

Original Research Article

Bioethanol production from various lignocellulosic materials by encapsulated *Saccharomyces cerevisiae* NCIM 3095.

ABSTRACT

Bioethanol has a greater promise for environmental safety and energy security than fossil fuels. The alternate source required to meet the fuel's requirements can be provided by bioethanol. Untapped sugar-rich sources, like cellulose-rich household wastes, industrial wastes, and agricultural wastes, can all be used to make bioethanol at a minimal cost. The study's objective was to determine whether *saccharomyces cerevisiae* cells from the encapsulated NCIM 3095 strain of *Saccharomyces cerevisiae* could be used to make low-cost ethanol from a variety of lignocellulosic wastes, including newspaper, banana leaves, gram straw, soybean straw, and cow dung. To reduce bacterial contamination and serve as an external growth stimulator, benzathine penicillin G and ammonium sulfate were added to each sample broth containing calcium alginate-encapsulated yeast cells. The samples were fermented for ten days. The ethanol content was evaluated every three days. The largest yield of bioethanol was produced by soybean straw (10.0%), while the lowest was by cow dung (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 [1]. However, it is difficult to convert lignocellulosic material into sugar, which necessitates advanced equipment and could raise operational costs. The earlier research concentrated on several pretreatments for the release of free reducing sugar as well as the utilization of free yeast cells for fermentation. An ongoing effort has been undertaken to develop and enhance a method that would use inexpensive feedstocks to create a sustainable transportation biofuel [2]. 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 [5]. 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 into ethanol, need to be pretreated [6]. 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 [11]. 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* [14]. Encapsulated microbial cells can be used to combat the effects of these inhibitory chemicals, which also lessens issues with filtration and cell biomass separation [16].

The primary goal of the current study is to evaluate the potential of various lignocellulosic agricultural wastes to serve as a source of raw materials for genetically modified and encapsulated yeast cells. Pretreatment that is combined rather than used separately produces superior results. Lignocellulosic wastes could be used to produce bioethanol at a low cost.

2. MATERIAL AND METHODS

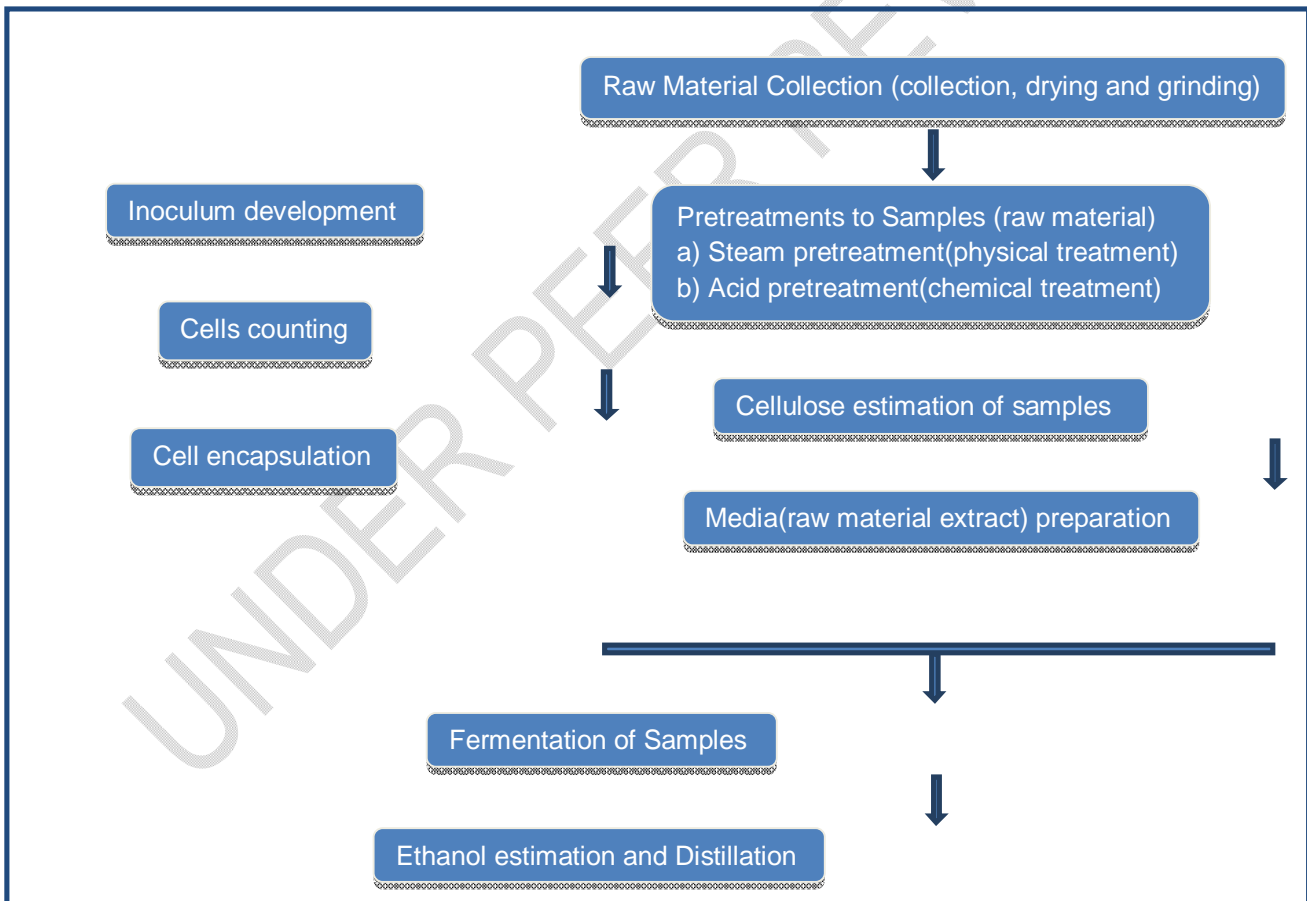




Fig. 1. Flowchart of experimental steps

2.1 COLLECTION OF RAW MATERIALS



Soybean Straw



Gram Straw



Banana Leaves



Cow Dung



News Paper

Fig. 2. Collection of raw materials (Soybean straw (*Glycine max*), Gram straw (*Cicer arietinum*), Banana leaves (*Musa acuminata*), Cow dung (Jersey cow), and Newspaper.

Soybean straw (*Glycine max*), Gram straw (*Cicer arietinum*), Banana leaves (*Musa acuminata*), and Cow dung (Jersey cow), samples were collected from agricultural fields and Newspapers from a 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 overnight at 60°C. The dried samples were ground into powdered, and packed in a polyethylene bag. For all samples, the fermentation broth was prepared by adding 20 gm of sample in 300 ml of distilled water in five different conical flasks, wrapped and labeled properly to avoid any mistakes.

2.3 PRETREATMENT OF SAMPLES

To check the effect of combined pretreatment i.e., steam and acid pretreatment on lignin breakdown and release of free cellulose from the cell wall, all samples were firstly treated with steam treatment in an 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, the insoluble portion after filtration was treated with 25 ml of 1.50 % sulphuric acid (H_2SO_4) for 21.66 mins at 91.02°C [3]. After hydrolysis, the insoluble portion is again separated by the soluble portion by filtration by cellulose paper. The filtrate was transferred to the previously filtered solution for the next process.

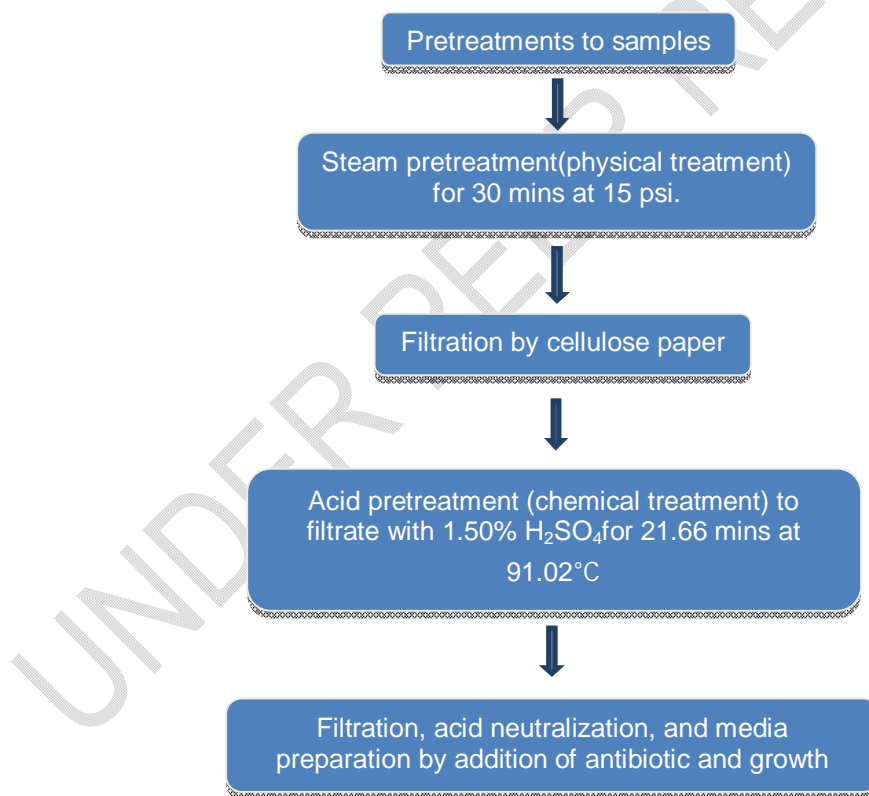


Fig. 3. Pretreatment to samples (steam & acid pre-treatment)

2.4 CELLULOSE ESTIMATION BY ANTHRONE METHOD

Anthrone reagent was prepared by dissolving 200 mg of Anthrone (97 %) in 100 ml of 95 % chilled sulphuric acid (H_2SO_4). The cellulose (99.16%) solution of 0.5 mg/ml concentration was used as a standard solution for comparison.

Sugar (cellulose) concentration of samples before and after pretreatments was estimated by Anthrone reagent to check the effectiveness of combined pretreatments. To get the perfect result by measuring absorbance, samples were diluted in a 1:2 (sample: distilled water) ratio. For each sample, 1 ml of sample extract and 3 ml of Anthrone reagent were added to the test tube. Test tubes were incubated for 17 mins at 90°C and reaction absorbance was measured at 670 nm in a colorimeter. An increase in free cellulose concentration after pretreatments than before pretreatments indicates 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 (99%) solution (0.05 gm cells in 5 % 10 ml of glucose solution) and kept on a shaker for 15-20 mins at 110 rpm [1]. The activated cells were streaked on MYGP media plates and slanted. Both plates and slants were incubated for 24 hrs. in a fungal incubator at 27°C. The final inoculum for fermentation was prepared by inoculating a 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 (98%) antibiotic was added to the inoculum media [1]. The inoculum media was kept on a 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 the hemocytometer method. For this 0.1 %, methylene blue (99.5%) was used for cell staining. 1 ml cell sample (dilution factor- 10) + 1 ml of methylene blue (99.5%) was mixed and 10 μ l of the mixed sample was added to the hemocytometer [8]. The mean of 5 center squares was calculated. The final cell concentration used for encapsulation by calcium alginate and sugar fermentation was 8.7×10^6 cells/ml.

2.7 CELL IMMOBILIZATION BY CALCIUM ALGINATE

Different inhibitors form during sulphuric acid pretreatment. Hence to protect cells from inhibitors, avoid the negative impact of inhibitors on cell metabolism, enhance ethanol production, and make it easy for cell separation from broth, inoculum cells were encapsulated in calcium alginate. For this, inoculum media and Sodium Alginate (98 %) ($NaC_6H_7O_6$), 1.5% were taken in the ratio of 1:0.5 [16]. This mixture was added dropwise to 2 % of 50 ml Calcium Chloride (96.20%) ($CaCl_2$) in Laminar airflow under aseptic conditions. 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 at a final volume of 250 ml in a 500 ml conical flask. Each sample was supplemented with 3 gm/lit. of Ammonium sulfate (98.50%) as an external growth factor for proper cell growth and metabolism of samples [9]. Samples were calibrated to pH 6.2 by 1N Sodium hydroxide (99.8 %) (NaOH). Samples were sterilized before inoculation of cell beads by autoclave for 10 min. at 121°C at 15 psi. After autoclave samples were allowed to cool and for each sample benzathine penicillin G (98%) was added to avoid contamination during fermentation. For each sample 20

ml inoculum gel beads were added separately under aseptic conditions. All conical flasks were wrapped and placed on a rotary shaker for 10 days at 120 rpm at room temperature.

2.9 ETHANOL ESTIMATION BY DICHROMATE REAGENT

To check the status of metabolic activity of yeast cells for conversion of sugar to ethanol, ethanol estimation was carried out after every 3 days of an interval by potassium dichromate reagent. The reagent was prepared by the addition of 10 % Potassium dichromate(99.50%)($K_2Cr_2O_7$) in 5M sulphuric acid(98 %)(H_2SO_4). Different concentrations of pure ethanol(99.50%) of 10%,20%,30%,40%,50% were taken as standard solution to measure optical density. For every 1 ml of sample 4 ml of potassium, dichromate reagent was added and incubated for 10 min. at 55°C. Dilution was carried out by the addition of 9 ml of distilled water in each tube to reduce the intensity of the formed color complex and to stop the reaction. Reaction absorbance was measured at 600 nm in the colorimeter.

2.9.1 ETHANOL DISTILLATION AND FINAL ESTIMATION

After completion of the incubation period, all samples were filtered by filtration using cellulose filter paper. The calcium alginate encapsulated cells reduce the difficulty of separation which face during the separation of free cells from the broth. Encapsulated cells separate easily from the broth. Each sample was distilled by adding in distillation assembly. Distillation was carried out at 78.37°C for 60 min[3], for each sample. The final product(ethanol) was collected in a separate bottle and evaluated for its 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, the unknown ethanol concentration of distilled ethanol samples was found.

3. RESULTS AND DISCUSSION

3.1 FREE CELLULOSE CONCENTRATION BEFORE AND AFTER COMBINED PRETREATMENT

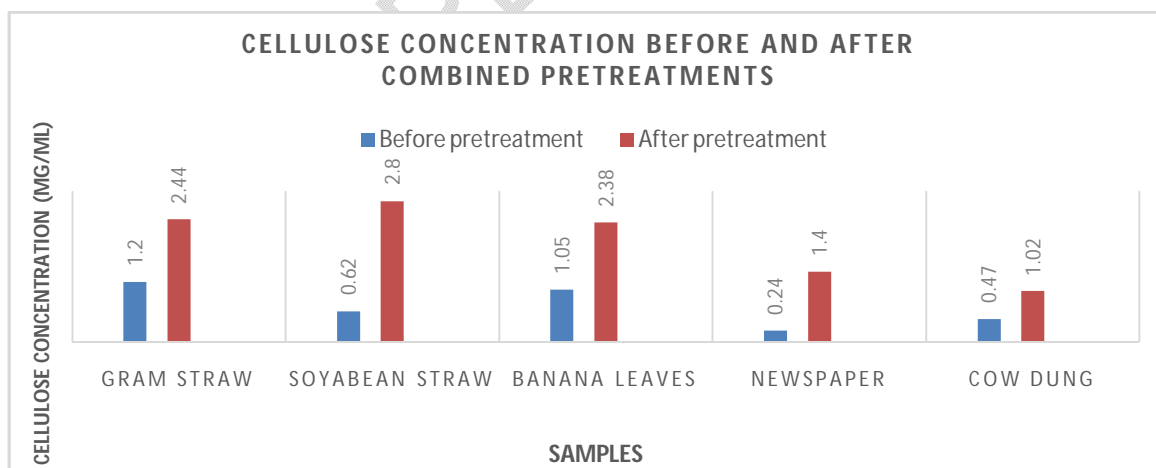


Fig.4. Free Cellulose Concentration Before and After Combined Pretreatment (steam pretreatment and acid pretreatment)

The blue color bars in the above fig. indicate the amount (in mg/ml) of reducing sugar (cellulose) before physical (steam) and chemical (H_2SO_4) pretreatment while the brown color bars indicate the amount of cellulose obtained when raw material samples treated with steam and sulphuric acid. The free reducing sugar (cellulose) concentration was determined

by anthron's reagent. The concentration of reducing sugar increased significantly for each raw material sample treated with combined steam and acid pretreatment which showed different bluish-green color intensities according to the free cellulose concentration present in each sample. We obtained the highest amount of free sugar after pretreatments in the soybean straw sample (2.8 mg/ml) while the lowest concentration of cellulose was obtained in the cow dung sample (1.02 mg/ml). In general, we obtained the highest concentration of reducing sugar after pretreatments indicating that the use of combined pretreatments is more effective than the use of a single one.

3.2 ETHANOL CONCENTRATION DURING FERMENTATION OR INCUBATION PERIOD

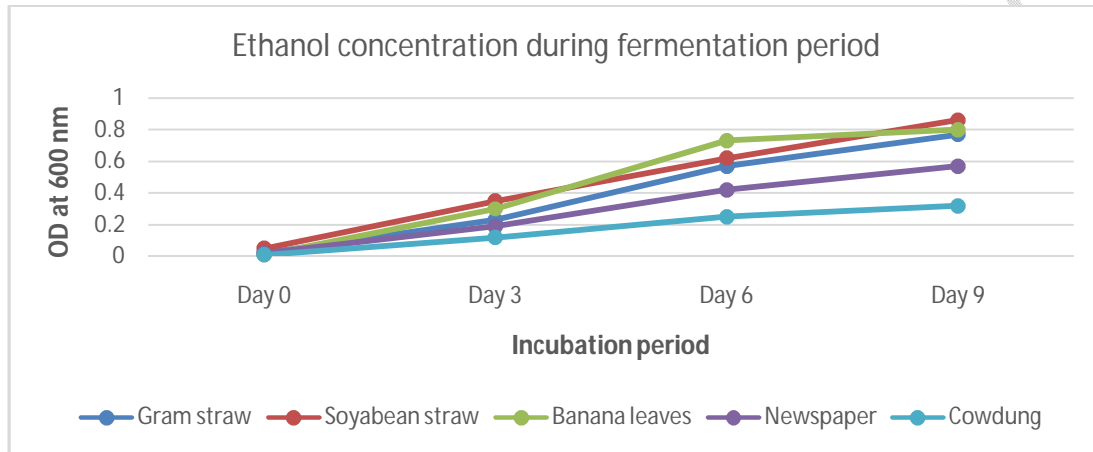


Fig. 5. Ethanol Concentration During Fermentation or Incubation Period at Different Time Intervals.

After the inoculation of calcium alginate encapsulated (immobilized) yeast cells in each sample, the ethanol concentration of each sample was determined by performing the chemical test with a potassium dichromate reagent. The intensity of the color complex after the reaction was proportional to ethanol concentration in the sample. The ethanol concentration of each sample was checked after every 3 days of the interval to check the metabolic activity status of encapsulated yeast cells and obtained results were labeled as day 0, day 3, day 6, and day 9.

In the above fig. ethanol concentration at day 0 is 0 % but it is elevating to the incubation period. The different color lines represent different samples. An increase in ethanol concentration in broth to increase in incubation period indicates the proper metabolic activity performed by yeast cells. Ethanol production was reduced on the 10th day and no further increase in ethanol concentration in broths indicates either nutrient unavailability, effects of various inhibitors formed during metabolic reactions in broth on yeast cell metabolic activity, and different environmental factors (pH, temperature, etc.) affect ethanol production in samples.

3.3 FINAL ETHANOL ESTIMATION

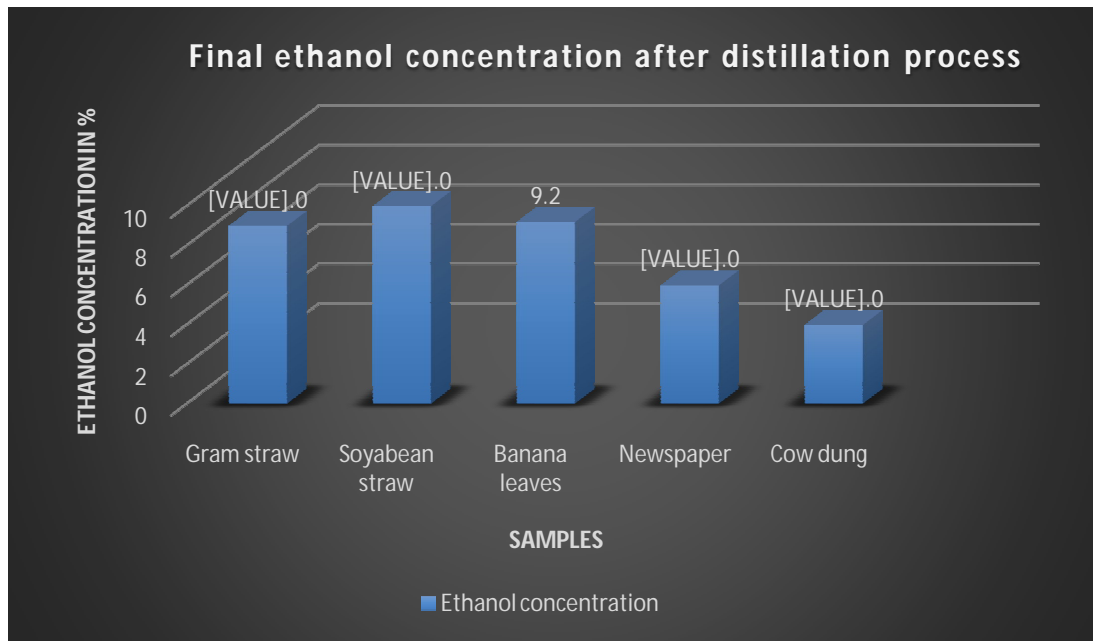


Fig. 6. Final Ethanol Concentration After Distillation Process

Following a 10-day incubation period, samples were distilled for 2 hrs. The final sample was then tested for the presence of bioethanol using a dichromate reagent and a colorimeter to measure absorbance at 600 nm.

Soybean straw produces maximum bioethanol yield (10.0%), followed by banana leaves (9.2%), gram straw (9.0%), newspaper (6.0%), and cow dung sample (4.0%) which give the lowest bioethanol yield, according to the result of the final concentration of bioethanol obtained after reaction with dichromate reagent and measurement by a colorimeter.

4. CONCLUSION

Free reducing sugar (cellulose) estimation before and after pretreatment by Anthrone reagent shows an 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 better metabolic activity (fermentation) than free cells and reduces the effect of inhibitors which produces during different metabolic reactions of cells during fermentation. Apart from that it also reduces cost and problems face during broth filtration and cell separation.

After completion of the incubation period and distillation, each sample was analyzed for its final ethanol content with a dichromate reagent. According to the results, soybean straw has the greatest potential among the various raw materials used in this study for use as a raw material for low-cost bioethanol production. Its availability and low cost also make it more suitable for use. Banana leaves and gram straw can also be utilized as a source for the synthesis of bioethanol, after soybean straw. Cow dung has the lowest potential to be used as a source of raw materials for the manufacturing of bioethanol, whereas the newspaper has some potential.

CONSENT AND ETHICAL APPROVAL

No informed consent and ethical approval are applicable.

REFERENCES

- 1) Utilization of unripe banana peel waste as feedstock for ethanol production, Ashish G. Waghmare, Shalini S. Arya*, Bioethanol 2016; 2: 146–156
- 2) Optimization of Banana Peels Hydrolysis for the Production of Bioethanol: Response Surface Methodology, Mebrahtom Gebresemati¹, a*, and Alula Gebregergs², International Letters of Natural Sciences Online: 2015-11-03 ISSN: 2300-9675, Vol. 48, pp 53-60 DOI: 10.18052/www.scipress.com/ILNS.48.53 CC BY 4.0. Published by SciPress Ltd, Switzerland, 2015
- 3) Bioethanol Production from Waste Banana Peel Madhumala Y1, Vijayalaxmi Naganuri², Megha Mathad³, INTERNATIONAL RESEARCH JOURNAL OF ENGINEERING AND TECHNOLOGY (IRJET) E-ISSN: 2395-0056 VOLUME: 07, SPECIAL ISSUE | JUNE 2020
- 4) Bioethanol production from banana peels using different pretreatments, 2 Yasir. Yousif, Hind S Abdulhey, Published: 30.12.2017
- 5) Bioethanol Production from Banana Peels Gaddafi I. Danmaliki* ¹, Auwal M. Muhammad², Abdullahi A. Shamsuddeen³ Bashir J. Usman⁴
- 6) Production of Biofuel (Bio-Ethanol) From Fruitwaste: Banana Peels Muhamad Ariff Amir Hamzah, AzilBahari Alias, Nor Elina Ahmad, International Journal of Engineering and Advanced Technology (IJEAT) ISSN: 2249 – 8958, Volume-9 Issue-1, October 2019
- 7) Production of bioethanol from the waste newspaper, Shruti A.Byadgi a*, P.B.Kalburgi^b,procedia Environmental Sciences 35(2016) 555-562
- 8) Microscopic yeast cell counting by hemocytometer, American Society of Brewing and Subcommittee on improved Microscopic Yeast Cell Counting, journal 46:123,1988
- 9) Bioethanol production from starchy part of the tuberous plant(potato) using Saccharomyces cerevisiae MTCC-170, Joginder Singh Duhan a*, Ashok kumar¹, Sunil Kumar Tanwar ^{2.}, African Journal of Microbiology Research
- 10) Selection of inoculum size and Saccharomyces cerevisiae strain for ethanol production in simultaneous saccharification and fermentation (SSF) of sugar cane bagasse, Maria Carolina de Albuquerque Wanderley, Mariana Lucena Soares, and Ester Ribeiro Gouveia
- 11) Sanchez O.J., Cardona C.A., Review: Trends in biotechnological production of fuel ethanol from different feedstocks, Bioresour. Technol., 2008, 99, 5270-5295.
- 12)Brethauer S., Wyman C.E., Review: Continuous hydrolysis and fermentation for cellulosic ethanol production, Bioresour. Technol., 2010, 101, 4862-4874.
- 13)Muhammad Nasidi M., Agu R., Deeni Y., Giginyu I.B., Walker G., Bioconversion of degraded husked sorghum grains to ethanol, Bioeth., 2015, 2, 1-11. [9] Yamashita Y., Sasaki C., Nakamura Y., Effective enzyme saccharification and ethanol production from Japanese cedar using various pre-treatment methods, J. Biosci. Bioeng., 2010, 110, 79-86.
- 14) Campo I.D., Alegria I., Zazpe M., Echeverria M., Echeverria I., Diluted acid hydrolysis pre-treatment of agri-food wastes for bio-ethanol production, Ind. Crop. Prod., 2006, 42, 214-221.
- 15)Pramanik K., Rao D.E., Kinetic study on ethanol fermentation of grape waste using Saccharomyces cerevisiae yeast isolated from toddy, IE (I) Journal, 2005, 85, 53-58.
- 16)Bioethanol Production Using Saccharomyces cerevisiae with Different Perspectives: Substrates, Growth Variables, Inhibitor Reduction and Immobilization, Bhadana and Chauhan, Ferment Technol 2016, 5:2 DOI: 10.4172/2167-7972.100013118) Khan N., Roes-Hill M., Welz P.J., Grandin K.A., Kudanga T., Van Dyk J.S., et al., Fruit waste streams in South Africa and their potential role in developing a bio-economy, S. Afr. J. Sci., 2015, 111, 5-16.

- 17) Choia I.S., Leea Y.G., Khanal S.K., Park B.J., Bae H.J., A low-energy, cost-effective approach to fruit and citrus peel waste processing for bioethanol production, *Appl. Energy.*, 2015, 140, 65-74.
- 18) Wyman C.E., Twenty years of trials, tribulations, and research progress in bioethanol technology, *Appl. Biochem. Biotechnol.*, 2001, 91, 5-21.
- 19) Hammond J.B., Egg R., Diggins D., Coble C.G., Alcohol from bananas, *Bioresour. Technol.*, 1996, 56, 125-130.
- 20) Guneseelan N.V., Biochemical methane potential of fruits and vegetables solid waste feedstocks, *Biomass Bioenerg.*, 2004, 26, 389-399.
- 21) Oberoi H.S., Vadlani P.V., Saida L., Bansal S., Hughes J.D., Ethanol production from banana peels using statistically optimized simultaneous saccharification and fermentation process, *Waste Manag.*, 2011, 31(7), 1576-84.
- 22) AOAC (Association of Official Analytical Chemists), *Official methods of analysis*, 16th ed. Washington, 2001.
- 23) Miller G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, 1956, 31, 426-428.
- 24) Branyikova I., Marsalkova B., Doucha J., Branyik T., Bisova K., Zachleder V., Vitova M., Microalgae-novel highly efficient starch producers, *Biotechnol. Bioeng.*, 2011, 108, 766-776.
- 25) Takeshita T., Ota S., Yamazaki T., Hirata A., Zachleder V., Kawano S., Starch and lipid accumulation in eight strains of six *Chlorella* species under comparatively high light intensity and aeration culture conditions, *Bioresour Technol.*, 2014, 158, 127-34.
- 26) Gera R., Dhamija S.S., Gera T., Singh D., Intergeneric ethanol producing hybrids of thermotolerant *Kluyveromyces* and non-thermotolerant *Saccharomyces cerevisiae*, *Biotechnol Lett.*, 1997, 19(2), 189-194.
- 27) Larsson S., Palmqvist E., Hahn-Hagerdal B., Tenengeborg C., Stenberg K., Zacchi G., et al. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb. Technol.*, 1999, 24, 151-15.