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RECOVERY OF FERMENTABLE SUGARS FROM PALM OIL MILL EFFLUENT VIA ENZYMATIC HYDROLYSIS

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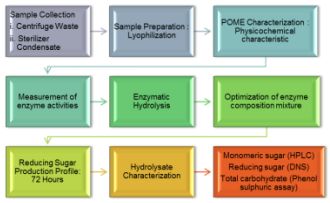
Graphical abstract



Abstract

In order to enhance the recovery of fermentable sugars from palm oil mill effluent (POME), experiments were carried out to analyze the effect of different combinations of enzymes and enzymatic hydrolysis retention time on the structural carbohydrate composition of POME originated from centrifugal waste and sterilizer condensate in the mill. The optimum combination of enzymes comprising of Celluclast 1.5 L (X_1), Novozyme 188 (X_2) and Viscozyme-L (X_3) and optimum incubation time for enzymatic hydrolysis were determined based on one-factor-at-a-time (OFAT). For hydrolysis of centrifugal waste, maximum yield of 34.3 g/L monomeric sugar concentration was achieved with ratio of enzymes at 0.33: 0.33: 0.33 (X_1:X_2:X_3), at enzyme loading of 3%, pH 4.8, and 48 hours of incubation at 50 °C. For sterilizer condensate, maximum yield of 6.5 g/L monomeric sugar concentration was achieved with ratio of enzymes at 0.42: 0.33: 0.25 (X_1:X_2:X_3) under similar processing condition.

Keywords: Cellulase, enzymatic hydrolysis, Palm Oil Mill Effluent (POME), reducing sugars

Abstrak

Dalam usaha untuk meningkatkan pemulihan gula penurun dari air sisa kilang kelapa sawit (POME), eksperimen telah dijalankan untuk menganalisis kesan kombinasi enzim dan masa tahanan hidrolisis enzim terhadap komposisi karbohidrat POME yang berpunca dari proses pengemparan dan sterilisasi. Gabungan enzim yang optimum terdiri daripada *Celluclast 1.5 L* (X_1), *Novozyme 188* (X_2) dan *Viscozyme-L* (X_3) serta masa pengeraman optimum untuk hidrolisis enzim telah ditentukan berdasarkan kaedah pengoptimuman satu faktor pada satu masa (OFAT). Bagi hidrolisis sisa pengemparan, hasil maksimum kepekatan gula monomerik iaitu 34.3 g / L dicapai dengan nisbah enzim pada 0.33: 0.33: 0.33 (X_1:X_2:X_3), pemuatan enzim sebanyak 3%, pH 4.8, pengeraman selama 48 jam pada 50 °C. Bagi kondensat sterilisasi, hasil maksimum kepekatan gula monomerik sebanyak 6.5 g/L dicapai dengan nisbah enzim pada 0.42: 0.33: 0.25 (X_1:X_2:X_3) melalui keadaan pemprosesan yang sama.

Kata kunci: Selulase, hidrolisis enzim, Air sisa kilang kelapa sawit (POME)

1.0 INTRODUCTION

With the most suitable climatic conditions for oil palm cultivation, Malaysia has become one of the world's largest producers and exporter of palm oil, accounting for 11% of oils and fats production and 26% of fats and oils export trade, worldwide [1]. However, the sprawling palm oil industry has not only been a catalyst to industrialization, but has led to large generation of liquid waste known as palm oil

mill effluents (POME) which has to be treated before discharged to any water intake. Processing of 100 kg of fresh fruit bunches (FFB) generates 67 kg of wastewater (POME), which amounted to 60.3 million tons, annually [2,3]. POME is a brown colloidal suspension, containing 95-96% water, 0.6-0.7% oil and 4-5% of suspended solids [4]. It is rich in lignocellulosic material (cellulose, hemicellulose and lignin). Besides, being abundant and of non-food nature, it has been the choice of lignocellulosic biomass substrates in biofuel industry for suitable conversion into fermentable sugars. By converting POME into useful products, a cost-effective treatment process and utilization of POME as higher value renewable feedstock can be developed leading to sustainability of the oil palm industry.

Through POME treatment technologies, a variety of useful products such as enzymes, sugar, carbohydrates, proteins, organic acids, biological pesticides, activated carbon, fertilizer, biodiesel, methane, and other products can be recovered [5]. Studies have shown that POME can act as a high potential fermentation medium for the production of antibiotics, solvent acetone-butanol-ethanol, hydrogen, citric acid, cellulose etc [6-10]. Polysaccharides in lignocellulosic materials, e.g., in cellulose, consist of long homopolymer chain of glucose units connected by a beta acetyl linkage and in hemicellulose, a branched heteropolymer of pentoses (xylose and arabinose), hexoses (glucose, galactose, mannose) and sugar acids (acetic). However, the digestibility of lignocellulosic biomass to recover fermentable sugars is low owing to its inherent structural features such as lignin content, acetyl groups and crystallinity. Therefore, use of physical and chemical pretreatment processes followed by enzymatic hydrolysis is vital to improve the accessibility of the sugar components. Pretreatments employing strong acids and alkali could result in reduction of sugar yield due to loss of composition and formation of inhibitory by-products. This also leads to hazards of chemical handling and complications in recycling the large quantities of chemicals [11].

Enzymatic hydrolysis of cellulose involves three types of enzymes viz., cellulase (endoglucanase, cellobiohydrolase and β-glucosidase), which work simultaneously [12]. Endoglucanase (EC 3.2.1.4) randomly integrate β-1,4 glycosidase linkage of cellulose, followed by cellobiohydrolase (EC 3.2.1.91) degrading end of cellulose chains to produce individual units of cellulose to cellobiase (glucose dimer connected by bonds β-1,4 glycosidase) and finally β-glucosidase (EC 3.2.1.21) hydrolysecellobiose to two molecules of glucose. Cellobiose is a strong inhibitor of both cellobiohydrolase and endocellulase, and β-glucosidase action can reduce its impact. On the other hand, Viscozyme-L is a multi enzyme complex consisting of arabinose, cellulase, β-glucanase, hemicellulase, xylanase. It is also actively used against the branched pectin-like substances. Studies have proven Viscozyme-L to be an efficient biocatalyst for carbohydrate removal with sugars yield of 80%, as glucose equivalent units [13].

However, hydrolysis and further recovery of value added products from highly viscous liquids have a negative impact on downstream processing. High viscosity level of any processing liquids limits the dry substance level in the process, increasing energy and water consumption thus lowering product yield. Non-starch polysaccharides eventually reduce the efficiency of separation, evaporation and heat exchange leading to an economically unfavorable and non-sustainable process in long runs [14]. The use of Viscozyme-L in hydrolysis of POME is likely to increase the dry matter levels which in turn reduce the quantity of water that will have to be heated, cooled and evaporated in downstream processing. Less viscous stream in processes allows a better heat exchange operations providing reduction in overall operating costs. Nevertheless, high cost of enzymes and low rate of hydrolysis are potential drawbacks in enzymatic hydrolysis, thus, sugar productivity can be improved by formulating better enzyme mixtures and optimizing the hydrolysis process parameters [15]. In this study, POME collected from two different sources in palm oil mill viz. centrifugal waste and sterilizer condensate has been characterized prior to enzymatic hydrolysis. Different combinations of Viscozyme L and other cellulases were used in the enzymatic hydrolysis process to maximize the yield of reducing sugar. The appropriate composition of enzyme mixture and retention time for optimal reducing sugar recovery from POME was also investigated

2.0 EXPERIMENTAL

2.1 Substrate Collection

POME were obtained from Sime Darby East Palm Oil Mill, Carey Island, Selangor from two different sources which are sterilizer condensate and centrifugal waste and were kept refrigerated at 4 °C prior to use. Samples were solidified by lyophilization method using a freeze dryer, model Martin Christ Alpha 1-4LSC at -40 °C and under vacuum condition. Later, the dried sample were crushed to powder form using mortar and pestle and kept in air tight container.

2.2 Enzymes

Three different types of enzymes were used to perform hydrolytic degradation on structural carbohydrate composition of POME. These were cellulase from *Trichodermareesei* ATCC 26921 (78.72 FPU/mLwith protein concentration of 42.03 mg/ml), cellobiase from *Aspergillus niger* (1543.3 CBU/mL with protein concentration of 47.49 mg/mL) and *Viscozyme®L* from *Aspergillus aculeatus* (69.4 FPU/mL with protein concentration of 29.11 mg/mL) purchased from Novozyme A/S, Denmark. One FPU

unit is considered as the amount of enzyme required to release a fixed amount of glucose equivalent from 50 mg Whatman no.1 filter paper in 1 min. The protein concentration was determined by dye-binding assay of Bradford using bovine serum albumin (BSA) as standard and the absorbance of solution was measured at wavelength of 595 nm using spectrophotometer [16]. Analytical grade reagents were used as received for all analyses. Enzyme activities of cellulase and *Viscozyme®L* were determined by measuring the reducing sugars produced from 1 x 6 cm^2 strip of Whatman filter paper No.1 50 mg as substrate [17] where else cellobiase activity was determined based on method described by Yeoh et al. [18].

2.3 Enzymatic Hydrolysis of POME

The enzymatic hydrolysis of POME was carried out in a 250 mL conical flask (at a ratio of 100 mL raw POME to 3 mL of enzyme mixture). The hydrolysis process was conducted with different combinations of enzymes to maximize the reducing sugar production. The total volume of enzyme used was 3 mL. Table 1 shows the ratio and the volume of enzyme used in the experiment. The pH of POME was adjusted to 4.8 using sodium hydroxide (0.1 M). Conical flasks were placed in an incubator shaker at 50 ˚C and agitation speed of 150 rpm for 64 hours. After 64 hours, the samples were placed in a water bath at 100 ˚C or 10 min to deactivate the enzyme activity. Samples were then centrifuged at 6500 rpm for 10 minutes in order to remove the residual solids remaining after enzymatic hydrolysis. The procedure above was employed to create a reducing sugar profile to identify the optimum time for reducing sugar production with the best enzyme mixture. Samples were taken at the 2nd, 4th, 8th, 24th, 36th, 48th, and 72nd hour with a pipette and prepared for sugar analysis.

Table 1 Ratios and respective volume of enzyme mixtures at 3% enzyme loading in four different combinations

Combination code	Ratio (%)		
	Cellulase (X_1)	Cellobiase (X_2)	Viscozyme (X_3)
a	0.33	0.33	0.33
b	0.50	0.50	0
c	0.42	0.33	0.25
d	0.33	0.42	0.25

2.4 Analytical Method

Physicochemical characteristics of samples were determined prior to hydrolysis process. Reducing sugar in the medium was determined using *dinitrosalicyclic* (DNS) assay [19], while phenol-sulphuric acid assay [20] was used to determine total carbohydrate and the Chemical Oxygen Demand (COD) was measured using HACH reagents. Total Suspended Solid (TSS) and Volatile Suspended Solid (VSS) were measured using standard method [21]. Sugar compositions were quantified using HPLC (Agilent Technologies, USA) equipped with refractive index detector and column of Rezex (ROA Organic acids H+ (8%) 4E, 7.8 mm × 300 mm). The HPLC was operated at 60 °C using mobile phase of 0.005 N sulphuric acid with flow rate of 0.6 mL/min.

3.0 RESULTS AND DISCUSSION

3.1 POME Characteristics

Precise quantification of biomass constituents is very important in determining the product conversion efficiency and process economics of any type of feedstock. The composition of structural carbohydrate and other major components in centrifugal waste and sterilizer condensate are given in Table 2.

Table 2 Characteristics of POME obtained from centrifugal waste and sterilizer condensate

Parameters	Centrifugal waste	Sterilizer condensate
pH	4.54 ± 0.1	4.82 ± 0.1
Total suspended solid, TSS (mg/L)	86,417 ± 7000 (8.6 %)	19,783 ± 2600 (2 %)
Total volatile solid, VSS (mg/L)	79,700 ± 5500 (8 %)	18,339 ± 2600 (1.8 %)
COD (mg/L)	46,000 ± 40 (4.6 %)	27,050 ± 26 (2.7 %)
Total reducing sugar (g/L)	10.78 ± 0.1	6.46 ± 0.2
Total carbohydrate (g/L)	21.94 ± 0.5	12.98 ± 0.5
Hexose: glucan (g/L)	6.83 ± 0.03	5.32 ± 0.05
Pentose: xylan (g/L)	2.28 ± 0.01	0.96 ± 0.01
Pentose: arabinan (g/L)	0.85 ± 0.01	0.28 ± 0.01

Typically, the pH of palm oil mill effluent water is low due to organic acids produced during the natural fermentation process [22]. In the present study, the pH value of the centrifugal waste (CW) was slightly lower than that from sterilizer condensate (SC). However, these two samples showed wide variation especially in terms of TSS, VSS and COD values probably due to point of origin. The higher suspended solids content in CW could be attributed by pieces of palm husk derived from high speed centrifugation. On the other hand, sterilizer condensate is a steam condensate from sterilization process that uses high-pressure steam to separate the kernel from the bunch. Significant differences in the compositional value, mainly TSS, VSS and COD can be noticed in comparison to previously reported data [23-25]. The characteristics of palm oil mill effluent varied widely from day to day and from plant to plant, depending on the quality of fresh fruit bunches (FFB), seasonal differences and the efficiency of the machines involved during the

extraction process as well as the operational control. Therefore, the treatment efficiency of POME using different samples may not be the same due to the variations in characteristics. In this study, it was found that reducing sugar content in the raw CW was higher than SC by 79%. Reducing sugar in the liquid waste was determined to identify the effectiveness of enzymatic hydrolysis process. Among the most common reducing sugars produced from lignocellulosic wastes are glucose, xylose, xylitol, cellobiose, arabinose, and galactose.

3.2 Optimum Enzyme Combination

In the present study, enzymatic hydrolysis of CW and SC were performed with four different combinations of enzyme mixtures and were optimized on the basis of the reducing sugar and total carbohydrates content. Usually, the optimum temperature for the reaction and the stability of three components: endoglucanase, cellobiohydrolase and beta-glucosidase in *Aspergillus niger* and *Trichoderma Sp*. is between 50-60°C [26]. Therefore, enzymatic hydrolysis were carried out for 64 hours at temperature of 50 °C and at pH 4.8 and stirring speed of 150 rpm. Conversion efficiency of cellulosic carbohydrate polymers to fermentable sugars can be improved by adjusting time and temperature of hydrolysis [27]. Figure 1(a) shows the yield of total carbohydrate and reducing sugar content of POME hydrolysate collected from centrifugal waste. Enzyme mixtures of cellulose, cellobiase and viscozyme in the ratio of 1:1:1 (78.72 FPU / ml: 1,543.3 CBU / ml: 69.4 FPU / ml) resulted in the highest yield of reducing sugar which was 30 g/L while total carbohydrate content was 30.2 g/L. The similarity in these two values can be regarded that initial polysaccharides of higher molecular weight are all broken down to pentoses and hexoses, which exist as readily available monomeric sugars after complete hydrolysis. Similarly, with enzyme combination of (b) and (d), the reducing sugar and total carbohydrate content remained almost same but the quantity was lower than that of (a). The absence of viscozyme in combination (b) doesn't compromise sugar extraction yield but the hydrolysate was less viscous. Centrifugation process on hydrolysate from (b) for further analysis was much easier compared to serial centrifugation required for hydrolysates from other combinations. The reducing sugar yield for (b), (c) and (d) is 28 g/L, 28.6 g/L and 25.5 g/L, respectively. These values of reducing sugar are much higher than the initial carbohydrate value of 21.9 g/L reflecting the efficient conversion of cellulose to fermentable sugars.

Figure 1(b) illustrates the yield of monomeric sugars namely glucose, xylose and arabinose. Glucose accounts for the highest constituents of monomeric sugar, in the range of 57% to 63%, followed by xylose while arabinose was present in little. The yield of monomeric sugars for enzyme combinations (a), (c) and (d) is almost similar which is 20 g/L for glucose, 11 - 12 g/L for xylose and 3 g/L for arabinose. It can be concluded that, the mixture of enzymes with viscozyme had a better effect on the hydrolysis reaction by reduction of viscosity. In presence of viscozyme, pectin and hemicellulose could be hydrolyzed to a greater level, however, increasing the dosage of viscozyme did not show any significant increase in monomeric sugar concentration. Enzymatic hydrolysis with (b) combination of cellulose and cellobiase (78.72 FPU/ml and 1,543.3 CBU/ml) resulted in a lower extraction value with 17.5 g/L of glucose, 8 g/L xylose and 2.3 g/L arabinose. Overall saccharification efficiency for POME hydrolysate from centrifugal waste was the highest for enzyme mixture of (a) which is 67% for glucose, 88% for xylose and 70 % for arabinose. These readily available fermentable sugars can be recovered for further fermentation for the production of biofuels.

The effect of enzyme mixtures on POME collected from sterilizer condensate in terms of total carbohydrate and monomeric sugar extracted are shown in figure 2(a). In contrast with POME hydrolysate from CW, value of total carbohydrate after enzymatic hydrolysis was much lower compared to the initial carbohydrate content. However, analyzing the reducing sugar content in hydrolysate shows an increase for (a), (c) and (d) combinations. Cellulose degradation with enzyme combination of (c) with ratio of 1:0.8:0.6 (78.72 FPU/ml: 1,234.64 CBU/ml: 41.64 FPU/ml) showed effective reaction with yield of 11.96 g/L reducing sugar which is very close to the initial total carbohydrate content of 13 g/L. Based on this data, the most efficient hydrolysis for SC is with 45% of reducing sugar yield yet the conversion is much lower than that of centrifugal waste. In a previous study, enzymatic hydrolysis on sterilizer condensate had resulted in yield of 97.4% with production of 2.01 g/L reducing sugar [28]. Even though, the reducing sugar content is much lower compared to the present study yield, the extremely lower initial value of reducing sugar have given a greater yield percentage responding to effective conversion. Likewise, enzyme combination of (b) without viscozyme showed negative reaction where only 0.4 g/L increment in reducing sugar was obtained.

Results for the yield of monomeric sugars from sterilizer condensate after enzymatic hydrolysis are shown in figure 2(b). The constituents of reducing sugar in SC hydrolysate are glucose and xylose with distribution of 70% and 30%, respectively. Maximum yield of 3.26 g/L glucose and 1.38 g/L of xylose were achieved for enzyme combination of (a). This relatively very poor conversion of reducing sugar from SC revealed that, it is not feasible to utilize sterilizer condensate alone for enzymatic hydrolysis regardless of any optimum enzyme combination or processing condition. In previous researches involving extraction of sugars from biomass, chemical or physical pretreatment such as acid, alkali and thermal treatment were employed prior to enzymatic

hydrolysis to achieve maximum saccharification [29,30]. Chaturvedi & Verma [31] summarized the various pretreatment technologies employed for conversion of lignocellulosic biomass into biofuels and value added products and concluded that no technology could offer 100% conversion of fermentable sugars. However, with new efficient or upgraded existing process, a more sustainable biomass utilization approach can catalyst the renewable energy supplement. The challenge behind lignocellulosic based biofuel production is the recovery of fermentable sugars from biomass, where an optimum enzymatic hydrolysis process without any need of pretreatment could serve as the most economical operational condition for larger scale processing of palm oil mill effluent.

3.3 Optimum Retention Time

Figure 3 shows reducing sugar profile for a period of 72 hours using the optimum enzyme composition. The reducing sugar yield at three different stages, namely initial, stimulating and declining were estimated as shown in Figure 3. It is obvious that the increase in yield of reducing sugar in CW hydrolysate was far more significant compared to SC. For CW, after two hours of incubation, 14.4 g/L of reducing sugar is obtained which increased to 21.7 g/L at 48th hour. After 24 hours, the reducing sugar content remained same indicating the end to the hydrolytic interaction between enzymes and cellulose.

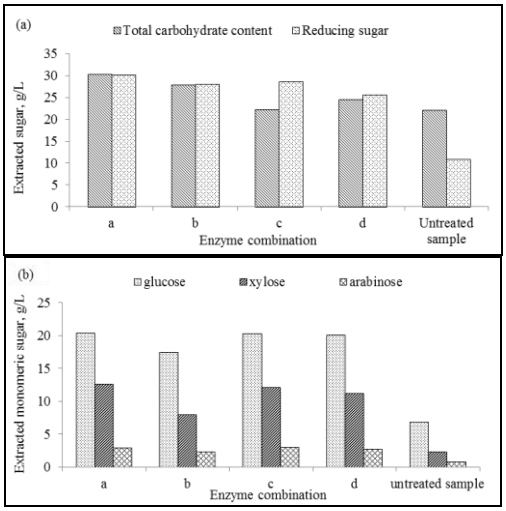


Figure 1 Sugar yield for hydrolysis of centrifugal waste with different enzyme mixtures. (a) Yield of reducing sugar and total carbohydrate after hydrolysis of 64 hours, (b) Yield of monomeric sugars of glucose, xylose and arabinose after hydrolysis of 64 hours.

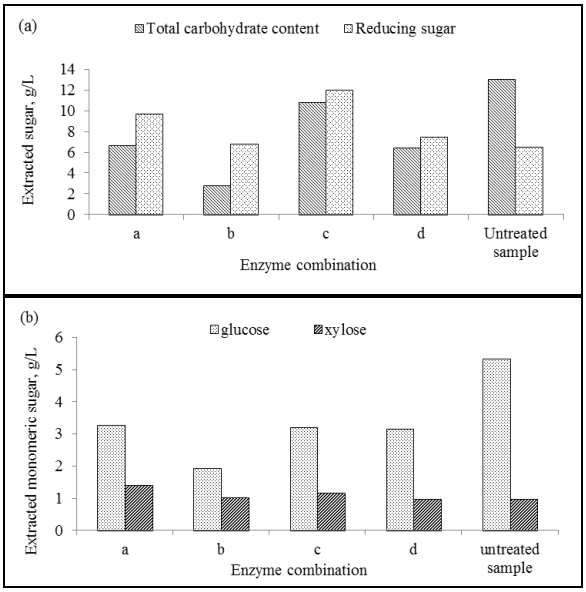


Figure 2 Sugar yield for hydrolysis of sterilizer condensate with different enzyme mixtures. (a) Yield of reducing sugar and total carbohydrate after hydrolysis of 64 hours, (b) Yield of monomeric sugars of glucose, xylose and xylose after hydrolysis of 64 hours.

Hydrolysis rate decreases probably due to the diffusion into and entrapment of enzyme molecules in the small pores present in cellulose when hydrolysis process continues [32]. Thereupon, the adsorbed enzyme, after deconstructing few cellulose linkages, remains restrained at a site being unable to proceed with potential attacks.

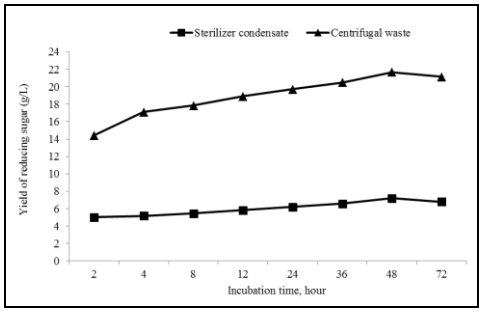


Figure 3 Comparison of enzymatic hydrolysis for centrifugal waste and sterilizer condensate over a time frame of 72 hours enzymatic hydrolysis in response to the yield reducing sugar.

Nevertheless, similar trend on yield of reducing sugar for SC was observed where a gradual increase starting from 2nd hour to an optimum of 7.2 g/L at 48th hour. However, after that, a decrease in reducing sugars was observed which could be due to fatigue amorphous cellulose substrates and accumulation of strong inhibitory products such as cellobiose [33]. According to Tsai & Meyer [34], in most enzyme facilitated degradations, the reaction rates decline significantly after 12 hours and the hydrolytic

efficiency beyond 48 hours demonstrates the overall hydrolysis performance. Hydrolysis for 48 hours is regarded as the most common and acceptable industrial processing times for lignocellulosic hydrolysis in bioethanol processing. Figure 4 summarizes the enzymatic saccharification for CW and SC based on the monomeric sugar yield. For hydrolysis of CW, at the 48th hour, total monomeric sugar as high as 34.3 g/L was achieved with distribution of 58%, 32% and 10% of glucose, xylose and arabinose, respectively. Similarly in SC, a gradual increase of glucose and xylose from the initial stage with slight fluctuation was observed till the optimum reaction stage at 48th hour with yield of 6.5 g/L and decreased to 4.8 g/L at 72nd hour. Thus, 48 hours of incubation was chosen as optimum time for enzymatic hydrolysis for CW and SC. Saifuddin & Refal [35] have reported that microwave assisted alkaline pretreatment of POME prior to enzymatic hydrolysis enhances the reducing sugar yield by increasing the rate of enzymatic reaction. However, the drawback is that it doesn't promise a sustainable process and it is a non-environmentally friendly system due to the excessive usage of chemicals, increase in operational cost and high energy consuming for large scale pretreatment of biomass. Although the combinations of several pretreatment processes have shown promising results, it is often expensive and contributes to about 20% of the overall lignocellulosic ethanol processing cost [36].

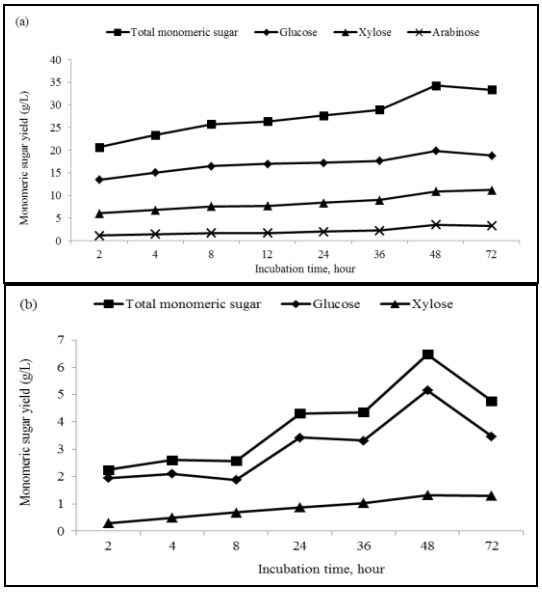


Figure 4 Yield of total monomeric sugars (glucose, xylose and arabinose) for (a) centrifugal waste and (b) sterilizer condensate over a time frame of 72 hours enzymatic hydrolysis.

4.0 CONCLUSION

This study examined the feasibility of utilizing raw POME from sterilizer condensate and centrifugal waste for recovery of fermentable sugars. The current study has successfully shown the effectiveness and potential of enzymatic hydrolysis process without prior pretreatment in extracting fermentable sugars. It can be concluded that, hydrolysis process with equal ratio (0.33:0.33:0.33) of cellulase, cellobiose and viscozyme has resulted in extraction of 34 g/L monomeric sugar in POME hydrolysate from centrifugal waste, while in sterilizer condensate, enzyme mixture (c) with ratio of (0.42:0.33:0.25) resulted in 6.5 g/L monomeric sugars.

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