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Full Length Research Paper

Development of pretreatment of empty fruit bunches for enhanced enzymatic saccharification

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To achieve an accomplished optimized condition for enzymatic saccharification of palm oil mill empty fruit bunches (EFB) for higher yield of sugar hydrolysis, a comprehensive pretreatment of EFB was carried out using the laboratory produced cellulase enzyme through bioconversion of palm oil mill effluent (POME) by the fungal strain, *Trichoderma reesei* RUT C-30. This study was conducted by using two different types of agents (physical and chemical). Heating, boiling and steaming are among the physical agents and different concentrations of nitric acid, sulfuric acid and sodium hydroxide (NaOH) were the chemical agents used for the pretreatment of EFB to enhance the enzymatic saccharification of EFB. NaOH was proved to be the best among all the pretreatment agents and 3% NaOH was far higher and 2.35 fold increment was achieved on the yield of reducing sugar (175.03 mg/g of EFB) after 96 h of saccharification. A maximum of 41.82% yield of reducing sugar was achieved with 5% (w/v) of EFB and 7% (v/v) of enzyme after 120 h of saccharification when eight important parameters, namely saccharification duration, EFB size, EFB dose, enzyme dose, Tween 80, triton 100, agitation and incubation temperature, were examined in an OFAT (one factor at-a-time) design.

Key words: Empty fruit bunches (EFB), palm oil mill effluent (POME), one factor at-a-time (OFAT), saccharification, pretreatment.

INTRODUCTION

Lignocellulosic materials are the most abundant biopolymer in nature. About 120 to 150 billion tones dry matter of lignocellulosic biopolymer is produced globally (Rajoka and Malik, 1996). This mostly important carbon sources can play very significant role in the carbon cycle through its degradation and subsequent utilization (Niranjane et al., 2007). Utilization of these lignocellulosic wastes can be tremendously increased if these materials are first hydrolyzed chemically or enzymatically to glucose and other soluble sugars which can be subsequently used for making sweeteners, single-cell protein (SCP), energy materials (alcohols) or other fermentation products (Rajoka and Malik, 1996). Many researchers studied the feasibilities in the production and bioconversion of different bioproducts such as composting (Kausar et al., 2010), enzyme (Xia and Shen, 2004), single-cell protein (SCP) (Gibriel et al., 1981), activated carbon (Keith et al., 2005), energy materials-bioethanol (Sharma et al., 2004), food products (Kumar et al., 2003), etc by using the lignocellulosics.

Among them, energy material such as, bioethanol, is of special interest, because the fossil fuel reserve is depleted. In the wake of the current global fuel reserve status, the United states alone targeted to blend 7.5 billion gal of renewable fuels into gasoline by 2012, and by far, the most common renewable fuel is ethanol following the US energy policy act, 2005 (Gray et al., 2006). Therefore, efficient enzymatic saccharification of the most abundant renewable carbon sources in the world has a high economic potential for the production of bio-ethanol. Many researchers used different waste

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Abbreviations: EFB, Empty fruit bunches; **POME,** palm oil mill effluent; **OFAT,** one factor at-a-time.

materials such as woody materials (Ballesteros et al. 2004), water hyacinth biomass (Aswathy et al., 2010), food wastes (Jung et al., 2008), etc for the evaluation of the lab-scale saccharification and enzymatic saccharification was investigated on sunflower stalks (Sharma et al., 2002), rice hull (Badal and Cotta, 2008), rice straw (Ma et al., 2009) etc.Malaysia earned foreign exchange of about $20,000 million from export of palm oil and palm oil-based products in 2008 (DOS, 2009). Last couple of decades, agriculture industry has significantly contributed to the economy and oil palm is the largest plantation sector in Malaysia. It accounts for about 17.08 million tones of lignocellulosic materials in the form of empty fruit bunch (EFB) which are produced from the oil palm processing industries (Chew and Bhatia, 2008). Some part of this waste are being utilized as biofertilizer, however, most of the portion is disposed off or incinerated (Hussein et al., 1985).

Using the lignocellulosic solid wastes of the palm oil mill industry, many researchers have already come up with some laboratory scale output of different products such as composting (Yahya et al., 2010), activated carbon (Alam et al., 2007), enzyme (Alam et al., 2009), citric acid (Bari et al., 2009), gasification (Tomoko et al., 2010), etc. Cellulose is the main constituent of the lignocellulosic materials which is just above 50% of the total in EFB. Therefore, it is a very suitable material to be hydrolysed enzymatically to produce saccharides. The main challenge of saccharification of the EFB is the hemicellulose (about 22%) and lignin content (21%) (Bari et al., 2009). So, an effective pretreatment of these lignocellulosic materials is inevitable to enhance the hydrolysis process through reduction of the lignin and hemicellulose content, cellulose crystallinity and increase porosity (Zhu et al., 2006).

Pretreatment methods can be physical, chemical or sometimes both (Hsu, 1996). Steam and water are not considered as chemical agents for pretreatment since extraneous chemicals are not added to the biomass. Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size), steam explosion and hydrothermolysis.

Comminution, including dry, wet and vibratory ball milling (Millett et al., 1979; Rivers and Emert, 1987; Sidiras and Koukios, 1989) and compression milling (Tassinari et al., 1982) is sometimes needed to make material handling easier through subsequent processing steps. High yield of the saccharification of EFB require optimization of its process parameters.

In this study, the cheaper cellulase enzyme used was produced through bioconversion of palm oil mill effluent (POME), a very cheaper and abundant liquid waste. So, in this study, different physical and chemical pretreatment agents were used to evaluate the effect of pretreatment on the enzymatic saccharification of EFB and the pretreated EFB was investigated under different process parameters and accelerating chemicals to enhance the sugar production yield.

MATERIALS AND METHODS

Enzyme preparation

Sample of crude cellulase enzyme was collected from the Environmental Engineering Laboratory Stock of International Islamic University Malaysia (IIUM). Crude cellulase are the broth of the bioconversion product of POME by *Trichoderma reesei* RUT C-30 using optimized media and process conditions in a 30 L bioreactor. POME with 2% (w/v) total suspended solids (TSS) was used as a basal medium including the supplementary nutrients such as peptone (0.5%, w/v), cellulose (0.5%, w/v) and Tween 80 (0.2%, v/v). A 7.5% culture inoculum was inoculated into the bioconversion medium and incubated for 5 days where temperature, aeration, agitation and pH were 30°C, 1.5 VVM, 200 rpm and 7, respectively. The fermentation broth of cellulase was first filtered using the bag filter with porosity of 250 µm and stored at -20°C and thawed at 4°C overnight before micro- and ultra-filtration were started. After thawing, the enzyme solution was centrifuged at 4°C with 10,000 g. The supernatant was used as feed in the cross flow micro- and ultra-filtration system.

Collection and preparation of EFB

Sample of EFB was collected from East Oil Mill, Sime-Darby, Banting, Kuala Lumpur, Malaysia. Collected sample was preserved in the cold room at 4°C to avoid the unwanted bio-degradation by any microorganisms. Collected EFB sample was washed with distilled water vigorously to remove all mud, dust and other unwanted substances. Washed sample was dried in oven at 105°C for 24 h to get constant dry weight. Dried EFB fiber was ground with milling machine to obtain desired particle size (0.25, 0.5, 1, 2 and 3 mm down graded).

Effect of pretreatment with different physical agents

Different physical agents were examined to evaluate the effect of the pre-treatment of EFB on the saccharification. In this study, heating in woven and water bath and steaming in water bath among the physical agents as pretreatment were used. In each of the pre-treatment, 500 g cleaned EFB was used.

Dry heating in the woven was performed in two ways. The shredded EFB was dried, then milled to the specified size and the other way was the drying of the milled EFB of specific particle size. In both ways of this pre-treatment, 1, 2 and 4 h of heating were used. During heating in the water bath, EFB of specific particle size was submerged in container with distilled water and the container with submerged EFB was placed in boiling water bath. Effect of boiling of EFB on saccharification was studied on different interval of boiling such as, 1, 2 and 4 h. To study the effect of the pre-treatment of steam, EFB of specific particle size was placed on a platform in a boiling water bath with different intervals (1, 2 and 4 h), then dried in 105°C woven for 2 h.

Effect of chemical pretreatment

Chemical pre-treatment was carried out by using nitric acid (HNO_3), sulphuric acid (H_2SO_4) and sodium hydroxide (NaOH). This pre-treatment was performed in two stages. At first, the concentration of the chemical agent was examined, and then the ratio of the selected agent with the substrate, EFB, was studied.

Chemical pretreatment was carried out by submerging EFB with specific particle size in aqueous solution of 0.5, 1, 3 and 5% of the earlier mentioned chemical pre-treatment agents. Pre-treatment was carried out in boiling water bath at room temperature (29 ±

2℃) with different time intervals of 1, 2 and 4 h. 25% (w/v) of EFB particle was used. After pre-treatment, the samples were washed and then dried at 105℃ for 4 h and then saccharification was studied. Washing of these samples was performed in two ways to study the effect on saccharification. One set of washing was done with distilled water until it reached the pH at neutral and was then dried. In another set of experiment, all the pretreated samples were neutralized first by using weak acid/bases and then washed three times with distilled water before drying.

After selection of the best chemical agent of pre-treatment, the best ratio of the chemical agent and the substrate was studied by varying the ratio of EFB in the aqueous solution of the selected chemical agent (12.5 to 50 %, w/v) and then the saccharification was done to study the effect.

Enzymatic saccharification

The enzymatic saccharification of the EFB was performed by using 50 mM sodium acetate buffer (pH 4.0) in 100 ml Erlenmeyer flasks with the working volume of 50 ml. The flasks were shaken at 150 rpm and 50℃. 5% (w/v) of EFB with 1 mm (downgraded) particle size and 1% (v/v) of cellulase enzyme broth containing activity of CMCase 60 U/ml was used in the saccharification medium. Samples were withdrawn after intervals of 4, 8, 12, 24, 48, 72, 96 and 120 h, centrifuged at 5000 rpm for 20 min and the supernatant was analyzed for reducing sugars.

Effect of the parameters for enzymatic saccharification of EFB

The process parameters for the enzymatic saccharification of EFB were performed in two steps. The parameters were tested using the one-factor-at-a-time (OFAT) method to determine the possible optimum levels. Particle size of substrate, substrate dose, enzyme dose, agitation, saccharification duration, Tween 80 and triton 100 are the parameters optimized in the OFAT design with the ranges of 0.5 to 3.0 mm, 1.0 to 10.0% (w/v), 1.0 to 5.0% (v/v), 50 to 250 rpm, 4 to 120 h, 0.1 to 0.7% (v/v) and 0.1 to 0.7 % (v/v), respectively for the enzymatic saccharification of the EFB.

Analytical analysis

Reducing sugar of the assayed samples was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). Residual cellulase activity of the reaction broth was determined by CMC (carboxy methyl cellulose) assay (CMCase) where CMC was used as a substrate. The method of determination of degradation of lignin, cellulose and hemicellulose in EFB during saccharification was described.

The sequential fractionation of lignocellulosics was carried out according to Datta (1981) with slight modifications. One gram of sample was suspended in 100 ml distilled water, kept at 100℃ for 2 h in a water bath and filtered on a tare crucible, and residue was dried at 90℃ till constant weight. Loss was considered as water soluble part. Dried residue was suspended in 100 ml of 0.5 M H_2SO_4 and after keeping for 2 h at 100℃ in a water bath, the contents were filtered, dried and weighed as described in the first step and loss in weight was represented as hemicellulose content. For cellulose and lignin estimations, 10 ml of 72% (v/v) H_2SO_4 was added to the earlier mentioned dried residue and kept at 30℃ for 1 h on a rotary shaker at 200 rpm. After incubation, the mixture was diluted up to 4% (v/v) of H_2SO_4 and autoclaved at 1.06 kg/cm^2 for 40 min. The contents were filtered, dried and weighed. The loss in weight was treated as cellulose, and the left over residue was considered as lignin.

For estimating the residual ash content, 1 g of sample was kept at 550℃ for 5 h in a tare crucible and reweighed to calculate the residual ash content.

Degradations of lignin, cellulose and hemicellulose contents of EFB after saccharification by cellulase enzyme were determined by subtracting the remaining quantity from the initial quantity and expressed in percentage. Initial fractions of lignin, cellulose and hemicellulose were determined for the EFB before saccharification and the remaining contents were determined in EFB sample after saccharification.

RESULTS AND DISCUSSION

Effect of pretreatment with different physical agents

Drying, boiling and steaming were introduced to pretreat the EFB and to examine the effect on its enzymatic saccharification. Result of the effect of EFB pre-treatment by these physical agents on saccharification are shown in Table 1. It was found from the result that all the physical agents had positive effect on the saccharification to some extent. More than 30% increase in saccharification was observed in the case of drying the shredded and milled EFB (Table 1). But the second type of pre-treatment, drying the milled EFB was found to be the most successful among the physical agents, because the saccharification performance was increased by 33.4%, that is, from 76.16 to 101.6 mg/g of EFB, after 2 h of pre-treatment and 96 h of saccharification (Table 1). Usually, the physical agents are moderately effective in removing the lignin layer of the lignocellulosic materials (Mosier et al., 2005). It is likely that the saccharification yield would be moderate when the substrate is pretreated with the physical agents.

Effect of chemical pretreatment on the saccharification of EFB

Nitric acid (HNO_3), sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH) were evaluated with the concentration ranges of 0.5 to 5.0% as a pre-treatment agent to examine the saccharification performance of EFB (Table 2). 3.0% NaOH was used to pretreat the EFB for two hours which gave rise to the best saccharification result (Table 2). It was observed from the result that the saccharification was sharply increased by 2.35 fold (175.03 mg/g of EFB) after 96 h of saccharification. But the degree of saccharification decreased as compared to the control when HNO_3 and H_2SO_4 were used as the pretreatment chemical agent.

Pretreatment with NaOH was studied in detail to get a comprehensive pre-treatment protocol in order to achieve a complete functional saccharification method together with pre-treatment strategy. In the earlier mentioned pre-treatment, hot water bath was used. So, the pre-treatment performance at room temperature (28 ± 2℃) at the maximum achieving combination (3% NaOH for 2 h) was studied (Figure 1) and 24.46% of reduction was found in terms of reducing sugar released during saccharification.

Table 1. Evaluation of saccharification under different conditions of different physical agents.

Pretreatment Type	Pretreatment Period (hr)	Saccharification Hour						
		4	**8**	**12**	**24**	**48**	**72**	**96**
Control	**0**	29.34	36.90	44.40	55.46	64.77	76.16	74.45
Dried the shredded EFB	**1**	22.28	27.85	41.78	43.89	55.56	59.80	60.30
	2	30.32	37.90	56.85	51.39	64.35	70.84	71.64
	4	28.24	35.30	52.95	48.62	58.91	61.73	68.30
	8	33.40	41.75	62.63	54.32	65.22	69.32	73.73
	12	32.18	40.23	60.34	53.86	62.94	72.89	71.53
	24	32.36	40.45	60.68	53.79	66.47	77.91	76.16
	48	45.40	56.75	85.13	78.25	89.72	99.06	**100.09**
Dried the Milled EFB	**1**	24.12	30.15	45.23	44.60	49.25	62.24	72.44
	2	40.32	50.40	75.60	78.80	88.70	97.30	**101.60**
	4	36.35	45.44	68.16	70.40	80.70	85.50	90.80
	8	32.60	40.75	61.13	60.40	70.80	73.40	87.80
	12	30.40	38.00	57.00	57.90	69.20	74.60	80.90
	24	30.80	38.50	57.75	55.00	63.90	72.60	78.80
	48	25.36	31.70	47.55	45.58	55.80	61.78	65.90
Boilling the EFB	**1**	36.16	45.20	67.80	74.07	79.20	90.18	91.47
	2	34.25	42.81	64.22	70.13	85.31	91.58	91.28
	4	37.30	46.63	69.94	70.92	80.64	87.52	87.06
	8	34.70	43.38	65.06	66.80	71.40	78.08	84.08
	12	33.20	41.50	62.25	64.12	68.60	72.47	77.60
	24	48.56	60.70	91.05	61.46	60.09	61.31	62.94
	48	44.24	55.30	82.95	60.07	56.80	50.80	55.60
Steaming	**1**	36.80	46.00	69.00	66.80	74.67	80.60	85.00
	2	41.06	51.33	76.99	78.40	81.08	83.40	86.00
	4	45.22	56.53	84.79	73.84	86.64	90.94	93.75
	8	41.50	51.88	77.81	80.60	82.40	85.09	88.08
	12	42.30	52.88	79.31	81.60	81.40	84.09	88.90
	24	35.00	43.75	65.63	65.80	68.90	72.60	76.60
	48	36.60	45.75	68.63	70.90	71.66	74.05	78.50

Process condition: 5% (w/v) of EFB of 1 mm (downgraded) particle size and 1% (v/v) of enzyme

Furthermore, NaOH and EFB ratio and the washing method were studied during pre-treatment with NaOH. The result (Figure 2) suggests that the maximum saccharification production (165.38 mg of reducing sugar/g of EFB) was achieved after 96 h of incubation when the concentration of EFB in NaOH during pretreatment was 25% (w/v). Three different types of washing method were examined after the pre-treatment was finished (data not shown) and the result suggested that the pretreated content was neutralized first followed by washing with water and drying for 4 h at 105°C before saccharification.

Zhang and Cai (2008) also reported better performance of enzymatic hydrolysis on alkali pretreated rice straw. In the lignocellulosic bio-material, alkali pretreatment may give improved results because alkali can remove the lignin layer more efficiently than acid. On the other hand, acid pretreatment mainly hydrolyze the hemicellulose layer than lignin.

Effect of some parameters on the enzymatic saccharification of EFB

In this study, saccharification of the pretreated EFB was examined in an OFAT design to evaluate possible optimum levels of the parameters. The parameters were saccharification duration, EFB size, EFB dose, enzyme dose, Tween 80, triton 100, agitation and incubation temperature. Every single parameter was varied in a range to evaluate the performance of saccharification. The results of the investigation of saccharification time (data not shown) suggested that even after 120 h of saccharification, the trend increased but not too much. However, until 96 h of incubation, the rate of saccharifi-

Table 2. Evaluation of saccharification under different conditions of different chemical agents.

Pretreatment Agent	Agent Conc. (%, v/v)	Heating Hour	Saccharification Hour						
			4	**8**	**12**	**24**	**48**	**72**	**96**
Control	**0**	**0**	29.34	36.90	44.40	55.46	64.77	76.16	74.45
HNO3	0.5	1	11.50	14.38	21.56	37.45	50.67	52.60	54.93
	0.5	2	13.60	17.00	25.50	40.04	49.95	55.40	59.94
	0.5	4	18.80	23.50	35.25	37.79	46.72	54.55	58.72
	1.0	1	8.70	10.88	16.31	31.68	42.50	50.40	54.47
	1.0	2	12.50	15.63	23.44	35.59	47.69	56.75	60.40
	1.0	4	1.40	1.75	2.63	34.64	46.42	55.24	52.68
	3.0	1	12.50	15.63	23.44	31.79	42.60	50.70	64.30
	3.0	2	10.80	13.50	20.25	26.25	35.17	41.85	41.90
	3.0	4	18.60	23.25	34.88	33.81	45.30	53.91	59.80
	5.0	1	8.60	10.75	16.13	22.94	30.74	36.58	52.50
	5.0	2	6.70	8.38	12.56	23.09	30.95	36.83	45.60
	5.0	4	12.40	15.50	23.25	21.58	28.91	34.40	60.80
H2SO4	0.5	1	12.50	15.63	23.44	34.76	46.57	55.42	60.20
	0.5	2	12.90	16.13	24.19	36.09	48.35	57.54	59.80
	0.5	4	19.90	24.88	37.31	35.17	47.13	56.09	70.40
	1.0	1	12.30	15.38	23.06	38.55	48.54	0.00	56.80
	1.0	2	13.50	16.88	25.31	39.35	46.65	53.79	56.71
	1.0	4	18.08	22.60	33.90	37.38	49.04	52.61	55.80
	3.0	1	8.06	10.08	15.11	31.95	42.81	50.94	51.43
	3.0	2	8.60	10.75	16.13	29.55	39.60	47.12	55.70
	3.0	4	10.10	12.63	18.94	24.77	33.19	39.49	37.98
	5.0	1	8.02	10.03	15.04	29.36	39.35	46.82	47.22
	5.0	2	6.40	8.00	12.00	21.61	28.96	34.46	22.64
	5.0	4	6.30	7.88	11.81	19.37	25.96	30.89	27.35
NaOH	0.5	1	17.80	22.25	33.38	42.92	57.52	68.44	63.40
	0.5	2	20.20	25.25	37.88	38.06	51.00	60.69	51.77
	0.5	4	20.60	25.75	38.63	39.50	52.94	62.99	56.98
	1.0	1	28.08	35.10	52.65	57.02	76.40	70.40	65.80
	1.0	2	32.40	40.50	60.75	58.31	78.13	72.50	68.40
	1.0	4	35.50	44.38	66.56	56.45	75.64	74.80	72.10
	3.0	1	75.60	94.50	108.60	109.81	123.73	131.08	144.66
	3.0	2	70.10	85.60	115.30	136.75	142.67	155.69	**175.03**
	3.0	4	80.20	100.25	111.80	110.69	132.21	131.24	161.15
	5.0	1	55.08	68.85	103.28	104.76	140.38	145.50	155.90
	5.0	2	70.40	88.00	103.20	104.61	140.18	147.80	150.20
	5.0	4	78.80	98.50	101.50	101.46	135.95	161.78	160.80

Process condition: 5% (w/v) of EFB of 1 mm (downgraded) particle size and 1% (v/v) of enzyme

cation was high. The total reducing sugar content after 96 h of incubation increased (12.42%) from 72 h of incubation, whereas the total reducing sugar content after 120 h of incubation increased (5.93%) only from the saccharification after 96 h of incubation. Badal and Michael (2008) studied 72 h enzymatic saccharification of rice hulls, while Huan et al. (2009) did the enzymatic saccharification of rice straw for 100 h. The result of EFB size study (Table 3) showed that the rate of saccharification is reciprocal to the size of EFB and the highest release of reducing sugar was observed in the saccharification of 0.5 mm EFB after 120 h of incubation. The rate of change of saccharification was high, from 3.0 mm (89.28 mg/g of EFB) to 1.0 mm (176.42 mg/g of EFB) of EFB, but the rate of saccharification of 0.5 mm of EFB was not too higher than that of 1.0 mm and, the reducing sugar yield was 197.84, the increment was only 12.14%. Huan et al. (2009) used 0.45 to .85 mm of rice straw in

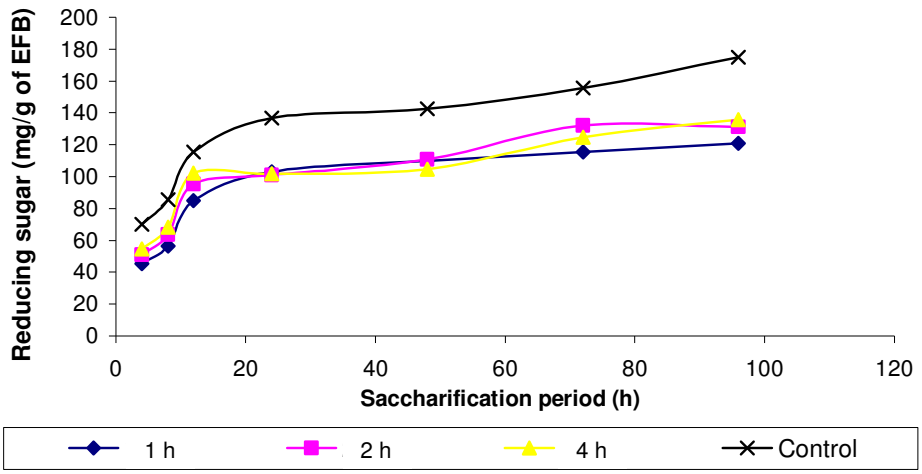

Figure 1. Effect of pretreatment at room temperature on the saccharification.

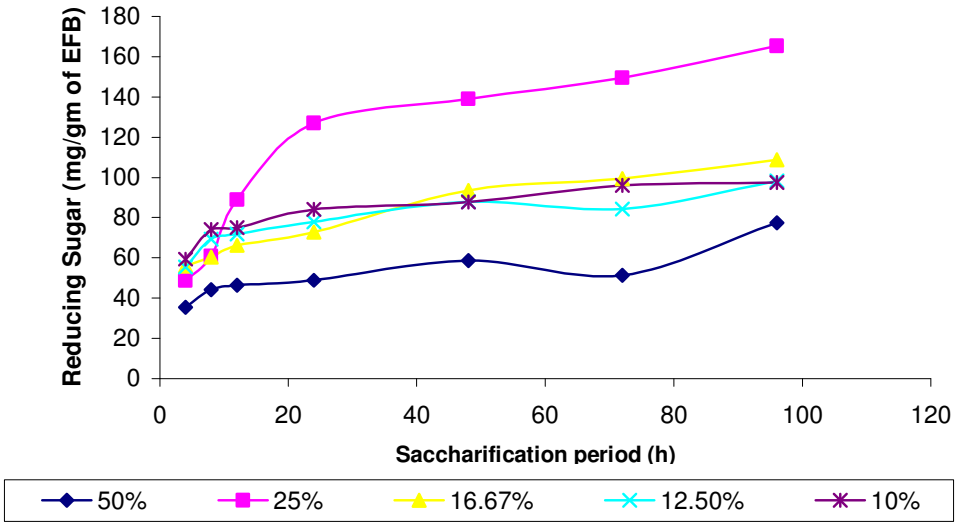

Figure 2. Effect of pretreatment on saccharification at different ratio of NaOH and EFB.

their optimization study.

The results of EFB dose were discussed from two aspects. When the saccharification was explained considering the liberation of reducing sugar, it was found from Figure 3a that the rate of saccharification was directly proportional to the dose of EFB. The maximum and minimum reducing sugar content was 1229.55 mg/100 ml and 540.20 mg/100 ml of saccharification medium when the EFB dose was 10 and 2.5% (w/v), respectively. However, in terms of yield, the results (Figure 3b) were found to be reversed and the maximum and minimum yield was 216.08 mg/g of EFB and 122.96 mg/g of EFB when the EFB dose was 2.5 and 10 % (w/v), respectively. In the case of rice straw, Qiuzhuo and Weimin (2008) and Huan et al. (2009) in different studies used 1% (w/v) substrate, while Aswathy et al. (2010) used 5% (w/v) of water hyacinth during the evaluation study of enzymatic saccharification.

Cellulase enzyme having activity of 50 CMC U/ml was used in varying ranges of 1 to 7% where 5% (w/v) EFB was used to evaluate the rate of saccharification of EFB. The result (Table 3) shows that the rate of saccharification is directly proportional to the enzyme dose. Reducing sugar of 409.13 and 176.42 mg/g of EFB was assayed where 7 and 1% of cellulase enzyme were used, respectively. Enzyme loading depends on the activity of the enzyme solution. Jung et al. (2008) optimized the enzymatic saccharification of food waste with 0.16% (v/v) enzyme concentration having 400 AGU/g of *Aspergillus* glucoamylase.

Surfactants were used in the saccharification process as a carrier of enzyme during the enzyme-substrate inter-

Table 3. Effect of different factors on the saccharification.

Studied Parameters		Saccharification Period				
		24	48	72	96	120
Particle Size (mm)	0.5	126.12	129.65	156.37	178.88	197.84
	1.0	117.71	117.42	142.16	170.02	176.42
	2.0	110.32	101.22	129.36	152.39	150.40
	3.0	77.56	66.81	97.24	89.28	97.52
Enzyme Dose (%, v/v)	1.0	117.71	117.42	142.16	170.02	176.42
	3.0	166.72	203.34	225.18	235.07	249.80
	5.0	262.14	304.22	317.58	339.76	395.77
	7.0	286.36	326.96	387.24	401.74	409.13
Tween 80 (%, v/v)	0.1	102.22	117.98	125.10	130.40	153.43
	0.3	100.93	121.21	138.50	160.08	161.80
	0.5	104.74	123.40	138.32	160.79	162.86
	0.7	104.61	122.30	131.80	144.80	154.85
Triton 100 (%, v/v)	0.1	94.79	110.62	120.50	131.70	149.62
	0.3	97.70	115.33	130.06	144.60	151.80
	0.5	99.51	114.43	132.51	163.31	141.68
	0.7	97.77	115.20	140.80	150.80	159.69
Agitation (rpm)	50	94.62	107.36	136.47	147.39	153.03
	150	100.08	119.55	144.84	154.85	185.42
	250	99.90	121.73	147.75	157.03	181.05
Temperature (ºC)	35	136.81	141.31	169.07	177.13	180.01
	50	117.71	117.42	142.16	170.02	176.42
	65	89.00	110.35	111.19	105.60	98.08

action of the reaction process. In this study, Tween 80 and triton 100 was used but no improvement was observed in terms of saccharification when compared with the control (176.42 mg of reducing sugar/g of EFB after 120 h of saccharification) (Table 3).

The maximum reducing sugar liberated was 162.86 mg/g of EFB after 120 h of saccharification, where 0.5% (v/v) of Tween 80 was used. Surfactants are known to enhance the enzymatic saccharification of cellulosics (Eriksson et al., 2002). Saha and Cotta (2007) found an increase in the saccharification of wheat straw by Tween 20, Aswathy et al. (2010) optimized the concentration of Tween 80 to 0.15% (v/v), to achieve the maximum saccharification. In the OFAT study of agitation, it was found that moderate agitation could be operated to keep the saccharification rate upward. The result (Table 3) shows that maximum of 185.42 mg of reducing sugar was liberated/g of EFB after 120 days of saccharification when 150 rpm was operated, whereas lower (50 rpm) and higher (250 rpm) agitation resulted with the reducing sugar of 153.03 and 181.05 mg per g of EFB, respectively. These are lower than the saccharification obtained for 150 rpm. It was found from the previous studies that during the enzymatic saccharification, the agitation was moderate. Many of the researchers reported using about 100 rpm of agitation (Badal and Michael, 2008; Huan et al. 2009; Aswathy et al. 2010) and Zhang and Cai (2008) found 180 rpm of agitation to be optimized for maximum saccharification of rice straw.

Lastly temperature was studied under the OFAT method to evaluate the performance of the cellulolytic saccharification of EFB. It was observed from the result (Table 3) that saccharification was highest, 180.01 mg/gm of EFB when the saccharification was employed at 35℃. When the saccharification was employed at 50℃, the result (176.42 mg/g of EFB) was similar with that of 35℃, but it was very low (98.08 mg/g of EFB), when the temperature increased to 65℃. It was found from the literature that hydrolysis was optimized within the temperature range of 35 to 50℃ (Badal and Michael, 2008; Bommarius et al., 2008).

After analyzing the OFAT results thoroughly and based

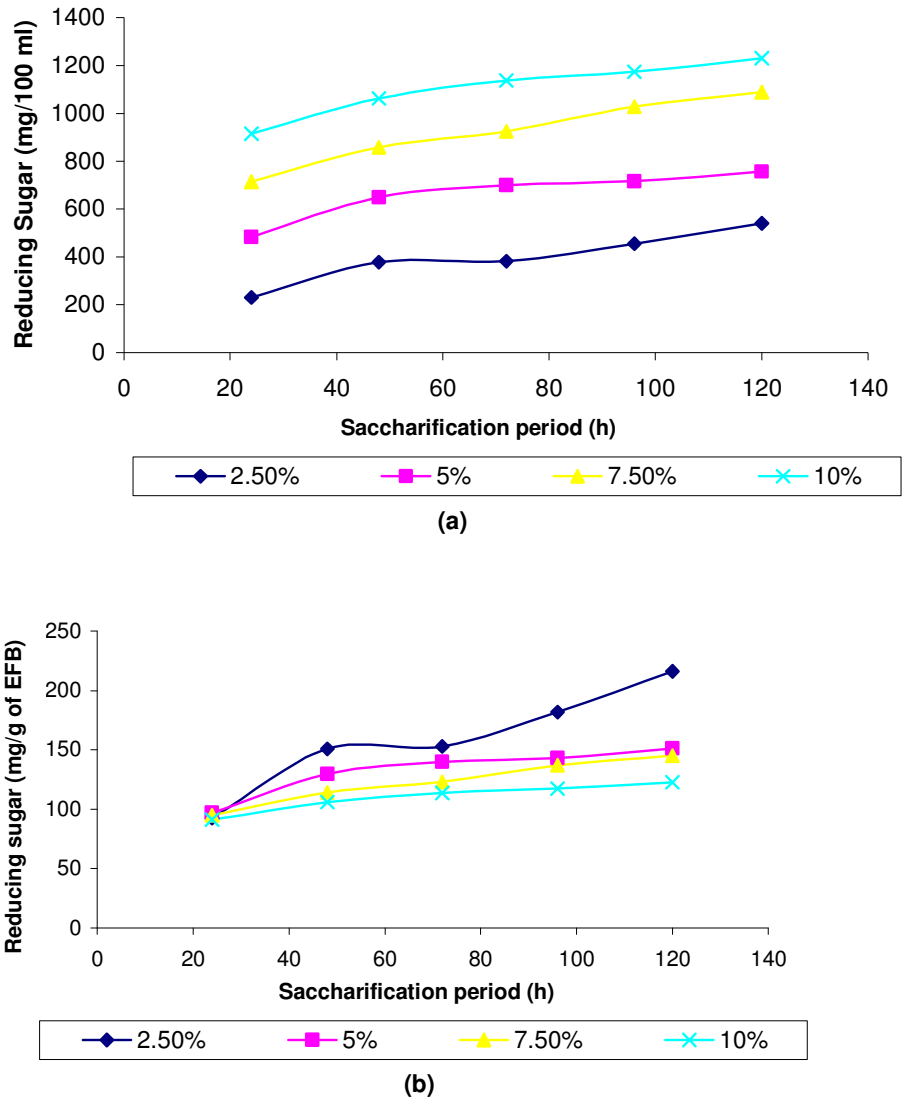

Figure 3. Effect of EFB dose on saccharification in term of, a) sugar quantity; b) sugar yield.

on the literature review, the saccharification duration, EFB dose with the size of 1.0 mm and enzyme dose was further studied using statistical method of FCCCD under response surface methodology (RSM). Based on the OFAT study, 50℃ of temperature and 150 rpm of agitation were fixed as operational condition for the saccharification process of EFB during RSM study. The surfactants Tween 80 and triton 100 was discarded from the design of saccharification process since there was no improvement.

Enzymatic saccharification of different lignocellulosic biomasses, such as food waste (Jung et al., 2008), rice straw (Huan et al., 2009), rice hulls (Badal and Michael, 2008), sunflower stalks (Sharma et al., 2002), waste paper (Wyk, 1999), water hyacinth (Aswathy et al., 2010), etc, were carried out. Among them, 32% yield was achieved from rice hulls (Badal et al., 2008); 57.8% saccharification was gotten from sunflower stalks (Sharma et al., 2002) and 30.3% yield was recorded by Huan et al. (2009) during saccharification of rice straw.

Kinetic study of the saccharification process

The process parameters of the enzymatic hydrolysis of the EFB were optimized through the OFAT. 1.0 mm of EFB pretreated with 3% NaOH at 100℃ water bath for 2 h was found to hydrolyzed 409.13 mg/g of EFB having 5 and 7% of EFB and enzyme broth, respectively. This optimized process conditions were examined through the kinetic study. Yield of reducing sugar was monitored from zero to 168 h of saccharification where degradation of cellulose, hemicellulose and lignin were correlated with the yield of reducing sugar together with the residual enzyme activity.

Characterization of the treated and raw EFB is shown

Table 4. Comparison of EFB composition before and after pretreatment.

	Hemicellulose (%)	Cellulose (%)	Lignin (%)	Ashes (%)
Untreated	30.41	46.02	19.96	3.61
Pretreated	22.37	57.26	15.57	4.80

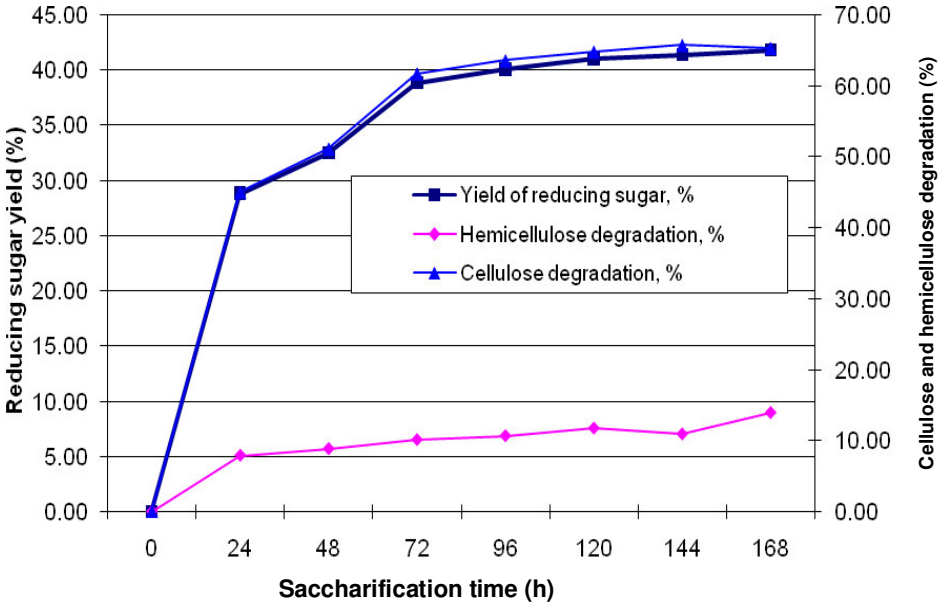

Figure 4. Effect of cellulose and hemicellulose degradation on saccharification.

in Table 4. The result shows that the hemicellulose and lignin content were reduced by 26.43 and 21.99%, respectively whereas cellulose content was increased by 24.42%. In the pre-treatment process, 26 mg/g of EFB of total carbohydrate was hydrolyzed and released out through washing after pre-treatment was done. In the saccharification of cellulosic biomass, cellulose and hemicellulose are of principle subject of interest and in the current study, it was found that the cellulose and hemicellulose were 57.26 and 22.37%, respectively. It was also found that after pretreatment of the EFB, the total weight was reduced by 28.5%. Zhang and Cai (2010) and Umikalsom et al. (1997) reported similar trend of increment and reduction of the rice straw and EFB, respectively during pre-treatment.

During the kinetic study, it was found that the rate of degradation of cellulose was higher than that of hemicellulose. The result in the Figure 4 showed that 7.92% of hemicellulose was degraded after 24 h of saccharification, whereas 13.94% degradation was noticed after a long period of saccharification (168). The pattern of degradation is quite similar, though the degradation is higher than that of hemicellulose. After 24 h of saccharification, 45.03% of the cellulose content was degraded, but after 144 h of saccharification, maximum of 65.77% degradation was achieved. Figure 4 shows that the rate of hydrolysis was very high within 24 h but after that it progressed slowly until 120 h and later on, it became almost stationary. Trend of saccharification, in terms of yield of reducing sugar, also followed the trend of the degradation of cellulose and hemicellulose. 28.84% of yield of reducing sugar was obtained just after 24 h of saccharification, whereas the maximum yield, 41.82%, was achieved after 168 h of saccharification. Several researchers (Ortega et al., 2001; Zhang and Cai, 2010; Umikalsom, 1997) reported similar saccharification trend during their investigation. The residual enzyme activity (CMCase) was also analyzed during the study and no activity prevailed even after 24 h of saccharification until the end of the investigation. The enzymes got adsorbed very quickly after being loaded into the cellulosic materials. Singh et al. (1991) reported that within 10 min, the loaded enzymes were adsorbed onto the cellulosic materials during saccharification.

Conclusions

This study shows that among the physical and chemical agents, 3% NaOH was more effective on the saccharifi-

cation of EFB, and 129.82% (175.03 mg/gm of EFB) increase (2.35 folds) was achieved on the yield of reducing sugar after 96 h of incubation. During optimization of the process conditions, eight important parameters namely, saccharification duration, EFB size, EFB dose, enzyme dose, Tween 80, triton 100, agitation and incubation temperature, were examined in an OFAT design to evaluate possible optimum levels of the parameters. From the investigation, the maximum yield 409.13 mg/g of EFB of reducing sugar was achieved with 5% (w/v) of EFB and 7% (v/v) of enzyme after 120 h of saccharification. It is evident from the kinetic study that the maximum yield (41.82 %) was achieved due to the reduction of lignin and hemicellulose layer of the EFB during the pretreatment and the increase of enzyme dose.

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