Effect of neutralizing agents in the preparation of succinic acid from oil palm trunk

N A Bukhari1,2, S K Loh1, A A I Luthfi2,3, P M Abdul2,3, S Harun2,3 and J M Jahim2,3

1 Energy and Environment Unit, Engineering & Processing Research Division, Malaysian Palm Oil Board (MPOB), 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia
2 Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
3 Centre for Sustainable Process Technology (CESPRO), Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Email: adel@mpob.gov.my

Abstract. Neutralization is an important process to control the pH required for enzymatic saccharification of pretreated biomass followed by fermentation for biochemical conversion. In this study, the production of succinic acid as a potential C4 building block was investigated by utilizing lignocellulosic biomass in the form of oil palm trunk (OPT). The effect of different neutralizing agents (NaOH, KOH and NH4OH) on the enzymatic saccharification of oxalic acid-pretreated OPT and subsequent succinic acid fermentation by Actinobacillus succinogenes ATCC 55618 was investigated. The results showed that all neutralizing agents tested were able to assist in the recovery of fermentable sugars with concentrations ranging from 38.1 to 39.6 g/L. However, during succinic acid fermentation, it was found that the soluble NH4-oxalate salt formed severely inhibited succinic acid fermentation compared to Na and K, thereby decreasing the succinic acid production from 14.0 g/L (using NaOH) to 1.0 g/L (using NH4OH). In particular, Na- and K-oxalate did not exhibit apparent inhibition for both the saccharification and fermentation processes. Hence, the choice of neutralizing reagent is essential to prevent inhibition in the preparation of succinic acid from lignocellulosic biomass.

1. Introduction
Growing concern over environmental and ecological protection globally has shifted the effort to manufacture industrially important chemicals from renewable resources. Among the promising bio-based chemicals, succinic acid has been identified as one of the most versatile chemicals that can be produced via biological transformation. Succinic acid finds a broad range of applications and is a key component to produce various industrially important products, including detergent and surfactant, food additives, biopolymers, fungicides, herbicides, etc. [1].

Conversion of bio-based succinic acid from lignocellulosic agro-industrial residues appears as an attractive approach in promoting green technology [2,3]. Accordingly, Actinobacillus succinogenes is the most preferred biocatalyst because of its capability to utilize a wide spectrum of carbon sources including hexose and pentose sugars under anaerobic conditions [3]. The utilization of lignocellulosic
feedstocks is sustainable, promising, and eco-friendly approach towards the production of succinic acid since it completely avoids the consumption of food crops [2].

Lignocellulosic material is mainly made up of cellulose, hemicellulose, and lignin, which form a complex structure to withstand the chemical or microbial degradation [4]. In Malaysia, one of the available lignocellulosic materials is oil palm trunk (OPT) amounting to approximately 21.6 million tonnes (dry weight basis) based on a replanted rate of 5% from 5.9 million ha of oil palm plantations in 2019 [5]. OPT consists of 35 - 40 % cellulose, 25 - 30 % hemicellulose and 20 - 25 % lignin [6,7]. Nevertheless, lignocellulosic OPT currently needs to be pretreated to achieve high sugar yields for the microorganisms to synthesize the succinic acid. Pretreatment is the first step in bioconversion to disrupt the heterogeneous structure of lignocellulose, solubilize hemicellulose and/or lignin, and increase the surface area and porosity of the respective biomass [8]. Dilute acid pretreatment is one of the most widely used processes, which yield promising amounts of digestible substrates for successful enzymatic hydrolysis. This is followed by enzymatic saccharification, where the pretreated lignocellulose is converted into fermentable sugars [7].

Prior to enzymatic saccharification, neutralization step is a prerequisite for adjusting the acidic pretreated hydrolysate or slurry to the required pH favorable for the enzyme reactivity. Generally, alkaline neutralizers such as potassium hydroxide (KOH), sodium acetate (NaCH₃COO), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium sulfate (Na₂SO₄), calcium hydroxide (CaH₂O₂), calcium carbonate (CaCO₃) and ammonium hydroxide (NH₄OH) are able to neutralize the pretreated acid hydrolysates to the desired pH. However, soluble salts formed during the neutralization step might affect the efficiency of saccharification and fermentation [9]. Several soluble salts, i.e. Na₂SO₄, NaCH₃COO and NaCl are detrimental to microorganisms thus inhibit the cell growth and product formation [9,10].

In this study, different neutralizing agents were used for pH adjustment of acid hydrolysates aiming at exploring the most suitable neutralizer which does not inhibit the subsequent biocatalytic activities. Different alkaline neutralizers were selected i.e. KOH, NaOH and NH₄OH and their influences were investigated on (1) enzymatic saccharification of OPT pretreated with acid and (2) fermentation by A. succinogenes to produce succinic acid. In addition, the effects of soluble salts co-exist with complex nutrient and defined mineral salts supplementation during fermentation were also performed to assess the extent of microbial inhibition.

2. Materials and methods

2.1. Oil palm trunk fibers
The OPT sample was ground to achieve a mean particle size of <10 mm using a laboratory stainless steel grinder (Pulverisette 15, Fritsch GmbH, Germany). The OPT fiber, previously characterized, has 56.70 % holocellulose (30.86 % cellulose and 25.84 % hemicellulose), 24.29 % lignin and 2.29 % ash [11].

2.2. Dilute oxalic acid pretreatment
Ground OPT solid at 10% (w/v) loading were loaded in 250-mL screw-cap Erlenmeyer flasks containing 1% (w/v) oxalic acid. The samples were further pretreated at 120 °C for 3 h as previously optimized [12].

2.3. Neutralization with different neutralizing agents
In order to compare the effects of different neutralizing agents on saccharification and subsequent fermentation, the whole slurry of acid pretreated samples were tailored to meet the required pH 5.0 by three different bases i.e., 5 M KOH, 5 M NaOH and 30% (v/v) NH₄OH as detailed in table 1.
Table 1. Bases used during neutralization step of oil palm trunk acid hydrolysate.

| Base/Neutralizing agent | Formula | Physical appearance | Molarity/concentration | Solubility in water | Volume required (mL) |
|-------------------------|---------|---------------------|------------------------|---------------------|---------------------|
| Potassium hydroxide     | KOH     | White solid, deliquescent | 5 M                   | Soluble             | 5                   |
| Sodium hydroxide        | NaOH    | White, waxy, opaque crystals | 5 M                   | Soluble             | 4                   |
| Ammonium hydroxide      | NH₄OH   | Colourless aqueous solution | 30% (v/v)             | Miscible            | 2                   |

2.4. Enzymatic saccharification
The neutralized whole slurry of pretreated OPT was enzymatically saccharified using 15 filter paper units (FPU) of commercial cellulase (Cellic CTec2, Novozyme, Denmark) per gram of cellulose, aided by 0.5% (v/v) Triton X-100 [11]. The samples were incubated at 50 °C, 150 rpm for 72 h. The recovered sugars after enzymatic saccharification were analysed using high performance liquid chromatography (HPLC). The enzymatic hydrolysates were collected and used for subsequent fermentation.

The sugar yield was calculated as follows equation (1):

\[
\text{Sugar yield (g g}^{-1}\text{)} = \frac{\text{Glucose + xylose in enzymatic hydrolysate}}{\text{Holocellulose content in OPT}}
\]  

(1)

2.5. Fermentation of OPT hydrolysate
The effect of mineral salts (MS) (per litre of medium: 0.2 g MgCl₂·6H₂O, 0.2 g CaCl₂·2H₂O, 3.0 g KH₂PO₄, 1.0 g NaCl) with or without nitrogen source (yeast extract, YE) supplementation (15.0 g per litre) on succinic acid fermentation was also investigated using different acid hydrolysates. Nine types of media were prepared as follows: 1) KOH-oxalate supplemented with YE and MS; 2) KOH-oxalate supplemented with YE without MS; 3) Non-supplemented KOH-oxalate; 4) NaOH-oxalate supplemented with YE and MS; 5) NaOH-oxalate supplemented with YE; 6) Non-supplemented NaOH-oxalate; 7) NH₄OH-oxalate supplemented with YE and MS; 8) NH₄OH-oxalate supplemented with YE and 9) Non-supplemented NH₄OH-oxalate. All samples were supplemented with 30.0 g MgCO₃. The medium was dispensed into a 100-mL serum flask, capped with a butyl rubber stopper and clamped with an aluminum seal. Fermentation was performed anaerobically in a 100-mL serum bottle by adding 10% (v/v) inoculum of A. succinogenes ATCC 55618 into 50 mL medium. The culture was cultivated at 37 °C, 200 rpm for 72 h.

The succinic acid yield was calculated as follows equation (2):

\[
\text{Succinic acid yield (g g}^{-1}\text{)} = \frac{\text{Succinic acid in fermentation broth}}{\text{Glucose + xylose consumed}}
\]  

(2)

2.6. Analytical methods
The sugars and organic acids recovered were quantified using HPLC (Waters 2707, USA) equipped with Phenomenex Rezex™ ROA column (Sunnyvale, USA) as previously described [11]. The column was maintained at 60 °C and the samples were eluted with 0.0025 M H₂SO₄ at 0.6 mL/min. The apparatus was integrated with an auto sampler, refractive index detector (Waters 2414, USA) set at 40 °C and isocratic HPLC pump (Waters 1515, USA).
3. Results and discussion

3.1. Effects of neutralizing agents on enzymatic saccharification of pretreated OPT

The dilute oxalic acid pretreatment of OPT was shown to effectively hydrolyze hemicellulose into monomeric xylose (table 2), releasing 15.17 ± 0.18 g/L xylose in the hydrolysate. Only a small amount of glucose (2.05 ± 0.04 g/L) was detected in the acid hydrolysate derived from the solubilization of hemicellulose. Conversely, acetic acid, one of the derivatives of lignocellulosic component, was also present in the hydrolysate at 4.67 ± 0.10 g/L, mainly due to hydrolysis of acetyl groups of hemicellulose.

| Neutralizing agent | Glucose (g/L) | Xylose (g/L) | Total sugar (g/L) | Acetic acid (g/L) |
|--------------------|---------------|--------------|-------------------|------------------|
| -                  | 2.05 ± 0.04   | 15.17 ± 0.18 | 17.22 ± 0.22      | 4.67 ± 0.10      |

B. After enzymatic saccharification

| Neutralizing agent | Glucose (g/L) | Xylose (g/L) | Total sugar (g/L) | Acetic acid (g/L) |
|--------------------|---------------|--------------|-------------------|------------------|
| KOH                | 20.80 ± 0.25  | 17.34 ± 0.27 | 38.14 ± 0.52      | 4.49 ± 0.06      |
| NaOH               | 20.62 ± 0.50  | 17.48 ± 1.16 | 38.10 ± 1.65      | 4.44 ± 0.06      |
| NH₃OH              | 19.66 ± 3.17  | 19.91 ± 0.86 | 39.58 ± 4.03      | 4.53 ± 0.11      |

The acid hydrolysates were neutralized with different bases to find the most promising neutralizing agent that has no inhibitory effect to enzyme activity and subsequent fermentation. In this study, three bases i.e. KOH, NaOH and NH₃OH that have better solubility in water were investigated. As shown in table 1, the whole slurry of pretreated acid hydrolysate required about 5, 4 and 2 mL of 5 M KOH, 5 M NaOH and 30 % (v/v) NH₃OH, respectively, during pH adjustment to around pH 4.8 - 5.0.

After enzymatic saccharification, the total sugar released from all samples gave similar results in the range of 38.1-39.6 g/L (table 2) with a satisfactory sugar yield (67-69 %). Cations may influence the catalytic activity of cellulase by acting as cofactor or inhibitor. In previous study, Bin and Hongzhang (2010) found that the K⁺, Ca²⁺, Cu²⁺, Mn²⁺, Mg²⁺, Zn²⁺, Al³⁺ and Fe³⁺ showed inhibitory effects on cellulase activity during enzymatic saccharification of rice straw at different levels. The higher ash content of rice straw had contributed to an overall higher concentration of these leached cations, which was responsible for the enzymatic inhibitions [13]. In another study using oil palm empty fruit bunch, the tested Ca²⁺, Co³⁺, Cu²⁺, Mn²⁺, Ni²⁺, Fe³⁺ and Zn²⁺ all showed otherwise stimulative effects [14]. The result in this study showed that K⁺, Na⁺ and NH₄⁺ did not demonstrate any inhibitory effects on cellulase activity in the whole slurry hydrolysate of OPT. This indicated that cellulase might act differently in response to the varying chemical compositions of different types of biomass (substrates).

3.2. Effects of salts from different neutralizing agents on succinic acid fermentation

In common medium consisting of nitrogen source and mineral salts (YE+MS), the highest succinic acid of 13.22 ± 0.22 g/L was achieved from K-oxalate containing hydrolysate. This was followed closely by Na-oxalate containing hydrolysate with 12.82 ± 0.19 g/L (figure 1). However, fermentation of NH₄-oxalate containing hydrolysate inhibited succinic acid accumulation by A. succinogenes as only 1.22 ± 0.32 g/L was detected in the medium.

The fermentation experiments in OPT hydrolysate containing different neutralization salts with and without supplementation of YE and MS were also performed to investigate their performance on succinic acid production by A. succinogenes. As shown in figure 1, the highest succinic acid production of 13.99 ± 0.16 g/L was attained using Na-oxalate hydrolysate supplemented with YE without MS, which increased succinic acid titer by 9 % as compared to the MS supplemented hydrolysate. On the
other hand, 10.72 ± 1.56 g/L succinic acid was produced from K-oxalate containing hydrolysate without MS supplementation, showing 23% reduction as compared to that of MS supplementation. Meanwhile, the succinic acid production was strictly inhibited in all NH₄-oxalate containing hydrolysates, producing very poor succinic acid titer (<1.5 g/L) (figure 1).

![Figure 1. Succinic acid production using oil palm trunk hydrolysates neutralized using different agents. Cells were grown with or without the supplementation of 15 g/L yeast extract (YE) and 4.4 g/L mineral salts (MS) at 37°C, 200 rpm for 60 h. NS: non-supplemented.](image)

Evidently, this showed that fermentation of *A. succinogenes* required YE to synthesis succinic acid while MS supplementation is optional depending on the selection of base (i.e. NaOH) during neutralization. *A. succinogenes* is auxotrophic to several amino acids and vitamins [15], hence, supplementation of complex nutrients such as YE is essential for cell proliferation. It is noteworthy that sufficient MS was required in OPT hydrolysate for bioconversion in this study. Furthermore, the NaOH added during neutralization step of the whole slurry of acid hydrolysates might be sufficient for cellular metabolism to accumulate succinic acid. Owing to this, supplementation of Na could be eliminated, which will benefit the overall process cost besides simplifying medium preparation step [16].

The selection of NH₄OH during neutralization was intentionally made to assess the capability of NH₄⁺ to function as nitrogen source to complement the basic requirement of *A. succinogenes* fermentation for succinic acid production. However, the results showed otherwise. The succinic acid yield was negligible in all NH₄OH-containing hydrolysates (table 2). This indicates that inorganic nitrogen source such as NH₄OH was not preferred by *A. succinogenes* in succinic acid fermentation. The poor performance of NH₄ salt was probably related to its toxicity mainly caused by free ammonia (NH₃) to the anaerobic fermentation of *A. succinogenes*. This finding corresponded with Liu et al. (2008), reported that inorganic nitrogen source in the form of NH₄Cl inhibited *A. succinogenes* fermentation [17]. Regardless of the presence of the supplemented YE and MS, the soluble salts (NH₄⁺-oxalate) had strictly inhibited the succinic acid fermentation.

Table 2 shows the total sugar consumption during succinic acid production. The highest total sugar consumption was 93.1% using Na-oxalate (YE without MS) containing hydrolysate, followed closely by 92.5 %, 91.3 % and 90.2 % from those of K-oxalate (YE without MS), Na-oxalate (YE + MS) and K-oxalate (YE + MS), respectively. In Na-oxalate (YE without MS) hydrolysate, only 0.9 g/L of glucose and 1.7 g/L of xylose (table 3) remained in the culture from their original base of 20.6 g/L and 17.5 g/L (table 2), respectively, demonstrating that the cells were capable of metabolizing xylose as effectively as glucose. In terms of the formation of by-products, higher formic acid and acetic acid were observed.
in YE (without MS) hydrolysates compared to those supplemented with MS in both the Na-oxalate and K-oxalate containing hydrolysates. This is possibly because some MS can act as cofactor in A. succinogenes metabolic pathway which has led to a higher production of succinic acid and lower by-products formation.

Table 3. Concentrations of sugars and acids in the oil palm trunk hydrolysates after fermentation.

| Hydrolysate | Residual glucose (g/L) | Residual xylose (g/L) | Total consumed sugar (%) | Succinic acid yield (g/g) | By-products |
|-------------|------------------------|-----------------------|-------------------------|---------------------------|-------------|
| K-oxalate   | 1) YE + MS<sup>a</sup>  | 0.73 ± 0.17           | 3.04 ± 1.03             | 90.15                     | 0.38        |
|             | 2) YE<sup>b</sup>       | 0.82 ± 0.14           | 2.04 ± 1.41             | 92.51                     | 0.30        |
|             | 3) NS<sup>c</sup>       | 11.01 ± 0.97          | 14.57 ± 1.26            | 32.46                     | 0.09        |
|             | 4) YE + MS<sup>a</sup>  | 1.06 ± 0.12           | 2.10 ± 1.63             | 91.33                     | 0.39        |
|             | 5) YE<sup>b</sup>       | 0.98 ± 0.07           | 1.72 ± 0.88             | 93.14                     | 0.38        |
|             | 6) NS<sup>c</sup>       | 10.72 ± 0.47          | 15.00 ± 0.73            | 33.30                     | 0.11        |
| Na-oxalate  | 7) YE + MS<sup>a</sup>  | 9.50 ± 0.01           | 9.33 ± 0.27             | 54.33                     | 0.05        |
|             | 8) YE<sup>b</sup>       | 5.84 ± 0.84           | 9.97 ± 1.26             | 58.33                     | 0.05        |
|             | 9) NS<sup>c</sup>       | 6.79 ± 2.34           | 8.99 ± 1.46             | 40.98                     | 0.07        |

<sup>a</sup> Yeast extract with mineral salts
<sup>b</sup> Yeast extract without mineral salts
<sup>c</sup> Non-supplemented

The overall performance in terms of sugar and succinic acid yield is summarized in figure 2. Clearly, the enzymatic saccharification was hardly affected by the neutralizing agent. On the other hand, the NH₄-oxalate salt formed during neutralization was detrimental to A. succinogenes in the subsequent fermentation. Therefore, the utilization of NH₄OH in succinic acid fermentation by A. succinogenes should be avoided.

Figure 2. Sugar and succinic acid yields during enzymatic saccharification and fermentation by A. succinogenes using oil palm trunk hydrolysates neutralized with different agents.

4. Conclusion
Neutralizing agents such as NaOH, KOH and NH₄OH were shown to affect saccharification and fermentation involving OPT biomass. Based on the findings, NaOH might be the first choice to be considered for designing bioprocess for the preparation of succinic acid from lignocellulosic OPT acid hydrolysates by A. succinogenes. Interestingly, supplementation of mineral salts is not required in the subsequent fermentation when incorporating NaOH as the neutralizing agent. A proper selection of suitable neutralizing agent is perhaps wise to reduce any inhibitory effects caused by alkali salts
formation during neutralization process. The study provides a scientific basis for adjusting bioprocessing conditions to enhance the feasibility of succinic acid production from lignocellulosic biomass.

Acknowledgments
The authors would like to thank the Director-General of the Malaysian Palm Oil Board (MPOB) for permission to publish this article.

References
[1] Yang Q, Wu M, Dai Z, Xin F, Zhou J, Dong W, Ma J, Jiang M and Zhang W 2020 Comprehensive investigation of succinic acid production by Actinobacillus succinogenes: A promising native succinic acid producer Biofuels, Bioprod. Biorefining 14 950–64
[2] Luthfi A A I, Jahim J M, Harun S, Tan J P and Mohammad A W 2017 Potential use of coconut shell activated carbon as an immobilisation carrier for high conversion of succinic acid from oil palm frond hydrolysate RSC Adv. 7 49480–9
[3] Dessie W, Xin F, Zhang W, Jiang Y, Wu H, Ma J and Jiang M 2018 Opportunities, challenges, and future perspectives of succinic acid production by Actinobacillus succinogenes Appl. Microbiol. Biotechnol. 102 9893–910
[4] Abdul P M, Md. Jahim J, Harun S, Markom M, Hassan O, Mohammad A W and Asis A J 2013 Biohydrogen production from pentose-rich oil palm empty fruit bunch molasses: A first trial Int. J. Hydrogen Energy 38 15693–9
[5] Parveez G K A, Hishamuddin E, Loh S K, Ong-Abdullah M, Salleh K M, Bidin M N I Z, Sundram S, Hasan Z A A and Idris Z 2020 Oil palm economic performance in Malaysia and R&D progress in 2019 J. Oil Palm Res. 32 159–90
[6] Loh S K 2017 The potential of the Malaysian oil palm biomass as a renewable energy source Energy Convers. Manag. 141 285–98
[7] Bukhari N A, Jahim J M, Loh S K, Bakar N A and Luthfi A A I 2019 Response surface optimisation of enzymatically hydrolysed and dilute acid pretreated oil palm trunk bagasse for succinic acid production BioResources 14 1679–93
[8] Abdul P M, Jahim J M, Harun S, Markom M, Lutpi N A, Hassan O, Balan V, Dale B E and Mohd Nor M T 2016 Effects of changes in chemical and structural characteristic of ammonia fibre expansion (AFEX) pretreated oil palm empty fruit bunch fibre on enzymatic saccharification and fermentability for biohydrogen Bioresour. Technol. 211 200–8
[9] Yang M, Wang J, Nan Y, Zhang J, Li L, Liu G, Vepsäläinen J, Kuittinen S and Pappinen A 2019 Effect of salts formed by neutralization for the enzymatic hydrolysis of cellulose and acetone-butanol-ethanol fermentation RSC Adv. 9 33755–60
[10] Qureshi N, Ezeji T C, Ebener J, Dien B S, Cotta M A and Blaschek H P 2008 Butanol production by Clostridium beijerinckii. Part I: Use of acid and enzyme hydrolyzed corn fiber Bioresour. Technol. 99 5915–22
[11] Bukhari N A, Jahim J M, Loh S K, Nasrin A B, Harun S and Abdul P M 2020 Organic acid pretreatment of oil palm trunk biomass for succinic acid production Waste and Biomass Valorization 11 5549–59
[12] Bukhari N A, Loh S K, Luthfi A A I, Nasrin A B, Abdul P M, Harun S and Jahim J M 2021 Oil palm trunk biomass pretreatment with oxalic acid and its effect on enzymatic digestibility and fermentability Mater. Today Proc. 42 119–23
[13] Bin Y and Hongzhang C 2010 Effect of the ash on enzymatic hydrolysis of steam-exploded rice straw Bioresour. Technol. 101 9114–9
[14] Nurul Adela B, Loh S K and Nasrin A B 2015 The improvement on enzymatic hydrolysis of oil palm (Elaeis guineensis) empty fruit bunch lignocellulose Malaysian Appl. Biol. 44 95–100
[15] McKinlay J B, Laivenieks M, Schindler B D, McKinlay A A, Siddaramappa S, Challacombe J F, Lowry S R, Clum A, Lapidus A L, Burkhart K B, Harkins V and Vieille C 2010 A genomic
perspective on the potential of *Actinobacillus succinogenes* for industrial succinate production

*BMC Genomics* **11** 680

[16] Bukhari N A, Loh S K, Nasrin A B, Luthfi A A I, Harun S, Abdul P M and Jahim J M 2019 Compatibility of utilising nitrogen-rich oil palm trunk sap for succinic acid fermentation by *Actinobacillus succinogenes* *Bioresour. Technol.* **293** 122085

[17] Liu Y P, Zheng P, Sun Z H, Ni Y, Dong J J and Zhu L L 2008 Economical succinic acid production from cane molasses by *Actinobacillus succinogenes* *Bioresour. Technol.* **99** 1736–42