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# Steam Explosion and Sequential Steam Explosion – Dilute Acid Pretreatment Optimization of Banana Pseudostem for Polyhydroxybutyrate (PHB) Production

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**Polyhydroxybutyrate (PHB) is a suitable biodegradable alternative to non-renewable petroleum-based plastics. Readily available agricultural lignocellulosic residues such as banana pseudostem can be utilized as a substrate to reduce the production cost of PHB and improve its feasibility for commercialization. Accordingly, pretreatment of banana pseudostem is needed to efficiently convert the substrate to PHB. In this study, optimization of two pretreatment methods for banana pseudostem – namely, steam explosion and sequential steam explosion – dilute acid pretreatment – were optimized to improve the digestibility of the biomass and consequently increase the production of reducing sugars in the hydrolysate during enzymatic saccharification. Response surface methodology (RSM)-designed experiments showed that among all factors investigated, for both pretreatment methods, the steam explosion temperature had the strongest positive impact on reducing sugar production. Optimum conditions of steam explosion pretreatment were 219.31 °C steam explosion temperature and 10 min of pretreatment time, producing 7.33 g/L (48.87% yield) of reducing sugars in the enzymatic hydrolysate. For sequential pretreatment, optimum conditions were 220 °C steam explosion temperature, 135 °C dilute acid temperature, 44 min of dilute acid reaction time, and 1.57% w/v $H_2SO_4$, with a corresponding reducing sugar concentration of 13.02 g/L (86.79% yield). A 90% increase in reducing sugar yield was observed after dilute acid pretreatment of steam-exploded banana pseudostem. Using the hydrolysate from sequentially-pretreated banana pseudostem, PHB (2.64 g/L) was successfully synthesized after 12 h of bacterial fermentation. Hence, sequential pretreatment was proven effective in producing enzymatic hydrolysates from banana pseudostem for PHB production.**

Keywords: banana pseudostem, enzymatic saccharification, polyhydroxybutyrate, response surface methodology, sequential steam explosion – dilute acid pretreatment, steam explosion

## INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable polymers that are usually produced by microorganisms through bacterial fermentation (Getachew and Woldesenbet 2016). These polymers exhibit material properties comparable to non-biodegradable petrochemical plastics (Madkour *et al*. 2013). They produce non-toxic degradation products and are usually derived from renewable biomass sources (Verlinden *et*

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*al*. 2007; Jain *et al*. 2010). Due to these attributes, PHAs are currently explored as a sustainable alternative to conventional plastics.

PHB is the first type of PHA discovered during the 1920s, appearing as intracellular granular structures in the cytoplasm of *Bacillus megaterium* (Singh 2015). Similar to other PHAs, PHB is produced by various microorganisms in response to conditions of physiological stress, serving as carbon and energy storage for future consumption (Obruca 2015). Most works on PHB are commonly focused on its use as commodity plastic, together with its application in medicine and controlled drug release systems (Martino *et al*. 2014).

Different bacteria such as *Pseudomonas* spp., *Bacillus* spp., and *Cupriavidus* spp. have already been used for PHB production (Tan *et al*. 2014). However, Gram-negative bacteria are known to release lipopolysaccharides that have an endotoxic effect in humans (Khiyami *et al*. 2011). Removal of these endotoxins during PHB extraction entails additional cost. In this case, Gram-positive bacteria such as *B. megaterium* may be more advantageous to use. Additionally, *B. megaterium* can be easily grown by utilizing a wide spectrum of cheap substrates, from simple sugars to complex industrial wastes (Mohapatra *et al*. 2017). These attributes make the microorganism suitable for commercial production of PHB.

The price of PHB is still high in comparison to conventional plastics due to its expensive production cost. High production cost is attributed to the expensive cost of substrates, which accounts for about 40% of the total production cost (Kourmentza *et al*. 2015). To lower the production cost, inexpensive substrates such as sugarcane molasses (Dalsasso *et al*. 2019), oils and glycerols (Rao *et al*. 2010), effluents and co-products from ethanol (Davila *et al*. 2016) and dairy industries (Bosco and Chiampo 2010), as well as food wastes (Nielsen *et al*. 2017) were already explored as raw materials for PHB production. Another option is to utilize agricultural by-products such as lignocellulosic residues. These materials are exceptionally attractive as feedstocks because they are relatively inexpensive, abundant, and sustainable (Du *et al*. 2012). In general, lignocellulosic materials are composed of cellulose, hemicellulose, and lignin that are present in varying compositions. A wide range of compounds may be generated from these materials – including fermentable sugars such as glucose, xylose, mannose, galactose, and arabinose, which can be further converted to value-added products such as PHB (Kim 2017).

As an agricultural country, the Philippines generates large volumes of lignocellulose (Requiso *et al*. 2018). One of the most abundant lignocellulosic agricultural residues in the Philippines is banana pseudostem. The biomass contains about 60–85% lignocellulose on a dry weight basis (Jayaprabha *et al*. 2011). Banana pseudostems are fibrous in nature and contain lower amounts of lignin compared to other agricultural residues (Gabhane *et al*. 2014). However, after banana production, the pseudostem ends up only as agricultural waste.

PHB production involving lignocellulosic biomass such as banana pseudostem usually starts with the release of fermentable sugars through pretreatment and enzymatic saccharification. Lignocellulosic materials are recalcitrant and often require pretreatment to disrupt the lignin complex to liberate the sugars contained embedded in plant cell walls (Chen and Liu 2015). Effective pretreatment is the most critical operation in the use of lignocellulosic biomass as feedstock for the biosynthesis of various products (Jönsson and Martín 2016). An ideal pretreatment process renders lignocellulosic materials completely vulnerable to enzymes during saccharification (Sun *et al*. 2015). It should minimize sugar losses and the formation of by-products that inhibit the action of enzymes and the growth of fermentative microorganisms (Chen and Liu 2015). Most importantly, the process should have low capital and operational cost and must be applicable to a wide variety of lignocellulosic materials (Jönsson and Martín 2016).

The type of pretreatment process and its corresponding efficiency are greatly influenced by the nature of the lignocellulosic biomass. Steam explosion and dilute acid pretreatment are two of the most common pretreatment methods for lignocellulosic biomass (Agbor *et al*. 2019). Steam explosion is a physicochemical process that breaks down the fibers by treating the biomass with saturated steam, which is followed by a sudden reduction of pressure causing explosive decompression. This process causes the defibration of cellulose bundles, which results in better cellulose accessibility (Chen and Liu 2015). Commonly, steam explosion is combined with acid pretreatment to further increase the susceptibility of the biomass to enzymes (Guerrero *et al*. 2017). Acid pretreatment – which can be done using sulfuric acid ($H_2SO_4$), hydrochloric acid, mineral acids (phosphoric and nitric acid), and some organic acids (formic acid) – is relatively cost-effective and more destructive towards the biomass, yielding appreciable amounts of sugar upon saccharification (Lenihan *et al*. 2010). Combining these two pretreatment methods can result in higher sugar yields at reduced operating costs (Silva *et al*. 2017). As to the author's knowledge, there had been no published work about optimizing steam explosion and/or acid pretreatment of banana pseudostem for PHB production.

In this study, two pretreatment methods for banana pseudostem – namely, steam explosion and sequential steam explosion – dilute sulfuric acid pretreatment

– were optimized *via* RSM. Optimization was based mainly on the extent of the digestibility of pretreated biomass, as quantified by the concentration of reducing sugars in the hydrolysate produced after enzymatic saccharification. For the steam explosion, the effects of steam explosion temperature and pretreatment time were investigated, while steam explosion temperature, dilute acid reaction temperature, dilute acid reaction time, and acid concentration were tested for sequential pretreatment. Finally, the fermentability of the hydrolysate as an alternative carbon source for biodegradable plastic production produced was assessed *via* flask fermentation.

## METHODOLOGY

### Materials

Banana pseudostem was provided by DIWANG Adhika Enterprise based in San Jose, Morong, Rizal, Philippines and was immediately processed at UPLB-BIOTECH, Laguna, Philippines. Collected biomass was sun-dried to reduce its initial moisture content. It was cut into pieces (approximately 1 $in^2$) and oven-dried at 60–70 °C for 2–3 d. The particle size of banana pseudostem was further reduced to approximately 5 mm using a Model H-12 Hammer Mill (Hosokawa Micron Corporation, Osaka, Japan). Then, the biomass was oven-dried again at 60–70 °C for 2–3 d to further lower its moisture content to approximately 2% w/w and prevent spoilage. After drying, the biomass was milled using Retsch SM300 Cutting Mill (Retsch GmbH, Haan, Germany) to attain an average particle size of 0.5 mm. Powdered banana pseudostem was placed in plastic bags and stored under ordinary room conditions. On a dry basis, the banana pseudostem sample was analyzed to contain (w/w): 33.82% cellulose, 14.74% hemicellulose, 9.45% lignin, and 18.07% extractives.

Sulfuric acid (98%, analytical grade) (RCI Labscan Limited, Thailand) was used to prepare the dilute acid for sequential pretreatment. VISCAMYL™ Flow Cellulase (DuPont, Genencor Company, USA) was used as the enzyme for the saccharification of pretreated banana pseudostem. *B. megaterium* PNCM 1890, obtained from the Philippine National Collection of Microorganisms (PNCM) was used as the fermentation microorganism for flask-level PHB production. PHB (Santa Cruz Biotechnology, Inc., Dallas, Texas) was used as the standard for the Fourier transform infrared (FTIR) spectroscopy analysis of PHB produced from fermentation.

### Experimental Design and Data Analysis for Steam Explosion and Sequential Pretreatment

A face-centered central composite design (FCD) was used as the RSM-designed experiment to optimize the steam explosion. Factors investigated were steam explosion temperature and pretreatment time. Meanwhile, the experiments for sequential pretreatment involved a Split-Plot FCD. Three hard-to-change factors (steam explosion temperature, dilute acid temperature, and dilute acid reaction time) and an easy-to-change factor (acid concentration) were tested for optimization. In both optimization experiments, the concentration of reducing sugars (*RS*) in the hydrolysate produced after enzymatic saccharification of pretreated biomass was considered as the response. In sequential pretreatment, the steam explosion was performed prior to dilute acid pretreatment to avoid possible corrosion of the steam exploder. Since steam explosion temperature affects *RS* more significantly than pretreatment time, it was the only factor considered for the steam explosion. The steam explosion was held constant for 10 min. Experimental runs for both pretreatment methods were generated using Design Expert v11.0 (Stat-Ease, Minnesota, USA).

Upon analysis of data, recommended models that relate the significant factors to *RS* were generated using Design Expert v11.0. Models were generated based on the analysis of the sequential model sum of squares, which involved lack-of-fit (LOF) assessment, *p*-value calculations as well as computations of coefficients of regression ($R^2$). The significance ($p < 0.05$) of each term in the generated recommended model, as well as the model's LOF statistics, were generated *via* ANOVA. LOF must be desirably insignificant, coefficients of regression must be high (close to 99%), and the difference between the adjusted and predicted $R^2$ values must be less than 0.2 (Anderson and Whitcomb 2017). Furthermore, assumptions of ANOVA are checked through three diagnostic plots – namely, normality of residuals, homogeneity of variance residuals, and independence of residuals. Graphs were generated for a visual representation of the effects of significant factors once the models were statistically validated. Lastly, numerical optimization was done using the software to determine the pretreatment conditions that maximize the concentration of reducing sugars in the hydrolysate. Predictions made were confirmed *via* experimental verification. Optimization was considered successful if the actual values from experimental verification were within the 95% prediction interval generated by the software, with less than 10% difference from the predicted value.

### Steam Explosion and Sequential Pretreatment Methods

For the steam explosion, banana pseudostem was subjected to high temperature and high-pressure saturated steam using QBS-80 Steam Exploder (Hebi-Bioenergy,

Shanghai, China). About 35 g of banana pseudostem was loaded to the reaction chamber of the exploder for every experimental run. Steam explosion temperature and pretreatment time were varied according to the experimental design, as shown in Table 1. After the explosion, the banana pseudostem slurry was collected from the equipment. For sequential pretreatment, steam-exploded banana pseudostem was pretreated with dilute $H_2SO_4$. Dilute acid pretreatment was done in Technicon BD-40 Heating Unit (Technicon Corporation, New York, USA) using 25-mL screw cap tubes with a solids loading of 1.5 g dry pretreated biomass per 15 mL dilute $H_2SO_4$ solution. Acid concentration, as well as dilute acid temperature and dilute acid reaction time, were adjusted according to the experimental design presented in Table 3.

Pretreated banana pseudostem slurry was separated from the pretreatment liquor by filtration through a 0.25-mm pore size cloth. Collected solids were sufficiently washed with tap water until the washings attain a neutral pH of pH 7.0. Finally, the solids were stored in a freezer at 0 °C until enzymatic saccharification.

**Enzymatic Saccharification**

Enzymatic saccharification was done to determine the maximum extent of the digestibility of pretreated banana pseudostem, measuring the effectiveness of the pretreatment (Adney and Baker 2008; Selig *et al*. 2008). Briefly, about 0.15 g of dry pretreated banana pseudostem was mixed with 5 mL sodium citrate ($Na_3C_6H_5O_7$) buffer (0.1 M, pH 4.8). To prevent the growth of microbial contaminants during saccharification, 0.1 mL of 20.0 g g/L sodium azide solution ($NaN_3$) was added to the reaction mixture. Enzyme loading was set at 60 FPU per g of dry pretreated banana pseudostem. Assuming that pretreated banana pseudostem has a density of 1 g/mL, an adequate amount of distilled water was added to the mixture such that the total reaction volume after the addition of enzyme would be 10 mL. Then, the tubes were incubated at 50 °C in Thomas Shaking Incubator MI-699 (Thomas Kagaku Co. Ltd., Tokyo, Japan) for 72 h. To stop the reaction, the enzyme was inactivated by heating the samples at 100 °C for 5 min. The liquid hydrolysate was separated from the solids by centrifugation at 10,000 rpm at 25 °C for 10 min using Combi 514R Centrifuge (Hanil Science Industrial, South Korea). The hydrolysate was stored at 0 °C in a freezer until analysis.

**Flask-level PHB Production**

A modified PHA production medium (Mineral Medium H-3) supplemented with glucose was used as a seed medium (Atlas 2010). A liter of seed medium contained 20.0 g glucose ($C_6H_{12}O_6$), 2.3 g potassium dihydrogen phosphate ($KH_2PO_4$), 2.3 g sodium hydrogen phosphate ($Na_2HPO_4$), 1.6 g sodium nitrate ($NaNO_3$), 0.5 g sodium hydrogen carbonate ($NaHCO_3$), 0.5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.1 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 20.0 mL aqueous ferric citrate ($C_6H_5FeO_7$) solution (2.5 g/L), and 5.0 mL trace elements solution. A liter of trace elements solution contained 0.1 g zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 0.03 g manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 0.3 g boric acid ($H_3BO_3$), 0.2 g cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 0.01 g copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), 0.02 g nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$), and 0.03 g sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$). Ferric citrate and trace elements solutions were sterilized separately to avoid reactions that may occur at high temperatures.

A 24-h old slant culture of *B. megaterium* PNCM 1890 was inoculated to the seed medium. The seed culture was incubated in a shaking incubator for 18 h at 35 °C and 150 rpm. An initial $OD_{600}$ of 0.50 was used for the fermentation runs. Bacterial cells needed for inoculation were separated from the seed broth by centrifugation at 2000x g for 15 min using Swing Type Centrifuge CD-50SR (Tomy Seiko, Japan) and was inoculated to the hydrolysate medium. A liter of the hydrolysate medium, which was produced from the optimized pretreatment conditions, was supplemented with the same amount of salts and minerals as that of the seed medium. Fermentation experiments with glucose (11.80 g/L) as the carbon source were also conducted for comparison. Fermentation flasks were incubated in a shaker at 35 °C and 150 rpm. Samples were withdrawn to determine cell growth (in terms of dry cell weight), residual reducing sugar concentration, and PHB concentration.

**Analytical Methods**

The concentration of reducing sugars in the samples was measured using the modified 3,5-dinitrosalicylic acid (DNS) method with spectrophotometry using a UV-1800 spectrophotometer (Shimadzu Corporation, Tokyo, Japan) (Adney and Baker 2008).

For the determination of dry cell weight, a 10-mL fermentation broth sample was centrifuged at 2,000x g for 15 min. The supernatant was stored at 0 °C in a freezer for reducing sugar analysis. Meanwhile, the deposited cell pellet was washed twice with distilled water to remove excess medium components that may interfere with the analysis. After washing, the resulting cell pellet was resuspended in distilled water, and the produced cell suspension was dried in an oven at 105 °C until constant weight.

PHB was extracted by the dissolution of non-PHB cell material using sodium hypochlorite and purification

using acetone (Berger *et al*. 1989; Porras *et al*. 2018). Ten (10) mL of culture medium sample was centrifuged at 2000x g for 15 min. The recovered cell pellet was washed twice with distilled water and resuspended in 10 mL of commercial sodium hypochlorite solution (NaOCl) (Clorox, 6% NaOCl, pH 9.8). Then, the mixture was incubated at 37 °C in a water bath for 1 h. After incubation, the solids were separated from the supernatant by centrifugation for 30 min at 2,000x g to remove excess NaOCl containing non-PHB cell material. The solids were washed twice with distilled water. After washing, the pellet was washed with approximately 5 mL of acetone to dissolve the remaining lipids. Finally, the sample was dried to constant weight in an oven at 60 °C.

The chemical structure of the extracted PHB was investigated using the FTIR spectrophotometer (Shimadzu IR Prestige-21, Japan) equipped with attenuated total reflectance (ATR) unit. The samples were placed as films in the germanium crystal of the ATR unit. Scans were performed in the range of 400–4000 $cm^{-1}$.

**Severity Factor, Combined Severity Factor, and Reducing Sugar Yield**

Severity factor ($R_o$) (Equation 1) quantifies the aggression of steam explosion towards disrupting the lignocellulosic structure of the material and is calculated using the values of steam explosion pretreatment time (*t*, min) and steam explosion temperature (*T*, °C). The combined severity factor (*CSF*) (Equation 2) shows the combined effects of steam explosion and acid pretreatment (expressed using the pH of dilute acid) (Wyman and Yang 2017). Reducing sugar yield (*RSY*, % w/w) per unit mass of saccharified biomass was calculated using Equation 3.

$$R_o = t \times e^{\frac{T-100}{14.75}} \quad (1)$$

$$\log CSF = \log R_o - pH \quad (2)$$

$$RSY = \frac{\text{reducing sugars in hydrolysate (g)}}{\text{saccharified biomass in dry weight basis (g)}} \text{ x } 100\% \quad (3)$$

## RESULTS AND DISCUSSION

**Steam Explosion of Banana Pseudostem**

The values of reducing sugar concentrations and yields corresponding to varying steam explosion conditions and severity factors are presented in Table 1. Reducing sugar production from steam-exploded banana pseudostem is significantly improved with increased severity factor. At low pretreatment severities ($R_o$ < 1,200), only little improvements in reducing sugar concentration (3.49–4.98 g/L) and yield (23–33.20%) were observed. In contrast, at severities greater than 3,400, both reducing sugar concentration (5.41–7.59 g/L) and yield (36.09–50.58%) increased abruptly. A steam explosion at 220 °C and 10 min ($R_o$ = 34,138) produced the highest reducing sugar yield of 50.58% (7.59 g/L) while the lowest yield of 23.02% (3.45 g/L) was obtained at 120°C and 1 min ($R_o$ = 4). Overall, as steam explosion conditions were increased, reducing sugar production also correspondingly increased. This agrees with the results of a study related to the production of biofuels from lignocellulosic banana wastes (Santa-Maria *et al*. 2013), wherein a higher glucose concentration (8 g/L) was produced at severe steam explosion conditions (200 °C and 64 min) compared to

**Table 1**. Reducing sugar concentrations and yields obtained from the optimization of the steam explosion of banana pseudostem.

| Standard run | Steam explosion temperature (°C) | Pretreatment time (min) | Severity factor ($R_o$) | Reducing sugar | |
|---|---|---|---|---|---|
| | | | | Concentration (g/L) | Yield (%) |
| 1 | 120 | 1 | 4 | 3.45 | 23.00 |
| 2 | 220 | 1 | 3,414 | 5.41 | 36.09 |
| 3 | 120 | 10 | 39 | 3.76 | 25.07 |
| 4 | 220 | 10 | 34,138 | 7.59 | 50.58 |
| 5 | 120 | 5.5 | 21 | 4.15 | 27.66 |
| 6 | 220 | 5.5 | 18,776 | 6.24 | 41.63 |
| 7 | 170 | 1 | 115 | 4.36 | 29.06 |
| 8 | 170 | 10 | 1,151 | 4.85 | 32.32 |
| 9 | 170 | 5.5 | 633 | 4.98 | 33.20 |
| 10 | 170 | 5.5 | 633 | 4.63 | 30.84 |
| 11 | 170 | 5.5 | 633 | 4.68 | 31.21 |
| 12 | 170 | 5.5 | 633 | 4.34 | 28.92 |
| 13 | 170 | 5.5 | 633 | 4.25 | 28.33 |

the concentration (4.2 g/L) obtained at low pretreatment severity (190 °C and 10 min).

Generally, increasing both steam explosion temperature and pretreatment time increases the severity of a steam explosion. However, the severity factor is apparently more dependent on the steam explosion temperature than on pretreatment time (Table 1). An 880-fold increase in severity factor was observed when the steam explosion temperature was increased from 120 °C to 220 °C (at 10 min). Meanwhile, prolonging the pretreatment time from 1 min to 10 min (at 220 °C) resulted only in a 10-fold increase in severity. A steam explosion at higher temperatures releases more energy during steam decompression, increasing the tendency of biomass particle collisions. This enhances the disruption of the lignocellulosic complex of the biomass, exposing more surface area of cellulose to enzymes during saccharification, eventually leading to increased reducing sugar yields (Pielhop *et al*. 2016).

**Optimization of Steam Explosion of Banana Pseudostem**

Table 2 shows the significance of the inverse model relating *RS* to steam explosion conditions. The desirable insignificance of LOF in the optimization of the steam explosion of banana pseudostem was confirmed *via* ANOVA. Also, post-ANOVA analysis showed high values of regression coefficients ($R^2$ = 0.8988, adjusted $R^2$ = 0.8785, and predicted $R^2$ = 0.8226). These results imply that the model explains the majority of the effects of steam explosion conditions to *RS* and could be used to make predictions about the response for given levels of the factors.

Both main effects (*A* – steam temperature and *B* – pretreatment time) significantly affected *RS*. The coded model relating *RS* to the factors is shown in Equation 4. Based on the model, steam temperature (with a coefficient of –0.0533) had the largest (positive) indirect effect on *RS*. The positive coefficients of both factors suggest that *RS* increased as the factors are increased. Thus, at a high steam explosion temperature of 220 °C and a prolonged pretreatment time of 10 min, banana pseudostem was efficiently pretreated.

$$\frac{1}{RS} = 2.00A + 1.55B + 7.69 \qquad (4)$$

Figure 1 shows the three-dimensional (3D) graphical representation of the model. Reducing sugar concentration may probably still increase beyond 220 °C and 10 min. However, due to the limitations on the operating conditions of the exploder, steam explosion experiments beyond 220 °C were not performed anymore.

With the goal to maximize the yield of reducing sugars during enzymatic saccharification, the steam explosion of banana pseudostem was optimized. The optimum conditions for the steam explosion were at a steam temperature of 219.3 °C and a pretreatment time of 10 min, with a predicted reducing sugar concentration of 6.89 g/L (45.93% yield). Reducing sugar concentration of

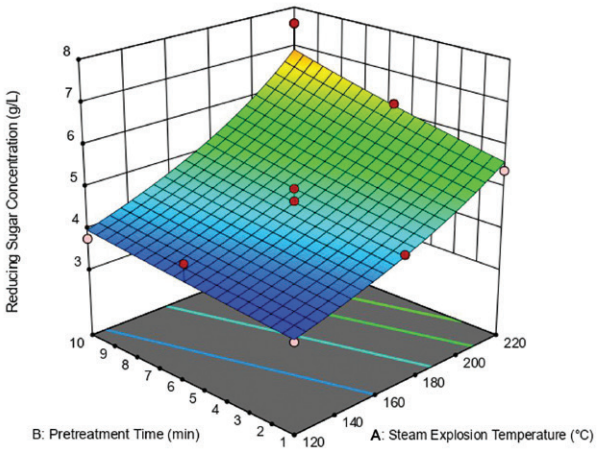


**Figure 1**. 3D surface plot of reducing sugar concentration against steam explosion temperature and pretreatment time in the optimization of the steam explosion of banana pseudostem.

**Table 2**. Results of ANOVA from the optimization of the steam explosion of banana pseudostem.

| Source | Sum of squares | Degrees of freedom | Mean square | *F*-value | *p*-value | Remarks |
|---|---|---|---|---|---|---|
| **Model** | 0.0187 | 2 | 0.0094 | 44.40 | < 0.0001 | Significant |
| *A* – Steam explosion temperature | 0.0171 | 1 | 0.0171 | 80.88 | < 0.0001 | |
| *B* – Pretreatment time | 0.0017 | 1 | 0.0017 | 7.93 | 0.0183 | |
| **Residual** | 0.0021 | 10 | 0.0002 | | | |
| Lack of fit | 0.0013 | 6 | 0.0002 | 1.17 | 0.4587 | Not significant |
| Pure error | 0.0008 | 4 | 0.0002 | | | |
| **Cor total** | 0.0208 | 12 | | | | |

7.33 g/L (48.86% yield) obtained from actual experiments was very close to the predicted value, with only a 6.37% difference. The reducing sugar yield is lower than the yield obtained from a similar study (92%) on the production of fermentable sugars from acid-catalyzed steam-exploded banana pseudostem, pretreated at 177 °C for 5 min, with a 2.2% v/v $H_2SO_4$ catalyst (Guerrero *et al*. 2017). This suggests that the steam explosion may still be insufficient in pretreating and improving the digestibility of banana pseudostem for saccharification. Hence, a steam explosion coupled with an additional pretreatment method (*i.e*. dilute acid pretreatment) was also explored in this study.

## Sequential Pretreatment of Banana Pseudostem

To simplify the experimental design, pretreatment time during the steam explosion was set at 10 min since it was proven to have less prominent effects on the severity of pretreatment and on *RS*, based on the results of the steam explosion. Reducing sugar yield values corresponding to different sequential pretreatment conditions is presented in Table 3.

Combined severity factor values increase as steam explosion temperature and acid concentration are increased. At severe sequential pretreatment conditions (*CSF* > 1,740), relatively higher reducing sugar yields ranging from 55–86% were produced, while less severe pretreatment conditions (*CSF* < 8) only yielded 32–47% of reducing sugars. After the dilute acid pretreatment of steam-exploded banana pseudostem, the highest yield obtained was 86.67%, produced at high levels of all factors: 220 °C steam explosion temperature, 140 °C dilute acid temperature, 45 min of dilute acid reaction time, and 2% w/v dilute acid concentration.

## Optimization of Sequential Pretreatment of Banana Pseudostem

Table 4 shows the ANOVA obtained from sequential pretreatment, while Equation 5 shows the model relating *RS* to the significant factors. Values of regression coefficients are relatively high at $R^2 = 0.8212$ and adjusted $R^2 = 0.7573$. All main effects (*a* – steam explosion temperature, *b* – dilute acid temperature, *c* – dilute acid reaction time, and *D* – acid concentration), two two-way

**Table 3**. Reducing sugar concentrations and yields obtained from the optimization of sequential pretreatment of banana pseudostem.

| Standard run | Steam explosion temperature (°C) | Dilute acid temperature (°C) | Dilute acid reaction time (min) | Acid concentration (% w/v) | Combined severity factor (*CSF*) | Reducing sugar | |
|---|---|---|---|---|---|---|---|
| | | | | | | Concentration (g/L) | Yield (%) |
| 1 | 120 | 80 | 15 | 0.5 | 2 | 4.84 | 32.24 |
| 2 | 120 | 80 | 15 | 2 | 8 | 6.15 | 41.00 |
| 3 | 220 | 80 | 15 | 0.5 | 1,740 | 7.68 | 51.23 |
| 4 | 220 | 80 | 15 | 2 | 6,961 | 8.34 | 55.61 |
| 5 | 120 | 140 | 15 | 0.5 | 2 | 4.90 | 32.66 |
| 6 | 120 | 140 | 15 | 2 | 8 | 5.90 | 39.34 |
| 7 | 220 | 140 | 15 | 0.5 | 1,740 | 9.09 | 60.61 |
| 8 | 220 | 140 | 15 | 2 | 6,961 | 11.22 | 74.82 |
| 9 | 120 | 80 | 45 | 0.5 | 2 | 5.09 | 33.91 |
| 10 | 120 | 80 | 45 | 2 | 8 | 5.18 | 34.54 |
| 11 | 220 | 80 | 45 | 0.5 | 1,740 | 9.08 | 60.51 |
| 12 | 220 | 80 | 45 | 2 | 6,961 | 10.02 | 66.82 |
| 13 | 120 | 140 | 45 | 0.5 | 2 | 5.29 | 35.29 |
| 14 | 120 | 140 | 45 | 2 | 8 | 6.17 | 41.13 |
| 15 | 220 | 140 | 45 | 0.5 | 1,740 | 11.70 | 78.02 |
| 16 | 220 | 140 | 45 | 2 | 6,961 | 13.00 | 86.67 |
| 17 | 170 | 110 | 30 | 1.25 | 147 | 6.65 | 44.34 |
| 18 | 170 | 110 | 30 | 1.25 | 147 | 5.88 | 39.23 |
| 19 | 170 | 110 | 30 | 1.25 | 147 | 8.72 | 58.11 |
| 20 | 120 | 110 | 30 | 1.25 | 5 | 6.68 | 44.55 |
| 21 | 120 | 110 | 30 | 1.25 | 5 | 7.06 | 47.05 |
| 22 | 220 | 110 | 30 | 1.25 | 4,351 | 9.30 | 61.97 |

**Table 3**. continuation

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 23 | 220 | 110 | 30 | 1.25 | 4,351 | 11.95 | 79.70 |
| 24 | 170 | 80 | 30 | 1.25 | 147 | 6.26 | 41.73 |
| 25 | 170 | 80 | 30 | 1.25 | 147 | 8.34 | 55.61 |
| 26 | 170 | 140 | 30 | 1.25 | 147 | 8.36 | 55.71 |
| 27 | 170 | 140 | 30 | 1.25 | 147 | 7.56 | 50.39 |
| 28 | 170 | 110 | 15 | 1.25 | 147 | 5.65 | 37.64 |
| 29 | 170 | 110 | 15 | 1.25 | 147 | 5.63 | 37.55 |
| 30 | 170 | 110 | 45 | 1.25 | 147 | 8.99 | 59.95 |
| 31 | 170 | 110 | 45 | 1.25 | 147 | 8.96 | 59.76 |
| 32 | 170 | 110 | 30 | 0.5 | 59 | 5.65 | 37.67 |
| 33 | 170 | 110 | 30 | 2 | 235 | 7.29 | 48.62 |
| 34 | 170 | 110 | 30 | 1.25 | 147 | 6.18 | 41.22 |
| 35 | 170 | 110 | 30 | 1.25 | 147 | 8.63 | 57.50 |
| 36 | 170 | 110 | 30 | 1.25 | 147 | 9.19 | 61.26 |
| 37 | 170 | 110 | 30 | 1.25 | 147 | 5.96 | 39.75 |
| 38 | 170 | 110 | 30 | 1.25 | 147 | 8.87 | 59.15 |
| 39 | 170 | 110 | 30 | 1.25 | 147 | 6.34 | 42.26 |

**Table 4.** Results of ANOVA from the optimization of sequential pretreatment of banana pseudostem.

| Source | Term degrees of freedom | Error degrees of freedom | *F*-value | *p*-value | Remarks |
|---|---|---|---|---|---|
| **Whole plot** | 7 | 30.00 | 18.89 | < 0.0001 | Significant |
| *a* – Steam explosion temperature | 1 | 30.00 | 97.71 | < 0.0001 | |
| *b* – Dilute acid temperature | 1 | 30.00 | 7.48 | 0.0104 | |
| *c* – Dilute acid reaction time | 1 | 30.00 | 9.95 | 0.0036 | |
| *ab* | 1 | 30.00 | 4.96 | 0.0336 | |
| *ac* | 1 | 30.00 | 3.56 | 0.0690 | |
| $a^2$ | 1 | 30.00 | 8.53 | 0.0066 | |
| $D^2$ | 1 | 30.00 | 4.96 | 0.0336 | |
| **Subplot** | 1 | 30.00 | 5.52 | 0.0256 | Significant |
| *D* – Acid concentration | 1 | 30.00 | 5.52 | 0.0256 | |

interaction effects (*ab* – steam explosion temperature x dilute acid temperature, and *ac* – steam explosion temperature x dilute acid reaction time), and two quadratic effects ($a^2$ – squared term for steam explosion temperature, and $D^2$ – squared term for acid concentration) were significant in the production of reducing sugars.

$$RS = 7.43 + 2.21a + 0.6105b + 0.7040c + 0.5528D + 0.5556ab + 0.4706ac + 1.30a^2 - 0.9951D^2 \quad (5)$$

Based on the factor coefficients, the steam explosion temperature – with a coefficient of (+)2.21 – has the largest positive impact on *RS* during sequential pretreatment. Interestingly, increasing almost all factors, except acid concentration, leads to an increase in the response. Further increase in acid concentration eventually led to a significant reduction in *RS*, as implied by the negative coefficient [(–)0.9951] of the quadratic term of the factor. Very high acid concentrations may have catalyzed the degradation of sugars and the formation of unwanted by-products during dilute acid pretreatment.

Figure 2 shows that the effect of dilute acid temperature (A) and dilute acid reaction time (B) on *RS* largely depended on the steam explosion temperature. At higher steam explosion temperature, increasing both dilute acid temperature and dilute acid reaction time increased the production of reducing sugars. This agrees with the previous observation that the steam explosion temperature predominates among all factors affecting *RS*. Low

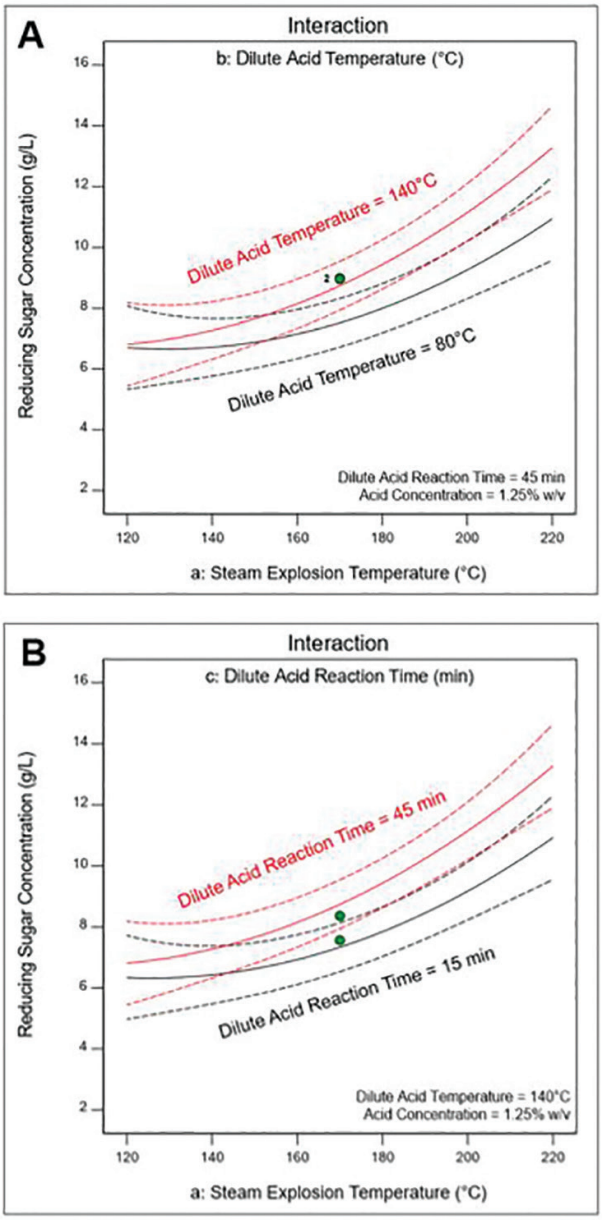


**Figure 2**. Interaction effects plot of steam explosion temperature with (A) dilute acid temperature and (B) dilute acid reaction time on the optimization of sequential pretreatment of banana pseudostem.

operating conditions for dilute acid pretreatment are enough to increase *RS*, provided that a steam explosion is conducted at high temperatures. Therefore, high steam explosion temperature should be coupled with dilute acid pretreatment to effectively disrupt the recalcitrance of biomass, increasing its susceptibility to enzymes during saccharification.

3D surface graphs relating to reducing sugar concentration to steam explosion temperature and dilute acid temperature at different levels of dilute acid reaction time and acid concentration on the optimization of sequential pretreatment of banana pseudostem is presented in Figure 3. It shows that the effect of dilute acid concentration on the production of reducing sugars is highly influenced by the duration of dilute acid pretreatment.

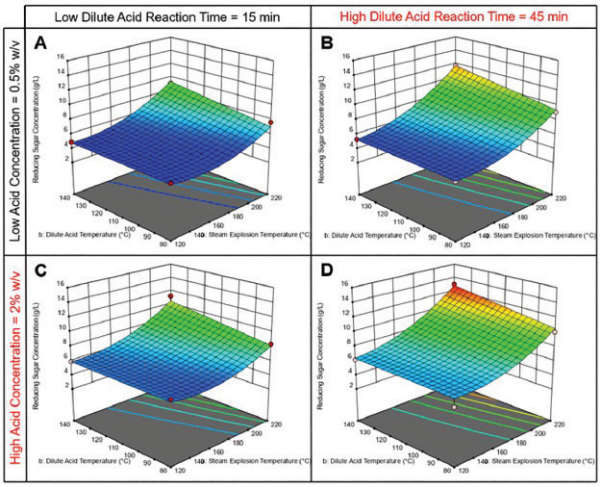


**Figure 3**. 3D surface plots of reducing sugar concentration against steam explosion temperature and dilute acid temperature at different levels of dilute acid reaction time and acid concentration on the optimization of sequential pretreatment of banana pseudostem.

Figures 3A and 3C show that if the reaction time is too short, any concentration of acid could not effectively pretreat the biomass. Although sugar degradation can be significantly avoided at shorter reaction times, the production of reducing sugars was significantly compromised. On the other hand – as shown in Figures 3B and 3D – at prolonged dilute acid pretreatment, acid molecules had sufficient time to break the lignin-carbohydrate matrix of the biomass, producing more sugars during saccharification. In Figure 3D, as the concentration of acid present in the system is increased, a larger portion of the biomass is pretreated in given reaction time. Increasing the dilute acid concentration resulted in a large increase in the combined severity factor, which improves the production of reducing sugars. Therefore, in order to yield high amounts of reducing sugars, dilute acid pretreatment should be carried out at high steam explosion temperature, high dilute acid temperature, longer dilute acid reaction time, and high acid concentration.

The optimum conditions for sequential pretreatment were at steam explosion temperature of 220 °C (pretreatment time of 6.5 min), 135 °C dilute acid temperature, 44 min dilute acid reaction time, and 1.573% w/v acid concentration, with a predicted reducing sugar concentration of 13.02 g/L (86.79% yield). An actual

reducing sugar concentration of 11.80 g/L (60.93% yield) obtained from confirmatory experiments was also close to the predicted value, with a 9.84% difference.

Pretreating banana pseudostem by steam explosion only yielded 45.93% of reducing sugars, while sequential pretreatment generated an 86.79% yield in the hydrolysate. Further pretreatment of steam-exploded biomass by dilute acid increased the reducing sugar yield by approximately 90%. In conclusion, sequential pretreatment is more effective than a sole steam explosion pretreatment. Dilute acid pretreatment may have reinforced the effect of the steam explosion in destroying the recalcitrance of banana pseudostem.

### Fermentation of Banana Pseudostem Hydrolysate from Optimum Pretreatment Conditions

Sequential pretreatment was employed in the production of pretreated banana pseudostem for enzymatic saccharification since it was proven to be more effective in terms of reducing sugar production. Fermentation results showed that PHB was successfully synthesized using the actual hydrolysate. Figures 4A and 4B show the residual reducing sugar concentration, cell growth and PHB production profiles of *B. megaterium* PNCM 1890 grown in fermentation medium with banana pseudostem hydrolysate and glucose as carbon sources, respectively.

Based on Figure 4A, bacterial exponential growth was already observed from the start until the 12th h of fermentation. During the exponential phase, a dramatic decrease in the concentration of reducing sugars occurred, suggesting that cells were rapidly consuming them for growth and PHB production. After 12 h, with about 90% of the reducing sugars in the medium consumed, cell growth proceeded to stationary phase. Then, the death phase started after 36 h of fermentation. Interestingly, the PHB concentration profile is highly resembled the cell growth profile, implying that PHB production *via B. megaterium* was growth-associated. Therefore, PHB production is at its peak when cell concentration is maximum. The highest dry cell weight (3.31 g/L) and PHB (2.64 g/L) concentrations were obtained after 12 h of fermentation using the hydrolysate.

As shown in Figure 4B, similar trends were observed when glucose was used as a carbon source. Maximum dry cell weight (4.58 g/L) and PHB (3.67 g/L) concentrations were produced at 24 h. Although dry cell weight and PHB concentrations were relatively lower, desirable shorter peak time was exhibited when actual banana pseudostem hydrolysate was used as a carbon source. This suggests that there could be components in the actual hydrolysate that triggered an immediate production of PHB. Further studies to completely understand the nature and effects of these components of the hydrolysate is an interesting area of research to improve and optimize the production of PHB using banana pseudostem.

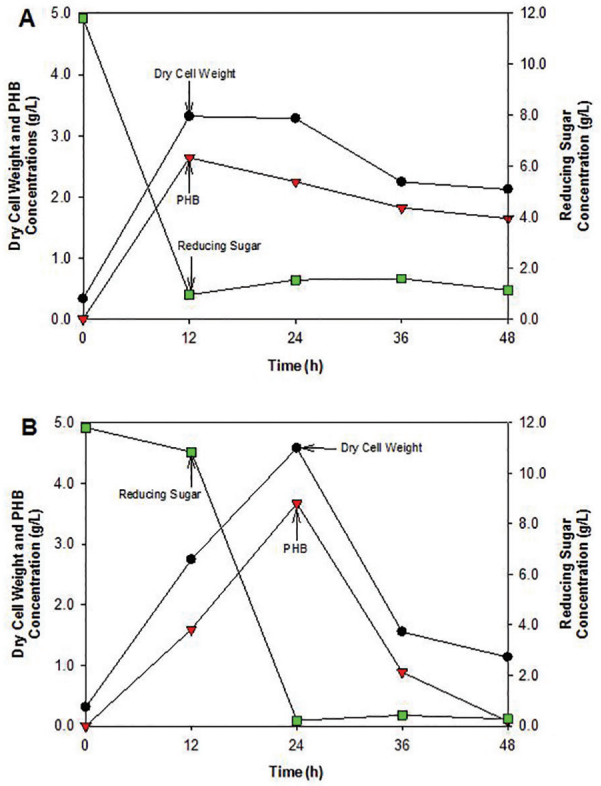


**Figure 4**. Reducing sugar consumption, cell growth (in terms of dry cell weight concentration) and PHB production profiles of *B. megaterium* PNCM 1890 grown in medium with (A) banana pseudostem hydrolysate and (B) glucose as carbon sources.

### Characterization of PHB from Optimized Fermentation Conditions

Figure 5A shows the FTIR spectrum of the extracted PHB. Peaks at 1277 $cm^{-1}$ and 1720 $cm^{-1}$ represent rotations around carbon atoms specific to certain functional groups (Ramezani *et al*. 2015). Adsorption bands at 1277 $cm^{-1}$ and 1720 $cm^{-1}$ indicate a –CH group and an ester carbonyl group (C=O) in the molecule of the polymer, respectively. Series of bands between 978 $cm^{-1}$ and 1379 $cm^{-1}$ corresponds to the stretching of the ester carbonyl bond, while bands occurring between 2876 $cm^{-1}$ and 2934 $cm^{-1}$ indicate the presence of a $–CH_3$ group. The peaks at 1380 $cm^{-1}$ and 1470 $cm^{-1}$ represent symmetric and asymmetric bending $–CH_3$ (or $–CH_2$) groups, respectively. Finally, the bands at 3402 $cm^{-1}$ are related to a terminal –OH group, or possible water adsorption in the sample. The peaks observed were similar to that of standard PHB, indicating that the extracted polymer is PHB (Dañez *et al*. 2020).

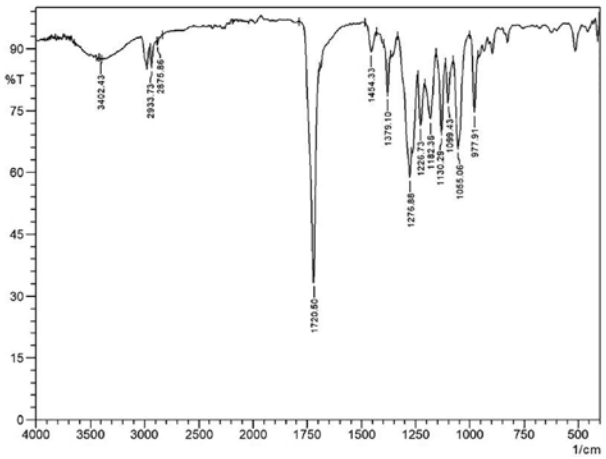


**Figure 5**. FTIR spectra of PHB from the fermentation of banana pseudostem hydrolysate medium.

## CONCLUSIONS

Steam explosion and sequential steam explosion – dilute acid pretreatment of banana pseudostem were optimized to disrupt the recalcitrance of the biomass and consequently improve its digestibility during enzymatic saccharification. Enhancing the digestibility of the biomass increases the production of fermentable sugars that can be converted to PHB by bacterial fermentation.

For the steam explosion, increasing both steam temperature and pretreatment time increased the production of reducing sugars. For sequential pretreatment, reducing sugar yield is maximized at high steam explosion temperature, high dilute acid reaction time, high dilute acid temperature, and high acid concentration. With reducing sugar yield doubled after dilute acid pretreatment of steam-exploded banana pseudostem, it was proven that sequential pretreatment is a more effective pretreatment method for banana pseudostem.

PHB was successfully produced using the hydrolysate produced from optimally steam-exploded banana pseudostem, with *B. megaterium* PNCM 1890 as the fermentation microorganism. Optimization of enzymatic saccharification, fermentation conditions and PHB extraction may further improve the feasibility of PHB production using banana pseudostem. This study could be used as a basis to further explore the use of banana pseudostem and the potential of sequential pretreatment of different agricultural residue feedstocks for a low-cost commercial PHB production.

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