Asian Journal of Scientific Research

ISSN 1992-1454

science alert
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Asian Journal of Scientific Research 5 (4): 285-289, 2012
ISSN 1992-1454 / DOI: 10.3923/ajsr.2012.285.289

Production of Ethanol from Water Hyacinth (*Eichhornia crassipes*) by *Zymomonas mobilis* CP4: Optimization Studies

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ABSTRACT

Ethanol is produced from water hyacinth, an aquatic weed plant, comprising of cellulose, hemicelluloses and lignin by a two-step method. Water hyacinth was pretreated with different concentration of sulfuric acid, detoxified with CaOH and NaOH and then fermented using *Zymomonas mobilis* CP4 (a recombinant strain). Batch fermentation and Simultaneous Saccharification and Fermentation (SSF) were performed and the ethanol yields were compared. Central Composite Design (CCD) is conducted to optimize the production conditions like pH, temperature, enzyme concentration and substrate concentration for higher ethanol yield. Under optimum conditions ethanol yield was found to be 68.3 g L^{-1}.

Key words: Water hyacinth, *Zymomonas mobilis* CP4, simultaneous saccharification and fermentation, central composite design

INTRODUCTION

Fossil fuels are formed by anaerobic decomposition process. Fossil fuels provide the major share of the world's energy/power demand. As the demand for the fuels is ever increasing and the resources are depleting it is very much necessary to identify alternate energy sources. To overcome these problems, the vastly available lignocellulosic materials are used as source of ethanol, an alternate to fossil fuel. Commercial ethanol is produced by both synthetic and biological method (Isarankura-Na-Ayudhya *et al*., 2007). The synthetic ethanol production is commonly done by catalytic hydration of ethylene in vapor phase. The ethanol produced from this process is mostly used as a solvent (60%) and chemical intermediate (40%). The ethanol is produced by fermentation of sugars extracted mostly from lignocellulosic materials (Purwadi, 2006). Lignocellulosic biomass comprises of cellulose, hemicellulose and lignin. Hemicellulose is a heteropolymer with a xylose backbone which branched with other sugars (Olsson and Hahn-Hagerdal, 1996).

Water Hyacinth (WH) is one of the lignocellulosic materials used for production of ethanol. It is a fast growing, free floating, aquatic plant. Hemicellulose is the major component in the WH. Water hyacinth is pretreated (acid hydrolysis and detoxification) before fermentation process, because it is difficult to convert hemicellulose to xylose. During acid hydrolysis treatment, by-products like phenolic compounds, furfural, formic acid etc are generated. These compounds are the potential inhibitors for micro organisms. To reduce or to remove the amount of by-products, detoxification process is carried out using CaOH and NaOH (Purwadi, 2006). After acid hydrolysis and detoxification fermentation was carried out. In simultaneous saccharification and

fermentation (SSF) method, saccharification and fermentation process are done simultaneously. Major advantages of this method are: (1) end-product inhibition of the hydrolysis can be avoided, (2) risk of contamination is reduced and (3) product yield is increased. Micro-organism used in fermentation process of ethanol is *Zymomonas mobile* which has the ability to utilize sucrose, glucose and fructose. But they are unable to ferment xylose to ethanol. For this conversion, a recombinant strain *Z. mobilis* CP4 is used as fermenting organism which is able to convert xylose to ethanol (Yamada *et al.*, 2002).

MATERIALS AND METHODS

Water hyacinth, Sulfuric acid (5, 10 and 30%), Whatman filter paper No.1, CaOH, NaOH, Xylose (100mg/100mL), O-Toluidine. Absolute ethanol, Potassium dichromate, Microorganism *Z. mobilis* CP4 and *Aspergillus niger* were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Media preparation for culturing microorganisms: *Z. mobilis* CP4 was maintained in Regeneration Medium (RM) (Yeast extract = 10 g L^{-1}, Agar = 1.5 g L^{-1}, were dissolved in water and autoclaved for 120°C for 15min. To this, 100 mL of 20% (w/v) glucose solution and stock solutions of $MgCl_2$ (10 g L^{-1}), $(NH_4)_2SO_4$ (10 g L^{-1}), KH_2PO_4 (10 g L^{-1}) were added) and incubated for 2 days. Subculture was then performed on RM broth prior to fermentation. *Aspergillus niger* was cultured in potato dextrose broth (2.4 g of potato dextrose broth powder was dissolved in 100 mL of distilled water (24 g L^{-1}). The initial cell concentration was measured at 640 nm and the culture was incubated in a shaker. At regular interval (every 24 h), the cell concentration was measured. Enzyme and glucose concentrations were measured by DNS method (Miller, 1959). After 7 days of incubation, the broth was centrifuged at 2600 rpm for 5 min. The supernatant was collected and used a cellulase enzyme source.

Preparation of water hyacinth: Fresh water hyacinth was collected from an open pond in Madurai in December 2010. It was washed with running tap water. Then it was chopped into small pieces and dried in a hot air oven at 105°C. The dried material was stored in room temperature until further use.

Preparation of acid hydrolysate: Ten gram of dried water hyacinth was mixed with 100 mL of sulfuric acid in different concentrations. Then it was autoclaved at 121°C, 15l bs for 15 min. It was cooled down to room temperature and filtered using Whatman filter paper No. 1. The hydrolysate was collected.

Detoxification of acid hydrolysate: Acid hydrolysate was heated to 50°C for 15 min to reduce the volatile components concentration. The 34 g L^{-1} calcium hydroxide was added to the hydrolysate and agitated for 30min in order to detoxify harmful materials presented in the hydrolysate (Taherzadeh, 1999; Sun and Cheng, 2002). Then it was filtered through the Whatman filter paper. Calcium sulphate is removed as sludge. Addition of calcium hydroxide to the acid hydrolysate increased the pH of the solution, so pH of the CaOH treated hydrolysate was adjusted by using 10 M NaOH.

Simultaneous saccharification and fermentation (SSF): 10 grams of pretreated WH was dissolved in 100 mL of distilled water and sterilized at 121°C and 1atm for 15 min. 12.5 mL of *Z. mobilis* CP4 was inoculated in 100 mL of pretreated solution. Cellulase enzyme of 5 mL was added to the fermentation medium and kept in shaker. Samples were withdrawn for every 24 h, centrifuged at 5000 rpm and supernatants were analyzed for glucose and ethanol concentrations (Vogel, 1951).

Central composite design: The CCD experiment was conducted to study the effect of enzyme concentration, pH, temperature, substrate concentration and incubation time on ethanol yield. CCD experiment was conducted in 27 different combinations of process parameters and inoculated with *Z. mobilis* CP4 broth and incubated at different conditions. During this experiment, pH of the medium was monitored and maintained. After incubation, the medium was distilled and ethanol content was measured.

RESULTS AND DISCUSSION

In enzyme hydrolysis, cellulose was used to convert hemicellulose and cellulose into xylose and glucose by the addition of enzymes. After the addition of cellulase enzyme the readings were taken at 24 h regular time interval. At the end of enzyme hydrolysis step, *Z. mobilis* CP4 was added to convert the already formed glucose and xylose into bioethanol. The samples were withdrawn at 24 h regular time interval and pH and enzyme concentration was estimated. After 4 days of incubation, it was distillated and ethanol content was determined using potassium dichromate assay as mentioned earlier.

In simultaneous saccharification and fermentation, enzyme hydrolysis (saccharification) and microbial fermentation were carried out simultaneously. pH, temperature, glucose concentration and final ethanol yield readings were taken at 24 h regular time interval.

Experiments based on Central Composite Design (CCD) were conducted to study the effect of substrate concentration, pH, temperature, enzyme concentration on ethanol concentration and the above parameters were optimized using Response Surface Methodology (RSM). The factors affecting the Simultaneous saccharification and fermentation of water hyacinth with cellulase enzyme and *Z. mobilis* CP4 culture was studied using CCD experiments. The substrate concentration (g L^{-1}), the pH, time (°C), the enzyme concentration (g L^{-}), were chosen as the independent variable. Ethanol concentration (Y) was chosen as the dependent output variable. Twenty seven experiments based on the CCD were carried out with different combinations of variables. A quadratic model was employed to study the effect various factors employed. The experimental results were analyzed using statistical software Minitab 15 and the results were tabulated in Table 1. Factors with p-values greater than 0.05 were considered to be statistically not significant and hence they were eliminated from the regression model (Ponnusami *et al*., 2008; Pratibha *et al*., 2010). Accordingly three factors were removed from the model and reduced model was regressed. Results are shown in Table 1. High R^2 value (0.9611) shows that the model described 96.11% variations of the response variable leaving only 3.89% to residuals.

Substituting the regression coefficients in the model following equation was obtained:

$$Y = 22.52+1.87\ pH+4.97\ C+2.1\ Enz-3.28\ time+3.97\ pH^2+8.70\ C^2+10.18\ enz^2-4.4\ time^2-5.00\ pH\ C-5.98\ pH\ enz$$

$$R^2 = 0.9611$$

Table 1: Estimated regression coefficients for the reduced model for the production of ethanol

Term	Coef.	SE Coef.	t-value	p-value
Constant	22.52	1.206	22.521	0.000
pH	1.87	0.603	1.866	0.082
C	4.97	0.603	4.967	0.000
Enz	2.10	0.603	2.102	0.053
Time	-3.28	0.603	-3.283	0.005
pH^2	3.97	0.905	3.965	0.001
C^2	8.70	0.905	8.692	0.000
Enz^2	10.18	0.905	10.176	0.000
$Time^2$	-4.40	0.905	-4.405	0.001
C×pH	-5.00	1.045	-5.009	0.000
C×Enz	5.73	1.045	5.481	0.000
pH×Enz	-5.98	1.045	-5.981	0.000

Table 2: ANOVA table for the production of ethanol from water hyacinth

Source	DF	Seq.SS	Adj.SS	Adj.MS	F-value	p-value
Regression	11	1619.37	1619.37	147.215	33.70	0.000
Linear	4	189.36	189.36	47.34	10.84	0.000
Square	4	1032.92	1032.92	258.23	59.11	0.000
Interaction	3	397.09	397.09	132.363	30.30	0.000
Residual error	15	65.53	65.53	4.369		
Lack-of-Fit	13	42.85	42.85	3.297	0.29	0.937
Pure error	2	22.67	22.67	11.337		
Total	26	1684.89				

Here the regression factor square of enzyme and substrate concentration had significant effect on ethanol yield that the interaction effect between all the four independent variables were not that much significant except regression factor square of enzyme and substrate concentration. Hence, these two parameters play a major role in order to get maximum bioconversion of water hyacinth to ethanol.

Analysis of variance (ANOVA) is shown in Table 2. The p-values less than 0.05 confirm that all the linear, square and interaction sources were significant. In addition, p-value (0.937) confirmed that lack-of-fit was not statistically significant.

The optimum values of substrate concentration, pH, time and enzyme concentration were then obtained by solving this equation and were found to be 200 g L^{-1}, 4.5, 3.25 days, 50 g L^{-1}, respectively. Under these conditions ethanol yield was 68.3 g L^{-1}.

CONCLUSION

Water hyacinth (*Eichhornia crassipes*) is a weedy lignocellulosic material which is used as a source for bioethanol production. Ethanol was produced by simultaneous saccharification and fermentation of detoxified, sulfuric acid treated water hyacinth. *Aspergillus niger* was used for hydrolysis (saccharification) and *Z. mobilis*CP4 (MTCC 2427) was used for fermentation. Single step SSF has an advantage over two step methods as it minimizes product inhibition. The parameters such as pH, time, substrate and enzyme concentrations were optimized by conducting Central Composite Design (CCD). A quadratic model was proposed to explain the variations in ethanol yield as a function of process variables chosen. The model was able to describe 96.11%

variation in ethanol yield leaving only 3.89% to residuals. The optimum values of substrate concentration, pH, time and enzyme concentration were then obtained by solving this model equation and were found to be 200 g L^{-1}, 4.5, 3.25 day, 50 g L^{-1}, respectively. Under these conditions (optimal conditions) ethanol yield was 68.3 g L^{-1}.

ACKNOWLEDGMENT

All the authors like to thank the SASTRA University management for providing necessary facilities to carry out this project and the continuous encouragements given.

REFERENCES

Isarankura-Na-Ayudhya, C., T. Tantimongcolwat, T. Kongpanpee, P. Prabkate and V. Prachayasittikul, 2007. Appropriate technology for the bioconversion of water hyacinth (*Eichhornia crassipes*) to liquid ethanol. EXCLI J., 6: 167-176.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Olsson, L. and B. Hahn-Hagerdal, 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb. Technol., 18: 312-331.

Ponnusami, V., N. Lavanya, M. Meenal, R.A.G. Raj and S.N. Srivastava, 2008. Application of nitric acid treated rice husk for sorption of reactive dye reactive black 5: Analysis using statistical experimental design. Pollut. Res., 27: 45-48.

Pratibha, R., P. Malar, T. Rajapriya, S. Balapoornima and V. Ponnusami, 2010. Statistical and equilibrium studies on enhancing biosorption capacity of *Saccharomyces cerevisiae* through acid treatment. Desalination, 264: 102-107.

Purwadi, R., 2006. Continuous Ethanol Production from Dilute-Acid Hydrolyzates: Detoxification and Fermentation Strategy. Chalmers University of Technology, Sweden, ISBN: 9789172918382, pp: 1-72.

Sun, Y. and J. Cheng, 2002. Hydrolysis of lignocellulosic material from ethanol production: A review. Bioresour. Technol., 83: 1-11.

Taherzadeh, M.J., 1999. Ethanol from lignocellulose: Physiological effects of inhibitors and fermentation strategies. Ph.D. Thesis, Chalmers University of Technology, Sweden.

Vogel, A.I., 1951. Text Book of Quantitative Inorganic Analysis. 2nd Edn., Longman Scientific and Technical, New York.

Yamada, T., M.A. Fatigati and M. Zhang, 2002. Performance of immobilized *Zymomonas mobilis* 31821 (PZB5) on actual hydrolysate produced by Arkenol technology. Applied Biochem. Biotechnol., 98-100: 899-907.