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Degradation of Lignocellulosic Feed Stock by *Fibrobacter succinogenes* in the Production of Anhydrous Alcohol

Sampana Mutalik[1], C.S. VinodKumar[2], Subramanya Swamy[1] and S. Manjappa[1]

[1]Bapuji Institute of Engineering technology, Davangere, Karnataka, India.
[2]Department of Microbiology, S .S. Institute of Medical Sciences and Research Centre, Davangere - 577 004, India.

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Early in the twentieth century, petroleum derived fuels (fossil fuels) began to appear and quickly dominated the market. Low prices persisted for several decades until the advent of the "oil crisis" in the 1970, demanding for alternative to fossil fuel. Current ethanol production processes using crops such as sugar cane and corn are well-established; however, utilization of a cheaper substrate such as lignocellulose could make bioethanol more competitive with fossil fuel. The processing and utilization of this substrate is complex. Lignocellulosic biomass contains carbohydrate fractions that can be converted into ethanol. In order to convert these fractions, the cellulose and hemicelluloses must ultimately be converted or hydrolysed into monosaccharides; it is the hydrolysis that has historically proven to be problematic. Biologically mediated processes are promising for energy conversion, in particular for the conversion of lignocellulosic biomass into fuels. The objective of the present study is to optimise cellulosic ethanol production from bagasse and maize by using *Fibrobacter succinogenes* isolated from rumen of herbivores animals. In this process cellulose is converted into monosaccharides by *Fibrobacter succinogenes*. These monosaccharides were subjected to alcoholic fermentation by *Saccharomyces cerevisiae*. This process of fermentation was followed by distillation at 78°C for alcohol extraction. Optimum temperature, pH and substrate concentration for hydrolyses of bagasse and maize was 39^{0}C, 6 and 3.5% respectively for *Fibrobacter succinogenes*. For the feed stock of concentration 3.5% of bagasse and maize, ethanol yield of 16.8g/l for bagasse and 13.9 g/l for maize was obtained

Key words: Depolymerisation, Lignocellulose, *Fibrobacter succinogenes,* ethanol.

The bacterium *Fibrobacter succinogenes* (*Bacteroides succinogenes*) of one of the most widespread cellulolytic bacteria of the rumen. *Fibrobacter succinogenes* accounted for around 20% of the isolates recovered from a rumen of herbivorous animals. On first isolation, the cells are predominantly rod shaped, but on culture they can become coccoid to lemon shaped or oval. Most cells occur singly, but short chains and grape like clusters may be observed.

Lignocellulosic biomass can be utilized to produce ethanol, a promising alternative energy source for the limited crude oil. The important key technologies required for the successful biological conversion of lignocellulosic biomass to ethanol have been extensively reviewed. The biological process of ethanol fuel production utilizing lignocellulose as substrate requires: (1) delignification to liberate cellulose and hemicellulose from their complex with lignin[2], depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free

* To whom all correspondence should be addressed.
E-mail: vinodmicro@yahoo.com

sugars, and (3) fermentation of mixed hexose and pentose sugars to produce ethanol. The development of the feasible biological delignification process should be possible if lignin-degrading microorganisms, their ecophysiological requirements, and optimal bioreactor design are effectively coordinated. In this study, an attempt is made to optimize various dependent parameters to hydrolyse lignocellulosic feed stocks like bagasse and maize by using *Fibrobacter succinogenes* isolated from rumen of cattle.

MATERIALS AND METHODS

Raw materials

Bagasse from local sugar factory (Samson's Distilleries, Davangere) and maize by nearby farm. Raw materials were powdered and sieved into a 1mm seiver. Powder feed stocks was used as carbon source.

Microorganisms

Fibrobacter succinogenes was isolated from rumen of cattle. Isolation and characterization was done in anaerobic glove box as per the standard microbiological techniques[6,9 10]

Inoculum preparation

Fibrobacter succinogenes was inoculated onto blood agar plate. After 24-48 h, inoculum density of 10^9 CFU / ml was adjusted and used for later experiments.

Culture conditions

Culture media of baggase and maize (8 g each) was prepared in conical flasks containing 250 ml of CSV medium. The conical flasks were plugged with cotton and sterilized at 121^0 C for 20 minutes. The medium was inoculated with 5 ml of 10^9 CFU of *Fibrobacter succinogenes* strains. These flasks were incubated at 39^0C for 5days on an orbital shaker under anaerobic condition. For every 6h till five days, pH was monitored and for every 24h, 5ml samples were drawn and filtered. The filtrate was used for further studies[11].

The optimum temperature of depolymerisation was determined by incubating the reaction mixture at different temperature ranging from 27 to 51°C. The optimum pH was determined by adjusting the pH of the reaction mixture from 1 to 10. The optimum substrate concentration was determined by preparing substrate suspensions 1 to 5%.

Determination of total carbohydrate

The carbohydrate content of untreated and depolymerised raw materials in the culture broth was measured by phenol sulphuric acid method with glucose as standard[7,12].

Determination of reducing sugars

Reducing sugars in untreated and depolymerised raw material in the culture broth were determined by dinitrosalicylic acid (DNS) method with glucose as standard[12,13].

Determination of protein

The protein content of culture broth and depolymerised raw material was determined by Lowry et al. method with bovine serum albumin as standard[11].

FPU assay

Cellulase enzyme production was studied by FPU assay [12]

Fermentation

Saccharomyces cereviseae strain was inoculated into the culture filtrate and allowed for fermentation for 36h[10]. After fermentation it was filtered and subjected for distillation for ethanol at 78^0C.

Ethanol estimation by high pressure liquid chromatography

The injected volume was 1 µl and the retention time was 25 min. Identification and quantification was based on direct comparison of the high pressure liquid chromatogram response to ethanol standards.

Statistics

All the tests were laid in complete randomized design and each treatment was tested for five times. ANOVA analyses were carried out with Assistat 7.5 beta.

RESULTS AND DISCUSSION

Ethanol has been known for a long time, being perhaps the oldest product obtained through traditional biotechnology. Its current applications include potable, chemical, and fuel ethanol. Cars fuelled by ethanol were planned by Henry Ford in the 1880s, when he designed early model Ts that ran on "farm ethanol" made from corn. Early in the twentieth century, however, petroleum derived fuels (fossil fuels) began to appear and quickly dominated the market. Low prices persisted for several decades until the advent of the "oil crisis"

in the 1970, demanding for alternative to fossil fuel. Current ethanol production processes using crops such as sugar cane and corn are well-established; however, utilization of a cheaper substrate such as lignocellulose could make bioethanol more competitive with fossil fuel. The processing and utilization of this substrate is complex. Lignocellulosic biomass contains carbohydrate fractions that can be converted into ethanol. In order to convert these fractions, the cellulose and hemicelluloses must ultimately be converted or hydrolysed into monosaccharides; it is the hydrolysis that has historically proven to be problematic. Biologically mediated processes are promising for energy conversion, in particular for the conversion of lignocellulosic biomass into fuels

Total sugar, reducing sugar, non reducing sugar, organic carbon, Nitrogen, total solids, moisture content of bagasse and maize was determined. Initial composition of raw material is given in Table 1.

FPU activity of *F. succinogenes* for bagasse is given in Table 2.

Fibrobacter succinogenes the best cellulolytic anaerobic bacteria was isolated from rumen of herbivores animals and cultured on feed stock based broth medium for 6 days on shaker at 120 rpm. Aliquots of 5 ml were sampled at 6 h interval and assayed for enzyme activities. Fig. 1 illustrates the enzyme activities over 78 h period.

There was progressive increase in enzyme activity from 24 to 60th h after incubation. Cellulase is an induced enzyme and its production increased with increase in bacterial biomass over the incubation period and as simple sugar in the substrate diminished[11].

Depolymerisation of bagasse and maize powder over the 80 h is illustrated in Fig. 2. There was increase in saccharification from 0 to 60th h. The increase was steeper up to 12th to the 60th h. The slowdown in rate for hydrolysis must be due to the action of the enzymes been slowed down by obstacles that interfere with their path or a loss in activity and/or processivity making them less effective [14, 13]. Enhanced enzymatic activity was observed for bagasse compared to maize

The effects of substrate concentration, temperature and pH on release of reducing sugars were also carried out. The rate of depolymerisation is directly proportional to substrate concentration up to the optimal substrate concentration. This is because random collisions between the substrate and enzyme active sites happen more frequently. Beyond the optimum, the active sites are saturated so higher substrate concentration has no effect on rate of depolymerisation. Depolymerisation increased with substrate concentration as shown in Fig. 3. There was increase in reducing sugars with increase in substrate concentration. The highest mean glucose concentration of 16.8 mg/ml was recorded for substrate concentration of 3.5% for bagasse and 13.9mg/ml glucose concentration for 3.5% substrate concentration of maize and was significantly different. Substrate concentration of 1% released the least reducing sugars concentration. The glucose concentration for 3.5% substrate concentration was higher and significantly different from 2% substrate concentration, which suggests that anything less than 3.5% is or below optimum for both bagasee and maize substrate concentration.

Table 1. Initial composition of the raw materials

Parameters	Initial composition	
	Maize	Bagasse
Alpha cellulose (%)	35.62	39.24
Total sugar (mgg^{-1})	2.4	1.3
Reducing suagar (mgg^{-1})	0.145	0.175
Non reducing sugar (mgg^{-1})	0.118	0.125
Moisture (%)	1.83	8.34
Total solids (%)	83.42	91.66
Organic (%)	32.93	36.18
Nitrogen (%)	0.384	0.448

Table 2. Effect of *Fibrobacter succinogenes* treatment on feed stock

Parameters	Pre-treated composition	
	Maize	Bagasse
Alpha cellulose (%)	26.14	28.96
Total sugar (mgg^{-1})	28.74	30.0
Reducing suagar (mgg^{-1})	27.62	28.75
Non reducing sugar , (mgg^{-1})	0.92	1.25
Protein (mgg^{-1})	7.6	8.8
FPU ($IUml^{-1}$)	0.6	0.9
Ethanol (gl^{-1})	15.92	18.17

Temperature has complex effect on enzyme activity and hence saccharification. It affects the speed of molecules; the activation energy of the catalytic reaction and thermal stability of the enzyme. Generally saccharification increased with temperature up to the optimum after which it declines. The increase with temperature is due to corresponding increase in kinetic energy

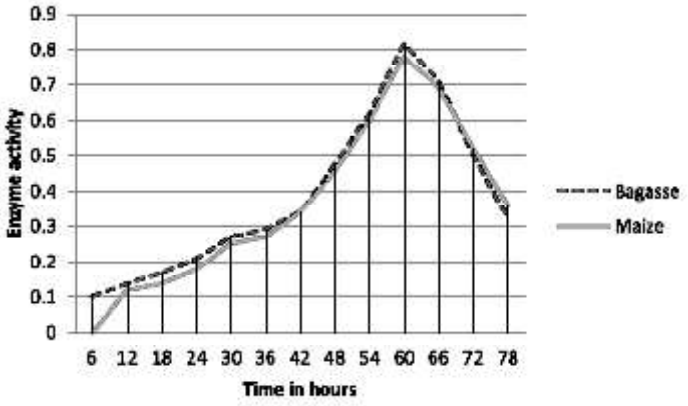

Fig. 1. Enzyme activity of *Fibrobacter succinogenes* over 78 h period

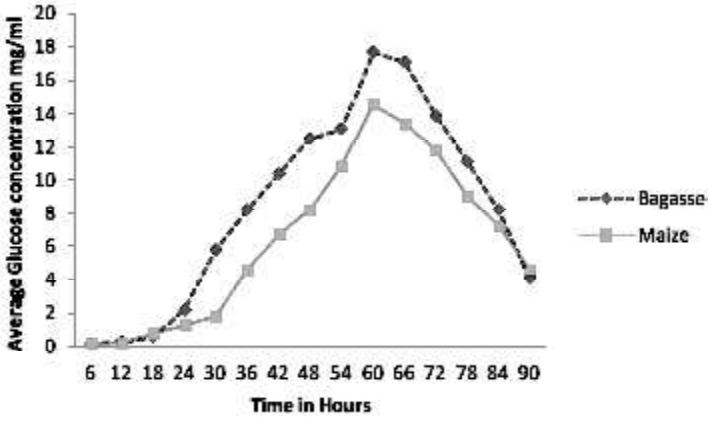

Fig. 2. Time course for Depolymerisation of Bagasse and Maize powder by *Fibrobacter succinogenes*

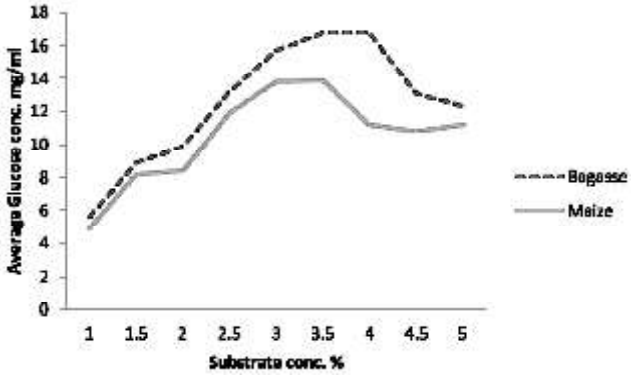

Fig. 3. Effect of substrate concentration on Depolymerisation by *Ruminococcus albus*.

and the decline after the optimum due enzyme denaturation [15, 16]. Effect of temperature on saccharification is shown on Fig. 4. Saccharification increased from 30°C to maximum at 39°C after which it decreased up to 60°C. Saccharification was least significant ($p<0.05$) at 60°C. The decrease of saccharification from 45 to 57°C was sharp due to the fact that enzyme denaturation is much faster [13,15,16]. Hence an optimum temperature of 39°C is maintained in this study. The highest mean glucose concentration of 16.7 mg/ml was recorded for bagasse and 13.9mg/ml glucose concentration for maize at 39°C

The pH of a solution has several effects on the structure and activity of enzymes and hence depolymerisation. Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups vary, according to their acid dissociation constants, with the pH of the solution. Thus pH affects the reactivity of the catalytically active groups[14,16].

Fig. 5 illustrates the effect of pH on release of reducing sugars from the substrates. Depolymerisation increased from pH 5 to 6, after which it decreased up to 10.0. The highest saccharification which was significantly different was recorded at pH 6. Saccharification was least significant at pH 4 and 10, thus the optimum pH was 6 was maintained for both bagasse and maize. The highest mean glucose concentration of 16.8 mg/ml was recorded for bagasse and 13.9mg/ml glucose concentration for maize at pH of 6.

Ethanol yield was 16.8g/l for bagasse and

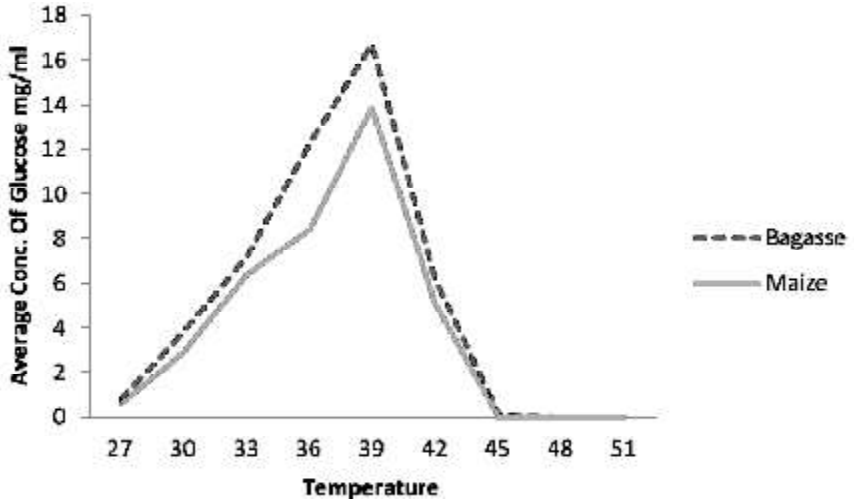

Fig. 4. Effect of temperature on Depolymerisation of bagasse powder by *Ruminococcus albus*

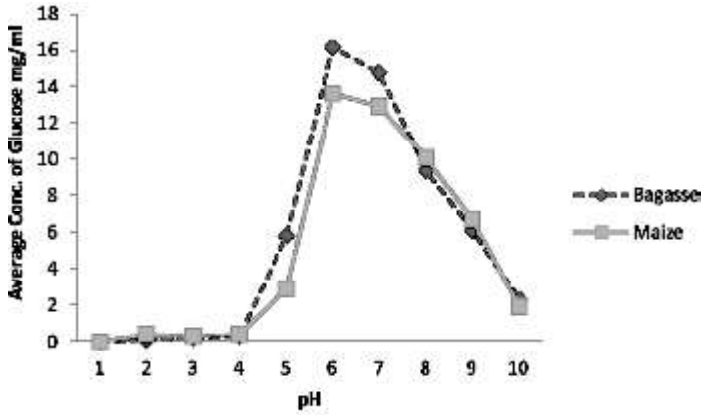

Fig. 5. Effect of pH on Depolymerisation of bagasse powder by *Ruminococcus albus*

13.9g/l for maize was obtained. Comparison to similar works in literature is difficult because ethanol concentration was not cited and they differ in either in type of pre-treatment if any and detoxification, substrate concentration, fermentation strain, temperature or mode of operation which affects the final ethanol concentration[14, 15].

CONCLUSION

The optimization test has shown that the *Fibrobacter succinogenes* is an efficient lignocellulosic depolymeriser. Without using physical or chemical methods of pre-treatment it was possible to efficiently depolymerise bagasse and maize to get the highest mean ethanol concentration of 16.8g/l and 13.9 g/l respectively using biological process.

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REFERENCES

1. Kadam K L, Forrest L H & Jacobson W A. Rice straw as a lignocellulosic resource collection, processing, transportation and environmental aspects. *Biomass Bioenergy*, 2000; **8**: 369-389.
2. Ballerini D, Desmarquest J P & Pourquie, J. Ethanol production from lignocellulosics; Large scale experimentation and economics. *Biores Tech*, 1994; **50**: 1723-1727.
3. Asant A. Assessment of food import and food aid against support for agricultural development, the case of Ghana. *Draft report for FAO Regional Office.* 2004; 10-25.
4. Mosier N, Wyman C, Dale B & Elander R. Features of promising technologies for pre-treatment of lignocellulosic biomass. *Biores Tech*, 2005; **96**: 673-686.
5. Bollok M & Reczey K. Cellulase enzyme production by various fungal strains on different carbon sources. *Acta Alimentaria*, 29 (2000) 155-168.
6. Abdul J K, Othman, OL & Shu F. Isolation of cellulolytic fungi from the Barino Highlands, Sarawak.In: ASEAN. *Review of biodiversity and environmental conservation,* 1999; **20**: 245-259.
7. Garleb K A, Borquin J T & Fahey Jr. Isolation and chemical analysis of nonfermented ûber fractions of oat hulls and cottonseed hulls. *J. Anim. Sci*, 1991; **69**: 1255-1271.
8. Ghose T K. Measurement of cellulase activities. *Pure and Appl. Chem*, 1987; **59**: 257-268.
9. Miller G.L. Use of DNS reagent for the measurement of reducing sugar. *Anal. Chem,* 1959; **31**: 426-428.
10. Sandhu H, Bajaj K L & Arneja J SBiochemical studies on bioconversion of rice straw to ethanol. *Ind. J. of Ecol*, 1998; **25**: 62-65.
11. Lynd L R & Weimer P J & Zyl W H. Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. *MMBR*, 2002; **66**: 506-577.
12. Yang B, Willies D M & Wyman C E. Changes in the enzymatic hydrolysis rate of avicel cellulose with conversion, *Biotechnol* 2006; 213-222.
13. Surender V J, Sindhu R, Binod P & Janu, K U. Lignocellulosic ethanol in India: Prospects, challenges and feedstock availability. *Bioresour Techno.* 2010; **101**: 4826-33.
14. Delgenes J P, Moletta R & Navarro, J M. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stiptis, and Candida shehatae. *Enzyme Microb Technol,*1996; 19: 220-225.
15. Ahring B K, Jensen K T, Bjerre A B & Schmidt A SPretreatment of wheat straw and xylose and xylan into ethanol by thermophilic anaerobic bacteria. *Bioresource Technol.* 1996; **58**: 107-113
16. Olofsson K, Bertilsson M & Liden G. A short review on SSF-an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol. for Biofuels*, 2008; **7**: 1-14.