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RESEARCH ARTICLE

PRODUCTION OF BIOETHANOL AFTER HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS INTO SUGARS USING *ZYMOMONAS MOBILIS* AND *SACCHAROMYCES CEREVISIAEISOLATED* FROM WINE SAMPLE

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ABSTRACT

Cellulase is an inducible enzyme complex, produced by a number of bacteria, actinomycetes and fungi including *Trichoderma* species and *Aspergillus* species etc. Corn husk which is composed mainly of cellulose. It is a major component of agricultural and domestic waste. It can be converted to bioethanol, alternative source of energy. Production of cellulase was induced in the fungi *Aspergillus nigerby* growing it in mineral salt medium and alkali pre-treated corn husk. The product of hydrolysis was fermented with the help of *Saccharomyces cerevisiae* and *Zymomonasmobilis*. The cellulosic hydrolysate yielded 1.78 gl^{-1} sugars, from which got 9.10 gl^{-1} ethanol, which obtained after fermentation using *Zymomonasmobilis*. This is compared to ethanol yield produce by *Saccharomyces cerevisiae* which was just 8.20 gl^{-1}. Thus, our study has shown that corn husk hydrolyzed with cellulase extracted from *A. niger* with the help of *Z. mobilis* and *S. cerevisiae* isolated from red wine could be a perfect source of bioethanol.

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INTRODUCTION

Among the all transportation fuel, the importance of ethanol is a clean and safe transportation fuel which has increased with the anticipated shortage of fossil fuel reserves and create less air pollution too. The continuous depletion of the fossil fuel reserves and consequent hike in their price has stimulated an extensive evaluation of alternative technologies to find out different substrates to meet the global energy demand (Cazetta *et al*., 2007). Ethanol can be readily produced from agriculture based renewable materials like sugarcane juice, molasses, cassava and sweet potato starch and bagasse (Agrawal *et al*., 2011). Corn huskproduces about 30 % of maize agro-wastes throughout the world. Bioethanol industry are the focus of many researches aimed at achieving an effective and efficient waste management scheme by using his corn husk maize waste (Anase *et al*., 2005). The corn husk are burnt as fuel in households of peasant rural farmers which leads to severe environmental pollution. Corn husk contain 32.3-45.6 % cellulose, 39.8 % hemicelluloses and 6.7-13.9 % lignin which can be converted to fermentable sugar for ethanol production via enzymatic and microbial action (Gunasekaran *et al*., 1999). So, the present research involved the production of bioethanol from cornhusk by the action of cellulolytic fungi *Aspergillus niger* and combined activity of *Zymomonasmobilis* and *Saccharomyces cerevisiae,* isolated from red wine.

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MATERIALS AND METHODS

Sample collection: A freshly tapped red wine was purchased from a wine market. The red wine was used fresh after it was tapped. A sterile polythene bag was used to collect a decaying corn husk at a dumpsite in Bangalurumetropolitan city. The cornhusk and red wine were taken to the microbiology laboratory, where they were further analyzed.

Isolation of microorganisms: The test organisms including *Aspergillus niger, Zymomonasmobilis* and *Saccharomyces cerevisiae* were isolated based on standard microbiology techniques (Cheesbrough, 2006). Briefly, Twenty (20) ml volume of the red wine was centrifuged at 5000 rpm for 10 minutes, and a loopful of the red wine sediment was aseptically collected using a wire loop, and this was inoculated

on already prepared Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) growth media supplemented with 300 mg of Ketoconazole and 500 mg of chloramphenicol to inhibit the growth of saprophytic fungi and bacteria respectively. After inoculation, the Petri dishes were packed in an anaerobic jar, and incubated at 37°C for 48 hrs for fungal growth and isolation. The suspect colonies were sub cultured onto freshly prepared culture media plates for the isolation of pure isolates. The identification and characterization of the pure bacterial isolates were based on morphological, physiological and biochemical tests as per Cheesbrough,2006 and Cazetta *et al*., 2007.

Isolation of *Aspergillus niger*from dumpsite cornhusk

A sterile polythene bag was used to collect a decaying cornhusk at a dumpsite in Bangaluru. The cornhusk was taken to the laboratory after which 10 g of corn husk was immersed in 100 ml of distilled water. A fourfold serial dilution of remnant of cornhusk water immersion was carried out and cultivation was done on potato dextrose agar (PDA). Pour plate method was used to inoculate the growth medium prepared with 1 ml of the diluted sample and were incubated for a period of 4 days at 37 ° C. Sub culturing was carried out until pure cultures of *Aspergillus niger* was obtained and the pure *Aspergillus niger* colony was used to produce cellulase. After 4 days of cultivation, a small portion of the mycelia growth was carefully picked with the aid of a sterile wire loop and placed on a drop of lacto phenol cotton blue on a microscope slide and covered with a cover slip. The slide was examined under the microscope with (40x) objective lens for morphological examination as described by Cheesbrough (2006).

Screening of *Aspergillus niger* for cellulase activity

Aloopful of growth culture of isolated colonies was inoculated on 3.9 g of Potato dextrose Agar (PDA) mixed with 0.2 g Carboxyl Methyl Cellulose (CMC), dissolved in 100ml distilled water. The PDA plates were incubated for 3 days at 37°C for *Aspergillus* isolate and observed for growth. To undertake ammonia steeping, 20 g of milled cornhusk of particle size of 2 mm was mixed with 100 ml 2.9 M NH_4OH solution in a 250 ml Erlenmeyer flask. The mixture was then incubated in a shaker for 24 hr at 30°C. The content was filtered using a 2µmWhatman filter paper into 250 ml Erlenmeyer flask. It was further rinsed twice using distilled water. The cornhusk were then dried at 30°C in an oven overnight (Cazetta *et al*., 2007).

Production of enzyme

Spores of *Aspergillus niger* were harvested by flooding 1 week old stock culture on agar slants with sterile distilled water (5 ml) as per Martin *et al.,* 2002. The inoculums medium (100 ml) comprised of Mandels mineral salt, was inoculated with the entire spore suspension and incubated on a rotary shaker (150 rpm) at room temperature for 72 hrs. Each 250 ml flask containing 100 ml of Mandels mineral salt medium, into which was incorporated 10 g of cellulosic materials i.e. alkali pre-treated and untreated corn husk; and Carboxyl methyl cellulose (CMC). The medium was inoculated with inoculums culture (5 ml) containing approximately 4.6 × 106 spores/ml of the fungi and incubated at 28°C on a shaker incubator (150 rpm) for 10 days and enzyme filtrates were obtained (Cazetta *et al*., 2007; Anase *et al*., 2005).Cultures were centrifuged and stored at -10°Cfor further use.

Assay for Cellulase activities

Reaction mixture comprising of 0.5ml carboxyl methyl cellulose (2 % w/v) in 0.05M citrate buffer, pH 4.8 and 0.5ml culture filtrate in test tubes. Mixture was incubated at 50°C for 1 hr. After incubation 1ml of di-nitrosalicyclic acid reagent (DNSA) was added to stop the reaction. The reactants in test tubes were boiled for 5 mins in boiling water bath and transferred to cold water bath. 10 ml of distilled water was added and absorbance was measured at 540 nm in spectrophotometer. Amount of reducing sugar was read from a curve obtained by plotting value of O.D. against concentration (Cazetta *et al*., 2007). One unit of enzyme activity is defined as the amount of enzyme that released 1 µm of reducing sugar.

Bioethanol production

Bioethanol production from the cornhusk was microbiologically analyzed using previous methodology; and the methods employed in this assay included enzyme hydrolysis, fermentation and distillation process as previously described by Anase *et al*., 2005; Oyeleke *et al*., 2012.

Hydrolysis of corn husk with the partially purified cellulose of isolated fungi

The method of Oyeleke *et al.* (2012) was demonstrated. Citrate buffer (100 ml) containing alkali treated corn husk (10 % w/v) in 250 ml Erlenmeyer flask was inoculated with 10 ml of the partially purified filtrate and incubated at 50°C for 7 hrs. Samples (1 ml) was withdrawn aseptically from flask at 1 hr interval and analyzed for reducing sugar by the DNSA method to determine the optimum time in hours for cellulose digestion of corn husk. The hydrolysate was then used for fermentation to produce ethanol.

Fermentation of the products of cornhusk digestion to ethanol

The fermentation of the products of cornhusk digestion to ethanol was carried out as perdescribed by Oyeleke *et al.,* 2012. Yeast peptone dextrose broth (100 ml) in 250 ml Erlenmeyer flask was inoculated with pure colonies of yeast from agar slant with the aid of an inoculating loop. This was incubated at 28°C on orbital shaker at 150 rpm for 48 hrs. 0.3 g malt extract, 0.3 g yeast extract, 2 g glucose, 0.5 g peptone and 0.002 g of acetone dissolved in water was inoculated with pure colonies of *Zymomonasmobilis* from agar slant with the help of inoculating loop. The fermentation broth (100 ml) comprising of (%w/v), Peptone, 8 g; yeast extract, 8 g; and the product of hydrolysis of cornhusk as the fermenting sugar. The broth (80 ml) was filled into a 100 ml of culture bottle, sterilized in an autoclave and inoculated with 10 ml of each of the inoculums. The bottles were sealed with the help of an adhesive tape and incubated at 28°C for a period of 8-48 hrs. Bottles were

removed at 8 hrs intervals to determine the amount of ethanol produced and the residual sugar in the medium.

Determination of ethanol concentration

The fermented broth was assayed for ethanol using the acidified dichromate-thiosulphate titration method. Tenml acid dichromate solution (0.01 M in 5.0 M sulphuric acid) was placed in 250 ml Erlenmeyer flask. This was connected to 10 ml of fermented broth in another 250 ml Erlenmeyer flask which was placed in water bath set at 80°C. The set up was allowed to stand for 3 hrs, during which ethanol produced by the fermentation of the broth evaporated into the acid dichromate solution. All the flasks were fixed with rubber stopper and sealed with wax to avoid leakages. After the incubation period, the set up was dismantled, 100 ml distilled water and 1.0 ml Potassium iodide (1.2 M) was added to the dichromate solution.

This was then titrated with Sodium thiosulphate (0.03 M) until the brown color turned to yellow, at that point 1% starch solution (1 ml) was added as indicator of iodine and further titrated until the blue colour fades. Three flasks consisting of 10 ml acid dichromate was set up as blanks and titrated at first so as to calculate the volume of thiosulphate required. The difference between thiosulphate used in the titration of the blank and that of the sample was used, finally help in calculating the amount of ethanol (Cazetta *et al*., 2007).

Determination of residual sugar in the fermentation medium

The amount of sugar in the fermentation medium after each period of fermentation was determined following the DNSA method of Martin (2003). Dinitrosalicylic acid (DNSA) reagent (1 ml) was added to an aliquot (1 ml) of the fermentation medium in a test tube and properly mixed. The mixture was boiled for 5 mins and cooled under running tap water. Five ml of distilled water was added to the mixture and O.D. taken in spectrophotometer at 540 nm. Amount of reducing sugar was calculated from a standard glucose curve and expressed as mg ml^{-1}finally.

RESULTS

Cellulase production by *Aspergillus niger*

The result of this investigation showed that there was progressive increase in enzyme activity from 24 to 140 thhr after incubation. The highest cellulase activity was obtained on the 5th day (3.80mg /ml/sec) and the least on the first day (0.42 mg /ml/ sec) this corresponds to the day, having the highest and lowest biomass activities (Fig 1).

Effect of cellulase dosage on hydrolysis of cornhusk powder

The hydrolysis experiments was performed with $100gl^{-1}$ substrate and different dosage of the crude cellulase at pH 4.5 and 40°C. The reducing sugar concentration increased sharply with an increase in the cellulase dosage (Fig 2).

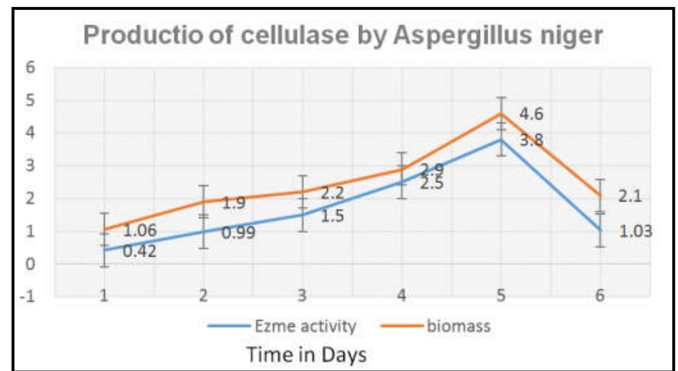


Fig. 1. Production of cellulase by *Aspergillus niger*

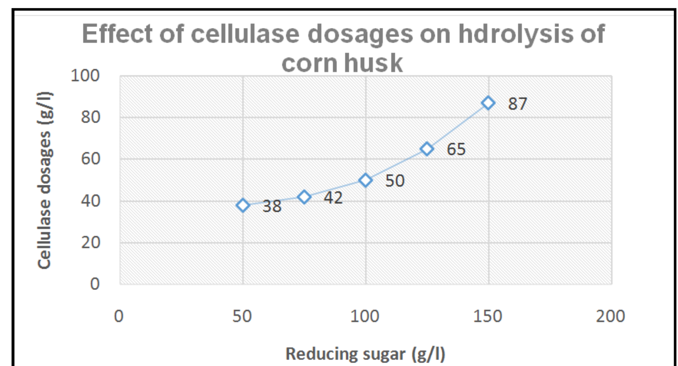


Fig. 2. Effect of cellulase dosages on hydrolysis of corn husk

Time course for saccharification of cornhuskpowder

The result of this experiment showed that there was an increase in saccharification of corn husk from 0 to 9th hrs. The increase was steeper up to 6th hr than from 6 to 7th hrs (Fig 3).

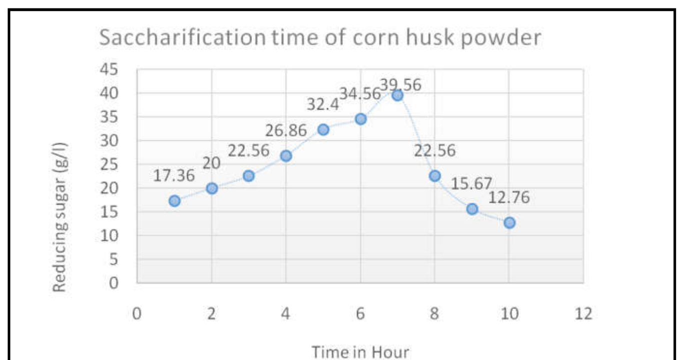
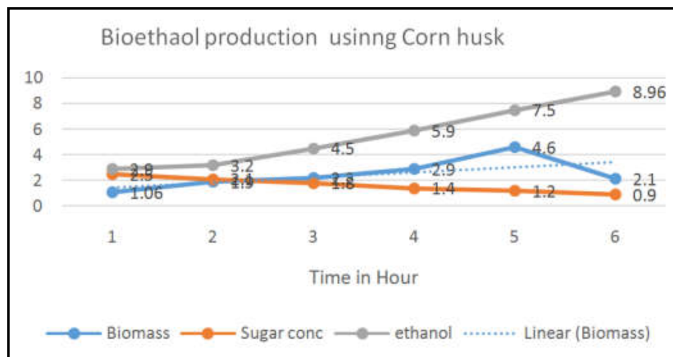


Fig. 3. Saccharification time of cornhusk powder

Production of bioethanol with the use of *Saccharomyces cerevisiae*

Our obtained result showed the production of ethanol with use of *S. cerevisiae*. The result showed that the value of the microbial cell density increased drastically from 8th hr up to the 32^{nd}hr, at which point a lag phase of its growth was observed. Also the result showed an increase in ethanol concentration from 0.9 gl^{-1} at the 8th hr to 12.3 gl^{-1} at the 40th hr, after which there was decrease in its concentration. The result also indicate that the sugar concentration inversely

decrease with an increase in ethanol concentration which was observed at 8-16 hrs and 24-48 hrs intervals, is the result of glucose utilization by *S. cerevisiae* either for biomass or ethanol production (Fig 4).



Fig, 4. Bioethanol production using cornhusk

DISCUSSION

Our study showed the production of bioethanol from corn husk which were hydrolysed by the cellulase activity of *A. Niger*. The hydrolyzed product then used as alternative source of feed stock for the production of bioethanol with the help of *Z. mobilis* and *S. cerevisiae,* were isolated from red wine. The results obtained from this study showed that *Saccharomyces cerevisiae* and *Zymomonasmobilis* are abundant in red wine. These organisms play critical roles in the conversion of sugar to alcohol (Cazetta *et al*., 2007). *Z. mobilis* found in red wine as a dominant alcohol producing organism; and it has also shown a high growth rate and tolerance to ethanol. *Zymomonas* species being an anaerobic, Gram negative, rod organism (Olsson *et al*., 1996). Fermentation process using *Zymomonas* species would require no aeration, whereas yeast require aeration (Lynd, 1996); and this helps to reduce fermentation cost. *A. niger*, isolated from cornhusk sample and was assayed for enzyme activity. The result showed a progressive increase in enzyme activity. Cellulase is an induced extracellular enzyme. Its production increased with increase in fungal biomass based on the incubation period and as simple sugar in the substrate utilized (Olofsson *et al*., 1996). Our result also showed that there was sharp increase in scarification from 0 to 10th hr. The increase was sharp up to 6th hr than from 6 to the 10th hr. The slowdown in rate for hydrolysis predicted to be due to the effect of the enzymes became slowed because of negative feedback activity (Olofsson *et al.,* 1996; Jacek, 2000; Jan, 2014). The rate of Saccharification is directly proportional to substrate concentration and usages till optimal substrate utilization. This is due to random collisions between the substrate and enzyme active sites (Solomon *et al.,* 2007). During fermentation, the ethanol as product increased, while the reducing sugar concentration decreased which indicate the fact that during fermentation by yeast (*S. cerevisiae*), utilized the sugar as a source of carbon and energy, and ethanol is produced as a result (Oyeleke *et al*., 2012, Cao *et al*., 1997; Solomon *et al.,* 2007). There was increase in ethanol production as increase in saccharificationdue to fermentation, which was responsible for making glucose available to the microorganisms (*S. cerevisiae* and *Z. mobilis*) for fermentation. Several similar studies showed that enzymatic hydrolysis of the solid fraction has a large control over the total rate of ethanol production as simultaneous saccharification and fermentation takes place (Olofsson *et al.,* 2008; Hansen *et al*., 2005; Lin *et al*., 2006). A high ethanol yield (49 %) was achieved in the fermentation of biomass hydrolysate by *Z. mobilis* compared to the yield obtained in presence of *S. cerevisiae* which (43 %) in our study. *S. cerevisiae*, however, consumes sugar for growth and production of other metabolites. Ethanol production inour study is comparable to previous results reported, where varying rates of ethanol where produced from local agro-wastes (Olofsson *et al*., 2008; Oyeleke *et al*., 2012).

Conclusion

In conclusion to say, ethanol was produced from cornhuskagro-wastes via the combined fermentative and activities of *A. niger, Z. Mobilis, S. Cerevisiae*. The cornhusk was found as viable feedstock for energy production, it hydrolyzed by cellulases of *A. niger* which was productive in terms of ethanol yield.

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