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Oil palm empty fruit bunches a promising substrate for succinic acid production via simultaneous saccharification and fermentation

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ABSTRACT

Oil palm empty fruit bunch (EFB), a plentiful agricultural waste in Malaysia was evaluated for the production of succinic acid (SA) via simultaneous saccharification and fermentation (SSF) using *A. succinogenes* ATCC 55618. In the current study, EFB was pretreated using 2 different pretreatment methods; autoclave alkali (AA) and sequential dilute acid microwave alkali (DA-MWA). The pretreated EFBs were hydrolyzed enzymatically and they were also used as a substrate for SA production. During enzymatic hydrolysis different ratios of *cellulase* and *cellobiase* were used. Results revealed the best yield of glucose accumulation (31.4 g L^{-1}) was obtained when the ratio of *cellulase* and *cellobiase* was kept to 7:1. Subsequent findings also showed maximum concentration, (33.4 g L^{-1}), yield (30.47 g g^{-1}substrate) and productivity 0.69 g L^{-1} h^{-1} of SA was achieved when using sequential DA-MWA pretreated EFB sample. The results suggested that agriculture waste EFB has the potential to be an alternative substrate for efficient and economic SA production.

1. Introduction

Several researchers have identified the potential use of low price lignocellulosic biomass for the production of biofuels and high value added organic acids [1–5]. Malaysia being the world's second largest producer of oil palm has approximately 59 million tons/year of the total lignocellulosic biomass agricultural waste. The oil palm biomass comprised of empty fruit bunches (EFB), oil palm fronds (OPF), palm kernel shells (PKS) and oil palm trunks (OPT). Recently, there are studies that suggest on the use of lignocellulosic biomass for production of industrially important compound succinic acid. Succinic acid is a carbon intermediate chemical that is used for food and pharmaceutical products, surfactants and detergents, green solvents and ingredients to plant and animal growth stimulants [6–9].

Different pretreatments methods were required for various lignocellulosic biomass to produce fermentable sugars which resulted in the production of industrially important compounds. During the last decade, many studies have reported on the combine use of physical and chemical pretreatments on lignocellulose materials. The microwave heating pretreatment for the degradation of lignocellulose into fermentable sugars has the potential to replace the current conventional autoclave heating. Microwave alkali (MWA) pretreatment method and its application were applied on a variety of lignocellulose such as: rice straw [10], switch grass [11] and empty fruit bunches.

Organic acids such as SA can be produced via separate hydrolysis and fermentation (SHF) or SSF. In SHF, enzymatic hydrolysis and fermentation are performed in separate steps to produce glucose in hydrolysate from lignocellulosic biomass followed by fermentation. Several studies suggested that the optimal temperature for enzymatic hydrolysis was 50 °C and pH 4.8 while the optimal fermentation temperature was 37 °C [12–15] for corn straw, corn fiber, corn stover, corn stalk and cotton stalk, orange peel and wheat straw, rapeseed meal and cane molasses hydrolysate using *A. succinogenes* in SA production. However, when using SSF the two steps enzymatic hydrolysis and fermentation are combined in a single process where SSF was carried out at 38 °C for SA production from corn fiber [16] and rapeseed meal [17] by *A. succinogenes*.

In this study, the potential of using EFB for SA production via SSF process using *A. succinogenes* ATCC 55618 was studied for the first time. The enzymatic hydrolysis process was evaluated by studying the effect of enzyme loading, different ratio of *cellulase* to *cellobiase* and substrate concentration. The effects of pretreatment methods; autoclave alkali (AA) and sequential dilute acid microwave alkali (DA-MWA) pretreated EFB on enzymatic hydrolysis and SA

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production were studied in detail for SSF process.

2. Materials and methods

2.1. Raw materials

The EFB biomass was collected from FELDA palm oil mill Semenchu, Kota Tinggi, Johor, Malaysia. Prior to use the samples were grinded to 0.5 × 1.0 cm size using disk mill model FFC-15, from China. The samples were washed and dried under the sun and then at 70 °C in the oven for 24 h and stored in a container at room temperature.

2.2. Enzymes

Two commercial enzymes *cellulase* and *cellobiase* used in the hydrolysis process were purchased from Sigma Aldrich. The enzyme activity of *cellulase* was 75 FPU mL^{-1} (filter paper activity, FPU). The β 1-4 glucosidase activity of *cellobiase* was 132 CBU mL^{-1}. The enzyme activity of *cellulase* and *cellobiase* were quantified as described by Ghose [18]. All the chemicals used in the study were obtained from Merck Chemicals Malaysia.

2.3. Pretreatment of EFB

The procedures for the various pretreatment methods are as follows:

i) AA pretreatment was performed in an autoclave under the following conditions; EFB (20 g) was soaked in 2.5 M NaOH (20% w/v), heated in an autoclave at 121 °Cfor 2.0 h under 0.12 MPa pressure.
ii) Sequential DA-MWA pretreatment was carried under the following conditions: Dilute acid pretreatment: 20 g of dry EFB was soaked in 8.0% (v/v) H_2SO_4 solution, heated in an autoclave at 121 °C for 1 h. The dilute acid treated EPB (20 g) was then soaked in 200 ml of 2.5 N NaOH, placed in microwave (model MAS-II microwave, SINEO) at the following conditions: power 900 W, temperature 90 °C and time 20 min. The conditions selected were based on our previous findings [19].

2.4. Enzymatic hydrolysis of EFB

The untreated and the various pretreated EFB samples were then hydrolyzed enzymatically. The enzymatic hydrolysis were carried out in 150 ml flasks having 50 mL of citrate buffer solution at 50 °C and 180 rpm in a water bath. Substrate 70 g L^{-1} EFB and enzyme loading *cellulase* 25 FPU/g and *cellobiase* (Novozyme 188) 10CBU/g were added in the media. The effect of different ratios of *cellulase* and *cellobiase* 1:0, 1:1, 1:2, 2:1, 5:1, 7:1 and 10:1 on enzymatic hydrolysis were studied to determine the best enzyme ratio for glucose accumulation.

2.5. Microorganisms and SSF media preparation

A. succinogenes ATCC 55618 was obtained from the American Type Culture Collection (ATCC). The inoculum media of the cells were prepared in 250 mL flasks. The 50 mL preculture media consists of the following components (g L^{-1}): tryptone 17, NaCl 5, soya peptone 3, K_2HPO_4 2.5, glucose 2.5. The pH was kept at 7.5. The strain was stored in 50% glycerol solution at −80 °C. The SSF fermentation medium consists of the following components in (g L^{-1}): EFB 70, yeast extract (YE) 20, corn steep liquor (CSL) 20; sodium acetate 1.5, NaH_2PO_4 1.5; K_2HPO_4 1.5; MgCl 0.2, $CaCl_2$ 0.2. The pH was maintained at 6.5 by adding 65 g/L of $MgCO_3$. The fermentation medium was inoculated with 5% of the preculture broth in SSF medium. The O.D of the initial bacterial cell of the preculture broth was 0.566. SSF experiments were carried in the 150 ml flasks containing 50 mL of the fermentation medium for 48 h. *Cellulase* 25 FPU/g and *cellubiase* (Novozyme 188) 10 FPU/g were added into the fermentation after the seeds of *A. succinogenes* ATCC 55618 were inoculated. The fermentation was performed in an incubated shaker at 38 °C (210 rpm). The SA production by *A. succinogenes* was observed during the fermentation process. Samples were taken at 6, 12, 24, 36 and 48 h for HPLC analysis to quantify the products formed. All the experiments were carried out in triplicates and each data point showed an average value with an error bar. SA yield was determined as the amount of SA produced (g) from 1 g of EFB and was expressed as g/g.

2.6. Analytical methods

The amount of extractives was measured by soxhlet extraction (Tappi-1997; T 204 cm-97), Klason's lignin (Tappi 2002; T 222 cm-02), holocellulose content [20], α-cellulose content (T203 cm-09) with slight modification while, hemicellulose content of EFB sample was obtained by subtracting cellulose from holocellulose [20]. The growth of *A. succinogenes* ATCC 55618 was monitored by measuring the optical density at 660 nm. At 660 OD of 1, the concentration of *A. succinogenes*is was 0.626 dry cell weight per liter. After samples collections the enzymes were denatured by adding 2 mL of acetonitrile and 2 mL of methanol in1 mL of fermentation broth and kept at 4 °C overnight. The samples were centrifuged for 20 min at 9000 G and the supernatants were filtered through a 0.22-μm filter and analyzed by high-performance liquid chromatography (HPLC, Agilent). Rezex ROA-Organic Acid H^+ column (7.8 × 300 mm) was utilized. The column temperature was kept at 40 °C. The mobile phase was 0.005 N NH_2SO_4 at a flow rate 0.5 mL min^{-1} under isocratic conditions. The detection wave was fixed at 210 nm.

3. Results and discussion

3.1. Effect of different pretreatments on chemical composition

The effect of two pretreatment methods; AA and sequential DA-MWA pretreated EFB on the cellulose, hemicellulose and lignin contents are depicted in Table 1. The AA pretreatment method showed that cellulose content increased from 40.6 g/100 g to 53.3 g/100 g. The hemicellulose content was observed to decrease from 36.6 g/100 g to 28.7 g/100 g. The delignification of 39.8% was achieved as the amount of lignin decrease from 19.6 g/100 g−11.8 g/100 g. Pretreatment of lignocellulosic biomass disrupts the structure of lignin and hemicellulose and makes cellulose easily accessible for the enzymes [21]. Different types of alkali such as KOH, NaOH, $Ca(OH)_2$, anhydrous ammonia and hydrazine are used in conventional alkali pretreatment method. The mild condition used in alkali method increases the treatment time but it prevents degradation of sugar to furfural and also prevents lignin

Table 1
Chemical composition of raw and the differently treated EFB biomass (g/100 g biomass).

Pretreatment Type	(g/100 g biomass)			
	Cellulose	Hemicellulose	Lignin	Extractives
Raw	40.6 ± 1.4	36.6 ± 1.6	19.6 ± 0.25	3.5 ± 0.30
AA	53.3 ± 1.3	28.7 ± 1.3	11.8 ± 0.40	2.8 ± 0.04
DA-MwA	86.8 ± 1.4	3.4 ± 1.5	5.3 ± 0.16	1.7 ± 0.06