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Bioremediation of sago industry waste

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ABSTRACT

Bioremediation of cassava bagasse was investigated using individual and co-cultures. Two individual strains *Bacillus pumilus* and *Cellulomonas cellulans* with cellulolytic activity were examined. Concentration of reducing sugar and carboxy methyl cellulase (CMCase) activity by these microbes were determined regularly. Maximum CMCase activities achieved were 0.51 and 0.55 U/mL with *B. pumilus* and *C. cellulans*, respectively. Four different combinations, namely *B. pumilus + S. cerevisiae, B. pumilus+ Z. mobilis, C. cellulans + S.cerevisiae* and *C. cellulans+ Z. mobilis* were investigated for ethanol production by simultaneous saccharification and co-fermentation of cassava bagasse. The end product ethanol was found to be in the following order *C. cellulans+ Z. mobilis > B. pumilus+ Z. mobilis > C. cellulans + S. cerevisiae > B. pumilus + S. cerevisiae*. In the case of solid state fermentation with 80 % moisture, *Z. mobilis* showed better fermentation and ethanol yield of 4.3 g/g ethanol and 4.7 g/g ethanol in combination with *B. pumilus* and *C. cellulans* respectively.

KEYWORDS

Bioremediation;
Cassava bagasse;
Cellulase activity;
Ethanol production;
SSF.

INTRODUCTION

Agro based raw materials are used by several industries in India. Water and solid wastes from these industries pollute air, water as well as land. Smoke, particulate emission and odor released before and after processing of raw materials are principal air pollutants. Effluent and waste water discharged from these industries pose major threat through water pollution. Processed agro based raw materials are discharged as solid wastes; these wastes pollute the soil if they are not utilized or recycled properly. Curative strategies to minimize these pollution problems include (i) developing end-of-pipe treatment, (ii) minimizing waste and (iii) recycling waste as energy source for biofuel production. The treatment processes followed for solid waste management must be cost effective and feasible[1].

Cassava (*Manihot esculenta*), one of the drought resistant crop, has starch rich tubers and serves as a foremost staple food in Asian and African countries. High viscosity, clarity and freeze-thaw stability of cassava starch had found its application in food industry, pharmaceuticals, textile industry, beverages, adhesives and building materials. After separation of starch from

FULL PAPER

cassava tubers, the residue contains about 55-60% of starch that remain inseparable with cellulose fibers[2].

Owing to the processing cost and lack of technology, cassava based industries dispose the residue as land fills. Due to its high BOD and COD level, the disposed lands fills cause pollution and affect health and quality of soil[3]. The chemical and biological reaction between continuously rottening peel and residue affect the natural micro flora and soil quality. The residues are also mixed with cattle feeds as they cannot supplement nutrients directly to cattle due to very low nitrogen content. Researchers have tried to improve the nutritional content of residue for animal feed[4, 5]. A review by Pandey et al. (2000) discussed elaborately about cassava bagasse and extraction of value added products like enzymes, lactic acid, aroma compounds and ethanol[6].

Biomass generated from various agro based industries, lignocellulose rich plant biomass sugarcane molasses, paper and pulp industry waste, etc. have been used for the generation of renewable energy[7, 8]. Energy generated from biomass served as a cheap and easily available source benefiting economic growth and development of nation[9, 10]. The starch factory residue with high amount of starch, cellulose and low lignin content could be a suitable low cost substrate for production of many value added products[11]. Major constraint in utilization of lignocellulosic waste is break down of complex cellulosic substrates into simple fermentable sugars. Different physical, chemical, microbial and enzymatic methods had been attempted by researchers for complete utilization of lignocellulosic material[2, 12–14]. Physical, chemical and enzymatic methods were expensive and energy consuming process, whereas microbial degradation could benefit extraction of value added products at low cost.

This current paper focuses on solid-state fermentation of sago industry waste for enzyme and ethanol production. The work aimed at economical way of degrading cellulose by exploiting cellulolytic bacteria *Bacillus pumilus* and *Cellulomonas cellulans* for celllulase production. The hydrolysed product was simultaneously fermented using *Zymomonas mobilis* and *Saccharomyces cerevisiae*. Solid state fermentation was opted as the chosen coculture could ferment the sago waste under low-shear environment and require less capital investment. The cellulolytic activity and time taken for cellulose hydrolysis were investigated by simultaneous saccharification and fermentation (SSF) with co-cultures.

MATERIALS AND METHOD

Organisms and culture conditions

Bacterial strains for the present work were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. *Bacillus pumilus* (MTCC 7514) and *Cellulomonas cellulans* (MTCC 1769) lyophilized cultures were suspended in sterile distilled water and grown on nutrient agar (beef extract 1.0 g/L, yeast extract 2.0 g/L, peptone 5.0 g/L, NaCl 5.0g/L and agar 15.0g/L). Sub-cultures of these bacterial strains were prepared by suspending a loop full of seed culture in nutrient medium. *Zymomonas mobilis* (MTCC 90) was maintained on Yeast Extract Glucose Salt Agar (Yeast extract 10.0 g/L and Agar 15.0 g/L in 100 mL of sterilized glucose solution (20% w/v) and 10 mL each of stock solutions of $MgCl_2$, $(NH_4)_2SO_4$ and KH_2PO_4). Sub-culture of *Z. mobilis* in the liquid medium was prepared with same composition without agar. Commercially available baker's yeast (*Saccharomyces cerevisiae*) was purchased from market and grown in medium containing yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L. Crude α amylase from laboratory culture, *Aspergillus niger* was used for enzymatic hydrolysis of starch. All the media components were prepared using distilled water sterilized at 121 °C, 15 psi.

Preparation of raw material and fermentation medium

Cassava (*Manihot esculenta*) bagasse, a solid residue, was collected from a nearby Sago industry in Salem, Tamil Nadu. It was sun dried for 48 h, powdered and sieved with standard mesh size of BSS # +25-30 for further use. Mineral salt solution containing KCl 0.2 g/L, $(NH_4)_2PO_4$ 1 g/L, $MgSO_4$. $7H_2O$ 0.2 g/L, $CaCO_3$ 2 g/L, K_2HPO_4 0.5 g/L and KH_2PO_4 0.5 g/L was used to supplement mineral ions to microorganism. Cassava bagasse, mineral salt solution and water were sterilized separately at 121 °C, 15 psi for 15 min. Cassava bagasse was then treated enzymatically with α amylase at

80ÚC with pH of 5.8 for 1h and used throughout this study. Moisture content was varied as 20, 40, 60, 80 (v/v) percentages for solid state fermentation.

Cassava bagasse (10 g) was taken in 250 mL conical flask for batch mode of solid state fermentation and 10 mL of mineral salt solution was added to all flasks. Moisture content was adjusted with sterilized distilled water and pH was adjusted to 6.5 with 1 M NaOH solution. After sterilization, 5% innoculum (*B. pumilus* 10^8 CFU/ml, *C. cellulans* 10^7 CFU/ml, *Z. mobilis* 10^9 CFU/ml and *S. cerevisiae* 10^8 CFU/m) was added to the flask under sterile conditions. The flasks were maintained at temperature of 30 ± 3 °C in a sterile room and assayed periodically.

Analysis

Cassava bagasse was analysed for its monosaccharide and polysaccharide composition. Lane and Eynon constant titre method was used for detemination of Dextrose Equivalent (DE)[15]. Starch composition was determined according to method adopted by Hodge, J E et al. Total cellulose was estimated by anthrone method[16]. Ash content was determined by heating the bagasse at 550 °C for 2 h. Total reducing sugar was estimated using 5-dinitrosalicylic acid (DNS) method developed by Miller (1959)[17]. Amount of starch and cellulose in the sago industry cassava waste was determined by anthrone method[18]. As solid state fermentation is a heterogeneous process composite samples were taken and used for analysis. That is, every time samples from different locations were collected and mixed well to ensure homogeneity and that the sample taken is a true representative sample, Collected sample was then weighed (1 g) and mixed well with citrate phosphate buffer (pH 5) and centrifuged at 10000 rpm for 10 min. The supernatant was analyzed for reducing sugar colorimetrically using dinitro salicylic acid procedure[17]. Supernatant was also assayed for cellulase activity with carboxymethylcellulase (CMC). One percent CMC solution (1ml) and 1 ml of 0.1 M citrarte buffere were added to 1 ml of supernantant and maintained at 50 °C for 30 min. The reaction was stopped by adding 3 ml of DNS solution and analyzed by Milled method. One unit of CMCase is the amount of enzyme required to liberate 1 µmol of glucose equivalent in 1 ml of solution/min). Ethanol estimation was done according to the method of Caputi et al. (1968) with chromic acid[19]. Concentration of ethanol in the supernatant after fermentation was determined colorimetrically. Systronics spectrophotometer 2201 was used for recording the optical densities of above mentioned analysis.

EXPERIMENTAL

Cellulolytic bacteria namely *Bacillus pumilus* and *Cellulomonas cellulans* were inoculated to cassava bagasse taken in conical flasks with different moisture content (20 – 80%). They were analyzed for cellulase activity on hourly basis and time for maximum activity was recorded. The enzyme activity and total reducing sugar concentration were also determined. Simultaneous saccharification and fermentation was then carried out separately by inoculating cellulolytic bacteria and fermenting microorganisms namely *Zymomonas mobilis* and *Saccharomyce*. The fermented product was assayed for amount of ethanol produced. Temperature of 30 ± 3 °C and pH 6.5 were maintained during microbial hydrolysis and fermentation with varying moisture content from 20 – 80%.

RESULT AND DISCUSSION

COMPOSITION OF CASSAVA BAGASSE

Cassava bagasse from the sago industry was analyzed for its composition. The biochemical analysis shows that the residue was rich source of starch (59.1%) and cellulose (31.8%) with 7.4% moisture. It also contained about 68% carbon and 1.7% ash. Total reducing sugar estimated after enzymatic treatment was 5.7 g/g of dry substrate with DE 16. From the analysis cassava bagasse proved to be a value added agro-industry waste that could be used for production of value added products.

Cellulolytic activity of bacillus pumilus and cellulomonas cellulans

The cellulase produced by hydrolytic action of bacteria were examined. Figure 1(a) and 1(b) depict the CMCase activity of two cellulolytic bacteria. In both the figures the trends of enzyme activity were almost similar having exponential increase up to 12 h and then remained constant. From the CMCase activities, as

FULL PAPER

reported in the figure, it could be seen that the organisms degraded cellulose content of the cassava bagasse. It is hypothesized that the organisms initially grow by consuming the readily available carbon source, starch hydrolysate, and later produced cellulase to utilize cellulose.

From these results, cassava bagasse appeared to be a promising low cost substrate for cellulase enzyme production.

Cellulose hydrolysis and total reducing sugar concentration

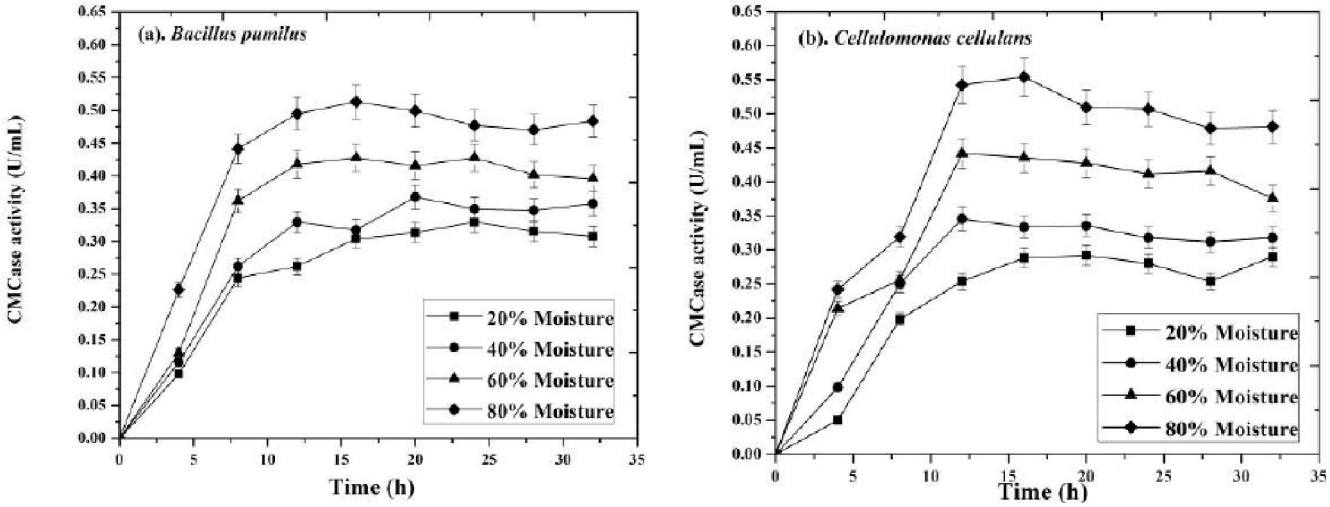

Figure 1 : CMCase activities of (a) Bacillus pumilus and (b) Cellulomonas cellulans. [Moisture content: 20 – 80 %; temperature: ambient temperature, 33±1 °C]

The moisture content of the substrate played a great role in influencing the microbial growth and metabolism. The moisture contents at 20 and 40% did not influence on bacterial growth whereas 60 and 80% moisture supported the growth and utilization of the substrate. This had resulted in the increase in enzyme activity at 80% moisture with 0.51 U/mL CMCase activities from *B. pumilus* and 0.55 U/mL from *C. cellulans*.

Cassava bagasse autoclaved at 121 °C, 15 psi for 15 min could be considered as a hydrothermal pretreatment of substrate[2]. During this stage, partial starch hydrolysis and weakening of cellulose fibers may take place. This facilitated the microbial degradation of the solid waste. *B. pumilus* and *C. cellulans* effectively acted on the bagasse and converted it to reducing sugars. Reducing sugar produced by the metabolic path-

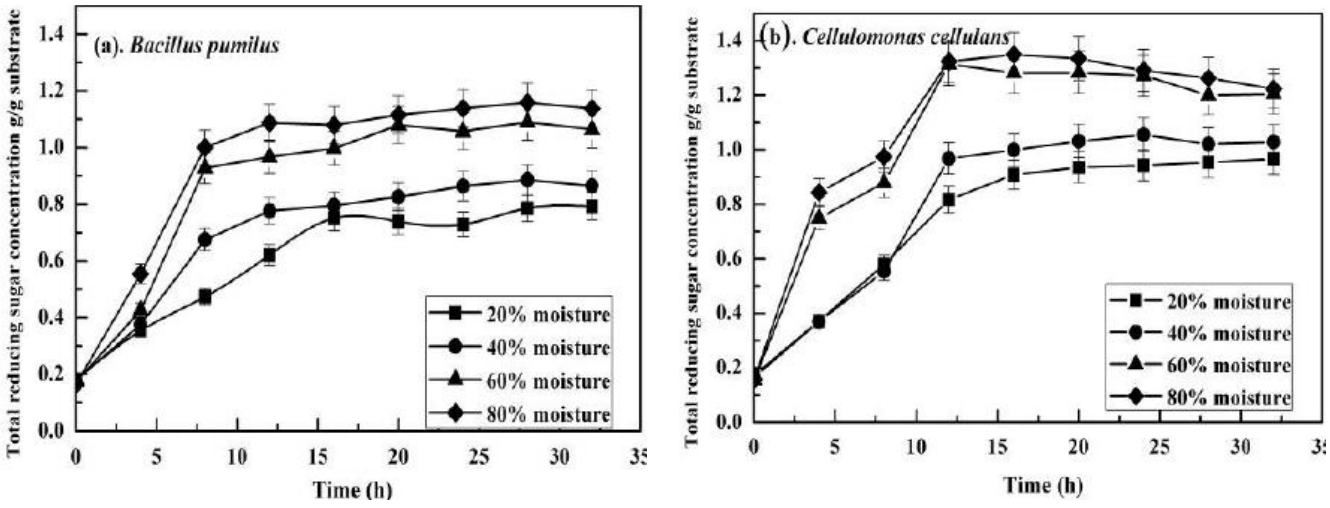

Figure 2 : Reducing sugar concentration of microbially hydrolyzed cassava bagasse by (a) Bacillus pumilus and (b) Cellulomonas cellulans [Moisture content: 20 – 80 %; temperature: ambient temperature, 33±1 °C]

way of *B. pumilus* and *C. cellulans* are shown in Figure 2 (a) and (b) with initial reducing sugar from enzymatic treatment taken as blank.

Maximum concentration of reducing sugar was attained with 80 % moisture content after 12 h of degradation. 1.1 and 1.3 g/g of substrate from B. pumilus (Figure 3(a)) and C. cellulans (Figure 3(b)), respectively, moisture content played a significant role in microbial hydrolysis by solid state fermentation. The 80% moisture had nearly increased concentration of reducing sugar by two folds when compared with 20% moisture. Both bacterial species were efficient in converting cellulosic fibers to simple sugars[11, 20, 21].

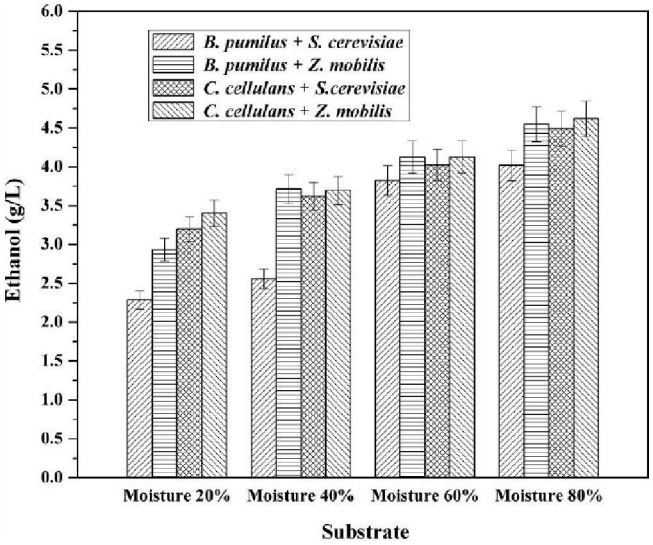

Figure 3 : Comparison of ethanol yield from SSF of cassava bagasse with four co-cultures after 48 h 20- 80% moisture, pH 6.5 at 30 °C

Simultaneous saccharification and fermentation

Ethanol production by SSF was investigated in solid state fermentation. Ethanol yield at 48 h is shown in Figure 3.

Figure 3 shows that SSF of cassava bagasse with co-culture of *Z. mobilis* with two cellulolytic bacteria resulted in higher ethanol yield than those with *S.cerevisiae* under similar conditions.. The 80% moisture had favored ethanol production in all the four co-cultures stated above. Maximum yields obtained were: 3.7 g/g ethanol with *B. pumilus + S. cerevisiae*, 4.3 g/g ethanol with *B. pumilus+ Z. mobilis*, 4.1 g/g ethanol with *C. cellulans + S.cerevisiae* and 4.7 g/g ethanol with *C. cellulans+ Z. mobilis*. Theoretical ethanol yield was estimated to be 5.3 g/g ethanol for dry cassava substrate.

The fermentation profile was different in case of fermentation with *S.cerevisiae* and *Z. mobilis*. After 48 h, rate of ethanol production started to decrease in *S.cerevisiae* co-cultures as ethanol inhibited yeast growth[22]. But in case of *Z. mobilis* the production pathway remained constant even up to 95 h (data not shown). Theoretical yield of ethanol from substrate with 80% moisture was tabulated in TABLE 1. It was clearly understood with these results that using co-cultures of *Z. mobilis* and *C. cellulans* or *B. pumilus*, better conversion of cassava bagasse could be achieved.

TABLE 1 : Observed and theoretical yield of ethanol from cassava bagasse with 80% moisture

Microbial culture combination	Observed ethanol (g)	Theoritical yield (%)
B. pumilus + S.cerevisiae	3.7	69.8
B. pumilus + Z. mobilis	4.3	81.1
C. cellulans + S.cerevisiae	4.1	77.4
C. cellulans + Z. mobilis	4.7	88.7

CONCLUSION

Bioremediation of cassava bagasse was investigated using individual and co-cultures. Two individual strains *Bacillus pumilus* and *Cellulomonas cellulans* were examined for their cellulolytic activity. Both organisms showed CMCase activity upto 0.51 and 0.55 U/mL by *B. pumilus* and *C. cellulans*, respectively. Maximum concentration of reducing sugar was attained with 80 % moisture content after 12 h of degradation at 1.1 g/g of substrate for *B. pumilus* and 1.3 g/g of substrate for *C. cellulans*. Among four co-cultures, maximum ethanol production was obtained from SSF of cassava bagasse with *C. cellulans+ Z. mobilis* followed by *B. pumilus+ Z. mobilis, C. cellulans + S.cerevisiae* and *B. pumilus + S. cerevisiae*.

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FULL PAPER

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