Optimization of substrate composition for pectinase production from Satkara (Citrus macroptera) peel using Aspergillus niger-ATCC 1640 in solid-state fermentation

Tanvir Ahmeda, Md Rahmatuzzaman Ranaa,⁎, Wahidu Zzaman a, Rowshon Araa, Mohammad Gulzarul Aziz b

a Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet, 3100, Bangladesh
b Department of Food Technology and Rural Industries, Bangladesh Agricultural University, Mymensingh, 2202, Bangladesh

ARTICLE INFO

Keywords:
Box-Behnken design
Enzymatic hydrolysis
Pectinase
Response surface methodology
Utilization of Satkara peel

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, (NH₄)₂PO₄, NH₄NO₃, KH₂PO₄, ZnSO₄, and MgSO₄.7H₂O 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 (NH₄)₂SO₄ significantly affected the pectinase production. RSM results indicated that the predicted response for pectinase production was in good agreement with experimental data (R² = 0.9836). Under the optimized condition of Satkara peel (8.4 g/L), urea (0.5 g/L), and (NH₄)₂SO₄ (2.7 g/L), the pectinase activity was predicted to be 0.6178 μmol/mL. In the present study, the experimental pectinase production achieved 0.6045 μmol/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 reported Satkara as a potential pharmacological agent [8, 9]. Besides, in a previous study, Islam &...
Rahman-Un-Nasir [10] have found the low methoxyl pectin content ranged from 2.35 ± 0.18 to 1.89 ± 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 exoglucanases and endoglucanases 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 Satkara peel as a sole carbon substrate. At the same time, urea, (NH₄)₂SO₄, NH₂NO₃, KH₂PO₄, ZnSO₄, and MgSO₄·7H₂O 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 °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) (34–38 °C) for 30 min to ensure complete hydration. Later, the hydrated material was vortexed (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 °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 30–40 °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 dextrose 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 °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 CaCl₂ (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, the suspension medium composed of 0.25 g/L CaCl₂ (mineral salt) and 0.5 g/L KH₂PO₄ (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.

2.7. 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 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, R² = 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, (NH₄)₂SO₄, NH₄NO₃, KH₂PO₄, ZnSO₄, and MgSO₄·7H₂O). 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.

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 (NH₄)₂SO₄ (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 (NH₄)₂SO₄ 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 (X₁), urea (X₂), and (NH₄)₂SO₄ (X₃), 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.

\[ Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_i^2 X_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} B_{ij} X_i X_j \]  

where, Y is the predicted pectinase yield; K is the number of factors studied; Xᵢ and Xⱼ are the independent variables; B₀ is a constant; and Bᵢ, Bᵢᵢ, and Bᵢⱼ 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

| Variables | Levels |
|-----------|--------|
| Satkara peel (g/L) | Low (-1) | High (+1) |
| Urea (g/L) | 0.50 | 0.75 |
| (NH₄)₂PO₄ (g/L) | 2.5 | 3.0 |
| NH₄NO₃ (g/L) | 0.5 | 1.0 |
| KH₂PO₄ (g/L) | 0.1 | 0.2 |
| ZnSO₄ (g/L) | 0.01 | 0.02 |
| MgSO₄·7H₂O (g/L) | 0.5 | 1.0 |

Table 1. Screening components and their levels for the Plackett-Burman Design.
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–Y1043. 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 significant nitrogen source with urea, multiple investigations have found NH4NO3 and (NH4)2SO4 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 KH2PO4 by PBD as mineral salt that influenced the pectinase production. Dilipkumar et al. [48] and Parveen et al. [49] showed the significant effect of ZnSO4 as a metal ion on the activity of inulinase and cellulase enzyme, respectively. The same trend was also recorded in the case of MgSO4·7H2O. 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 MgSO4·7H2O showed the maximum pectinase activity in both fermentation processes among six metals salts. Similar positive effects of MgSO4·7H2O on the xylanase enzyme production were also reported by Graciano et al. [50] and Sakhishelvan et al. [51].

In the present study, in order to identify the most significant components for pectinase production, a total of seven components, namely, Satkara peel as carbon source, urea, (NH4)2SO4, and NH4NO3 as nitrogen source, KH2PO4, ZnSO4, and MgSO4·7H2O 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 to 0.5670 μmol/mL, which indicated the requirement of further medium components optimization. Additionally, the coefficient of determination (R2) for pectinase activity was R2 = 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 2. Plackett-Burman Design for screening the major medium components influencing the pectinase production.

| Trial No. | Satkora peel (g/L) | Urea (g/L) | (NH4)2PO4 (g/L) | NH4NO3 (g/L) | KH2PO4 (g/L) | ZnSO4 (g/L) | MgSO4·7H2O (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                 |

---

### 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   |
| (NH4)2SO4 (g/L)   | 1  | 0.0187 | 0.0187 | 8.03    | 0.047   |
| NH4NO3 (g/L)      | 1  | 0.0069 | 0.0069 | 2.97    | 0.160   |
| KH2PO4 (g/L)      | 1  | 0.0159 | 0.0159 | 6.85    | 0.059   |
| ZnSO4 (g/L)       | 1  | 0.0002 | 0.0002 | 0.10    | 0.764   |
| MgSO4·7H2O (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.
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 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 that the optimization of fermentation conditions increased the activity of polygalacturonase (27 U/mL) and polygalacturonate lyases (40 U/mL), respectively, 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 (NH4)2SO4 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 (X1) (5.0–10.0 g/L), urea (X2) (0.5–1.0 g/L), and (NH4)2SO4 (X3) (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.

\[
Y(\text{Total pectinase, \mu mole / mL}) = -0.3937 + 0.2374 \times X_1 - 0.0145
\]
\[
\times X_2 + 0.0424 \times X_3 - 0.0743 \times X_1 \times X_2 + 0.0054 \times X_1 \times X_3 + 0.0948 \times X_2 \times X_3
\]
\[
-0.0124(X_1)^2 + 0.2074(X_2)^2 - 0.0281(X_3)^2
\]

Eq. 2

According to the experimental response, the amount of pectinase enzyme produced by Aspergillus niger ATCC 16404 varied from 0.4335 to 0.6173 \(\mu\text{mol/mL}\) (Table 5). Moreover, the maximum production of pectinase (0.6173 \(\mu\text{mol/mL}\)) was noticed at Satkara peel (7.5 g/L), urea (0.5 g/L), and (NH4)2SO4 (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 4. Coded values of the selected medium components used in the experimental design of Box-Behnken.

| Medium components | Symbols | Coded levels |
|-------------------|--------|-------------|
| Satkara peel (g/L) | X1     | -1 0 1      |
| Urea (g/L)        | X2     | 5.0 7.5 10.0|
| (NH4)2SO4 (g/L)  | X3     | 0.5 0.75 1.0|

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

| Trial number | Medium components | Total pectinase (\(\mu\text{mol/mL}\)) | Experimental response |
|--------------|-------------------|--------------------------------------|-----------------------|
|              | X1 (Satkara peel) | X2 (Urea) | X3 ((NH4)2SO4) |                              |
| 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                             |

**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 production...
also observed a strong similarity between the predicted value and actual values of pectinase activity. Aklilu [58] and jackfruit waste [59] have 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 and are 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. 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 ±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.

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

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   | 0.046E-003  | 33.40   | 0.0006  |          |
| X1           | 7.381E-003     | 1   | 7.381E-003  | 54.20   | 0.0007  |          |
| X2           | 2.808E-004     | 1   | 2.808E-004  | 2.06    | 0.105   |          |
| X3           | 4.410E-004     | 1   | 4.410E-004  | 3.24    | 0.1318  |          |
| X1X2         | 8.630E-003     | 1   | 8.630E-003  | 63.37   | 0.0005  |          |
| X1X3         | 1.822E-004     | 1   | 1.822E-004  | 1.34    | 0.2996  |          |
| X2X3         | 5.617E-003     | 1   | 5.617E-004  | 4.12    | 0.0980  |          |
| X1          | 0.0022         | 1   | 0.022       | 162.35  | <0.0001 |          |
| X2          | 6.208E-004     | 1   | 6.208E-004  | 4.56    | 0.0859  |          |
| X3          | 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  |             | 9.836   |         |          |
| CV%         | 2.13           |     |             |         |         |          |
| R²          | 0.9836         |     |             |         |         |          |
| Adjusted R² | 0.9542         |     |             |         |         |          |
| Predicted R²| 0.7899         |     |             |         |         |          |
| Adeq Precision | 17.469  |     |             |         |         |          |

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' 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, X1 (Satkara peel), interactions like X1X2 (Satkara peel and urea), X1X3 (urea and (NH4)2SO4), X12 (Satkara peel)2, X22 (urea)2 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 relatives to the pure error, the value is not

![Figure 1](image-url)
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 interactions 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 (NH$_4$)$_2$SO$_4$, the highest pectinase response was obtained with a minimum concentration.

A non-significant lack of fit for 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 (NH$_4$)$_2$SO$_4$. Although keeping the urea at the mid level (5.0 g/L), the highest pectinase activity (0.6173 μmol/mL) was obtained at the mid-level of Satkara peel (7.5 g/L) and low level of

![Figure 2](image)

**Figure 2.** (a) Pareto chart illustrating the standardized effects of three variables: Satkara peel ($X_1$), urea ($X_2$), (NH$_4$)$_2$SO$_4$ ($X_3$) and their interaction effects on the pectinase production. (b) Main effects plot of Satkara peel, urea, and (NH$_4$)$_2$SO$_4$ on pectinase production.
(NH₄)₂SO₄ (2.5 g/L). This result is in agreement with the report of El Enshasy et al. [52]. They found that (NH₄)₂SO₄ was less effective on pectinase production during the interaction between pectin and (NH₄)₂SO₄ by keeping K₂HPO₄ concentration constant. Similarly, Hours et al. [72] evaluated the effects of (NH₄)₂SO₄ on the pectinase production in solid-state cultures from apple pomace. At lower concentrations, (NH₄)₂SO₄ was observed to have no effect on the production of pectinases. One possible reason behind the decreased pectinase activities is that the Aspergillus niger-ATCC 16404 strain presumably failed to hydrolyze the (NH₄)₂SO₄ 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 (NH₄)₂SO₄. The contour plot in the form of a saddle shape indicates somewhat unusual results. Both plots illustrate that low urea and low (NH₄)₂SO₄ content contribute to enhancing pectinase activity. In this study, as shown in Table 5, the highest level of pectinase activity (0.6173 μmol/mL) was achieved at low-level urea of 0.5 g/L and low (NH₄)₂SO₄ of 2.5 g/L, which validate Figure 3e, f. In a study, Mru-dula et al. [74] found that the second-highest pectinase production (208.7 U/g DMS) was obtained by a combination of urea and (NH₄)₂SO₄ (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 (NH₄)₂SO₄ 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.
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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ 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 (R² = 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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄. 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 is solid-state fermentation.

Declarations

Author contribution statement

Tanvir Ahmed: Performed the experiments; Analysed and interpreted the data; Wrote the paper.
Md Rahmatuzzaman Rana: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Wahiduzzaman: Analyzed and interpreted the data.
Rowshan Ara: Contributed reagents, materials, analysis tools or data.
Mohammad Gulzarul Aziz: Conceived and designed the experiments.

Funding statement

This work was supported by Shahjalal University of Science and Technology (AS/2017/1/22) research center.

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.

Acknowledgements

The authors express gratitude to the Food Engineering and Tea Technology (FET) laboratory of Shahjalal University of Science and Technology (SUST) for technical assistance and other supports throughout the research work.

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.
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.

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.

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.

M. Shahedi, M. Yousefi, Z. Halabi, M. Mohammadzadi, 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.

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.

Y. Li, F. Cai, 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.

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.

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.

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) 1289–1296.

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.

A.-L. Kjøniksen, M. Hiorth, B. Nyström, Association under shear flow in aqueous solutions of pectin, Eur. Polym. J. 41 (2005) 761–776.

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 (2020) (2019) 421.

R.A. Hours, C.E. Vogel, R.J. Ertola, Some factors affecting pectinase production from apple pomace in solid-state cultures, Biol. wastes. 24 (1988) 147–157.

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.

S. Murdula, 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://doi.org/gjbb/gjb6(2011)/4.pdf.

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.