International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.7, No.5, pp 2287-2295, 2014-2015

Microwave Assisted lime pretreatment of banana pseudostem for ethanol production

Farjana Begum. A[1] , Chittibabu. S[1]*

[1]Department of Biotechnology, Periyar Maniammai University, Vallam, Thanjavur-613403, Tamil Nadu, India.

Abstract: Lignocellulosic biomass is considered as the best alternative feedstock for the ethanol production. In the present study, banana pseudo stem pretreated by microwave assisted lime treatment was tested as a substrate for ethanol production. Pretreatment parameters viz., lime concentration, liquid-solid ratio, time and temperature were selected as operation variables and ethanol concentration after 64 h of fermentation was considered as response variable for optimization using response surface methodology. Optimal pretreatment conditions, were found to be temperature 86˚C, microwave irradiation time 460 s, lime concentration 0.5 kg m^{-3}, solid-liquid ratio 4.8 (v/v). The resulting ethanol concentration was 19 g $L^{-1.}$
Keywords: fuel ethanol; banana pseudo stem; lime pretreatment; microwave heating; simultaneous saccharification and fermentation (SSF); response surface methodology.

Introduction

Global demand for transport and industrial fuels is increasing, but oil reserves are diminishing, so a serious imbalance of demand and supply is imminent for conventional fuels. Worldwide energy consumption is projected to increase by 49%, and so, the need to identify more sustainable and environmentally acceptable sources of energy is paramount. Renewable energies including water, wind, solar, geothermal, and especially biofuels have received much political support recently to help solve this problem. Biofuels, such as ethanol, obtained from biomass represent an attractive alternative to fossil fuels to reduce dependence on foreign oil and to decrease CO_2 emissions, the main cause of climatic change. Ethanol is one of the important substitute for the conventional petroleum based transportation fuels because of its easy adaptability to existing engines (1). The Government's decision on mandatory blending of ethanol has created huge demand for fuel grade ethanol. For ethanol production, feedstock availability, its variability and sustainability are the main issues to be addressed (2, 3). At present, ethanol is produced from sugarcane molasses and starch based feed stocks and these feed stocks are directly connected to the food chain. Hence it is not possible to spare these feed stocks for fuel ethanol production. Ethanol from lignocellulose materials present important advantages as the raw material is less expensive than conventional agricultural feedstock and requires lower input. Biofuels such as ethanol from lignocellulose generate low net greenhouse gas emissions, reducing environmental impacts, and they might also provide employment in rural areas (4). However, the technology for ethanol production from lignocellulosic biomass is complex and more challenging. Though the biomass is cheap, the cost of processing is relatively higher (5). Banana pseudostem (BPS) is one ofthe most abundantly available agricultural residues in subtropical and tropical regions. India is the largest producer of banana, contributing 27% of the world's production. Banana pseudostem is a lignocellulosic residue, an attractive feedstock for the production of second generation bioethanol. Various bottle necks in such technologies include pretreatment of biomass, effective enzymatic saccharification of the pretreated biomass and fermentation of hexose and pentose sugars released by the saccharification (6, 7, 8). Each of these problems requires substantial research and development efforts for improved efficiency and process economics.

Microwave heated lime pretreatment process was used for the pretreatment of BPS. Lime ($Ca(OH)_2$) is an inexpensive reagent and it can be easily recovered as calcium carbonate by neutralization with carbon dioxide, which is already available in the ethanol plants as byproduct of fermentation. Microwave irradiation generates rapid intense heating of polar substances but non-polar substances do not heat up because they do not absorb the radiation (9). This selective heating has been utilized efficiently in heterogeneous reactions to heat selectively a polar catalyst. It was reported that microwave assisted pretreatment process was more efficient than the convection based pretreatment (10).

Simultaneous saccharification and fermentation (SSF) has been established as a promising option for ethanol production from lignocellulosic materials. The overall ethanol yield in SSF has been reported to be higher than if the enzymatic hydrolysis and fermentation are carried out separately (SHF) (11). However, the ethanol concentration is important too, because the distillation costs decrease as a function of the final ethanol concentration. To increase the ethanol concentration, a high content of substrate is needed in the SSF process (12). However, a high WIS content leads to high viscosity of the medium, making it more difficult to stir. Substrate concentration, incubation time, and temperature are important parameters affecting the SSF process. On the other hand, SSF has not yet been applied as a strategy for ethanol production from Banana pseudostem (BPS) pre treated with Microwave and lime treatment together.

Materials and Methods

Rawmaterial

Banana pseudostem (BPS) of *Musa sapientum* species collected from Banana farms in Thanjavur, India (10˚ 48′N, 79˚09′E), elevation 77 m above mean sea level (MSL). It is part of Cauvery river delta region with mean annual rainfall 1078 mm. The samples about 0.3 m above the ground to 1 m height were collected from the banana plant after a day of harvesting the fruits, collected BPS was chopped into small pieces, thoroughly washed with water, squeezed and sun dried for 5 days. The sun dried BPS was ground and screened. The fractions that passed through the 40 mesh screen were stored in a dark and dry place until use.

Experimental setup

Microwave oven was used to heat the BPS suspended in solution with known lime concentration. The microwave oven had a maximum power of 1000 W with two settings high and low. Low microwave power was used to heat the BPS to the designated temperature. 500 ml Erlenmeyer flask fitted with stopper was used for the pretreatment of BPS. The stopper is made up of microwave plastic. Thermocouple and vent tube with valve were fixed in the stopper. The microwave oven was calibrated using water for microwave power settings, exposure time and temperature. The time at which the contents in the flask reached the designated temperature was set as zero.

Experimental procedure

Lime pretreatment

BPS content was maintained constantly at 50 g for all the batch experiments. The liquid-solidratio (LSR), lime concentration, pretreatment temperature and irradiation time were varied according to the Central composite design (CCD). The actual factor levels and coded values of independent variables used for pretreatment of BPS are given in table 1. The pretreated BPS was washed with water until the pH of the water is neutral and pressed to remove excess water. The wet BPS with 20% moisture was used for further SSF experiments.

Table.1. Codes of the independent variables and their corresponding values used for the optimization.

Independent variables	Coded symbol	Coded values and actual factor levels				
		-2	**-1**	**0**	**+1**	**+2**
Liquid – solid ratio (v/v)	x_1	1.5	3	4.5	6	7.5
Lime concentration (kg/m^3)	x_2	0	0.2	0.4	0.6	0.8
Pretreatment time (s)	x_3	270	360	450	540	630
Pretreatment temperature (°C)	x_4	45	60	75	90	105

Simultaneous Saccharification and Fermentatio

The SSF experiments were performed in a 500 ml Erlenmeyer flask with working volume of 200 ml. 100 g/L of BPS concentration was maintained in all the flasks. Medium containing yeast extract – 1% w/v, peptone – 2% w/v was inoculated using 1% inoculums of *saccharomyces cerevisae*. Mixture of cellulase enzymes - 25 FPU/g of cellulose and β-glucosidase - 6% of the volume of cellulose enzymes added, diluted in 1 M citrate buffer were added aseptically into the SSF flask. Bubble traps were connected to the SSF flask to maintain anaerobic conditions. The pH was maintained at 5.0 and the flasks were incubated at temperature 40°C in a shaker incubator with 130 rpm for a period of 96 h (13). 10 ml of samples were withdrawn for every 12 h and centrifuged at 6000 rpm for 5 min. The supernatant was filtered (0.45 µm syringe filters) and stored at 4˚C for ethanol analysis. Theoretical ethanol yield was calculated using the following formula given in the Equation 1 (14).

$$\text{Theoretical ethanol yield (\%)} = \frac{[\mathrm{EtOH}]_f - [\mathrm{EtOH}]_i}{0.51(f\,[\mathrm{biomass}]1.111)} \times 100 \qquad (1)$$

where, $[EtOH]_f$ - Final ethanol concentration in g/L, $[EtOH]_i$-Initial ethanol concentration in g/L, f-cellulose fraction of dry biomass =0.39, [biomass]- dry biomass concentration at the beginning of the fermentation in g/L, 0.51-conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, 1.111-conversion factor for cellulose to equivalent glucose.

Experimental Design

The optimization of microwave heated lime pretreatment were carried out using four factors, three-level full factorial with total 16 Cube points (7 center points and 8 axial points) as described by Central Composite Design (CCD) (15). Solid-Liquid ratio (SLR), lime concentration, pretreatment time and pretreatment temperature were the independent variables and ethanol concentration after 64 h of fermentation was considered as the response variable. The actual range of variables for these experiments was selected based on our preliminary studies. Thirty-one experimental trials were performed as per the experimental design given in Table 2.

A quadratic model was developed to fit the response variable in order to correlate the response variable to the independent variables. The behavior of the system was explained by the polynomial equation (2):

$$y = \beta_0 + \beta_1.x_1 + \beta_2.x_2 + \beta_3.x_3 + \beta_4.x_4 + \beta_{11}.(x_1)^2 + \beta_{22}.(x_2)^2 + \beta_{33}.(x_3)^2 + \beta_{44}.(x_4)^2 + \beta_{12}.(x_1x_2) + \beta_{13}(x_1x_3) + \beta_{14}.(x_1x_4) + \beta_{23}.(x_2x_3) + \beta_{24}.(x_2x_4) + \beta_{34}.(x_3x_4) \qquad (2)$$

where, y - calculated ethanol concentration (kg/m^3) x_1- LSR (v/v), x_2- Lime concentration (kg/m^3), x_3-Pretreatment time (s), x_4- Pretreatment temperature (°C), β_0- the intercept term (constant), β_i-i^{th} linear coefficient, β_{ii}- quadratic coefficient and β_{ij} -ij^{th} interaction coefficient.

MINITAB-15 software was used to perform regression analysis, analysis of variance (ANOVA) and to plot response surfaces. Each experimental runs were performed in triplicates and average of the three was considered as result.

Microorganisms

Saccharomyces cerevisiae (MTCC-172) was purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India and used for ethanol production. The yeast was grown in YP media (3% glucose, 1% yeast extract, 2% peptone) at 37°C and 200 rpm for 16 h in a shaker incubator. For pre-culture, approximately 10 ml yeast was transferred to 2 l YP media. The inoculated media was incubated at 30 °C for 24 h at 200 rpm (14).

Analytical procedures

Cellulase enzymes activity was determined according to the standard IUPAC procedures (16). NREL laboratory analytical procedures were followed for the determination of extractives, ash content, structural carbohydrates and lignin contents present in the BPS (17). Ethanol content was analyzed using gas chromatography equipped with flame ionization detector (FID) and Propak QS column 100/120 mesh. Nitrogen gas was used as carrier gas. Isopropanol was used as internal standard (18).

Results and Discussion

Composition of Banana pseudostem

The moisture content of the fresh BPS was found to be 94% (by wt). The chemical composition of the BPS was analyzed. It contains 39.06% cellulose, hemicelluloses 32.8%, acid insoluble lignin 8.38%, acid soluble lignin 1.9%, ash content 8.4%, and extractives 3.1% (by wt). The chemical composition of BPS reveals that the holocellulose (cellulose + hemicelluloses) content is much higher than the other agricultural residues such as rice straw, wheat straw etc and also it contains very low lignin when compared to woody biomass residues. But the lignin content of BPS is lower than many of the potential lignocellulosic biomasses used for ethanol production like softwood, rice straw, sugarcane bagasse, corn straw etc. Low lignin content leads to less chemical usage in pretreatment processes and improves the efficiency of enzymatic hydrolysis process, because lignin limits the rate and extends the enzymatic hydrolysis by acting as shield, preventing the digestible parts of the substrate to be hydrolyzed (20). The presence of ash and extractives in BPS were higher than that of softwoods and lower than the straw. Even though the ash content is not taking part in the reaction, its presence occupies considerable volume of the biomass, which resulted in low solid to liquid ratio during pretreatment.

Optimization of microwave heated lime pretreatment processes

The ethanol concentration for each run as per the experimental design for microwave heated lime pretreatment processes were given in Table 2. The responses of the central composite design (CCD) were fitted in second order polynomial Equation (2) and the coefficients were listed in Table 3.The statistical significance of model equation was evaluated using F-test and analysis of variance (ANOVA) for the fitted quadratic polynomial was summarized in Table 4.

Table.2. Experimental design table in coded values and ethanol concentration.

Run No.	Liquid– solid ratio (vv^{-1})	Lime concentration (kgm^{-3})	Time (s)	Temperature (˚C)	Ethanol concentration (kgm^{-3})
1	1	-1	1	-1	13.89
2	-1	1	-1	1	16.00
3	0	0	0	0	18.37
4	0	0	0	0	18.29
5	-1	1	-1	-1	15.81
6	0	0	0	0	18.25
7	-1	-1	1	-1	14.31
8	0	-2	0	0	14.41
9	0	0	-2	0	15.52
10	-1	-1	-1	-1	13.55
11	-1	1	1	1	16.48
12	0	0	0	0	18.86
13	1	-1	-1	1	16.62
14	1	-1	1	1	16.46
15	1	1	1	1	17.36
16	0	0	0	0	18.04
17	0	0	0	0	18.67
18	1	1	1	-1	15.56
19	0	0	0	0	19.05
20	0	0	0	2	17.84
21	1	-1	-1	-1	14.01
22	1	1	-1	-1	16.03
23	0	2	0	0	16.12
24	0	0	0	-2	16.80
25	-2	0	0	0	15.19
26	-1	-1	-1	1	15.13

27	2	0	0	0	17.07
28	0	0	2	0	16.48
29	-1	1	1	-1	16.04
30	1	1	-1	1	16.91
31	-1	-1	1	1	15.74

Table.3. Regression Coefficients using coded values.

Term	Coefficient	SE[a] Coefficient	T	P
Constant	18.5043	0.19986	92.586	0.000
Liquid - solid ratio	0.3142	0.10794	2.911	0.010
Lime concentration	0.5792	0.10794	5.366	0.000
Time	0.1542	0.10794	1.428	0.172
Temperature	0.5658	0.10794	5.242	0.000
Liquid - solid ratio × Liquid - solid ratio	-0.6869	0.09888	-6.947	0.000
Lime concentration × Lime concentration	-0.9032	0.09888	-9.133	0.000
Time × Time	-0.7194	0.09888	-7.275	0.000
Temperature × Temperature	-0.3894	0.09888	-3.938	0.001
Liquid - solid ratio × Lime concentration	-0.0450	0.13220	-0.340	0.738
Liquid - solid ratio × Time	-0.1487	0.13220	-1.125	0.277
Liquid - solid ratio × Temperature	0.2637	0.13220	1.995	0.063
Lime concentration × Time	-0.0250	0.13220	-0.189	0.852
Lime concentration × Temperature	-0.3050	0.13220	-2.307	0.035
Time × Temperature	0.0613	0.13220	0.463	0.649

Table.4. ANOVA for microwave heat lime pretreated Banana Pseudostem.

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	F	P
Regression	14	65.5490	65.5490	4.6821	16.74	0.000
Linear	4	18.6737	18.6737	4.6684	16.70	0.000
Square	4	43.8174	43.8174	10.9544	39.18	0.000
Interaction	6	3.0579	3.0579	0.5096	1.82	0.158
Residual Error	16	4.4738	4.4738	0.2796		
Lack-of-Fit	10	3.6778	3.6778	0.3678	2.77	0.112
Pure Error	6	0.7960	0.7960	0.1327		
Total	30	70.0228				

[a] Degrees of freedom, [b] Sequential sum of squares, [c] Adjusted sequential squares, [d] Adjusted mean squares.

The P_{model}>F value for the model showed that the model was statistically significant at the probability level of $\alpha = 0.05$. However, the lack of fit was observed to be insignificant implying that the regression models were adequate to represent the experimental data. The fitness of the models was also expressed by the coefficient of determination R^2, the values are $R^2 = 93.6\%$ and $R^2(adj) = 88.0\%$. The R^2 values indicate the percentage of variability in the responses, which was explained by the model [15]. The 3-D response surface plots (figures 1,2,3,4,5 and 6) were obtained by plotting the response variable (ethanol concentration) on the Z-axis against any two variables while keeping other variables at its ‘0’ level (middle values). Even though the ethanol concentration was observed at 12 h intervals, the ethanol concentration after 64 h of SSF was taken as the response variable.

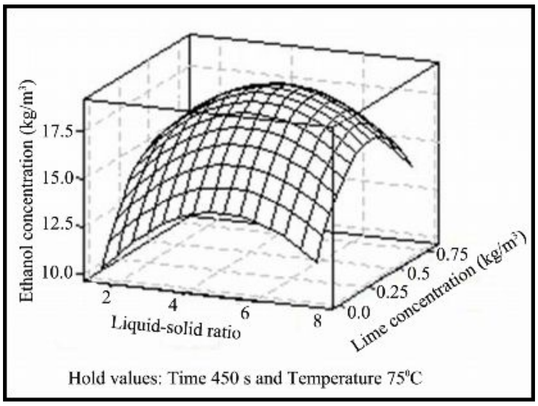

Fig.1. Effect of liquid-solid ratio and lime concentration on ethanol concentration.

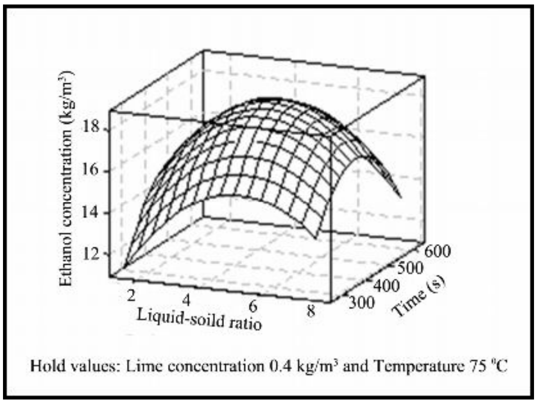

Fig.2. Effect of liquid-solid ratio and time on ethanol concentration.

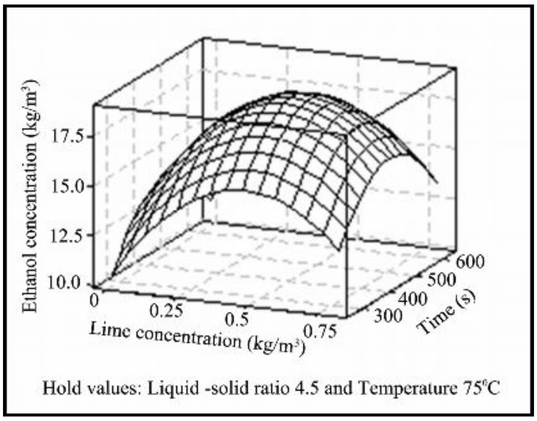

Fig.3. Effect of lime concentration and time on ethanol concentration

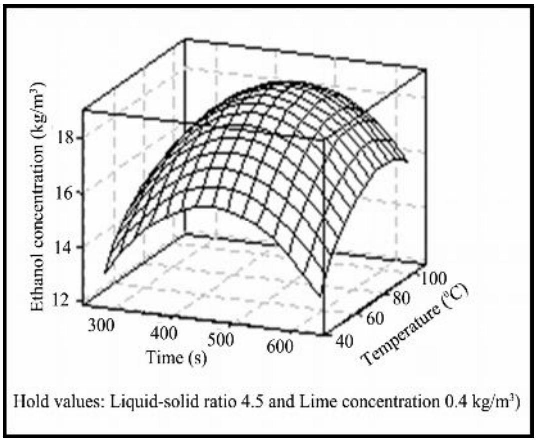

Fig.4. Effect of time and temperature on ethanol concentration

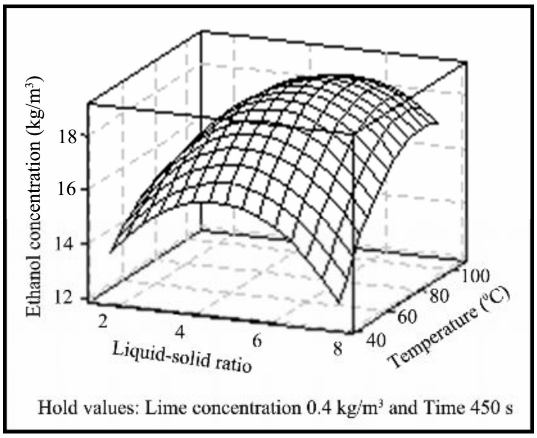

Fig.5. Effect of liquid-solid ratio and temperature on ethanol concentration.

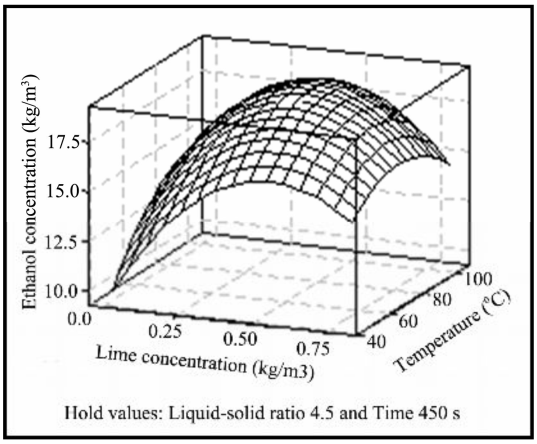

Fig.6. Effect of lime concentration and temperature on ethanol concentration

Table 3 displays the Student's t-distribution and the probability (P) values which were used to check the significance of each coefficient. A larger magnitude of the t-test and smaller p-value denote greater significance of the corresponding coefficient. From the P values (table 3 & 4), it was found that the interaction effects were not significant; hence the interaction terms in equation (2) were neglected. The first derivative of equation (2) was found out for each independent variable and the coded values for each variable were calculated. The modified equation of equation (2) was given as equation (3).

$$y = \beta_0 + \beta_1.x_1 + \beta_2.x_2 + \beta_3.x_3 + \beta_4.x_4 + \beta_{11}.\ (x_1)^2 + \beta_{22}.\ (x_2)^2 + \beta_{33}.\ (x_3)^2 + \beta_{44}.\ (x_4)1^2 \quad (3)$$

For liquid-solid ratio,

$$dy/dx_1 = 0.3142 - 1.3738x_1 = 0 \quad (4)$$

For lime concentration,

$$dy/dx_2 = 0.5792 - 1.8064x_2 = 0 \quad (5)$$

For time,

$$dy/dx_3 = 0.1542 - 1.4388x_3 = 0 \quad (6)$$

For temperature,

$$dy/dx_4 = 0.5658 - 0.7788x_4 = 0 \quad (7)$$

Equation used for the conversion of coded values to uncoded values of independent variables,

$$x_i = (X_i - X_{cp})/(\Delta X_i) \quad (8)$$

where x_i – coded value of i^{th} variable ; X_i – uncoded value of i^{th} variable; X_{cp} – uncoded values at central points; ΔX_i – difference between the levels

The optimized pretreatment parameters were found out from the first derivative equations (4), (5) (6) and (7). The second derivatives of these equations were negative and hence the points obtained were maximal points. The uncoded values of independent variables were calculated from the equation 8 and tabulated in table 5. The ethanol concentration using the equation 2 was 18.5 kg/m^3 at the optimized pretreatment conditions. These pretreatment conditions from the experimental studies were used for the pretreatment of BPS to validate the optimized conditions from the response surface plots. The ethanol concentration obtained after 64 h of fermentation was 19 kg/m^3 and the theoretical ethanol yield was 85% of cellulose.

Table.5. Optimum conditions for microwave heat lime pretreatment process

Pretreatment parameters	Optimized parameters used for the validation	
	Coded values	Uncoded values
Liquid – solid ratio	0.228	4.8 (vv^{-1})
Lime concentration	0.3206	0.5 kgm^{-3}
Time	0.1071	460 s
Temperature	0.7265	86 °C

High LSR can significantly decrease the cost of cellulosic ethanol because of handling high biomass per batch. For lime pretreatment, the optimum LSR was found to be 5:1. However, when LSR was lower than 3:1, it was hard to keep the reaction system homogeneous because of reduced liquid content and swelling nature of the fibers. Ethanol concentration was decreased due to poor degradation of cellulosic fibers and over exposure of cellulosic fibers to the microwave irradiation. High proportion of water favors the adsorption of microwave irradiation energy because the energy absorption is based on oscillation of water molecules. Hence microwave heat chemical pretreatment process requires high water content in the pretreatment process. This leads to increase the load of the effluent treatment processes for microwave heated chemical pretreatment processes. Lime pretreatment removes lignin which increases the enzyme effectiveness by reducing non-productive adsorption sites for enzymes. Lime pretreatment has been proven successfully at temperatures from 85-150°C and for 3 – 13 hrs with corn stover (21). High lime concentration (above 0.5 kg/m^3) resulted in low ethanol concentration. This is due to the formation of inhibitory compounds such as furan derivatives, acetic acid, etc., and degradation of cellulose fibers in the pretreatment process.

The results of SSF experiment for the optimization of microwave heat pretreatment shows that the time taken for the pretreatment of BPS was reduced drastically compared to convection mode of heating. From the literature, it was learnt that the time taken for the chemical pretreatment is inversely proportional to the temperature. Convection heating chemical pretreatment processes took more than two hours at moderate temperature conditions (22). Microwave irradiation reduced the pretreatment time of BPS within 8 min at same temperature, chemical concentration and solid loadings. This was due to selective heating of lignocellulosic biomass by microwaves (23).

In the kinetics studies of SSF, microwave heat pretreated BPS took 64 hrs to reach the maximum ethanol concentration. The decline in the ethanol concentration was noticed after 64 h of fermentation may be due to exhaustion of released glucose and the transition of the yeast metabolism towards utilization of ethanol as carbon source (24).

Conclusion

Ethanol production from the lignocellulosic biomass necessitates the production technology to be cost effective and environmentally sustainable. From the results, it was concluded that the banana pseudostem could be used as a potential feedstock for the production of ethanol like other lignocellulosic agricultural by products viz., rice straw, wheat straw, baggasse etc. Microwave irradiation has been widely used in many areas because of its high heating efficiency and easy operation. Microwave heated lime pretreatment process was optimized. It was concluded that the microwave heat lime pretreatment process could be the most suitable pretreatment process for the production of ethanol by SSF using BPS because the highest theoretical ethanol yield of 85% was obtained. The analysis of variance for the response variable revealed that the models developed were adequate to fit the data. Therefore, these models could be used to optimize response variables at their maximal values. Power requirement and increase in capital costs are inevitable in microwave pretreatment methods. Therefore, an economic evaluation considering the total process from biomass to ethanol is needed for further comparison.

References

1. Bayraktar H. Experimental and theoretical investigation of using gasoline-ethanol blends in spark ignition engines. Renew. Energy., 2005, 30, 1733-1747.

2. Hamelinck CN, Hooijdonk GV, Faaij APC. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass Bioenergy., 2005, 28, 384–410.
3. Sanchez OJ, Cardona, CA. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour. Technol., 2008, 99, 5270 – 5295.
4. Mohapatra D, Mishra S, Sutar N. Banana and its by-product utilization: an overview. J. Sci. Ind. Res., 2010, 69, 323-329.
5. Jorgensen H, Kristensen JB, Felby C. Enzymatic conversion of lignocelluloses into fermentable sugars: challenges and opportunities. Biofuels. Bioprod. Bioref., 2007, 1; 119-134.
6. Taherzadeh MJ, Karimi K. Enzyme based hydrolysis processes for ethanol from lignocellulosic materials- a review. Bioresources, 2007, 2, 707 – 738.
7. Chen M, Zhao J, Xia L. Comparison of four different chemical pretreatments of corn stover for enhancing enzymatic digestibility. Biomass Bioenergy., 2009, 33(10), 1381-1385.
8. Ballesteros M, Oliva J, Negro MJ, Manzanares P, Ballesteros, I. Ethanol from lignocellulosic materials by simultaneous saccharification and fermentation process with *Kluveromyces marxianus* CECT 10875. Process Biochem., 2004, 39(12), 1843-1848.
9. Gong G, Liu D, Huang, Y. Microwave-assisted organic acid pretreatment for enzymatic hydrolysis of rice straw. Bioprocess Biosyst. Eng., 2010, 107, 67-73.
10. Hu Z, Wen Z. Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment. Biochem. Eng. J., 2008, 38, 369-378.
11. Hoyer K, Galbe M, Zacchi, G. Production of fuel ethanol from softwood by simultaneous saccharification and fermentation at high dry matter content. J Chem Technol Biotechnol., 2009*,* 84, 570–577.
12. Hari Krishna S, Chowdary GV. Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic biomass. J Agric Food Chem*.,* 2000, 48, 1971–1976.
13. Dowe N, McMillan J. SSF Experimental Protocols – Lignocellulosic biomass hydrolysis and fermentation, Laboratory analytical procedures (LAP), Technical Reports NREL/TP-510-42630, National Renewable Energy Laboratory, Golden, Colarado. 2008.
14. Salvi DA, Aita GM, Robert D, Bazan V. Ethanol production from sorghum by a dilute ammonia pretreatment. J. Ind. Microbiol. Biotechnol., 2010, 37, 27-34.
15. Huang L, Lu Z, Yuan Y, Lii F, Bie X. Optimization of a protective medium for enhancing the viability of freeze-dried *Lactobacillus delbruceckii* subsp. *Bulgaricus* based on response surface methodology, J. Ind. Microbiol. Biotechnol., 2006, 33, 55-61.
16. Ghose TK. Measurement of cellulsae activities. Pure. Appl. Chem., 1987, 59; 257 – 268.
17. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of Ash in biomass, Laboratory analytical procedures (LAP), Technical Reports NREL/TP-510-42622, National Renewable Energy Laboratory, Golden, Colarado.2008
18. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of Structural Carbohydrates and Lignin in Biomass, Laboratory analytical procedures (LAP), Technical Reports NREL/TP-510-42618, National Renewable Energy Laboratory, Golden, Colarad. 2010.
19. Najafpour G, Younesi H, Ismail KSK. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisia.* Bioresour. Technol., 2004, 92, 251–260.
20. Chang VS. & Holtzapple, M.T.: Fundamental factors affecting enzymatic reactivity, Appl. Biochem. Biotechnol., 2000, 84-86, 5-37.
21. Kim S, Holtzapple MT. Delignification kinetics of corn stover in lime pretreatment,. Bioresour. Technol.2006, 97, 778–785.
22. Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol., 2002, 83, 1-11.
23. Zhang X, Lee C, Mingos DMP, Hayward DO. Effects of Microwave Dielectric Heating on Heterogeneous Catalysis. Catal. Lett. 2003, 88, 33-38.
24. Galbe M, Zacchi G. A review of the production of ethanol from softwood, Appl. Microbiol. Biotechnol.,2002, 59, 618–628.

*****