Regular Article

Depolymerization of bagasse by *Ruminococcus albus* in the production of eco-friendly fuel

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Ethanol production by fermentation of lignocellulosic biomass-derived sugars involves a fairly ancient art and an ever-evolving science. Production of ethanol from lignocellulosic biomass is not avant-garde, and wood ethanol plants have been in existence since at least 1915. Most current ethanol production relies on starch- and sugar-based crops as the substrate; however, limitations of these materials and competing value for human and animal feeds is renewing interest in lignocellulose conversion. 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 by using *Ruminococcus albus* isolated from rumen of herbivores animals. The processing and utilization of the lignocellulosic substrate is complex, differing in many aspects from crop-based ethanol production. Since the scientific dogma states that the breakdown or depolymerisation of lignocellulose can be achieved only by chemical or by enzymatic approach; in the present study we isolated *Ruminococcus albus* from rumen animals which was depolymerise cellulose and hemicellulose as well as to tolerate stress conditions. Optimum temperature, pH and substrate concentration for depolymerization were 39[0]C, 8.8 and 3.5% respectively for *Ruminococcus albus*. For the feed stock of concentration 3.5%, ethanol yield of 19.8g/l was obtained.

Key words: Depolymerisation, Lignocellulose, *Ruminococcus albus,* ethanol

Bioethanol produced from renewable biomass has received considerable attention in current years. Using ethanol as a gasoline fuel additive as well as transportation fuel helps to alleviate global warming and environmental pollution (Assant, 2004) In the last decade, most research has tended to focus on developing an economical and ecofriendly ethanol production process. Much emphasis is being given to the production of ethanol from agricultural and forestry residues and other forms of lignocellulosic biomass (Kadam *et al*., 2000). Changes in how agricultural field residues are managed further complicate farming economies. In the past, disposal of straw by burning was an accepted practice. This practice is now being challenged due to concern over the health effects of smoke from burning fields.

Further the cellulosic plant material represents an as–of–yet untapped source of fermentable sugars for significant use, especially non-food lignocellulosic waste products like wheat straw, rice straw, baggasse, rice husk etc. In these waste products, the polysaccharides, cellulose and hemicellulose are intimately associated with lignin in the plant cell wall (Balterini *et al.*, 1994). The lignin component acts as a physical barrier and must be removed to make the carbohydrates available for further transformation processes. Therefore, the pre-treatment is a necessary process for utilization of lignocellulosic materials to obtain ultimately high degree of fermentable sugars. Bio-conversion of cellulosic biomass into fermentable sugar, for production of ethanol using microorganisms makes bioethanol production economic and environmental friendly.

Cellulose is the major constituent of organic matter of plant origin. Lignocellulosic materials are most abundant and renewable resources on earth, which makes them attractive for production of ethanol. Pre-treatment is an important tool for practical cellulose conversion processes. Pre-treatment is required to alter the structures of cellulosic biomass to make more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars (Mosier *et al.*, 2005) and to cellulase producing microorganisms. There are several ways to increase the digestibility of cellulose before it is exposed to enzyme or microbial conversion: mechanical, physical chemical or biological pretreatment, as well as the combination of these methods (Bollok *et al.*, 2000).

In this study, an attempt is made to optimize various dependent parameters to depolymerise bagasse by using *Ruminococcus albus* isolated from rumen of herbivores animals

Materials and Methods

Raw materials **:** Bagasse from local sugar factory (Samson's Distilleries, Davangere). Raw material was powdered and sieved into a 1mm seiver. Powder bagasse was used as carbon source.

Microorganisms: *Ruminococcus albus* was isolated from rumen of herbivorous animals. Isolation and characterization was done as per the standard microbiological techniques

*Inoculum preparation***:** Ruminococcus albus 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***:** 8g maize was taken in conical flask 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 *Ruminococcus albus* strains. These flasks were incubated at 37^0C 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 (Abdul *et al.*, 1999). 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 (Thimmaiah *et al.*, 1999).

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 (Ghose, 1987).

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 (Thimmaiah *et al.*, 1999).

FPU assay: Cellulase enzyme production was studied by FPU assay (Miller, 1959)

Fermentation: Saccharomyces cereviseae strain was inoculated into the culture filtrate and allowed for fermentation for 36h (Sandhu *et al.*, 1998). After fermentation it was filtered and subjected for distillation for ethanol at 80^0C.

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. 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 (Statistical Assistant, 2008).

Results & Discussion
Total sugar, reducing sugar, non reducing sugar, organic carbon, Nitrogen, total solids, moisture content of bagasse was determined. Initial composition of raw material is given in the table 1. FPU activity of *Ruminococcus albus* for bagasse is given in the table-2. *Ruminococcus albus* the best cellulolytic anaerobic bacteria was isolated from rumen of herbivores animals and cultured on bagasse 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. Figure 1 illustrates the enzyme activities over 78 h period. There was progressive increase in enzyme activity from 24 to 72 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 (Lynd *et al.,* 2002).

Depolymerisation of bagasse powder over the 80 h is illustrated in Figure 2. There was increase in saccharification from 0 to 66th h. The increase was steeper up to 12th to the 66th 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 (Xang *et al.*, 2006). 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 Figure 3. There was increase in reducing sugars with increase in substrate concentration. The highest mean glucose concentration of 17.4 mg/ml was recorded for substrate concentration of 3.5% 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 substrate concentration.

Table 1: Initial composition of the raw materials

Bagasse (Raw material)	Initial composition
Alpha cellulose (%)	39.24
Total sugar (mgg^{-1})	1.3
Reducing sugar (mgg^{-1})	0.175
Non reducing sugar (mgg^{-1})	0.125
Moisture (%)	8.34
Total solids (%)	91.66
Organic (%)	36.18
Nitrogen (%)	0.448

Table 2: Effect of *Ruminococcus albus* treatment on feed stock

Bagasse (Raw material)	Composition after treatment with bacteria
Alpha cellulose (%)	28.96
Total sugar (mgg^{-1})	30.0
Reducing sugar (mgg^{-1})	28.75
Non reducing sugar (mgg^{-1})	1.25
Protein (mgg^{-1})	8.8
FPU (IUml^{-1})	0.9
Ethanol (gl^{-1})	19.82
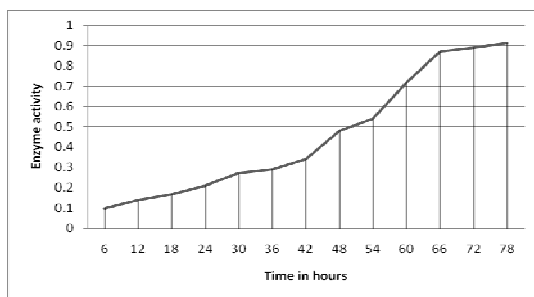
Figure 1. Enzyme activity of *Ruminococcus albus* in Bagasse broth over 78 h period
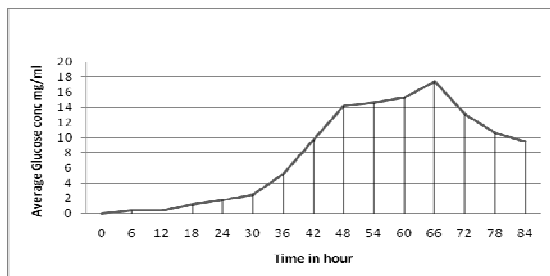
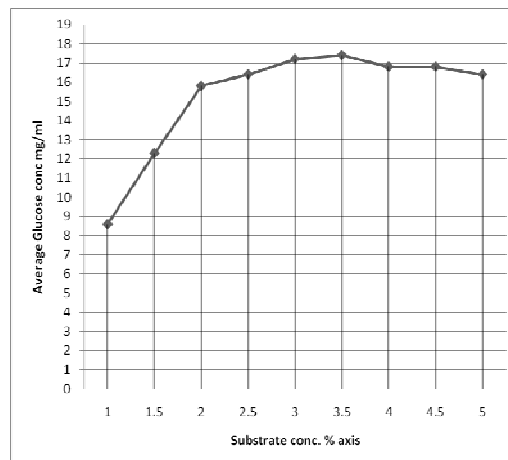**Figure 2.** Time course for Depolymerisation of Bagasse powder by *Ruminococcus albus*

Figure 3. Effect of Bagasse powder substrate concentration on Depolymerisation by *Ruminococcus albus*
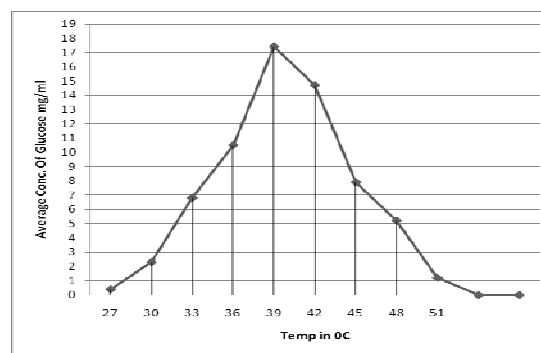

Figure 4. Effect of temperature on Depolymerisation of bagasse powder by *Ruminococcus albus*
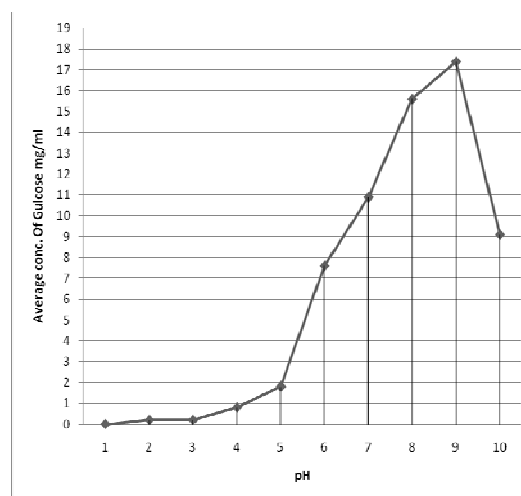

Figure 5. Effect of pH on Depolymerisation of bagasse powder by *Ruminococcus albus*

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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 and the decline after the optimum due enzyme denaturation (Shuler *et al.*, 1997, Chaplin *et al.*, 1998). Effect of temperature on saccharification is shown on Figure 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 (Nester *et al.*, 2001, Chaplin *et al.*, 1998). Hence an optimum temperature of 39°C is maintained in this study.

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 (Nester *et al.*, 2001, Chaplin *et al.*, 1998). Figure 5 illustrates the effect of pH on release of reducing sugars from the substrate. Depolymerisation increased from pH 8.5 to a maximum of 8.8 after which it decreased up to 10.0. The highest saccharification which was significantly different was recorded at pH 8.8. This makes the isolate halophilic (Olofssom *et al.,* 2008). Saccharification was least significant at pH 5 and 10, thus the optimum pH was 8.8 was maintained in our study.

Ethanol yield was 19.82g/l for bagasse (Fig-7, table-2). 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 (Olofssom *et al.,* 2008).
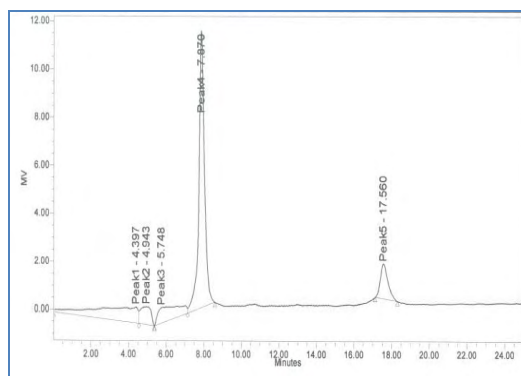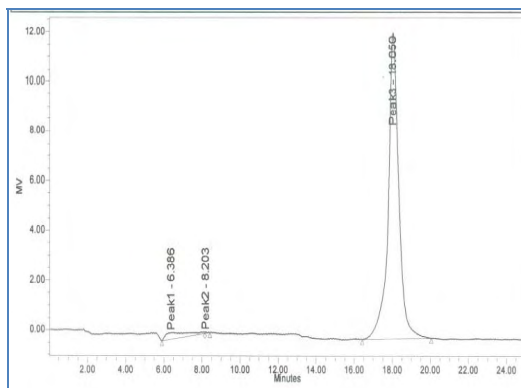

Fig 6: HPLC result showing Glucose concentration

Fig 7: HPLC result showing Ethanol concentration

Conclusion

The optimization test has shown that the *Ruminococcus albus* is an efficient lignocellulosic depolymeriser. Without using physical or chemical methods of pre-treatment it was possible to efficiently depolymerise bagasse to get the highest mean ethanol concentration of 19.8g/l using biological process.

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