

A method of pre-treating biowasteTechnical Field

The present invention relates to a method of pre-treating biowaste.

5 Background

Cellulose is a major component of biowaste. However, cellulose is entangled in a cross-linked matrix of lignin and hemicellulose and is not easily accessible for breakdown. Therefore, various pre-treatment steps are required to improve the breakdown of biowaste. Accordingly, in biowaste management, pre-treatment of
10 biowaste is essential to enhance enzymatic digestibility.

Current methods for pre-treatment of biowaste include heat treatment, acid treatment, alkaline treatment, and oxidising agent-based treatment. However, heat treatment such as steam explosion, and oxidising agent-based treatment, are energy-intensive as high temperatures are required. Further, for acid treatment, highly concentrated acid is
15 used, which is corrosive, not environmentally friendly, and produces inhibitory compounds which are detrimental to subsequent processing. On the other hand, alkaline treatment is a slow process which takes several days to weeks.

There is therefore a need for an improved method for pre-treating biowaste.

Summary of the invention

20 The present invention seeks to address these problems, and/or to provide an improved method for pre-treating biowaste.

According to a first aspect, the present invention provides a method of pre-treating biowaste, the method comprising mixing biowaste with an alkaline solution, an oxidising agent and a synthetic catalyst to form pre-treated biowaste.

25 The biowaste may be any suitable biowaste. For example, the biowaste may comprise lignocellulosic biomass.

The synthetic catalyst may be any suitable synthetic catalyst. For example, the synthetic catalyst may comprise a metal complex with a metal ion and surrounded by a synthetic ligand. In particular, the synthetic ligand may comprise at least one nitrogen.

According to a particular aspect, the metal ion comprised in the synthetic catalyst may be a transition metal ion.

The mixing may be carried out under suitable conditions. For example, the mixing may be carried out at a temperature of 20-100°C.

- 5 According to a particular aspect, the mixing may comprise adding the biowaste to a solution comprising the alkaline solution, the oxidising agent and the synthetic catalyst. A suitable amount of biowaste may be added to the solution. For example, the adding may comprise adding 1-50 wt% biowaste based on the total weight of the solution. The mixing may be carried out at a temperature of 20-80°C. The mixing may be carried out
10 for ≤ 24 hours.

According to another particular aspect, wherein the mixing may comprise:

- mixing biowaste with an alkaline solution for a pre-determined period of time and at a pre-determined temperature to form a biowaste solid; and
- adding the biowaste solid to a solution comprising the oxidising agent and
15 the synthetic catalyst.

The pre-determined period of time may be any suitable period of time. For example, the pre-determined period of time may be 1-72 hours. The pre-determined temperature may be any suitable temperature. For example, the pre-determined temperature may be $\leq 100^\circ\text{C}$. In particular, the pre-determined temperature may be 20-100°C. Even
20 more in particular, the pre-determined temperature may be room temperature.

The solution may comprise a suitable amount of oxidising agent and synthetic catalyst. For example, the solution may comprise 0.1-10 vol % oxidising agent based on the total volume of the solution. For example, the solution may comprise 0.1-1000 ppm
25 synthetic catalyst.

The method may further comprise treating the pre-treated biowaste. The treating may comprise any suitable type of treatment. In particular, the treating may comprise fermenting, enzymatic saccharification, or a combination thereof of the pre-treated biowaste.

According to a particular aspect, the treating the pre-treated biowaste comprises fermenting the pre-treated biowaste in the presence of a bacteria. The fermenting may be carried out for a suitable period of time. In particular, the fermenting may be carried out for a period of 24-195 hours.

5 Brief Description of the Drawings

In order that the invention may be fully understood and readily put into practical effect there shall now be described by way of non-limitative example only exemplary embodiments, the description being with reference to the accompanying illustrative drawings. In the drawings:

10 Figure 1 shows a schematic workflow of the pre-treatment on biowaste and subsequent tests conducted;

Figure 2 shows soluble lignin concentrations after pre-treatment of biomass with three different methods; Figure 2A shows incubation in 1% NaOH for 1 day, prior to the pre-treatment (Experiment Set 1); Figure 2B shows 2 days incubation in 1% NaOH, prior to the pre-treatment (Experiment Set 1); Figure 2C shows phenolic concentrations after pre-treatment of biomass with alkaline peroxide (AP) method and catalytic alkaline peroxide (CAP) method;

Figure 3 shows scanning electron microscopy (SEM) images of biomass after different pre-treatment methods; Figure 3A shows control; Figure 3B shows alkaline pre-treatment (Experiment Set 1); Figure 3C shows alkaline peroxide pre-treatment (Experiment Set 1); Figure 3D shows catalytic alkaline peroxide pre-treatment (Experiment Set 1); Figure 3E shows alkaline peroxide pre-treatment (Experiment Set 2); Figure 3F shows catalytic alkaline peroxide pre-treatment (Experiment Set 2);

Figure 4 shows changes after enzymatic saccharification of the pre-treated biomass; Figure 4A shows reduction in biomass (Experiment Set 1); Figure 4B shows glucose concentration (Experiment Set 1); Figure 4C shows reducing sugars concentration (Experiment Set 2);

Figure 5 shows changes after fermentation of soluble lignin using *Clostridium sp.* G117; Figure 5A shows a gas chromatogram results of the fermentation products of the soluble lignin after 24 hours of fermentation; Figure 5B shows time-course fermentation

profile of three main products, acetone, butanol, and butyric acid; Figure 5C shows utilization of lignin and glucose by *Clostridium sp.* G117;

Figure 6 shows the total methane produced after methanogenesis with three pre-treatment methods, control, alkaline peroxide (AP), and catalytic alkaline peroxide (CAP);

Figure 7 shows the yield of fermentation products from pre-treated food wastes, using *Clostridium acetobutylicum* BOH3;

Figure 8 shows HPLC analysis on oxidation product from vanillin with control, only H₂O₂, and H₂O₂ with catalyst; and

Figure 9 shows NMR analysis on products extracted from the aromatic liquor of biomass.

Detailed Description

As explained above, there is a need for an improved method for pre-treating biowaste.

In general terms, the invention relates to a method for pre-treating biowaste so as to further breakdown the components of the biowaste, thereby increasing their biodegradability. The method of the present invention is also an eco-friendly method which does not use extreme heat and temperatures and also uses less chemicals. The method also does not affect subsequent treatment of the pre-treated biowaste. In particular, the method of the present invention is energy efficient and environmentally friendly.

According to a first aspect, the present invention provides a method of pre-treating biowaste, the method comprising mixing biowaste with an alkaline solution, an oxidising agent and a synthetic catalyst to form pre-treated biowaste.

The biowaste may be any suitable biowaste. For the purposes of the present invention, biowaste may be defined as biodegradable organic waste. The biowaste may comprise residual biomass, food waste, agricultural waste, waste sludge such as from wastewater treatment plants, and the like. According to a particular aspect, the biowaste may comprise lignocellulosic biomass. In particular, the biowaste may comprise cellulose which may be a cross-linked matrix of lignin and hemicellulose.

The synthetic catalyst may be any suitable synthetic catalyst. For example, the synthetic catalyst may comprise a metal complex with a metal ion and surrounded by a synthetic ligand. The metal ion may be any suitable metal ion. According to a particular aspect, the metal ion comprised in the synthetic catalyst may be a transition metal ion.

5 In particular, the metal ion may be an ion of, but not limited to, iron, copper, or alloys thereof.

The synthetic ligand may comprise at least one nitrogen. For example, the synthetic ligand may comprise one or more of, but not limited to, diglycyl-glycine (GGG), Histidyl-glycyl-glycine (HGG), glycyl-glycyl-histidine (GGH), phthalocyanine (Pc), 2,2'-bipyridine (bpy), ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), trans-1,2-
 10 diaminocyclohexane-N,N,N',N'-tetraacetic acid (Cy-DTA), diethylenetriamine-N,N,N',N'',N'''-pentaacetic acid (DTPA), N-(2-hydroxyethyl)ethylenediamine-N',N',N'-triacetic acid (EDTA-OH), triethylenetetraamine-N,N,N',N'',N''',N''''-hexaacetic acid (TTHA), O,O'-bis(2-aminoethyl) ethyleneglycol- N,N,N',N'-tetraacetic acid (GEDTA),
 15 ethylenediamine-N,N'-dipropionic acid dihydrochloride (EDDP), tetraamidomacrocyclic ligand (TAML) and 2,2'-bipyridine (BPY).

According to a particular aspect, the synthetic catalyst may be, but not limited to, Cu^{II}-(bpy), Cu^{II}-GGG, Cu^{II}-GGH, Cu^{II}-DTPA, Cu^{II}-BPY, and Fe^{II}-Pc. In particular, the synthetic catalyst may be iron-tetraamidomacrocyclic ligand (Fe-TAML).

20 The oxidising agent may be any suitable oxidising agent. For example, the oxidising agent may be, but not limited to, dioxygen, ozone, fluorine, chlorine, perchlorate, hypochlorite, permanganate compounds, hydrogen peroxide (H₂O₂) and peroxides such as benzyl peroxides. According to a particular aspect, the oxidizing agent may be H₂O₂.

25 The alkaline solution may be any suitable alkaline solution. For example, the alkaline solution may have a pH of 10-14. The alkaline solution may be, but not limited to sodium hydroxide, potassium hydroxide, magnesium hydroxide, sodium orthosilicate, sodium metasilicate, sodium carbonate, ammonia, ammonium hydroxide, calcium carbonate, or a combination thereof.

30 The mixing may be carried out under suitable conditions. According to a particular aspect, the mixing may be carried out at a temperature of 20-100°C. For example, the

mixing may be carried out at a temperature of 22-95°C, 25-90°C, 28-85°C, 30-80°C, 35-75°C, 40-70°C, 42-68°C, 45-65°C, 50-60°C, 55-58°C. In particular, the temperature may be 42-100°C.

The mixing may be by any suitable method. For example, the mixing of the alkaline solution, oxidising agent and the synthetic catalyst may be carried out simultaneously or sequentially.

According to one particular aspect, the mixing may comprise simultaneous mixing. For example, the mixing may comprise adding the biowaste to a pre-treatment solution comprising the alkaline solution, the oxidising agent and the synthetic catalyst. Accordingly, the method may further comprise preparing the pre-treatment solution prior to the mixing. The preparing the pre-treatment solution may comprise mixing a suitable amount of an alkaline solution, oxidising agent and synthetic catalyst. Water may also be added to form the pre-treatment solution. The alkaline solution, oxidising agent and the synthetic catalyst may be as described above.

In particular, the preparing may comprise mixing 0.1-10 vol % alkaline solution based on the total volume of the pre-treatment solution to be prepared. For example, the preparing may comprise mixing 0.5-9.5 vol %, 1.0-9.0 vol %, 1.5-8.5 vol %, 2.0-8.0 vol %, 2.5-7.5 vol %, 3.0-7.0 vol %, 3.5-6.5 vol %, 4.0-6.0 vol %, 4.5-5.5 vol %, 5.0-5.2 vol % alkaline solution based on the total volume of the pre-treatment solution to be prepared. Even more in particular, the preparing may comprise mixing 0.5-2.0 vol % alkaline solution based on the total volume of the pre-treatment solution to be prepared.

The preparing may comprise mixing 0.1-10 vol % oxidising agent based on the total volume of the pre-treatment solution to be prepared. For example, the preparing may comprise mixing 0.5-9.5 vol %, 1.0-9.0 vol %, 1.5-8.5 vol %, 2.0-8.0 vol %, 2.5-7.5 vol %, 3.0-7.0 vol %, 3.5-6.5 vol %, 4.0-6.0 vol %, 4.5-5.5 vol %, 5.0-5.2 vol % oxidising agent based on the total volume of the pre-treatment solution to be prepared. Even more in particular, the preparing may comprise mixing 0.5-3.0 vol % oxidising agent based on the total volume of the pre-treatment solution to be prepared.

The preparing may comprise mixing 0.1-1000 ppm synthetic catalyst based on the total volume of the pre-treatment solution to be prepared. For example, the amount of

synthetic catalyst may be 0.5-900 ppm, 1.0-750 ppm, 5-500 ppm, 10-400 ppm, 15-350 ppm, 20-300 ppm, 50-150 ppm, 75-100 ppm. Even more in particular, the preparing may comprise mixing 0.5-5.0 ppm catalyst based on the total volume of the pre-treatment solution to be prepared.

- 5 A suitable amount of biowaste may be added to the pre-treatment solution. For example, the adding may comprise adding 1-50 wt % biowaste based on the total volume of the pre-treatment solution. In particular, the amount of biowaste added may be 5-45 wt %, 10-40 wt %, 15-35 wt %, 20-30 wt %, 25-28 wt %. Even more in particular, the amount of biowaste may be 5-20 wt % based on the total volume of the
10 pre-treatment solution to be prepared.

The mixing may be carried out at a suitable temperature. For example, the temperature may be 20-80°C. In particular, the temperature may be 25-75°C, 30-60°C, 40-58°C, 45-55°C, 48-50°C. Even more in particular, the temperature may be 40-50°C.

- The mixing may be carried out for a suitable period of time. For example, the mixing
15 may be for ≤ 24 hours. In particular, the mixing may be for 5-22 hours, 10-20 hours, 12-18 hours, 15-16 hours. Even more in particular, the mixing may be for 3-8 hours.

- According to another particular aspect, the mixing may comprise sequential mixing. For example, the mixing may comprise a first mixing of the biowaste with a first solution comprising the alkaline solution for a first pre-determined period of time and at a first
20 pre-determined temperature to form a biowaste solid. Subsequently, the mixing may comprise mixing of the biowaste solid with a second solution comprising the oxidising agent and the synthetic catalyst for a second pre-determined period of time and at a second pre-determined temperature. The biowaste may be washed between the first mixing and the second mixing. For example, the biowaste may be mixed with water
25 between the first mixing and the second mixing.

- The first solution may have a suitable pH. For example, the pH of the first solution may be about 10-14. The first pre-determined period of time may be any suitable time. For example, the first pre-determined period of time may be 1-72 hours. In particular, the first pre-determined period of time may be 6-60 hours, 12-48 hours, 18-42 hours, 24-36
30 hours. Even more in particular, the first pre-determined period of time may be 12-24 hours.

The first pre-determined temperature may be any suitable temperature. For example, the first pre-determined temperature may be $\leq 100^{\circ}\text{C}$. In particular, the first pre-determined temperature may be $20-100^{\circ}\text{C}$, $25-90^{\circ}\text{C}$, $30-75^{\circ}\text{C}$, $45-70^{\circ}\text{C}$, $50-60^{\circ}\text{C}$. Even more in particular, the first pre-determined temperature may be about $20-25^{\circ}\text{C}$.

- 5 The second solution may comprise a suitable amount of the oxidising agent and the synthetic catalyst. For example, the second solution may comprise 0.1-10 vol % oxidising agent based on the total volume of the second solution. In particular, the second solution may comprise 0.5-9.5 vol %, 1.0-9.0 vol %, 1.5-8.5 vol %, 2.0-8.0 vol %, 2.5-7.5 vol %, 3.0-7.0 vol %, 3.5-6.5 vol %, 4.0-6.0 vol %, 4.5-5.5 vol %, 5.0-5.2 vol
10 % oxidising agent based on the total volume of the second solution. Even more in particular, the second solution may comprise 0.5-2.5 vol % oxidising agent based on the total volume of the second solution.

- The second solution may comprise 0.1-1000 ppm synthetic catalyst based on the total volume of the second solution. For example, the second solution may comprise 0.5-
15 900 ppm, 1.0-750 ppm, 5-500 ppm, 10-400 ppm, 15-350 ppm, 20-300 ppm, 50-150 ppm, 75-100 ppm synthetic catalyst. Even more in particular, the second solution may comprise 0.5-3.0 ppm catalyst based on the total volume of the second solution.

- The second pre-determined temperature may be any suitable temperature. For example, the second pre-determined temperature may be $\leq 100^{\circ}\text{C}$. In particular, the
20 second pre-determined temperature may be $20-100^{\circ}\text{C}$, $25-90^{\circ}\text{C}$, $30-75^{\circ}\text{C}$, $45-70^{\circ}\text{C}$, $50-60^{\circ}\text{C}$. Even more in particular, the second pre-determined temperature may be about $20-25^{\circ}\text{C}$.

- The second pre-determined period of time may be any suitable time. For example, the second pre-determined period of time may be 1-72 hours. In particular, the second pre-
25 determined period of time may be 6-60 hours, 12-48 hours, 18-42 hours, 24-36 hours. Even more in particular, the second pre-determined period of time may be 12-24 hours.

- The method of the present invention enables the oxidising agent and the synthetic catalyst to break down the recalcitrant structure of biomass comprised in the biowaste without forming inhibitory compounds which may be detrimental to subsequent
30 processing or treatment steps of the pre-treated biowaste. In particular, the metal complex comprised in the synthetic catalyst may bind to the oxidizing agents and

catalyse oxidation of organic molecules such as lignin comprised in the biowaste by breaking down the cell walls and releasing lignins from the biowaste.

The method of the present invention may further comprise treating the pre-treated biowaste. The further treating may enable transformation of the pre-treated biowaste
5 into value added products such as sugars and biofuels. In particular, lignin released from the biowaste following the pre-treatment of the biowaste may be converted into chemicals such as acetone, ethanol, butanol. Further, subjecting the pre-treated biowaste to a fermentation process may enable higher conversion of biowaste to biofuels since the pre-treated biowaste is able to be digested more easily.

10 The treating may comprise any suitable type of treatment. In particular, the treating may comprise fermenting, enzymatic saccharification, or a combination thereof of the pre-treated biowaste.

According to a particular aspect, the further treating may comprise enzyme saccharification of the pre-treated biowaste to form sugars such as, but not limited to,
15 glucose, xylose, fructose, galactose, lactose, maltose and sucrose. Any suitable enzyme may be used for the enzyme saccharification such as, but not limited to, endoglucanases, cellulase, hemicellulose or mixtures thereof. In particular, the enzyme may be cellulase. The sugar may be subjected to further processing, such as fermentation, to form biofuel.

20 According to another particular aspect, the further treating may comprises fermenting the pre-treated biowaste in the presence of a bacteria to form value-added products such as, but not limited to, acetone, ethanol, butanol. The bacteria may be any suitable bacteria. For example, the bacteria may be, but not limited to, yeast, streptococcus, lactobacillus, bacillus, escherichia, salmonella, clostridium, or a combination thereof. In
25 particular, the bacteria maybe clostridium. Even more in particular, the bacteria may be *Clostridium sp.* BOH3.

The fermenting may be carried out in a suitable culture medium with the pre-treated biowaste as the substrate for the culture medium. In particular, the culture medium may comprise a substrate loading of 30-200 /L of pre-treatment biowaste.

The fermenting may be carried out for a suitable period of time. In particular, the fermenting may be carried out for a period of 24-195 hours. In particular, the fermenting may be for a period of 36-192 hours, 48-180 hours, 60-156 hours, 72-144 hours, 96-120 hours.

- 5 Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration, and is not intended to be limiting.

Example

Example 1 – Pre-treatment of biomass

10 *pH optimisation*

The effect of pH on the reducing sugar concentration was investigated. Biomass was pre-treated using catalytic alkaline peroxide (CAP) solution (1% H₂O₂ and 1 ppm Fe-TAML catalyst) with different pH. After the pre-treatment, the pre-treated sample was subjected to enzymatic hydrolysis for 24 hours to determine how much reducing sugar
15 could be obtained from the biomass. When CAP method was performed at pH 3, the reducing sugar concentration was only 15.9 ± 0.56 g/L, suggesting that a weakly acidic condition was not suitable for CAP. Similarly, when the CAP method was conducted in a neutral condition at pH 7, the reducing sugar concentration was 16.7 g/L. When the CAP method was conducted in an alkaline condition at pH 10, the reducing sugar
20 concentration increased to 19 g/L.

However, further increase of the pH to 11.5 did not lead to an improvement in reducing sugar concentration, which remained at 18.4 g/L. Lastly, at pH 13, the highest reducing sugar concentration around was 24.5 g/L. However, the high pH required a large amount of sodium hydroxide, which was not environmentally friendly. Since pH 10 was
25 the optimized pH for the Fe-TAML and less sodium hydroxide was needed, pH 10 was used in the following examples, to identify a reaction condition at pH 10 which can lead to a reducing sugar concentration higher than 24.5 g/L.

Materials and methods

Experiment Set 1:

Dry biomass (corn stover) samples containing about 18 wt. % of lignin were used in the study. The sample was ground and sieved with a uniform particle size. 2 g of biomass was immersed in 20 mL of 1 vol. % sodium hydroxide (NaOH) solution, for one day or two days (Stage 1).

- 5 Thereafter, the solutions were centrifuged at 7,000 rpm for 4 minutes and residual solid was recovered. The recovered solid was mixed with 20 mL of hydrogen peroxide (H_2O_2) solution (5000 ppm) to form a mixture. In the case of catalytic alkaline peroxide pre-treatment, iron-tetraamido macrocyclic ligand (Fe-TAML) was added to the mixture, to a final concentration of 0.5 ppm. The pH of the mixture solution was adjusted to 10
10 by using 1 M of NaOH solution. Subsequently, the mixture was placed inside an incubator at 50 °C for 24 hours (Stage 2).

Experiment Set 2:

- Corn stover was used as model biomass in Experiment Set 2. The mean size of the corn stover was about 3 mm. It was stored in a dry cabinet before use. Different
15 alkaline H_2O_2 (0.5–2 vol. %) liquor was prepared by adding 20 vol. % H_2O_2 solution in deionized water and adjusting pH to 10 with a 1 M NaOH solution. Then, 2 g of biomass was added into 20 mL of liquor to a final concentration of 100 g/L. Next, the catalyst (5 ppm) was added. The liquor was placed inside an incubator with constant shaking of 155 rpm at 50 °C for different pre-treatment times. After the pre-treatment,
20 the liquor was filtered and all remaining solids were collected and dried at 55 °C for 10 hours.

After the pre-treatment, samples were subjected to soluble lignin test, and the remaining solid residue was further digested by using Cellic CTec 2 enzyme. A schematic flow chart of the process is shown in Figure 1.

25 Soluble lignin test

- Folin–Ciocalteu (FC) reagent was diluted in a 1:9 ratio. 0.3 mL of the diluted FC reagent was added to 0.3 mL of sample after the pre-treatment step. After 5 minutes, 0.6 mL of 15 vol. % sodium carbonate solution was added into the sample for colour development. After 1 hour, absorbance of the sample was measured by using an UV-
30 vis spectrophotometer to determine the concentration of lignin.

Enzymatic saccharification/hydrolysis for residual solid

Residual solid after the pre-treatment was mixed with 20 mL of Cellic Ctec 2 solution (100U) in 12% (w/v) sodium acetate buffer (pH = 4.8). The sample was placed in an incubator (T = 50 °C). After 24 hours, the remaining solid was separated from the supernatant and then dried. The amount of reducing sugar in the supernatant was determined by using dinitrosalicylic acid (DNS) method.

DNS reagent was prepared by mixing 100 mL of distilled water with 2 g of 3,5-dinitrosalicylic acid. Then, 60 g of Rochelle salts (sodium potassium tartrate) was added to the solution until it fully dissolved. NaOH solution (3.2 g in 40 mL of water) was added into the reagent and mixed well.

To determine reducing sugar concentration, 2 mL of DNS reagent was added to 1 mL of sample solution in a tube. The mixture was placed in boiling water for 5 minutes. The mixture was cooled to room temperature and 7 mL of distilled water was added to the tube. Absorbance value of the sample at 540 nm was measured by using an UV-vis spectrophotometer to determine the reducing sugar concentration. Finally, the concentration of the reducing sugar was determined from a calibration curve.

Fermentation of lignin and reducing sugar

The microorganism used in this study was *Clostridium* sp. G117. A commercial reinforced clostridial medium (RCM) was used for culturing strain G117.

20 mL of RCM was dispensed into a 120-mL serum bottle while the bottle was purged with nitrogen gas to remove oxygen. The bottle was sealed with a rubber septum and an aluminium cap to provide an anaerobic condition. Subsequently, the bottles containing RCM were autoclaved at 121 °C for 20 minutes. Meanwhile, the hydrolysate was also sealed with a rubber septum and an aluminium cap and autoclaved. The hydrolysate was added to RCM separately just before the inoculation.

After the inoculation, the bottle was incubated at 37 °C under constant shaking (140 rpm). After 24 hours of fermentation, 1 mL of medium was collected from the bottle and centrifuged at 9,000 rpm for 10 minutes. A clear supernatant obtained was used for the determination of fermentation products.

Analysis of fermentation products

The supernatant was analysed by using gas chromatography (GC; model 7890A; Agilent technologies, U.S.A) equipped with a flame ionization detector (FID). Fermentation products including acetone, butanol, butyric acids, etc. were detected based on their retention times.

Results

Three different pre-treatment methods, alkaline (NaOH), alkaline peroxide (NaOH + H₂O₂), catalytic alkaline peroxide (NaOH + H₂O₂ + catalyst), were compared in two different experiment sets, using the experimental conditions listed in Table 1.

10

Experiment Set No.	Pre-treatment Condition	Control	Alkaline (A)	Alkaline Peroxide (AP)	Catalytic Alkaline Peroxide (CAP)
1	NaOH (1%)	-	+	+	+
	H ₂ O ₂ (5000 ppm)	-	-	+	+
	Fe-TAML (0.5 ppm)	-	-	-	+
2	NaOH (1%)	-	N.A.	+	+
	H ₂ O ₂ (4000 ppm)	-	N.A.	+	+
	Fe-TAML (5 ppm)	-	N.A.	-	+

Table 1: Pre-treatment conditions for different sets of pre-treatment

Soluble lignin test

After the pre-treatments, soluble lignin concentrations in the solution were measured. Figure 2A shows that when the biomass was only treated with NaOH and the incubation time was 1 day, the lignin concentration in the solution was only 43.6 g/L, showing that NaOH was able to remove some lignin from the biomass. In contrast, when both NaOH and H₂O₂ (5000 ppm) were used, the soluble lignin concentration further increased to 50.4 g/L. When all three components (NaOH, H₂O₂, and catalyst) were added per Experiment Set 1, the soluble lignin concentration increased to 51.6 g/L. The result shows that more lignin was released from the biomass by using the catalytic alkaline peroxide (CAP) method. Removal of lignin from the biomass helps to disrupt the cellulose-hemicellulose and produces more glucose for fermentation.

When the incubation time in NaOH was 2 days, the lignin concentration (as shown in Figure 2B) was almost the same as for the incubation period of 1 day. However, when CAP pre-treatment was used, the lignin concentration was only 47.9 g/L, which was lower than that for the incubation period of 1 day. Some lignin was oxidized by the H₂O₂ to form reducing sugar.

As seen in Figure 2C, when the biomass was soaked in NaOH, the phenolic concentration after the AP method was higher than that after the CAP method. This might be due to the oxidation of phenolic compounds in the presence of Fe-TAML. Because lignin is made of phenolic compounds, the presence of Fe-TAML can accelerate the oxidation and dissolution of lignin. As a result, more lignin was removed by using the CAP method. After oxidation of biomass, the soluble phenolic compounds were further oxidized by H₂O₂ in the presence of Fe-TAML, resulting in a lower phenolic concentration shown.

The results, when combined, suggest that the use of H₂O₂ and Fe-TAML overall led to an increase in the soluble lignin concentration.

Physical appearance

The physical appearances of the samples of Experiment Set 1 were observed after pre-treatment. In the control and the alkaline pre-treatment samples, the biomass remained largely intact. No apparent change was observed. In contrast, after the alkaline peroxide and catalytic alkaline peroxide pre-treatments, the samples were whitened by the H₂O₂. Moreover, the biomass became fragile and broke up easily. In particular, when the CAP method was used, the biomass sample became white and powdery, which is a good indication that the pre-treatment was very effective.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to study the microscopic images of the biomass after the pre-treatments to better understand transformation of biomass under the microscopic level. As seen in Figure 3A, there were no surface destruction of the control sample. The surface was rigid and tightly packed with little porosity.

Figure 3B shows that the alkaline pre-treated sample surface in Experiment Set 1 was damaged with some cracks and holes on the surface. The result show that NaOH was

able to hydrolyze the biomass to some extent, but overall the biomass remained intact. In Figure 3C, some cylindrical fibres could be observed on the surface of the alkaline peroxide pre-treated sample in Experiment Set 1. This result indicates that the AP method enhanced the delignification process and that led to the detachment of
5 holocellulose fibers from the matrix. Figure 3D shows that the CAP method in Experiment Set 1 caused a very rough surface and some parts of the surface were carved out, and some cylindrical fibres and a deep valley also appeared on the surface.

Figure 3E shows numerous cylindrical fibers on the surface after the AP pre-treatment in Experiment Set 2. This further shows that the alkaline peroxide enhanced the pre-
10 treatment process leading to delignification. Figure 3F shows that after the CAP pre-treatment in Experiment Set 2, the sample surface exhibited distorted internal structures with mostly cylindrical fibers on the surface. Based on the observation of clear and more fragmented fibrous structure after CAP pre-treatment, it was evident that the CAP method improves enzymatic hydrolysis efficiency and a higher reducing
15 sugar concentration could be obtained.

X-ray diffractometer (XRD)

An X-ray diffractometer (XRD) was used to characterize the pre-treated biomass samples of Experiment Set 2. The crystallinity indices of control, AP pre-treated and CAP pre-treated samples were 58.36%, 60.17%, and 67.02%, respectively. An
20 increment of crystallinity index was observed after pre-treatment which may be due to hydrolysis of glycosidic linkages in the cellulose accessible regions that leads to higher exposure of crystalline cellulose region to the enzyme.

Enzymatic saccharification/hydrolysis for residual solid

After the pre-treatments, the pre-treated solid biomass was subjected to enzymatic
25 saccharification to determine how much biomass could be hydrolysed into reducing sugars. After incubation with Cellic CTec 2 for 24 hours, the residual biomass which cannot be hydrolysed during the process was separated from the liquid. After drying, the residual biomass was weighed and the result is as shown in Figure 4A.

From Figure 4A, it can be observed that the reduction in biomass increased from
30 71.5% to 92% after the addition of H₂O₂. The result suggests that H₂O₂ is able to

oxidize the lignin of the biomass and release more holocellulose for enzymatic hydrolysis. Moreover, when the catalyst was used, more than 98% of the solid biomass was digested and became soluble in water. Only 2% of the biomass remained as solid after the CAP pre-treatment method. The high digestion efficiency (> 92%) for the AP-
5 or CAP-pre-treated biomass also implies that Cellic CTec 2 enzyme was not inhibited by residual H₂O₂ and the catalyst.

Reducing sugar concentrations

For the liquid portion after the enzymatic hydrolysis, DNS test was carried out to determine the amounts of reducing sugars in the liquid, as shown in Figures 4B and
10 4C. The results were used to assess the effectiveness of three pre-treatment methods.

Reducing sugar concentrations in the liquid portion after the enzymatic hydrolysis showed a similar trend. In Experiment Set 1, the presence of 5000 ppm of H₂O₂ during the pre-treatment, the reducing sugar concentration was more than 50.9 g/L whereas in the absence of H₂O₂, the reducing sugar concentration was only 37.8 g/L. The highest
15 reducing sugar concentration was 58.7 g/L produced by the biomass pre-treated with the CAP method (Figure 4B).

In Experiment Set 2, the reducing sugar concentration obtained from the AP method was 23.71 g/L where the glucose concentration is 23.46 ± 0.03 g/L and xylose concentration is 0.250 ± 0.002 g/L, which was 2 times higher than that in the control.
20 The result shows the synergistic effect of NaOH and H₂O₂ in the pre-treatment process. Figure 4C also shows that if the CAP method was used, the reducing sugar concentration further increased to 27.91 g/L where the glucose concentration is 27.58 ± 0.15 g/L and xylose concentration is 0.326 ± 0.002 g/L (Figure 4C). The result suggests that the addition of Fe-TAML is beneficial to the pre-treatment process.

25 The higher reducing sugar concentration was due to two factors. Firstly, the dissolution of lignin caused the biomass to become more porous and susceptible to enzymatic hydrolysis. Secondly, some phenolic compounds such as vanillin are potent enzyme inhibitors, which were oxidized by H₂O₂ and Fe-TAML in the CAP method. In other words, the CAP method is also a detoxification method for the removal of potent
30 inhibitors during the pre-treatment.

Generally, a reducing sugar concentration of about 50-60 g/L is ideal for acetone-butanol-ethanol (ABE) fermentation. A rough mass balance between the solid and liquid portions showed that additional 7-8 g/L of the pre-treated biomass with the CAP method could be converted to reducing sugar after enzymatic hydrolysis.

5 *Fermentation of lignin and reducing sugar*

After the pre-treatment methods were carried out, soluble lignin and residual sugar were released. In order to utilize it as a useful substrate, fermentation was carried out with the pre-treated samples from Experiment Set 1. Soluble lignin was used as a carbon source for fermentation. Approximately 14 g/L of lignin was added to RCM
10 culture medium.

Initially, after the inoculum process, dark colour of fermentation broth can be observed. After 24 hours of fermentation, the fermentation broth turned lighter in colour and became cloudy. Bubbles were formed, which indicate that the *Clostridium* strain G117 is able to grow under soluble lignin and residual glucose as substrate. Figure 5A shows
15 the products formed during the fermentation process. From the retention time, it can be seen that acetone, butanol and butyric acid were formed. From Figure 5B, the concentrations of acetone, butanol and butyric acid increased over the course of fermentation, but stopped increasing after 100 hours of fermentation. Figure 5C shows that the soluble lignin and glucose were consumed rapidly by *Clostridium sp.* G117
20 during the course of fermentation.

It is known that inhibitors such as phenol, furfural and hydroxymethylfurfural are produced from lignin after acid or alkaline pre-treatments. These inhibitors can inhibit the growth of microorganism and compromise the yield of fermentation products. However, there was no indication of inhibitory compounds present in the soluble lignin
25 obtained from the CAP pre-treatment method.

Optimization of pre-treatment method

The effects of H₂O₂ concentration, catalyst concentration, and time on the reducing sugar concentration were analyzed and optimised using response surface methodology (RSM) using the conditions of Experiment Set 2. The experimental results were
30 visualized in three-dimensional response surface plots and contour plots were also

generated to investigate the effects of any two processed variables at a time while holding the other variable as constant at the central level. In the design boundary, each response surface plot had a clear peak, implying the maximum concentration of reducing sugar could be obtained within the boundary.

- 5 The effect of H_2O_2 concentration and time on the reducing sugar concentration at a fixed catalyst concentration at 2.75 ppm was studied. With an increasing concentration of H_2O_2 , the concentration of reducing sugar increased with the length of pre-treatment time. However, there was a maximum reducing sugar concentration at 38.66 g/L. This shows that the excessive use of H_2O_2 leads to the production of enzyme inhibitors and
10 lower the reducing sugar concentration.

The effect of catalyst concentration and time on reducing sugar concentration at a fixed H_2O_2 concentration of 12,500 ppm was investigated next. With a higher catalyst concentration and a longer pre-treatment time, a higher concentration of reducing sugar was obtained. However, when the reducing sugar concentration reached a
15 maximum value of 38.66 g/L, a further increase in the catalyst concentration or pre-treatment time only led to a decrease in the reducing sugar concentration. This could be due to the catalase activity of the catalyst and decomposition of H_2O_2 when the catalyst concentration was too high. The excessive amount of catalyst may inhibit the enzymatic activity and cause a reduction in the reducing sugar concentration.

- 20 The effect of H_2O_2 concentration and catalyst concentration on reducing sugar concentration when the pre-treatment time was fixed at 4.5 hours was studied. The increase in reducing sugar concentration was more sensitive to the H_2O_2 concentration than the catalyst concentration. However, there was an optimal H_2O_2 concentration at about 12,500 ppm. Beyond this point, the reducing sugar concentration remained
25 constant. This is because the catalyst was not fast enough to catalyse all H_2O_2 molecules within the pre-treatment time and that led to excessive H_2O_2 in the system.

The optimum H_2O_2 concentration, catalyst concentration, and time on the reducing sugar concentration were found to be 8850 ppm, 0.91 ppm, and 4.44 hours, respectively, by using RSM. Applying the new optimized condition generated by the
30 Design Expert, the maximum reducing sugar obtained was 36.2 g/L, which is 36.6% higher than that reducing sugar concentration obtained at pH 13. Experiments with two replicates were conducted to validate the optimum conditions, a result consistent with

the predicted values. The mean value of the reducing sugar concentration was 37.31 ± 0.08 g/L where the glucose concentration was 36.54 ± 0.08 g/L and xylose concentration was 0.769 ± 0.002 g/L which is 3.08% higher than the optimized sugar concentration. The xylose concentration was low probably due to the enzyme used
5 being ineffective for the hydrolysis of hemicellulose. Finally, the prediction accuracy was 96.92%, which is acceptable.

Example 2 – Pre-treatment of sludge

Materials and methods

Sludge samples were collected from a municipal wastewater treatment plant in
10 Singapore. Sludge digestion was carried out with three treatment methods, including control, alkaline peroxide and catalytic alkaline peroxide, on samples before and after anaerobic digestion (breaking down of organic matter in the sludge using bacteria).

For alkaline peroxide treatment, 20 mL of sludge samples were mixed with 2 mL of
20% H_2O_2 solution, and the pH of the mixture solution was adjusted to 10 by using 1.0
15 M NaOH solution. For catalytic alkaline peroxide, Fe-TAML was also added to the mixture to a final concentration of 1 ppm. The mixtures were placed inside a water bath at 50 °C for treatment. The pre-treated sludge underwent methanogenesis after 1 hour. The biogas from methanogenesis was analysed by using gas chromatography (GC; model 7890A; Agilent technologies, U.S.A) equipped with a flame ionization detector
20 (FID). Biogas (methane) was detected.

Results

These treatment methods are important for sludge digestion as solubilization of carbon in the sludge into dissolved organics will further enhance methane production. For
sludge sampled before anaerobic digester, the appearance of sludge became slightly
25 lighter after treatment. This indicated some sludge was digested during the process, however, the changes were not obvious as the sludge sampled before anaerobic digester was highly concentrated and viscous.

The appearance of the sludge sampled after the anaerobic digester became lighter after the alkaline peroxide and catalytic alkaline peroxide pre-treatment methods. This
30 was probably because the sludge was less concentrated after it went through the

anaerobic digester. Some sludge solid may have been digested by the anaerobes to produce methane during the process. Furthermore, there were obvious changes after the pre-treatment. For both alkaline peroxide and catalytic alkaline peroxide treatments, a clear layer of supernatant appeared after the pre-treatment. The layer of liquid could be separated from the solid sediment easily. Moreover, the best efficiency of sludge digestion was observed for the catalytic alkaline peroxide treatment, as the liquid volume was greater than that after the alkaline peroxide treatment. This is due to the addition of the catalyst that helps in the oxidation process of hydrogen peroxide.

Methanogenesis

Methanogenesis was carried out to determine how much methane was produced after pre-treatment. As seen in Figure 6, the total methane produced increased from 7.46 mmol to 12.5 mmol after the addition of H_2O_2 . The result suggests that H_2O_2 can assist in the digestion of waste sludge. The highest methane production was 19.3 mmol, produced by the pre-treated sludge with the CAP method. This shows that the CAP method improves the digestibility and has not inhibited the methanogens in the production of methane gas.

Example 3 – Fermentation of food waste

Materials and methods

The fermentation medium in the bioreactor consisted of (per liter): 0.5 g KH_2PO_4 ; 0.5 g K_2HPO_4 ; 0.2 g $MgSO_4$; 2.2 g CH_3COONH_4 ; 0.05 g $MnSO_4$; 0.01 g $FeSO_4 \cdot 7H_2O$; 1 g $NaCl$; 3 g yeast extract, and 1 mL of a trace element solution at a concentration of (per liter): 0.006 mg H_3BO_3 , 0.024 mg $NiCl_2 \cdot 6H_2O$; 0.1 mg $ZnCl_2$; 1.9 mg $CoCl_2 \cdot 6H_2O$; 0.036 mg $Na_2MoO_4 \cdot 2H_2O$; and 0.05 mg $CuCl_2 \cdot 2H_2O$.

For microbial seed cultivation, the medium was first boiled with an addition of 0.25 mL resazurin solution (0.1%) and cooled to room temperature under nitrogen flow. Anaerobic medium (pH 6.5) was prepared with an addition of 0.0242 g/L of L-cysteine, and 0.048 g/L of $Na_2S \cdot 6H_2O$, respectively. 5-mL glucose sterile stock solution (300 g/L) and 1-mL yeast extract sterile stock (150 g/L) were added to the medium. Subsequently, active cells (4%, vol/vol) were inoculated and incubated for 24 to 30 hours at 37 °C and stirred at 150 rpm on a rotary shaker.

Three-stage supplementation of carbon source was performed: initial dosage of 80 g/L food waste, the second dosage of 80 g/L food waste at 28 to 36 hours, and the third dosage of 60 g/L food waste at 48 to 60 hours (220 g/L of food waste is equivalent to ~100 g/L starch).

- 5 1 g/L of CaCO_3 (Ca^{2+} as a metal cofactor for α -amylase in *Clostridium* strain BOH3) was supplemented in the food waste-based fermentation medium to enhance α -amylase activity for starch hydrolysis and butanol production.

- 10 Tryptophan-induced redox modulation of ABE fermentation by strain BOH3: 1g/L of L-tryptophan, a precursor in de novo synthesis of NADH and NADPH, was added to the defined medium to enhance the reducing force to redirect the flux via triggering the availabilities of NADH and NADPH. Synthetic catalyst with a concentration of 1-10 ppm was also added.

- 15 A two-stage pH-shift strategy was adopted: pH was first controlled at 6.0 during the first 6 hours (excluding lag phase), then allowed to drop to 5.0 as the culture progressed. Subsequently, pH was automatically maintained at 5.0-5.3 for onward reactions.

Results

- 20 Fed-batch fermentation process containing *Clostridium acetobutylicum* BOH3 can directly ferment a broad spectrum of pre-treated food wastes with high yield. As seen in Figure 7, the process produced 16.5 and 24.1 g/L butanol and total solvents (ABE), corresponding to ~ 16.5% of butanol yield and 0.229 g/(L·h) of butanol productivity by applying a two-stage pH-shift strategy, cofactor availability, redox modulation and three-stage feeding strategy. The experiment results show that the resultant strain has a yield and production rate better than the known strains. Therefore, the wild type *Clostridium acetobutylicum* strain BOH3 is able to ferment a wide range of low-cost and readily available carbon sources (which in many cases are obtained from wastes) with high yield and productivity, and it is easily cultured and developed for commercial purposes.

Example 4 - Detoxification of potential inhibitors (vanillin)

Materials and methods

Detoxification was carried out with three treatment methods, including control, only H_2O_2 , and catalytic alkaline peroxide which included H_2O_2 and Fe-TAML catalyst. These treatment methods are important as these potential inhibitors might exist after the pre-treatment process which would inhibit enzymatic activities.

- 5 10 mg/mL of vanillin was dissolved in ethanol/deionized water (ratio=1:9). The pH of the solution was adjusted to 10 with a 1 M NaOH solution to obtain an inhibitor solution. For the method with only H_2O_2 , 100 mg/mL hydrogen peroxide liquor was prepared by adding 20% hydrogen peroxide solution to deionized water, and added to the inhibitor solution. For catalytic alkaline peroxide method, 0.01 mg/mL of Fe-TAML catalyst was
10 also added to the inhibitor solution.

After 1 hour, the oxidation products were further analyzed by using High-Performance Liquid Chromatography (HPLC) (Agilent, U.S.A) equipped with a photodiode array detector (PDA, 280 nm).

Results

- 15 From the HPLC analysis (Figure 8), no change was observed in the control set. With only H_2O_2 , vanillin has been oxidized into 4.096 mg/mL of vanillic acid. For the catalytic alkaline peroxide method, there were no peaks observed, which indicated successful detoxification using the model inhibitor.

- Enzymatic hydrolysis was further carried out to determine the amount of sugar that can
20 be produced after the detoxification process. From Table 2, it can be seen that the total sugar produced did not change much from 5.91 g/L to 5.36 g/L after the addition of H_2O_2 . The result suggests that the existence of vanillic acid still inhibits the enzymatic activity after using oxidation solely H_2O_2 . The highest sugar production was 22.06 g/L, produced by the detoxification method with CAP. This shows that the CAP method is
25 able to removal potential inhibitors and have not inhibited the enzymes in the production of sugar.

Run	Control	H ₂ O ₂	H ₂ O ₂ + catalyst (CAP)
Time (hours)	1	1	1
H ₂ O ₂ (mg/mL)	-	100	100
Catalyst (mg/mL)	-	-	0.01
Vanillin (mg/mL)	9.134	0.0141	Not detected
Reduction in Vanillin (%)	0	97.68	100
Vanillin Acid (mg/mL)	-	4.096	Not detected
Precipitate (Y/N)	N	N	Y
Sugar concentration (g/L)	5.91	5.36	22.06

Table 2: Oxidation of vanillin at 10 mg/mL

Example 5 - Production of polyphenolic acid from aromatic liquor

Soluble lignin was precipitated using 3M of sulfuric acid. The acidified lignin (precipitate) is then filtered and aromatic liquor was subject to solvent extraction using ethyl acetate. After solvent extraction, the polyphenolic acid was further purified using column chromatography. Thereafter, crystallization is used to obtain the pure polyphenolic acid in solid form.

Different polyphenolic acids were obtained. For instance, pure p-coumaric acid was obtained from corn stover biomass (Figure 9). This shows that all side products from the treatment processes can be utilised to produce valuable polyphenolic acids. In addition to that, it is easily separated, produced and can be further developed for commercial purposes.

Whilst the foregoing description has described exemplary embodiments, it will be understood by those skilled in the technology concerned that many variations may be made without departing from the present invention.

Claims

1. A method of pre-treating biowaste, the method comprising mixing biowaste with an alkaline solution, an oxidising agent and a synthetic catalyst to form pre-treated biowaste.
- 5 2. The method according to claim 1, wherein the biowaste comprises lignocellulosic biomass.
3. The method according to claim 1 or 2, wherein the synthetic catalyst comprises
10 a metal complex with a metal ion and surrounded by a synthetic ligand.
4. The method according to claim 4, wherein the synthetic ligand comprises at least one nitrogen.
- 15 5. The method according to claim 3 or 4, wherein the metal ion is a transition metal ion.
6. The method according to any preceding claim, wherein the mixing is carried out at a temperature of 20-100°C.
- 20 7. The method according to any preceding claim, wherein the mixing comprises adding the biowaste to a solution comprising the alkaline solution, the oxidising agent and the synthetic catalyst.
- 25 8. The method according to claim 7, wherein the adding comprises adding 1-50 wt % biowaste based on the total weight of the solution.
9. The method according to claim 7 or 8, wherein the mixing is carried out at a temperature of 20-80°C.
- 30 10. The method according to any of claims 7 to 9, wherein the mixing is carried out for ≤ 24 hours.

11. The method according to any of claims 1 to 6, wherein the mixing comprises:
- mixing biowaste with an alkaline solution for a pre-determined period of time and at a pre-determined temperature to form a biowaste solid; and
 - adding the biowaste solid to a solution comprising the oxidising agent and the synthetic catalyst.
12. The method according to claim 11, wherein the pre-determined period of time is 1-72 hours.
13. The method according to claim 11 or 12, wherein the pre-determined temperature is $\leq 100^{\circ}\text{C}$.
14. The method according to claim 13, wherein the pre-determined temperature is 20-100°C.
15. The method according to claim 13 or 14, wherein the pre-determined temperature is room temperature.
16. The method according to any of claims 7 to 15, wherein the solution comprises 0.1-10 vol % oxidising agent based on total volume of the solution.
17. The method according to any of claims 7 to 16, wherein the solution comprises 0.1-1000 ppm synthetic catalyst.
18. The method according to any preceding claim, wherein the method further comprises treating the pre-treated biowaste.
19. The method according to claim 18, wherein the treating comprises fermenting, enzymatic saccharification, or a combination thereof of the pre-treated biowaste.
20. The method according to claim 19, wherein the fermenting comprises fermenting the pre-treated biowaste in the presence of a bacteria.

21. The method according to claim 19 or 20, wherein the fermenting is carried out for a period of 24-195 hours.