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Study on the biodegradability of alkaline pretreated custard apple peel (*Annona squamosa Linn*.) by using local cellulolytic bacteria

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ABSTRACT

This study investigated custard apple peel (*Annona squamosa* Linn.) as a novel raw material for sugar production through a combination of chemical and biological hydrolysis. Thermal-alkaline pretreatment was first optimized using one-factor-at-a-time (OFAT) experiments, followed by Box–Behnken Design (BBD) with Response Surface Methodology (RSM). The optimal conditions, with a NaOH concentration of 2.5 g/L, a temperature of 90 °C, and a treatment time of 70 min, yielded 3440 mg/L of reducing sugars, which is 1.8 times higher than the result obtained from single-factor optimization (1862 mg/L). The model showed a strong fit ($R^2 = 0.9774$, $P < 0.05$), confirming the reliability of the predicted values. In the biological approach, four cellulolytic bacterial strains, namely *Alcaligenes* sp. KHM19, *Staphylococcus gallinarum* VC10, *Bacillus safensis* HL04, and *Bacillus velezensis* KH-08 were assessed for their enzymatic potential. Among them, *B. velezensis* KH-08 displayed the highest FPase activity (19.56 U/L) and produced up to 365.97 mg/L reducing sugars after 6 days. Overall, the findings provide the first evidence for the valorization of custard apple peel through integrated hydrolysis strategies and identify *B. velezensis* KH-08 as a promising novel strain for future biotechnological applications.

Keywords: alkaline hydrolysis, cellulolytic bacteria, custard apple peel, FPase activity, reducing sugars concentration.

INTRODUCTION

Bioethanol derived from lignocellulosic biomass is widely recognized as a promising renewable and sustainable energy source, offering an alternative to fossil fuels and contributing to the reduction of greenhouse gas emissions (Adeniyi et al., 2024; Dutta et al., 2021; Ghosh et al., 2021). Lignocellulose, the principal structural component of plant cell walls, consists mainly of cellulose, hemicellulose, and lignin, with cellulose accounting for approximately 30–40% of the total (Abdel-Hamid et al., 2013; Kirui et al., 2022; Silva et al., 2023; Troncoso et al., 2023; Zoghlami and Paes, 2019). In the production process, cellulose must first be hydrolyzed into glucose, which can then be fermented by microorganisms to yield ethanol (Ganguly et al., 2021; Tri et al., 2024; Vasic et al., 2021; Xiros et al., 2012).

Among the various pretreatment strategies for lignocellulosic biomass, alkaline pretreatment using sodium hydroxide (NaOH) has demonstrated considerable effectiveness in delignification and in improving subsequent enzymatic hydrolysis. Several studies have shown that NaOH treatment disrupts lignin structures,

enhances cellulose accessibility, and markedly increases sugar yields. For instance, Kim and Holtzapple reported that NaOH pretreatment significantly improved the digestibility of corn stover for fermentation (Kim and Holtzapple, 2006). Similarly, Zhang et al. applied NaOH pretreatment to wheat straw and obtained high ethanol yields following enzymatic saccharification and fermentation (Zhang et al., 2013). In a more recent study, bamboo pretreated with alkaline NaOH was successfully used for butanol production via co-culture of the wood-rotting fungus *Phlebia* sp. MG-60-P2 and the bacterium *Clostridium saccharoperbutylacetonicum*, indicating the effectiveness of NaOH pretreatment in facilitating fermentation (Tri et al., 2024). Additionally, alkaline-pretreated sugarcane bagasse was utilized for bioethanol production through consolidated bioprocessing using *Phlebia* sp. MG-60, further demonstrating the potential of NaOH in enhancing the bioconversion efficiency of various lignocellulosic (Khuong et al., 2014).

Although alkaline pretreatment is effective in enhancing cellulose accessibility, its limitations, such as chemical recovery costs and environmental concerns, underscore the need for complementary approaches. Biological hydrolysis using cellulolytic microorganisms offers an eco-friendly alternative that can further improve lignocellulosic conversion. Custard apple peels (*Annona squamosa* Linn.), extensively cultivated for their economic value, generate large quantities of peel residues during processing, most of which are discarded or left to accumulate, causing environmental burdens and resource underutilization. Although some studies have investigated bioactive compounds from the peel (Rojas-Garcia et al., 2022; Shivamathi et al., 2019; Tai et al., 2022), systematic research on cellulolytic strains capable of degrading this custard apple peel residue for fermentable sugar production remains scarce. Given its high cellulose content, custard apple peel represents a promising yet underexplored feedstock that could be valorized through integrated pretreatment and microbial hydrolysis. To achieve efficient conversion, however, systematic optimization of hydrolysis conditions is required, for which RSM provides a powerful framework to evaluate multiple variables, reduce experimental effort, and enhance process accuracy. To address these gaps, the present study integrates chemical pretreatment with microbial hydrolysis and employs RSM to systematically optimize the conversion of custard apple peel into fermentable sugars, thereby providing a potential route for subsequent bioethanol production.

MATERIALS AND METHODS

Materials

Custard apple peels were collected from An Sinh commune, Dong Trieu City, Quang Ninh, Vietnam (21°10′18″ N, 106°31′24″ E). The peels were separated and cleaned by removing leftover flesh, and then they were washed with purified water to remove any remaining sugars. Next, the peels were dried at 50 °C for 5 days. After drying, the peels were ground using a BQ-3KW-220V grinder to a size of ≤ 0.01 mm.

Four bacterial strains isolated at Quang Ninh Province capable of producing FPase and degrading custard apple peel after thermal-alkaline pretreatment were identified based on 16S rRNA gene sequences. The sequences have been deposited in GenBank under the following accession numbers: *Alcaligenes* sp. KHM19 (accession number: MT211505, link: https://www.ncbi.nlm.nih.gov/nuccore/MT211505), *Staphylococcus gallinarum* VC10 (accession number: PV834039, link https://www.ncbi.nlm.nih.gov/nuccore/PV834039, *Bacillus safensis* HL04 (accession number: PQ864774, link https://www.ncbi.nlm.nih.gov/nuccore/PQ864774, and *Bacillus velezensis* KH-08 (accession number: PV936483, link: https://www.ncbi.nlm.nih.gov/nuccore/PV936483). The availability of this data allows for independent verification of our findings and helps to further future study in this sector.

Biomass thermal-alkaline pretreatment by the OFAT method

The conversion of custard apple peel powder was carried out following the OFAT approach. In this method, dilute NaOH solutions at concentrations of 1.25, 2.5, 3.75, 5.0, and 6.25 g/L were applied while maintaining an NaOH-to-biomass ratio of 1:10 (w/v). Thermal pretreatment was then performed in a Memmert UN110 device at 60, 90, 120, 150, and 180 °C for residence times of 40, 70, 100, 130, and 150 min, respectively. Subsequently, filter paper was used to separate the treated biomass residue from the liquid fraction. The

filtrate was centrifuged at 12,000 rpm for 15 min to determine the efficiency of thermal-alkaline hydrolysis and the reducing sugar content. The pretreated custard apple peel residue was washed with tap water until neutral pH, dried at 50 °C for 48 h, and stored in sealed zip bags for subsequent biological decomposition experiments. The reducing sugar content was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, 1 mL of the sample was mixed with 1 mL of DNS reagent. The samples were heated at 100 °C for 5 min to stop the reaction. It was read using a UV-VIS Spectrophotometer at an absorbance of 540 nm. The amount of reducing sugars generated directly correlates with the reaction mixture's color intensity. A standard curve graph of pure glucose can be used to calculate the sample's reducing sugar concentration.

Optimization of the thermal-alkaline pretreatment by RSM

The effects of three independent variables on the conversion of custard apple peel into reducing sugars for ethanol fermentation were evaluated using RSM based on BBD after the influencing factors were identified and the required levels were established using the OFAT technique. The effects of three experimental parameters (NaOH concentration, temperature, and treatment time) at three different levels (low, medium, and high) represented by the defined values of -1, 0, and +1, respectively, were examined for this purpose. Design Expert statistical software (Version 11.1.2.0, StateEase Inc., USA) was used to determine the coefficients of the quadratic regression models, statistically analyze the multivariate equations, and determine the effects of factors on the variables (Anggrainy et al., 2023). Three independent variables at different levels were used in BBD (Table 1). RSM uses a limited number of trials to achieve a response through a predetermined number of planned experiments. This statistical method provides the link between several parameters for ideal operating conditions and is suitable for multi-factor studies (Ravikumar et al., 2005; Sahu et al., 2015). The equation provides the number of trial runs (N) in RSM.

$$N = 2k(k-1) + C_o \quad (1)$$

where: N – number of experimental runs, K – number of variables, and C_o – Centre point.

The behavior of the system is explained by the following quadratic equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ij} {x_i}^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where: Y – anticipated reaction, the model's error is represented by ε, whereas β_0, β_i, β_{ii}, and β_{ij} are constant, linear, quadratic, and interactive coefficients, respectively (Acharya et al., 2018; Krishna and Sree, 2013).

The range and levels of the independent variables used in the BBD were NaOH concentration (1.25 to 3.75 g/L), temperature (60 to 120 °C), and treatment time (40 to 100 min) (Table 1). The BBD comprised 17 experimental runs, including 12 independent runs and 5 replicates at the center runs to evaluate model adequacy and experimental precision (Table 2). All experimental independent runs of the program were performed in triplicate, and the data were analyzed as means (Sharma et al., 2023).

Fermentation by FPase-producing bacterial strains

The four bacterial strains, including *Alcaligenes* sp. KHM19, *Staphylococcus gallinarum* VC10, *Bacillus safensis* HL04, and *Bacillus velezensis* KH-08 were cultured at 37 °C for 24 hours while being shaken at 120 rpm in a nutritional medium that contains (g/L) 5.0 peptone, 3.0 yeast extract, 5.0 NaCl, and pH 7.0 (Khuong et al., 2025). Then,

Table 1. Experimental range and independent variable levels

Independent variable	Unit	Symbols	Range and levels		
			-1	0	+1
NaOH concentration	mg/L	A	1.25	2.5	3.75
Temperature	°C	B	60	90	120
Treatment time	min	C	40	70	100

2.0 mL of the activated bacterial culture was added to 18.0 mL of fermentation medium that contained (g/L): 10.0 pretreated custard apple peels, 10.0 carboxymethyl cellulose (CMC), 0.5 K_2HPO_4, 0.5 KH_2PO_4, 1.0 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.1 $CaCl_2$, 6.0 NaCl, and 0.1 yeast extract (the medium was made based on a previously published medium with minor adjustments (Khuong et al., 2025)). Fermentation broth was collected on 2, 4, 6, and 8 days of incubation at 37 °C and 120 rpm. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant (crude enzyme) was used for FPase and reducing sugar concentration assays.

FPase assay

FPase was measured by combining 0.5 mL of crude enzyme extract with 0.5 mL of 0.05 M sodium citrate buffer (pH 4.8) and Whatman No.1 filter paper 10 × 60 mm (50 mg). After an hour of incubation at 50 °C, the reaction was stopped by adding 0.5 mL of DNS reagent to 1.0 mL of the reaction mixture. To quantify reducing sugars, absorbance was measured at 540 nm with a UV-Vis spectrophotometer (Miller, 1959). A unit of FPase activity (U) is defined as the quantity of enzyme that releases 1 μmol of glucose per minute under test conditions. The negative control samples included a crude enzyme solution without substrate.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was conducted using ANOVA in Microsoft Office 2019. A probability value of $P < 0.05$ was considered statistically significant. Error bars in the figures represent SD values.

RESULTS AND DISCUSSION

Effect of individual factors on pretreatment conditions

Effect of NaOH concentration

Figure 1 shows that the hydrolysis efficiency of custard apple peels increases with the concentration of NaOH. Corresponding to the increase in concentration from 1.25 to 3.75 g/L, the hydrolysis efficiency of custard apple peels increases gradually from 12.48 to 15.84%. The amount of reducing sugar obtained increases correspondingly with the hydrolysis efficiency. However, when the concentration of NaOH increases from 3.75 to 6.25 g/L, the hydrolysis efficiency and the content of reducing sugar tend to decrease. In the study by Wang et al., it was reported that the reducing sugar (RS) yield of wheat straw increased with rising NaOH concentration, reaching a maximum of 80.65% at 1.0% (w/w) NaOH (≈10 g/L). However, further increasing the NaOH concentration to 4.0% (w/w) (≈40 g/L) did not enhance the RS yield; instead, a marked decline was observed (Wang et al., 2021). An extended soak in a high concentration of NaOH dissolves pentose, reducing RS recovery (R Kataria and Ghosh, 2014), and also makes it more difficult to wash away residual alkalinity from substrates such as custard apple peel, which may inhibit subsequent enzymatic hydrolysis. Similarly, Kataria et al. demonstrated that alkali pretreatment of Kans grass with NaOH yielded significantly higher reducing sugars, 350 mg/g, compared to acid pretreatment, 69.08 mg/g (Kataria et al., 2013). Consistently, Nath et al. reported that sequential pretreatment with 1% (w/v) NaOH at 50 °C for 2 h, followed by organosolv treatment (phosphoric acid and acetone), resulted in 66.1% cellulose recovery, 83.2% lignin removal, and a total reducing sugar yield of 230 mg/g bagasse (Nath et al., 2021). Collectively, these findings highlight that NaOH pretreatment, particularly at relatively low concentrations, is generally more effective than acid pretreatment, as it promotes delignification while minimizing carbohydrate loss, thereby enhancing enzymatic accessibility and improving overall saccharification efficiency. Therefore, this study chose the NaOH concentration of 3.75 g/L because it showed the highest reducing sugar content of 1695 mg/L to design the following experiments.

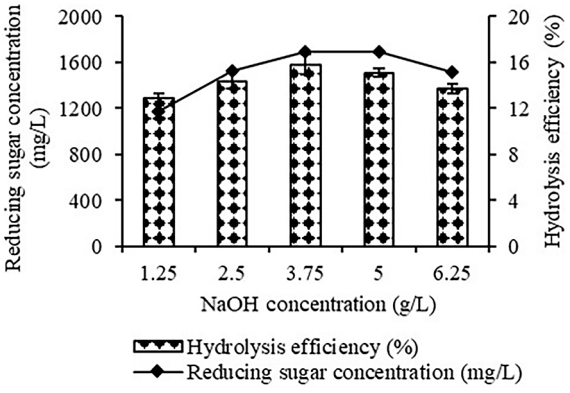

Figure 1. The effect of NaOH concentration on reducing sugar concentration and hydrolysis efficiency

Effect of treatment temperature

As shown in Figure 2, both reducing sugar concentration and hydrolysis efficiency increased with temperature up to an optimum at 120 °C, where the maximum sugar concentration of 1755 mg/L and hydrolysis efficiency of 15.8% were achieved. At lower temperatures, such as 60 °C (1310 mg/L, 12.3%), the pretreatment was insufficient to effectively disrupt the lignocellulosic structure, resulting in lower sugar release. Conversely, at higher temperatures (150 to 180 °C), a decline in sugar concentration, from 1590 to 1390 mg/L, and hydrolysis efficiency of 14 to 13% was observed. In the study by Chen et al., temperature was identified as the second most important parameter affecting corn stover hydrolysis, with optimal ranges of 103 to 106 °C for glucose and 93 to 97 °C for xylose release (Chen et al., 2013). According to Jiang et al., optimization by RSM revealed that reaction temperature significantly influenced fermentable sugar recovery during alkali pretreatment of sorghum pith. At 40 °C, lignin and xylan removal was enhanced, thereby improving enzymatic accessibility and resulting in maximum glucose and xylose yields of 90.5% and 57.7%, respectively (Jiang et al., 2019). In a study of Vietnamosasa pusilla, pretreatment with 2% NaOH at 120 to 130 °C increased glucose and xylose release compared to 110 °C due to better lignocellulose disruption and enzyme accessibility (Wongleang et al., 2023). However, at higher temperatures, glucan and xylan recovery was significantly reduced, resulting in poorer sugar yields due to polysaccharide breakdown into inhibitory by-products. Moreover, higher temperatures increase carbohydrate loss due to random chain breakage and peeling processes, which can significantly lower total sugar yield (McDonough, 1996). Furthermore, harsh pretreatment conditions can cause lignin condensation processes that produce carbon-carbon bonds between lignin subunits, restricting their removal and hence lowering glucan and xylan conversion (Pan et al., 2004). These results indicate that 120 °C represents the optimal pretreatment temperature for maximizing reducing sugar yield and enzymatic hydrolysis efficiency of custard apple peel.

Effect of treatment time

Figure 3 shows that the hydrolysis efficiency increases with increasing treatment time. The amount of hydrolyzed custard apple peel powder

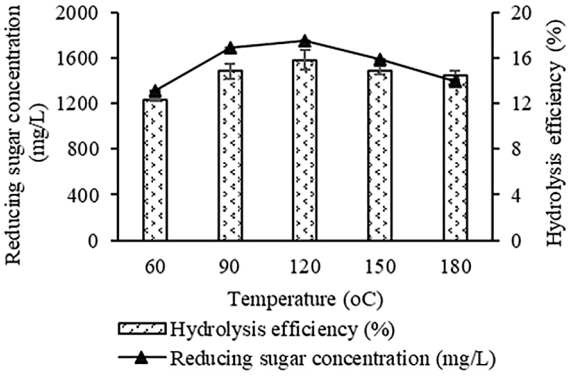

Figure 2. The effect of temperature on reducing sugar concentration and hydrolysis efficiency

increases from 1410 to 1862 mg/L when the time increases from 40 to 100 min. However, after 100 min of treatment, the hydrolysis efficiency does not increase significantly, and the reducing sugar content decreases. Pretreatment time is a critical factor influencing the reducing sugar yield from wheat straw. Using 1.0% (w/w) NaOH, a duration of 60 min was found to be optimal, achieving the highest RS yield of 86.63%, whereas longer durations resulted in decreased yields due to glucan solubilization and degradation (Wang et al., 2021). In the study by McIntosh and Vancov, pretreatment time played a critical role in sugar recovery from wheat straw. A 30-minute treatment with 2% NaOH at 121 °C increased yields by 6.3-fold, while extending the time to 60 min with 1% NaOH achieved glucose yields of about 480 mg/g dry biomass (McIntosh and Vancov, 2011). These results highlight that optimizing pretreatment duration is essential for maximizing sugar reduction. Therefore, the appropriate treatment time chosen for the next experiment was 100 min.

ANOVA and regression model analysis

Using the program Design Expert, an ANOVA was performed to ascertain the statistical significance of the polynomial model equation (Table 3). A second-order polynomial equation was fitted to the produced response Equation 3.

$$Y = 3074.57 - 152.4A - 391.13B - 272.94C + \\ + 281.69AB - 62.03AC - 60.9BC - 810.33A^2 - \\ - 1099.43B^2 - 389.15C^2 \quad (3)$$

where: Y – reducing sugar concentration (mg/L), A – NaOH concentration (mg/L), B – temperature (°C), and C – treatment time (min).

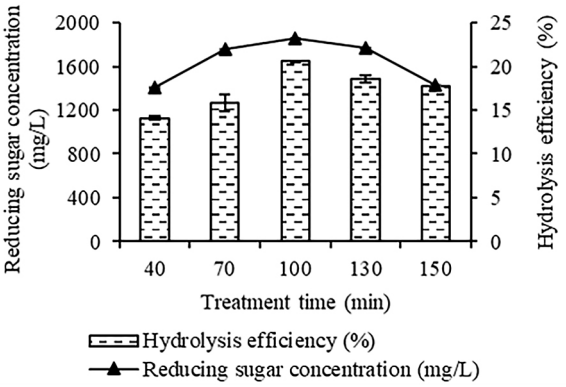

Figure 3. The effect of treatment time on reducing sugar concentration and hydrolysis efficiency

This equation was used to link the impact of experimental parameters with the reduction in sugar concentration. Several linear regressions were used to forecast the quadratic model's coefficients, and those with $P<0.05$ (P is the result of a statistical hypothesis test) were considered as significant. The maximum reducing sugar concentration in the BBD experimental design was found to be 3440 mg/L at NaOH concentration 2.5 mg/L, temperature 90 °C, and treatment time 70 min of conversion of custard apple peels as shown in Table 2.

The data were analyzed using ANOVA, and Table 3 displays the design matrix together with the actual experimental and expected responses. The close reduction in sugar content between the model and the actual response, as determined by the experimental results, proved the validity of the regression quadratic model. The F-value determines whether the test is statistically significant and the equation has been revealed to be 33.65, which implies that the quadratic model is important. The "Lack of fit F-value" of 0.25 indicated that the experimental data fit the model and that the lack of fit is not substantial in relation to the pure error, which is the desired characteristic. One can evaluate the quadratic model generated by RSM for goodness of fit by calculating the R^2 (coefficient). The model's R^2 value in the current analysis was 0.9774, whilst the adjusted and predicted R^2 values were 0.9484 and 0.9132, respectively. As a result, the current R^2 value indicated that the model is positive and stable for determining reducing sugar content and recreated a good fit between the expected and observed responses. Additionally, the results suggested that the quadratic model was the best fit for response prediction.

3-D plot response surface

Concerning reducing sugar content, the correlations between the individual factors-specifically, specifically NaOH concentration, temperature, and treatment time, were illustrated using

Table 2. RSM design using both expected and observed values

Std	Run	A: NaOH concentration (mg/L)	B: Temperature (°C)	C: Treatment time (min)	Reducing sugar concentration (mg/L)	
					Actual	Predicted
7	1	1.25	90	100	1860.98	1816.57
1	2	1.25	60	70	2047.08	1990.02
6	3	3.75	90	40	2013.25	2057.65
13	**4**	**2.5**	**90**	**70**	**3440.00**	**3074.57**
4	5	3.75	120	70	845.90	902.96
16	6	2.5	90	70	3191.87	3074.57
17	7	2.5	90	70	2876.07	3074.57
10	8	2.5	120	40	1630.16	1528.69
12	9	2.5	120	100	879.02	861.01
15	10	2.5	90	70	2932.46	3074.57
3	11	1.25	120	70	581.97	644.39
2	12	3.75	60	70	1184.26	1121.84
8	13	3.75	90	100	1426.75	1387.71
5	14	1.25	90	40	2199.34	2238.39
14	15	2.5	90	70	2932.46	3074.57
11	16	2.5	60	100	1663.61	1765.07
9	17	2.5	60	40	2171.15	2189.16

Table 3. Analysis of variance for the Box-Behnken methodology's experimental findings for the quadratic model (R^2=0.9774, R^2 [pred]=0.9132, R^2 [adj]=0.9484)

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	11664158	9	1296018	33.64662	6.07E-05	significant
A-NaOH concentration	185810	1	185810	4.823915	0.064076	
B-Temperature	1223869	1	1223869	31.77353	0.000785	
C-Treatment time	595977.3	1	595977.3	15.47249	0.00565	
AB	317393.8	1	317393.8	8.240034	0.023973	
AC	15392.27	1	15392.27	0.399607	0.547375	
BC	14835.12	1	14835.12	0.385143	0.554523	
A^2	2764808	1	2764808	71.77868	6.31E-05	
B^2	5089485	1	5089485	132.1309	8.48E-06	
C^2	637646	1	637646	16.55427	0.004757	
Residual	269629.6	7	38518.51			
Lack of fit	42536.39	3	14178.8	0.249744	0.858178	not significant
Pure error	227093.2	4	56773.29			
Cor total	11933787	16				

3-D response surface graphs (Figure 4a-c). With all other parameters held constant at zero (0), the impact of two different factors was shown by the reaction surface plot. The link between the three parameters and reducing sugar concentration is visually represented by the response surface. Identifying each variable's optimal level to achieve the highest reducing sugar value requires an understanding of the interplay between components (Saeed et al., 2023). The surface plots that demonstrate the relationship between NaOH concentration, temperature, treatment time, and reducing sugar concentration are shown in Figure 4.

ANOVA analysis showed that the interaction between NaOH concentration and temperature (AB) was statistically significant with P = 0.0239 < 0.05 (Table 3), indicating that this is a pair of factors that significantly affect the concentration of reducing sugar obtained. Observations from the surface response diagram (Figure 4a) show that the reducing sugar yield increases with increasing temperature in the range from 60 °C to about 100 °C, especially at low NaOH levels (1.25 to 2.5 mg/L). However, at higher temperatures, the yield tends to be saturated or slightly decreased, especially at high NaOH concentrations (3.75 mg/L). This trend can be explained by the fact that increasing the temperature helps to break down the lignocellulose structure, releasing polysaccharides that are easily converted into reducing sugars (Janga et al., 2012; Mosier et al., 2005). However, at high NaOH concentrations combined with high temperatures, sugar decomposition or the formation of inhibitory by-products may occur, leading to a decrease in yield. This confirms that NaOH and temperature should not be increased simultaneously too much, but should be optimally adjusted within the average range to avoid negative effects due to side reactions. Previous research revealed similar findings. Uzunlu et al. adjusted the alkaline pretreatment of poppy stalks and discovered that glucose production increased with NaOH concentration and temperature up to an optimum of 2.4% NaOH at 80 °C for 70 minutes, after which additional increases resulted in a drop in sugar recovery owing to carbohydrate breakdown (Uzunlu et al., 2014). The interaction between pretreatment time and NaOH concentration was statistically significant ($p < 0.05$) in another study on sweet sorghum bagasse. It demonstrated that longer pretreatment times and higher alkali loading led to a greater removal of lignin, but that excessive conditions decreased the amount of hydrolyzable carbohydrate fraction available for sugar release (Utoro et al., 2023). Similarly, in alkali-pretreated sugarcane bagasse, the ANOVA model revealed that NaOH concentration and its interaction with time significantly influenced reducing sugar production; response surface plots revealed that moderate NaOH levels combined with moderate temperature and time increased sugar yield, whereas severe conditions promoted sugar decomposition and inhibitor formation (Yoon et al., 2012). In summary, these studies provide credence to the current finding that while temperature and NaOH

are necessary for efficient delignification, their combined effect becomes harmful when applied at excessively high levels, highlighting the importance of carefully balancing these two variables within the ideal range.

The interaction between NaOH concentration and processing time (AC) was not statistically significant with P = 0.5473 > 0.05 (Table 3), indicating that the simultaneous influence of these two factors on the reducing sugar concentration was insignificant. However, the response diagram (Figure 4b) still showed some potential trends: when the processing time was short (about 40 min), increasing the NaOH concentration significantly improved the sugar yield. On the contrary, at a long processing time (100 min), increasing the NaOH concentration could slightly reduce the yield. This phenomenon can be explained by the fact that NaOH needs enough time to separate lignin and break down the cellulose structure, but if the time is too long, especially with high alkali concentration, it can cause sugar destruction (Alwi et al., 2023; Uzunlu et al., 2014). Although not statistically significant, this interaction still suggests that a balance between time and chemical dosage is needed to avoid overtreatment. According to Zhou et al., Box-Behnken optimization of tea stalk pretreatment demonstrated that high NaOH concentration and prolonged residence time harmed lowering sugar production by destroying the lignocellulosic structure and producing degradation products (Zhou et al., 2025). An efficient pretreatment for industrial use should strike a compromise between delignification and carbohydrate preservation to enhance fermentable sugar yield. At modest alkali loading and a shorter residence period, this balance can dramatically increase reducing sugar production.

The interaction between temperature and processing time (BC) was also not statistically significant with P = 0.5545 > 0.05 (Table 3), reflecting that these two factors did not have a strong synergistic effect on the concentration of reducing sugars. However, the response diagram (Figure 4c) showed that the sugar yield increased as both temperature and time increased to a medium-high level (about 90 to 100 °C and 70 min). After this level, if the time or temperature continued to increase, the yield tended to decrease. The

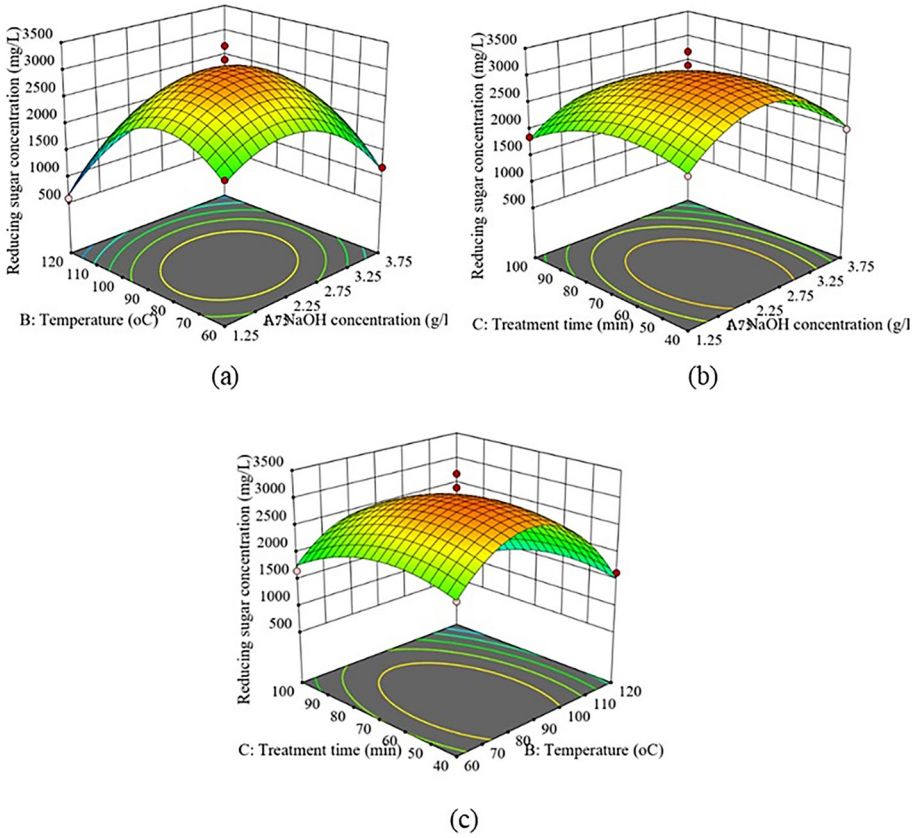

Figure 4. Response surface 3-D plots revealing correlation effects of (a) NaOH concentration and temperature, (b) NaOH concentration and treatment time, and (c) temperature and treatment time

mechanism may be because long temperature and time help to break down the cell wall structure, increasing the ability to hydrolyze polysaccharides (Lin et al., 2020; Lu et al., 2018). However, when the optimal limit is exceeded, sugar degradation, loss by evaporation, or formation of undesirable compounds may occur. This suggests that temperature and time should be controlled independently within the optimal limit, instead of increasing simultaneously. Similar observations were reported by Pedersen et al. during wheat straw pretreatment optimization. They found that raising the temperature from 100 to 170 °C increased glucose recovery from 20% to over 80% after enzymatic hydrolysis. However, when the residence time exceeded 20 min at 170 °C, glucose yield declined due to the accumulation of furfural and 5-hydroxymethylfurfural (Pedersen et al., 2011). A similar pattern was reported by Ahmad et al. during the pretreatment of *Sida cordifolia* biomass. Using response surface methodology, the authors identified the optimal conditions at 80 °C for 60 min with 3.5% acid, which yielded 15.8 g/L of reducing sugars. However, extending the time to 90 min or increasing the temperature to 100 °C reduced the yield to below 12 g/L (Ahmad et al., 2023). Wong et al. also reported a clear interaction between temperature and time during dilute acid hydrolysis of oil palm trunk. Xylose peaked at 32.4% at 115 °C for 30 min but declined to 28% at 60 min, while glucose reached 15.3% at 100 °C for 60 min, yet dropped to 7 to 9% at 130 °C for the same duration (Wong et al., 2022). These results demonstrate that exceeding the optimal window of temperature and time conditions leads to carbohydrate degradation and inhibitor formation, highlighting the necessity of simultaneous control of both parameters.

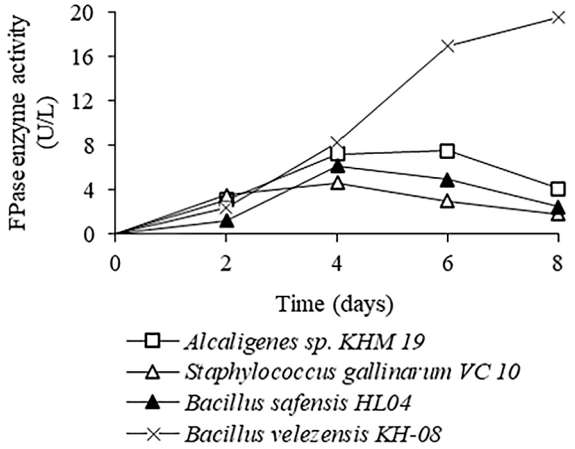

Figure 5. FPase activity of bacterial strains during biodegradation

Biological degradation of pretreated custard apple peels

Previous research has demonstrated that NaOH at high temperatures can decompose lignin and break down the cellulose structure (Andhika et al., 2021; Utoro et al., 2023; Yang et al., 2019). Therefore, biological agents such as enzymes or microorganisms quickly decompose the NaOH-treated residues. In this study, the biodegradability of custard apple peels continues to be evaluated through biological decomposition with four bacterial strains.

Figure 5 demonstrates that all four bacterial strains, including *Alcaligenes* sp. KHM19, *Staphylococcus gallinarum* VC10, *Bacillus safensis* HL04, and *Bacillus velezensis* KH-08 were capable of hydrolyzing thermal-alkaline pretreatment custard apple peels, as evidenced by their FPase activity. At the initial stage (2 days), the enzyme activity remained relatively low, ranging from 1.19 U/L in *B. safensis* HL04 to 2.34 U/L in *B. velezensis* KH-08, suggesting the adaptation of the strains to the lignocellulosic substrate. As the incubation progressed, a clear divergence in enzyme productivity was observed.

By day four, *B. velezensis* KH-08 had a nearly twofold increase in FPase activity (8.22 U/L) compared to *S. gallinarum* VC10. Enzymatic activity continued to climb until 6 days, when *B. velezensis* KH-08 outperformed the other strains, reaching 16.89 U/l. In contrast, *Alcaligenes* sp. KHM19 remained moderately active at 7.49 U/L, whereas *B. safensis* HL04 (4.89 U/L) and *S. gallinarum* VC10 (2.99 U/L) persisted at lower levels.

After 8 days of incubation, *B. velezensis* KH-08 achieved the highest FPase activity of 19.56 U/L, confirming its strong cellulolytic potential. *Alcaligenes* sp. KHM19 followed with 4.06 U/L, whereas *B. safensis* HL04 and *S. gallinarum* VC10 showed limited increases, reaching only 2.47 U/L and 1.77 U/L, respectively. These results clearly indicate that although all tested strains were able to degrade cellulose from thermal-alkaline pretreatment of custard apple peels, *B. velezensis* KH-08 was the most efficient cellulase producer. The superior performance of *B. velezensis* KH-08 may be attributed to its higher capacity for secreting a complete cellulase system, which effectively

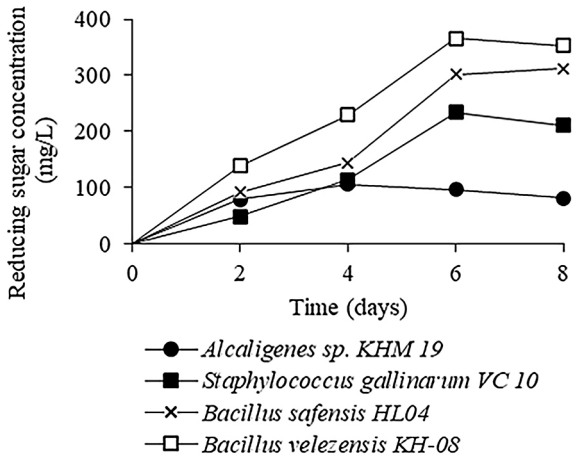

Figure 6. Reducing sugar concentration of bacterial strains during biodegradation

breaks down the complex lignocellulosic structure of custard apple peels. Meanwhile, the relatively lower activities of *S. gallinarum* VC10 and *B. safensis* HL04 suggest a more limited enzymatic machinery, resulting in slower cellulose hydrolysis. Overall, these findings highlight the potential of *B. velezensis* KH-08 as a promising candidate for bioconversion processes using agricultural residues such as custard apple peels. Furthermore, statistical analysis revealed a significant difference ($P = 0.0015$) in the FPase enzyme activity across the bacterial strains.

Figure 6 illustrates the production of reducing sugars during the biodegradation of thermal-alkaline pretreatment custard apple peels by four bacterial strains. All strains exhibited the ability to release reducing sugars, confirming their cellulolytic potential, although the yield varied significantly among them.

At 2 days, reducing sugar concentrations ranged from 78.52 mg/L in *S. gallinarum* VC10 to 137.95 mg/L in *B. velezensis* KH-08. By day four, sugar levels increased steadily, with *B. velezensis* KH-08 achieving 229.30 mg/L, which was approximately 2.2-fold higher than *S. gallinarum* VC10 (105.89 mg/L) and *Alcaligenes* sp. KHM19 (122.63 mg/L). *B. safensis* HL04 also showed a reduced sugar concentration (186.57 mg/L).

The maximum sugar release was observed on 6 days, when *B. velezensis* KH-08 reached 365.97 mg/L, followed by *B. safensis* HL04 at 301.41 mg/L. In contrast, *S. gallinarum* VC10 and *Alcaligenes* sp. KHM19 produced much lower levels of reducing sugars, only 233.43 mg/L and 96.195 mg/L, respectively. Interestingly, after 6 days, the reducing sugar concentration of *B. velezensis* KH-08 slightly decreased to 352.57 mg/L, while *B. safensis* HL04 slightly increased to 312.67 mg/L. This reduction could be attributed to the consumption of sugars by bacterial metabolism or further conversion into secondary metabolites. The decrease observed in *B. velezensis* KH-08 may be associated with the utilization of sugars for bacterial metabolism or their further conversion into secondary metabolites.

Overall, these results demonstrate that all strains contributed to cellulose hydrolysis, but *B. velezensis* KH-08 exhibited the highest efficiency in releasing fermentable sugars from thermal-alkaline pretreatment of custard apple peels. The performance of *B. safensis* HL04 was moderate, whereas *S. gallinarum* VC10 and *Alcaligenes* sp. KHM19 showed comparatively weak activity. Statistical analysis demonstrated that reducing sugar content differed significantly across bacterial strains, $P = 0.0002$. The strong correlation between FPase activity (Figure 5) and sugar release (Figure 6) further confirms that FPase production is a critical determinant of biodegradation efficiency. Thus, *B. velezensis* KH-08 represents the most promising candidate for bioconversion applications aimed at generating fermentable sugars from agricultural residues.

CONCLUSIONS

This study demonstrated the feasibility of valorizing custard apple peel (*Annona squamosa* Linn.) into a sugar-rich solution through integrated chemical and biological hydrolysis. Thermal-alkaline pretreatment followed by microbial degradation using four cellulolytic bacterial strains, including *Alcaligenes* sp. KHM19, *Staphylococcus gallinarum* VC10, *Bacillus safensis* HL04, and *Bacillus velezensis* KH-08 confirmed the effective biodegradability of the pretreated biomass. Among the tested strains, *B. velezensis* KH-08 exhibited the highest FPase activity (19.56 U/L) and released the maximum reducing sugar concentration (365.97 mg/L) after 6 days of incubation, highlighting its strong cellulolytic efficiency. In comparison, *B. safensis* HL04 showed moderate activity with a sugar yield of 312.67 mg/L after 8 days of incubation mg/L, whereas *Alcaligenes* sp. KHM19 and *S. gallinarum* VC10 displayed relatively lower hydrolytic capacities.

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