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Pretreatment of Cocoa Waste for Bioethanol Production Using Ionic Liquid

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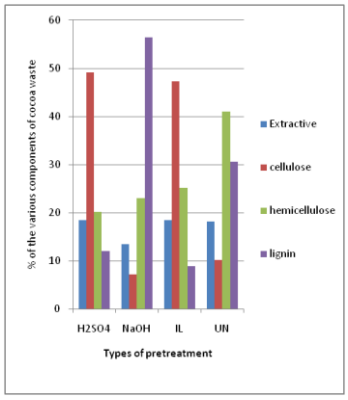
Graphical abstract



Abstract

One of the major advantages of biofuel over fossil fuel is that it is environmentally friendly but unfortunately most of the chemicals used in the pretreatment of lignocelluloses biomass to produce biofuel can cause adverse effects to the environment. In this study, ionic liquid was used for the pretreatment of cocoa waste. Its effectiveness in the treatment process was compared to the alkalis and acids used in the conventional pretreatment media. The effectiveness of pretreatment using ionic liquid, H_2SO_4 and NaOH was based on the reduction of biomass, production of reducing sugar and also bioethanol. Ionic liquid pretreatment was found to show minimal biomass loss of only 31% after pretreatment compared to H_2SO_4 and NaOH which showed loss of 61% and 79% respectively. The untreated biomass has 10% amount of cellulose but upon pretreatment with ionic liquid, H_2SO_4 and NaOH, significant amount of cellulose was detected compared to NaOH which produced only 7% of cellulose. Two types of yeasts were also isolated from Malaysian local fermented food, the *tapai ubi* which were tested for the abilities to ferment the reducing sugar produced. Using the DNS method for determining reducing sugar, ionic liquid pretreatment was shown to produce 6.3×10^{-2}g/L of reducing sugar while the untreated, H_2SO_4 and NaOH pretreatment produced 2.87×10^{-2}g/L, 7.4×10^{-2}g/L and 3.37×10^{-2}g/L respectively at the end of 24 hours of incubation. Bioethanol produced during the fermentation was analysed using gas chromatography. Ionic liquid produced a total of 7.885g/L, H_2SO_4 produced 7.911g/L NaOH produced 6.824g/L and untreated cocoa waste produced 5.116g/L of ethanol at the end of 24 hours incubation.

Keywords: Cocoa waste; pretreatment; lignocelluloses; biomass; cellulose; hemicelluloses; lignin

Abstrak

Antara kelebihan utama bahan api bio berbanding bahan api fosil adalah ianya mesra alam. Tetapi malangnya kebanyakan bahan kimia yang digunakan dalam prarawatan biojisim lignoselulosa membawa kesan negatif terhadap alam sekitar. Dalam kajian ini, penggunaan cecair ionik digunakan dalam kaedah prarawatan, Kesan efektif penggunaan cecair ionik dibandingkan dengan media konvensional seperti penggunaan asid dan juga alkali. Hasil keberkesanan cecair ionik dalam prarawatan dibandingkan dengan prarawatan H_2SO_4, prarawatan NaOH dan biojisim tidak dirawat dikenalpasti melalui kemusnahan biojisim, penghasilan gula penurun dan juga penghasilan bioetanol. Prarawatan cecair ionik telah ditemui dan menunjukkan kemusnahan biojisim yang minimum 30.77% selepas prarawatan manakala H_2SO_4 menunjukkan 61.18% dan NaOH menunjukkan kemusnahan biojisim 78.89% selepas prarawatan. Biojisim tidak dirawat mempunyai jumlah 10.23% selulosa tetapi prarawatan dengan cecair ionik mendedahkan jumlah ini sehingga 47.30%, H_2SO_4 untuk 49.13% dan NaOH mengurangkan amaun ini ke 7.150%. Dua jenis yis juga telah diasingkan daripada Tapai Ubi untuk menjalankan penapaian.Menggunakan kaedah DNS untuk menentukan mengurangkan gula, prarawatan cecair Ionik menghasilkan 6.3×10^{-2}g/L, tidak dirawat, prarawatan H_2SO_4 dan prarawatan NaOH menghasilkan 2.87×10^{-2} g/L, 7.4×10^{-2}g/L dan 3.37×10^{-2}g/L masing-masing pada akhir 24 jam pengeraman. Bioethanol yang dihasilkan semasa penapaian yang telah dianalisis menggunakan kromatografi gas. Cecair ionik telah mengeluarkan sejumlah 7.885g/L, H_2SO_4 menghasilkan 7.911g/L NaOH dihasilkan 6.824g/L dan sisa koko tidak dirawat menghasilkan 5.116g/L etanol pada akhir 24 jam.

Kata kunci: Hasil buangan koko; pra-rawatan; lignoselulosa; biojisim; selulosa; hemiselulosa; lignin

1.0 INTRODUCTION

Bioethanol is a promising alternative fuel which can be produced biologically from a variety of feedstock and wastes. Feedstock used in bioethanol production such as soybeans, palm, canola, rapeseed, animal fats, vegetable oil, starch and sugar are considered first generation feedstock. The disadvantage of the first generation feedstock is that they could be used alternatively to make food for humans. As such they posed serious threat to human food chain and supply. Production of ethanol from non-food feedstock (lignocelluloses biomass) provides an alternative solution towards the crisis. Lignocellulosic biomass (LB) is one of the most abundant biomass from non-food bio-feedstock. Examples of this feedstock are wood and agricultural crop residues such as cocoa wastes, straw and sugar beet pulp. Apart from eliminating competition for food, a mixture of different crops and agricultural wastes can be used. But conversion of LB to ethanol is significantly hindered by the structural and chemical complexity of biomass, which makes these materials a challenge to be used as feedstock for ethanol production. To produce ethanol from such feedstock a pretreatment step is therefore necessary. Pretreatment refers to the solubilisation and separation of one or more components of the biomass (hemicelluloses, cellulose, and lignin) to make the remaining solid biomass more accessible to further chemical or biological treatment. The goal of pretreatment is to remove the lignin and hemicelluloses, reduce cellulose crystallinity and increase the porosity of the biomass [1]. Various chemical substances such acids and alkaline that are used in the pretreatment process are harsh to the environment. To minimise this problem and other environmental impact of these chemicals, the concept of green chemistry must be applied in the pretreatment process.

Green chemistry involves the use of chemicals substances and chemical processes that are designed to eliminate or reduce negative environmental impacts. This will unknowingly eliminate pollution and other environmental problems before we even know they exist. The principles of green chemistry involve the substitution of hazardous chemical process (substances) with less hazardous ones in synthetic processes. This concept is very popular among scientists today, especially the exploration of novel solvents that can replace the volatile organic compounds that is used in synthesis, catalysis, and separation processes [2].One of such examples is to substitute the volatile organic solvent with ionic liquids. Ionic liquids (ILs) are ionic, salt-like materials that are liquid below 100°C. ILs at ambient temperature possess many attractive properties such as negligible volatility, non-flammability, high thermal stability, and controllable hydrophobicity [3]. ILs have solvent properties and are miscible with organic solvents and water. Their non-flammability and negligible vapour pressure make them not readily lost to the environment. In recent years, ILs has been extensively studied as an alternative to organic solvent. They have been widely examined as extracting phases in liquid-liquid extraction systems and show good extraction performance and separation ability for metal ions when compared to organic solvents [3]. Study has shown that the extraction of alkaline and alkaline metal in ILs and achieved high extraction efficiency compared to that of ordinary organic solvent. The extraction behaviour of strontium into imidazolium cation based ILs was also examined and obtained a high distribution coefficient compared to that of ordinary organic solvent [4]. From these studies, it can be deduced that apart from being environmentally benign, ILs also show high extraction performance compared to conventional organic solvents. This study was aimed at demonstrating the use of IL in pretreatment of cocoa wastes for production of bioethanol. To determine its effectiveness, ILs will be compared to the conventional chemicals used in pretreatment of lignocellulosic biomass as discussed in the next section.

2.0 PROBLEM STATEMENT

To achieve enzymatic degradation in production of ethanol, a pretreatment process is necessary. The purpose of all pretreatments is to remove lignin and hemicellulose, reduce cellulose crystalinity and increase the porosity of the materials. Accordingly, all kinds of pretreatment process must meet the following requirements which involved production of reactive cellulosic fibre for enzymatic attack, avoiding destruction of hemicelluloses and cellulose, avoiding formation of possible inhibitors for hydrolytic enzymes and fermenting microorganisms, minimizing the energy demand, reducing the cost of size reduction for feedstocks, reducing the cost of material for construction of pretreatment reactors, producing less residues, consumption of little or no chemical and usage of cheap chemical [5].

Most of the pretreatment methods fail to meet all the above requirements, thus there is need to exploit some other alternative methods which are environmentally friendly and are in line with the green chemistry. Hence the significance of this study was to exploit any possible alternative method(s) for pretreatment of lignocellulosic biomass that is environmentally friendly and may possibly meet the above requirements.

3.0 EXPERIMENTAL WORK AND METHODOLOGY

The study was designed to determine the effect of cocoa wastes pretreated by IL. The amount of cellulose, hemicelluloses and lignin of the cocoa waste before and after the pretreatment process will be measured as well as reducing sugar and ethanol yielded were analysed and compared with the present conventional methods that involves acid and alkali pretreatment.

3.1 Ionic Liquid (IL) Pretreatment

10g of the ground biomass was mixed with 50% and 80% of IL and in this study 100 ml ionic liquid {1-ethyl-3-methylimidazolium methanesulfonate ($C_7H_{14}N_2O_3S$)} was used. The mixture was left at room temperature for two hours before it was autoclaved for 15 minutes at 121°C. It was allowed to cool before rinsing with distilled water and finally pressed through cheese cloth and dried in a furnace until constant weight was obtained. The same procedure was applied to 0.5M and 1M of H_2SO_4 and NaOH.

3.2 Determination of Cellulose, Hemicelluloses and Lignin

3.2.1 Determination of the Amount of Extractive

180 ml of acetone was poured into 3g of dried biomass and the temperature held at 90°C for two hours in a furnace. The sample was later dried at 105°C until constant weight was obtained. The weight difference before and after the extraction is the amount of the extractives [6].

3.2.2 Determination of Hemicelluloses

10 ml of the 0.5M of NaOH was added to 1g of the extractive-free dried biomass. The sample was held at 80°C for 3.5 hours and later washed using hot deionised water until the pH value approached 7. The sample was dried at 105°C until a constant

weight was obtained. The amount of hemicelluloses was obtained from the difference in weight before and after the treatment [6].

3.2.3 Determination of Lignin

30 ml of H_2SO_4 (98%) was added to each 1g of extractive- free dried biomass. The sample was held at ambient temperature for 24 hours. It was later boiled at 100°C for 1 hour after which it was filtered and dried to constant temperature at 105°C. The weight of the residue was recorded as lignin [6].

3.2.4 Determination of Cellulose

The content of the cellulose was determined by the difference of the components of the lignocellulosic material; assuming extractive, hemicelluloses, lignin and cellulose are the only components of the entire biomass [6].

3.3 Isolation of Yeast from "Tapai Ubi"

For the fermentation of sugar into bioethanol , a local homemade fermented food "tapai ubi" bought from local shop in Skudai, Johor was used for the isolation of yeast. "Tapai ubi" is fermented tapioca and is known to contain strains able to break down starch to fermentable sugars and longer period of fermentation will yield ethanol and aromatic esters. For this work, 10g of "tapai ubi" was mixed and blended with 100 ml of 0.85% NaCl, using a blender. 10 ml of the blended solution was added to 100 ml PYS medium without substrate in a 250 ml flask. The solution was incubated in shaker at 30°C and agitated at 200 rpm for 24 hours. One loopful of the solution was streaked on the PDA agar plate using inoculating loop and incubated for 24 hours at 30°C.

3.4 PYS Medium

Four different PYS media were prepared. The chemical composition of all the media were the same except the source of carbon which were cocoa wastes that had undergone different pretreatment process; untreated cocoa waste; the others were pre-treated with ionic liquid, sulphuric acid, and sodium hydroxide treated cocoa wastes. Table 2.1 shows the composition of the PYS medium ; the components were mixed with 500 ml distilled water and stirred until the mixture was dissolved completely prior to autoclaving for 15 min at 121°C.

Table 2.1 Composition of PYS medium

Components	Quantity (g/L)
Yeast extract	2.5
Peptone	2.5
NH_4Cl	1
$KHPO_4$	0.5
$MgSO_4$	0.15
Substrate (pretreated cocoa wastes)	3

3.5 Fermentation Process

One colony of the yeast from PDA plate was inoculated into PYS medium having different kind of pretreated substrate. The fermentation was carried out in 250 ml flask which was covered with cotton wool and agitated at 200 rpm, for 24 hours at 30°C. The pH was adjusted using NaOH or HCl to maintain at pH 5. A 5ml aliquot of the sample was taken out every 3 hours over a duration of 24 hours and centrifuged at 4000 rpm and supernantant was used for the analysis of reducing sugar and bioethanol.

3.6 Determination of Reducing Sugar using DNS

The DNS method was used to determine the concentration of glucose by detecting the reducing end of the monosaccharide. This group converts the oxidized form of 3, 5-dinitrosalicylic acid (DNS), to the reduced form which is absorbed at 540 nm. The standard solution for DNS was prepared using 0.01g of glucose in 10 ml distilled water. To detect the reducing sugar, 1 ml of sample was mixed with 1ml DNS solution and 2 drops of 0.1M NaOH was added. The mixture was boiled in a water bath for about 5 minutes and allowed to cool. 10 ml of distilled water was added and mixed evenly and the absorbance was taken at 540nm.

3.7 Ethanol Analysis

Agilent Technology 6890N gas chromatography was used to determine the ethanol concentration. The carrier gas was helium and the temperature held at 40°C for 4 minutes. The temperature was set with increment of 10°C to 100°C. Detector temperature and injector temperature was set at 250°C. Peak area of the compound was integrated against external standard. The compound was separated by using a non-polar capillary column. A standard curve using appropriate concentration of ethanol was plotted. 1 ml of the sample was placed into centrifuge tube and a drop of (5% v/v) of HCl was added. This was followed by addition of 0.5 ml dichloromethane. The mixture was mixed thoroughly before it was centrifuged at 3000 rpm for 30 minutes. The top layer was discarded while the bottom layer was transferred to GC vial for analysis.

■4.0 RESULTS AND DISCUSSION

4.1 Effect of Different Pretreatment Methods on Cocoa Waste

Pretreatment has significant effect on the composition of biomass; it alters the composition of the various components of the biomass when compared to the untreated biomass. This alteration brought about changes in the amount of biomass obtained after pretreatment. In this study the amount of biomass used before and after pretreatment was obtained by weight difference before and after pretreatment. The percentage of the total solid obtained after pretreatment and the percentage of solid biomass lost during pretreatment were also determined. Pretreatment was carried out in duplicate using concentrations of 1M and 0.5M for both sulphuric acid and sodium hydroxide, and 80% and 50% of the ionic liquid. Results obtained from the pretreatments showed that 0.5M and 80% ionic liquid have minimal lost of solid biomass. Ionic liquid pretreatment also has the highest total solid biomass recovered compared to sulphuric acid and sodium hydroxide.

4.1.1 Effect of IL Pretreatment on Cocoa Waster

In this study IL pretreatment has the minimal solid biomass lost as compared to H_2SO_4 and NaOH pretreatment. Using 80% IL 69% solid biomass was recovered and only 31% of the biomass was lost. Using 50% IL 66.69% solid biomass was recovered while 33% was lost. This may be attributed to the fact that IL is not corrosive and consists of only ions; as such it provided a mild condition for the reaction. Since IL consists of entirely ions, they are non-flammable, have high thermal stability and controllable hydrophobicity [3]. Another possible reason could be the viscosity of ionic liquids compared to that of 0.5M and 1M of NaOH and H_2SO_4. The ionic liquids are viscous, similar to that of oil. The viscosity restricts flow of the IL thereby providing more time for reaction. This effect was also observed when the concentration was increased from 50% to 80%; the total solid biomass recovered also increased. Table 4.1 gives the percentage of solid biomass recovered after pre-treament and that of biomass lost during the pretreatment.

4.1.2 Effect of Sulphuric Acid Pretreatment on Cocoa Waste

Dilute acid pretreatment of lignocellulosic biomass is one of the most effective pretreatment methods which predominantly affect hemicelluloses with little impact on lignin degradation [7]. From the study 1M of H_2SO_4 has 34% of solid biomass recovered after pretreatment and 66% of solid biomass lost during pretreatment While using 0.5M, the solid biomass lost was lowered to 61% and the solid biomass recovered after pretreatment was higher up to 39% Compared to IL, H_2SO_4 has higher solid biomass lost and less solid biomass recovered after pretreatment. This is due to H_2SO_4 being a strong mineral acid and is corrosive while ionic liquid as salt, consists of only ion and is not corrosive.

4.1.3 Effect of Sodium Hydroxide Pretreatment on Cocoa Waste

The pretreatment with NaOH has the highest solid biomass lost during of 85% and only 15% of solid biomass was recovered after pretreatment using concentration of 1M. Using 0.5M, 79% of solid biomass was lost during pretreatment and 21% of solid was recovered after pretreatment. This may be due to the concentrations and the temperature used during the pretreatment. Previous work on the effect of temperature on NaOH showed that temperature was only significant when the residence time was 90 minutes at 1 % and 2 % NaOH with the highest biomass recovery [8].

Table 4.1 Effect of different kinds of pretreatments on percentage loss of cocoa wastes

Pretreatment type	Concentrations	% of solid after pretreatment	% of solid lost
IL($C_7H_{14}N_2O_3S$)	80%	69	31
	50%	67	33
H_2SO_4	1M	34	66
	0.5M	39	61
NaOH	1M	15	85
	0.5M	21	79

Generally it can be deduced from Table 4.1 that as concentration were reduced, less solid biomass was lost except for ionic liquid. This means that pretreatment at lower concentration gives minimal solid lost and vice versa. The results obtained were similar to the findings which stated that elevated temperature, residence time and acid concentration reduced solid recovery after pretreatment [8]. The rate of the biomass lost will definitely have effect on the amount of sugar to be produced by each pretreatment, which in turn will have effect on the production of bioethanol.

4.2 Analysis of Cellulose, Hemicelluloses and Lignin

The lignocelluloses biomass consists of cellulose, hemicelluloses and lignin. In addition to these, there are plant-specific chemicals in the matrix, called extractives (resins, phenolics, and other chemicals), and minerals (calcium, magnesium, potassium, and others) that will leave ash when biomass is burned .The amounts of all the four components present in the cocoa waste were determined. Results showed that there was variation in amount of the respective components due to the activities of different chemicals used during pretreatment as seen in Figure 4.1

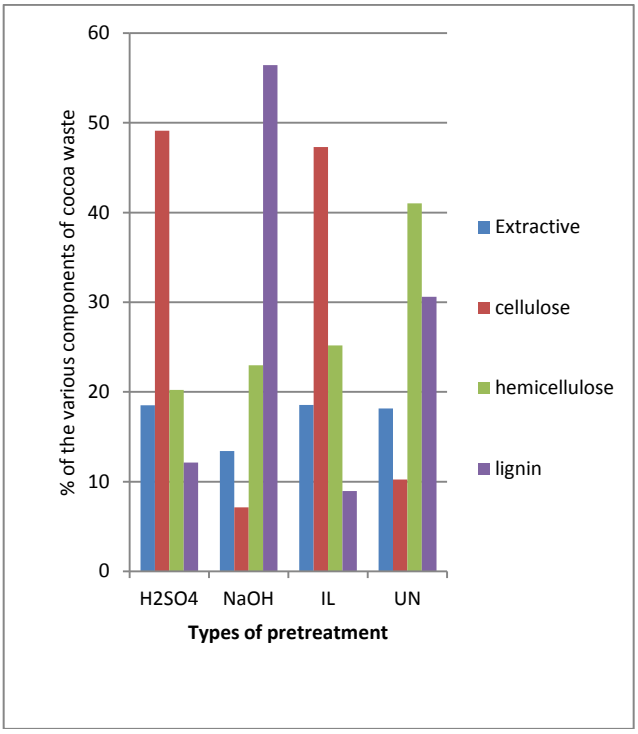


Figure 4.1 Analysis of cellulose, hemicelluloses and lignin in cocoa waste after pretreatment with hydrochloric acid (H_2SO_4), sodium hydroxide (NaOH), ionic liquid (IL) with untreated sample as control

There is no significant difference between the amounts of extractive from sulphuric acid, ionic liquid and the untreated biomass, which is approximately 18%. This is because the target of any pretreatment is not the extractive but rather the lignin. On the other hand there is a significant difference in the amount of cellulose produced by different pretreatment. The untreated cocoa waste contained 10% of cellulose. This was increased to 47% after pretreatment with IL. H_2SO_4 pretreatment also increased the amount to 49.13%. Treatment with NaOH however decreased this amount to 7%. This showed that H_2SO_4 was more effective in exposing the cellulose followed by IL. NaOH however was ineffective in this process. This may be attributed to the concentrations and the temperature used during pretreatment or

the type of biomass used in the process. This is in agreement with the findings of Millet *et al* [7] where it was indicated that pretreatment with dilute NaOH has no effect on softwood with lignin content greater than 26%. But on contrary, the digestibility of NaOH–treated hardwood increased from 14% to 55% with decrease in lignin content from 24% - 55% to 29% [8]. This indicated that NaOH was more effective on hardwood than softwood.

The hemicelluloses content of the cocoa waste was also altered after different kinds of pretreatment methods. The amount of hemicelluloses of untreated biomass was 41%. This amount was reduced to 25% by IL pretreatment, 23% by NaOH and 20% by H_2SO_4. This showed that IL has less effect on exposing the hemicelluloses than the other forms of pretreatment used in this study. H_2SO_4 has the highest removal rate of hemicelluloses compared to others.

The lignin content of the cocoa wastes differed when using different pretreatment methods. The untreated biomass has 31% lignin, but after pretreatment with IL the amount was significantly reduced to 9%. H_2SO_4 reduced this amount to 12%. This finding is in line with the findings of Li *et al* [9] where dilute acid pretreatment of switchgrass with ionic liquid pretreatment in terms of delignification, saccharification efficiency and reducing sugar yields were compared. They found that lignin removal by IL was more effective than that of acid pretreatment. During ionic liquid pretreatment, switchgrass cellulose undergoes dissolution and precipitation by an anti-solvent, resulting in reduced cellulose crystallinity and increased surface area, and a glucan yield of 96 % [9].

However NaOH pretreatment increased the amount of lignin to 56 %. This shows that treatment with NaOH destroyed the cellulose and hemicelluloses, as a result the percentage of lignin increased more than the untreated cocoa waste. Table 4.2 gives the summary of the percentage of each component of the cocoa wastes.

Table 4.2 Analysis of cellulose, hemicelluloses and lignin

Pretreatment	Extractive %	Cellulose %	Hemicelluloses%	Lignin %
H_2SO_4	19	49	20	12
NaOH	13	7	23	56
Ionic Liquid (IL)	19	47	25	9
Untreated (UN)	18	10	41	31

4.3 Isolation of Yeast from "Tapai Ubi"

A home made "tapai ubi" bought from a local shop in Skudai was isolated using the PDA media. After blending with 0.85% NaCl and incubating at 30°C for 24 hours in PYS media without a substrate, it was later streaked on the PDA plate. Visual observation showed the presence of two different colonies. Both of the colonies appeared white in colour but one was lighter than the other and spread throughout the plate. A pure culture was then made to separate the two colonies. One colony appeared to be filamentous, transparent with smooth surface and texture, termed as Tapai Ubi A. The other colony also appeared filamentous but translucent with rough surface and texture was termed as Tapai Ubi B.

To examine the yeast under the light microscope, methylene blue was used to visualise the yeast cells. Yeast cells that are alive appeared opaque because their enzymes are actively metabolizing (breaking down) the methylene blue. Cells that were not living turned blue because they were unable to metabolize the stain. There was little spreading for cells of Tapai Ubi A, hence when viewed under the microscope, they appeared to be smaller. They were also not connected by hyphae. The cells of Tapai Ubi B however spread widely and appeared to be larger than Tapai Ubi A. There was also thread-like formation that connects budding cells and these are referred to as pseudohyphae, or false hyphae. Figure 4.2 gives the pure culture the microscopic image and the description of the yeast.

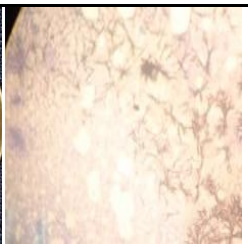
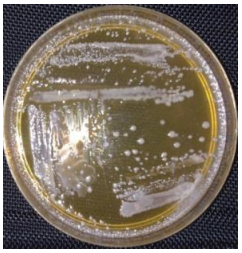
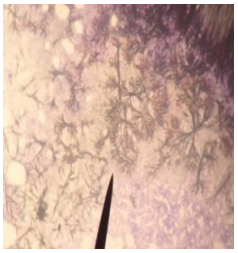
Yeast plate	Microscopic image	Description
		*Tapai Ubi A *Smooth surface *Colony spread little under the microscope *Smooth texture *Bipolar budding
		*Tapai Ubi B *Colony spread widely on the plate *Multiple budding *lager colony *compact under microscope *more whitish

Figure 4.2 Yeast isolated from tapai ubi

4.4 DNS Analysis of Reducing Sugar

4.4.1 Reducing Sugar Produced by Tapai Ubi A

In this study,the amount of sugar produced from different kinds of pretreatment using both isolated yeasts were measured and compared to untreated biomass. The initial concentration of reducing sugar was different as shown in Figure 4.3 as the biomass was used in continuity of the pretreatment process carried out. The total amount of reducing sugar produced by untreated biomass using Tapai Ubi A was 0.0286g/L. This corresponds to the amount produced at 0 hour since there was not enough time for contact between the yeast and the sugar to produce ethanol. At every three hours interval the amount was shown to be reduced until it reached 0.0149g/L at 24 hours. The reduction in the amount of sugar over time was an indication that the yeast utilized the sugar into ethanol besides using it for growth. It was observed that the amount of sugar produced at 24 hour using untreated cocoa waste was more than that of all the chemical methods used in this study. This may attributed to the fact that the untreated biomass contained more hemicelluloses than cellulose. Figure 4.3 gives the amount of reducing sugar produced by the different kinds of pretreatment.

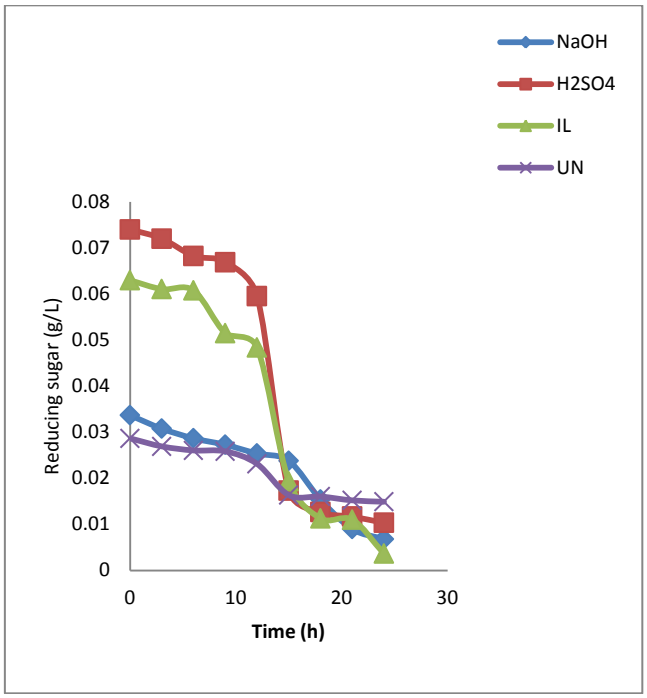


Figure 4.3 Reducing Sugar produced by Tapai Ubi A yeast after incubation at 30^0C , shaking at 200 rpm

Sulphuric acid was found to produce highest concentration of reducing sugar production compared to other forms of pretreatment methods used in this study. It produced more than 0.070g/L of sugar. This amount was more than three times compared to the untreated cocoa wastes (control). The IL pretreatment also produced high amount of reducing sugar which amount up to 0.063g/L. Sodium hydroxide pretreatment produced less amount of sugar compared to other forms of pretreatment methods. It produced 0.0337 g/L of sugar which may be attributed to destruction of cellulose during pretreatment. This was indicated in the reduction in the amount of cellulose obtained after pretreatment. Table 4.2 gives the reducing sugar produced by Tapai A yeast.

The production of higher amount of reducing sugar by H_2SO_4 and IL is related to the amount of cellulose obtained after pretreatment process. The H_2SO_4 has 49 % and IL has and 47 % of cellulose after pretreatment while NaOH and UN have 7 % and 10% respectively. Thus it can be concluded that the amount of sugar produced is directly related to the amount of cellulose.

In the hydrolysis reaction, the cellulose molecule is cleaved by the addition of a water molecule. The equation for this reaction is as follows: $(C_6 H_{10} O_5)_n + {}_n H_2O \rightarrow {}_n C_6 H_{12} O_6$. In this process cellulose is hydrolysed into its glucose. The composition of the PYS medium used consists of 500ml of water which broke down the cellulose to its glucose units as described by the above equation. The presence of cellulase can also helped the hydrolysis of the cellulose.

4.4.2 Reducing Sugar Produced by Tapai Ubi B

The total amount of reducing sugar produced by Tapai Ubi A yeast was higher than Tapai Ubi B over the 24h period. This may be due to the difference in the metabolism rate between two types of the yeasts. The total reducing sugar produced by untreated biomass using Tapai Ubi B yeast was 0.017g/L as compared to 0.0286g/L of Tapai Ubi A yeast. NaOH pretreatment yielded of yeast 0.023g/L, H_2SO_4 0.052g/L and IL 0.0453g/L .All these amounts are lower compared to the amount produced by Tapai Ubi A yeast. Figure 4.4 gives the detail of the reducing sugar produced by the Tapai Ubi B yeast.The best solution to compare yield between 2 types of yeast would be to determine the production of glucose over the dry weight of yeast which was not carried out in this study.

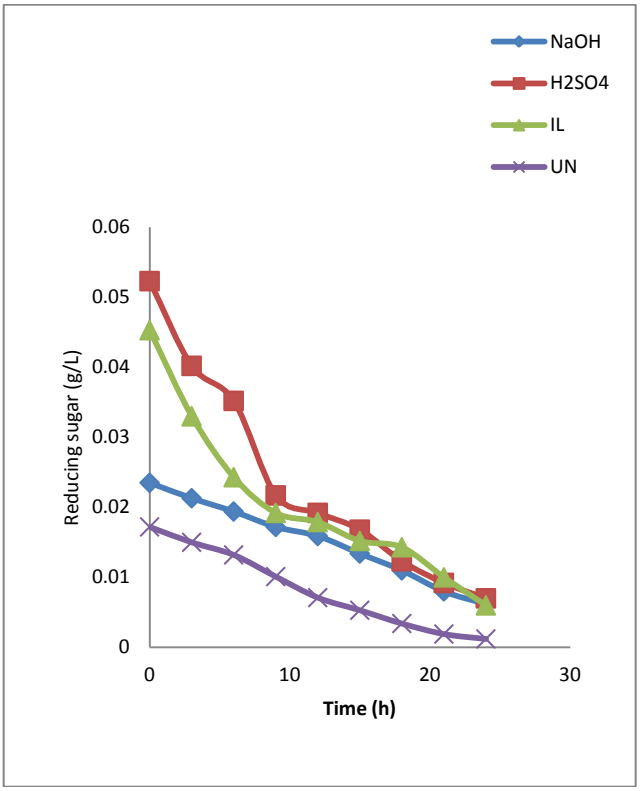


Figure 4.4 Reducing Sugar produced by Tapai Ubi B yeast

4.5 Ethanol Analysis Using Gas Chromatography

Bioethanol production depends solely on the metabolic activities of the microorganism and the amount of fermentable sugars available. Some microbes can ferment only six carbon sugars (hexose) while others can ferment both five (pentose) and six carbon sugar. As such pretreatment is evaluated based on sugar yield not on ethanol yield. This is in agreement with the statement of name of Zhu and Pan [10] who reported that evaluation of pretreatment technologies in the literature has been primarily focused on sugar yield. For these reasons, this study only detected the presence of ethanol as evidence that fermentation has taken place but the data cannot be consistently used to determine the type of pretreatment that produced the highest amount of ethanol. To effectively determine the amount of bioethanol produced by each pretreatment, the different kinds of sugar produced by the cocoa waste should be determined and also whether the yeasts isolated from “tapai ubi” can ferment all of these sugars. The sugars present should also be a fermentable type. In addition, the volatility of ethanol and the storage method before analysis may also influence accuracy of ethanol detrmination. Furthermore, the sampling stopped at the end of 24 hours while there is still production of ethanol. This may also affect the overall quantification of the ethanol produced by each pretreatment

method. Hence, the amount of ethanol produced in each pretreatment was reported having considered the experimental constraints.

4.5.1 Ethanol Produced By Tapai Ubi A

To produce ethanol, yeasts break down sugar molecules into carbon dioxide gas and ethanol (ethyl alcohol). This begins as the growing population of the yeast produces enzymes to break dissacharides into monossacharides if needed and then convert the single molecule sugars into ethanol. Yield of ethanol approach a limit as the yeast either consume all of the fermentable sugars or the products and by-products of fermentation inhibit the yeast from further fermenting the sugars.

Tapai Ubi A yeast was able to produce a total of 27.706g/L ethanol at the end of the 24 hours. Figure 4.5 gives the detail amount of ethanol produced by different kinds of pretreatment.

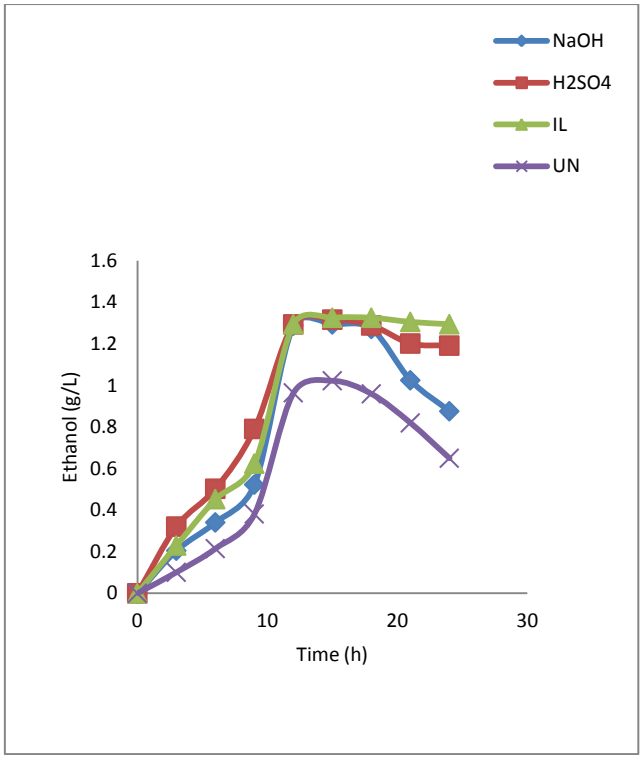


Figure 4.5 Ethanol produced by Tapai Ubi A yeast

In this study Tapai Ubi A yeast was capable of fermenting the sugars produced by different kinds of pretreatment into ethanol as shown in Figure 4.5. Sulphuric acid pretreatment produced the highest amount of ethanol of 7.911g/L at the end of 24 hours. This was followed by ionic liquid pretreatment which produced 7.855g/L and NaOH pretreatment with the lowest ethanol yield of 6.824g/L at the end of 24 hours. The untreated cocoa waste produced 5.116g/L using this yeast.

4.5.2 Ethanol Produced By Tapai Ubi B

Tapai ubi B yeast also produced ethanol with all the different kinds of pretreatment. The amount of ethanol produced was lower compared to Tapai Ubi A. A total of 24.123g/L of ethanol was produced by this yeast as compared to 27.706g/L produced by Tapai Ubi A. Similar to the Tapai Ubi A yeast pretreatment with H_2SO_4 , Tapai Ubi B produced the highest amount of ethanol compared to other pretreatment used in this study when H_2SO_4 was used. It produced a total of 6.535g/L at the end of 24 hours while IL and NaOH produced a total of 6.506g/L and 6.354g/L respectively.The untreated cocoa waste produced a total of 4.726g/L at the end of the 24 hours. Figure 4.6 gives the detail amount of ethanol produced by Tapai Ubi B yeast.

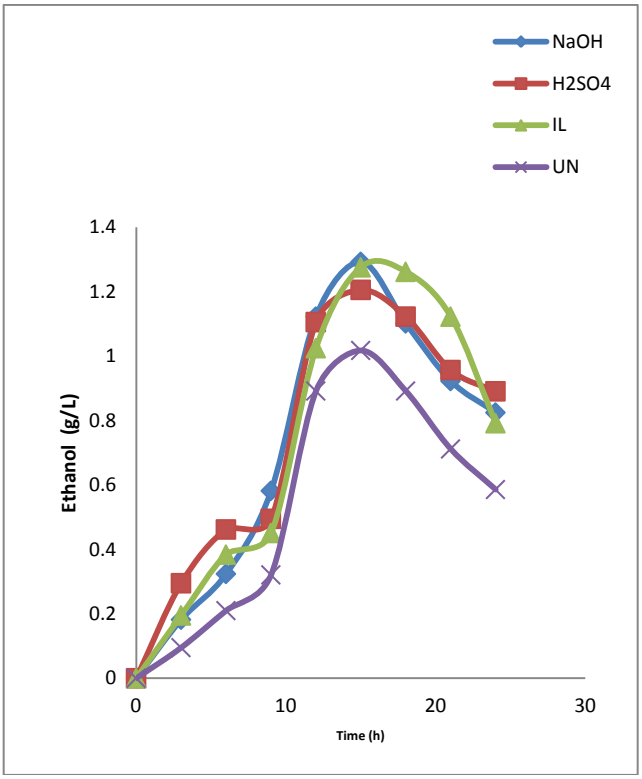


Figure 4.6 Ethanol produce by Tapai Ubi B yeast

The amount of ethanol produced by Tapai Ubi A was higher in all of the pretreatments to the amount produced by Tapai Ubi B. Similar finding was made in the amount of reducing sugar produced. This may be attributed the differences in the metabolic activities of the two yeasts. It is possible that Tapai Ubi A has higher metabolic activity than Tapai ubi B.

5.0 CONCLUSION

It can be concluded that IL showed good potential as an alternative in the pretreatment of cocoa wastes that is environmentally friendly having distinct advantages over the use of strong acids and alkalis. Lesser amount of cocoa wastes was required to produce reducing sugar compared to the other pretreatment methods, hence IL may be considered to be more economical. IL produced the highest amount of reducing sugar after H_2SO_4. Comparatively, *Tapai Ubi* yeast A produced high amount of reducing sugar than *Tapai Ubi* yeast B. Yeast A was also effective in the delignification of cocoa wastes. IL pretreatment produced high amount of reducing sugar, exposed the cellulose to enzymatic activities, removes both lignin and hemicelluloses with minimal destruction of biomass.

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