*Original Research Article*

# Utilization of Different Cellulosic Wastes for Low-Cost Ethanol Production by Immobilized Yeast Cells


## ABSTRACT

In comparison to fossil fuels, bioethanol has a larger potential for environmental safety and energy security. Bioethanol can provide the alternative source needed to meet the fuel's requirements. There are many untapped sugar-rich sources, such as cellulose-rich agricultural wastes, household wastes, industrial wastes, etc., that can be used to produce bioethanol at a low cost. The goal of the study was to investigate the use of various lignocellulosic wastes, such as soybean straw, gram straw, banana leaves, newspaper, and cow dung, treated with various pretreatments (steam and acid), to produce inexpensive ethanol using *Saccharomyces cerevisiae* NCIM 3095 strain cells that have been encapsulated. Each sample received the combined pretreatments (steam treatment and acid treatment). Following both pretreatments, the concentration of free sugar (cellulose) increased, indicating that the pretreatment was successful. Each sample broth containing calcium alginate-encapsulated yeast cells was supplemented with benzathine penicillin G to lessen bacterial contamination and ammonium sulphate to act as an external growth stimulator. Ten days were spent fermenting the samples. Every three days, the ethanol content was estimated. Soybean straw produced the highest output of bioethanol (10.0%), whereas cow dung produced the lowest yield (4.0%).



*Keywords: Cellulosic Wastes; Ethanol Production; Biofuel; Bioethanol*


## 1. INTRODUCTION

A renewable energy source created by the fermentation of sugars is ethanol. In addition to corn and sugarcane, numerous additional agricultural raw materials are used globally as feedstocks for ethanol production. However, it is difficult to convert lignocellulosic material into sugar, which necessitates advanced equipment and could raise operational costs. An ongoing effort has been undertaken to develop and enhance a method that would use inexpensive feedstocks to create a sustainable transportation biofuel. The only option to meet the demand for ethanol is to produce it from relatively less expensive sources of raw materials like domestic and agricultural wastes utilizing effective fermentative microorganisms. Other biomasses, such as starches and lignocelluloses, need to be processed to break down the tough cell walls and make sugars available for the following fermentation stage. Simple sugars, which can be immediately turned to ethanol, need to be pretreated. A mixture of fermentable sugars and possible inhibitory substances found in the

biomass structure are released during pretreatment and hydrolysis. The most efficient way to release simple sugars from cellulosic materials is through hydrolysis employing the right enzymes, however process rates must be improved and enzyme production costs must be reduced. Widely employed, diluted acid hydrolysis is a quick and practical method. However, due to the breakdown of sugar, dilute-acid hydrolysis is carried out at high temperatures and pressures and results in the creation of several byproducts. In the ensuing fermentation stage, these byproducts can have strong inhibitory effects on microorganisms like Saccharomyces cerevisiae. Encapsulated microbial cells can be used to combat the effects of these inhibitory chemicals, which also lessens issues with filtration and cell biomass separation.

## 2. MATERIAL AND METHODS

### 2.1 COLLECTION OF RAW MATERIALS

Soyabean straw (*Glycine max),* Gram straw *(Cicer arietinum),* Banana leaves *(Musa acuminata)*, Cow dung (Jersey cow), samples were collected from agriculture field and Newspaper from local shop.

### 2.2 PREPARATION OF RAW MATERIAL

The collected samples were chopped into small pieces and washed with distilled water to remove dust particles. They were shed dried for 24 hrs. And oven dried for overnight at 60ºC.The dried samples were grinded into powdered, packed in polyethylene bag. For all samples, fermentation broth was prepared by adding 20 gm of sample in 300 ml of distilled water in five different conical flasks, wrapped and labelled properly to avoid any mistake.

### 2.3 PRETREATMENT OF SAMPLES

To check effect of combined pretreatment i.e., steam and acid pretreatment on lignin breakdown and release of free cellulose from cell wall, all samples were firstly treated with steam treatment in autoclave for 30 mins at 15 psi. pressure. After steam treatment the samples were allowed to cool and the soluble portion was separated from the insoluble portion by using filtration by cellulose filter paper. For acid pretreatment, insoluble portion after filtration was treated with 25 ml of 1.50 % sulphuric acid($H_2So_4$) for 21.66 mins at 91.02 ºC. After hydrolysis, insoluble portion again separated by soluble portion by filtration by cellulose paper. Filtrate was transfer to previously filtered solution for next process.

### 2.4 CELLULOSE ESTIMATION BY ANTHRONE METHOD

Anthrone reagent was prepared by dissolving 200 mg of Anthrone in 100 ml of 95 % chilled sulphuric acid($H_2So_4$). The cellulose solution of 0.5 mg/ml concentration was used as standard solution for comparison.

Sugar (cellulose) concentration of samples before and after pretreatments was estimated by Anthrone reagent to check effectiveness of combined pretreatments. To get perfect result by measuring absorbance, samples were diluted in 1:2 (sample: distilled water) ratio. For each sample, 1 ml of sample extract and 3 ml of Anthrone reagent was added to test tube. Test tubes were incubated for 17 mins at 90ºC and reaction absorbance was measured at 670 nm in colorimeter. Increase in free cellulose concentration after pretreatments than before pretreatments indicate effective pretreatments.

### 2.5 INOCULUM DEVELOPMENT

The genetically modified yeast strain *(Saccharomyces cerevisiae* NCIM 3095) for ethanol production was procured from National Chemical Laboratory (NCL) Pune. The dry yeast cells were activated by inoculating in glucose solution (0.05 gm cells in 5 % 10 ml of glucose

solution) and kept on shaker for 15-20 mins at 110 rpm. The activated cells were streak on MYGP media plates and slant. Both plates and slants were incubated for 24 hrs.in fungal incubator at 27ºC. The final inoculum for fermentation was prepared by inoculating 4-5 loopful culture of *Saccharomyces cerevisiae* NCIM 3095 (48 hrs. Old Culture) in 100 ml of autoclaved MYGP media. To avoid contamination 0.05 gm of benzathine penicillin G antibiotic was added to inoculum media. The inoculum media was kept on rotary shaker at 120 rpm for 12-18 hrs. at room temperature for proper growth.

## 2.6 CELL COUNT BY HEMOCYTOMETER

After incubation, cells in inoculum media were calculated by hemocytometer method. For this 0.1 % methylene blue was used for cell staining. 1 ml cell sample (dilution factor- 10) + 1 ml of methylene blue was mixed and 10 µl of mixed sample was added in hemocytometer. Mean of 5 center square was calculated. The final cell concentration used for encapsulation by calcium alginate and sugar fermentation was 8.7 ×10□ cells/ml.

## 2.7 CELL IMMOBILIZATION BY CALCIUM ALGINATE

Different inhibitors form during sulphuric acid pretreatment. Hence to protect cells from inhibitors, avoid negative impact of inhibitors on cell metabolism, enhance ethanol production and to make easy for cell separation from broth, inoculum cells were encapsulated in calcium alginate. For this, inoculum media and Sodium Alginate ($NaC_6H_7O_6$) 1.5% was taken as in ratio of 1:0.5. This mixture was added dropwise to 2 % of 50 ml Calcium Chloride ($CaCl_2$) in Laminar air flow under aseptic condition. The encapsulated beads were washed with distilled water to remove impurities. For each sample,20 ml of inoculum was used to prepare Calcium Alginate beads.

## 2.8 FERMENTATION OF SAMPLES

Acid and steam combined pretreated samples were taken as final volume of 250 ml in 500 ml conical flask. Each sample was supplemented with 3 gm/lit. of Ammonium sulfate as external growth factor for proper cell growth and metabolism of samples were calibrated to 6.2 by 1N NaOH (sodium hydroxide). Samples were sterilized before inoculation of cell beads by autoclave for 10 min. at 121 ºC at 15 psi. After autoclave samples were allowed to cool and for each sample benzathine penicillin G (0.5 gm/lit) was added to avoid contamination during fermentation. For each sample 20 ml inoculum gel beads were added separately under aseptic condition. All conical flasks were wrapped and placed on rotary shaker for 10 days at 120 rpm at room temperature.

## 2.9 ETHANOL ESTIMATION BY DICHROMATE REAGENT

To check status of metabolic activity of yeast cells for conversion of sugar to ethanol, ethanol estimation was carried out after each 3 days of interval by potassium dichromate reagent. The reagent was prepared by addition of 10 % Potassium dichromate($K_2Cr_2O_7$) in 5M sulphuric acid ($H_2So_4$). Different concentrations of pure ethanol (10%,20%,30%,40%,50%) were taken as standard solution to measure optical density. For each 1 ml of sample 4 ml of potassium dichromate reagent was added and incubated for 10 min. at 55ºC. Dilution was carried out by addition of 9 ml of distilled water in each tube to reduce intensity of formed color complex and to stop reaction. Reaction absorbance was measured at 600 nm in colorimeter.

### 2.9.1 ETHANOL DISTILLATION AND FINAL ESTIMATION

After completion of incubation period, all samples were filtered by filtration by using cellulose filter paper. The calcium alginate encapsulated cells reduce the difficulty of separation which face during separation of free cells from broth. Encapsulated cells separate easily from broth. Each sample was distilled by adding in distillation assembly. Distillation was carried out at 78.37ºC for 2 hrs. for each sample. The final product(ethanol)was collected in

separate bottle and evaluated for their ethanol content by plotting a graph of OD (optical density) measured by colorimeter versus % of ethanol. From OD (optical density) for known ethanol concentration solution, unknown ethanol concentration of distilled ethanol samples was found out.

## 3. RESULTS AND DISCUSSION

### 3.1 CELLULOSE CONCENTRATION BEFORE AND AFTER COMBINED PRETREATMENT

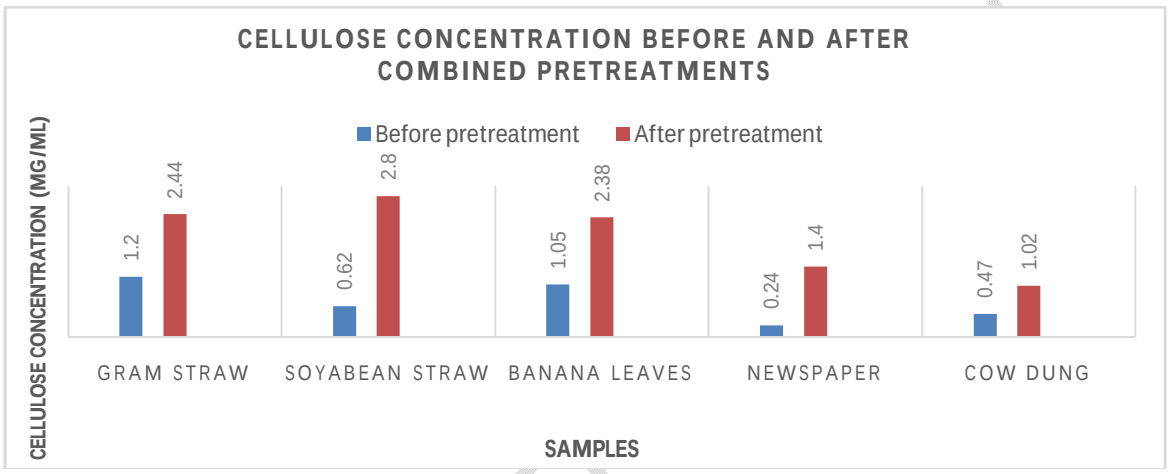


**Fig. 1. Cellulose Concentration Before and After Combined Pretreatment (steam pretreatment and acid pretreatment)**

Increase in free cellulose concentration after combined pretreatments (steam pretreatment and acid pretreatment) indicate effective pretreatments for different samples used in experiment for low-cost bioethanol production.

### 3.2 ETHANOL CONCENTRATION DURING FERMENTATION OR INCUBATION PERIOD

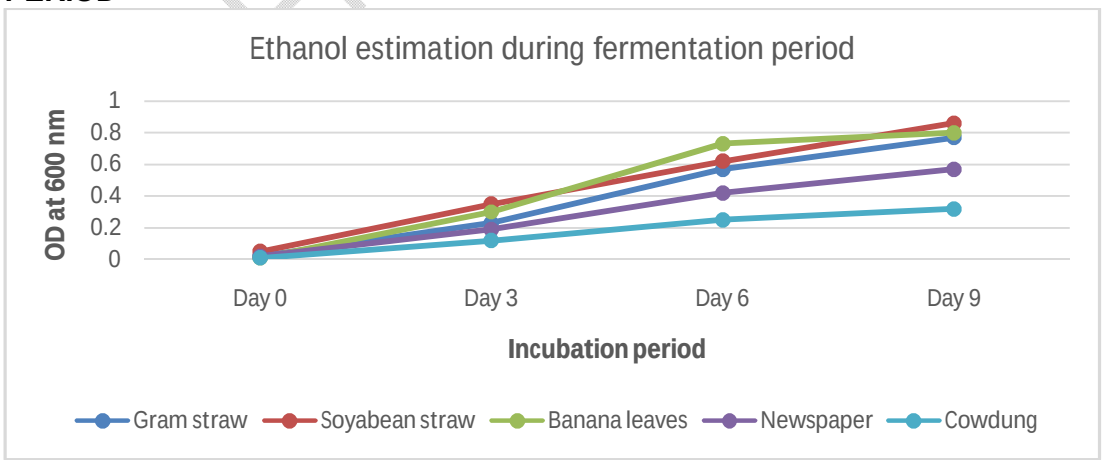


**Fig. 2. Ethanol Concentration During Fermentation or Incubation Period at Different Time Intervals.**

After addition of calcium alginate encapsulated yeast cells, ethanol concentration of each sample was determined by reaction with dichromate reagent and labelled as 'Day 0'. Ethanol concentration was checked after each 3 days interval to check metabolic activity of encapsulated yeast cells.
Increase in ethanol concentration with respect to increase in incubation period shows proper metabolic activity performed by yeast cells. Ethanol production was reduced on 10$^{th}$ day and no increase in much ethanol concentration indicate either nutrient unavailability, effects of inhibitors on yeast cell metabolic activity and different environmental factors affect ethanol production in samples.

### 3.3 FINAL ETHANOL ESTIMATION

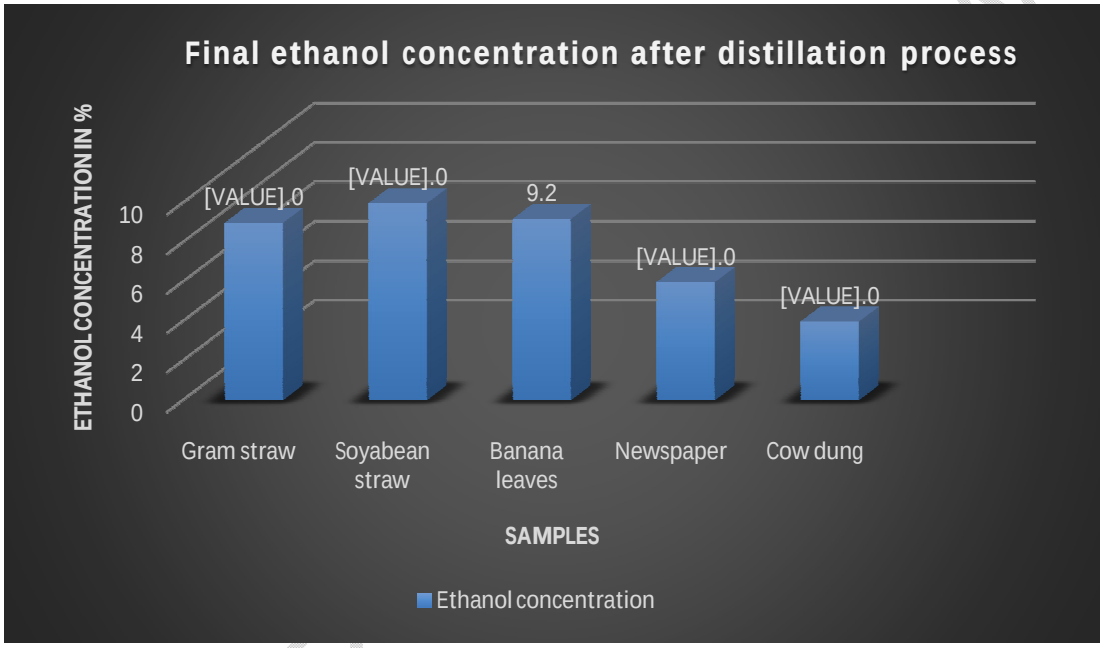


**Fig. 3. Final Ethanol Concentration After Distillation Process**

After incubation period of 10 days, samples were distilled at 78.37ºC for 2 hrs. or 60 min. After distillation final sample was analyzed for % presence of ethanol by dichromate reagent by measuring absorbance at 600 nm in colorimeter.
From the result of final concentration of ethanol obtained after estimation by dichromate reagent it is concluded that soyabean straw gives highest ethanol yield of 10.0% while cow dung gives lowest ethanol yield of 4.0%.

### 4. CONCLUSION

Cellulose estimation before and after pretreatment by Anthrone reagent shows increase in free sugar yield after steam and acid pretreatments. From this it is concluded that steam and acid treatment is effective to obtain better sugar yield from cellulosic wastes.
Encapsulation of yeast cells in calcium alginate shows the better metabolic activity than free cells. From this it is concluded that encapsulation enhance cell metabolic activity and reduce effect of inhibitors on cell, form during fermentation. Apart from that it also reduces cost and problems face during broth filtration.

After completion of incubation period and distillation, each sample analyzed for its final ethanol content by dichromate reagent. From the obtained result it is concluded that soyabean straw has potential to be used as a substrate for low-cost bioethanol production.

## CONSENT AND ETHICAL APPROVAL

No Informed Consent and Ethical Approval is Applicable.

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