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# Optimisation of Xylanase–Pectinase Cocktail Production with *Bacillus amyloliquefaciens* ADI2 Using a Low-Cost Substrate via Statistical Strategy

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**Abstract:** An effective statistical tool for increasing and boosting the production of xylanase and pectinase by *Bacillus amyloliquefaciens* ADI2 during submerged fermentation (SmF) appears to be the response of surface methodology (RSM) using the central composite design (CCD). Optimum production was achieved under fermentation conditions of a temperature of 28 °C, pH of 8.38, inoculum size of 4% (*w*/*v*) and agitation speed of 94 rpm for 48 h. The experimental responses demonstrated a near agreement with the expected responses under optimum conditions of independent variables, suggesting the model's validity. The optimised CCD model had a 1.34-fold, 159 ± 6 U/mL greater xylanase and 5.96-fold, 205 ± 9 U/mL greater pectinase production than the one factor at a time (OFAT) approach. The production of concurrent enzymes of xylanase–pectinase resulted in a ratio of 1:1.3.

**Keywords:** RSM; xylanase; pectinase; *Bacillus*; production

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## 1. Introduction

Xylanases and pectinases have a broad range of applications in biotechnology, such as in wastewater treatment, the digestion of animal feed, food processing, brewing technology, biofuels and textiles, as well as the pulp and paper industries [1–6]. It is crucial to study the effects of pH and temperature on their functional aspects and stabilities, which significantly influence their potential usages. For example, in the pulp and paper industry, which demand that the enzymes used must be stable in the industrial process spectrum, with a wide pH and temperature range [7,8].

Other than that, the main bottleneck of the inclusive use of enzymes in the industry is attributable to the high cost of production. To obtain economic feasibility, it is essential that the production of enzymes and microbial growth be optimised. There are varieties of optimal parameters that need to be studied in enzymes' biosyntheses, such as media compositions, environmental conditions and variations of the producing strains. The enzymes produced under submerged and solid-state conditions are influenced by various nutritional and fermentation parameters. These parameters must, therefore, be designed to maximise the production of the enzymes. In submerged fermentation (SmF), microorganisms are grown in suspension in a basal medium, wherein different nutrients are dissolved or suspended in several commercial media as particulate solids [9].

SmF is the most suitable method for the industrial production of enzymes. This is because SmF systems offer easier sterilisation and involve a less-laborious process. Nevertheless, to produce microbial enzymes at a lower operating cost and capital investment, solid-state fermentation (SSF) could be an alternative system [10–13]. Since SmF utilises free-flowing liquid substrates, it would be the best method for bacterial work as bacteria require a high moisture content, unlike SSF, which is implemented in the absence of free-flowing water [9,14]. In comparison, to study the physiological aspects of enzyme synthesis and secondary metabolite production, the SmF system is highly recommended [15,16].

Proportionally, most enzymes (about 90%) are generated industrially using SmF technology. In other words, SmF is preferable on account of several benefits compared to the other fermentation modes. One of the benefits is that the production process requires a short fermentation time while simultaneously reducing the mixed microbial contamination. The conversion level of the raw medium substrates is also higher due to the regulated growth cycle, well advanced and improved scale-up method, thus reducing the use of the production-monitoring lab [17,18]. Furthermore, the environmental control of SmF is easy to monitor as the homogenous microbial suspension, nutrient solution and products are in a liquid state.

Generally, fundamental research on industrial applications aims to minimise enzyme production costs by optimising the fermentation medium [19]. This, in turn, will aid in a cost-effective manufacturing process. In general, "one factor at a time" (OFAT) and RSM strategies were used in this study. The purpose of this study was to evaluate the optimal conditions for xylano-pectinolytic enzyme production with *Bacillus amyloliquefaciens* ADI2 using the statistical approach and RSM.

## 2. Materials and Methods

### *2.1. Bacterial Suspension Preparation*

*Bacillus amyloliquefaciens* ADI2 (accession number MG726535) used in this study was isolated from soil and molecularly identified by the Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia, Malaysia. *Bacillus amyloliquefaciens* ADI2 was inoculated in nutrient broth and kept in an incubator shaker under 150 rpm at 30 °C for 12 h, which turned the culture into the log growth phase period, where the pre-specified volume of 12 h of grown cultures (each maintained at $3.2 \times 10^8$ CFU/mL) was used for inoculation of the fermentation medium. Optical density (λ 600 nm) was used to monitor the bacterial growth, and the concentration of bacterial suspension was determined using a UV-VIS spectrophotometer [20]. Dilution was performed to create the bacterial suspension of the desired concentration.

### *2.2. Production of Enzymes*

Under SmF, xylano-pectinolytic enzymes were produced in 250-mL Erlenmeyer flasks, which contained 50 mL of basal medium (g/L: peptone, 5; yeast extract, 5; $KNO_3$, 5; $KH_2PO_4$, 1; $MgSO_4 \cdot H_2O$, 0.1; pH 8.5). The medium was supplied with 2 carbon sources, 2% oil palm trunk and orange peel [21,22]. Prior to use, substrates were washed, dried overnight, ground and screened to collect small particles. Crude enzymes were extracted by centrifugation at 10,000× *g* for 20 min at 4 °C using a refrigerated benchtop centrifuge, and the clear supernatant was used for xylanase and pectinase assays, quantification of reducing sugars, and the estimation of protein [23].

### *2.3. Enzymes Assay*

A mixture of 10 µL of an enzyme solution and 490 µL of respective substrates (birchwood xylan, 1%; polygalacturonic acid, 0.5%; both supplied by Sigma-Aldrich, Darmstadt, Germany, ≥90% purity) in 0.01 M glycine-NaOH buffer (pH 8.5) was used to evaluate the activity of xylanase and pectinase [23], and the reaction was performed at 30 °C for 20 min. The 3,5-dinitrosalicylic acid (DNS) assay approach was used to calculate the release of reducing sugar according to Miller et al. (1959) [24]. The reaction was terminated by adding

1.5 mL of 3,5-dinitrosalicyclic acid (DNS) reagent. The contents were boiled for 15 min, and after cooling, the absorbance was read at λ 540 nm. The activity of xylanase and pectinase was expressed as μmol of reducing sugar released per min per millilitre (U/mL). Xylose and galacturonic acid were used as a standard for the respective enzymes.

### *2.4. Effect of Culture Conditions on Bacillus amyloliquefaciens ADI2 Xylano-Pectinolytic Enzymes Production*

The effect of initial pH of the media, temperature, inoculum concentration of bacterial suspension, agitation speed, and different substrates on *Bacillus amyloliquefaciens* ADI2 growth and xylanase–pectinase enzymes production were analysed using the OFAT approach. The pH of the medium was adjusted from pH 5.0 to pH 11.0 at 0.5-unit increments (before autoclaving) by using 1 N NaOH and 1 M HCl. The temperature ranged from 20 °C to 70 °C (20, 30, 37, 40, 50, 60 and 70 °C) and was set and maintained in different shaking incubators. Different inoculum concentrations were used from 1% to 10% ($v/v$) in 1% increments. The inoculum concentrations above 10% ($v/v$) are not preferable in industrial fermentation. Various agitation speeds were used in 50-unit increments from static to 250 rpm. To study the effect of different substrates, each agricultural waste was locally obtained from the domestic market, with different 2% ($w/v$) agro-wastes, such as oil palm trunk (OPT), oil palm leaf (OPL), empty fruit bunch (EFB), orange peel (OP), papaya peel (PP), pomegranate peel (POP), mango peel (MP), grape pomace (GP), banana peel (BP), apple pomace (AP) and corn stalks (CS). They were washed, dried overnight, ground and screened through 0.30 mm mesh to collect small particles to be used as a solid substrate in submerged fermentation. Fermentation was performed for 72 h, and samples were collected for pH and optical density (OD) analysis. Crude xylanase and pectinase were extracted for 20 min at 4 °C by centrifugation at 10,000× $g$, and the clear supernatant was used for enzymes' assay, reducing sugar quantification and protein estimation.

### *2.5. Optimisation of Xylano-Pectinolytic Enzymes Production Using Central Composite Design (CCD)*

The effects of the initial pH of the media, temperature, inoculum concentration, and agitation speed on the xylanase and pectinase activities were investigated using the CCD at three different levels (−1, 0, +1), including 6 replicates of the centre points and 8 axial points. Table 1 lists the coded and uncoded ranges and the levels of the experimental variables. Design-Expert software (Version 10, Stat-Ease, Inc., Minneapolis, MN, USA) was used to conduct data analysis and response graphics. The experimental data were introduced for the polynomial equation of the second order, including each variable of individual, quadratic and cross-effects according to Equation (1).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where $Y$ = predicted response, $\beta_0$ = offset term, $\beta_1$ = linear effect, $\beta_{11}$ = square effect, $\beta_{12}$ = interactive effect and A–C = variables.

**Table 1.** Coded and uncoded ranges of variables of CCD for xylanase and pectinase production.

| Variables | Values of CCD Variables | | | | |
|---|---|---|---|---|---|
| | $-\alpha$ | −1 | 0 | +1 | $+\alpha$ |
| A. Initial pH of media | 5.5 | 7 | 8.5 | 10 | 11.5 |
| B. Temperature (°C) | 11.5 | 20 | 28.5 | 37 | 45.5 |
| C. Inoculum concentration (%) | 2 | 3 | 4 | 5 | 6 |
| D. Agitation speed (rpm) | 0 | 50 | 100 | 150 | 200 |

A table of variance analysis (ANOVA) was developed, and individual linear, quadratic and interaction terms were calculated for their effects and regression coefficients. The significance of all polynomial terms was determined in comparison to *p*-values lower than 5% significance level [25].

### *2.6. Validation of the Model*

As proposed by the software, the optimum values of the chosen variables were obtained. The optimal conditions suggested were used to validate the adequacy and validity of the predictive model.

## 3. Results and Discussion

### *3.1. Effect of pH on Xylanase and Pectinase Production*

The initial pH of the media greatly affected the secretion and synthesisation of enzymes as well as the transportation through the cell membrane of many enzyme species [26,27]. The fermentation media were inoculated with a 4% inoculum, an agitation speed at 150 rpm, a temperature of 30 °C, and 2% oil palm trunk and orange peel as the carbon source for 48 h. In this study, it was observed that beyond the pH of 10, there was a slight drop in enzyme activities. This could mean that the enzyme activities and the growth of *Bacillus amyloliquefaciens* ADI2 slowed down. In addition, it was found that pH can also affect bacterial physiology during the regulation and maintenance of homeostasis. It is a well-known fact that bacterial cells are flexible, extensible and elastic as a result of their peptidoglycan layers [28,29]. Therefore, changes in pH could result in the damage or autolysis of the peptidoglycans and thereby the loss of the ability to provide mechanical support to the bacteria [30]. Additionally, the contraction and expansion of the bacteria cell walls due to changes in pH may result in an increase or a decrease in cellular lysis, which affects its overall performance [30].

However, the culture was capable of producing xylanase at pH values of pH 5.5 to pH 9.5 and pectinase at pH 8.5 to pH 9.5 (above 40 U/mL), and maximum production of xylanase and pectinase was observed at a pH of 8.5 (Figure 1a). The results indicated that *Bacillus amyloliquefaciens* ADI2 is a facultative alkalophilic bacteria.

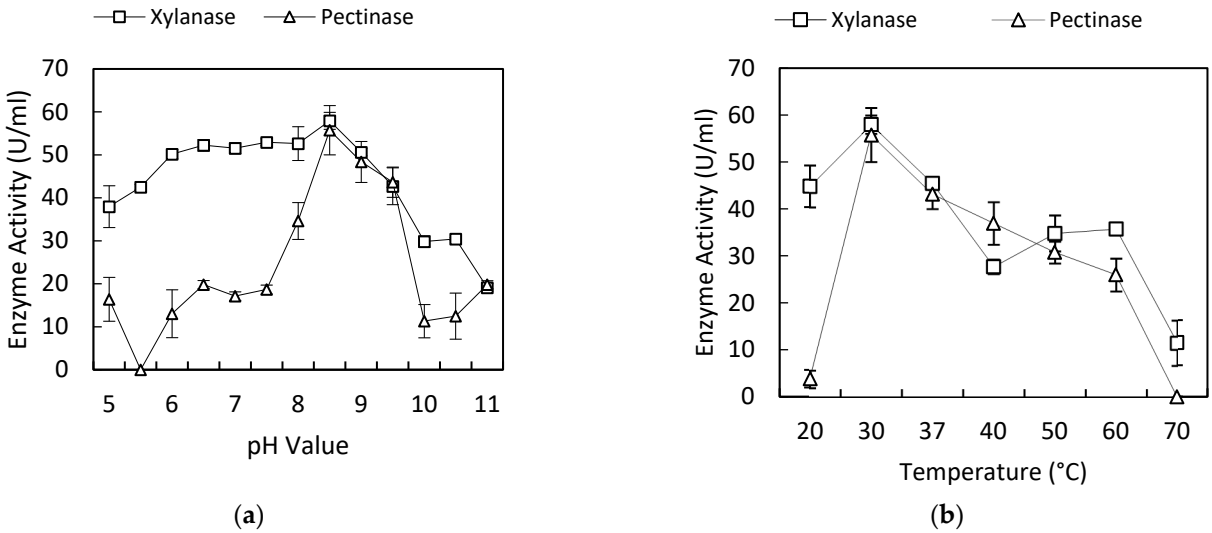


**Figure 1.** *Cont.*

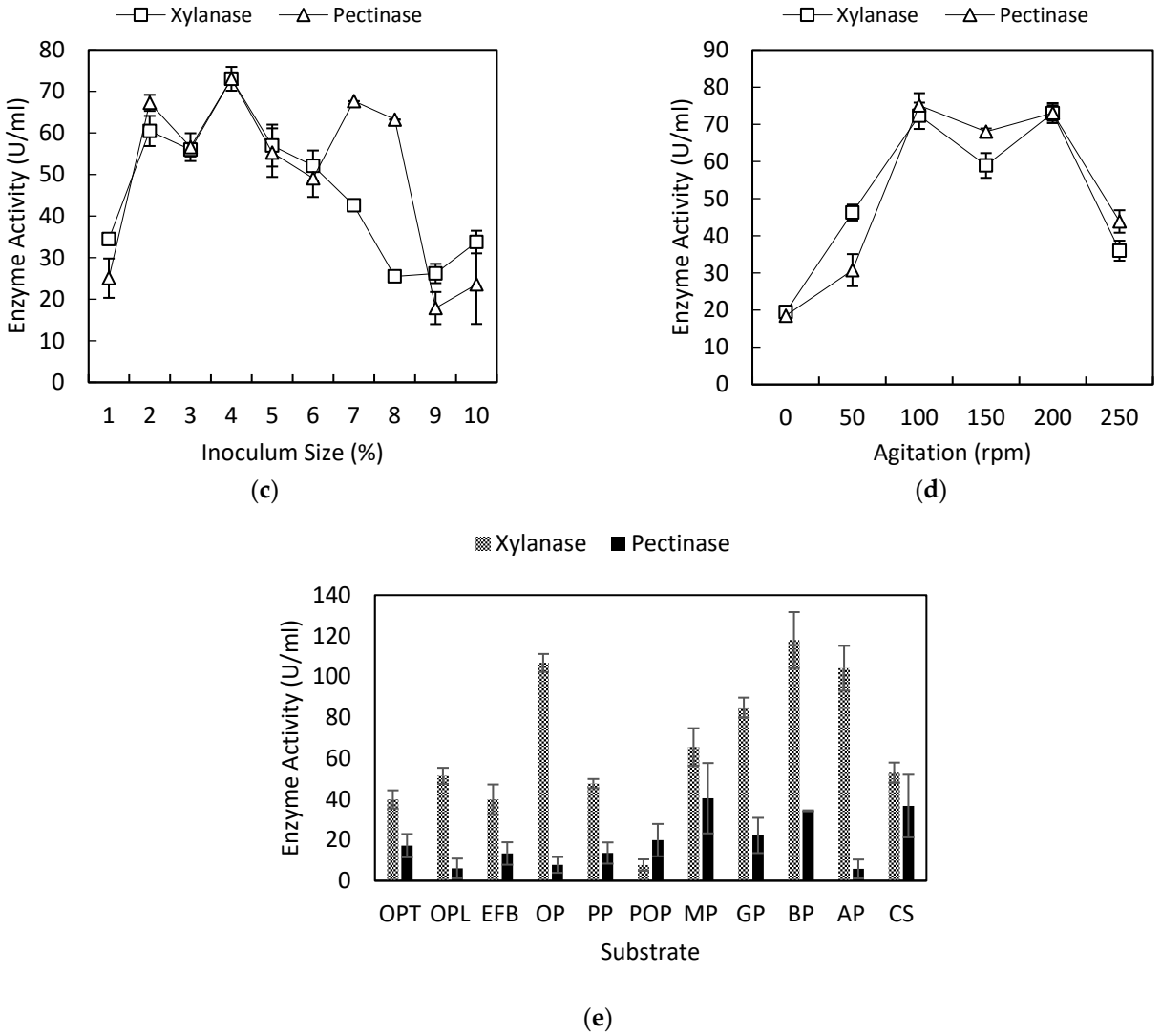


**Figure 1.** The pattern of enzyme activity in basal medium with different (**a**) pH values, (**b**) temperatures, (**c**) inoculum concentrations, (**d**) agitation speeds, (**e**) and substrates on xylanase and pectinase production by *Bacillus amyloliquefaciens* ADI2 after 48 h. Oil palm trunk (OPT); oil palm leaf (OPL); empty fruit bunch (EFB); orange peel (OP); papaya peel (PP); pomegranate peel (POP); mango peel (MP); grape pomace (GP); banana peel (BP); apple pomace (AP); corn stalks (CS).

### *3.2. Effect of Temperature on Xylanase and Pectinase*

During the study on the effect of temperature, where the inoculum size, agitation speed and substrate used were similar as in Section 3.1, and the pH was kept constant at an optimum value of pH 8.5, it was observed that the maximum production of xylanase and pectinase was at the temperature within the range of 20 °C to 70 °C. It was also observed that the optimal production of xylanase and pectinase was at 30 °C, and the temperature was within the optimum temperature for other *Bacillus* spp. such as *Bacillus mojavensis* AG137 [31], *Bacillus subtilis* [32] and *Bacillus halodurans* M29 [33]. For xylanase, it was observed that the production at 20 °C and 37 °C was functionally 77.35% and 79% greater in comparison to enzyme production at 30 °C. Increasing the temperature to 40 °C, 50 °C and 60 °C resulted in the lowering of the production to an average level of 56.62% (Figure 1b). Likewise, it was also observed that pectinase production also slightly decreased to 77.41% at 37 °C. The production continued to decline steadily to 66.31% at 40 °C, 55.21% at 50 °C and then totally stopped at 70 °C. It was evident that temperature impacts the various biological processes. The secretion of the extracellular enzyme may have been influenced

by the change in the physical properties of the cell membrane [34]. At a high incubation temperature, there might be denaturation of extracellular enzyme activities. Generally, it was observed that this phenomenon occurred after the activities of the enzymes had reached their peak, probably after growth had ceased [35].

Conclusively, *Bacillus amyloliquefaciens* ADI2 could grow and produce both xylanase and pectinase at a range of temperatures between 30 °C to 60 °C, and therefore, this organism could be classified as a thermotolerant bacterium capable of producing enzymes at a temperature up to 50 °C. It is also a fact that thermotolerant bacteria grow best at a temperature above 45–50 °C [36]. In addition, it was found that the best production of xylanase and pectinase was achieved when the temperature was 30 °C.

### *3.3. Effect of Inoculum Concentration on Xylanase and Pectinase Production*

Several studies indicated that the concentration of inoculum and its physiological conditions were among the most significant variables influencing the cost and yield of enzyme production and organism fermentation profile [37,38]. The inoculum concentration was optimised for 48 h in a range of 1% to 10%. At this point, the values of the pH and temperature were adjusted based on the optimised values obtained from previous experiments. The agitation speed and substrates remained the same as described in Section 3.1. A study on the effect of different inoculum concentrations at 1% to 10% (with 1% interval, *v*/*v*) on the production of xylanase and pectinase in *Bacillus amyloliquefaciens* ADI2 was conducted. The concentration of inoculum above 10% (*v*/*v*) was not preferred in industrial fermentation [39,40]. Figure 1c shows that the highest xylanase production was at 4% (*v*/*v*) (73.04 U/mL), and the second-highest was at 2% (*v*/*v*), at 60.47 U/mL (82%). There have been several studies of higher production of xylanase and pectinase by *Bacillus* spp. at a lower inoculum concentration [41,42]. As for pectinase, the best production was achieved when the inoculum concentration was at 4% (*v*/*v*) and was closely followed by a concentration at 2% (*v*/*v*).

It was noted in this study that a 1% (*v*/*v*) inoculum concentration could not increase the production of enzymes. This may be due to the low concentration of cell biomass in the basal medium. Higher inoculum concentrations may have induced the compounds, which had an adverse effect on the production of xylanases and pectinases [39,40]. In this study, it was evident that increases in inoculum concentration resulted in a decreased production of enzymes. This may be attributed to cell clumping, which may have decreased the rate of sugar and oxygen absorption as well as the release of the enzyme [43–45]. In other words, competition for space and nutrients causes a decrease in the growth of the bacteria. Additionally, competition for nutrients could result in the accumulation of toxic products generated from the death of some of the bacteria, hence inhibiting the growth and enzyme production by surviving cells.

Conclusively, the best production of both xylanase and pectinase was achieved when the inoculum concentration was at 4% (*v*/*v*) compared to 2% (*v*/*v*), which was 146.08 U/mL compared to 127.73 U/mL.

### *3.4. Effect of Agitation on Xylanase and Pectinase Production*

The agitation process examined the oxygen requirement during the fermentation period. Erlenmeyer flasks were inoculated with 4% inoculum and placed onto a rotary shaker at different rpms (static, 50, 100, 150, 200 and 250 rpm) at 30 °C for 48 h. An increase in agitation can lead to the dissolution of oxygen in the medium, which is important for uniform distribution of the medium contents, such as catabolites and foodstuffs, and essential for microbial cell membrane components [46,47]. Another factor improving the rate of oxygen transmission is providing a large gas–liquid surface area, but this might induce a shear effect on the circulating cells. Mechanical agitation is important in SmF because it can prevent the cells from clumping and improve the mixing of the contents of the media [48]. With the agitation speed of 200 rpm, the maximum production of xylanase achieved was equal to 73.03 U/mL, which was close to 100 rpm with 72.33 U/mL. However,

low xylanase production resulted in agitation rates below 50 rpm. Similarly, xylanase production decreased when shaken at 250 rpm, while the lowest xylanase production when agitation was static. Another phenomenon that was observed was that pectinase production showed optimal activity at an agitation rate of 100 rpm, at 75.10 U/mL, followed by 200 and 150 rpm (with 97.26% and 90.58% compared to the optimal production) (Figure 1d). At static conditions, pectinase showed the lowest enzyme production.

A reason for this was that oxygen diffusion was limited in the stationary (or low-agitation-rate) culture, thus affecting the growth of the *Bacillus amyloliquefaciens* ADI2. High agitation rates also affected the biomass concentration, which caused the limitation of nutrient levels for cell growth to stagnate. This, in turn, influenced xylanase and pectinase production. Other than that, this may have been due to enzyme denaturation caused by high-speed agitation. Bacterial cells may have been damaged by high-speed agitation, thus resulting in a reduction in *Bacillus amyloliquefaciens* ADI2 in the medium and lowered production of xylanase and pectinase [49]. In comparison to xylanase and pectinase production rates, the highest total enzyme activity in the concurrent production of xylanase and pectinase was at 100 rpm, with 147.43 U/mL. Thus, it was considered as the optimum agitation rate for concurrent enzyme production. Other investigators also used an agitation speed between 100 to 150 rpm for xylanase production [50] and 100 to 200 rpm for pectinase production [51].

### *3.5. Effect of the Substrate on Xylanase and Pectinase Production*

The effect of the various substrates on the growth of *Bacillus amyloliquefaciens* ADI2 and the total production of xylanase and pectinase were studied by cultivating the bacteria isolated from 11 different agro-waste substrates: oil palm trunk, oil palm leaf, rice bran, sugarcane bagasse, empty fruit bunch, orange peel, papaya peel, pomegranate peel, mango peel, grape pomace, banana peel, apple pomace and corn stalks, while other conditions, such as pH, temperature, agitation speed and inoculum concentration, were adjusted according to optimised values. Figure 1e shows the effect of different agro-wastes on the total xylanase and pectinase enzymes production.

The highest total xylanase and pectinase enzyme production was obtained when banana peel was used as the carbon source (152.32 U/mL), followed by orange peel (114.51 U/mL) and apple pomace (109.99 U/mL). BP was found to be suitable for significant production of xylanase (117.97 U/mL) and a moderate amount of pectinase produced (34.35 U/mL) among the different substrates (Figure 1e). Most substrates, except for BP, were unable to promote the remarkable biosynthesis of xylanase and pectinase simultaneously. This could be attributed to banana peels having a higher pectin and hemicellulose content (10–21%) than the other agro-wastes used in the study. Furthermore, protein, essential amino acids, foodstuff fibre, polyunsaturated fatty acids, iron and potassium, were also rich in banana peels [52]. The banana peels included a high concentration of dopamine and antioxidants (80–560 mg per 100 g in the peel), as well as 9.14% nitrogen [53], which might be responsible for boosting pectinase production. There were also some reports using banana wastes for xylanase and pectinase production, using *Pleurotus* sp. [54]. *Aspergillus niger* was used to study pectinase production with banana peels as the substrate [55].

### *3.6. Central Composite Design Analysis*

Table 1 displays independent variables with their respective high and low levels used during optimisation. Table 2 provides the CCD matrix of independent variables in coded units, together with predicted and experimental response values.

**Table 2.** CCD, including the experimental and predicted values of dependent variables.

| Run Order | A | B | C | D | Xylanase (U/mL) | | Pectinase (U/mL) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted | Experimental | Predicted |
| 1 | 0 | 0 | 0 | 0 | 180.36 | 155.11 | 208.75 | 220.73 |
| 2 | −1 | +1 | +1 | +1 | 11.98 | 11.70 | 61.70 | 47.89 |
| 3 | −1 | −1 | +1 | −1 | 67.94 | 62.71 | 58.69 | 50.35 |
| 4 | +α | 0 | 0 | 0 | 24.13 | 28.32 | 42.97 | 30.62 |
| 5 | 0 | 0 | 0 | 0 | 168.46 | 155.11 | 229.34 | 220.73 |
| 6 | −1 | +1 | −1 | +1 | 4.72 | 6.65 | 0.00 | −6.54 |
| 7 | −1 | +1 | +1 | −1 | 53.05 | 50.57 | 38.16 | 36.33 |
| 8 | 0 | +α | 0 | 0 | 15.25 | 8.08 | 0.00 | −7.21 |
| 9 | −1 | +1 | −1 | −1 | 40.23 | 46.90 | 18.57 | 36.56 |
| 10 | 0 | 0 | 0 | +α | 24.37 | 24.47 | 2.86 | 12.88 |
| 11 | 0 | 0 | −α | 0 | 26.87 | 23.83 | 38.90 | 25.15 |
| 12 | +1 | −1 | +1 | +1 | 36.68 | 27.33 | 74.49 | 54.01 |
| 13 | 0 | 0 | 0 | 0 | 166.64 | 155.11 | 216.40 | 220.73 |
| 14 | +1 | −1 | +1 | −1 | 48.63 | 43.85 | 0.00 | 9.99 |
| 15 | −α | 0 | 0 | 0 | 39.72 | 41.05 | 47.21 | 58.60 |
| 16 | −1 | −1 | −1 | +1 | 27.22 | 26.04 | 0.00 | −0.87 |
| 17 | +1 | +1 | −1 | −1 | 30.78 | 29.83 | 47.35 | 51.01 |
| 18 | −1 | −1 | +1 | +1 | 24.88 | 22.98 | 96.66 | 96.45 |
| 19 | +1 | −1 | −1 | −1 | 43.59 | 41.19 | 4.60 | 15.91 |
| 20 | 0 | 0 | 0 | 0 | 157.31 | 155.11 | 222.13 | 220.73 |
| 21 | 0 | 0 | +α | 0 | 22.97 | 31.53 | 60.86 | 73.65 |
| 22 | 0 | 0 | 0 | 0 | 125.14 | 155.11 | 215.88 | 220.73 |
| 23 | +1 | +1 | −1 | +1 | 10.24 | 12.79 | 0.00 | 5.84 |
| 24 | +1 | +1 | +1 | +1 | 22.05 | 24.92 | 0.00 | 11.68 |
| 25 | +1 | +1 | +1 | −1 | 42.09 | 40.59 | 3.82 | 2.20 |
| 26 | +1 | −1 | −1 | +1 | 23.66 | 23.30 | 0.00 | 5.28 |
| 27 | 0 | −α | 0 | 0 | 18.05 | 30.73 | 0.00 | 6.25 |
| 28 | 0 | 0 | 0 | −α | 75.81 | 81.24 | 22.94 | 11.96 |
| 29 | −1 | −1 | −1 | −1 | 72.86 | 67.14 | 15.93 | 7.70 |
| 30 | 0 | 0 | 0 | 0 | 132.73 | 155.11 | 231.91 | 220.73 |

The production of xylanase was predicted by Equation (2):

$$\text{Xylanase (U/mL)} = 155.11 - 3.18A - 5.66B + 1.93C - 14.19D + 2.22AB + 1.77AC + 5.80AD + 2.02BC + 0.21BD + 0.34CD - 30.11A^2 - 33.92B^2 - 31.86C^2 - 25.56D^2 \quad (2)$$

The production of pectinase was predicted by Equation (3):

$$\text{Pectinase (U/mL)} = 220.73 - 7.00A - 3.36B + 12.13C + 0.23D + 1.56AB - 12.15AC - 0.52AD - 10.72BC - 8.63BD + 13.66CD - 44.03A^2 - 55.30B^2 - 42.83C^2 - 52.08D^2 \quad (3)$$

Run No. 1 produced the highest xylanase activity (Table 2) at a pH of 8.5, a temperature of 28.5 °C, inoculum concentration of 4% ($v/v$), and agitation at 100 rpm. In addition, Run No. 30, which also had the same optimal conditions, produced the highest pectinase activity.

Tables 3 and 4 provide an analysis of variance (ANOVA) for the quadric model of xylanase and pectinase. The *F*-value model of 28.58 indicates that the model is significant. A large "Model *F*-value" only has a 0.01% probability of occurring due to noise. "Prob > *F* < 0.05" values suggest the model terms are significant. Within this circumstance, D, $A^2$, $B^2$, $C^2$, and $D^2$ are significant model terms (Table 3). According to Table 3, the main effects of variables are in following order: agitation > temperature > pH > inoculum size.

**Table 3.** ANOVA for xylanase activity as a function of independent variables.

| Source | Sum of Squares | df | Mean Square | *F*-Value | *p*-Value Prob > *F* | |
|---|---|---|---|---|---|---|
| Model | 78,687.82 | 14 | 5620.58 | 28.58 | <0.0001 | Significant |
| A | 242.82 | 1 | 242.82 | 1.23 | 0.28 | |
| B | 769.41 | 1 | 769.41 | 3.91 | 0.07 | |
| C | 88.98 | 1 | 88.98 | 0.45 | 0.51 | |
| D | 4834.05 | 1 | 4834.05 | 24.58 | 0.00 | |
| AB | 78.83 | 1 | 78.83 | 0.40 | 0.54 | |
| AC | 50.29 | 1 | 50.29 | 0.25 | 0.62 | |
| AD | 538.51 | 1 | 538.51 | 2.74 | 0.12 | |
| BC | 65.59 | 1 | 65.59 | 0.33 | 0.57 | |
| BD | 0.73 | 1 | 0.73 | 0.00 | 0.95 | |
| CD | 1.89 | 1 | 1.89 | 0.01 | 0.92 | |
| $A^2$ | 24,859.25 | 1 | 24,859.25 | 126.41 | <0.0001 | |
| $B^2$ | 31,566.79 | 1 | 31,566.79 | 160.52 | <0.0001 | |
| $C^2$ | 27,834.88 | 1 | 27,834.88 | 141.55 | <0.0001 | |
| $D^2$ | 17,925.08 | 1 | 17,925.08 | 91.15 | <0.0001 | |
| Residual | 2949.75 | 15 | 196.65 | | | |
| Lack of Fit | 597.12 | 10 | 59.71 | 0.13 | 0.99 | Not significant |
| Pure Error | 2352.63 | 5 | 470.52 | | | |
| Corr. Total | 81,637.57 | 29 | | | | |

$R^2$: 0.9639, adj. $R^2$: 0.9301, predicted $R^2$: 0.9164, CV: 24.20%, adeq. precision: 14.971; df = degree of freedom; highly significant, $p \leq 0.0001$; significant, $p \leq 0.05$; non-significant, $p > 0.05$.

**Table 4.** ANOVA for pectinase activity as a function of independent variables.

| Source | Sum of Squares | df | Mean Square | *F*-Value | *p*-Value Prob > *F* | |
|---|---|---|---|---|---|---|
| Model | 198,070.57 | 14 | 14,147.90 | 73.31 | 3.27 | Significant |
| A | 1174.60 | 1 | 1174.60 | 6.09 | 0.03 | |
| B | 271.72 | 1 | 271.72 | 1.41 | 0.25 | |
| C | 3528.71 | 1 | 3528.71 | 18.29 | 0.00 | |
| D | 1.29 | 1 | 1.29 | 0.01 | 0.94 | |
| AB | 38.86 | 1 | 38.86 | 0.20 | 0.66 | |
| AC | 2360.65 | 1 | 2360.65 | 12.23 | 0.00 | |
| AD | 4.30 | 1 | 4.30 | 0.02 | 0.88 | |
| BC | 1839.38 | 1 | 1839.38 | 9.53 | 0.01 | |
| BD | 1192.77 | 1 | 1192.77 | 6.18 | 0.025 | |
| CD | 2986.84 | 1 | 2986.84 | 15.48 | 0.001 | |
| A2 | 53,177.47 | 1 | 53,177.47 | 275.57 | <0.0001 | |
| B2 | 83,890.04 | 1 | 83,890.04 | 434.72 | <0.0001 | |
| C2 | 50,323.44 | 1 | 50,323.44 | 260.78 | <0.0001 | |
| D2 | 74,392.74 | 1 | 74,392.74 | 385.51 | <0.0001 | |
| Residual | 2894.62 | 15 | 192.97 | | | |
| Lack of Fit | 2507.70 | 10 | 250.77 | 3.24 | 0.1032 | Not significant |
| Pure Error | 386.92 | 5 | 77.38 | | | |
| Corr. Total | $2.01 \times 10^5$ | 29 | | | | |

$R^2$: 0.9856, adj. $R^2$: 0.9722, predicted $R^2$: 0.9254, CV: 21.26%, adeq. precision: 23.206; df = degree of freedom; highly significant, $p \leq 0.0001$; significant, $p \leq 0.05$; non-significant, $p > 0.05$.

The 0.13 "Lack of Fit *F*-value" indicates that the Lack of Fit is insignificant compared with the pure error. Thus, for the model to fit, the non-significant Lack of Fit is necessary. Multiple coefficients of correlation closer to 1 imply a good correlation between experimental and predicted values and prove the model has a good response. A low CV (24.20%) in the present study points to the very reliability and precision of the experiments performed. The "Predicted $R^2$" of 0.9164 is in good accordance with the "adj. $R^2$" of 0.9301. The 14.971 ratio of "adeq. precision" demonstrates an acceptable signal since the value is higher than 4. The design space can be navigated by this model.

The independent variables A, C, AC, BC, BD, CD, $A^2$, $B^2$, $C^2$ and $D^2$, as shown in Table 4, had a significant effect on pectinase production ($p < 0.05$). According to Table 4, the main effects of variables are in following order: inoculum size > pH > temperature > agitation. There was no significant interactions between pH and temperature (AB), pH and agitation (AD), and temperature and agitation (BD) ($p > 0.05$). Data were fitted to six central points of the quadratic model. The equation was checked for statistical significance, with the determination coefficient ($R^2$) of 0.9856 calculated to indicate that the model explained 98% of the variability in the response. Additionally, the *F* test (73.31 times greater than the listed *F*-value at a 90% confidence level) was satisfactory for predicting the model used to describe the enzyme activity's response surface plot. The enzyme activity had a higher experimental value in the central point at a pH of 8.5, a temperature of 28.5 °C, inoculum concentration of 4% (*v*/*v*), and agitation speed of 100 rpm, respectively. There was no significant *p*-value for the "Lack of Fit" test, which showed that the model was reasonably reliable to predict the responses of the factors in the ranges studied. The 3.24 "Lack of Fit *F*-value" indicated that the Lack of Fit was insignificant compared to the pure error.

There was a possibility that the "Lack of Fit *F*-value" of this large (10.32%, $p = 0.1032$) was likely to result in noises. The "Predicted $R^2$" of 0.9254 was near the "adj. $R^2$" of 0.9722 (difference below 0.2), which indicated a reasonable agreement. "adeq. precision" evaluated the signal-to-noise ratio. The 23.206 ratio of "adeq. precision" demonstrated an acceptable signal since the value was higher than 4. The design space could be navigated by this model.

As the plot was obtained in a straight line, as Figure 2a indicates, the normality assumption versus internally Studentised was found to be satisfactory, where the normal probability plot of the residuals is close to being linear; this supports the fact that the error terms are normally distributed. Figure 2b represents the interaction of temperature and pH. The interaction between inoculum concentration and temperature is shown in Figure 2c, while the interaction between inoculum concentration and agitation is shown in Figure 2d. Figure 2b–d illustrate an approximately circular shape of the 3D surface plots. The presence of a peak suggested the mutual relationship between these variables, with the centre of the system representing the point where the maximum xylanase production was achieved [56]. A clear peak observed in the 3D surface plot indicates that the optimum point is within the boundary [57].

Figure 2b depicts a significant effect on xylanase production when pH and temperature are at their low levels. The maximum xylanase activity was recorded at the middle level of pH values (8.2–8.8) and temperatures (23.4–30.2 °C).

Interaction between temperature (B) and inoculum size (C) on xylanase activity is shown in Figure 2c. Figure 2c reveals the optimum point between 26.8 °C to 30.2 °C and 3.5% to 4.5%. Both variables showed increases in xylanase production at their respective middle levels. As for Figure 2d, the circular shape of the 3D response surface shows significant decreases in xylanase activity at lower and higher inoculum size concentrations (C) and agitation (D), where an inoculum concentration between 3.5% and 4.5% and agitation speed of 70 to 90 rpm indicates the optimum point for xylanase production.

The same phenomenon was observed for pectinase due to the straight-line plot, as shown in Figure 3a, where the normality assumption versus internally Studentised residuals was satisfactory. Figure 3b,d illustrate an approximately circular shape, while Figure 3c shows a slightly elongated elliptical shape of the 3D surface plots. Nevertheless, these elliptical 3D surface plot shapes indicated a mutual interaction of two variables, with the system's centre representing the point of maximum production.

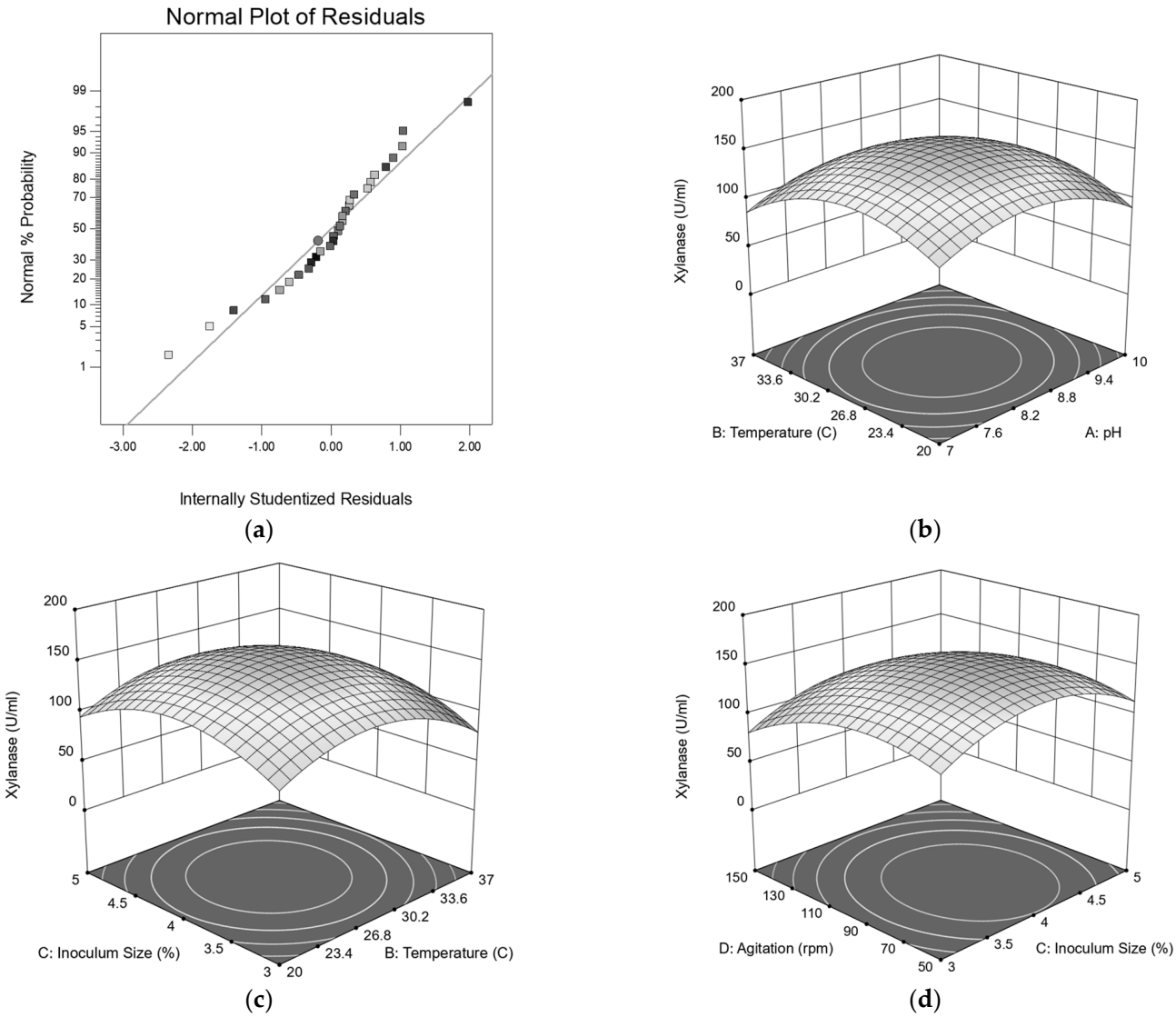


**Figure 2.** (**a**) The normality assumption versus internally Studentised residuals of xylanase activity. Response surface plot showing the interaction between (**b**) temperature and pH, (**c**) inoculum size and temperature and (**d**) agitation and inoculum size on xylanase activity.

Figure 3b illustrates that pH (A) and temperature (B) at their respective middle levels showed the highest pectinase production. Based on Figure 3b, the 3D surface plot shows that the optimum condition for *Bacillus amyloliquefaciens* ADI2 was between a pH of 8.2 and 8.8 and temperatures of 26.8 °C to 30.2 °C. As for the interaction between temperature and the inoculum size of pectinase production, Figure 3c reveals an increase in enzyme production from the middle levels of temperature (26.8–30.2 °C) and middle to higher levels of inoculum concentration (3.9–4.4%) since the 3D surface plot shows an elongated elliptical shape, while Figure 3d also illustrates a significant increase in pectinase production at the middle level of inoculum concentration (4%) and agitation (100 rpm).

### *3.7. Validation of the Model*

The optimised conditions suggested by the RSM model for xylanase and pectinase production were a pH of 8.38, temperature of 28 °C, inoculum size of 4% (*v*/*v*) and agitation at 94 rpm. The validation of the predicted optimisation was conducted in the optimised condition based on the variables mentioned above. The xylanase-dependent response was shown to be 159.08 U/mL, which was very similar to the 156.60 U/mL of the predicted

response. It was the same for pectinase, which experimentally obtained 204.86 U/mL, which was close to the predicted response of 221.05 U/mL. On the contrary, the lowest response of 4.72 U/mL, the RSM optimisation, showed a 97% enhancement in the response of xylanase and a 100% enhancement in the response of pectinase. The optimised conditions revealed that the response values were close to the predictions, demonstrating the validation strategy for the production of enzymes. The $R^2$'s reasonable accepted values describe the actual behaviour of the statistical system that can be used in the experimental domain for interpolation.

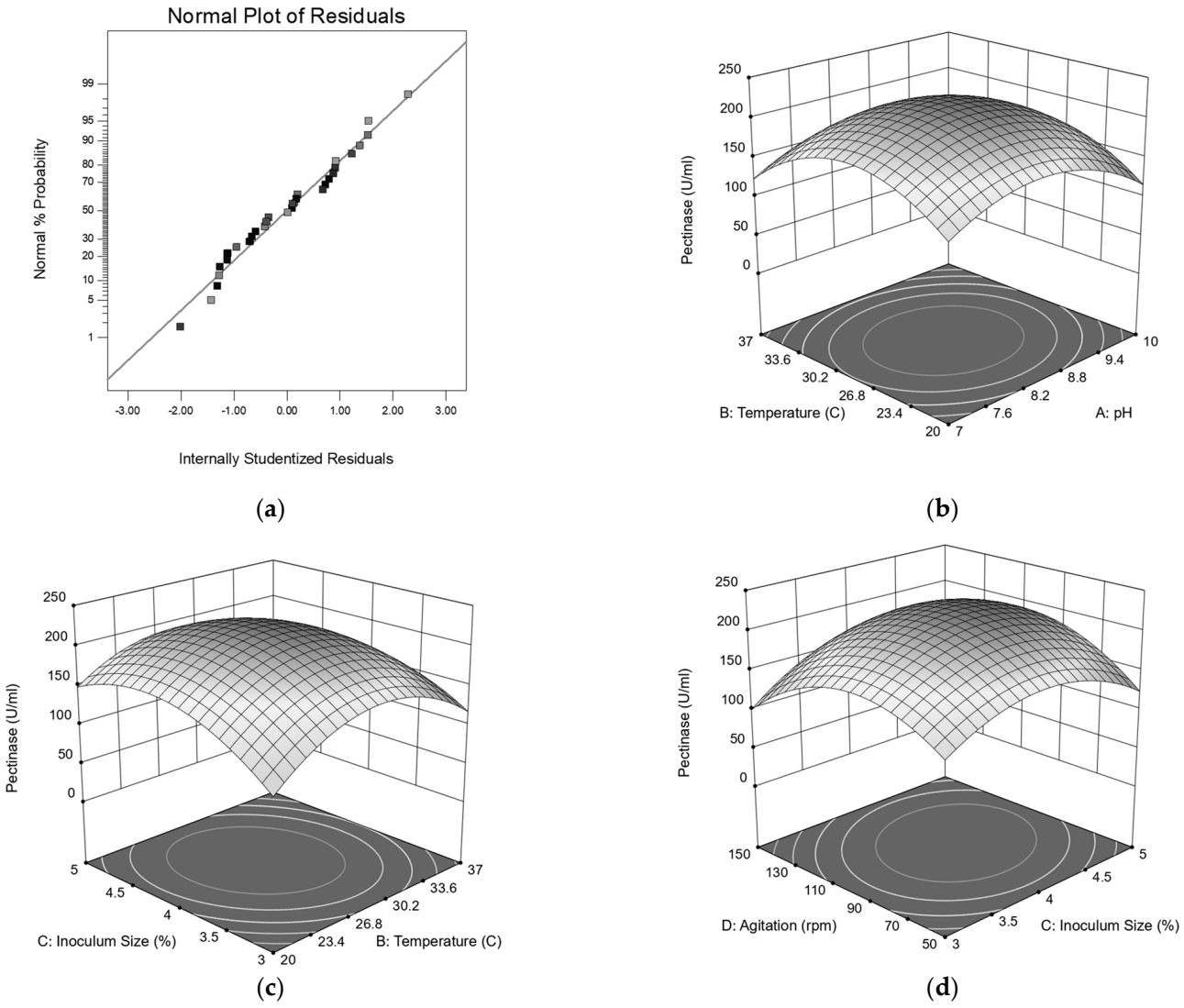


**Figure 3.** (**a**) The normality assumption versus internally Studentised residuals of pectinase activity. Response surface plot showing the interaction between (**b**) temperature and pH, (**c**) inoculum size and temperature and (**d**) agitation and inoculum size on pectinase activity.

Thus, from this optimisation study, it was found that the production of xylanase from *Bacillus amyloliquefaciens* ADI2 was enhanced 1.34-fold (159.08 ± 5.82 U/mL) by the response surface optimisation using CCD compared to the OFAT method. However, for pectinase, the production was significantly enhanced by 5.96-fold (204.86 ± 8.95 U/mL) after RSM optimisation, giving a xylanase–pectinase production ratio of 1:1.3. There was literature reporting that RSM had been successfully used to optimise the culture conditions for maximum production of the desired bioproducts [58–60].

The statistical method used in the optimal production of xylanase and pectinase demonstrated the effect of four independent variables at five different levels. The results indicated that the observed and predicted values of both responses were in close agreement, which demonstrated that the RSM method was effective and reliable to optimise the parameters available for the production of the enzyme.

The model adjustment with observed responses to xylanase and pectinase activity was demonstrated by the ANOVA (*F* test). The lesser CV value calculated from the responses showed higher accuracy and reliability of the model, which was further verified by the correlation coefficient (R) and the measurement coefficient ($R^2$). Subsequently, in this case, the assumption of normality against the internally Studentised residual plot that existed in a straight line demonstrated a better model accuracy. The interactive effect of variables on the response was shown as a perturbating graph for the production of xylanase and pectinase from *Bacillus amyloliquefaciens* ADI2. In the current context, statistical optimisation was successfully and effectively applied to submerged fermentation, which overcomes the drawbacks of the one factor at a time (OFAT) method. The interaction between the independent variables such as pH and temperature, pH and inoculum size, pH and agitation, temperature and inoculum concentration, temperature and agitation and inoculum concentration and agitation demonstrated a significant effect on both of the responses.

The improved production of both enzymes (xylanase and pectinase) showed mutual interconnection at their respective middle values between the variables. Previous literature also reported the successful use of RSM to optimise the culture conditions for maximum production of desired bioproducts [58–60]. RSM maximises the amount of information that can be obtained while limiting the number of individual experiments required.

After statistical optimisation, the experimental data collected resulted in enhanced production of xylanase and pectinase via SmF, and the current study found that *Bacillus amyloliquefaciens* ADI2 can also be used to enhance xylanase and pectinase production using an inoculum size of 4% (*v*/*v*) in pH 8.38 of media and agitation of 94 rpm at 28 °C.

A comparison of xylanase and pectinase production from *Bacillus amyloliquefaciens* ADI2 revealed that the enzyme titre value was higher than other strains from other reported *Bacillus* sp. (Tables 5 and 6). It was remarkable to acquire two biotechnologically essential enzymes in comparatively high amounts from a single micro-organism.

**Table 5.** Comparison of xylanase production by *Bacillus* sp.

| Organisms | Methods | Carbon Sources | Xylanase Activity (U/mL) | References |
|---|---|---|---|---|
| *B. amyloliquefaciens* ADI2 | CCD, RSM | Banana peel | 159.08 | Present investigation |
| *B. tequilensis* ARMATI | CCD, RSM | Birchwood xylan | 86.82 | [61] |
| *Bacillus* sp. | Non-statistical | Birchwood xylan | 49.00 | [62] |
| *B. subtilis* | Non-statistical | Pineapple peel | 18.87 | [63] |
| *Bacillus* sp. | CCD, RSM | Corn husk | 2.50 | [64] |
| *B. subtilis* | CCD, RSM | LB-Xylose | 119.00 | [65] |
| *B. atropheaus* | CCD, RSM | Xylan | 85.16 | [66] |
| *B. subtilis* SD8 | CCD, RSM | Xylan | 8.18 | [42] |
| *B. mojavensis* A21 | CCD, RSM | Barley bran | 7.45 | [67] |
| *B. cereus* BSA1 | Taguchi OA | Xylan | 7.40 | [68] |
| *Bacillus* sp. 2129 | CCD, RSM | Oat | 2.39 | [69] |
| *B. tequilensis* UD-3 | Non-statistical | Rice straw | 8.54 | [70] |
| *B. substilis JJBS250* | CCD, RSM | Sugarcane bagasse | 98.16 | [71] |

**Table 6.** Comparison of pectinase production by *Bacillus* sp.

| Organisms | Methods | Carbon Sources | Pectinase Activity (U/mL) | References |
|---|---|---|---|---|
| *B. amyloliquefaciens* ADI2 | CCD, RSM | Banana peel | 204.86 | Present investigation |
| *B. subtilis* | CCD, RSM | Hazelnut shell | 5.60 | [72] |
| *B. pumilus* | CCD, RSM | Sugar beet pulp | 33.43 | [58] |
| *B. cereus* | CCD, RSM | Pectin | 3.37 | [73] |
| *B. mojavensis* I4 | CCD, RSM | Carrot peel | 64.80 | [51] |
| *Bacillus* sp. Y1 | CCD, RSM | Starch, sucrose, wheat bran | 40.00 | [74] |
| *Bacillus* spp. | Non-statistical | Apple pomace | 11.25 | [75] |
| *B. cereus* | Non-statistical | Pectin | 44.00 | [76] |
| *Bacillus* sp. CH15 | Non-statistical | Pectin | 0.31 | [77] |
| *B. pumilus* AJK | CCD, RSM | Pectin, wheat bran | 109.19 | [78] |
| *B. subtilis* BTK27 | Non-statistical | Pectin | 66.30 | [79] |
| *Bacillus* sp. DT7 | Non-statistical | Pectin | 53.00 | [80] |
| *B. subtilis* BK-3 | CCD, RSM | Citrus peel | 31.8 | [81] |

## 4. Conclusions

The optimised model through CCD showed 1.34-fold greater xylanase and 5.96-fold greater pectinase production after conventional and statistical optimisation. The optimum conditions were as follows: pH = 8.38, temperature = 28 °C, inoculum size = 4% (*v*/*v*), and agitation = 94 rpm. The main effects of variables for xylanase production are in the following order: agitation > temperature > pH > inoculum size. Meanwhile, for pectinase: inoculum size > pH > temperature > agitation. The study demonstrated a novel approach where one single medium was optimised based on a quadratic model developed through CCD-RSM for concurrent production of xylanase and pectinase enzymes from *Bacillus* amyloliquefaciens ADI2 individually, plus using agro-industrial waste as an economical substrate, which helps the production of the enzymes to be cost-effective for a plethora of industrial applications, such as pulp and paper and enzyme-feed-additive industries.

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## Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| CCD | Central composite design |
| CV | Coefficient of variation |
| OD | Optical density |
| OFAT | One factor at a time |
| RSM | Response surface methodology |
| SmF | Submerged fermentation |
| SSF | Solid-state fermentation |

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