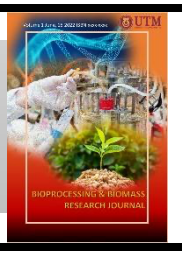
Bioprocessing and Biomass Technology

Journal homepage: https://bioprocessing.utm.my



Research Article

Optimization of Temperature, Cellulase Concentration and pH value of Enzymatic Saccharification for Producing Sugar from Ozone Pre-treated Oil Palm Empty Fruit Bunch

Nur Zahidah Abd Majid[a], Amnani Shamjuddin[a*], Umi Aisah Asli[a], Nur Alia Farhin Mohd Fauzi[a], Sharifah Nurain Hussain[a], Asiah Nusaibah Masri[b, c], Nur Hidayah Zainan[d], Nardiah Rizwana Jaafar[d]

[a] *Chemical Reaction Engineering Group (CREG), Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia*
[b] *UTM-MPRC Institute for Oil & Gas, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia*
[c] *Energy Management Group, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia*
[d] *Department of Bioprocess & Polymer Engineering, Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310, Johor Bahru, Johor, Malaysia*

ARTICLE INFO

Article History:
Received 01 June 2025
Received in revised form 21 July 2025
Accepted 22 July 2025
Available online 31 July 2025

Keywords:
Optimization,
Enzymatic saccharification,
Oil palm empty fruit bunch,
Response surface methodology,
Ozonolysis

ABSTRACT

Enzymatic saccharification is a crucial step in biomass conversion, where cellulase enzymes break down cellulose into fermentable sugars. This study focuses on optimizing the enzymatic saccharification process for total reducing sugar (TRS) production from ozone pre-treated oil palm empty fruit bunches (OPEFB). A Face-Centred Central Composite Design (FCCD) was employed to evaluate the effects of three key process parameters: temperature (30 °C to 60 °C), cellulase concentration (0.5 mg/mL to 1.5 mg/mL), and pH (3 to 7). A total of 20 experimental runs were conducted. The response variable measured was the concentration of total reducing sugar (TRS) produced. The highest TRS yield achieved was 3.598 mg/mL under the optimized conditions of 39.157 °C, 1.177 mg/mL cellulase concentration, and pH 6.824. These findings demonstrate that ozone pre-treatment significantly enhances the enzymatic hydrolysis efficiency of OPEFB. The optimized conditions suggest a promising pathway for the efficient production of TRS, supporting future upscaling and potential commercialization of bio-based products.

INTRODUCTION

Oil palm empty fruit bunch (OPEFB) generated by oil palm mill is an abundant lignocellulosic biomass that is easily accessible in Malaysia. OPEFB has high cellulose content that can be converted into higher value-added chemicals such as fermentable sugars. Before the production of fermentable sugars, the biomass pre-treatment process is significantly important to extract the cellulose component from OPEFB. Pre-treatment is a critical step in biomass conversion. In this study, ozonolysis was selected as the pre-treatment method for OPEFB to improve enzymatic saccharification efficiency (Chen *et al.*, 2023). Ozonolysis pre-treatment appears as a green technique for extracting cellulose components as it can effectively remove lignin and hemicellulose components with zero toxic waste and minimal detrimental effects on cellulose structures (Ab Rasid *et al.*, 2021). To address the growing dependence on fossil fuels, lignocellulose is recognized as a promising renewable substrate for the generation of fermentable sugars, which can be biochemically converted into biofuels and high-value chemicals through advanced catalytic and enzymatic processes (Gonzales et al, 2019). Enzymatic saccharification is widely recognized for its effectiveness in converting pre-treated lignocellulosic biomass into total reducing sugar (Postiaux *et al.*, 2025). For instance, Rizal *et al.* (2018) conducted enzymatic hydrolysis of OPEFB at 50 °C and 150

*Corresponding Author
E-mail address: amnani.shamjuddin@utm.my
DOI address: 10.11113/bioprocessing.v4n1.73

rpm, collecting hydrolysates over 1–72 hours and quantifying TRS using the dinitrosalicylic acid (DNS) method. Enzymatic hydrolysis is a very selective method of breaking down polysaccharides. Enzymatic hydrolysis produces monosaccharides by facilitating the multistep depolymerization of cellulose in the presence of water using a system of enzymes called cellulases. These enzymes function by dispersing throughout the biomass, attaching themselves to the polysaccharides, and rupturing glycosidic bonds at specific sites. This process eventually occurs because of the many enzyme processes. Enzymes break glycosidic bonds by a complicated and extremely specific method (Brown et al., 2024). Efficient saccharification of lignocellulosic biomass requires the best pre-treatment method that maximizing the cellulose exposure (Ling et al., 2013). In enzymatic saccharification, the enzymes cleave ether and ester bonds to produce fermentable sugars. The product of enzymatic saccharification is total reducing sugar (TRS) and analysed by DNS method using a UV-Vis Spectrophotometer. Hence, in this study, the production of TRS from ozone pre-treated OPEFB via the enzymatic saccharification is optimized. Enzymatic saccharification utilizes cellulases for hydrolysing the pre-treated OPEFB aimed at maximizing the TRS production. A Face-Centred Central Composite Design (FCCD) was employed to evaluate the effects of three key process parameters: temperature (30 °C to 60 °C), cellulase concentration (0.5 mg/mL to 1.5 mg/mL), and pH (3 to 7). A total of 20 experimental runs were conducted. The response variable measured was the concentration of total reducing sugar (TRS) produced. The highest TRS yield achieved was 3.598 mg/mL under the optimized conditions of 39.157 °C, 1.177 mg/mL cellulase concentration, and pH 6.824. These findings demonstrate that ozone pre-treatment significantly enhances the enzymatic hydrolysis efficiency of OPEFB.

MATERIALS AND METHOD

Oil Palm Empty Fruit Bunch Fibre

The OPEFB sample was obtained from Felda Taib Andak Palm Oil Mill, Kulai. It was first physically pre-treated through sun drying for 8 hours daily over the course of a week to remove moisture. After that, the sample was oven-dried at 100 °C, followed by a grinding process using a cutting mill (SM-100, Germany) with an outlet mesh size of 0.8 mm. The ground OPEFB was then subjected to a sieving process using a mechanical sieve shaker (Biobase BK-TS200, China) equipped with mesh sizes of 1.0, 0.8, 0.5, 0.3, and 0.25 mm to obtain the desired particle size of 0.3 mm.

Ozonolysis Pre-treatment of OPEFB Fibre

The ozonolysis pre-treatment of OPEFB was carried out using the OzBioNY 2.0 reactor at UTM. A 500 g of OPEFB was charged into the reactor and moistened using distilled water. 60 g/m^3 ozone concentration was then supplied into the reactor for completing the reaction about 90 minutes.

Enzymatic Hydrolysis of Pre-treated OPEFB Fibre

Two grams sample of OPEFB was weighed and put into 250 mL conical flask. The enzymatic saccharification of OPEFB is conducted in a 0.05 M sodium acetate buffer with a pH of 5 and the incubator shaker was maintained at a temperature of 45 °C. Cellulase concentration of 1.5 mg/mL was added by using a micropipette into the conical flasks that already contain the OPEFB substrate. Then, the sample solution was put into the incubator shaker. The agitation speed is set at 250 rpm for 24 hours. After 24 hours, the samples were put into a water bath at 90 °C for 5 minutes to deactivate enzyme activity (Lee et al., 2020). The samples were filtered by using a 0.2 µm membrane syringe filter.

Design of Experiment and Statistical Analysis

The experimental design and statistical analysis for the optimization study were performed using Design Expert software (Stat-ease Inc., USA). **Table 1** show the independent variables which are temperature (℃), enzyme concentration(mg/ml), pH while the total reducing sugar concentration (mg/ml) is dependent variable. 20 sets of generated experiments are shown in **Table 2** by Response Surface Method (RSM) three-factor of face-centred central composite design (FCCD). The fixed parameters where agitation speed is set at 250 rpm for 24 hours. The enzymatic saccharification experiments were repeated by following the parameter values from the design of the experiment in **Table 2**. The actual and predicted response was analyzed through analysis of variance (ANOVA) by Design Expert Software.

Table 1 Independent variables and their corresponding levels

Variables	-1	0	+1
Temperature (℃)	30	45	60
Enzyme concentration(mg/ml)	0.5	1	1.5
pH	3	5	7

Analysis of Total Reducing Sugar using DNS Method

Approximately 3 mL of the hydrolysate from each run, resulting from the enzymatic saccharification process, was combined with 3 mL of DNS reagent and heated to a boil for 5 minutes. Following boiling, the mixture was promptly cooled to room temperature before UV–vis analysis at 540 nanometers. The concentration of total reducing sugars (TRS) in the hydrolysate filtrate was determined in mg/mL using a calibration curve based on glucose concentration as the standard curve.

RESULTS AND DISCUSSION

Table 2 shows the actual and predicted response for total reducing sugar (TRS) concentration for 20 sets of experiment design, while **Table 3** shows the analysis of variance (ANOVA) for TRS responses. The highest yield of TRS (3.53 mg/ml) in **Table 2** was observed at 60 °C temperature, 0.5 mL enzyme concentration, and pH 3 of buffer. The enzymatic saccharification experiments were conducted within the temperature range of 30–60 °C to evaluate the effect of temperature on the hydrolysis efficiency of the ozonolysis-pretreated empty fruit bunch (EFB). The upper limit of 60 °C was selected based on previous studies reporting that certain commercial cellulase blends, such as Cellic® CTec2, retain significant activity up to 60 °C for short durations, despite their optimum activity being around 50 °C (Lee et al., 2020). Including 60 °C in the experimental design provides insight into enzyme stability and performance

under thermally stressed conditions, which is valuable for process optimization and potential scale-up.

The modelling prediction equation for TRS was generated by the Design Expert software as **Equation (1)**:

$$TRS = -3.84647 + 0.323717A - 6.37207B + 1.44831C + 0.051167AB - 0.027875AC + 0.778750BC - 0.002877A^2 - 0.109091B^2 - 0.090568C^2 \quad \textbf{Equation (1)}$$

Table 2 Design matrix experiment from Design Expert

Run	T* (℃)	EC* (mg/ml)	pH	TRS* (mg/ml)	
				Actual	Predicted
1	30	0.5	3	3.02	3.02
2	60	0.5	3	3.53	3.22
3	30	1.5	3	0.32	0.30
4	60	1.5	3	2.02	2.04
5	30	0.5	7	3.42	3.40
6	60	0.5	7	0.24	0.26
7	30	1.5	7	3.49	3.80
8	60	1.5	7	2.19	2.19
9	30	1.0	5	3.29	3.02
10	60	1.0	5	2.05	2.32
11	45	0.5	5	3.18	3.49
12	45	1.5	5	3.40	3.1
13	45	1.0	3	2.51	2.82
14	45	1.0	7	3.40	3.09
15	45	1.0	5	3.32	3.32
16	45	1.0	5	3.38	3.32
17	45	1.0	5	3.30	3.32
18	45	1.0	5	3.21	3.32
19	45	1.0	5	3.36	3.32
20	45	1.0	5	3.32	3.32

*T-Temperature (℃), EC-Enzyme Concentration (mg/ml), TRS-Total Reducing Sugar Concentration

The ANOVA result in **Table 3** indicates that the TRS model is statistically significant, with a P-value of 0.0001, with a high coefficient of determination (R^2 = 0.9602) and an adjusted R^2 of 0.9244, demonstrating a strong fit between the experimental and predicted values. The TRS concentration model has R^2 value of 0.9602. Based on **Table 3**, temperature, enzyme concentration, and pH value are significant parameters affecting TRS production, as all the P-values are less than 0.05. Among the linear terms, temperature (A) and enzyme concentration (B) significantly influenced the response (p = 0.0022 and 0.0445, respectively), whereas pH (C) showed no significant effect individually (p = 0.1492). However, interaction terms AC and BC exhibited strong significance (p <0.0001), indicating synergistic effects between temperature and pH, and between enzyme concentration and pH, respectively. The AB interaction was also significant (p = 0.0025), further supporting the relevance of combined variable effects. Quadratic terms A^2 and C^2 were significant or borderline significant (p = 0.0027 and 0.0511, respectively), suggesting curvature in the response surface for temperature and pH. On the other hand, B^2 (p = 0.8709) was not significant, indicating a linear response to enzyme concentration within the tested range. Despite the significance of the lack-of-fit (p = 0.0005), which could indicate model deviations, the high R^2 values suggest that the model still adequately describes the system behaviour. Overall, temperature and its interaction with other variables play a crucial role in determining saccharification efficiency.

Table 3 ANOVA table for the enzymatic saccharification model including their interactions and quadratic terms on the sugar yield

Source	Sum of Squares	DF*	Mean Square	F-value	p-value	
Model	17.75	9	1.97	26.82	< 0.0001	significant
A*	1.23	1	1.23	16.75	0.0022	
B*	0.3881	1	0.3881	5.28	0.0445	
C*	0.1796	1	0.1796	2.44	0.1492	
AB	1.18	1	1.18	16.02	0.0025	
AC	5.59	1	5.59	76.07	< 0.0001	
BC	4.85	1	4.85	65.97	< 0.0001	
A^2	1.15	1	1.15	15.67	0.0027	
B^2	0.0020	1	0.0020	0.0278	0.8709	
C^2	0.3609	1	0.3609	4.91	0.0511	
R^2	0.9602					
Adj R^2	0.9244					
Lack of Fit	0.7179	5	0.1436	40.90	0.0005	significant

*A-Temperature, B-Enzyme Concentration, C-pH value, DF-Degrees of freedom

Analysis of Response Surface Plot

Figures 1, 2, and **3** show the relationship between the two parameters fixed. The trend in **Figure 1** shown that the TRS concentration increases with enzyme concentration. As enzyme concentration increases from 0.5 to around 1.3–1.5 mg/mL, the production of TRS concentration increases significantly, especially at moderate temperatures. Temperature has a peak effect as temperature increases from 30 °C to around 45–50 °C, TRS production also increases, but beyond that point, the TRS production starts to decrease, could be possibly due to enzyme denaturation at higher temperatures. The highest region on the surface (the red-orange peak) corresponds to a temperature of around 45–50 °C and an enzyme concentration of about 1.3–1.5 mg/mL. This suggests that these are the optimal conditions for maximizing total reducing sugar yield. Enzyme concentration has a strong positive effect on TRS

production. Temperature affects TRS production non-linearly, there's an optimum point (45–50 °C), after which enzyme activity may decline. The graph helps in identifying the best operating conditions for enzymatic saccharification.

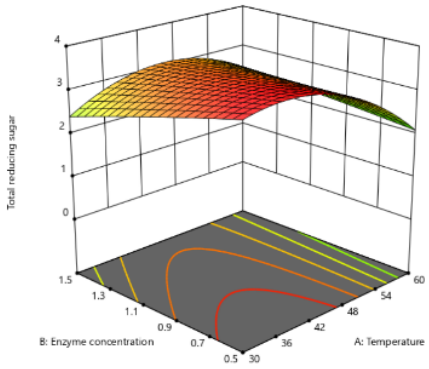


Figure 1 3D response surface plot of total reducing sugar (TRS) as a function of enzyme concentration and temperature of enzymatic saccharification.

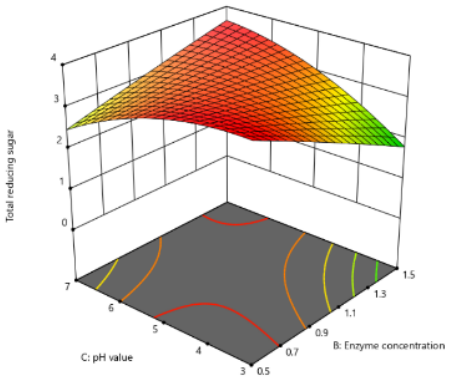


Figure 2 3D response surface plot of total reducing sugar (TRS) as a function of pH value and enzyme concentration of enzymatic saccharification.

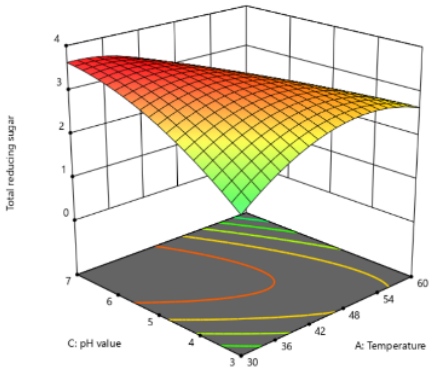


Figure 3 3D response surface plot of total reducing sugar (TRS) as a function of enzyme concentration and temperature of enzymatic saccharification.

In **Figure 2**, enzyme concentration has a strong positive effect. As enzyme concentration increases from 0.5 to 1.5 mg/mL, the production of total reducing sugar consistently increases. This is a common trend, as more enzyme means more catalytic activity for hydrolysing cellulose into sugars. Besides, pH has an optimal range. TRS production increases as pH rises from 3 to around 5. Beyond pH 5, the production either plateaus or slightly decreases. This suggests that pH 5 is the optimal pH for the enzymatic hydrolysis under these conditions, likely the point where cellulase activity is maximized. The peak of the surface (top of the red-orange area) is where enzyme concentration is high (1.5 mg/mL) and pH is around 5. This indicates the best combination of conditions for maximizing TRS concentration. We can see that enzyme concentration has a direct, strong positive correlation with TRS concentration. Besides, pH affects enzyme activity non-linearly, with pH 5 being the most favourable. The graph identifies the optimal zone for maximizing total reducing sugar yield in enzymatic saccharification using cellulase enzyme (Guo et al, 2018).

Figure 3 shows the TRS concentration increases with pH. At a fixed temperature, TRS concentration increases as pH rises from 3 to around 5–6. This suggests that slightly acidic conditions (around pH 5) are optimal for enzyme activity, which is typical for cellulase enzymes. TRS concentration decreases with higher temperatures. As temperature increases from 30 °C to 60 °C, TRS concentration generally declines. This indicates that higher temperatures negatively affect enzyme activity, likely due to enzyme denaturation. The lowest TRS concentration is observed at low pH 3 and high temperature 60 °C. These are both stressful conditions for enzymes, leading to reduced hydrolysis efficiency. The highest TRS concentration is observed at low temperature 30 °C and moderate to high pH 6–7. This region reflects the most favourable conditions for enzyme stability and function in this setup (Uchegbu *et al.*, 2022). The curve patterns show that pH has a stronger effect than temperature across the range. The contour lines are more curved along the pH axis than the temperature axis, indicating pH is a more critical variable in this interaction. The current findings show that pH has a positive effect on TRS concentration up to around pH 6. Temperature has a negative effect when it exceeds the cellulase enzyme stable operating range. The optimal condition lies at low temperature 30–36 °C and moderate pH 5–6. Previous study has reported (Maryana et al., 2024) production of reducing sugar 8.3% which is higher than this study which is 3.6 mg/mL (Chen *et al.*, 2023).

CONCLUSION

This study successfully optimized the enzymatic saccharification conditions for the production of total reducing sugars (TRS) from ozone pre-treated oil palm empty fruit bunch (OPEFB) using a Face-centred Central Composite Design (FCCD). The optimal conditions identified is temperature of 39.157 °C, cellulase concentration of 1.177 mg/mL, and pH 6.824, yielded a maximum TRS concentration of 3.598 mg/mL. These findings highlight the potential of ozone pre-treated OPEFB as a viable and efficient feedstock for fermentable sugar production. The results provide a strong foundation for further scale-up and commercialization of this process, contributing to the sustainable conversion of agricultural waste into valuable bio-based products.

Acknowledgement

This work was supported by the Ministry of Education Malaysia under the Fundamental Research Grant Scheme

(FRGS)(FRGS/1/2022/TK05/UTM/02/11) and Universiti Teknologi Malaysia, UTM CG, Q.J130000.3046.05M00.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

Ab Rasid, N. S., Shamjuddin, A., Rahman, A. Z. A., & Amin, N. A. S. (2021). Recent advances in green pre-treatment methods of lignocellulosic biomass for enhanced biofuel production. *Journal of Cleaner Production*, *321*, 129038.

Brown, J., Lindstrom, J. K., Ghosh, A., Rollag, S. A., & Brown, R. C. (2024). Production of sugars from lignocellulosic biomass via biochemical and thermochemical routes. *Frontiers in Energy Research*, *12*, 1347373.

Chen, Z., Chen, L., Khoo, K. S., Gupta, V. K., Sharma, M., Show, P. L., & Yap, P. S. (2023). Exploitation of lignocellulosic-based biomass biorefinery: A critical review of renewable bioresource, sustainability and economic views. *Biotechnology Advances*, *69*, 108265.

Gonzales, R. R., Kim, J. S., & Kim, S. H. (2019). Optimization of dilute acid and enzymatic hydrolysis for dark fermentative hydrogen production from the empty fruit bunch of oil palm. *International Journal of Hydrogen Energy*, *44*(4), 2191-2202.

Guo, H., Chang, Y., & Lee, D. J. (2018). Enzymatic saccharification of lignocellulosic biorefinery: research focuses. *Bioresource technology*, *252*, 198-215.

Lee, K. M., Zanil, M. F., Chan, K. K., Chin, Z. P., Liu, Y. C., & Lim, S. (2020). Synergistic ultrasound-assisted organosolv pretreatment of oil palm empty fruit bunches for enhanced enzymatic saccharification: an optimization study using artificial neural networks. *Biomass and Bioenergy*, *139*, 105621.

Ling, T. P., Hassan, O., Badri, K., Maskat, M. Y., & Mustapha, W. A. W. (2013). Sugar recovery of enzymatic hydrolysed oil palm empty fruit bunch fiber by chemical pretreatment. *Cellulose*, *20*(6), 3191-3203.

Maryana, R., Bardant, T. B., Ihsan, D. M., Das, A. K., Irawan, Y., Rizal, W. A., ... & Sudiyani, Y. (2024). Reducing sugars and bioethanol production from oil palm empty fruit bunch by applying a batch and continuous pretreatment process with low temperature and pressure. *Biomass Conversion and Biorefinery*, *14*(10), 11155-11164.

Postiaux, A., Putra, F. J. N., Kahar, P., Richel, A., & Ogino, C. (2024). Maleic acid-butanol pretreatment to enhance cellulose accessibility for enzymatic hydrolysis and ethanol production from oil palm empty fruit bunch. *ACS Environmental Au*, *5*(1), 76-85.

Rizal, N. F. A. A., Ibrahim, M. F., Zakaria, M. R., Abd-Aziz, S., Yee, P. L., & Hassan, M. A. (2018). Pre-treatment of oil palm biomass for fermentable sugars production. *Molecules*, *23*(6), 1381.

Uchegbu, N. N., Ude, C. N., Okoyeuzu, C. F., Nwadi, O. M. M., Rasaq, W. A., Ogbonna, C. U., ... & Okpala, C. (2022). Optimisation of enzymatic fermented glucose production of wild cocoyam starch using response surface methodology. *Glob. NEST J*, *24*, 351-361.