hydrolysis. Enzyme activity was assessed in units per gram of dry substrate (gds).

Ethanol quantification by gas chromatography (GC)

Ethanol content was assessed using the GC method established by **Anthony (1984)**. The analysis employed an Agilent system model 6890 GC with a 7673 B/C injector. The process utilized a glass-packed GC column with a 5% Carbowax 20M and matrix 80/120 Carbopack B-AW support. The column dimensions were 2m x 1/4in x 2mm in configuration A, adhering to Agilent standards. The analysis

involved various gases, including a constant airflow of 400 mL/min of air, nitrogen as a carrier gas at 20 mL/min, and hydrogen as the fuel gas at 40 mL/min. Before injecting the sample, the injector port was set at 150 °C, and the GC column oven and detector were maintained at 120 ºC and 200 ºC, respectively. Detection employed a flame ionization detector (FID) employing n-propanol as an internal reference to improve measurement precision.

Box-Behnken design (BBD)

The use of the RSM with the BBD was used to ascertain the optimal mixtures of watermelon juice, lemon peel powder, KH_2PO_4, $(NH_4)_2SO_4$, and $MgSO_4$, denoted as A, B, C, D, and E, respectively. This investigation aimed to improve the synthesis of FPase, CMCase, and β-glucosidase (measured in U/gds). RSM facilitates a numerical depiction of independent procedural factors.

$$Y = bo + \Sigma biXi + \Sigma biiXi^2 + \Sigma bijXiXj \quad (1)$$

In the equation mentioned above, Y symbolizes the predicted response derived from experimentation. The term b0 signifies the response function, whereas Xi and Xj stand for experimentally encoded variables as detailed in Table 3. By utilising the subsequent relationship equation, these process-encoded variables are linked to their uncoded counterparts

$$Xi = \frac{2(ai - bi)}{di} \quad (2)$$

The relationship described in Eq. (2) involves variables where "bi" represents the average of maximum and minimum values of certain factors, "ai" stands for the estimated value of the dependent variable for the ith observation, and "di" indicates the variance among the maximum and minimum values of the "ai" variable. The specific variables corresponding to this relationship are outlined in Table 3. In this research, a regression design encompassing 5 linear factors (A to E), 5 quadratic factors (A^2 to E^2), 10 interaction factors (AB to DE), and a constant block term "b_0" was employed. To analyze the comprehensive second-order polynomial mathematical and statistical connection between the response variable "Y" and the five variables under investigation—namely, watermelon juice, lemon peel powder, KH_2PO_4, $(NH4)_2SO_4$, and $MgSO_4$—the quadratic equation (3) can be effectively utilized.

$$Y = bo + b_1A + b_2B + b_3C + b_4D + b_5E + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 + b_{55}E^2 + b_{12}AB + b_{13}AC + b_{14}AD + b_{15}AE + b_{23}BC + b_{24}BD + b_{25}BE + b_{34}CD + b_{35}CE + b_{45}DE \quad (3)$$

The experimental design involved testing different levels of watermelon juice (2-10 mL/L), lemon peel powder (2-8 g/L), KH_2PO_4 (2-8 g/L), $(NH_4)_2SO_4$ (3-6 g/L), and $MgSO_4$ (5-15 g/L) to measure enzymatic activities. A total of 46 experiments were conducted fallowing the design outlined in Table 4. The trial setup and statistical evaluation were performed utilising the **Design-Expert (v11.0.4, 2018)** from Stat-Ease USA, employing the response surface method for analysis.

RESULTS

The major process variables were analyzed and adjusted to enhance cellulase enzyme production in SSF using *Cladosporium* sp. (NCIM 901) on pretreated sugarcane bagasse. To facilitate enzyme action on the plant's cell wall lignin, the raw bagasse substrate underwent treatment with PAA at room temperature. PAA, a potent oxidizing chemical, exhibited selective action on lignin without significant carbohydrate removal or furfural formation during lignin oxidation **(Hu et al. 2022)**.

Comparing untreated bagasse, the PAA pretreatment led to improved proportions of cellulose and hemicelluloses at 58.2±0.6% and 32.4±0.2%, respectively, while lignin content reduced to 10.6±0.6% (Table 1). Proximate testing of fruit waste revealed specific characteristics (Table 2). Dried lemon peel exhibited crude fat (4.2±0.03%), crude fiber (14.20±0.02%), and crude protein (8.90±0.06%). In contrast, watermelon displayed lower values of crude fat (0.19±0.04%) and crude fiber (0.16±0.06%), along with crude protein (0.36±0.04%). Lemon peel contained more cellulose than watermelon, resulting in a higher overall carbohydrate content in lemon peel.

Table 1 Composition of the bagasse (on dry weight basis)

Substrate	Cellulose %	Hemicellulose %	Lignin %
Raw bagasse	42.7±0.8	24.6±0.4	18.4±0.4
Pretreated bagasse	58.2±0.6	32.4±0.2	10.6±0.6

*****Values are mean of two replicates.

Table 2 Proximate analysis and composition of fruit waste (Based on dry weight basis %)

Substrate	Moisture	Ash	Crude protein	Crude fiber	Crude fat	Total carbohydrates
Watermelon	86.2±0.04	0.42±0.02	0.36±0.04	0.16±0.06	0.19±0.04	6.5±0.02
Lemon peel powder	68.6±0.04	6.04±0.08	8.90±0.06	14.20±0.02	4.2±0.03	7.8±0.01

*****Data represents the mean ± SEM, n=3.

Table 3 Box-Behnken design (BBD) of actual and coded levels of variables for the optimization of medium consequents

Factor	Name	Units	Low level	Middle level	High level	Low coded level	Middle coded level	High coded level
A	Watermelon juice	mL/L	2	6	10	-1	0	1
B	Lemon peel powder	g/L	2	5	8	-1	0	1
C	KH_2PO_4	g/L	2	5	8	-1	0	1
D	$(NH4)_2SO_4$	g/L	3	4.5	6	-1	0	1
E	$MgSO_4$	g/L	5	10	15	-1	0	1

*A, B, C, D and E represents the process parameters denoted as X_1, X_2, X_3,X_4 and X_5 respectively, for regression equation.

Each SSF trial was conducted for period of 5 days, following the specified experimental design. To enhance cellulase enzyme production by the fungus during SSF, a mixture of food additives was introduced into the medium. Through a systematic alteration of five additives or variables, guided by statistical experimental design, optimization was pursued. Table 3 presents the coded and actual values of nutritional variables within the BBD, alongside the corresponding response variables. This examination of interplays among these factors aided in determining the optimal values.

Optimization of medium conditions for FPase production

Table 4 outlines the trial designs and outcomes utilising the BBD. Following a multiple regression test on the existing model, a quadratic equation (Eq. 4) was formulated to describe FPase production based on the experiment's examined components. The linear coefficient for $MgSO_4$ concentration stood notably high at 3.68, highlighting its significant and positive influence on FPase activity. Sequentially, the $(NH_4)_2SO_4$ level displayed an ascending trend alongside $MgSO_4$, as indicated by the linear correlation coefficient, resulting in increased enzyme activity. While a few other variables also exhibited a significant positive impact, their effect on FPase production remained marginal. Conversely, the comprehensive process variables exhibited an inhibitory effect on released enzyme activity, evident from the negative quadratic equation coefficient for these variables. The peak activity emerged beyond a certain point in this process variable continuum. The response surface was shaped by a combination of two independent variables long with a third variable set at a constant level.

$$Y_1 = 11.8 + 0.84A + 0.91B + 0.51C + 1.20D + 3.68E - 2.30A^2 - 2.55B^2 - 2.48C^2 - 1.29D^2 - 0.71E^2 + 0.25AB + 0.71AC + 0.74AD + 0.83AE + 0.063BC + 0.39BD + 1.19BE + 0.65CD + 1.52CE + 1.86DE \quad (4)$$

A, B, C, D, and E encode the process variables, and Y_1 is the independent (predicted) response (FPase activity, U/gds) in the equation above.

The design underwent statistical testing using Design-Expert software. Table 5 presents the F-test results for the ANOVA of FPase activity. The ANOVA indicates the model's significance, with an F-value of 13.04 and P>F value < 0.05. The fitted model explains 91.25% variance (R^2 = 0.9125), capturing 8.75% of overall variance. This suggests accurate process representation. "Pred R-squared" (0.6501) and "adj R-squared" (0.8425) align well. Fitted and observed values correlate (R^2=1.0). The CV value of 10% indicates experiment accuracy, a lower CV enhances reliability. The model's Prob > F < 0.05 signifies significance. The term for an insignificant fit issue is "lack of fit" (p > 0.05) - non-significant. The quadratic design suffices **(Behera et al. 2018)**. Table 5 provides P values for variables, interactions, quadratic terms. Low P values denote high-significance terms. Significant design terms have "Prob>F-value" < 0.05 for A, B, C, D, E, AE, BD, A^2, B^2, C^2, D, E. Terms with value > 0.1 are irrelevant **(Design-Expert 2018)**.

Table 4 Experimental design with coded values of variables and experimental and predected responses of the Box-Behnken design (BBD) model

Std	A	B	C	D	E	FPase (U/gds)	CMCase (U/gds)	β-glucosidase (U/gds)
1	2	2	5	4.5	10	7.02 (7.47)	9.82 (10.06)	4.36 (5.46)
2	10	2	5	4.5	10	8.63 (10.62)	10.5 (11.67)	5.84 (6.63)
3	2	8	5	4.5	10	8.52 (8.74)	9.02 (9.26)	4.84 (6.78)
4	10	8	5	4.5	10	9.85 (11.61)	12.8 (13.97)	7.32 (8.95)
5	6	5	2	3	10	8.06 (8.98)	10.2 (10.54)	6.13 (6.96)
6	6	5	8	3	10	7.89 (9.27)	9.12 (10.84)	5.86(6.68)
7	6	5	2	6	10	9.24 (9.74)	11.2 (10.65)	6.64 (8.08)
8	6	5	8	6	10	10.8 (11.77)	12.6 (13.43)	8.95 (10.38)
9	6	2	5	4.5	5	7.96 (6.96)	8.43 (7.4)	5.25 (5.15)
10	6	8	5	4.5	5	8.82 (7.87)	10.6 (10.28)	5.84 (4.59)
11	6	2	5	4.5	15	11.6 (12.41)	15.6 (15.4)	9.45 (10.12)
12	6	8	5	4.5	15	12.9 (13.76)	13.5 (14.0)	14.8 (14.32)
13	2	5	2	4.5	10	8.24 (7.4)	9.82 (9.1)	8.05 (6.38)
14	10	5	2	4.5	10	9.94 (9.87)	10.2 (10.58)	7.23 (6.65)
15	2	5	8	4.5	10	9.02 (8.02)	9.86 (8.95)	6.64 (5.99)
16	10	5	8	4.5	10	11.8 (11.57)	13.6 (13.79)	8.62 (9.06)
17	6	5	5	3	5	6.82 (7.47)	7.42 (8.98)	4.64 (6.78)
18	6	5	5	6	5	8.02 (9.1)	9.05 (9.99)	5.89 (5.48)
19	6	5	5	3	15	13.6 (13.14)	14.4 (14.5)	9.92 (10.42)
20	6	5	5	6	15	14.8 (14.77)	16.7 (16.19)	18.6 (16.54)
21	6	2	2	4.5	10	8.26 (8.13)	8.74 (9.22)	6.25 (5.41)
22	6	8	2	4.5	10	9.02 (8.85)	10.4 (10.69)	7.86 (7.11)
23	6	2	8	4.5	10	9.24 (8.89)	11.6 (11.48)	6.96 (6.3)
24	6	8	8	4.5	10	10.8 (10.41)	11.8 (11.49)	8.82 (8.25)
25	2	5	5	3	10	8.63 (8.11)	10.2 (9.41)	8.02 (6.91)
26	10	5	5	3	10	11.8 (11.21)	13.6 (13.02)	8.93 (7.11)
27	2	5	5	6	10	10.2 (9.83)	11.8 (11.21)	7.64 (7.84)
28	10	5	5	6	10	13.2 (12.76)	14.3 (13.92)	11.5 (10.99)
29	6	5	2	4.5	5	6.86 (6.76)	8.75 (7.97)	5.69 (5.95)
30	6	5	8	4.5	5	7.46 (7.28)	9.82 (8.44)	4.84 (3.92)
31	6	5	2	4.5	15	11.9 (11.79)	12.2 (12.77)	8.96 (10.26)
32	6	5	8	4.5	15	13.8 (13.6)	15.4 (15.37)	14.2 (14.32)
33	2	5	5	4.5	5	5.98 (7.35)	7.42 (9.05)	4.94 (5.12)
34	10	5	5	4.5	5	8.64 (7.77)	9.02 (8.41)	5.02 (5.13)
35	2	5	5	4.5	15	9.74 (10.43)	10.2 (11.11)	10.8 (10.81)
36	10	5	5	4.5	15	17.6 (16.04)	19.4 (18.07)	14.2 (14.14)
37	6	2	5	3	10	11.5 (10.7)	13.6 (12.68)	7.02 (6.23)
38	6	8	5	3	10	8.92 (8.34)	11.4 (9.97)	7.86 (7.28)
39	6	2	5	6	10	9.82 (8.85)	10.2 (10.58)	8.02 (7.87)
40	6	8	5	6	10	14.2 (13.45)	14.9 (14.77)	10.4 (10.46)
41[a]	6	5	5	4.5	10	13.6 (13.6)	14.2 (14.2)	11.8 (11.8)
42[a]	6	5	5	4.5	10	13.6 (13.6)	14.2 (14.2)	11.8 (11.8)
43[a]	6	5	5	4.5	10	13.6 (13.6)	14.2 (14.2)	11.8 (11.8)
44[a]	6	5	5	4.5	10	13.6 (13.6)	14.2 (14.2)	11.8 (11.8)
45[a]	6	5	5	4.5	10	13.6 (13.6)	14.2 (14.2)	11.8 (11.8)
46[a]	6	5	5	4.5	10	13.6 (13.6)	14.2 (14.2)	11.8 (11.8)

*The values in parenthesis are the predicted values; Std = standerd run order; [a] = Central value

**A: Watermelon juice (mL/L), B: Lemon peel powder (g/L), C: KH_2PO_4 (g/L), D: $(NH_4)_2SO_4$ (g/L), E: $MgSO_4$ (g/L)

Table 5 Analysis of variance (ANOVA) for quadratic model for FPase production

Source	Sum of Squares	DF	Mean Square	F-value	p-value Prob > F
Model	287.18	20	14.36	13.04	< 0.0001
A-Watermelon juice (mL/L)	36.33	1	36.33	32.99	< 0.0001
B-Lemon peel powder (g/L)	5.06	1	5.06	4.6	0.0419
C-KH_2PO_4 (g/L)	5.39	1	5.39	4.9	0.0362
D-$(NH_4)_2SO_4$ (g/L)	10.66	1	10.66	9.68	0.0046
E-$MgSO_4$ (g/L)	128.71	1	128.71	116.89	< 0.0001
AB	0.02	1	0.02	0.018	0.8949
AC	0.29	1	0.29	0.26	0.6114
AD	7.23	1	7.23	6.56	0.9361
AE	6.76	1	6.76	6.14	0.0203
BC	0.16	1	0.16	0.15	0.7063
BD	12.11	1	12.11	11	0.0028
BE	0.048	1	0.048	0.044	0.8356
CD	0.75	1	0.75	0.68	0.4176
CE	0.42	1	0.42	0.38	0.5412
DE	5.68	1	5.68	5.16	1
A^2	32.24	1	32.24	29.28	< 0.0001
B^2	37.32	1	37.32	33.89	< 0.0001
C^2	52.9	1	52.9	48.04	< 0.0001
D^2	12.52	1	12.52	11.37	0.0024
E^2	14.33	1	14.33	13.01	0.0013
Residual	27.53	25	1.1		
Lack of Fit	27.53	20	1.38		
Pure Error	0	5	0		
Cor Total	314.71	45			

Coefficient of variation = 10 %, R^2 =0.9125, Adjusted R^2 = 0.8425, Pred R^2 = 0.6501, Mean = 10.49.

To construct 3D response surface plots, the Z-axis represented enzyme activity. Two variables were compared, while a third stayed at its '0' level. Surface plots (3D) and iso-response contours (solid lines) depicted SSF requirement enhancement for enzyme activity. Figure 1A illustrated interaction effects between supplemented watermelon juice and $MgSO_4$, highlighting their significant association impacting FPase activity. Elevating watermelon juice (mL/L) elevated enzyme production. At $MgSO_4$ and watermelon juice concentrations of 15 and 10 mL/L, enzyme activity was 17.6 (actual) and 16.04 U/gds (predicted).

Figure 1B illustrates the interaction between lemon peel powder and $(NH_4)_2SO_4$ and its impact on FPase activity. The response plot highlights a significant interaction between the studied variables. Evidently, FPase activity was markedly high at $(NH_4)_2SO_4$ and orange peel powder concentrations of 5 and 4.5 g/L, respectively. These findings affirm that optimizing these variable values can substantially enhance enzyme activity.

Figure 1C clarifies the impact of $MgSO_4$ and $(NH_4)_2SO_4$ on enzyme activity. Response surface designs indicate insignificant interaction between the investigated factors. The study demonstrated that enzyme activity reached its optimal levels within an initial $MgSO_4$ concentration range of 7-8 g/L, regardless of the $(NH_4)_2SO_4$ concentration. Changes in $MgSO_4$ concentration didn't affect the $(NH_4)_2SO_4$ concentration range of 3-4.5 g/L for optimal results, implying no interaction. The optimal $(NH_4)_2SO_4$ and $MgSO_4$ concentrations fell within the ranges predicting a maximum activity of 16.04 U/gds. The designed medium, comprising watermelon juice (10 mL/L), lemon peel powder (5 g/L), KH_2PO_4 (5 g/L), $(NH_4)_2SO_4$ (4.5 g/L), and $MgSO_4$ (15 g/L), yielded higher FPase activity.

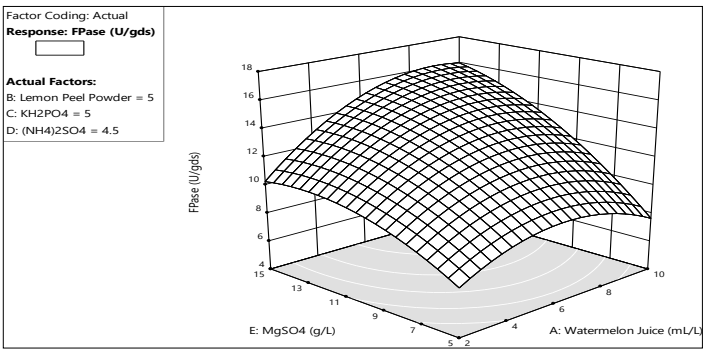

Figure 1A Response surface graph of FPase (U/gds) between the interaction of watermelon juice and $MgSO_4$

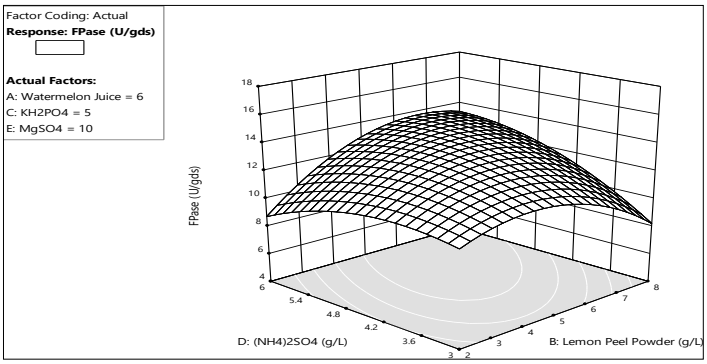

Figure 1B Response surface graph of FPase (U/gds) between Interaction of lemon peel powder and $(NH_4)_2SO_4$

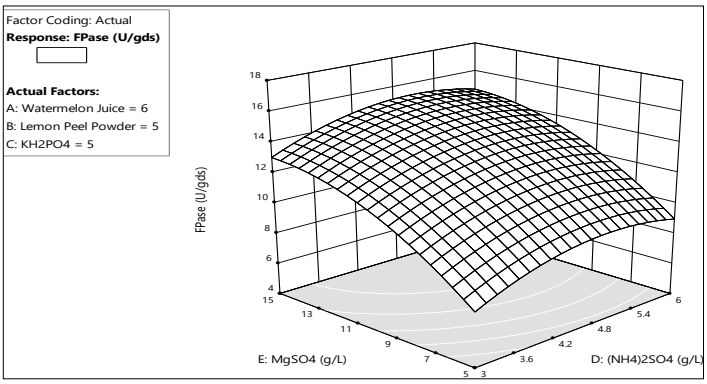

Figure 1C Response surface graph of FPase (U/gds) between the interaction of $(NH_4)_2SO_4$ and $MgSO_4$

Optimization of medium conditions for CMCase production

Enzyme titers ranged from 7.42 to 19.4 U/gds, showcasing the substantial influence of the investigated culture conditions on CMCase synthesis. Table 4 provides the actual and predicted responses for the 46 trial observations. We employed the second-order polynomial quadratic equation (Eq. 5) to fit the BBD, enabling us to model the trial data and elucidate CMCase production based on the studied individual factors.

$$Y_2 = 13.6 + 1.51A + 0.56B + 0.58C + 0.82D + 2.84E - 1.92A^2 - 2.07B^2 - 2.46C^2 - 1.2D^2 - 1.28E^2 - 0.07AB + 0.27AC - 0.05AD + 1.30AE + 0.20BC + 1.74BD + 0.11BE + 0.43CD + 0.33CE + 0.10DE \quad (5)$$

Here, Y_2 represents the dependent variable (CMCase activity, U/gds), Whereas A, B, C, D, and E represent the encoded process variable values.

The predicted responses, as displayed in Table 4, were determined using the regression equation. A substantial impact of the variable is indicated by the larger coefficient value of 2.84 for $MgSO_4$ concentration in the linear coefficient (Eq. 5). The linear correlation coefficient suggests that increasing $MgSO_4$ concentration enhances enzyme activity more than raising $(NH_4)_2SO_4$ concentration, implying reduced $(NH_4)_2SO_4$ requirement.

In Table 6, the ANOVA results for CMCase activity reveal a significant F-test value below 0.05, underscoring the value of design terms. The design F value of 13.32 further highlights this significance in the quadratic regression model as illustrated in the table. The R^2 estimate of 0.9142 signifies the design's ability to account for 91.4% variability, leaving 8.6% unexplained. If "Prob > F" values are below 0.05, model terms like A, D, C, E, AE, BD, A^2, B^2, C^2, D^2, and E^2 are likely significant **(Design-Expert, 2018)**. The lower CV value (8.76%) indicates higher reliability of the trials.

Table 6 Analysis of variance (ANOVA) for quadratic model for carboxy methyl cellulase (CMCase) production

Source	Sum of Squares	DF	Mean Square	F-value	p-value Prob > F
Model	286.4	20	14.32	13.32	< 0.0001
A-Watermelon juice (mL/L)	39.94	1	39.94	37.16	< 0.0001
B-Lemon peel powder (g/L)	2.2	1	2.2	2.04	0.1651
C-KH_2PO_4 (g/L)	9.44	1	9.44	8.78	0.0066
D-$(NH_4)_2SO_4$ (g/L)	7.3	1	7.3	6.79	0.0152
E-$MgSO_4$ (g/L)	137.42	1	137.42	127.85	< 0.0001
AB	2.4	1	2.4	2.24	0.1474
AC	2.82	1	2.82	2.63	0.1177
AD	0.2	1	0.2	0.19	0.668
AE	14.44	1	14.44	13.43	0.0012
BC	0.53	1	0.53	0.5	0.4879
BD	11.9	1	11.9	11.07	0.0027
BE	4.56	1	4.56	4.24	0.0500
CD	1.54	1	1.54	1.43	0.2429
CE	1.13	1	1.13	1.06	0.3141
DE	0.11	1	0.11	0.1	0.7493
A^2	20.6	1	20.6	19.17	0.0002
B^2	17.7	1	17.7	16.46	0.0004
C^2	36.94	1	36.94	34.36	< 0.0001
D^2	5.25	1	5.25	4.88	0.0365
E^2	8.86	1	8.86	8.24	0.0082
Residual	26.87	25	1.07		
Lack of Fit	26.87	20	1.34		
Pure Error	0.00	5	0		
Cor Total	313.27	45			

Coefficient of variation = 8.76 %, R^2 =0.9142, Adjusted R^2 = 0.8456, Pred R^2 = 0.6569, Mean = 11.83.

Figure 2A and 2B display response surface curves for various factors. Figure 2A illustrates the interaction impact between watermelon juice (10 mL/L) and $MgSO_4$ (15 g/L), underscoring the importance of their association. Optimal levels of these two factors result in heightened CMCase activity. Enzyme activity rise links to elevated watermelon juice concentrations due to increased $MgSO_4$ levels. In Figure 2B, the substantial interaction between lemon peel powder and $(NH_4)_2SO_4$ level on enzyme activity is depicted. The graph suggests that enzyme activity rises with increased $MgSO_4$ levels. The maximum actual and predicted CMCase activities were 19.4 and 18.07 U/gds, respectively. The optimized medium, comprising watermelon juice (10 mL/L), lemon peel powder (5 g/L), $(NH_4)_2SO_4$ (4.5 g/L), KH_2PO_4 (5 g/L), and $MgSO_4$ (15 g/L), yields elevated CMCase activity.

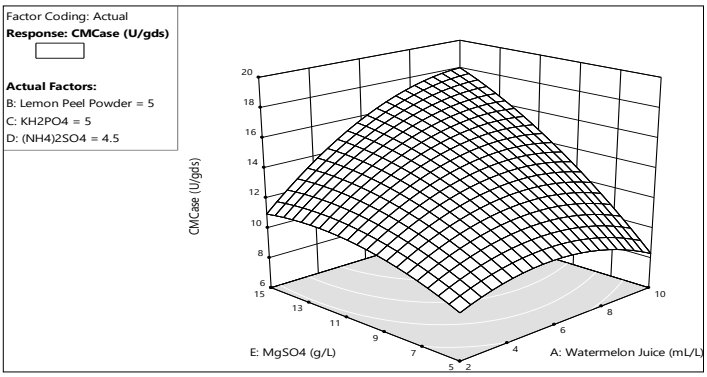

Figure 2A Response surface graph of CMCase (U/gds) between the interaction of watermelon juice and $MgSO_4$

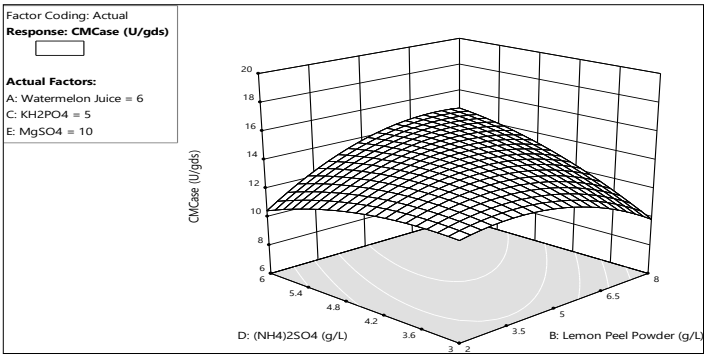

Figure 2B Response surface graph of CMCase (U/gds) between the interaction of lemon peel powder and $(NH_4)_2SO_4$

Optimization of β-glucosidase production medium conditions

The studied culture conditions exerted a significant influence on β-glucosidase activity, resulting in enzyme titers spanning 4.36 to 18.6 U/g. Actual and predicted enzyme activity yields are listed in Table 4. The BBD outcomes were fitted to a quadratic equation to elucidate the impact of medium components on β-glucosidaseactivity.

$$Y_3 = 14.2 + 1.58A + 0.37B + 0.77C + 0.68D + 2.93E - 1.54A^2 - 1.42B^2 - 2.06C^2 - 0.78D^2 - 1.01E^2 + 0.78AB + 0.84AC - 0.23AD + 1.90AE - 0.36BC + 1.73BD - 1.07BE + 0.62CD + 0.53CE + 0.17DE \quad (6)$$

Table 7 Analysis of variance (ANOVA) for quadratic model for β-glucosidase production

Source	Sum of Squares	DF	Mean Square	F-value	p-value Prob > F
Model	411.29	20	20.56	13.01	< 0.0001
A-Watermelon juice (mL/L)	11.17	1	11.17	7.07	0.0135
B-Lemon peel powder (g/L)	13.3	1	13.3	8.42	0.0076
C-KH_2PO_4 (g/L)	4.08	1	4.08	2.58	0.1206
D-$(NH_4)_2SO_4$ (g/L)	23.18	1	23.18	14.67	0.0008
E-$MgSO_4$ (g/L)	216.24	1	216.24	136.83	< 0.0001
AB	0.25	1	0.25	0.16	0.6942
AC	1.96	1	1.96	1.24	0.276
AD	2.18	1	2.18	1.38	0.2517
AE	2.76	1	2.76	1.74	0.1986
BC	0.016	1	0.016	9.89	0.9216
BD	0.59	1	0.59	0.38	0.5457
BE	5.66	1	5.66	3.58	0.07
CD	1.66	1	1.66	1.05	0.3146
CE	9.27	1	9.27	5.87	0.023
DE	13.8	1	13.8	8.73	0.0067
A^2	46	1	46	29.11	< 0.0001
B^2	56.79	1	56.79	35.93	< 0.0001
C^2	53.82	1	53.82	34.06	< 0.0001
D^2	14.52	1	14.52	9.19	0.0056
E^2	4.34	1	4.34	2.74	0.1101
Residual	39.51	25	1.58		
Lack of Fit	39.51	20	1.98		
Pure Error	0	5	0		
Cor Total	450.8	45			

Coefficient of variation = 14.69 %, R^2 =0.9124, Adjusted R^2 = 0.8422, Pred R^2 = 0.6494, Mean = 8.56.

In the equation, A, B, C, D, and E serve as coded process variable, and Y_3 predicts the response (β-glucosidase activity, U/gds).

The predicted responses included in Table 4 were generated using the regression equation. The notably high linear correlation coefficient of 2.93 for $MgSO_4$ concentration (Eq. 6) indicates its significant and positive impact on β-glucosidase activity. The linear coefficient reveals that elevated levels of KH_2PO_4 and $MgSO_4$ correspond to increased enzyme activity.

Table 7 depicts the ANOVA results for β-glucosidase activity. The model's current F-value of 13.01 confirms its validity, with a minimal 0.01% likelihood of occurring because of noise. Design terms A, B, D, E, CE, DE, A^2, B^2, C^2, and D^2 are essential if "Prob > F" values are below 0.0500 **(Design-Expert, 2018)**. The R^2 value 0.9124 signifies stronger alignment between predicted and actual responses, nearing 1. Practical harmony exists between the "pred R-squared" of 0.6494 and the "adj R-squared" of 0.8422. The lower CV (14.69%) underscores the robust dependability of the conducted trials. The absence of a significant lack-of-fit value confirms the appropriateness of the quadratic design for this experiment.

The factor response curves are showcased in Figures 3A and 3B. Figure 3A illustrates the interaction between $MgSO_4$ and KH_2PO_4, revealing that the increased enzyme activity is at the elevated concentration of $MgSO_4$ and a medium concentration of KH_2PO_4. Notably, a rise in enzyme activity is linked with a rise in $MgSO_4$ without necessitating a KH_2PO_4 concentration increase. Figure 3B demonstrates that when $(NH_4)_2SO_4$ and $MgSO_4$ concentrations are at their peak, a stable interaction occurs between these variables, leading to elevated β-glucosidase enzyme activity. Overall activity benefits from an enhanced $(NH_4)_2SO_4$ concentration in conjunction with increased $MgSO_4$. The maximum actual and predicted β-glucosidase activities were recorded as 18.6 and 16.54 U/gds, respectively. Optimized fermentation conditions incorporating watermelon juice (6 mL/L), $MgSO_4$ (15 g/L), $(NH_4)_2SO_4$ (6 g/L), lemon peel powder (5 g/L), and KH_2PO_4 (5 g/L) yield substantial β-glucosidase activity.

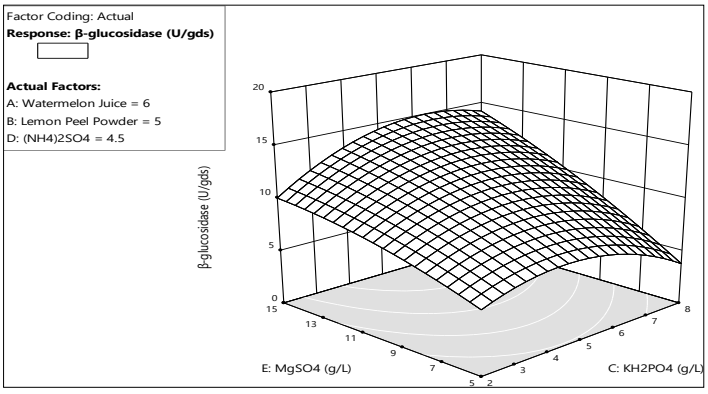

Figure 3A Response surface graph of β-glucosidase (U/gds) between the interaction of KH_2PO_4 and $MgSO_4$

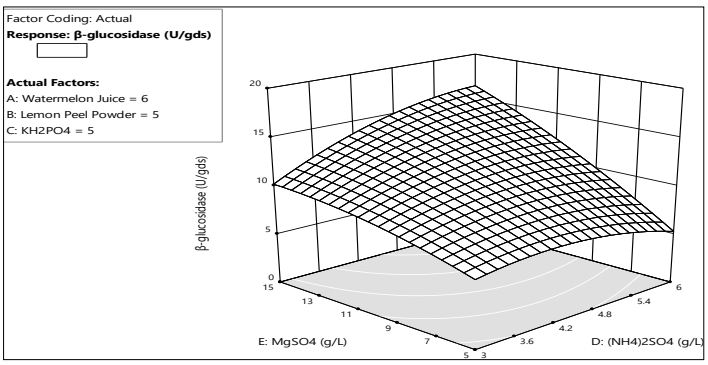

Figure 3B Response surface graph of β-glucosidase (U/gds) between the interaction of $(NH_4)_2SO_4$ and $MgSO_4$

Model validation

Three verification tests were executed within the experimental range specified in Table 8 to validate the model's adequacy. Statistical analysis was utilised for validation run data to assess the alignment between actual and predicted values. The R^2 value, at 0.9146, indicates a strong match between observed and expected values, affirming the model's accuracy.

Table 8 Model validation experiments

Trial No.	A	B	C	D	E	FPase (U/gds)	CMCase (U/gds)	β-glucosidase (U/gds)
1	2	5	8	4.5	10	9.62 (8.45)	9.24 (8.60)	6.20 (5.23)
2	10	5	8	4.5	10	11.4 (11.12)	13.2 (13.62)	8.76 (9.26)
3	6	5	2	4.5	5	6.79 (6.68)	8.70 (7.96)	5.36 (5.68)

*The values in parenthesis are the predicted values; **A: Watermelon juice (mL/L), B: Lemon peel powder (g/L), C: KH_2PO_4 (g/L), D: $(NH_4)_2SO_4$ (g/L), E: $MgSO_4$ (g/L).

Fermentation of ethanol by SsF

Additionally, the crude cellulase extract from the 36th SSF run was used for the SsF of both untreated and pretreated bagasse to ethanol (Figure 4). The peak ethanol concentration (26 g/L) was achieved using pretreated bagasse fermented by yeast, with the highest ethanol production occurring within a mere 4 days. In contrast, the untreated sugarcane bagasse culture yielded lower ethanol generation (18 g/L) after a 4-day incubation period. The decrease in yeast cell viability in both cultures likely contributed to the reduced ethanol production after 4 days.

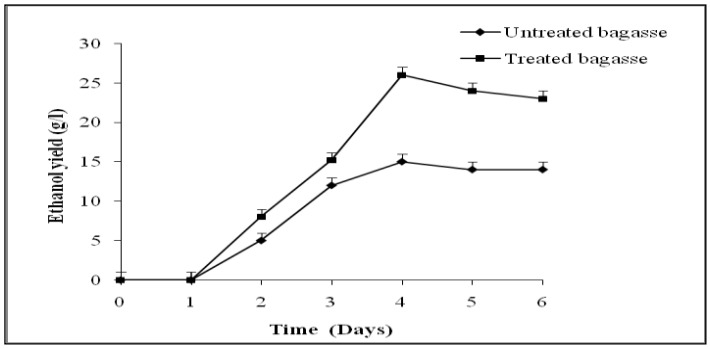

Figure 4 SsF of ethanol of crude cellulase with *Saccharomyces bayanus* on PAA untreated and treated bagasse

DISCUSSION

The price of cellulase enzymes significantly impacts the cost-effectiveness of ethanol generation from cellulosic biomass. Additionally, the choice of growth medium influences the manufacturing expense of industrial enzymes, including cellulases, which could be lowered by using more economical substrates. With these considerations in mind, the research focuses on investigating the feasibility of utilising accessible and cost-effective substrate materials—such as sugarcane bagasse, watermelon juice, and lemon peel powder—as inducers to increase the cellulolytic enzyme synthesis using the SSF method. Cultural factors notably affect the synthesis of cellulases by different fungi. In the ongoing study, the research team combines natural inducer substrates (watermelon juice, lemon peel powder) with KH_2PO_4, $(NH_4)_2SO_4$, and $MgSO_4$ in the MM medium. This combination, along with a PAA-treated bagasse substrate, is employed to culture the *Cladosporium* sp. mold, with the aim of enhancing cellulase production.

Sugarcane bagasse subjected to PAA treatment exhibited a reduction in lignin percentage along with increased hemicellulose and cellulose content, as indicated in Table 1. The utilization of this method further stimulates the creation and ease of access of cellulose-degrading enzymes towards cellulose. The research performed by **Gill et al. (2021)** and **Wi et al. (2015)** indicates that decreasing the substrate's particle size leads to improvements in both surface area and bulk density. This reduction in particle size also decreases the substrate's degree of polymerization and crystallinity, rendering it more conducive for fungal utilization. Consequently, higher levels of cellulase enzymes are released, facilitating the breakdown of cellulosic components.

In this current investigation, the fungus exhibited maximum FPase (17.6 U/gds) and CMCase (19.4 U/gds) activities during the 36th run, where the level of $MgSO_4$ and watermelon juice were 10 mL/L and 15 g/L, respectively. Existence of $(NH_4)_2SO_4$ (4.5 g/L) and lemon peel (5 g/L) also had a major impact on enzyme synthesis (Tables 5 and 6). Predicted values for FPase and CMCase were 16.4 and 18.07 U/gds, respectively. Although cellulase enzyme generation is inducible, the incorporation of other nutritional components enhanced its synthesis. In the experiment conducted by **Soumita et al. (2011)**, the optimal induction of exoglucanase synthesis was achieved with 1% (w/v) sweet lime, while dried flower at 0.75% (w/v) was proved to be the effective inducer of exoglucanase production. While most other media supplements performed optimally, higher concentrations of watermelon juice and $MgSO_4$ further increased enzyme synthesis.

The greatest enzymatic activity was achieved through the incorporation of intermediate-level (5 g/L) lemon peel powder into the medium. In all observed experimental designs, altering lemon peel powder concentrations, either higher or lower, resulted in reduced enzymatic activity. Research conducted by **Prashanth et al. (2008)** demonstrated that, following 7 days of fermentation, cellulolytic enzyme synthesis increased significantly. The study achieved the highest CMCase production level at 1.59 U/mL and its highest β-glucosidase synthesis at 1.82 U/mL in a medium supplemented with citric acid, cultured at 37 ºC, and maintained at an initial pH of 6.5. This finding is matches with the current study, which affirms that lemon peel also contributes to the improved synthesis of enzymes.

The peak CMCase activity (19.4 U/gds) was attained on the 5th day of fermentation, surpassing other enzyme activity levels because of the impactful supplements. Among the medium-level factors influencing enzyme synthesis, several were operating at their mid-level. The reduction in enzyme activity could be ascribed to higher concentrations of solid substrate, such as lemon peel powder and bagasse, impeding oxygen mass transfer. In the research by **Aggarwal et al. (2017)**, utilising *Asperigillus niger* for CMCase synthesis with inducer substrate, the highest production in the specified medium was 12.0 U/gds, which is comparable to the yield attained in the current study. **Septiani et al. (2019)** research indicates that cellulase synthesis is facilitated by the existence of carboxymethylcellulose, while soluble sugars encourage microbial growth. In the ongoing research, watermelon juice contained free amino acids and 7-10% (w/v) immediately fermentable sugars (fructose, sucrose, and glucose). Thus, in the ongoing study, watermelon juice also acted as a nitrogen supplement, facilitating robust fungal growth. The existence of nitrogenous compounds in the substrate significantly impacted cellulase synthesis in the medium **(Hussain et al., 2023)**.

In the 20th run, with KH_2PO_4 and $MgSO_4$ concentrations set at 5 and 15 g/L respectively, and notable impacts from $(NH_4)_2SO_4$ (6 g/L) and lemon peel powder (5 g/L), the fungus exhibited its highest β-glucosidase activity at 18.6 U/gds. As per **Yang et al. (2015)**, SSF demonstrated its highest enzyme synthesis with a basal medium comprising KH_2PO_4 and $(NH_4)_2SO_4$. Inducers and basal media required for cellulose enzyme complex synthesis vary among organisms. Cellulase enzyme synthesis is notably affected by the nitrogen and carbon-to-nitrogen ratio in the cultivation medium (Aggarwal *et al*., 2017). The introduction of ammonium salts to the medium notably boosted β-glucosidase synthesis. The peak level of $(NH_4)_2SO_4$ (6 g/L) in the medium highlighted its strong impact on enzyme synthesis. This can be ascribed to the evidence that the medium's nitrogen content was inadequate for consistent growth, necessitating the supplementation of proper nitrogen sources to support maximum enzyme activity, similar to observations in *A .niger* and *T. reesei* **(Septiani et al., 2019)**. By conducting trials under optimal conditions, researchers validated the current model's accuracy and found the measured enzyme activity to align closely with model-predicted values.

For identifying the superior substrate (sugarcane bagasse) for ethanol production, untreated and PAA-pretreated substrates were compared, with the pretreated substrate yielding better results. SsF was employed for ethanol generation with crude cellulase (run 36) followed by Saccharomyces bayanus fermentation. Unlike enzyme generation from the native substrate, **Aggarwal et al. (2017)** highlighted that alkali-assisted acid pretreated rice straw as the fermentation substrate exhibited high cellulase activity. The findings underscore that the substantial amount of fermentable sugars in the pretreated bagasse medium facilitated the conversion of most sugars into ethanol, resulting in an elevated ethanol yield (26 g/L) compared to untreated bagasse.

CONCLUSION

This study is a considerable achievement in cellulase enzyme synthesis, utilising accessible and cost-effective substrates. Our unique contribution involves introducing watermelon juice and powdered lemon peel into fermentation media, enhancing cellulolytic enzyme generation by *Cladosporium* sp. NCIM 901 fungus through SSF. Supplementary micronutrients like $MgSO_4$ and $(NH_4)_2SO_4$ also play a positive role. By reducing reliance on synthetic inducers like lactic acid and citric acid, our work highlights the possibilities of utilising fruit waste as a fermentation-based enzyme supplement. Furthermore, the study showcases the promise of SsF for ethanol generation from lignocellulosic substrates, utilising the crude cellulase we've developed.

Conflict of interest: No conflicts of interest declared for this new study's publication.

Ethical approval: This study was carried out in compliance with the established ethical guidelines.

REFERENCES

Aggarwal, N.K., Goyal, V., Saini, A., Yadav A., & Gupta, R. (2017). Enzymatic saccharification of pretreated rice straw by cellulases from *Aspergillus niger* BK01. 3 Biotech, 7: 158. https://doi.org/10.1007/s13205-017-0755-0

Anthony, J.C. (1984). Malt beverages and malt brewing materials: Gas chromatographic determination of ethanol in beer. Journal of Association off Analytical Chemists, 67: 192–193. https://doi.org/10.1093/jaoac/67.1.192

AOAC. (1990). Official methods of analysis of the association of official chemists. 15th ed. In: Helrich K., ed., AOAC Inc., Arlington, VA., USA.

Aydar, A.Y. (2018). Utilization of response surface methodology in optimization of extraction of plant materials. Statistical approaches with emphasis on design of

experiments applied to chemical processes. In Tech Open. DOI: 10.5772/intechopen.73690
Behera, S.K., Meena, H., Chakraborty, S., & Meikap, B.C. (2018). Application of response surface methodology (RSM) for optimization of leaching parameters for ash reduction from low-grade coal. International Journal of Mining Science and Technology, 28: 621–629. https://doi.org/10.1016/j.ijmst.2018.04.014
Cunha, F.M., Esperanca, M.N., Zangirolami, T.C., Badino, A.C., & Farinas, C.S. (2012). Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulose. Bioresource Technology, 112: 270–274. https://doi.org/10.1016/j.biortech.2012.02.082
Design-Expert. (2018). Design-Expert® Software. Version 11 user's guide.
Dotaniya, M.L., Datta, S.C., Biswas, D.R., Dotaniya, C.K., Meena, B.L., Rajendiran, S., Regar, K.L., & Lata, M. (2016). Use of sugarcane industrial by-products for improving sugarcane productivity and soil health. International Journal of Recycling of Organic Waste in Agriculture, 5: 185–194. https://doi.org/10.1007/s40093-016-0132-8
Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28: 350–356. https://doi.org/10.1021/ac60111a017
Ellila, S., Fonseca, L., & Uchima, C., Cota, J., Goldman, G.H., Sloheimo, M., Sacon, V., & Siika-aho, M. (2017). Development of a low-cost cellulase production process using *Trichoderma reesei* for Brazilian biorefineries. Biotechnology for Biofuels, 10: 30. https://doi.org/10.1186/s13068-017-0717-0
Ferreira, F.L., Antonia, C.B.D., Shiga, E.A., Alvim, L., & Pesson, R.A.B. (2018). Sugarcane bagasse as a source of carbon for enzyme production by filamentous fungi. Hoehnea, 45(1): 134–142. https://doi.org/10.1590/2236-8906-40/2017
Gares, M., Benaissa, A., Hiligsmann, S., & Cherfia, R. et al. (2023). Box-Behnken design optimization of xylanase and cellulase production by *Aspergillus fumigatus* on *Stipa tenacissima* biomass. Mycologia, 115(4):437-455. https://doi.org/10.1080/00275514.2023.2205331
Ghose, T.K. (1987). Measurement of cellulase activities. Pure and Applied Chemistry, 59: 257–268. https://doi.org/10.1351/pac198759020257
Gill, M.K., Kocher, G.S., & Panesar, A.S. (2021). Optimization of acid-mediated delignification of corn stover, an agriculture residue carbohydrate polymer for improved ethanol production. Carbohydrate Polymer Technologies and Applications, 2: 100029. http://dx.doi.org/10.1016/j.carpta.2020.100029
Goering, H.K., & Van Soest, P.J. (1971). Forage fiber analysis; apparatus, reagents, procedures, and some applications. USDA-ARS Agric. Handbook No. 379. U. S. Gov. Print. Office: Washington, DC.
Gokhale, D.V., Puntambekar, U.S., Deobagkar, D.N., & Peberdy, J.F. (1988). Production of cellulolytic enzymes by mutant of *Aspergillus niger* NCIM 1207. Enzyme and Microbial Technology, 10: 442–445.
Hu, M., Chen, J., Yu, Y., & Liu, Y. (2022). Peroxyacetic acid (PAA) pretreatment: a potentially promising strategy towards lignocellulose biorefinery. Molecules, 27: 6359. https:// doi.org/10.3390/molecules27196359
Huang, T.T., & Wages, J.M. (2016). New-to-nature sophorose analog: a potent inducer for gene expression in *Trichoderma reesei*. Enzyme and Microbial Technology, 85:44–50. doi: 10.1016/j.enzmictec.2016.01.003.
Hussain, M.I., Ijaz, I., Mustafa, M.A., Sabar, N., Memon, A., Najeeb, M., & Khan, M. (2023). Fungal production of cellulase using fruit waste as economical growth substrates. Pakistan Journal of Medical and Health Sciences, 17 (01): 43–47. https://doi.org/10.53350/pjmhs202317143
Irfan, M., Mushtaq, Q., Tabssum, F., Shakir, HA., & Qazi, J.I. (2017). Carboxymethyl cellulase production optimization from newly isolated thermophilic *Bacillus subtilis* K-18 for saccharification using response surface methodology. AMB Express, 7: 29. DOI: 10.1186/s13568-017-0331-3
Jampala, P., Tadikamalla, S., Preethi, M., Ramanujam, S., & Uppuluri, K.B. (2017). Concurrent production of cellulase and xylanase from *Trichoderma reesei* NCIM 1186: enhancement of production by desirability-based multi-objective method. 3 Biotech, 7(1):14. https://doi.org/10.1007/s13205-017-0607-y
Jeffries, T.W. (1996). Production and applications of cellulase. Laboratory procedures hand book, US Army Materials Laboratories, pp: 1–10.
Konde, K.S., Nagarajan, S., Kumar, V., Patil, S.V., & Ranade. V.V. (2021). Sugar cane bagasse based biorefineries in India: potential and challenges. Sustainable Energy and Fuels, 5: 52–78. https://doi.org/10.1039/D0SE01332C
Kumar, P., Barrett, D.M., Delwiche, M.J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial and Engineering Chemistry Research, 48: 3713–3729. https://doi.org/10.1021/ie801542g
Li, Y., Zhang, X., Xiong, L., Mehood, M.A., Zhao, X., & Bai, F. (2017). On-site cellulase production and efficient saccharification of corn stover employing cbh2 overexpressing *Trichoderma reesei* with novel induction system. Bioresource Technology, 238: 643–649. doi: 10.1016/j.biortech.2017.04.084
Miller, G.L. (1959). Use of dinitrosalicyclic acid reagent for determination of reducing sugar. Biotechnology and Bioengineering Symposium, 5: 193–219. DOI: 10.1021/ac60147a030
Minitab. (2021). What are response surface designs, central composite designs, and Box-Behnken designs?. Minitab HK, Hongkong.
Mohan, P.R., Pradeep, S., & Reddy, O.V.S. (2013). A comparative study on simultaneous saccharification and fermentation of agricultural wastes to bio-ethanol using two *Saccharomyces* strains. Chiang Mai Journal of Sciences, 40(3): 307–320.
Moharram, A.M., Zohri, A.A., Hesham, A.E., Maher, M.A., & Al-Bedak, O.A.M.S. (2021). Production of cocktail enzymes by three *Cladosporium* isolates and bioconversion of Orange peel wastes into valuable enzymes. Journal of Pure and Applied Microbiology, 15(4):2336–2346. https://doi.org/10.22207/JPAM.15.4.58
Moharram, A.M., Zohri, A.N.A., & Hesham, A.E.L. et al. (2022). Production of cold-active pectinases by three novel *Cladosporium* species isolated from Egypt and application of the most active enzyme. Science Reports, 12: 15599. https://doi.org/10.1038%2Fs41598-022-19807-z
Neelima, G., Balvindra, S., Supriya, V., & Sanjay, K. (2021). Enzyme production from different fruit and vegetable waste using lactic acid fermentation. Eco-friendly Agriculture Journal, 16(1):74–76.
Ozcelik, B., & Yavuz-Duzgun, M. (2016). Handbook of functional beverages and Human health. Taylor & Francis Group, LLC, pp. 553–562.
Pandey, A.K., Edgard, G., & Negi, S. (2016). Optimization of concomitant production of cellulase and xylanase from *Rhizopus oryzae* SN5 through EVOP-factorial design technique and application in Sorghum Stover based bioethanol production. Renewable Energy, 98: 51–56. https://doi.org/10.1016/j.renene.2016.05.071
Parameswaran, B. (2009). Sugarcane Bagasse. In: Singh nee Nigam P, Pandey A (eds) Biotechnology for Agro-Industrial Residues Utilisation. Springer, Dordrecht.
Passoth, V., & Sandgren, M. (2019). Biofuel production from straw hydrolysates: current achievements and perspectives. Applied Microbiology and Biotechnology, 103: 5105–5116. https://doi.org/10.1007/s00253-019-09863-3
Pham, T., Nguyen, N.T.P., Dinh, D.V., Kieu, N.T.H., Bach, L.G., Phong, H.X., Muoi, N.V., & Truc, T.T. (2020). Evaluate the chemical composition of peels and juice of seedless Lemon (*Citrus latifolia*) grown in Hau Giang province, Vietnam. The IOP Conference Series: Materials Science and Engineering, 991: 012127. http://dx.doi.org/10.1088/1757-899X/991/1/012127
Prasanna, H.N., Ramanjaneyulu, G., & Reddy, B.R. (2016). Optimization of cellulase production by *Penicillium* sp. 3 Biotech, 162:1–11. https://doi.org/10.1007%2Fs13205-016-0483-x
Prashanth, S., Madhumathi, M., & Mathivanan. N. (2008). Biosynthesis of cellulolytic enzymes by *Tricothecium roseum* with citric acid mediated induction. African Journal of Biotechnology, 7: 3917–3921.
Raajeswari, P.A., & Nischala. (2017). Dehydration, nutrient analysis and shelf life assay of Citrus fruit peel. Nutrition and Food Science International Journal, 4(2): 555634. http://dx.doi.org/10.19080/NFSIJ.2017.04.555634
Rastogi, M., & Shrivastava, S. (2017). Recent advances in second generation bioethanol production: An insight to pretreatment, saccharification and fermentation processes. Renewable and Sustainable Energy Reviews, 80: 330–340. http://dx.doi.org/10.1016/j.rser.2017.05.225
Satyamurthy, P., Jain, P., Karande, V.S., & Nadanathangam, V. (2016). Nanocellulose induces cellulase production in *Trichoderma reesei*. Process Biochemistry, 51(10): 1452–1457. http://dx.doi.org/10.1016/j.procbio.2016.06.016
Septiani, D., Surydi, H., Munim, A., & Mangunwardoyo, W. (2019). Production of cellulase from *Aspergillus niger* and *Trichoderma reesei* mixed culture in carboxymethylcellulose medium as sole carbon. Biodiversitas, 20(12): 3539–3544. https://doi.org/10.13057/biodiv/d201211
Soumita, M., Moumita, K., & Rani, R. (2011). Production of extra cellular exoglucanse by *Rhizopus oryzae* from submerged fermentation of agro wastes. Recent Research in Science and Technology, 3(3): 69–75. https://updatepublishing.com/journal/index.php/rrst/article/view/635
Straathof, A.J.J. (2014). Transformation of biomass into commodity chemicals using enzymes or cells. Chemical Reviews, 114: 1871–1908. https://doi.org/10.1021/cr400309c
Verma, N., & Kumar, V. (2019). Application of Box–Behnken design for the optimization of cellulase production under solid-state fermentation. SN Applied Sciences, 1:1733. https://doi.org/10.1007/s42452-019-1779-3
Wang, T., & Lu, F. (2021). Chapter 8 - Overcome saccharification barrier: Advances in hydrolysis technology. Advances in 2nd Generation of Bioethanol Production. Woodhead Publishing Series in Energy, 137–159. http://dx.doi.org/10.1016/B978-0-12-818862-0.00005-4
Wi, S.G., Cho, E.J., Lee, D.S., Lee, S.J., Lee, Y.J., & Bae, H.J. (2015). Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials. Biotechnology for Biofuels, 8: 228. https://doi.org/10.1186/s13068-015-0419-4
Yang, F., Gong, Y., Liu, G., Zhao, S., & Wang, J. (2015). Enhancing cellulase production in thermophilic fungus *Myceliophthora thermophila* ATCC42464 by RNA interference of cre1 gene expression. Journal of Microbiology and Biotechnology, 25(7): 1101–1107. doi: 10.4014/jmb.1501.01049.
Zhao, X.B., Wang, L., & Liu, D.H. (2007). Effect of several factors on peracetic acid pretreatment of sugarcane bagasse for enzymatic hydrolysis. Journal of

Chemical Technology and Biotechnology, 82: 1115–1121. http://dx.doi.org/10.1002/jctb.1775