Simultaneous Saccharification and Fermentation of *Jatropha curcas* (Linn.) Seed Cake for Production of Bioethanol

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**Abstract:** The simultaneous saccharification and fermentation (SSF) of pretreated *Jatropha curcas* seed cake for the production of bioethanol was reported. *Trichoderma reesei* and *Zymomonas mobilis* were selected for further fermentation processes. Various parameters like pH, enzyme loading, temperature, substrate concentration and working volume were selected and optimized using D-optimal statistical approach. Enzyme saccharification rate was evaluated prior to SSF studies. The enzyme works for 96.65% conversion of the polymeric carbohydrate into monomeric carbohydrate at pH = 4.5 treated with NaOH at 50 °C within 36 h. The pretreated substrate was then subjected to SSF process using *T. reesei* and *Z. mobilis*. Hence, the optimized condition pH = 4, enzyme load of 0.25%, pretreated substrate concentration of 8%, incubation temperature of 31 °C and working volume of 100 mL were found to obtain 78.52% conversion to ethanol in 24 h of incubation time. *Z. mobilis* gave the maximum conversion of bioethanol within 24 h. This study shows that *Z. mobilis* has potential for commercial production of ethanol from lingo-cellulose in SSF processes in industry.

**Keywords:** *Jatropha curcas*; seed cake; bioethanol; biofuel; *Zymomonas mobilis*; *Trichoderma reesei*; Bioenergy.
1. Introduction

Lignocellulosic materials are commonly found plant cells, which have secondary thickenings. These cell walls of plant contain cellulose, hemicellulose, lignin and pectin. Such lignocelluloses containing biomass is a potential renewable source for the production of bioethanol. Bioethanol can be produced from such lignocellulosic biomass in a various process that includes pretreatment, hydrolysis, fermentation and dehydration (Mosier et al., 2005).

Simultaneous saccharification and fermentation (SSF) process has been reported several times as a method of choice for bioethanol production from lignocellulosic material (Spindler et al., 1991). In this process sugar accumulation in the system can be minimized; feedback inhibition by product sugars is reduced and higher hydrolysis rates and yields can be possible than for saccharification without fermentation. In this process, increased yield can be favored by long residence times, low substrate and product concentrations, and optimum enzyme loading. Thus, there is a tradeoff between yield, product concentration, and enzyme consumption and the optimum combination of other parameters should be determined.

To avoid such competition, recent biotechnological developments have led on utilization of lignocellulosic biomass to produce bioethanol (Pandey et al. 2000). However, lignocellulosic substrates have complex chemical structure and high pentose fraction makes tough to enzymatic hydrolysis. Hence the developments of an integrated process combining optimized pretreatment, enzymatic saccharification and fermentation have been reported (Buaban et al. 2010). This study thus aimed at to investigate the potential use of a simple integrated process for the production of bioethanol from Jatropha seed cake. The enzyme saccharification and seed cake component analysis were determined. Optimization of the condition for maximum recovery of ethanol and reducing sugar release were also evaluated. This work can be helpful for further bioenergy alternative system for production of biofuel, bioprocessing of chemicals and biotechnological studies to understand and exploit the potential of Jatropha seed cake for various applications.

2. Material and Methods

2.1. Sample Preparation and Component Analysis

The seed of Jatropha curcas was procured from Suntan, Tamil Nadu, India. The sample was dried and ground to a 10-mesh size and taken for further investigation. Ash, lignin, cellulose and hemicellulose contents of the seed cake were determined using appropriate method. The dried samples of 0.5 g were boiled in 5 mL of 75% w/w H₂SO₄ solution for 6 h in order to hydrolyse the cellulose and hemicellulose. The suspension formed was filtered through a crucible and the solid residue was
dried at 105 °C for 24 h and weighed (W1).

The residue was heated at 600 °C for 6 h and ash content (W2) was determined. Acid insoluble lignin was then calculated by the difference (W1 - W2). The filtrate from the H₂SO₄ treatment that contained the sugars released from cellulose and hemicellulose was thoroughly stirred and homogenized. Glucose was determined using the DNS method against the standard (Miller, 1959).

2.2. Substrate and Pretreatment

The dried seed cake was pretreated with 2.5% NaOH and 4% NaOCl (Gould, 1983; Ghose 1987). The 5 g of the biomass was suspended in 50 mL solution of 2.5% NaOH and 4% NaOCl in a flask at a room temperature for 4 h which maintain the ratio of solid to liquid as 1:10 (w/v). Thereafter, the solid residue was collected by filtration and washed extensively with distilled water until neutral pH. Subsequently, this pretreated biomass was dried in the oven at 60 °C to maintain a constant weight to be used as the substrate for enzymatic hydrolysis (Eveleigh et al., 2009).

2.3. Enzymatic Saccharification

This experiment was carried out in stoppered conical flasks (100 mL) in the presence of 0.01% sodium azide. The pH was adjusted to 4.8 with 0.05 M citrate buffer, and cellulase was added to the pretreated substrate (2.5%, dry weight basis) in a total volume of 50 mL reaction medium. The flasks were incubated at 50 °C on an orbital shaker agitated at 150 rpm. Aliquots of 2 mL were taken periodically, centrifuged and the supernatants were analyzed for reducing sugars. The percentage of saccharification was calculated according to the literature (Ghose, 1987). Cellulase from Trichoderma viride (Celluclast ®) was kindly provided from Novo Nordisk, Denmark.

2.4. Preparation of Microbial Inoculum

Trichoderma reesei and Zymomonas mobilis were obtained from NCIM (National Chemical Laboratories, Pune, India). To prepare the inoculum the spores on the PDA slant were suspended in 2 mL medium and then pipetted into a 250 mL Erlenmeyer flask containing 50 mL of growth medium and incubated in a shaking bed (180 rpm) at 30 °C. The initial pH value of the medium was adjusted to 7 before being autoclaved at 121 °C for 15 min. After 24 h growth the medium was used as the inoculum for enzymatic hydrolysis. The amount of inoculum used was 5% (v/v) of the medium in the SSF. Zymomonas mobilis were also been used in this study. The cultures were routinely grown as described in the literature (Lee and Huang, 1995).

2.5. Simultaneous Saccharification and Fermentation (SSF)
Simultaneous saccharification and fermentation was reaction mixtures consisted of treated substrate. The composition of the hydrolysis medium was as follows: various concentration of the pretreated biomass. The SSF mixture was autoclaved and studies were carried out in 250 mL conical flasks with in various working volume. The culture in the Erlenmeyer flask was incubated at in various temperature, pH and enzyme loading. Thereafter culture broth was centrifuged and the supernatant was analyzed for reducing sugars, ethanol and FPA value for enzyme activity periodically.

2.6. Analytical Methods

*Jatropha curcas* seed cake was analyzed for cellulose, hemicelluloses, acid insoluble lignin and ash (Ververis *et al.*, 2007). Potential glucose, sugars and lignin in the substrate were determined after total hydrolysis with H$_2$SO$_4$ using DNS method and total carbohydrate was determined using anthrone reagent. Cellulase activity was assayed as FPU (filter paper units) (Eveleigh *et al.*, 2009). Ethanol was estimated by micro-dichromate method using microplate reader (Wang *et al.*, 2003; Xiao *et al.*, 2004). Isopropanol was used as an internal standard (Cazetta *et al.*, 2007).

2.7. Experimental Design and Data Analysis

The experiment was designed using optimal method which shows various interactions of multiple independent variables with the yield using RSM. The results of each D-optimal design were analyzed using Design Expert software version 7.1.5. All experiments were conducted in triplicate. The results were analyzed statistically using SPSS vers. 16 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Seed Cake Composition, Enzyme Saccharification and Cellulase Activity

The *Jatropha curcas* seed biomass was dried over night at 50 °C in an oven, ground, sieved and then taken for the study. Each time 50 g powder was packed in Soxhlet and defatted with petroleum ether (60-80 °C) for 30-35 complete cycles. The petroleum ether extract was concentrated using Buchi-Rotavapor at reduced pressure and temperature. The seed cake was then made ready for further study. Thereafter total carbohydrate, lignin, cellulose, hemicellulose and ash contents were determined using the appropriate methods. The seed cake in this study contained cellulose (13.6 ± 0.95), hemicellulose (22.567 ± 1.3), and lignin (10.47 ± 0.89) in the percentages respectively (Fig. 1). The ash content was found to be (4.6 ± 0.88)% and total carbohydrate contain was (46.63 ± 3.07)%.

From other studies the composition of seed cake like hemicellulose, cellulose, and lignin as 5.55%, 20.3%, and 19.46%, respectively have been reported (Staubmann *et al.*, 1997). In addition, the total carbohydrate content of
seed cake from India has been found to be 49.5% (Rakshit et al., 2008). The differences in composition could be owing to different analytical procedures and different varieties of Jatropha.

![Figure 1. Percentage composition of lignocellulosic material of Jatropha seed cake](image)

The cellulase activity and enzyme saccharification rate were determined using a modified filter paper assay technique (Eveleigh et al., 2009). In last couple of decades various pretreatment methods have been developed. These methods have been proven to provide high sugar yields from lignocellulosic materials (Galbe and Zacchi, 2007). The cellulase activity was calculated against the standard and the optimum was found to be 40 FPU/g substrate. The enzyme works for 96.65% conversion of the polymeric carbohydrate into monomeric carbohydrate at pH = 4.5 treated with NaOH at 50 °C within 36 h as shown in the Fig. 2. The seed cake was also treated with NaOCl and found to be less time consuming as compared with 2.5% NaOH. NaOH was found to be efficient in change the complex nature of the carbohydrate and open the condition for easy access for the enzyme cellulase. The pretreated sample using 2.5%NaOH was made ready for further fermentation study.

3.2. Strain Selection for Simultaneous Saccharification and Fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) was carried out using two microbial strains Zymomonas mobilis and Trichoderma reesei. These strains are known to produce intraclular enzymes that can covert carbohydrate in to simplest form. In addition they are tolerant to alcohol and have high utilization of sugar. Zymomonas mobilis, a Gram-negative bacterium, has been attracting increasing attention for fuel ethanol. It is an osmo- and ethanol- tolerant bacterium and it has shown higher specific rates of glucose uptake and ethanol production (Rogers et al., 1997). During one-factor-at a time experimental approach the T. reesei was found to be bulky and less resistant to alcohol than Z. mobilis. Moreover, T. reesei has taken more time of fermentation. Hence we used Z. mobilis for
further optimization study. *Zymomonas mobilis* is active fermentation to produce ethanol in SSF process using seed cake of *Jatropha* as substrate. This means that there is no sludge component that prevents the use of biological conversion techniques as well. Among the factors selected for the cultivation of the microbes the five factors: incubation temperature, pH, enzyme load, substrate concentration and working volume were found to be superiors and considered for further optimization process.

![Graph](image-url)

**Figure 2.** Percent of enzyme saccharification against reaction time

### 3.3. Optimization and Validation Study for Production of Bioethanol

The experiment was performed with various parameters like pH, enzyme loading, temperature, substrate concentration and working volume using optimal RSM statistical approach. In the study the temperature ranges of 25-37 °C, acid, neutral and alkaline pH values, enzyme load ranges from 0.25-0.75%, substrate concentration from 4-8%, and working volume ranged from 50 mL-100 mL were used. Hence, the optimized condition using D-optimal pH = 4, enzyme load of 0.25%, substrate concentration of 8%, incubation temperature of 31 °C and working volume were found to obtain 78.52% conversion of lingocellulosic material to alcohol in 24 h of incubation time. The pH of the media was found to be raised from 4 to 5.5 as shown in Fig. 3.

Since many variables may potentially affect the production of bioethanol in the process, a small type D-optimal design, response surface methodology (RSM) was employed in this study to determine the effects of independent variables on the response and the factor interactions (Myers and Montgomery, 2002). A total of 27 runs with five variables at various levels were used to optimize the condition for the maximum yield and simple sugar conversion. Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent variable, been described by a quadratic polynomial equation and fitted to the experimental data (Fig. 4A-D).
**Figure 3.** The bioethanol production and the change of pH at the optimal condition

**Figure 4.** A) 3D-surface plot for production of ethanol, B) contour plot (C) Normal plot, (D) Perturbation plot for bioethanol production by *Zymomonas mobilis*
The fit of the models was evaluated by the determination of coefficient $R^2 = 0.935$.

$$\text{Ethanol} = 35.23 + 4.71 \times A - 3.27 \times B - 0.76 \times C + 7.38 \times D - 5.40 \times E - 8.21 \times A\times B - 5.26 \times A\times C + 6.82 \times A\times D - 0.76 \times A\times E - 2.56 \times A^2 - 0.27 \times B\times C - 0.37 \times B\times D - 9.69 \times B\times E + 0.81 \times C\times D + 6.82 \times C\times E - 5.40 \times D\times E + 14.53 \times A^2 - 18.95 \times B^2 - 4.24 \times C^2 + 9.14 \times D^2 - 1.76 \times E^2$$

Where the independent variables represented as A: pH, B) Enzyme load, C) Substrate concentration, D) Working volume, and E) Incubation temperature.

Further validation study was undertaken using the optimal condition and found to be consistent with the optimized condition (Fig. 5).

**Figure 5.** Validation study on the production of alcohol and release of reducing sugar

4. Conclusions and Recommendation

Lignocellulosic biomass derived biofuels are essential to overcome excessive dependence on petroleum. Biological conversion of such materials using microorganism into simple form also provides various valuable chemicals as well. The results of this study showed that *Z. mobilis* is an important microorganism for simultaneous saccharification and fermentation of *Jatropha* seed cake to produce bioethanol in short time frame. Hence the perfect microbe that provides broad substrate utilization, give high ethanol yields and tolerant to the harsh conditions after chemical pretreatment is recommended. Reduction in process costs, by integrating process engineering tools with metabolic engineering should be searched further taking this result as a stepping stone.

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