



## Research article

Optimization of substrate composition for pectinase production from Satkara (*Citrus macroptera*) peel using *Aspergillus niger*-ATCC 1640 in solid-state fermentationTanvir Ahmed<sup>a</sup>, Md Rahmatuzzaman Rana<sup>a,\*</sup>, Wahidu Zzaman<sup>a</sup>, Rowshon Ara<sup>a</sup>,  
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## ABSTRACT

Pectinase is an enzyme having a broad industrial and commercial application. However, higher production costs may be the major constraint for the wide-scale application of pectinase. Therefore, researchers are trying to reduce the pectinase production cost for subsequent application in the industrial processes by using a unique substrate and optimizing the fermentation medium components and process conditions. The main purpose of the current study was to optimize medium composition for pectinase production using *Aspergillus niger*-ATCC 1640 in the solid-state fermentation.

The Response Surface Methodology (RSM) was performed to evaluate the effects of variables, specifically the concentrations of Satkara peel, urea,  $(\text{NH}_4)_2\text{PO}_4$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{ZnSO}_4$ , and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  on pectinase production in the solid substrate. Firstly, a two-factorial design, Plackett-Burman design (PBD) was applied to screen the variables that significantly influenced the pectinase production. After finding the critical variables, 15 experimental runs were carried out using a Box-Behnken design (BBD) to derive a statistical model for optimizing the concentrations of the selected variables.

The PBD model revealed that Satkara peel, urea, and  $(\text{NH}_4)_2\text{SO}_4$  significantly affected the pectinase production. RSM results indicated that the predicted response for pectinase production was in good agreement with experimental data ( $R^2 = 0.9836$ ). Under the optimized condition of Satkara peel (8.4 g/L), urea (0.5 g/L), and  $(\text{NH}_4)_2\text{SO}_4$  (2.7 g/L), the pectinase activity was predicted to be 0.6178  $\mu\text{mol}/\text{mL}$ . In the present study, the experimental pectinase production achieved 0.6045  $\mu\text{mol}/\text{mL}$ . The study revealed that optimization through RSM could improve the pectinase production from Satkara peel.

## 1. Introduction

Microbial enzymes have a wide range of commercial uses. Pectinase, one of the enzymes, is often recognized as a heterogeneous group capable of hydrolyzing pectin [1]. They have broad applications in various industrial settings such as juice and oil extraction, fermentation, animal feed, coating, wastewater treatment, pulp and paper, and pharmaceutical industries [2, 3]. Several studies have found a large amount of pectin in different varieties of citrus peel. Moreover, citrus is one of the most

cultivated, economically viable, and popular crops for nutritional advantages as well as secondary metabolites [4, 5]. Therefore, the citrus peel can be exploited as a critical substrate for producing different enzymes, i.e., pectinase [6, 7].

*Citrus macroptera*, locally known as Satkara fruit, is a native species of the *Rutaceae* family from the hilly region of Sylhet, Bangladesh. Local people use the peel of this traditional fruit as curries and pickles. Furthermore, numerous studies have been reported Satkara as a potential pharmacological agent [8, 9]. Besides, in a previous study, Islam &

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Rahman-Un-Nasir [10] have found the low methoxyl pectin content ranged from  $2.35 \pm 0.18$  to  $1.89 \pm 0.26$  g/100g of fresh Satkara, which can be used as a food ingredient. However, considering the industrial importance of the pectinase enzyme and Satkara fruit as a potential agent in both the food and pharmaceutical industries, further process optimization is required to produce a cost-effective pectinase enzyme from this traditional fruit.

Considering the above discussed issue, pectinase enzymes are produced commercially by using different microorganisms because of their ability to use the citrus peel as a substrate, increase the yield, and reduce the production cost using either submerged culture fermentation or solid-state fermentation [11]. Recent year, fungal enzyme production has attracted wide attention. Particularly, numerous studies utilized *Aspergillus* genus to produce commercial enzymes using different types of substrates as well agricultural wastes. For example, Santos et al. [12] employed *Aspergillus niger* to optimize exogluconases and endogluconases enzymes production using the waste of mango. In another study, Sethi et al. [13] demonstrated that *Aspergillus* NCFT 4269.10 enhanced pectinase production when banana peel was applied as a substrate. Besides, the *Aspergillus* strain can produce safe metabolites, multiply and colonize the solid substrates [14, 15]. Moreover, the solid-state fermentation process is often preferred to produce pectinase by filamentous fungi over submerged fermentation due to its ability to improve nutritional value, increase enzyme yield with high commercial value, less contamination, and cost-effectiveness [16, 17, 18].

Nevertheless, it has been shown that the composition of the substrate and the fermentation conditions have a significant impact on pectinase production by microorganisms [19, 20]. Although fermentation conditions affect the pectinase production, the nutritional composition sources (nitrogen, carbon, and mineral salts) are critical factors for the industry since one-third of the cost of enzyme production was primarily related to the expense of medium culture [21]. On that account, it is critical to select suitable medium components with adjustable concentration and understand how the components interact. Thus, medium composition optimization is also a crucial factor, which helps to minimize production costs and increase production yield.

In the last decades, statistical tools are frequently used to optimize the industrial process to reduce production costs. Mostly, the optimization process begins with identifying significant variables affecting the enzyme production, followed by the determination of their optimal concentration. Nevertheless, optimization of culture conditions and medium through a one-factor-at-a-time (OFAT) approach has some limitations in interaction accuracy between the variables or factors [22]. Hence, statistical-based strategies provide better cost-effective alternatives for the study of the interaction between factors. In particular, using a two-level full factorial design–Plackett–Burman design (PBD), a well-known and widely used statistical tool, researchers can identify process variables that have a significant impact on enzyme production while eliminating insignificant variables, resulting in a smaller, more manageable set of variables [23, 24]. After finding the significant variables, when two or three significant variables need to study for the further optimization process, several studies have used a three-level full factorial design like Box-Behnken design (BBD) with Response Surface Methodology (RSM) to optimize the actual value of significant variables [25, 26, 27, 28].

Therefore, RSM was employed in this study to optimize the solid-state fermentation medium components based on nitrogen, carbon, and mineral salts sources for production of pectinase utilizing *Aspergillus niger*-ATCC 16404. First, PBD was applied to screen the medium by identifying the key components for pectinase production. Later, the concentration of the selected medium components was optimized by BBD. Satkora peel was considered carbon source to screen the medium components because no previous study has been reported to optimize pectinase production using RSM, especially with Satkora peel as a sole carbon substrate. At the same time, urea,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{ZnSO}_4$ , and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  were selected for screening as nitrogen sources and mineral salts,

respectively, based on their positive results on the pectinase production from the previous studies [19, 29, 30, 31, 32].

## 2. Materials and methods

### 2.1. Substrates and chemicals

Fresh Satkara fruits were collected from the Citrus Research Institute, Jaintiapur, Sylhet, Bangladesh, during March–April 2017. Lyophilized pellets of *Aspergillus niger*-ATCC 16404 was collected from Sunman-Birdem Pharma Ltd., Gazipur, Bangladesh. All the chemicals used in this study were of analytical grade and collected from Sigma-Aldrich (US) and Merck (Germany).

### 2.2. Preparation of Satkara peel

Satkara fruits were washed with running tap water to remove extraneous materials and dust. The surface water was removed using blotting paper, and then the peel was separated from the fruit samples. After that, all peels were sliced thinly and dried in a freeze dryer (Model: LyoQuest-55, Telstar, Spain) at  $-60^\circ\text{C}$  (vacuum pressure, 7 mTorr) for 48 h. The dry sample was crushed to a fine powder and sieved through a mesh (0.05 mm) to obtain homogenous particles. The peel powder was weighed on a digital scale (Model: ML3002.E, Mettler Toledo, Switzerland) and kept in a desiccator (Model: S3119, Star Scientific Glass Co., India) until further use.

### 2.3. Maintenance and growth of *Aspergillus niger*-ATCC 16404

*Aspergillus niger*-ATCC 16404 strains were prepared by transferring two pellets to the 2mL vial of hydrating fluid with sterile forceps. The vial was recapped with the hydrated material and placed into an incubator (Model: BIFG-101, Biolab Scientific Ltd., Canada) ( $34\text{--}38^\circ\text{C}$ ) for 30 min to ensure complete hydration. Later, the hydrated material was vortex (Model: SI-P236, Scientific Industries, Inc., USA) until pellets had dissolved entirely and the suspension was homogenous.

19.5gm of sabouraud dextrose agar (SDA) powder was dissolved in an Erlenmeyer flask containing 500 mL distilled water. The flask was covered with a cotton plug and aluminum foil and sterilized by autoclaving (Model: LABEC 80L, CSK group, Australia) at  $121^\circ\text{C}$  for 40 min. Autoclaved agar medium was placed into each sterile Petri dish and allowed to solidify under laminar flow (Model: BBS13HGS, MRC Lab, UK) after cooling to room temperature. The resulting SDA plates were then used for the maintenance of the *Aspergillus niger*-ATCC 16404. A homogenized loop extract was streaked from liquid media to solid media under the Bunsen burner's flame. Following that, the plates were incubated at  $30\text{--}40^\circ\text{C}$  until visible colonies of *Aspergillus niger*-ATCC 16404 were observed. On separate plates, by repeated streaking and subculture, morphologically contrasting colonies were purified. This purification procedure was repeated until the pure isolates of *Aspergillus niger*-ATCC 16404 were acquired.

### 2.4. Preparation of spore suspension

The nutrient broth was prepared by adding 15% glycerin (75 mL) with 6.5 gm nutrient broth powder and increased the volume to 500 mL with distilled water. Besides, 10.0 g/L dextroses and 5.0 g/L tryptone were added to make a 500 mL sabouraud dextrose broth solution with distilled water. Afterwards, 25 mL sabouraud dextrose broth and 50 mL nutrient broth were taken separately in a 250 mL Erlenmeyer flask. After sterilization, flasks were cooled down in an optimum environment condition. Pure culture of *Aspergillus niger*-ATCC 16404 was then mixed with 25 mL sabouraud dextrose broth. 1 mL of nutrient broth and 0.5 mL of the mixture were placed to a vial tube and refrigerated at  $4^\circ\text{C}$ . During the process, the flask was shaken regularly to avoid precipitation.

## 2.5. Solid-state fermentation

The procedure mentioned previously by Oluwayemisi & Modupe [33] was followed in the present study with slight modification to carry out solid-state fermentation in 250 mL Erlenmeyer flasks. The basic fermentation medium composed of 0.25 g/L CaCl<sub>2</sub> (mineral salt) and 0.5 g/L pectin (carbon source). Other ingredients were added to the fermentation medium in accordance with the Plackett-Burman and Box-Behnken experimental design (Tables 2 and 5). After moistening with 80% distilled water, the mixture was autoclaved for 20 min at 121 °C. Following that, 1 mL spore suspension of *Aspergillus niger*-ATCC 16404 was added to the fermentation medium and incubated for 72 h at 30 °C.

## 2.6. Pectinase extraction

All the fermentation medium was mixed at a ratio of 1:4 w/v with phosphate buffer (pH 5.5) and kept at 40 °C overnight. The liquid was extracted by centrifugation of fermentation media at 500 rpm on a rotary shaker (Model: Innova 4000, New Brunswick Scientific, USA) for 15 min from fungal mycelia and the semi-solid substrate. Subsequently, following the procedure described by Dhillon et al. [34], Whiteman filter paper No.1 was used to filter the extract and the pectinase assay was performed using the filtered clear extract.

## 2.7. Total pectinase assay

The DNS (3,5-dinitrosalicylic acid) reagent test method explained by Preeti et al. [35] was used to achieve the pectinase assay, which was based on the detection of reducing sugars produced by enzymatic hydrolysis of pectin from Satkara peels. At first, 0.1 gm pectin was mixed with 100 mL phosphate buffer of pH 5.5. After that, a tube was filled with 400 µl of mixture solution and 100 µl of enzyme extract. The reaction mixture was incubated for 30 min at 50 °C and 800 rpm in a Thermo shaker incubator (Model: TT-100H, Hercuvan Lab Systems, UK), followed by the addition of 500 µl DNS solution to the tubes. The reaction was halted by heating the mixture in a water bath (Model: 286 43L 120V, Clarkson Laboratory and Supply Inc, USA) for 5 min at 90 °C. 2 mL distilled water was added to the contents of each tube after the reaction mixture was cooled, and the absorbance was measured at 550 nm against the appropriate blank using UV Spectrophotometer (Model-T60U, PG instruments Ltd., UK). A standard galacturonic acid calibration curve ( $Y = 0.388X - 0.1802$ ,  $R^2 = 0.9916$ ) was used to determine galacturonic acid concentration in the test sample. A unit of pectinase activity is the amount of enzyme required to efficiently separate one micromole of galacturonic acid per min. under the assay conditions. The activity of the pectinase enzyme was expressed in units of activity per milliliter.

## 2.8. Screening of the medium components using a Plackett-Burman design

Appropriate carbon, nitrogen, and mineral salts sources selection is critical in developing an effective and economical process. PBD is an efficient linear model for screening fermentation parameters, especially when many factors are available. Therefore, the significant medium components of pectinase production from carbon, nitrogen, and metal salts sources were identified in this study using a two-level PBD model. For screening carbon, nitrogen, and mineral salts sources, seven medium components were considered (Satkara peel, urea, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub>, KH<sub>2</sub>PO<sub>4</sub>, ZnSO<sub>4</sub>, and MgSO<sub>4</sub>·7H<sub>2</sub>O). The concentration range for each component was determined using data from numerous studies published in the literature. The design of individual components with lower (−1) and higher levels (+1) is shown in Table 1.

**Table 1.** Screening components and their levels for the Plackett-Burman Design.

Variables	Levels	
	Low (-1)	High (+1)
Screening components		
Satkara peel (g/L)	5.0	7.5
Urea (g/L)	0.50	0.75
(NH <sub>4</sub> ) <sub>2</sub> PO <sub>4</sub> (g/L)	2.5	3.0
NH <sub>4</sub> NO <sub>3</sub> (g/L)	0.5	1.0
KH <sub>2</sub> PO <sub>4</sub> (g/L)	0.1	0.2
ZnSO <sub>4</sub> (g/L)	0.01	0.02
MgSO <sub>4</sub> ·7H <sub>2</sub> O (g/L)	0.5	1.0

## 2.9. Optimization of significant medium components using a Box-Behnken design

Following PBD screening of the fermentation medium components, three significant components, i.e., urea (0.5–1.0 g/L), Satkara peel (5.0–10.0 g/L), and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (2.5–3.5 g/L) were selected for optimization. A response surface methodology was used to investigate the effects of Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> on pectinase yield. Response surface methodology (RSM) studies employed the Box-Behnken design [36] to optimize the fermentation medium components. The independent variables were selected as Satkara peel (X<sub>1</sub>), urea (X<sub>2</sub>), and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (X<sub>3</sub>), and the dependent response variable was pectinase activity (Y). For each variable, the lower, middle, and higher values were denoted by the digits -1, 0, and +1, respectively (Table 5). The effect of each variable as well as their interaction to get predicted yield and statistical analysis, were explained using the second-order polynomial Eq. (1) shown below.

$$Y = B_0 + \sum_{i=1}^k B_i X_i + \sum_{i=1}^k B_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k B_{ij} X_i X_j \quad \text{Eq. 1}$$

where, Y is the predicted pectinase yield; K is the number of factors studied; X<sub>i</sub> and X<sub>j</sub> are the independent variables; B<sub>0</sub> is a constant; and B<sub>i</sub>, B<sub>ii</sub>, and B<sub>ij</sub> are the linear, squared, and interaction coefficients, respectively.

## 2.10. Model validation

Additional experiments in triplicate under optimum fermentation conditions were done in order to validate the RSM-derived model.

## 2.11. Statistical analysis

The experimental design, regression, and graphical analysis of the generated data were performed using the software Design-Expert (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA). The model was statistically analyzed using analysis of variance (ANOVA).

## 3. Results and discussion

### 3.1. Screening of the significant components for pectinase production by PBD

Agriculture residue and fruits peels are the most promising sources of pectinase activity. In recent years, many researchers utilized citrus fruit peels in their study as fermentation raw material, which enhanced production and minimized the production cost. Dried citrus peels contain higher amounts of carbohydrates, pectin, and proteins; nevertheless, the fat content is comparatively low. Moreover, citrus fruits' middle lamella and cell walls naturally contain pectin, which can be produced by a

**Table 2.** Plackett–Burman Design for screening the major medium components influencing the pectinase production.

Trial No.	Satkora peel (g/L)	Urea (g/L)	(NH <sub>4</sub> ) <sub>2</sub> PO <sub>4</sub> (g/L)	NH <sub>4</sub> NO <sub>3</sub> (g/L)	KH <sub>2</sub> PO <sub>4</sub> (g/L)	ZnSO <sub>4</sub> (g/L)	MgSO <sub>4</sub> ·7H <sub>2</sub> O (g/L)	Total pectinase (μmol/mL)
1	-1	+1	+1	+1	-1	+1	+1	0.5670
2	-1	+1	+1	-1	+1	-1	-1	0.3710
3	-1	+1	-1	-1	-1	+1	+1	0.3545
4	+1	-1	+1	-1	-1	-1	+1	0.2549
5	-1	-1	-1	-1	-1	-1	-1	0.1856
6	-1	-1	+1	+1	+1	-1	+1	0.2581
7	+1	+1	+1	-1	+1	+1	-1	0.2137
8	+1	+1	-1	+1	+1	-1	+1	0.3112
9	+1	+1	-1	+1	-1	-1	-1	0.2847
10	+1	-1	+1	+1	-1	+1	-1	0.2110
11	-1	-1	-1	+1	+1	+1	-1	0.1509
12	+1	-1	-1	-1	+1	+1	+1	0.1147

diverse array of microorganisms. Many investigators used citrus peel as a raw carbon source for pectinase production in the fermentation medium. Garzón and Hours [37] reported that after 36 h of culture, *Aspergillus foetidus*-ATCC 16878 showed pectic enzyme activity of 1,600–1,700 U/g when cultivated in solid-state fermentation with citrus waste as carbon source. When *Aspergillus foetidus*-ATCC 16878 was grown in solid-state fermentation with citrus waste as the carbon source, it exhibited pectic enzyme activity of 1,600–1,700 U/g after 36 h of culture. Pectinase production was 25% higher than when apple pomace was used as a substrate for the same fungus strain and fermentation conditions. Maldonado et al. [38] investigated extracellular pectinase synthesis by *Aspergillus* sp. isolated from decaying lemons using differently pretreated lemon peel instead of pectin as the carbon source. When the unwashed fresh lemon peel was utilized in place of pectin, it was found that extracellular polygalacturonase and pectinesterase synthesis were almost the same, whereas pectin esterase production was substantially higher. Also, Larios et al. [39] investigated the production of endo-polygalacturonase by using citrus pectin and untreated lemon peel as carbon sources with *Aspergillus* sp. CH–Y-1043. Their findings showed that endo-polygalacturonase can be produced more efficiently using untreated lemon peel. Optimization of the culture media in terms of nitrogen source, phosphate concentration, and starting culture pH resulted in endo-polygalacturonase activity of 65.2 U/mL.

Following the carbon source, the next most abundant ingredient in the fermentation media is the nitrogen source. The type of nitrogen source is another key affecting cell growth and product formation. Microorganisms can be able to use both organic and inorganic nitrogen sources as their energy source. The production of enzyme and cell growth can be aided by both organic and inorganic sources of nitrogen. Additionally, several studies explored different nitrogen sources to investigate the impact of nitrogen sources on enzyme activity. For instance, in a report of Kiran et al. [40], PBD determined that urea was the most important variable among the seven variables in the synthesis of endo-polygalacturonase. Correspondingly, urea was identified most significant variable in the synthesis of xylanase and lipase enzymes by evaluating the nitrogen sources effect through PBD [41, 42]. Likewise to urea, multiple investigations have found NH<sub>4</sub>NO<sub>3</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the best nitrogen sources for fungal pectinase production [43, 44, 45, 46].

It is well documented that a microorganism requires an adequate supply of minerals along with carbon and nitrogen compounds in the fermentation medium for proper growth and efficient metabolism in order to produce the desired metabolites. However, the requirements and quantities of a particular mineral vary for various species, strains, and nutrients. The exact function of any mineral can be established only in a chemically defined medium that enables an accurate assessment of the involvement of individual minerals in growth and enzyme production. Numerous researches have been conducted to determine the effect of minerals on enzyme synthesis. Manesh et al. [47] conducted a study to

formulate a pectin extract medium for increased pectinase production using *Aspergillus niger* and identified KH<sub>2</sub>PO<sub>4</sub> by PBD as mineral salt that influenced the pectinase production. Dilipkumar et al. [48] and Parveen et al. [49] showed the significant effect of ZnSO<sub>4</sub> as a metal ion on the activity of inulinase and cellulase enzyme, respectively. The same trend was also recorded in the case of MgSO<sub>4</sub>·7H<sub>2</sub>O. A study by Oumer et al. [19] compared the production of pectinase in solid-state fermentation and submerged fermentation using *Bacillus subtilis* strain Btk 27. The authors found that MgSO<sub>4</sub>·7H<sub>2</sub>O showed the maximum pectinase activity in both fermentation processes among six metals salts. Similar positive effects of MgSO<sub>4</sub>·7H<sub>2</sub>O on the xylanase enzyme production were also reported by Graciano et al. [50] and Sakthiselvan et al. [51].

In the present study, in order to identify the most significant components for pectinase production, a total of seven components, namely, Satkora peel as carbon source, urea, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and NH<sub>4</sub>NO<sub>3</sub> as nitrogen source, KH<sub>2</sub>PO<sub>4</sub>, ZnSO<sub>4</sub>, and MgSO<sub>4</sub>·7H<sub>2</sub>O as mineral salts were selected based on the earlier studies. Later, their effects on the pectinase production were analyzed by a Plackett–Burman design (PBD). Table 2 shows different combinations of medium components with low (–1) and high value (+1). The experimental data from the performed total 12 trials depicted that the pectinase activity varied from 0.1147 – 0.5670 μmol/mL, which indicated the requirement of further medium components optimization. Additionally, the coefficient of determination (R<sup>2</sup>) for pectinase activity was R<sup>2</sup> = 0.9416, suggesting that the design was good-fit to the model.

However, the analysis of variance (ANOVA) for pectinase activity (Table 3) based on the PBD model was calculated, and the significance level of each medium component was evaluated based on the obtained

**Table 3.** Analysis of Variance (ANOVA) of Plackett–Burman Design model for the tested medium component.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	7	0.1504	0.0214	9.22	0.024
Linear	7	0.1504	0.0214	9.22	0.024
Satkora peel (g/L)	1	0.0205	0.0205	8.82	0.041*
Urea (g/L)	1	0.0715	0.0715	30.70	0.005*
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (g/L)	1	0.0187	0.0187	8.03	0.047*
NH <sub>4</sub> NO <sub>3</sub> (g/L)	1	0.0069	0.0069	2.97	0.160
KH <sub>2</sub> PO <sub>4</sub> (g/L)	1	0.0159	0.0159	6.85	0.059
ZnSO <sub>4</sub> (g/L)	1	0.0002	0.0002	0.10	0.764
MgSO <sub>4</sub> ·7H <sub>2</sub> O (g/L)	1	0.0163	0.0163	7.02	0.057
Error	4	0.0093	0.0023		
Total	11	0.1597			

SS: Sum of squares; DF: Degree of freedom; MS: Mean sum of squares.

\* Statistically significant at 95% of probability level.

**Table 4.** Coded values of the selected medium components used in the experimental design of Box-Behnken.

Medium components	Symbols	Coded levels		
		-1	0	1
Satkara peel (g/L)	X <sub>1</sub>	5.0	7.5	10.0
Urea (g/L)	X <sub>2</sub>	0.5	0.75	1.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (g/L)	X <sub>3</sub>	2.5	3.0	3.5

**Table 5.** Box-Behnken design with experimental responses for optimization of pectinase production.

Trial number	Medium components			Total pectinase (μmol/mL)
	X <sub>1</sub> (Satkara peel)	X <sub>2</sub> (Urea)	X <sub>3</sub> ((NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> )	Experimental response
1	0	0	0	0.5910
2	+1	0	+1	0.5372
3	+1	0	-1	0.5293
4	-1	-1	0	0.4335
5	+1	-1	0	0.5861
6	0	+1	-1	0.5922
7	-1	+1	0	0.5515
8	0	-1	-1	0.6173
9	0	0	0	0.5927
10	-1	0	-1	0.4810
11	-1	0	+1	0.4619
12	0	0	0	0.5766
13	0	+1	+1	0.5918
14	0	-1	+1	0.5795
15	+1	+1	0	0.5183

lower  $p$ -value ( $p < 0.05$ ) and higher model  $F$ -value. Besides, a variable is considered significant if its confidence level exceeds 95%. Furthermore, the  $p$ -value of Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were 0.041, 0.0005, and

production is to use agriculture, vegetables and fruits waste, which may increase pectinase production without increasing costs. Another approach is to optimize the conditions of fermentation to improve organism growth and pectinase production. Medium composition and interaction between components strongly influence the microorganisms' growth and pectinase production. Therefore, optimal medium composition and different cultural parameters are vital for opening low-cost and high pectinase production. ElEnshasy et al. [52] reported a 2.8-fold enhanced pectinase's volumetric productivity through statistical medium optimization. In another study, Guo and his colleagues [26] found the optimization of fermentation medium increased the activity of polygalacturonase (27 U/mL) and polygalacturonate lyases (40 U/mL), respective to 2-fold and 3.44-fold, and the fermentation time was reduced by 12 h (from 72 h to 60 h). The use of RSM to optimize the medium composition is a well-established method for achieving optimum conditions in systems with many variables. RSM encompasses experimental designs that investigate independent variables and optimize values of independent variables, which provide desirable variable responses. Despite possible flaws, RSM is capable of accurately simplifying complex problems and determining the response sensitivity of each variable. Few researchers have applied RSM design to enhance pectinase production with fewer experimental runs [53, 54, 55].

In the present study, based on the results of Plackett-Burman design (PBD), Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were selected as major ingredients for solid-state fermentation medium to observe their optimal combination effects on the pectinase production. Hence, the influence of each selected significant parameter and their interactions on pectinase production was scrutinized and optimized through the response surface methodology (RSM) with Box-Behnken design. A trial was obtained with 15 runs and performed with three different levels (-1, 0, +1) of Satkara peel (X<sub>1</sub>) (5.0–10.0 g/L), urea (X<sub>2</sub>) (0.5–1.0 g/L), and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (X<sub>3</sub>) (2.5–3.5 g/L) (Tables 4 and 5). The variables' levels were determined using the results of the previous PBD analysis. Based on the regression model's ANOVA results (Table 6), the following second-order polynomial proposed model (equation 2) under solid-state fermentation conditions for pectinase activities regressed by considering the significant terms.

$$\begin{aligned}
 Y(\text{Total pectinase, } \mu\text{mole / mL}) = & -0.3937 + 0.2374 \times X_1 - 0.0145 \\
 & \times X_2 + 0.0424 \times X_3 - 0.0743 \times X_1 \times X_2 \\
 & + 0.0054 \times X_1 \times X_3 + 0.0948 \times X_2 \times X_3 \\
 & - 0.0124(X_1)^2 + 0.2074(X_2)^2 - 0.0281(X_3)^2
 \end{aligned}
 \tag{Eq. 2}$$

0.047, respectively. Therefore, it is clear from the data that Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> significantly influenced the pectinase activity. The remaining four components indicated that their contributions to the response under investigation were insignificant ( $p > 0.05$ ) at the confidence level chosen for the study. So, all other insignificant components were neglected for the optimization study. The Response Surface Methodology (RSM) with Box-Behnken design (BBD) was applied to analyze further the optimum combinations of these three components (Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>).

### 3.2. Response surface methodology (RSM) and Box-Behnken design (BBD) for optimization study

The daily production of pectinase increases day by day as the demand for pectinase is rising in the industry. Still, the high production cost remains a limiting factor. So, the industry is trying to find ways to make their products more cost-effectively. One strategy to enhance pectinase

According to the experimental response, the amount of pectinase enzyme produced by *Aspergillus niger*-ATCC 16404 varied from 0.4335 to 0.6173 μmol/mL (Table 5). Moreover, the maximum production of pectinase (0.6173 μmol/mL) was noticed at Satkara peel (7.5 g/L), urea (0.5 g/L), and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (2.5 g/L) in run number 8. The predicted values fitted well with the actual values ( $R^2 = 0.9836$ ), further evidence of the model validity. It is also evident from the results that the selected components enhanced the pectinase activity compared with the earlier screening results.

### 3.3. Fitting the model

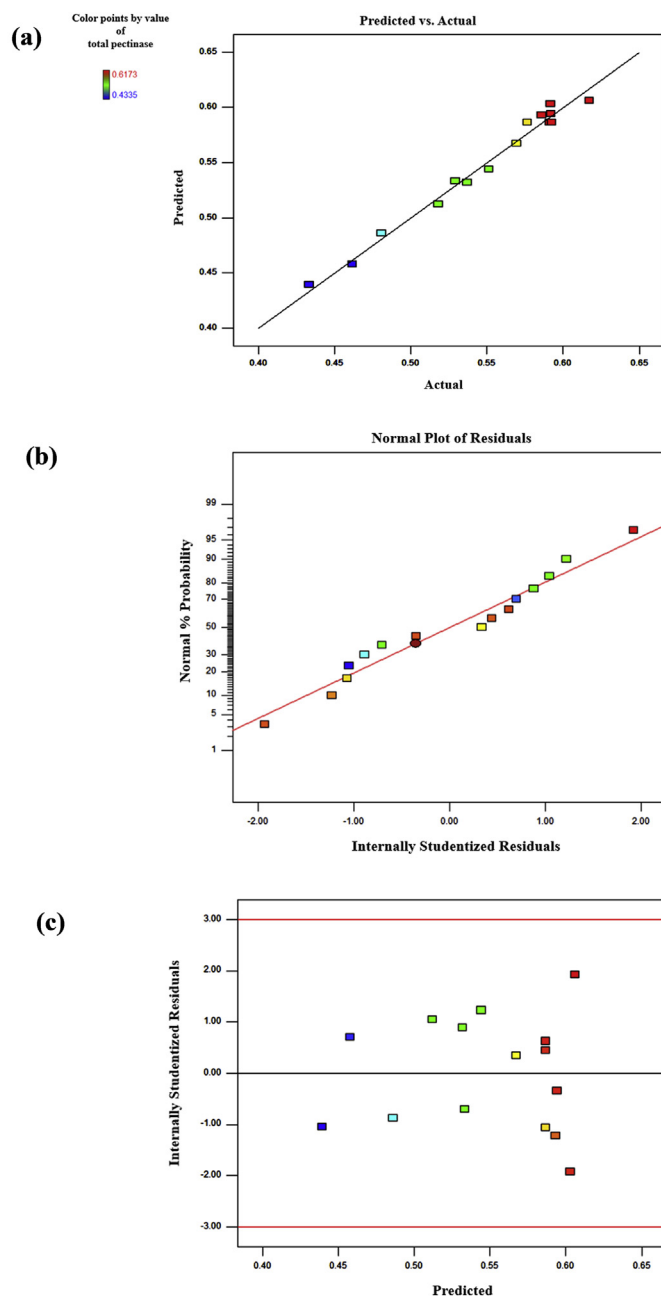
Furthermore, based on the previous studies [43, 56], the adequacy of the selected model was investigated by the diagnostic plot, namely, actual response versus predicted response, predicted versus internally studentized residuals, and normal probability plotted against internally

**Table 6.** Analysis of variance for the Box–Behnken Design experimental outcomes.

Source	Sum of squares	DF	Mean square	F-value	p-value Prob > F
Model	0.041	9	4.549E-003	33.40	0.0006
X <sub>1</sub>	7.381E-003	1	7.381E-003	54.20	0.0007
X <sub>2</sub>	2.808E-004	1	2.808E-004	2.06	0.2105
X <sub>3</sub>	4.410E-004	1	4.410E-004	3.24	0.1318
X <sub>1</sub> X <sub>2</sub>	8.630E-003	1	8.630E-003	63.37	0.0005
X <sub>1</sub> X <sub>3</sub>	1.822E-004	1	1.822E-004	1.34	0.2996
X <sub>2</sub> X <sub>3</sub>	5.617E-003	1	5.617E-004	4.12	0.0980
X <sub>1</sub> <sup>2</sup>	0.0022	1	0.022	162.35	<0.0001
X <sub>2</sub> <sup>2</sup>	6.208E-004	1	6.208E-004	4.56	0.0859
X <sub>3</sub> <sup>2</sup>	1.827E-004	1	1.827E-004	1.34	0.2991
Residual	6.809E-004	5	1.362E-004		
Lack of fit	5.245E-004	3	1.748E-004	2.23	0.3241
Pure error	1.565E-004	2	7.824E-005		
Cor. Total	0.042	14			
CV%	2.13				
R <sup>2</sup>	0.9836				
Adjusted R <sup>2</sup>	0.9542				
Predicted R <sup>2</sup>	0.7899				
Adeq Precision	17.469				

DF: Degree of freedom; Adeq: Adequate; \*Significant at 5% level ( $p < 0.05$ ).

studentized residuals. Figure 1a demonstrates the plot of predicted response versus actual pectinase production, which shows the points aligned to the fitted line and denotes a significant correspondence between the predicted pectinase production and the obtained results of the model. Similar to our results, Chandra Reddy & Saritha [29] found the applicability and accuracy of the RSM in optimizing culture media for pectinase production, which reflects a high degree of congruence between the predicted values and actual values of pectinase activity. Aklilu [57] also observed a strong similarity between the predicted value and the actual value of pectin yield from banana peel. The author found that the predicted outcomes were considerably similar to the observed results and were close to the straight line, demonstrating that the established model could accurately predict pectin yield. Similar investigations on pectin extraction from pomelo peels [58] and jackfruit waste [59] have been reported. Nevertheless, the normal probability plot of the residuals displays in Figure 1b. In regression analysis, the difference between the experimental and predicted value is known as residual. The normal residual probability plot means that the errors are normally distributed along a straight line and inconsequential [60]. As shown in Figure 1b, the residuals have a normal distribution. The resulting points near the diagonal line indicate that the model is well-fitted and reasonably congruent with experimental results. Recently, Tepe and Dursun [61] used the normal % probability plots to describe the effectiveness of the model for pectin lyase and endo-pectinase production using *Bacillus pumilus* from wheat bran, whether the residuals points were distributed normally and laid close to a straight line. Correspondingly, Purna et al. [29] explained the fitness of the model by the plots of normal % probability for pectinase production through optimizing culture media using *Enterobacter* sp. PSTB-1. Besides, Figure 1c plot the predicted response against internally studentized residuals. It was noticed that the standard residuals errors spread randomly and evenly below and above the center-line of zero in the range of  $\pm 3$  without an obvious pattern, indicating the constant variance between the residuals, which confirms the adequacy of the model. A similar trend was also observed by Li et al. [62] and Cao et al. [63] in terms of xylanase enzyme production using response surface methodology. In the present study, the data from the diagnostic plots concluded that the model accurately depicts pectinase activity by response surface.



**Figure 1.** (a) Diagnostic plot for model adequacy. Correlation between predicted and experimental values of pectinase activity. (b) Plots of normal % probability of internally studentized residual. (c) Plots of internally studentized residuals against predicted values.

The Fisher's test (F) and analysis of variance (ANOVA) were used to determine the statistical significance of the quadratic regression model, as shown in Table 6. The model with a higher F-ratio and smaller p-value is deemed to be more significant ( $p < 0.05$ ) [56]. Table 6 shows that the "Model F-value" value is 33.40, indicating that the model is significant. The chance of a "Model F-value" of this large being caused by noise is only 0.06%. Variables are considered statistically significant if "Prob > F" is less than 0.05. Nonetheless, variables are deemed non-significant if the value is greater than 0.10 [64]. In this case, X<sub>1</sub> (Satkara peel), interactions like X<sub>1</sub>X<sub>2</sub> (Satkara peel and urea), X<sub>2</sub>X<sub>3</sub> (urea and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), X<sub>1</sub><sup>2</sup> (Satkara peel)<sup>2</sup>, X<sub>2</sub><sup>2</sup> (urea)<sup>2</sup> were found to be significant model terms for increasing the production of pectinase. Moreover, the p-value for "Lack of fit" is also higher than 0.10. The F-value of "Lack of fit" is 2.23, which indicates that relative to the pure error, the value is not

significant. There is a 32.41% possibility that noises could contribute to this large "Lack of fit". A non-significant lack of fit represents the fitness of a model. The model's fitness is also assessed by the value of coefficient of determination ( $R^2$ ). The  $R^2$  value measures how much variability can be elucidated through the experimental variables as well as their inter-activities on the observed response values. When the value of  $R^2$  is close to 1, it indicates the better relationship among the predicted and actual response and the model makes more accurate prediction of the response. Hamilton et al. [65] recommended a minimum  $R^2$  of 0.80 for a well-fit model. Thus, the value of  $R^2$  for the present study model is 0.9836, as shown in Table 6, suggesting a well-fit model. The predicted model appears to have been more accurate in predicting the experimental results. As a result, the model adequately explained the responses. The adjusted  $R^2$  sets the  $R^2$  value in the model for the sample size and variable after eliminating the non-significant model terms. If the model contains many non-significant model terms, the adjusted  $R^2$  may be considerably smaller than the  $R^2$  [64]. The value of adjusted  $R^2$  for pectinase in this study is 0.9542, close to the value of the corresponding  $R^2$  (0.9836) in the model. A high adjusted  $R^2$  value also supports the model's significance for all responses. In this model, the predicted  $R^2$  (0.7899) is also reasonably agreed with the adjusted  $R^2$  (0.9542). The signal to noise ratio is measured by "Adequate precision", and a ratio greater than 4 is desirable. The signal to noise ratio of 17.469 in this model implies an adequate signal, and the model can be used for the navigation of the design space. The coefficient of variation (CV) shows the range to which the data are dispersed. The CV determines the residual variation in the data corresponding to the mean size. The smaller CV values provide better

reproducibility [66]. As shown in Table 6, the CV value of 2.13% in this model for the pectinase production indicates that the experimental findings are more accurate and reliable.

The estimated impact of each variable for the pectinase production is shown in the Pareto chart of the standardized effects diagram (Figure 2a). Standardized effects, shown as a vertical line, having a higher value than the t-limit (2.446) line, are considered statistically significant. From the Pareto chart analysis in Figure 2a, it could be assumed that the Satkara peel showed the most significant effect on the pectinase production, followed by the interaction between them and the interaction between Satkara peel and urea. On the other hand, the central effects diagram (Figure 2b) shows how the pectinase activity changes from low to high levels of individual factors. Figure 2b demonstrated that low or high concentrations of Satkara peel showed lower pectinase activity, but pectinase response increased with a mid-level concentration of Satkara peel. In contrast, the response of the pectinase was different in the case of urea. A low or high urea concentration exhibited the highest pectinase activity, while the lowest pectinase activity was obtained with a middle concentration of urea. However, in the case of  $(\text{NH}_4)_2\text{SO}_4$ , the highest pectinase response was obtained with a minimum concentration.

Apart from the individual effects from each of the main components, the interaction between the components also influenced the responses. In order to investigate the interaction among the different variables, 3D response surface plots were used, and their relative influences were evaluated. Response surface plots are usually used to simultaneously describe two independent variables while keeping the other factors fixed. So, these plots help us to comprehend the interaction of two factors. Figure 3 depicts the response surface plots (3D) and their respective two-dimensional (2D) contour plots representing the effects of Satkara peel, urea, and  $(\text{NH}_4)_2\text{SO}_4$  on the pectinase production.

The interaction between Satkara peel and urea is significant, with a  $p$ -value of 0.0005. Figure 3a, b shows the three-dimensional (3D) response surface plot and two-dimensional (2D) contour plot for Satkara peel and urea interaction, which indicates that a decrease in pectinase production was observed at low concentration of Satkara peel and urea when  $(\text{NH}_4)_2\text{SO}_4$  was kept at the central level (3.0 g/L). On the other hand, pectinase activity enhanced as the Satkara peel level increased from 5.0 g/L to 10.0 g/L and urea level from 0.5 g/L to 1.0 g/L. It is also comprehensible from Table 5, trial number 9 showed comparatively higher pectinase activity (0.5927  $\mu\text{mol/mL}$ ) at a high level of Satkara peel (7.5 g/L) and urea (0.75 g/L) when  $(\text{NH}_4)_2\text{SO}_4$  stayed constant (3.0 g/L). In a study, Mohan et al. [67] showed the interaction effect of citrus peel powder and urea on the CMCase (endoglucanase) production and reported higher enzyme activity at their middle concentration level. In another study, Ali et al. [68] reported high pectinase production with a higher concentration of *Citrus aurantium* L peel in the fermentation medium. A similar trend for urea was reported by Durairajan & Sankari [69], where a maximum pectinase production (5.58 mg/mL) in poly-methylesterase (PME) was achieved at 3% of urea compared to 1%, 2% of urea. These results demonstrated that interaction between Satkara peel and urea showed a significant effect on pectinase production. A possible explanation is that urea is a well-known denaturant and structure breaker in a wide variety of aqueous biopolymer systems. Urea has been reported to be capable of breaking the intermolecular hydrogen bonds between polysaccharide chains [70]. Therefore, the polarity of hydrogen bonding (N–H) in urea might be allowed it to permeate the cell wall of citrus peel with *Aspergillus niger*-ATCC 16404 and interacted with the pectin compounds, hence enhancing pectinase synthesis [71].

The interaction effects of Satkara peel and  $(\text{NH}_4)_2\text{SO}_4$ , as shown in Figure 3c, d, represent the moderate effect of both factors on pectinase activity. The contour line in Figure 3d is almost parallel and indicates a moderate impact. A low or high Satkara peel concentration decreased the pectinase activity, while not much remarkable effect on pectinase activity was observed in the case of  $(\text{NH}_4)_2\text{SO}_4$ . Although keeping the urea at the middle level (5.0 g/L), the highest pectinase activity (0.6173  $\mu\text{mol/mL}$ ) was obtained at the mid-level of Satkara peel (7.5 g/L) and low level of

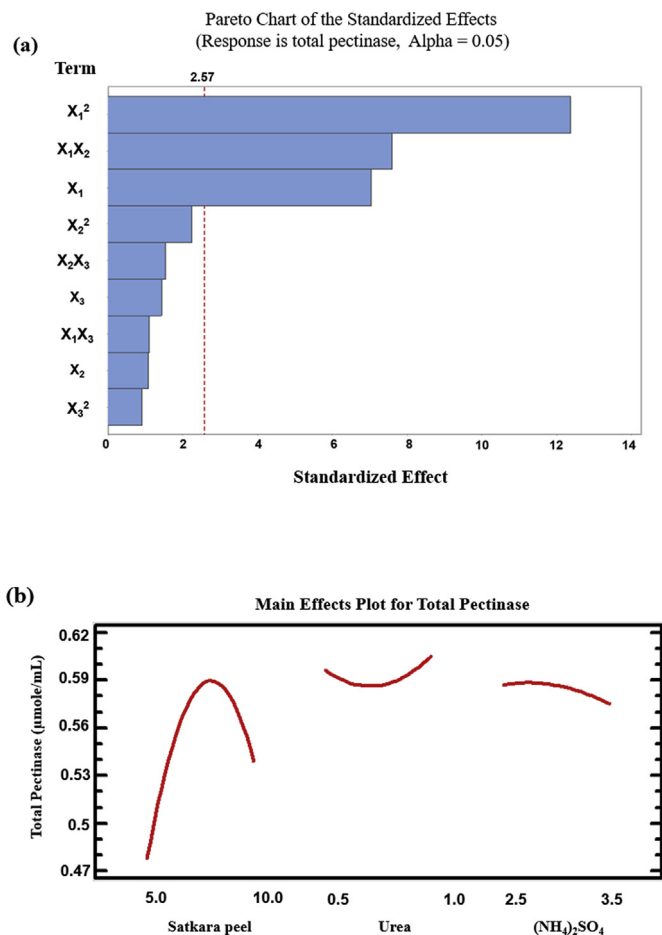
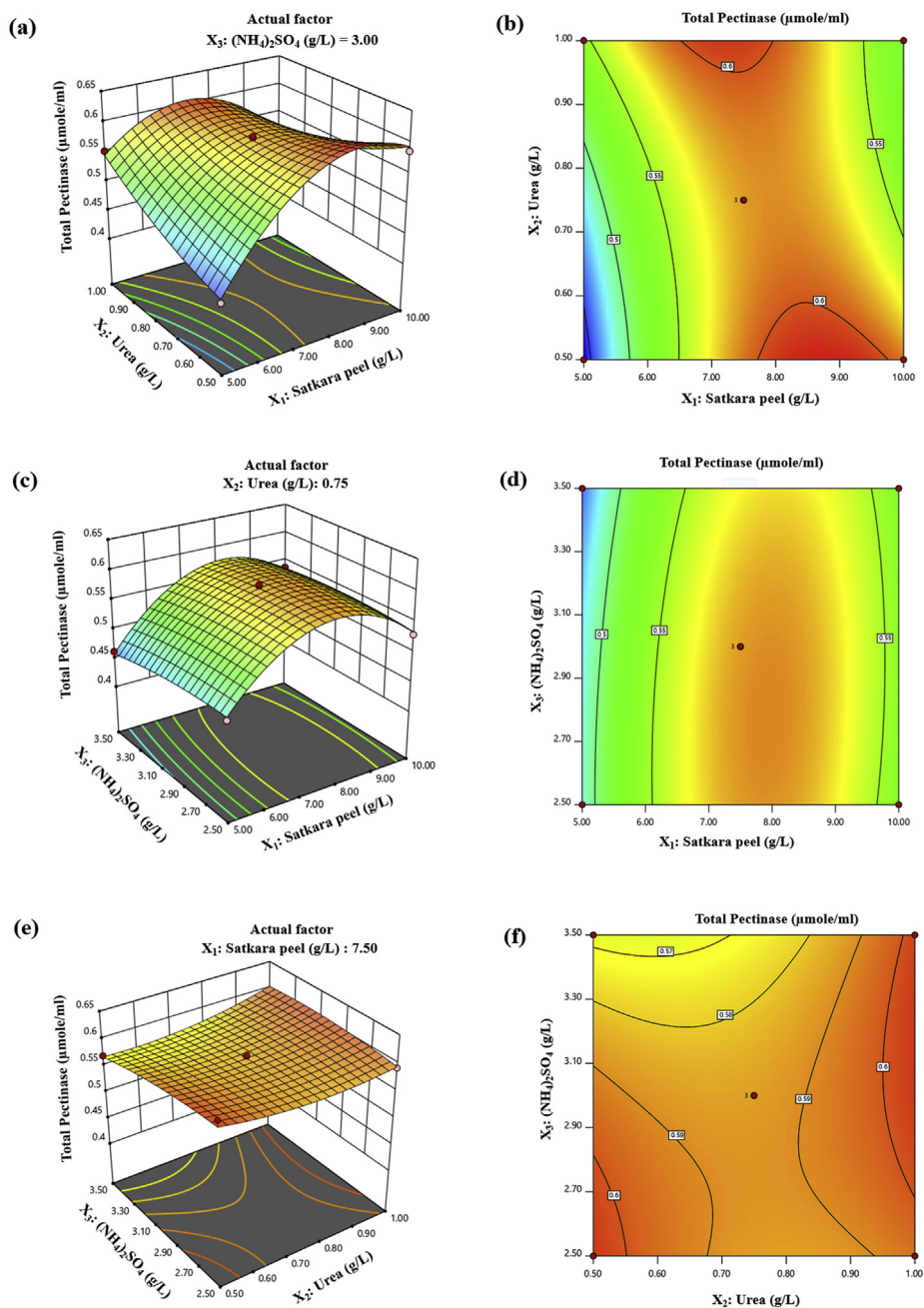


Figure 2. (a) Pareto chart illustrating the standardized effects of three variables: Satkara peel ( $X_1$ ), urea ( $X_2$ ),  $(\text{NH}_4)_2\text{SO}_4$  ( $X_3$ ) and their interaction effects on the pectinase production. (b) Main effects plot of Satkara peel, urea, and  $(\text{NH}_4)_2\text{SO}_4$  on pectinase production.



**Figure 3.** Response surface plots in three-dimensional (3D) and contour plot in two-dimensional (2D) illustrating the combined effects of (a,b) Satkara peel ( $X_1$ ) and urea ( $X_2$ ) (c,d) Satkara peel ( $X_1$ ) and  $(\text{NH}_4)_2\text{SO}_4$  ( $X_3$ ) (e,f) urea ( $X_2$ ) and  $(\text{NH}_4)_2\text{SO}_4$  ( $X_3$ ) on the pectinase production.

$(\text{NH}_4)_2\text{SO}_4$  (2.5 g/L). This result is in agreement with the report of El Enshasy et al. [52]. They found that  $(\text{NH}_4)_2\text{SO}_4$  was less effective on pectinase production during the interaction between pectin and  $(\text{NH}_4)_2\text{SO}_4$  by keeping  $\text{K}_2\text{HPO}_4$  concentration constant. Similarly, Hours et al. [72] evaluated the effects of  $(\text{NH}_4)_2\text{SO}_4$  on the pectinase production in solid-state cultures from apple pomace. At lower concentrations,  $(\text{NH}_4)_2\text{SO}_4$  was observed to have no effect on the production of pectinases. One possible reason behind the decreased pectinase activities is that because the *Aspergillus niger*-ATCC 16404 strain presumably failed to hydrolyze the  $(\text{NH}_4)_2\text{SO}_4$  during growth and pectinase production, converting its mineral components and other growth stimulants into easily absorbed elements [73].

The response curve in Figure 3e, f demonstrates the interaction between urea and  $(\text{NH}_4)_2\text{SO}_4$ . The contour plot in the form of a saddle shape indicates somewhat unusual results. Both plots illustrate that low

urea and low  $(\text{NH}_4)_2\text{SO}_4$  content contribute to enhancing pectinase activity. In this study, as shown in Table 5, the highest level of pectinase activity ( $0.6173 \mu\text{mol}/\text{mL}$ ) was achieved at low-level urea of 0.5 g/L and low  $(\text{NH}_4)_2\text{SO}_4$  of 2.5 g/L, which validate Figure 3e, f. In a study, Mrudula et al. [74] found that the second-highest pectinase production ( $208.7 \text{ U}/\text{g DMS}$ ) was obtained by a combination of urea and  $(\text{NH}_4)_2\text{SO}_4$  (1%, w/v) in the solid-state fermentation medium. In a different enzyme study, Mardawati et al. [75] reported that the production of xylanase enzyme by *Aspergillus niger* XY-1 increased with increasing urea and  $(\text{NH}_4)_2\text{SO}_4$  concentrations.

These results demonstrated that the ratio of Satkara peel, nitrogen, carbon, and mineral salts sources in the fermentation medium influenced pectinase production. The variations in the pectinase activity are inevitable because the enzyme activity is influenced by the nature of organisms, different culture conditions under the BBD and RSM,



**Table 7.** Optimized concentration of selected medium components for pectinase production.

Satkara peel (g/L) X <sub>1</sub>	Urea (g/L) X <sub>2</sub>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (g/L) X <sub>3</sub>	Target	Predicted Response (μmol/mL)	Experimental Response (μmol/mL)	Composite desirability (Out of 1)
8.4	0.5	2.7	Max.	0.6178	0.6045 ± 0.14	0.94

carbon, nitrogen, and mineral sources used as raw material. So, it is essential to adjust the concentrations of each component for the microorganism used in the pectinase enzyme production. The current study revealed that Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentrations influenced pectinase production. Satkara peel and the interaction between Satkara peel and urea showed a significant effect on pectinase production. In addition, in this study, urea exhibited better interaction with Satkara peel than (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in the solid-state fermentation medium. Each independent variable's optimal value and interaction could be observed from the response surface plots. The good correlation ( $R^2 = 0.9836$ ) between the experiment and predicted results confirmed the model's fitness.

### 3.4. Optimization and validation of the model

The optimal conditions for the selected medium components in the study for the pectinase production were validated through numerical optimization of the Design-Expert Software (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA) using Derringer's desirability function (d). The desirability function indicates whether the response is completely unacceptable, such as  $d = 0$ , or whether it matches the target value, such as  $d = 1$ . The value of  $d$  increases from 0 to 1 as the desirable response increases. Another way to assess the model's validation is to calculate the experimental error between the predicted and actual value.

After performing the numerical optimization with the significant variables, the optimized medium compositions under the solid-state fermentation were found as Satkara peel of 8.4 g/L, urea of 0.5 g/L, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> of 2.7 g/L (Table 7). Triplicate tests were carried out under the predicted experimental conditions to ensure the accuracy of the response surface model. The optimized medium composition was expected to produce 0.617 μmol/mL of pectinase. The actual pectinase production value (0.604 μmol/mL) was quite close to the predicted production value, thereby validating the model. In addition, Derringer's desirability function (d) was 0.94 out of 1, which indicated that the values observed in experiments were highly congruent with the regression model's prediction.

A study by El Enshasy et al. [52] investigated the optimization of pectinase production by factorial and response surface designs in a submerged cultivation system using *Aspergillus niger*. When the study compared the model results to actual runs, it was observed that the maximal pectinase production obtained experimentally (90 U/mL) was consistent with the model predicted production (92.48 U/mL), indicating a desirability value of 0.915. Thus, these results revealed that the model was validated, accurate and reliable. Similarly, in another study, Bibi et al. [56] reported a higher quantity of pectinase (219 U/mL) production from orange peel by *Bacillus licheniformis* with 95% desirability under the optimized conditions of temperature 37 °C, incubation period of 120 h, pH 9.5, nitrogen content 0.7%, and inoculum size 0.3 ml. Hence, the authors suggested that these conditions can produce the highest amounts of enzyme compared to less desirable conditions.

## 4. Conclusion

The present study optimized the medium composition for the solid-state fermentation in order to produce pectinase based on carbon, nitrogen, and mineral salts sources using *Aspergillus niger*-ATCC 1640. The Plackett-Burman design and the Box-Behnken design were used to carry

out the optimization process. The screening results showed that Satkara peel, urea, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentration significantly affected the pectinase production. The optimized fermentation medium for pectinase production by *Aspergillus niger*-ATCC 1640 was composed of 8.4 g/L Satkara peel, 0.5 g/L urea, and 2.7 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The maximum pectinase activity reached 0.604 μmol/mL under the optimized fermentation medium. It is also evident from the results that the selected components enhanced the pectinase activity under the optimized conditions compared with the earlier screening results. Additionally, this study indicated that Satkara peel may be a potential source of carbon for pectinase production. An increase in pectinase production is expected by adjusting other process variables in solid-state fermentation.

## Declarations

### Author contribution statement

Tanvir Ahmed: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Md Rahmatuzzaman Rana: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Wahidu Zzaman: Analyzed and interpreted the data.

Rowshon Ara: Contributed reagents, materials, analysis tools or data.

Mohammad Gulzarul Aziz: Conceived and designed the experiments.

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### Data availability statement

The data that has been used is confidential.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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## References

- [1] R. Abdollahzadeh, M. Pazhang, S. Najavand, V. Fallahzadeh-Mamaghani, A.R. Amani-Ghadim, Screening of pectinase-producing bacteria from farmlands and optimization of enzyme production from selected strain by RSM, *Folia Microbiol.* 65 (2020) 1–15.
- [2] N. Sharma, M. Rathore, M. Sharma, Microbial pectinase: sources, characterization and applications, *Rev. Environ. Sci. BioTechnol.* 12 (2013) 45–60.
- [3] O.J. Oumer, Pectinase: substrate, production and their biotechnological applications, *Int. J. Environ. Agric. Biotechnol.* 2 (2017) 1007–1014.

- [4] B. Satari, K. Karimi, Citrus processing wastes: environmental impacts, recent advances, and future perspectives in total valorization, *Resour. Conserv. Recycl.* 129 (2018) 153–167.
- [5] N. Mahato, K. Sharma, F. Nabybaccu, M.H. Cho, Citrus waste reuse for health benefits and pharma-/neutraceutical applications, *Era's J. Med. Res.* 3 (2016) 20–32.
- [6] I. Ahmed, M.A. Zia, M.A. Hussain, Z. Akram, M.T. Naveed, A. Nowrouzi, Bioprocessing of citrus waste peel for induced pectinase production by *Aspergillus Niger*; its purification and characterization, *J. Radiat. Res. Appl. Sci.* 9 (2016) 148–154.
- [7] Y. Guan, D. Wang, C. Lv, Y. Zhang, I. Gelbic, X. Ye, Archives of microbiology: screening of pectinase-producing bacteria from citrus peel and characterization of a recombinant pectate lyase with applied potential, *Arch. Microbiol.* 202 (2020) 1005–1013.
- [8] H. Rahman, M.C. Eswaraiyah, A. Dutta, Neuropharmacological activities of ethanolic extract of Citrus macroptera (Varanasiensis) fruit peels, *Global J. Pharmacol.* 8 (2014) 609–616.
- [9] M. Khalil, E.M. Tanvir, R. Afroz, S.A. Sulaiman, S.H. Gan, others, Cardioprotective effects of tualang honey: amelioration of cholesterol and cardiac enzymes levels, *BioMed Res. Int.* 4 (2015) 1–8.
- [10] G.M.R. Islam, A.K.M.A. Rahman-Un-Nasir, Isolation, characterization and industrial prospects of pectin isolated from citrus macroptera and citrus assamensis, *Int. J. Nutraceutical, Funct. Foods, Nov. Foods from Res. to Ind. Appl.* 16 (2017) 1–7.
- [11] S. Kc, et al., Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains, *Fermentation* 6 (2020) 59–68.
- [12] T.C. dosSantos, I.S. Cavalcanti, R.C.F. Bonomo, N.B. Santana, M. Franco, Optimization of productions of cellulolytic enzymes by *Aspergillus Niger* using residue of mango a substrate, *Ciência Rural.* 41 (2011) 2210–2216.
- [13] B.K. Sethi, P.K. Nanda, S. Sahoo, Enhanced production of pectinase by *Aspergillus terreus* NCFIT 4269.10 using banana peels as substrate, *Biotech* 6 (2016) 36–50.
- [14] I.G. Sandri, M.M. daSilveira, Production and application of pectinases from *Aspergillus Niger* obtained in solid state cultivation, *Beverages* 4 (2018) 48–58.
- [15] E.E. Perez, M.B. Fernández, S.M. Nolasco, G.H. Crapiste, Effect of pectinase on the oil solvent extraction from different genotypes of sunflower (*Helianthus annuus* L.), *J. Food Eng.* 117 (2013) 393–398.
- [16] T.C. Santos, G.A. Diniz, A.R.D.E. Brito, A.J.V. Pires, M. Franco, Effect of solid state fermentation on nutritional content and evaluation of degradability in cactus pear, *Rev. Caatinga.* 28 (2015) 248–254.
- [17] N.L. Rojas, G.E. Ortiz, D.J. Baroque, S.F. Cavalitto, P.D. Ghiringhelli, Production of heterologous polygalacturonase I from *Aspergillus kawachii* in *Saccharomyces cerevisiae* in batch and fed-batch cultures, *J. Ind. Microbiol. Biotechnol.* 38 (2011) 1437–1447.
- [18] H. Demir, N. Gogus, C. Tari, D. Heerd, M.F. Lahore, Optimization of the process parameters for the utilization of orange peel to produce polygalacturonase by solid-state fermentation from an *Aspergillus sojae* mutant strain, *Turkish J. Biol.* 36 (2012) 394–404.
- [19] O.J. Oumer, D. Abate, Comparative studies of pectinase production by *Bacillus subtilis* strain Btk 27 in submerged and solid-state fermentations, *BioMed Res. Int.* 2018 (2018) 1–10.
- [20] B.T. Hoa, P. VHung, Optimization of nutritional composition and fermentation conditions for cellulase and pectinase production by *Aspergillus oryzae* using response surface methodology, *Int. Food Res. J.* 20 (2013) 3269–3274. [http://www.ifrj.upm.edu.my/20\(06\)2013/42IFRJ20\(06\)2013RohmanHung241.pdf](http://www.ifrj.upm.edu.my/20(06)2013/42IFRJ20(06)2013RohmanHung241.pdf).
- [21] R. daGama Ferreira, A.R. Azzoni, S. Freitas, Techno-economic analysis of the industrial production of a low-cost enzyme using *E. coli*: the case of recombinant  $\beta$ -glucosidase, *Biotechnol. Biofuels* 11 (2018) 1–13.
- [22] U. Managamuri, M. Vijayalakshmi, S. Poda, V.S.R.K. Ganduri, R.S. Babu, Optimization of culture conditions by response surface methodology and unstructured kinetic modeling for bioactive metabolite production by *Nocardioopsis litoralis* VSM-8, *Biotech* 6 (2016) 219–237.
- [23] A.P. Karlapudi, S. Krupanidhi, R. Reddy, M. Indira, N.B. Md, T.C. Venkateswarulu, Plackett-Burman design for screening of process components and their effects on production of lactase by newly isolated *Bacillus* sp. VUVD101 strain from Dairy effluent, *Beni-Suef Univ. J. basic Appl. Sci.* 7 (2018) 543–546.
- [24] K. Vanaja, R.H. Shobha Rani, Design of experiments: concept and applications of Plackett Burman design, *Clin. Res. Regul. Aff.* 24 (2007) 1–23.
- [25] P.C. Oliveira, et al., Cocoa shell for the production of endoglucanase by *Penicillium roqueforti* ATCC 10110 in solid state fermentation and biochemical properties, *Rev. Mex. Ing. Química.* 18 (2019) 777–787.
- [26] F. Guo, X. Li, J. Zhao, G. Li, P. Gao, X. Han, Optimizing culture conditions by statistical approach to enhance production of pectinase from *Bacillus* sp, *Y1. Biomed Res. Int.* 1 (2019) 1–10.
- [27] A. Ahmed, M. Sohail, Characterization of pectinase from *Geotrichum candidum* AA15 and its potential application in orange juice clarification, *J. King Saud Univ.* 32 (2020) 955–961.
- [28] M. Yolmeh, M. Jafari, Applications of response surface methodology in the food industry processes, *Food Bioprocess Technol.* 10 (2017) 413–433.
- [29] M.P. Chandra Reddy, K.V. Saritha, Effects of the culture media optimization on pectinase production by *Enterobacter* sp. PSTB-1, *Biotech* 6 (2016) 1–11.
- [30] O.M. Adeyefa, O.A.T. Ebuehi, Isolation, identification and characterization of pectinase producers from agro wastes (citrus sinensis and ananas comosus), *Iris Publishers, World J. Agric. Soil Sci.* 4 (2020) 1–6.
- [31] I. Darah, S. Nor-hawani, L. Hong, A. Rosma, W. Haritharan, and others, Pomelo peels as alternative substrate for extracellular pectinase production by *Aspergillus Niger* HFM-8, *Malays. J. Microbiol.* 9 (2013) 308–316.
- [32] S. Islam, B. Feroza, A. Alam, S. Begum, Pectinase production by *Aspergillus Niger* isolated from decomposed apple skin, *Bangladesh J. Sci. Ind. Res.* 48 (2013) 25–32.
- [33] O.A. Oluwayemisi, A.O. Modupe, Influence of blanching on the production of pectinases from banana peels by *Aspergillus Niger*, *Int. J. Microbiol. Biotechnol.* 4 (2019) 8–11.
- [34] S.S. Dhillon, R.K. Gill, S.S. Gill, M. Singh, Studies on the utilization of citrus peel for pectinase production using fungus *Aspergillus Niger*, *Int. J. Environ. Stud.* 61 (2004) 199–210.
- [35] S. Preeti, T. Abhishek, K. Deeja, S. Suresh, and others, Isolation, screening and optimization of novel pectinase producing fungal strain for fruit juice clarification and extraction, *World J. Pharmaceut. Res.* 4 (2015) 2114–2126. [https://www.wjpr.net/index.php/dashboard/abstract\\_id/3032](https://www.wjpr.net/index.php/dashboard/abstract_id/3032).
- [36] G.E.P. Box, W.H. Hunter, S. Hunter, *And others. Statistics for Experimenters: an Introduction to Design, Data Analysis and Model Building*, 664, John Wiley & Sons, New York, 1978.
- [37] C.G. Garzón, R.A. Hours, Citrus waste: an alternative substrate for pectinase production in solid-state culture, *Bioresour. Technol.* 39 (1992) 93–95.
- [38] M.C. Maldonado, A. Navarro, D.A.S. Callieri, Production of pectinases by *Aspergillus* sp using differently pretreated lemon peel as the carbon source, *Biotechnol. Lett.* 8 (1986) 501–504.
- [39] G. Larios, J.M. Garcia, C. Huitron, Endo-polygalacturonase production from untreated lemon peel by *Aspergillus* sp. CH-Y-1043, *Biotechnol. Lett.* 11 (1989) 729–734.
- [40] R.R.S. Kiran, R. Konduri, G.H. Rao, G.M. Madhu, Statistical optimization of endo-polygalacturonase production by overproducing mutants of *Aspergillus Niger* in solid-state fermentation, *J. Biochem. Technol.* 2 (2010) 154–157. <https://jbiochemtech.com/storage/models/article/rwiQ4d6L5Zagx7SCCv9iD3p5Kpe6HHeRvSe8UOnz63ese0KHeYdf4q9qJT8/statistical-optimization-of-endo-polygalacturonase-production-by-overproducing-mutants-of-aspergil.pdf>.
- [41] S.B. Imandi, S.K. Karanam, H.R. Garapati, Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (*Brassica napus*), *Braz. J. Microbiol.* 44 (2013) 915–921.
- [42] Y. Xu, Y. Li, S. Xu, Y. Liu, X. Wang, J. Tang, Improvement of xylanase production by *Aspergillus Niger* XY-1 using response surface methodology for optimizing the medium composition, *J. Zhejiang Univ. - Sci. B.* 9 (2008) 558–566.
- [43] M. Palaniyappan, V. Vijayagopal, R. Viswanathan, T. Viruthagiri, Statistical optimization of substrate, carbon and nitrogen source by response surface methodology for pectinase production using *Aspergillus fumigatus* MTCC 870 in submerged fermentation, *Afr. J. Biotechnol.* 8 (2009) 6355–6363.
- [44] E.V.N. Raju, G. Divakar, Production of pectinase by using *Bacillus circulans* isolated from dump yards of vegetable wastes, *Int. J. Pharma Sci. Res.* 4 (2013) 2615–2622.
- [45] H. Abbasi, M.H. Fazaelpour, Pectinase Production in a Defined Medium Using Surface Culture Fermentation 1, 2010, pp. 5–10. <https://elmmet.ir/article/466056-42851/Pectinase-Production-in-a-Defined-Medium-Using-Surface-Culture-Fermentation>.
- [46] I. Darah, M.M.J. Taufiq, and S.H. Lim, Pomelo Citrus grandis (L.) osbeck peel as an economical alternative substrate for fungal pectinase production, *Food Sci. Biotechnol.* 22 (2013) 1683–1690.
- [47] N. Mahesh, R. Vivek, M. Arunkumar, S. Balakumar, Statistical designing of enriched pectin extract medium for the enhanced production of pectinase by *Aspergillus Niger*, *Int. J. Pharm. Pharmaceut. Sci.* 6 (2014) 666–672. <https://innovareacademi.in/journal/ijpps/Vol6Suppl2/8566.pdf>.
- [48] M. Dilipkumar, M. Rajasimman, N. Rajamohan, Optimization of inulinase production using copra waste by *K. marxianus* var. *marxianus*, *Chem. Ind. Chem. Eng. Q.* 16 (2010) 319–327.
- [49] H. Parveen, N. Bisht, L. Tewari, Optimization and biochemical characterization of thermo-alkalotolerant cellulase enzyme from *Aspergillus terreus* PPCF, *Cellul. Chem. Technol.* 54 (2020) 967–976. [https://www.cellulosechemtech.com.ro/pdf/CCT9-10\(2020\)/p.967-976.pdf](https://www.cellulosechemtech.com.ro/pdf/CCT9-10(2020)/p.967-976.pdf).
- [50] L. Graciano, et al., A thermotable xylanase from a new strain of *Aspergillus fumigatus* presents high ability to hydrolyze hemicellulose from corn straw, *Brazilian J. Dev.* 6 (2020) 69054–69077.
- [51] P. Sakthivelvan, B. Naveena, N. Partha, Effect of medium composition and ultrasonication on xylanase production by *Trichoderma harzianum* MTCC 4358 on novel substrate, *Afr. J. Biotechnol.* 11 (2012) 12067–12077.
- [52] H.A. ElEnshasy, E.A. Elsayed, N. Suhaimi, R. Abd Malek, M. Esawy, Bioprocess optimization for pectinase production using *Aspergillus Niger* in a submerged cultivation system, *BMC Biotechnol.* 18 (2018) 71–83.
- [53] C. Kuvvet, S. Uzuner, D. Cekmecelioglu, Improvement of pectinase production by co-culture of *Bacillus* spp. using apple pomace as a carbon source, *Waste Biomass Valorization* 10 (2019) 1241–1249.
- [54] D.D. Bhagat, P.R. Dudhagara, P. VDesai, Statistical approach for pectinase production by *Bacillus firmus* SDB9 and evaluation of pectino-xylanolytic enzymes for pretreatment of kraft pulp, *J. Microbiol. Biotechnol. Food Sci.* 5 (2016) 396–406.
- [55] A. Mohandas, et al., Production of pectinase from *Bacillus sonorensis* MPTD1, *Food Technol. Biotechnol.* 56 (2018) 110–116.
- [56] N. Bibi, S. Ali, R. Tabassum, Statistical optimization of pectinase biosynthesis from orange peel by *Bacillus licheniformis* using submerged fermentation, *Waste Biomass Valorization* 7 (2016) 467–481.

- [57] E.G. Aklilu, Modeling and optimization of pectin extraction from banana peel using artificial neural networks (ANNs) and response surface methodology (RSM), *J. Food Meas. Charact.* 15 (2021) 2759–2773.
- [58] Q. Le-Pham-Tan, A. Le-Thi-Lan, T. Mai-Vo-Thi-Kieu, T. Le-Thi, Optimization of the pectin extraction from pomelo peels by oxalic acid and microwave, *Banat. J. Biotechnol.* 5 (2014) 67–73.
- [59] A.A. Sundarraj, R.T. Vasudevan, G. Sriramulu, Optimized extraction and characterization of pectin from jackfruit (*Artocarpus integer*) wastes using response surface methodology, *Int. J. Biol. Macromol.* 106 (2018) 698–703.
- [60] M. Shahedi, M. Yousefi, Z. Habibi, M. Mohammadi, M.A. As' habi, Co-immobilization of *Rhizomucor miehei* lipase and *Candida Antarctica* lipase B and optimization of biocatalytic biodiesel production from palm oil using response surface methodology, *Renew. Energy* 141 (2019) 847–857.
- [61] T. Özlem, A.Y. Dursun, Optimization of endo-pectinase and pectin lyase production from wheat bran by *Bacillus pumilus* using response surface methodology, *Gazi Univ. J. Sci.* 34 (2021) 335–353.
- [62] Y. Li, F. Cui, Z. Liu, Y. Xu, H. Zhao, Improvement of xylanase production by *Penicillium oxalicum* ZH-30 using response surface methodology, *Enzym. Microb. Technol.* 40 (2007) 1381–1388.
- [63] Y. Cao, D. Meng, J. Lu, J. Long, Statistical optimization of xylanase production by *Aspergillus Niger* AN-13 under submerged fermentation using response surface methodology, *Afr. J. Biotechnol.* 7 (2008) 631–638.
- [64] O. Tepe, A.Y. Dursun, Exo-pectinase production by *Bacillus pumilus* using different agricultural wastes and optimizing of medium components using response surface methodology, *Environ. Sci. Pollut. Res.* 21 (2014) 9911–9920.
- [65] D.F. Hamilton, M. Ghert, A. Simpson, Interpreting regression models in clinical outcome studies, *Bone Joint Res* 4 (2015) 152–153.
- [66] S. Kumar, H.K. Sharma, B.C. Sarkar, Effect of substrate and fermentation conditions on pectinase and cellulase production by *Aspergillus Niger* NCIM 548 in submerged (SmF) and solid state fermentation (SSF), *Food Sci. Biotechnol.* 20 (2011) 1289–1298.
- [67] P.R. Mohan, P.V. Kumar, O.V.S. Reddy, Optimization of media and submerged fermentation conditions using central composite design for increased endoglucanase production by *Cladosporium* sp. NCIM 901, *Turkish J. Biochem. Biyokim. Derg.* 38 (2013) 385–395.
- [68] J. Ali, S.A. Jaffery, Q. Assad, A. Hussain, H. Abid, F. Gul, Optimization of pectinase enzyme production using sour oranges peel (*Citrus aurantium* L.) as substrate, *Pakistan J. Biochem. Mol. Biol.* 43 (2010) 126–130. [http://www.pjmb.org/pjmb/ages/PJBMBArchive/2010/PJBMB\\_43\\_3\\_Sep\\_2010/05.pdf](http://www.pjmb.org/pjmb/ages/PJBMBArchive/2010/PJBMB_43_3_Sep_2010/05.pdf).
- [69] B. Durairajan, P.S. Sankari, Optimization of solid state fermentation conditions for the production of pectinases by *Aspergillus Niger*, *J. Pharm. BioSci.* 2 (2014) 50–57. [https://www.speronline.com/jpbs/Articlefile/34\\_pdf.pdf](https://www.speronline.com/jpbs/Articlefile/34_pdf.pdf).
- [70] A.-L. Kjøniksen, M. Hiorth, B. Nyström, Association under shear flow in aqueous solutions of pectin, *Eur. Polym. J.* 41 (2005) 761–770.
- [71] R. Yusof, S.Z.A. Zaini, M.A. Azman, Characterization of pectin extracted from guava peels using deep eutectic, *Charting Sustain. Futur. ASEAN Sci. Technol. Proc. from 3rd Int. Conf. Futur. ASEAN 2* (2019) 421.
- [72] R.A. Hours, C.E. Voget, R.J. Ertola, Some factors affecting pectinase production from apple pomace in solid-state cultures, *Biol. wastes.* 24 (1988) 147–157.
- [73] A.D. Juwon, O.F. Emmanuel, and others. Screening of fungal isolates from Nigerian tar sand deposit in Ondo State for novel biocatalysts, *J. Biol. Sci.* 12 (2012) 57–61.
- [74] S. Mrudula, R. Anitharaj, Pectinase production in solid state fermentation by *Aspergillus Niger* using orange peel as substrate, *Glob. J. Biotechnol. Biochem.* 6 (2011) 64–71. [https://idosi.org/gjbb/gjbb6\(2\)11/4.pdf](https://idosi.org/gjbb/gjbb6(2)11/4.pdf).
- [75] E. Mardawati, T. Rialita, B. Nurhadi, and others. Optimization of moistening solution concentration on xylanase activity in solid state fermentation from oil palm empty fruit bunches, *IOP Conf. Ser. Earth Environ. Sci.* 141 (2018) 1–10.