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Bio-ethanol production from *Jatropha curcus*

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Abstract

Separate hydrolysis and fermentation (SHF) were employed to produce bio-ethanol from the jatropha stem and husk. This study investigates the favorable condition required to improve yield of monomeric sugars. Substrate was pretreated physically at first through cutter mill and subsequently by ball milling. Acremonium cellulase and optimash BG hydrolyzed the pretreated sample into fermentable sugars. In condition of 10% substrate concentration, ball milling for 60 min and 4 FPU/g enzyme loading and optimum sugar yield were observed. By comparison, jatropha stem is more favorable feedstock compared to jatropha husk in terms of both inherent sugar composition and sugar yield in enzymatic saccharification (hydrolysis). Yeast *Saccharomyces cerevisiae*, capable of converting hexose sugars into ethanol,was utilized in fermentation step. It was possible to extract 0.14 L and 0.20 L of ethanol per kg of dry substrate-based jatropha husk and jatropha stem, respectively.

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Introduction

Depletion of world's energy resources based on non-renewable fuels has forced to switch our focus on alternative sources of energy. Biomass energy can be a good candidate in compensating this energy crisis. It is estimated that biomass would complement solar, wind and other form of intermittent energy sources in future renewable energy sector. About 200?10^9 tons of plant biomass, of which 90% is lignocellulose, is being produced worldwide per year (Lin and Tanaka, 2006). This vast amount of biomass can be utilized as renewable energy sources.

Bio-ethanol is the most important product derived from biomass in terms of volume and market values (Taherzadeh and Karimi, 2007). Although use of food crop in bio-ethanol production contributes in global food shortages and price hike, this predicament can be eliminated largely through exploitation of non-food (non-edible) crops and biomass residues. Process based on consequent enzymatic saccharification (hydrolysis) and fermentation is considered now as a promising method of converting the lignocellulosic carbohydrates into ethanol with high yields and low production cost (Sassner *et al.*, 2006). Enzymatic hydrolysis can be carried out in mild conditions (at pH 4.8 and 45-50 °C), which minimizes utility cost and offers no corrosion problem. High yield, sometimes close to 100%, and no formation of inhibitory by-products make enzymatic hydrolysis attractive compared to acid or alkaline hydrolysis. In contrast, the drawbacks in this mode of hydrolysis are long time hydrolysis process and relatively high cost of enzyme (Balat *et al.*, 2008; Taherzadeh and Karimi, 2007).

In separate hydrolysis and fermentation (SHF) process, pretreated lignocellulosic materials first undergo hydrolysis by enzymes. These are degraded to monomeric sugars. Thereafter, these sugars are fermented to ethanol in a separate unit. The prime advantage of this process is that each step (saccharification and fermentation) can be performed at its optimal operating conditions (Sanchez and Cardona, 2008).

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Fermentation process can also be carried out in a continuous mode with yeast recirculation. This is made possible since lignin residue removal may occur prior to fermentation (Galbe *et al.*, 2005). Risk of contamination (Taherzadeh and Karimi, 2007) inhibitory effect of process end products (glucose and cellobiose) (Alfani *et al.*, 2000)and overall high capital cost sometimes impede SHF to be utilized on industrial scale.

Lignin mainly interferes with the enzymatic hydrolysis of lignocellulosic biomass (Karki *et al.*, 2011). Alongside lignin, hemicellulose also impedes (makes) the access of cellulase enzymes to cellulose difficult. Different physical, physico-chemical and chemical pretreatments aid to improve the efficiency of hydrolysis through removing lignin and hemicellulose, reducing cellulose crystallinity, and increasing available surface area and pore volume of the substrate (Alvira *et al.*, 2010; Sun *et al.*, 2002). Pretreatment is considered as an expensive processing step in bio-ethanol production. Introduction of cost-effective pretreatment process is of great importance in cellulose to ethanol technology (Chan *et al.*, 2007).

Physical pretreatment like ball milling at various conditions is responsible for effectively reduce in cellulose crystallinity of any lignocellulosic waste biomass and improve enzyme digestibility (Teramoto *etal.,* 2008; Mais *et al.,* 2002; Jamal *et al.,* 2011). Bioethanol production from various biomass with different physical pretreatments were practiced all over the world. In this consequence, ball milling pretreatment technique was employed to enhance saccharification and fermentation process and finally ethanol production from sugarcane bagasse (Jamal *et al.,* 2011; da Silva *et al.*, 2010), corn stover (Lin *et al.,* 2010), straw (da Silva *et al.*, 2010), Eucalyptus (Inoue *et al.,* 2008) etc.

Jatropha, mostly a tropical plant, produces fruit containing 37.5% shell and 62.5% seed on dry basis. Kernel occupies 58% portion of seed and the remaining is mainly hull/husk. Through mechanical extraction, 28-29% oil can be recovered from seed which on fruit weight basis is only 17-18%. This oil is non-edible in nature and can be converted into bio-ethanol with about 95-97% yield. While this oil is exclusively employed in bio-ethanol production, utilization of other parts of plant or fruit for this purpose was not given much attention (Singh *et al.*, 2008). Largeunused area of about 0.32 million hectare prevails in Bangladesh that can be utilized in Jatropha plantation. The agro-climatic condition prevailing in Bangladesh is also suitable for Jatropha cultivation. On average 2,500 jatropha tree can be planted in one hectare area (Nabi *et al.*, 2009). On using bio-ethanol from Jatropha plants, Bangladesh can reduce its dependency on import of petroleum oil.

This studyinvestigates the utilization of different parts of jatropha plants other than seed, as potential source of bio-ethanol production through SHF method. In this context, evaluation of effectiveness of physical pretreatment method and process optimization in enzymatic saccharification are conducted. This study also focuses on the maximum ethanol yield achievable from this raw material.

Materials and methods

Materials

Different parts of jatropha plant (*Jatropha curcas*) were used in this study. Jatropha stem (maturity of 12 years) and jatropha husk (fruit shell) were obtained from research filed of Bangladesh Council of Scientific and Industrial Research (BCSIR). The pictorial display of these materials is shown in Fig1. After collection, jatropha stem was chopped and sun dried. Sun drying was also performed in case of jatropha husk. Before pretreatment, all materialswere oven dried at 60°C for three days.

Fig. 1. Photograph of (a) jatropha stem and (b) jatropha husk

Pretreatment

Dried raw materials (jatropha wastes) were first treated by cutter mill (Fritsch, Germany) whereby their size was reduced into 2 mm (MM-2) and 0.2 mm (MM-0.2). Raw materials with 2 mm size were then subjected to ball milling (TI-300, CMT Co., Saitama, Japan) and those of 0.2 mm size were used in acid hydrolysis for determining sugar composition. In ball milling operation, raw materials were pretreated with ball for 10 min (MC-10), 30 min (MC-30), 60 min (MC-60) and 120 min (MC-120) at room temperature. The particle size of raw materials was reduced upto 20 µm during ball milling.

Analyses of chemical composition

Moisture and ash content

ASTM D 4442-07 was used to determine the moisture content of raw materials. About two grams of pretreated samples (ball milled for 60 min) were taken in a glass crucible and oven-dried at 105 ± 2°C. Weight measurement was carried out in a 3 hours interval and moisture content was expressed in percent wet basis. To determine ash content, over-dried samples were heated in a muffle furnace at 575 ± 25°C according to ASTM Standard E 1755-01. Ash content was expressed as a percentage of sample's oven-dried weight.

Sugar composition

Sugar composition of substrate (ball milled raw materials) was analyzed according to the procedure proposed by National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2008). According to this method, 0.3 ml of 72% H_2SO_4 has been added to hydrolyze the substrate in order to release monomeric sugars into the liquid fraction and 1% sugar (glucose, xylose, galactose, arabinose and mannose) solution is used as standard. Monomeric sugars in the hydrolysate were then analyzed by HPLC (column temp: 80 °C, column: Biorad Aminex HPX-87P; 300 ? 7.8 mm, stationary phase: lead ionic form supported on sulfonated divinyl benzene-styrene copolymer, mobile phase: degassed deionized water, flow rate: 1.0 ml/min, pressure: 10342 kPa, refractive index detector, Jasco, Japan).

Yeast inoculum preparation

Yeast strain *Saccharomyces cerevisiae*, also known as IR_2 was used to ferment sugars derived from enzymatic saccharification (Kuriyama *et al.*, 1985). Only hexose sugars can be converted into ethanol by this fermenting organism strain (Palmqvist *et al.*, 1996). Before inoculation, the yeast strain was isolated in a medium composed of glucose, peptone, yeast extract and agar (Difco). Yeast cell preculture, also known as YPD preculture was carried out in a 250 ml Erlenmeyer flask containing 60 ml culture medium containing 0.1% (w/v) yeast extract and 0.2% (w/v) peptone supplemented with 2% (w/v) glucose. The flask was kept at 30°C overnight in a non-gassed (microbiological) incubator and stirring was continued at that period. After cultivation, the yeast cells were separated from liquid medium by centrifuge at 10,000 rpm for 5 minutes. To utilize it in fermentation, yeast cells were washed with 0.9% sodium chloride and then dispersed into 0.9%sodium chloride solution by gentle shaking. Through this culture, the initial cell concentration i.e OD_{600} was found 5-10 cells/ml.

Separate hydrolysis and fermentation (SHF)

Enzymatic saccharification

Enzymatic saccharification (hydrolysis) was carried out to recover fermentable monomeric sugars (mainly glucose and xylose) from pretreated raw materials. The standard enzymatic saccharification reaction mixture consists of 80 FPU/ml enzyme acremonium cellulase and 10% optimash BG. Initially sample of zero hour of hydrolysis was taken for analysis. Hydrolysis was then performed in 50 mM acetate buffer (pH 5.0) at 50°C for 72 hours with continuous stirring. A portion of the hydrolysis product (usually 0.5 ml) of 3, 6, 24, 48 and 72 hours were removed periodically to determine sugar yield.

In this study, optimal substrate concentration was initially selected where MC-60 sample was saccharified with 4 FPU/g of dry substrate enzyme load and 5-15% substrate concentration. The effects of pretreatment type and time on enzymatic saccharification were then studied in which samples from different pretreatment conditions were hydrolyzed with the optimum substrate concentration and enzyme load of 4 FPU/g. Finally, the effects of different enzymatic loadings on saccharification process were considered by using favorable condition of pretreatment and substrate concentration. For this, a number of different enzyme loads per gram of substrate, such as 4 FPU/g, 8 FPU/g, 12 FPU/g and 20 FPU/g were used.

Adjustment of hydrolysate sample for fermentation

The pH of hydrolysate prepared through enzymatic saccharification for 72 hours was then adjusted before inoculation with yeast cells. For this purpose, NaOH was used to maintain pH at 6.0. The volume of hydrolysate was also adjusted by adding sterile water.

Fermentation

The pH adjusted hydrolysate was inoculated with 2 ml of YPD preculture of *Saccharomyces cerevisiae* (IR_2) in Fermentation vial (Aspect ratio: 8-10; Hydrolysis volume: 30

ml). After inoculation, 1 ml fermentation mixture of zero hour of fermentation was removed for analysis. Fermentation was then carried out at 30°C for 72 hours under incubation and constant stirring at 35 rpm. 1 ml solution each from 3, 6, 24, 48 and 72 hours of fermentation mixtures were taken out to determine ethanol production.Since increase of both substrate concentration and enzyme loading contributes in sugar yield augmentation, to obtain the highest amount of ethanol from jatropha waste, fermentation was performed with hydrolysate acquired through enzymatically hydrolyze MC-60 sample in condition of 15% substrate concentration and 20 FPU/gm enzyme loading. The process flow diagram for ethanol production from jatropha waste is shown in Fig. 2.

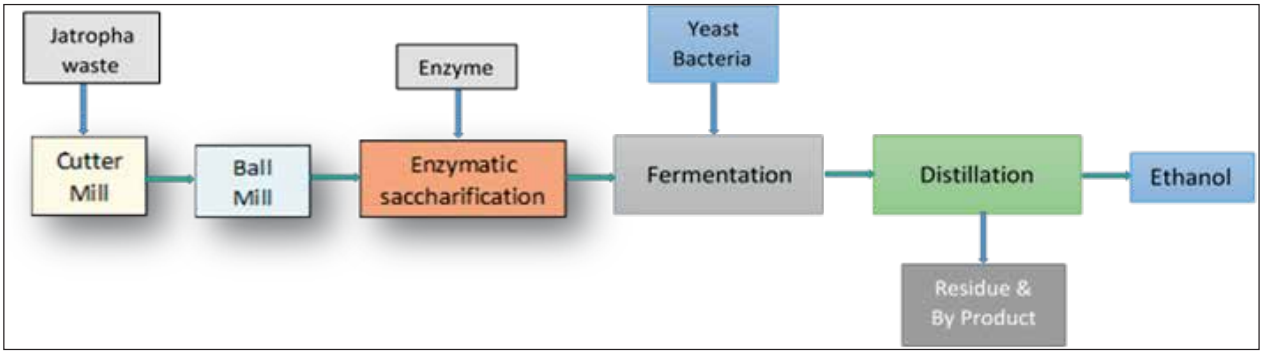

Fig. 2. Process-flow diagram for ethanol production from jatropha waste

Analysis of hydrolysate and fermentation products

Analysis of sugar mixture from enzymatic saccharification and ethanol production through fermentation was performed by HPLC using Aminex HPX-87P column and refractive index detector. Other specifications are identical with that used for substrate composition analysis obtained through acid hydrolysis.

Sugar yield in the liquid fraction of hydrolyzed substrate was determined by comparing its peak area detected by HPLC with that of 1% standard sugar solution. Theoretical yield of sugar was calculated on the basis of sugar yield obtained from acid hydrolysis.

Theoretical yield (%) = [Experimental yield / Yield by acid hydrolysis] x 100

Ethanol yield was calculated both as percentage basis by comparing to its theoretical ethanol conversion, and as liter of ethanol per kg of dry substrate basis.

Ethanol yield (%) = $[(E\text{x}0.79)/(S\text{x}0.514)]$ x 100

Ethanol yield (liter/kg of substrate) = $[(E\text{x}V_f)/(V_s\text{x}W)]$

Where, E is the ethanol concentration in fermentation mixture, S is initial glucose concentration, W is dry substrate weight, and V_f and V_s denote the initial volume of fermentation and final volume of saccharification respectively. Besides, 0.79 is the density of ethanol in g/ml and 0.514 is theoretical ethanol conversion from glucose.

Results and discussion

Chemical composition of substrate

In chemical composition analysis, sugars content, moisture and ash content of substrate were studied. The composition of jatropha stem and husk is shown in Table I. Glucose content was the higherin jatropha stem (40.5%) which is followed by jatropha husk (29.5%). In case of xylose content, jatropha stem also contains higher percentage of xylose (16.18%), followed by jatropha husk (9.34%). Other sugars namely galactose, arabinose and mannose are present in low amount (less than 3%). Moisture and ash content are higherin case of jatropha stem (3.65%) and jatropha husk (17.3%) respectively.

Enzymatic saccharification

Optimization of substrate concentration

To obtain the optimal substrate concentration, jatropha waste pretreated for 60 min was enzymatically hydrolyzed with 5, 10 and 15% substrate concentration using the same enzyme loading i.e., 4 FPU/g of dry substrate and the results are presented in Table II. In case of both jatropha husk and stem, glucose and xylose composition decreased with subsequent increase in substrate concentration but overall yield of fermentable sugars increased. With regard to jatropha waste, xylose composition reduced rather slowly compared to glucose content. Although

Table I. Composition of jatropha waste expressed as % of dry matter

Substrate	Glucose (%)	Xylose (%)	Galactose (%)	Arabinose (%)	Mannose (%)	Moisture (%)	Ash (%)
Jatropha husk	29.5	9.34	2.04	0.82	2.41	3.31	17.3
Jatropha stem	40.5	16.2	2.23	0.16	1.02	3.65	5.86

enzymatic activity changes in direct proportion with enzyme concentration, it becomes limiting at higher substrate concentration due to deficiency of active sites on enzyme. This low enzymatic activity at higher substrate concentration primarily contributes to reduction in hydrolysate sugar composition. Alternatively, high substrate loading is necessary to increase sugar yield and economize cellulosic ethanol production (Ong *et al.*, 2012). Taking these factors into consideration, 10% substrate concentration is chosen as optimum condition for succeeding experiments. During hydrolysis, arising mixing difficulties and accumulation of inhibitors in reaction medium also make 10% solid concentration the most adequate one (Sanchez and Cardona, 2008).

Table II. Variation of sugar yield with substrate concentration

Substrate	Sugar	Sugar composition (%) at substrate concentration		
		5%	10%	15%
Jatropha husk	Glucose	82.37	77.10	67.18
	Xylose	53.20	50.49	43.27
Jatropha stem	Glucose	83.69	80.73	75.45
	Xylose	51.32	48.68	44.76

Effect of pretreatment process on glucose and Xylose yield

At 10% substrate concentration and 4 FPU/g enzyme load, yield of glucose and xylose from jatropha husk and jatropha stem through enzymatic hydrolysis increased with extension of pretreatment time (in Fig. 3(a) and 3(b). Such physical pretreatment enhances effective surface area to enzymes by unfolding lignocellulosic constituents and it adds no additional value to raw materials (Duff *et al.*, 1996). With reference to the data presented in Figure 3(a), rapid increase in glucose concentration was observed between ball milling time of 30 and 60 min and improvement of glucose yield becomes insignificant after 60 min.

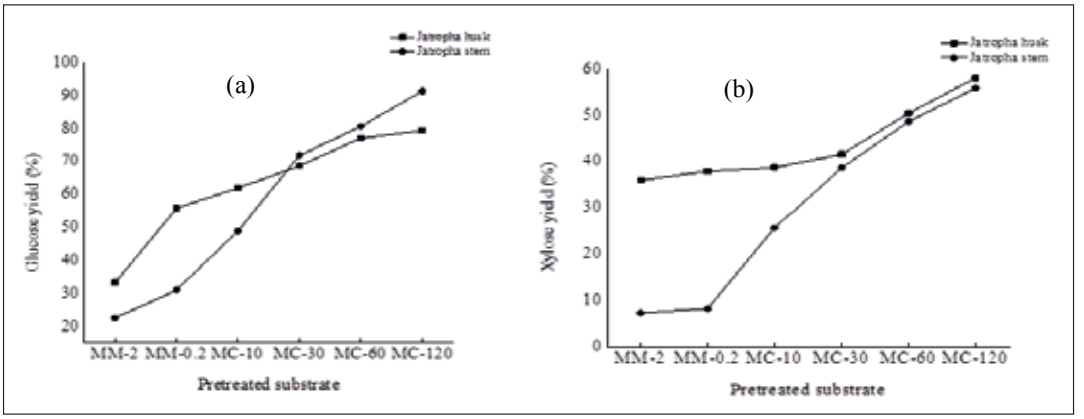

Fig. 3. Yield of (a) glucose (b) xylose from different pretreated jatropha substrate (10% substrate concentration and 4 FPU/g enzyme load)

It was also observed that enzymatic digestion was nearly complete in 6 hours for 2 mm cutter milled sample (MM-2), whereas this process almost came to a halt after 48 hours for other pretreated samples. Raw materials ball milled for 120 min (MC-120) gave highest glucose yield (79.46%) after 72 hours.

milled for 120 min gave maximum amount of xylose (55.90%). Considering the high energy requirement of ball milling pretreatment and corresponding yield of glucose and xylose, pretreatment time of 60 min was selected as optimum situation for both cases and used in subsequent experiments.

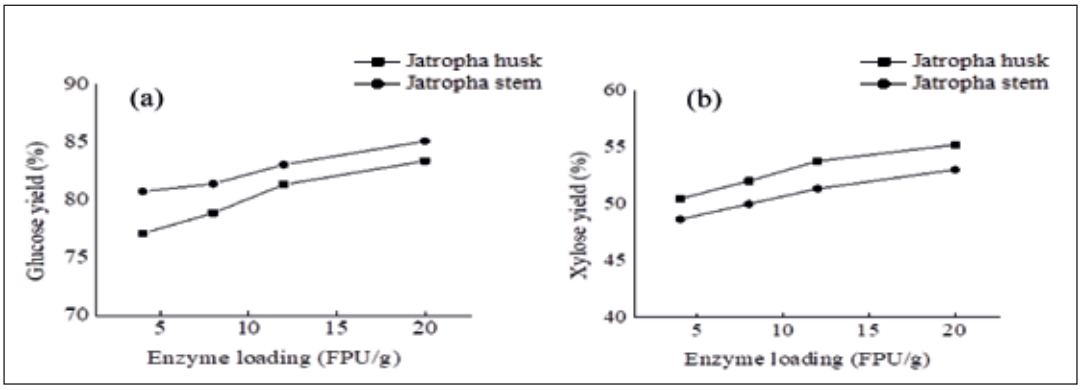

Fig. 4. Effect of enzyme loading on (a) & (b) glucose and xylose yield (MC-60 and 10% substrate concentration)

Jatropha stem pretreated by cutter mill are less susceptible to enzymatic saccharification in contrast to jatropha husk under identical conditions (Fig. 3(a)). Here abrupt increase in glucose yield was seen when pretreatment time during ball milling operation exceeded 10 min. In case of cutter milled sample (MM-2 and MM-0.2), glucose concentration decreased after 24 hours of hydrolysis which was followed by a steady increase afterwards. Sample ball milled for 10 min showed reduction in glucose content after 48 hours. End product (glucose and cellobiose) inhibition, which affects enzyme activity, may be the reason behind this anomaly (Mussatto *et al.*, 2008). In comparison with jatropha husk, jatropha stem ball milled for 30 min, 60 min and 120 min gave higher glucose yield (71.96%, 80.73% and 91.96% respectively) after 72 hours.

Xylose yield obtained from jatropha husk shows similar trend as that of glucose yield except for sample MC-120, in which considerable xylose was produced than preceding pretreated sample (MC-60) as shown in Fig. 3(b). Also, xylose concentration in the hydrolysate increased gradually after 48 hours of enzymatic digestion. Highest xylose content was found in case of ball milled sample of 120 min (58.10%).

Cutter mill pretreatment of jatropha stem gave low xylose yield (around 8.5%). Xylose concentration increased with ball milled substrate anddifferent ball mill pretreated samples showed large difference in xylose concentration. (Fig. 3 (b). In case of jatropha stem, increase in pretreatment time longer than 10 min did not give better xylose yield than jatropha husk as was observed regarding glucose yield. Substrate ball

Influence of enzyme load on sugar yield of jatropha waste

Effect of enzyme load on glucose and xylose yield was studied with substrate pretreated for 60 min (MC-60) at 10% substrate concentration. The results are presented in Figs. 4(a) & (b), respectively. Usually high enzyme loading contributes in enhancing sugar yield (Singh *et al.,* 2008). But in this study, no significant increase in glucose and xylose content was observed on varying enzyme load from 4 FPU/g to 20 FPU/g. For both the sugars, increase of yield was about 5%. At saturation level of substrate concentration, no additional enzyme activity is observed regardless of augmentation in enzyme load (Yang *et al.*, 2006). Enzyme inactivation due to accumulation of cellobiose may also contribute to small increase in sugar yield (Chen *et al.*, 2008). Since enzyme cost has considerable impact on economics of lignocellulosic bio-ethanol production (Klein-Marcuschamer *et al.*, 2012) enzyme load of 4 FPU/g is ideal for conducting enzymatic hydrolysis in case of jatropha substrate.

Fermentation

At hydrolysis condition of 15% substrate concentration and 20 FPU/g enzyme loading, pretreated jatropha husk sample MC-60 gave glucose concentration of 3.8% in hydrolysate after 72 hours. These glucose consumption and ethanol production during the course of fermentation for 72 hours are depicted in Fig. 5(a). Conversion of glucose to ethanol was almost complete within 24 hours. After 72 hours of fermentation, there still remains some glucose (about 0.2%) in the fermentation mixture. Oxygen free condition of

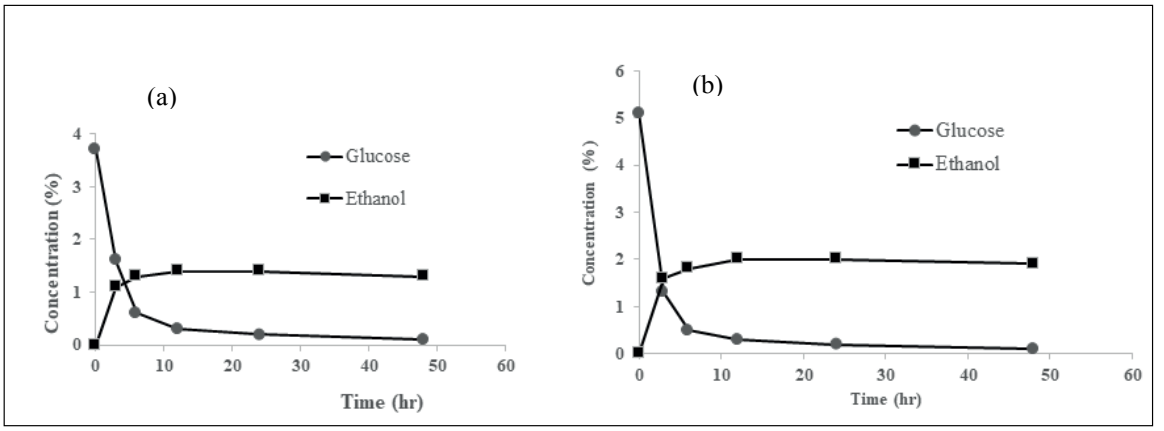

Fig. 5. Conversion of (a) jatropha husk and (b) jatropha stem hydrolysate into ethanol

fermentation slowly annihilates the microorganism population (Lynd, 1996). Furthermore, produced ethanol and some degradation products (furfural, hydroxymethyl furfural and phenolics) arising from pretreatment of lignocellulose inhibit the action of fermenting microorganisms (Yu and Zhang, 2003). These eventually give rise to this residual glucose. Ethanol yield of 87.5% or 0.14 liter/kg of ethanol based on dry jatropha husk was achieved.

Conclusion

This study illustrates that jatropha husk and stem can be considered as a viable bio-ethanol feedstock. Sugar composition analysis showed a higher fraction of glucose and xylose in jatropha stem than husk portion. Low percentage of sugar in jatropha husk may be attributed to its high ash content (17.3%). Economic sugar yield has led to the utilization of high substrate concentration (10%) and low enzyme loading (4 FPU/g).Considerable deviation in glucose and xylose content is observed between cutter mill and ball milling pretreatment.This variation did not follow any definite pattern. Substrate pretreated more than 60 min has proved to be unnecessary both with respect to required energy and resultant monosaccharide in enzymatic hydrolysis. In fermentation step, complete conversion to ethanol and rapidity of this conversion was also detected in case of jatropha stem hydrolysate. In contrast, alongside fermenting condition, formation of inhibitors due to lack of reaction rapidness inhibits complete conversion of jatropha husk hydrolysate.

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