



Optimization of Biological Delignification of Sugar Palm (*Arenga pinnata*) Stems Using *Streptomyces griseorubens* for Bioethanol Production

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Received: August 2026; Revised: September 2026; Accepted: September 2026; Published: September 2026

Abstract: This study aimed to optimize the biological pretreatment of sugar palm (*Arenga pinnata*) stem biomass using *Streptomyces griseorubens* through Response Surface Methodology (RSM), based on variations in pH, substrate loading, and incubation time. The lignocellulosic composition of the biomass was analyzed using the NREL method, while process optimization was performed using Design-Expert® version 13 with a Central Composite Design (CCD) comprising 20 experimental runs. Ligninolytic enzyme activities, including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, were evaluated. The pretreated biomass was subsequently subjected to saccharification followed by fermentation with *Saccharomyces cerevisiae* using the Separate Hydrolysis and Fermentation (SHF) approach. ANOVA showed that the model was significant for lignin degradation ($p = 0.012 < 0.05$). The optimum conditions were obtained at a substrate loading of 9 g, pH 4, and an incubation period of 21 days, resulting in 12.45% lignin degradation. Under these conditions, the cellulose content increased from 34.22% to 41.99%, and the hemicellulose content increased from 8.18% to 22.99%, whereas the lignin content decreased from 34.03% to 30.86%. Laccase, MnP, and LiP activities reached 11.32, 96.69, and 187.26 U/mL, respectively. Subsequent saccharification produced 1.847 g of reducing sugars from 5 g of biomass after 48 h, while fermentation yielded an ethanol concentration of 0.97% (w/w) after 72 h. These findings demonstrate the potential of *S. griseorubens*-mediated biological pretreatment to enhance the conversion of sugar palm stem biomass for second-generation bioethanol production.

Keywords: Sugar palm stem; bioethanol; lignocellulose; Response Surface Methodology; *Streptomyces griseorubens*

How to Cite: Hidayah, N., Susilowati, A., Sugiwati, S., & Utami, A. R. I. (2026). Optimization of Biological Delignification of Sugar Palm (*Arenga pinnata*) Stems Using *Streptomyces griseorubens* for Bioethanol Production. *Bioscientist: Jurnal Ilmiah Biologi*, 14(3), 1544–1557. <https://doi.org/10.33394/bioscientist.v14i3.22366>



<https://doi.org/10.33394/bioscientist.v14i3.22366>

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INTRODUCTION

Second-generation bioethanol is a promising alternative energy source because it utilizes non-food lignocellulosic biomass and therefore does not directly compete with food resources (Isikgor & Becer, 2015). One potential feedstock for second-generation bioethanol production is sugar palm (*Arenga pinnata*) stem biomass, which is relatively abundant in Indonesia. In 2024, the total cultivated area of sugar palm was reported to reach 2 million ha. West Java Province had the largest cultivated area in Indonesia, covering 15,227 ha with a production volume of 67,378 tons (Pertanian et al., n.d.). Despite its availability, sugar palm stem biomass remains underutilized as a value-added lignocellulosic feedstock. Imraan et al. (2023) reported the potential of sugar palm fibers as lignocellulosic materials for various applications, indicating that sugar palm biomass could also be further explored as a feedstock for bioethanol production.

Lignocellulosic biomass is primarily composed of cellulose, hemicellulose, and lignin, which form a complex structural matrix that limits enzymatic access to the

cellulose fraction (Bonnen et al., 1994). In sugar palm stems, the relatively high lignin content can hinder enzymatic hydrolysis and reduce the conversion of cellulose into fermentable sugars. Imraan et al. (2023) reported that the lignin content of sugar palm biomass is approximately 46.44%. This relatively high lignin content highlights the need for an effective pretreatment step to disrupt the lignocellulosic structure, reduce the barrier imposed by lignin, and improve cellulose accessibility to hydrolytic enzymes.

Various pretreatment strategies have been developed, including physical, chemical, and biological methods. Chemical pretreatment can effectively remove lignin; however, it may generate chemical waste and require relatively high processing costs (Kumla et al., 2020). Biological pretreatment using lignocellulolytic microorganisms therefore offers a potentially more environmentally friendly alternative because it can reduce chemical consumption while facilitating lignocellulose degradation. Among the microorganisms with potential for this purpose, *Streptomyces* species are capable of producing lignocellulolytic enzymes, including cellulases, hemicellulases, and ligninolytic enzymes (Prakash et al., 2013).

Streptomyces griseorubens has been reported as a promising microorganism for the biological pretreatment of lignocellulosic biomass. Zhang et al. (2015) showed that *S. griseorubens* JSD-1 applied to rice straw produced cellulase and xylanase, thereby supporting subsequent enzymatic saccharification. However, rice straw differs from sugar palm stems in physical structure and lignocellulosic composition; therefore, pretreatment conditions established for rice straw may not be directly applicable to sugar palm stem biomass. Differences in substrate structure, lignin content, and polysaccharide availability can influence microbial growth, enzyme production, and the extent of lignocellulose degradation. Previous studies have also indicated that the performance of *S. griseorubens* in lignocellulosic pretreatment depends on substrate characteristics and operational conditions (Astina et al., 2017; Utarti et al., 2021). Nevertheless, information on the optimization of *S. griseorubens*-mediated pretreatment specifically for sugar palm stem biomass remains limited. In particular, the combined effects of pH, substrate amount, and incubation time on lignin degradation in sugar palm stems have not been sufficiently characterized.

Response Surface Methodology (RSM) is a statistical approach widely used to evaluate the effects of multiple process variables and their interactions and to determine optimum operating conditions (Dewanti, 2018). In the present study, pH, substrate amount, and incubation time were selected as key variables because they can influence the growth and enzymatic activity of *S. griseorubens*, microorganism–substrate interactions, and the duration available for lignocellulose degradation. Simultaneous optimization of these factors is therefore important for identifying pretreatment conditions that are suitable for sugar palm stem biomass and capable of promoting effective lignin removal. Accordingly, these variables were optimized using Response Surface Methodology–Central Composite Design (RSM-CCD) to determine the optimum conditions for biological pretreatment (Bhatti et al., 2017; Sugiwati et al., 2020).

Therefore, this study aimed to optimize the biological pretreatment of sugar palm stem biomass using *S. griseorubens* through an RSM-CCD approach by varying pH, substrate amount, and incubation time. Pretreatment performance was evaluated based on changes in lignocellulosic composition and ligninolytic enzyme activities. The optimum pretreatment condition was subsequently validated through saccharification and fermentation using the Separate Hydrolysis and Fermentation (SHF) method by evaluating reducing sugar and ethanol production. This study provides information on

pretreatment conditions specifically tailored to sugar palm stem biomass and evaluates the potential of *S. griseorubens*-mediated biological pretreatment to support second-generation bioethanol production.

METHOD

Analysis of Lignocellulosic Components in Sugar Palm Stem Biomass

A total of 0.3 g of sugar palm stem biomass, ground to 40 mesh and with a moisture content of <10%, was used for lignocellulosic composition analysis (Triwahyuni et al., 2015). The biomass was hydrolyzed with 3 mL of 72% sulfuric acid (H_2SO_4) and incubated in a water bath at 30°C for 2 h. Subsequently, 84 mL of distilled water was added to obtain a total volume of 87 mL, and the mixture was autoclaved (Daihan Labtech Co., Ltd., Korea) at 121°C for 1 h (Maryana et al., 2022). The hydrolysate was then vacuum-filtered through a 0.45- μm membrane.

For lignin analysis, 0.25 mL of the filtrate was mixed with 3.75 mL of 4% H_2SO_4 , and the absorbance was measured at 205 nm using a UV spectrophotometer. The remaining biomass residue was incinerated in a furnace at 575°C for 3 h (Sudiyani, 2016). For cellulose and hemicellulose determination, 3 mL of the filtrate was neutralized with CaCO_3 to pH 7 (Sudiyani, 2016). The sample was subsequently analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87C column (250 × 4 mm) maintained at 65°C, with H_2O as the eluent. Cellulose and hemicellulose contents were calculated from glucose and xylose concentrations using anhydro correction factors of 0.90 for glucose and 0.88 for xylose, respectively (Sluiter et al., 2012; Sudiyani, 2016).

Revival and Preparation of *Streptomyces griseorubens*

The *Streptomyces griseorubens* INACC A884 isolate was obtained from the Indonesian Culture Collection, National Research and Innovation Agency of Indonesia. The isolate was revived using ISP-2 (International Streptomyces Project Medium No. 2). Liquid ISP-2 medium consisted of 0.8 g yeast extract, 2 g malt extract, 0.8 g dextrose, and 200 mL distilled water (Kurniati et al., 2019). The medium was sterilized by autoclaving at 121°C for 20 min.

A single *Streptomyces* colony from the original culture was transferred into the liquid medium and incubated in a shaker at 28°C for 5–7 days (Zhang et al., 2015). Subsequently, 1 mL of the culture suspension was transferred onto solid medium in a Petri dish and incubated at room temperature for 7 days (Kurniati et al., 2019). A 1-mL aliquot of the culture suspension was used as the inoculum for each treatment, and the inoculum volume was maintained constant across all treatments. The isolate was subsequently transferred to agar slants and incubated at 28°C for 7–14 days (Utarti et al., 2021).

Biological Pretreatment Screening

A total of 10 g of sugar palm stem biomass was mixed with 20 mL of distilled water at a biomass-to-water ratio of 1:2 and sterilized at 121°C for 20 min (Sugiwati et al., 2020). A suspension of *S. griseorubens* grown on agar slants was prepared in 10 mL of distilled water. One milliliter of the culture suspension was used as the inoculum for each treatment, and the samples were incubated at 28°C for 14 days (Astina et al., 2017; Utarti et al., 2021).

After incubation, sterile distilled water was added at a biomass-to-water ratio of 1:5 (w/v), corresponding to 10 g of substrate and 50 mL of distilled water. The residual biomass was then dried to a moisture content of <10% and subsequently analyzed for its lignocellulosic composition after pretreatment.

Response Surface Methodology (RSM)

Process optimization was performed using Response Surface Methodology (RSM) in Design-Expert® version 13 with a face-centered Central Composite Design (CCD; $\alpha = 1$). The experimental design consisted of eight factorial points, six axial points, and six center points, resulting in a total of 20 experimental runs. Each run was conducted with two biological replicates.

The independent variables were pH, incubation time, and substrate amount. The pH range was 4–8 and was adjusted using 1 mmol/L HCl or 1 mmol/L NaOH (Bhatti et al., 2017; Utarti et al., 2021). This factor was selected because pH may influence *Streptomyces* growth and the delignification process. Incubation time was varied from 7 to 21 days to evaluate the effect of prolonged interaction between *Streptomyces* and the biomass (Utarti et al., 2021). The substrate amount was varied from 5 to 9 g to evaluate the effects of biomass availability and microorganism–substrate contact (Sugiwati et al., 2020).

Table 1. Response Surface Methodology (RSM) experimental design with variations in substrate amount, pH, and incubation time generated using Design-Expert® version 13

Run	Substrate Amount (g)	pH	Incubation Time (days)
1	7	6	14
2	5	8	21
3	9	4	7
4	7	6	7
5	7	6	21
6	9	4	21
7	7	6	14
8	5	8	7
9	9	8	21
10	7	8	14
11	7	6	14
12	5	6	14
13	7	6	14
14	7	4	14
15	7	6	14
16	7	6	14
17	5	4	21
18	9	8	7
19	5	4	7
20	9	6	14

Following incubation, the *S. griseorubens* culture–substrate mixture was centrifuged at 9,000 rpm for 15 min at 4°C (Sugiwati et al., 2020). The supernatant was separated from the biomass and used as a crude enzyme extract for ligninolytic enzyme activity assays.

Ligninolytic Enzyme Activity Assays

Lignin peroxidase (LiP)

Lignin peroxidase (LiP) activity was determined based on the oxidation of veratryl alcohol to veratryl aldehyde (Barahani, 2015). A reaction mixture containing 0.2 mL of crude enzyme extract, 0.2 mL of 0.05 M tartrate buffer at pH 3, 0.1 mL of 8 mM veratryl alcohol, 0.05 mL of 5 mM H₂O₂, and 0.45 mL of distilled water was prepared (Sepwin et al., 2019; Swamy & Ramalingappa, 2015). LiP activity was measured spectrophotometrically at 310 nm (Bonnen et al., 1994; Swamy & Ramalingappa, 2015), with absorbance recorded at 0 and 30 min.

LiP activity was calculated using the following equation:

$$\text{LiP activity (U/mL)} = \frac{(A_t - A_0) \times V_{\text{total}} \times 10^6}{\varepsilon_{\text{max}} \times d \times V_{\text{enzyme}} \times t}$$

Where A_t is the absorbance at time t , A_0 is the initial absorbance, V_{total} is the total reaction volume, V_{enzyme} is the volume of enzyme extract, ε is the molar extinction coefficient of veratryl aldehyde (9,300 M/cm), d is the cuvette path length (1 cm), and t is the reaction time.

Manganese Peroxidase (MnP)

Manganese peroxidase (MnP) activity was determined based on the oxidation of guaiacol (2-methoxyphenol) (Barahani, 2015). The reaction mixture consisted of 0.25 mL of crude enzyme extract, 0.25 mL of citrate-phosphate buffer at pH 5.5, 0.5 mL of 4 mM guaiacol, 0.25 mL of 50 mM MnSO₄, and 0.25 mL of 50 mM H₂O₂ (Sepwin et al., 2019; Swamy & Ramalingappa, 2015). The reaction was monitored at 0 and 30 min, and MnP activity was measured spectrophotometrically at 450 nm.

The blank consisted of 0.5 mL of guaiacol, 0.25 mL of H₂O₂, 0.25 mL of citrate-phosphate buffer, 0.25 mL of crude enzyme extract, and 0.25 mL of distilled water (Bonnen et al., 1994; Swamy & Ramalingappa, 2015). MnP activity was calculated using the following equation:

$$\text{MnP activity (U/mL)} = \frac{(A_t - A_0) \times V_{\text{total}} \times 10^6}{\varepsilon_{\text{max}} \times d \times V_{\text{enzyme}} \times t}$$

Where ε is the molar extinction coefficient of guaiacol (12,100 M/cm), d is the cuvette path length (1 cm), and t is the reaction time.

Laccase

Laccase activity was determined by mixing 1 mL of crude enzyme extract obtained from the RSM treatments with 3 mL of sodium acetate buffer at pH 5 and 1 mL of 2 mM guaiacol as the substrate (Astina et al., 2017; Kalra et al., 2013). Laccase activity was measured spectrophotometrically at 450 nm (Sepwin et al., 2019). The blank consisted of 1 mL of 2 mM guaiacol, 3 mL of sodium acetate buffer, and 1 mL of distilled water (Bonnen et al., 1994; Swamy & Ramalingappa, 2015). The reaction mixture was incubated at room temperature for 15 min.

Laccase activity was calculated using the following equation:

$$\text{Laccase activity (U/mL)} = \frac{(A_t - A_0) \times V_{\text{total}} \times 10^6}{\varepsilon_{\text{max}} \times d \times V_{\text{enzyme}} \times t}$$

Where ε is the molar extinction coefficient stated in the original method as 12,100 M/cm, d is the cuvette path length (1 cm), and t is the reaction time.

Saccharification and Fermentation

The pretreated biomass residue was subjected to enzymatic saccharification at a solid loading of 20% (w/v), corresponding to 5 g of biomass in a total reaction volume of 25 mL. A total of 17.6 mL of 0.05 M citrate buffer at pH 4.8 was added (Saritha et al., 2013), and the mixture was sterilized at 121°C for 20 min. Subsequently, 1.79 mL of Cellic® CTec2 and 0.357 mL of Cellic® HTec2 were added, with the enzyme ratio reported as 1:5 (CTec2:HTec2) (Winarni & Bardant, 2017). The samples were incubated in a shaker incubator at 50°C and 150 rpm for 72 h (Shrestha et al., 2023; Rahmania et al., 2018).

Reducing sugar concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method. Absorbance was measured at 540 nm using a UV–Vis spectrophotometer, and reducing sugar concentration was calculated from a glucose standard curve. Following saccharification, 0.25 g of *Saccharomyces cerevisiae* (1% w/v) was added to the hydrolysate. Fermentation was conducted at 32°C for 72 h, with samples collected at 12-h intervals (Maryana et al., 2022; Sudiyani, 2016). Ethanol concentration was determined using a KEM RA-650 densitometer based on sample density and subsequently converted to ethanol content (%).

Data Analysis

Optimization data were analyzed using Response Surface Methodology (RSM) in Design-Expert® version 13 to generate the regression model and determine the optimum pretreatment conditions. Model significance and the effects of the independent variables on the response were evaluated by analysis of variance (ANOVA) using Design-Expert® (Feng et al., 2017). The statistical evaluation included the F-value, p-value, lack-of-fit, coefficient of determination (R^2), adjusted R^2 , and predicted R^2 . The optimum condition was determined based on the desirability function. Relationships between the independent variables and the response were visualized as three-dimensional response surface plots using Mathcad 14 (Elfghi & Amin, 2013).

RESULTS AND DISCUSSION

Sugar Palm Stem Biomass

The lignocellulosic composition of sugar palm stem biomass was analyzed using the National Renewable Energy Laboratory (NREL) method. Biological pretreatment was performed using *Streptomyces griseorubens*, which has the ability to degrade lignin and thereby potentially improve the accessibility of cellulose and hemicellulose. The lignocellulosic composition of sugar palm biomass may vary depending on plant variety, growth conditions, environmental factors, and the maturity of the biomass.

Table 2. Lignocellulosic composition of sugar palm stem biomass before and after pretreatment

Sugar palm stem	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated biomass	34.22	8.18	34.03
<i>Streptomyces griseorubens</i> pretreatment	37.42	13.63	31.40

As shown in Table 2, untreated sugar palm stem biomass contained 34.22% cellulose, 8.18% hemicellulose, and 34.03% lignin. Imraan et al. (2023) reported a lignin content of 46.44% in sugar palm biomass, which provides a useful comparison with the initial lignin content observed in the present study. Differences in lignin content

may arise from variations in raw-material characteristics, growing conditions, and analytical methods.

Following pretreatment with *S. griseorubens*, the cellulose and hemicellulose contents increased to 37.42% and 13.63%, respectively, whereas the lignin content decreased to 31.40%. The increase in the relative cellulose content after pretreatment may be associated with the partial removal of lignin surrounding the cellulose structure (Saritha et al., 2013). Cellulose consists of glucose chains organized into microfibrils through hydrogen bonding and intermolecular interactions (Barba-Ostria et al., 2022). Therefore, lignin degradation by *S. griseorubens* may contribute to disruption of the lignocellulosic matrix, thereby increasing the accessibility of cellulose and hemicellulose (Feng et al., 2017).

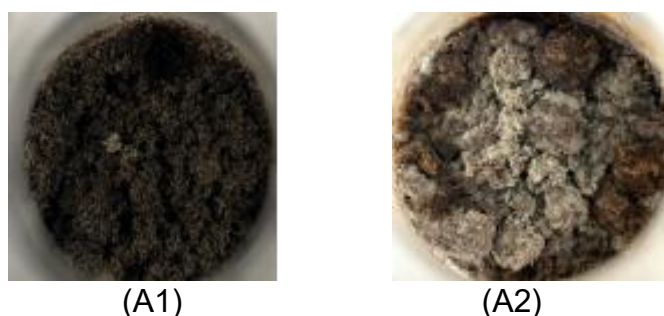


Figure 1. Visual appearance of sugar palm stem biomass after 14 days of pretreatment: (A1) without *Streptomyces* and (A2) with *Streptomyces griseorubens*

As shown in Figure 1, visual differences were observed between sugar palm stem biomass pretreated with *S. griseorubens* for 14 days and biomass without *Streptomyces* treatment. These visual changes were used as supporting observations of the pretreatment process. Disruption of the lignocellulosic structure was also associated with the apparent colonization of *Streptomyces* mycelia, which was observed from the fourth day of incubation. These observations suggest that *Streptomyces*-mediated pretreatment may improve substrate accessibility and support the subsequent bioconversion of lignocellulosic biomass (Sluiter et al., 2012; Sudiyani, 2016).

Optimization Using Response Surface Methodology (RSM)

The experimental design incorporated variations in substrate amount (5, 7, and 9 g), pH (4, 6, and 8), and incubation time (7, 14, and 21 days). Response Surface Methodology (RSM) was used to evaluate the responses generated by different combinations of these factors. During incubation, distilled water was added at a ratio of 2:1 relative to the amount of substrate. The lignin contents and percentages of lignin degradation obtained under the different pretreatment conditions are presented in Table 3.

Table 3. Lignin content and lignin degradation of sugar palm stem biomass after pretreatment with *Streptomyces griseorubens*

Run	Substrate (g)	pH	Incubation time (days)	Lignin content (%)	Lignin degradation (%)
Raw material	5	6	14	34.03	–
1	7	6	14	33.09	6.12 ± 0.03
2	5	8	21	33.74	2.43 ± 0.23
3	9	4	7	33.10	4.43 ± 1.08

Run	Substrate (g)	pH	Incubation time (days)	Lignin content (%)	Lignin degradation (%)
4	7	6	7	33.59	4.71 ± 0.25
5	7	6	21	34.27	2.79 ± 0.33
6	9	4	21	30.48	12.45 ± 0.02
7	7	6	14	33.30	5.52 ± 0.65
8	5	8	7	34.14	1.43 ± 0.51
9	9	8	21	31.78	5.81 ± 1.72
10	7	8	14	33.36	4.18 ± 1.05
11	7	6	14	32.11	8.90 ± 0.02
12	5	6	14	32.97	5.87 ± 1.06
13	7	6	14	32.12	8.88 ± 0.02
14	7	4	14	30.86	12.20 ± 0.51
15	7	6	14	31.67	10.15 ± 0.35
16	7	6	14	31.56	10.45 ± 1.81
17	5	4	21	34.40	2.39 ± 1.00
18	9	8	7	32.86	6.77 ± 0.10
19	5	4	7	33.69	4.42 ± 0.49
20	9	6	14	33.97	3.64 ± 0.53

RSM optimization showed that the highest lignin degradation was obtained at a substrate amount of 9 g, pH 4, and an incubation time of 21 days, resulting in 12.45% lignin degradation. In general, a higher percentage of lignin removal corresponded to a lower residual lignin content in the pretreated sugar palm stem biomass.

Table 4. Lignocellulosic composition of sugar palm stem biomass after RSM optimization

Sample	Analysis	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Sugar palm stem	RSM 6	41.99	22.99	30.48
	RSM 14	41.74	23.80	30.86

Following optimization, the biomass obtained under Run 6 contained 41.99% cellulose, 22.99% hemicellulose, and 30.48% lignin. Prakash et al. (2013) reported that acidic to near-neutral pH conditions are generally favorable for enzyme production by *Streptomyces*. The results of the present study also indicate that *S. griseorubens* was capable of maintaining ligninolytic activity under acidic conditions.

Statistical Analysis of Sugar Palm Stem Pretreatment

Response Surface Methodology–Central Composite Design (RSM-CCD) was used to evaluate the effects of pH, incubation time, and substrate amount on lignin degradation. Analysis of variance (ANOVA) showed that the combined effects of the three variables significantly influenced lignin degradation ($p = 0.012 < 0.05$), indicating that the fitted model was statistically significant. The relationship between the independent variables and lignin degradation was expressed using the following polynomial equation, which was subsequently used to generate three-dimensional response surface plots in Mathcad 16:

$$Y = -12.694 + 0.519X_1 + 5.092X_2 + 0.004X_3 - 0.028X_1^2 - 0.281X_2^2 - 0.013X_3^2 - 0.125X_1X_2 + 0.149X_1X_3 - 0.095X_2X_3$$

where:

Y = lignin degradation (%),

X₁ = substrate amount (g),

X₂ = pH, and

X₃ = incubation time.

The RSM-CCD analysis showed that the quadratic model was significant for lignin degradation, with an F-value of 4.708 and a p-value of 0.012 ($p < 0.05$). The model yielded an R value of 0.902, an R² of 0.813, and an adjusted R² of 0.644. The R² value indicates that the model explained 81.3% of the variation in lignin degradation. The lack-of-fit test produced an F-value of 0.858 and a p-value of 0.570 ($p > 0.05$), indicating that the lack of fit was not statistically significant.

A desirability value of 1.000 was obtained when lignin degradation was set as the response to be maximized. The best experimental condition was obtained with 9 g of substrate at pH 4 and an incubation time of 21 days, resulting in lignin degradation of $12.45 \pm 0.02\%$. The RSM model predicted a lignin degradation value of 12.30%, whereas experimental validation under the optimum conditions produced $12.45 \pm 0.02\%$, corresponding to an error of 1.22%. The close agreement between the predicted and experimental values indicates that the model adequately predicted lignin degradation within the range of pretreatment conditions investigated.

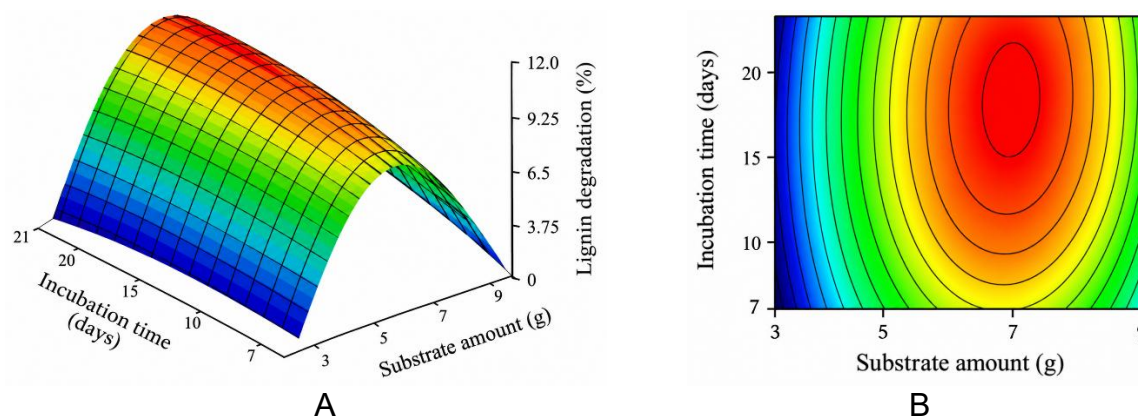


Figure 2. Three-dimensional response of lignin degradation in sugar palm stem biomass at pH 4 as a function of incubation time and substrate amount at 28°C: (A) Response surface plot, and (A) Contour plot

The response surface and contour plots shown in Figure 2 illustrate the region associated with maximum lignin degradation. Based on the experimental data, the highest lignin degradation of 12.45% was obtained with 9 g of substrate, pH 4, and an incubation time of 21 days. The response surface therefore indicates that, at pH 4, lignin degradation increased toward an incubation time of 21 days and a substrate amount of 9 g. The temperature was maintained at 28°C throughout the experiment and was not included as an independent variable in the RSM optimization.

Ligninolytic Enzyme Activity

Ligninolytic enzyme activities were evaluated in the crude enzyme extracts obtained from each RSM treatment to assess the enzymatic response of *Streptomyces griseorubens* under different pretreatment conditions. The activities of laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) are presented in Table 5.

Table 5. Ligninolytic enzyme activities in crude enzyme extracts obtained from sugar palm stem biomass

Crude Enzyme Extract	Laccase (U/mL)	MnP (U/mL)	LiP (U/mL)
Sugar palm stem 1	6.94	66.94	135.58
Sugar palm stem 2	5.62	11.16	37.74
Sugar palm stem 3	7.96	81.82	132.10
Sugar palm stem 4	7.69	63.22	95.81
Sugar palm stem 5	7.58	55.79	95.08
Sugar palm stem 6	11.32	96.69	187.26
Sugar palm stem 7	8.02	85.54	137.03
Sugar palm stem 8	6.28	11.16	29.03
Sugar palm stem 9	5.90	11.16	7.84
Sugar palm stem 10	5.76	7.44	21.63
Sugar palm stem 11	10.77	81.82	165.63
Sugar palm stem 12	9.50	40.91	143.71
Sugar palm stem 13	6.50	52.07	121.50
Sugar palm stem 14	11.21	89.26	184.35
Sugar palm stem 15	7.66	81.82	128.47
Sugar palm stem 16	8.15	74.38	124.14
Sugar palm stem 17	10.03	29.75	100.74
Sugar palm stem 18	5.84	3.72	14.95
Sugar palm stem 19	8.02	33.47	98.71
Sugar palm stem 20	7.82	33.47	88.40

The highest ligninolytic enzyme activities were observed in Run 6, corresponding to a substrate amount of 9 g, pH 4, an incubation time of 21 days, and a temperature of 28°C. Under these conditions, laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) activities reached 11.32, 96.69, and 187.26 U/mL, respectively. Run 6 also produced the highest lignin degradation, at 12.45%, with a final lignin content of 30.48%. The concurrence of high ligninolytic enzyme activities with the highest lignin degradation is consistent with the involvement of these enzymes in the delignification process.

LiP oxidizes phenolic and non-phenolic lignin compounds with relatively high redox potentials (Fisher & Fong, 2014), whereas MnP mediates substrate oxidation through Mn^{2+} in the presence of H_2O_2 as an electron acceptor (Madhavi & Lele, 2009). Laccase catalyzes the oxidation of phenolic compounds using molecular oxygen as the electron acceptor, thereby contributing to lignin depolymerization (Saini et al., 2015). The acidic conditions used in the present study were favorable for ligninolytic enzyme activity, particularly laccase, which generally exhibits high activity within the pH range of 4–5. The use of citrate buffer at pH 5 helped maintain pH stability during the enzymatic reaction and supported guaiacol oxidation, as indicated by the formation of a brown-colored oxidation product (Astina et al., 2017). Overall, the ligninolytic enzyme activity profile was consistent with the observed changes in lignocellulosic composition and supports the potential of *S. griseorubens* for biological delignification of sugar palm stem biomass.

Saccharification and Fermentation

To evaluate the effectiveness of enzymatic hydrolysis following biological pretreatment, the pretreated sugar palm stem biomass was subjected to saccharification using Cellic® CTec2 and Cellic® HTec2. Reducing sugar production was monitored over the hydrolysis period, and the results are presented in Figure 3.

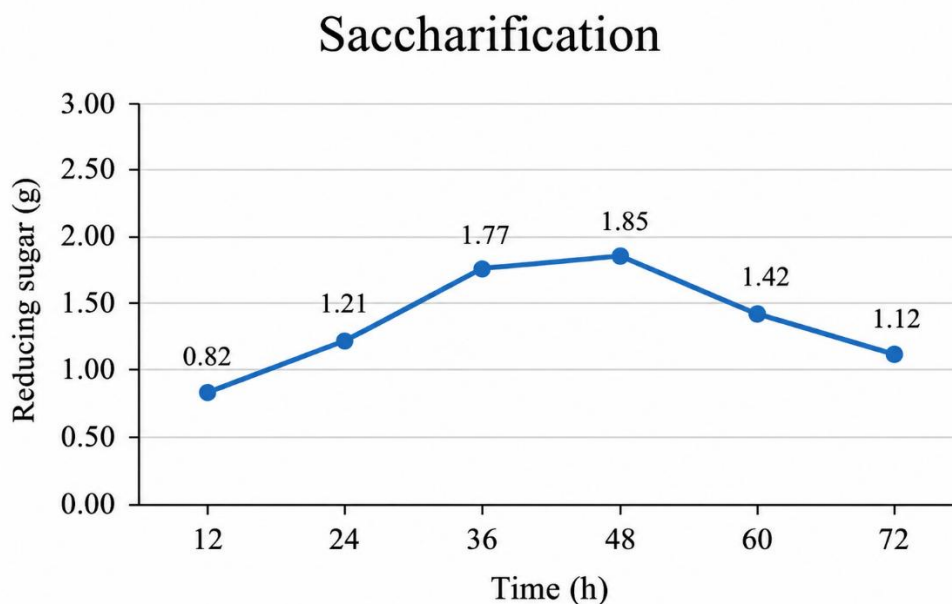


Figure 3. Reducing sugar production during enzymatic saccharification of *Streptomyces griseorubens*-pretreated sugar palm stem biomass

Saccharification was performed using Cellic® CTec2 and Cellic® HTec2 at 50°C and pH 4.8, with samples collected at 12-h intervals over 72 h. Reducing sugar production increased with hydrolysis time and reached its maximum value after 48 h (Figure 3). At this point, 1.847 g of reducing sugars was obtained from 5 g of biomass, corresponding to 36.94% relative to the initial biomass mass.

These results indicate that enzymatic saccharification converted a portion of the lignocellulosic components into reducing sugars. The pretreated biomass contained 41.74% cellulose, which contributed to reducing sugar formation during enzymatic hydrolysis, as also reported by Sudiyani (2016). The increase in reducing sugar production indicates that biological pretreatment with *S. griseorubens* improved the accessibility of cellulose to hydrolytic enzymes. The synergistic activities of endoglucanase, exoglucanase, and β -glucosidase promote the hydrolysis of cellulose into glucose and thereby enhance saccharification efficiency (Kumla et al., 2020; Sugiwati et al., 2020). After the maximum reducing sugar level was reached, the measured amount tended to decrease, which may be associated with substrate depletion and declining enzymatic activity (Kanti & A, 2005).

The hydrolysate obtained from saccharification was subsequently fermented with *Saccharomyces cerevisiae* using the Separate Hydrolysis and Fermentation (SHF) method for 72 h. Saccharification yielded 1.847 g of reducing sugars, which served as the substrate for the subsequent fermentation step. After 72 h of fermentation, an ethanol concentration of 0.97% (w/w) was obtained. Ethanol production is influenced by the amount of fermentable sugar generated during saccharification because glucose is a primary substrate converted to ethanol by *S. cerevisiae* (Maryana et al., 2022; Sudiyani, 2016). Although the ethanol concentration obtained in this study was

relatively low, the results demonstrate that biological pretreatment with *S. griseorubens* can facilitate reducing sugar production and thereby support the conversion of sugar palm stem biomass into second-generation bioethanol.

CONCLUSION

Biological pretreatment of sugar palm stem biomass using *Streptomyces griseorubens* effectively reduced lignin content and improved the accessibility of lignocellulosic components. The best experimental condition was obtained at a substrate amount of 9 g, pH 4, and an incubation time of 21 days, resulting in 12.45% lignin degradation and a final lignin content of 30.48%. Under these conditions, laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) activities reached 11.32, 96.69, and 187.26 U/mL, respectively. After pretreatment, the cellulose and hemicellulose contents were 41.99% and 22.99%, respectively. Subsequent saccharification produced a maximum of 1.847 g of reducing sugars from 5 g of initial biomass, while fermentation yielded an ethanol concentration of 0.97% (w/w). These findings indicate that *S. griseorubens* has potential as a biological pretreatment agent for enhancing the utilization of sugar palm stem biomass as a feedstock for second-generation bioethanol production.

RECOMMENDATION

Further studies should focus on optimizing the saccharification and fermentation stages to improve ethanol production and yield. In addition, mass balance, solid recovery, and carbohydrate recovery analyses are recommended to provide a more comprehensive evaluation of biomass conversion efficiency throughout the pretreatment, saccharification, and fermentation processes. Further optimization of key saccharification and fermentation parameters, including time, pH, temperature, and enzyme or microorganism concentration, is also needed to achieve higher ethanol yields.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Department of Biosciences, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret (FMIPA UNS), for providing research facilities during the master's program. The authors also thank the National Research and Innovation Agency of Indonesia (BRIN) for its additional support.

This research was partially supported by grants from the Directorate General of Higher Education, Ministry of Research, Technology, and Higher Education of the Republic of Indonesia, under Grant Nos. 106/E5/PG.02.00.PL/2024 (14 June 2024), 043/SP2H/RT-MONO/LL4/2024 (14 June 2024), and 041/LIT07/PPM-LIT/2024 (18 June 2024), as well as by the Control Systems and Energy Laboratory, Engineering Physics Study Program, Telkom University.

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