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Bioconversion of lignocellulosic fraction of water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate to ethanol by *Pichia stipitis*

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ABSTRACT

Fermentation of acid hydrolysate of water-hyacinth (*Eichhornia crassipes*), a free floating aquatic plant has been investigated for ethanol production. The dilute acid treatment has been applied to utilize the maximum hemicellulosic content of the water-hyacinth. The goal of this work was to investigate, both experimentally and theoretically using mathematical tools, a fermentative system utilizing water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate as a substrate for ethanol production using *Pichia stipitis*. It was found that 72.83% of xylose was converted to ethanol with a yield of 0.425 g_p/g_s and productivity of 0.176 g_p/L/h. An appropriate mathematical model was developed to explain theoretically the bioconversion of this hemicellulose acid hydrolysate to ethanol and the model was tested statistically to check the validity of the model.

1. Introduction

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Bioethanol, a renewable fuel is becoming increasingly important as a consequence of major concern for depleting oil reserves, rising crude oil prices and greenhouse effect (Sun and Cheng, 2002; Hu et al., 2008). Lignocellulosic feedstock is considered as an attractive raw material not only for the liquid transportation fuel but also for the production of chemicals and materials, i.e. the development of carbohydrate-based biorefineries (Herrera, 2006; Gray et al., 2006; Farrell et al., 2006) because of its availability in large quantities at low cost (Lynd, 1989; Parisi, 1989). Corn stover, wheat straw, sugar begasse, rice straw, rice hull, corn cob, oat hull, corn fiber, woodchip and cotton stalk have attracted the most interest of research (Chang et al., 2001; Chen and Liu, 2007; Esteghlalian et al., 1997; Moniruzzaman et al., 1997; Saha, 2003; Saha et al., 2005; Eklund and Zacchi, 1995; Jeffries, 2006; Sun and Chen, 2007).

Besides terrestrial plants, aquatic plants are also promising renewable resource. Aquatic plants have many advantages as they grow in water bodies without competing with arable lands for grains and vegetables; they are also used for water purification to extract nutrients and heavy metals. Especially, the vegetative form of free floating aquatic plants will facilitate their movement and harvest. Despite those advantages, no data on bioethanol production from aquatic plants are available except for water-hyacinth (*Eichhornia crassipes*) (Kahlon and Kumar, 1987; Nigam, 2002). Water-hyacinth (*Eichhornia crassipes*), which is widely prevalent aquatic weed in India having high content of hemicellulose (35–55% of dry weight), exceptionally fast growing plant and can provide hemicellulosic sugars for bioconversion to fuel ethanol.

The production of fuel ethanol from biomass involves hydrolysis, fermentation and distillation. The hydrolysate contain varying amounts of monosaccharides, both pentose and hexose, and a broad range of substances either derived from raw material or resulting as reaction products from sugar and lignin degradation. Many of these substances may have an inhibitory effect on the microorganisms in subsequent fermentation steps (Nigam, 2002).

The fermentation organism must be able to ferment all monosaccharides present and in addition, withstand potential inhibitors in the hydrolysates. The most commonly used ethanol producers, *Saccharomyces cerevisiae*, cannot ferment pentoses, which may constitute up to 45% of the raw material. Among the xylose fermenting yeasts *Pichia stipitis* has shown promise for industrial applications because it ferment xylose rapidly with a high ethanol yield and apparently produces no xylitol (Dominguez et al., 1993). This study investigated ethanol production from both the defined lab media i.e. synthetic hydrolysate media and pretreated-detoxified water-hyacinth hemicellulose acid hydrolysate using *P. stipitis* NCIM-3497 and the experimental facts explained by the proposed model.

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2. Methods

2.1. Microorganism and maintenance

Pichia stipitis NCIM-3497 used in this study was procured from the National Collection for Industrial Microorganisms, National Chemical Laboratory, Pune, India was grown and maintained at 30 ± 0.2 and 4 °C, respectively, on agar slants. The medium used for inoculum preparation contained (g/L): D-xylose 50; glucose 5; yeast extract 3; malt extract 3 and peptone 5, pH 5.0 ± 0.2. The media were sterilized by autoclaving at 121 °C for 15 min.

2.2. Production medium

2.2.1. Substrate preparation

Fresh water-hyacinth plant with long stem were collected and washed to remove adhering dirt and chopped in small pieces, dried, and powdered. The average composition of water-hyacinth was; total solids (TSs): 5.2–7.8 (% of wet weight), moisture 92.6–95 (% of wet weight), volatile solids (as % of TSs): 4.4–6.7 (84.0–85.9), hemicellulose (as % of TSs): 49.2 ± 0.024, cellulose (as % of TSs): 18.4 ± 0.018, lignin (as % of TSs): 3.55 ± 0.005, crude protein (as % of TSs): 12.60 ± 0.018.

2.2.2. Hydrolysate preparation

Hydrolysate was prepared by refluxing the dried powder with 10 volumes of (2% v/v) sulfuric acid for a period of 7 h at room temperature, in conical flasks, stirred at 250 rpm. Hydrolysate (liquid portion) was filtered to remove the unhydrolysed solid residue, and washed with warm water (60 °C). The filtrate and washings were pooled together.

2.2.3. Detoxification of acid hydrolysate

The acid hydrolysate (1 L) was heated to 100 °C, held at that temperature for 15 min to remove or reduce the concentration of volatile components. Any loss in volume during boiling was replaced with heated distilled water. The acid hydrolysate was then overlimed with solid $Ca(OH)_2$ up to pH 10.0, in combination with 0.1% sodium sulfite, filtered to remove insolubles and then reacidified to pH 6.0 ± 0.2, with 1 N sulfuric acid. The filtrate was concentrated under vacuum at 60 °C to achieve (5–6% w/v) of xylose concentration. The composition of the acid hydrolysate was then determined and was found as follows (g/L): D-xylose 54; D-glucose 3.5; L-arabinose 4.5; D-galactose 2.2; and D-mannose 3.3. Since the main fermentable sugar found was xylose, therefore, the acid hydrolysate is referred as hemicellulose acid hydrolysate hence forth. The resulting solution was stored at −10 °C for further use as substrate.

2.2.4. Water-hyacinth hemicellulose acid hydrolysate medium

Fermentation medium containing (g/L): yeast extract 1; $(NH_4)HPO_4$ 2; $(NH_4)SO_4$ 1; $MgSO_4 \cdot 7H_2O$ 0.25; and trace element solution 1 ml/L was supplemented with detoxified hemicellulose acid hydrolysate (Section 2.2.3). The trace element solution contained (g/L): $CuSO_4 \cdot H_2O$ 2.5; $FeCl_3 \cdot 6H_2O$ 2.7; $MnSO_4 \cdot H_2O$ 1.7; $Na_2Mo_2O_4 \cdot 2H_2O$ 2.42; $ZnSO_4 \cdot 7H_2O$ 2.87; $CaCl_2 \cdot 6H_2O$ 2.4 and medium pH was adjusted to 6.0 ± 0.2 with concentrated H_2SO_4 (98%) 0.5 ml/L.

2.2.5. Batch fermentation

Batch fermentation was conducted in a 500 ml conical flask with a working volume of 125 ml. The fermentation medium was inoculated with 5% v/v inoculum (20 h culture, 1×10^7 cells/ml). The fermentation temperature was kept constant at 30 ± 0.2 °C in an Incubation shaker (Lab Therm Kuhner, Switzerland). The broth was kept under agitation at 200 rpm. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol and residual sugars in the broth. All experiments were carried out in duplicate.

2.2.6. Recovery of ethanol

Fermented broth was diluted with water and distilled in a distillation assembly at 79 ± 1 °C. Temperature was controlled to prevent mixing of higher boiling distillate like water in the broth.

3. Analytical methods

Total solids (TSs), volatile solids, moisture and crude protein in water-hyacinth were determined according to standards (AOAC, 1975). Cellulose, hemicellulose and lignin contents were determined by the detergent extraction method (Robertson and van Soest, 1981).

3.1. Biomass estimation

Biomass growth was measured turbidometrically at 600 nm by diluting samples in the ratio 1:5 with 1 N HCl (to dissolve calcium salts), using a cuvette with 1 cm light path in Double Beam UV–vis Spectrophotometer (Electronic Corporation of India Ltd.) and culture dry weight was measured by centrifugation and drying at 105 °C, until no weight change between consecutive measurements was observed.

3.2. Sugar estimation

Total reducing sugar was estimated by using dinitrosalicylic acid (DNS) reagent (Miller, 1959), while pentose sugar was estimated by Roe and Rice method (Roe and Rice, 1948).

3.3. Ethanol estimation

Ethanol produced during the fermentation process was measured colorimetrically using potassium dichromate ($K_2Cr_2O_7$) reagent.

3.4. Software

The calculation of the parameters of the mathematical model and the statistical analysis followed by was done with the help of software package "Polymath" version 6.10 (CACHE Corporation, USA).

4. Results and discussion

4.1. Water-hyacinth hemicellulose acid hydrolysate preparation

Dilute sulfuric acid hydrolysis (2% v/v) under reflux was very effective in releasing good amount of sugar from water-hyacinth. After 7 h of reflux, reducing sugar yield was 18.8 g/100 g of dry biomass of which pentose sugar constituted 13.3 g/100 g, remaining was other reducing sugars. All these sugars were derived primarily from hemicellulosic fraction of water-hyacinth. The other reducing sugar yield was low, showing that cellulose remains practically unhydrolyzed. Dilute acid at moderate temperature effectively removes and recovers most of the hemicellulose as dissolved sugars (Lu et al., 2008). As the structure of cellulose is more complex than the hemicellulosic fraction in the plant material, therefore, requires much severe conditions for their degradation. Also water-hyacinth contains relatively high hemicellulose content compared to cellulose, this is fairly agreed with the data reported by Klass and Ghosh (1981). Besides sugars, the hydrolysate contained different and

varying amounts of toxic components, such as acetic acid, furfural, and soluble lignin derivatives, known as inhibitors to microorganisms.

4.2. Detoxification of the hemicellulose acid hydrolysate

The detoxification of hemicellulose acid hydrolysate by boiling and overliming generally results in better fermentability of the hydrolysate (Amartey and Jeffries, 1996). Volatile compounds, such as furfural and phenols are stripped by boiling, while overliming with $Ca(OH)_2$ remove and/or reduce the concentration of other acid components, e.g. acetic and tannic acid. Furthermore, the pH increase up to 10.0 due to overliming results in precipitation of heavy metals. The furfural is transformed into furfuryl acid, which condenses with other components of hydrolysate (Strickland and Beck, 1985). Overliming resulted in the loss of glucose (10%) and xylose (4%). To reduce losses, the shift to higher pH during the overliming process must be kept short to minimize pentose decomposition (McMillan, 1994). Very limited growth was observed when the organism was tried to grow in the hemicellulose acid hydrolysate medium prior the step of detoxification with lime (the data is not presented here). Similar observation was recorded by Amartey and Jeffries (1996).

4.3. Fermentation of pretreated water-hyacinth hemicellulose acid hydrolysate medium

The fermentation of pretreated hemicellulose acid hydrolysate medium and synthetic medium (where pretreated hemicellulose acid hydrolysate was replaced by equivalent amount of synthetic sugars) was done at initial pH 6.0 ± 0.2 and temperature of 30 °C. Glucose was utilized completely in both cases whereas only 72.83% of the xylose was utilized in pretreated hemicellulose acid hydrolysate medium. Utilization of different sugars has been reported by other authors also (Jeffries and Sreenath, 1988; Ferrari et al., 1992). Time-course for growth, utilization of sugars and ethanol production is shown in Figs. 1 and 2.

With 5% v/v (1×10^7 cells/ml) inoculum, fermentation was completed in 120 h for hemicellulose acid hydrolysate medium. The ethanol yield ($Y_{p/s}$), productivity (q_p), biomass yield ($Y_{x/s}$) and maximum specific growth rate (μ_{max}) were found to be 0.425 g_p/g_s, 0.176 g_p/L/h, 0.067 g_x/g_s and 0.076/h, respectively. These fermentation parameters for the detoxified hemicellulose acid hydrolysate were lower than those obtained with synthetic medium. The reason might be some left over toxic components in the detoxified hemicellulose acid hydrolysate that negatively affects the growth and ethanol producing ability of *P. stipitis*. The similar observations were also reported by Nigam (2002).

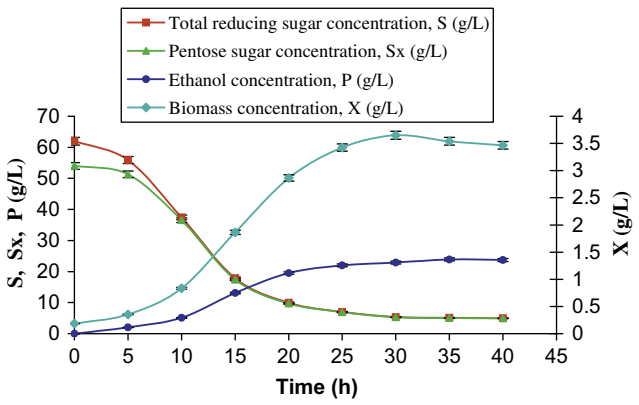

Fig. 1. Concentration profiles of total reducing sugar, pentose sugar, ethanol and biomass during fermentation of synthetic medium.

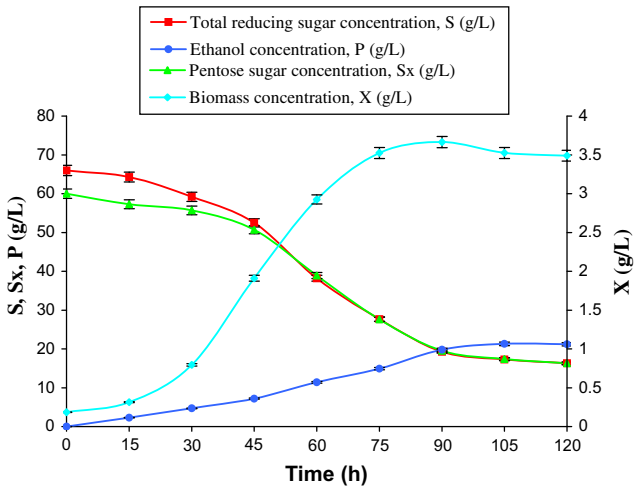

Fig. 2. Concentration profiles of total reducing sugar, pentose sugar, ethanol and biomass during fermentation of hemicellulose acid hydrolysate medium.

4.4. Modeling of ethanol formation

Interpretation of experimental results obtained by organized complex systems like microorganism requires an appropriate approach. The dynamics of the sugar metabolization is assumed to be the result of an autocatalytic process that depends on the concentrations of substrate and microorganism. Ethanol concentration acts as inhibitor.

The kinetics of xylose fermentation was quantified with the following assumptions: (a) the fermentation flasks were well mixed system and the conditions were uniform throughout the system, (b) the yeast cells did not die or become non-viable and (c) the agitation speed of 200 rpm was in excess of the need of fermentation to provide adequate mass transfer and uniform substrate availability. The systems can be described for rate of biomass growth (y) by Eq. (1), in which concentrations of sugar ($S(t)$), ethanol production ($P(t)$) and cell activation ($X(t)$) are linked:

$$Y = dX/dt = k_f \cdot S \cdot X - k_i \cdot X \cdot P \quad (1)$$

where k_f and k_i represents coefficients of cell formation and inhibition, respectively.

It is further assumed that the rate of product formation is related both to the rate of growth (dX/dt) and the concentration of microorganism present. The equation for product formation rate (z) can thus be written as follows (Eq. (2)):

$$Z = dP/dt = k_g \cdot (dX/dt) + k_p \cdot X \quad (2)$$

where, k_g and k_p represents coefficients of rate of growth and concentration of microorganism present respectively.

4.5. Calculation of kinetic parameters

The optimal values of the parameters of the models are estimated by non-linear regression technique using software package mentioned earlier (Section 3.4). The parameters were estimated using the experimental data of X, S, P and y (dX/dt) and z (dP/dt) at maximum concentration of X and the results are given as, k_f (L/g/h): 0.0010859, k_i (L/g/h): 0.0009699, k_g: 4.117547, and k_p (h^{-1}): −0.0009849 with 95% confidence.

4.6. Interpretation of regression models

The statistical parameters and the various plots can be used to assess the quality of the regression models. The plots of the calcu-

lated and experimental values of the dependent variables (y and z values) are showing the same trend (Fig. 3). If the differences between the experimental and calculated points are large, and no clear trend exists, this may indicate very noisy data (excessive experimental error), which can not accurately modeled.

4.6.1. Residual plot

The residual plots are used for judging the appropriateness of the model. The residual plot (Fig. 4) show the difference between the experimental and calculated values ($Ey = y$ experimental $- y$ calculated; $Ez = z$ experimental $- z$ calculated) of the dependent variables (y and z) as function of the experimental values. From the plots this can be interpreted that the proposed model is appropriate for this study as the residuals are randomly distributed around the line of zero error. If the residuals show a clear trend, then it indicates that an inappropriate model is being used.

4.6.2. R^2 and R^2_{adj}

The coefficient of determination (R^2) and coefficient of determination adjusted (R^2_{adj}) are frequently used to judge whether the model represents correctly the data, implying that if they are close to unity then the regression model is correct. The R^2 and R^2_{adj} values for the model for rate of biomass formation were 0.9854 and 0.9825, respectively and the same for rate of product formation were 0.9508 and 0.9438, respectively. Since the values are close to one, the proposed model is able to explain the experimental truths.

4.6.3. Variance and Rmsd

Just like coefficient of determination, these two statistical parameters, variance and root mean square deviation (Rmsd) are recommended to be used for model testing. A model with smaller variance and Rmsd, where in Rmsd calculation respective deviations were taken between experimental and calculated values for each data point (sampling time), represent the data more accurately than a model with larger values of these parameters.

The variance and Rmsd were found to be 0.0030777 and 0.0001096, respectively, for the rate of biomass formation, and these were 0.0118123 and 0.0016146, respectively, for rate of product formation. The R^2, R^2_{adj}, variance and Rmsd values were calculated by the same software mentioned earlier (Shacham et al., 1996).

5. Conclusions

As the water-hyacinth is rich in hemicellulosic content (49.2 ± 0.024% of TSs) the utilization of detoxified hemicellulosic fraction of water-hyacinth for ethanol production was evaluated in this study. Dilute acid at moderate temperature has been used to remove and recover the maximum sugars (especially xylose) from water-hyacinth while cellulosic fraction (18.4 ± 0.018% of TSs) was remained unhydrolysed under these conditions. The ethanol yield and productivity were 0.425 g_p/g_s and 0.176 g/L/h respectively, while 72.83% of xylose was utilized. The process of degradation of substrates, activation and inhibition of the cells and the ethanol production are strictly coupled. The used model is a simple representation and is good estimative for analyzing the behavior of fermentation process utilizing hydrolysate of water-hyacinth as substrate for ethanol production. The models allow us to evaluate the real level of the cellular activity, whose value is not correlated only to the concentration of biomass and is difficult to measure directly. The parameters of the models can be used as a tool for a comparison between microorganism performances. These models can constitute a basis for a deeper understanding of microorganism dynamics, permitting to identify variations in individual aspects of the degradation processes. The validity of the proposed models enables us to predict the optimal conditions for the strain cultivation for its maximum yield and the time to stop the process in order to avoid waste of time and energy at the final phase of the fermentation.

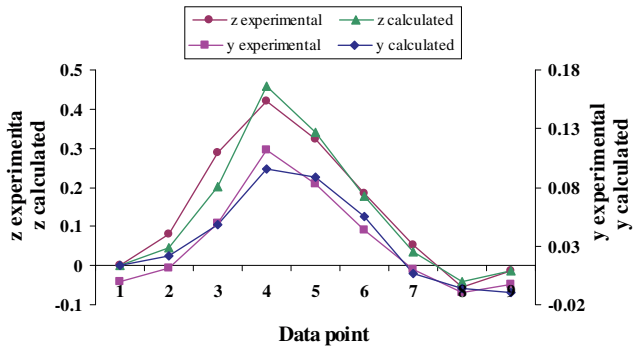

Fig. 3. Nonlinear plot of calculated and experimental values of biomass formation rate (y) and product formation rate (z) verses data point (sampling time) using hemicellulose acid hydrolysate medium.

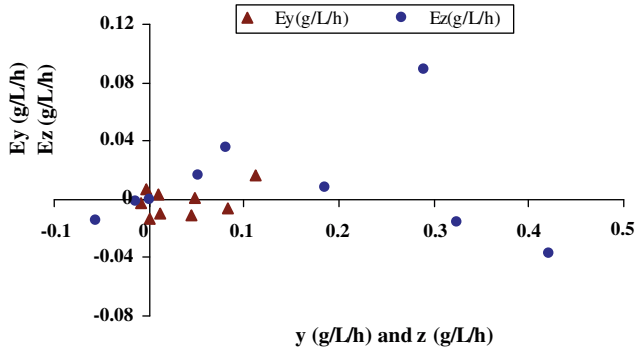

Fig. 4. Nonlinear residual plot of difference between experimental and calculated values of biomass formation rate (Ey) and product formation rate (Ez) verses experimental values of y and z, respectively, using hemicellulose acid hydrolysate medium.

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