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Bioethanol Over Production from Second Generation Feedstock using Rice Straw as Lignocellulosic waste

Reema Ningthoujam, Harish Kumar Dhingra*

Department of Biosciences, School of Liberal Arts and Sciences
Mody University of Science and Technology, Lakshmangarh - 332311 (Sikar), Rajasthan, India.
*Corresponding author: harishdhingra2000@gmail.com

Abstract

The efficient conversion of lignocellulosic biomass to fermentable sugars has shown several environmental and economic advantages in the ethanol production technology as compared to the bioethanol produced from food crops. In this study, rice straw which is a complete waste and which when burning give rise to pollution was used as the substrate. In this present investigation, the rice straw was pretreated with various physical (chipping, milling, grinding) and chemical (acid, alkali) methods to reduce the crystallinity content and remove lignin. A combination treatment of grinding along with 4% NaOH was found to be the most effective one. The production of bioethanol was carried out by using rice straw as a feedstock and by taking *Saccharomyces cerevisiae* as a fermentative microorganism. Yeasts were isolated from different food sources and were identified and selected based on the cell morphology, biochemical characterization, and ethanol tolerance capability. The potential isolates underwent enzyme saccharification by adding the cellulase enzyme followed by fermentation. Cultural conditions optimization was maintained by incubating the isolates for a period of two days. Also, the effect of temperature and pH on bioethanol production was checked at various ranges. In this investigation, the optimum parameters found for bioethanol production was an incubation time of 24 hrs, keeping the temperature and pH at 30°C and 4.5 respectively. After a week of fermentation, ethanol was obtained. For determining the ethanol concentration, the potassium dichromate method was used. The yeasts isolated from green and black grapes were found to synthesize a higher concentration of bioethanol. The highest yield of ethanol obtained in this study was found to be 18.2%.

Keywords: Lignocellulosic biomass, fermentable sugars, *Saccharomyces cerevisiae*, fermentation, bioethanol

Introduction

In the past few decades ethanol from renewable sources has been increased towards the development of alternative cleaner energy. Lignocellulosic biomass such as rice straws is a potential raw material in the production of high-value products like ethanol (1). Rice is one of the most staple food crops in Asia. Rice straw is generally produced after post-cultivation of the rice crop. It contains more than 50% of cellulose and hemicellulose and thus it is considered an attractive feedstock for bioethanol production (2). It is estimated that rice straw can produce 205 billion liters of bioethanol annually. Extensive research in the field of ethanol production has been widely studied (3), however, the manufacturing process for the production of ethanol has not been yet realized due to high cost in the transportation and collection from the rice field, energy-consuming pre-treatment steps, enzyme saccharification, and fermentation system, etc. Rice straw has a very complex structure where the cellulose is entangled with hemicellulose and lignin. A pre-treatment method is essential for breaking the bounded structure for bioconversion of the biomass to ethanol. The pre-treatment step is strictly required in achieving the best yield of fermentable sugar (4). For the conversion of the biomass to fermentable sugars, various processes such as pre-treatment, enzyme saccharification, followed by fermentation have been reported (5). There are various hydrolysis enzymes for effective hydrolysis however the selection of an appropriate enzyme-like cellulase is not an easy task due to structural difference in each biomass and difference in enzymatic activity (2). Hydrolysis is followed by fermentation and the best-suited microorganisms for ethanol production are the yeast species i.e. *Saccharomyces cerevisiae*. Yeast is widely distributed in nature and is commonly found on the skin of fruits, damaged and over-ripened fruits such as grapes, oranges, apples, plum, etc., plant leaves as well as rotten vegetables such as tomatoes. For ages, yeasts have been used in the production of various industrial products, alcoholic beverages, bread, cakes, etc. (6, 7, 8). They are also used in the petrochemical industries where it has been designed to produce biofuels like bioethanol (9). Bioethanol is a pure-burning renewable material that is produced after fermenting cellulosic biomass. It does not add an increase in atmospheric net CO_2 and hence there is no support for global warming (10). Thus, they are regarded as cleaner fuels.

This study aimed to evaluate the potential of rice straw as a feedstock for high bioethanol production by *Saccharomyces cerevisiae*. This work was concentrated on the fermentation of these reducing sugars to bioethanol. Parameters that influenced the production of bioethanol such as optimization of the incubation period, temperature, and pH were also deeply examined.

Materials and Methods

Collection of lignocellulosic wastes and pre-treatment of the biomass

Rice straw was collected from an agricultural field from the North Eastern region of India; Manipur, Imphal East. The collected biomass undergoes a combination of physical and chemical treatment. Physical treatment includes chipping, grinding, and milling. The finely ground biomass was washed with water to remove all the soluble contents present in it. The rinsing with water was continued until it reached a neutral pH. It was then kept at 65°C for 48 hrs for drying purposes (11). The physically treated biomass was then chemically treated as described by (12). The chemicals used were NaOH, HCl, and H_2SO_4. For treatment with NaOH, different concentrations in the

range of 1-10% were prepared and the biomass was soaked with aq. NaOH solution at a liquid to solid ratio of 10:1 (v/w) at room temperature for 3 hrs. and autoclaving at 121°C for 30 mins. For treatment with HCl and H_2SO_4, concentrations in the range from 1-10% were prepared as well and the biomass was soaked at 10:1 (v/w) for both the acids. It was then stored at room temperature for 10 hrs with an agitation of 150 rpm. Further, it was autoclaved at 121°C for 30 mins. The residues of all the 3 different chemically treated substrates were collected and washed extensively with tap water until neutral pH was reached, filtered, and then dried at 65°C for 48 hrs. Estimation of cellulose was then carried out.

Isolation and physiological characteristics of potential yeast isolates

Five different food samples were collected for yeast isolation from a nearby local market, Lakshmangarh, Rajasthan. The collected samples and their subcultures were coded with different names according to our convenience. GG: Green Grapes; BG: Black Grapes; OR: Orange; PP: Plum and AA: Apple. The samples were kept unwashed for few days and yeast was isolated from the skin of the over-ripened fruits as well as by serial dilution method also.

The isolated yeasts were spreaded on YEPDA media Pure colonies were obtained using the quadrant streaking method and subculture was done every 15 days for preservation and future use. Morphological characteristics were studied based on shape, color, texture, margin, elevation, and consistency. Simple staining was performed to observe the budding characteristics of the isolated yeasts.

The viable yeast was counted using the spot plate technique. 100µL of serially diluted sample was spread over the YEPDA media. The Petri plates were labeled according to the dilution factor and were incubated at 27°C for 24 hrs. Individual colonies in the most diluted samples were counted and the number of viable cells in the original culture was also calculated. Ethanol tolerance was checked upon the yeast isolates by inoculating the isolates in the YPG broth containing different ethanol concentrations ranging from 2-20%. It was then incubated at 27°C for 24 hrs. and the growth of the strains was checked at absorbance 600 nm.

Biochemical characterization

Carbohydrate fermentation test

Six sugar sources were utilized viz. glucose, fructose, sucrose, maltose, lactose, and galactose for the carbohydrate fermentation test. The test tubes having different sugar sources were autoclaved and inoculated with a loopful of 24 hrs old culture of the test organism. Daily observation for acid and gas production inside the Durhum tubes was checked (13).

Catalase test

Catalase test was performed by transferring a small amount of 24 hrs old culture on a clean slide with the help of a sterile inoculating loop. A drop of 2% hydrogen peroxide solution was poured over the slides and was observed for the appearance of gas bubbles which indicates the presence of catalase enzyme (14).

Urease test

A small amount of 24 hrs old culture was inoculated in a urease agar and was incubated at 27°C for 48 hrs and was checked for the development of red-pink color which indicates a positive result (15).

Hydrogen Sulfide Test

This test was performed to check their ability to produce hydrogen sulfide. Cells were inoculated on LA agar medium. Single streaking was done. It was then incubated for a week at 28°C. The Petri plates were observed for the appearance of black coloration along the line of stab inoculation that showed hydrogen sulfide production (16).

Amylase Production

To determine the ability of the yeast isolates to produce amylase enzyme this test was carried out. The isolates were grown on starch agar medium at 30°C for 48 hrs. After incubation, the plates were flooded with iodine for 30 secs. Plates were then observed for the formation of a clear zone around the line of growth (13).

Gelatinase test

To examine gelatinase activity of the isolates, petriplates and deep tubes containing gelatin agar were streaked and inoculated with a needle respectively and incubated at 30°C for 4 to 7 days. After incubation, the petriplates were examined for the formation of a clear zone after treatment with mercuric chloride. The deep tubes were observed for gelatin liquefaction after placing it at 4°C for 10 mins. (13).

Enzymatic hydrolysis and fermentation

Enzymatic saccharification of the pretreated rice straw was carried out in 100 mL Erlenmeyer's flask by incubating 5g of the biomass in 50mM citrate buffer. The pH was adjusted to 4.8. Hydrolysis was performed using a commercial cellulase enzyme. It was then followed by fermentation which was carried out by inoculating the potential isolates to the hydrolyzed biomass at 27°C for a week until it gave an alcoholic smell.

Optimization of culture condition

The yeast isolates were incubated at a different temperature ranging from 25, 27, 30, 37, and 40°C. Effect of pH on bioethanol production was also checked on various pH i.e. 4, 4.5, 5, 5.5, and 6. The yeast growth curve was also tested by incubating the isolates for a period of 48 hrs and the culture was tested at different time intervals of 0, 8, 16, 24, 32, 40 and 48 hrs.

Ethanol quantification and estimation

After a week of fermentation, distillation was carried out for purification of ethanol as it contains a lot of impurities. Ethanol was then collected for estimation using the potassium dichromate method. Absorbance at 600 nm was measured and was compared with the standard curve.

Results and Discussions

Size reduction of the lignocellulosic materials

The size of the materials after chipping was 10-20 µm. It was further reduced to 1-2 µm after milling. After pre-treating the substrates with acid and alkali, it was found out that 4% NaOH

release maximum cellulose and was chosen for further process.

Identification of potential yeast isolates and biochemical characterization

Table 1: Morphological characteristics of the isolated budding yeasts

Sample	Shape	Consistency	Elevation	Texture
GG	Spherical	Mucoid	Non-elevated	Smooth
BG	Spherical	Mucoid	Non-elevated	Smooth
OR	Spherical	Mucoid	Elevated	Smooth
PP	Spherical	Mucoid	Non-elevated	Smooth
AA	Round	Mucoid	Elevated	Smooth

Table 2: Colony count of viable yeasts by spot plate technique using Colony Counter

Sample	Dilution factor	Colony Count	cfu/mL
GG	10^5	108	1.08×10^8
BG	10^5	115	1.15×10^8
OR	10^6	75	7.5×10^8
PP	10^4	128	1.28×10^7
AA	10^3	164	1.64×10^6

Table 3: Biochemical characterization of yeasts isolates [(+): Positive, (-): Negative; AG: Acid and Gas].

Isolates/Test	GG	BG	OR	PP	AA
Glucose	AG	AG	AG	AG	AG
Fructose	AG	AG	AG	AG	AG
Sucrose	AG	AG	AG	AG	AG
Maltose	AG	-	-	-	-
Lactose	AG	AG	-	-	-
Galactose	AG	AG	-	-	AG
Catalase	+	+	+	+	+
Urease	-	-	-	-	-
H2S test	+	+	+	+	+
Amylase	+	+	-	+	+
Gelatinase	+	+	+	+	+

Morphological characteristics of the isolated yeasts were studied from the colonies that are isolated on the YEPDA media (Table 1). The budding stage was observed under the microscope after simple staining of the yeast cells. Counting of the viable yeast by spot plate method was performed so that the most dilute spots contain a lesser number of individual colonies. The colonies of each different sample were counted respectively (Table 2). Further, it was found that all the isolates were able to tolerate ethanol concentration up to 16% however less growth was observed at 18% ethanol concentration. The results of the biochemical characterization of the isolated microorganisms are given below (Table 3).

Fermentation, Culture Condition Optimization, and Ethanol Estimation

The hydrolyzed substrate underwent fermentation and the alcoholic smell was monitored in each flask containing the different isolates. There was a presence of alcoholic smell except in the flask that contains cultures isolated from AA: Apples. The effect of different temperatures (25, 27, 30, 37, and 40°C) was evaluated for the growth of *S. cerevisiae*. The best temperature for maximum bioethanol production was found to be at 30°C (Fig.1). The effect of different pH (4, 4.5, 5, 5.5, and 6) was evaluated to find out the optimum pH for bioethanol production. Fig. 2 shows that pH 4.5 was the best for maximum bioethanol production. A low yield of bioethanol was observed at pH 6 and found unsuitable for high yield. Also, with an increase in the incubation period, ethanol production increased and started decreasing after reaching a certain period (Fig.3). The hydrolyzed biomass after a week of fermentation was subjected to quantitative estimation of bioethanol. Absorbance at 600 nm of all the positive samples was measured by the potassium dichromate method. The absorbance of the sample was compared with the

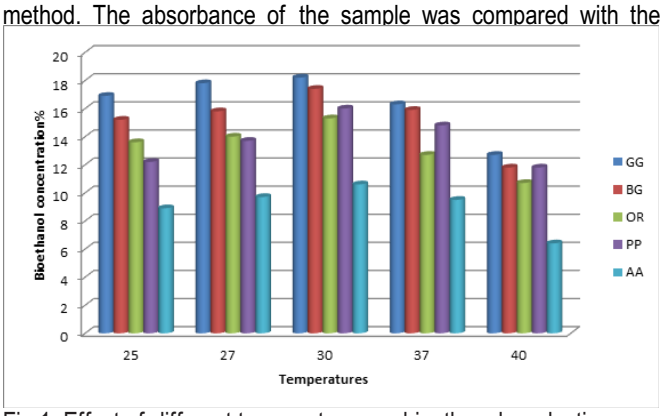

Fig 1: Effect of different temperatures on bioethanol production

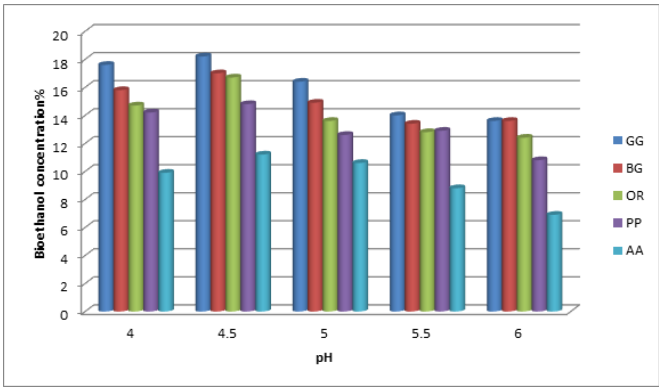

Fig.2. Effect of different pH on bioethanol production

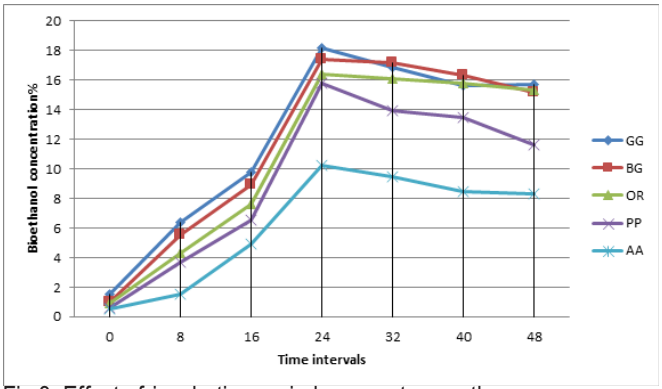

Fig.3. Effect of incubation period on yeasts growth

standard ethanol solution and was found that isolate GG: Green grapes produced the highest ethanol concentration of 18.2% followed by BG: Black grapes with an ethanol concentration of 17.4%.

The removal of lignin from the lignocellulosic biomass by various pretreatment methods is very much necessary to produce ethanol from the rice straw by the process of saccharification and fermentation as well. Alkali pre-treatment is the most favorite amongst the various types of pretreatment available as it can remove lignin very easily, fiberize and reduce the crystallinity of the cellulose (17). Due to the alkali pre-treatment, it was found that there is an increase in the content of the cellulose and also readily accessible to the fiber for enzymes as well as to the fermenting microorganisms. According to other studies, alkaline treatment with NaOH to rice straw resulted in multiple celluloses as well as hemicellulose content (2). Another study reports that treatment with 5% NaOH for 1 hr at 40°C and autoclaving at 121°C for 30 mins increased the cellulose content by 171% and decrease hemicellulose content by 65.9% (18). In the general process of bioethanol production, after pretreatment processing, saccharification is performed. Saccharification is one of the most crucial steps where complex carbohydrates are converted into simple monomers. The selection of the best-suited enzyme for particular biomass should be well considered. After the selection of the best-suited enzyme, the saccharified biomass is then used for fermentation by various microorganisms. However, not all microorganisms can ferment both hexose and pentose sugars and as a result, the utilization of the lignocellulosic biomass for bioethanol production is hindered (19). The best-known yeast and bacteria used in bioethanol production from hexoses are the *Saccharomyces cerevisiae* and the *Zymomonas mobilis* respectively. But *S.cerevisiae* cannot utilize xylose however there are native microorganisms such as *Pichia stipitis* that can utilize xylose but the ethanol production rate is very much lower as compared to *S.cerevisiae* (20). Thus, there is a need to search for favorable microorganisms as different microorganisms show different ethanol yields based on their monomer utilization. In the present work, different fermentative yeasts were isolated from various samples and were found that yeasts when grown in liquid broth follow a well-established microbial growth pattern. At the initial stage of the inoculation, yeast cells become acclimated to the new environment following a lag phase. The cells further enter the log phase where the number of cells increases exponentially along with high metabolic activities, making this phase a highly favorable phase for the generation of bioethanol.

Conclusion

A significant amount of bioethanol was obtained by the fermentation process using *S.cerevisiae* from sugars produced by the saccharification of rice straw. Ethanol yield was found to be maximum by yeast obtained from GG: Green Grapes maintaining temperature at 30°C. The growth rate continues to increase up to 35°C but decreased beyond it. pH is one important factor that affects fermentation. Studies report a maximum alcohol production with optimum pH ranging between 3-5. But in this study, the optimum pH was found to be at 4.5. Also, the best incubation period was found to be at 24 hrs. The amount of bioethanol produced by different organisms was found to differ. This may be due to the differences in the ethanol tolerance capability of the different microorganisms and their ability to utilize various sugars and ferment. Under the conditions as described in the present investigation, the highest yield of 18.2% bioethanol concentration chemically treated with 4% NaOH could be obtained.

References

1. Yoswathana, N., Phuriphipat, P., Treyawutthiwat, P. and Eshtiaghi, M.N. (2010). Bioethanol Production from Rice Straw. Energy Research Journal, 1(1): 26–31.

2. Takano, M. and Kazuhiro, H. (2018). Bioethanol production from rice straw by simultaneous saccharification and fermentation with statistical optimized cellulase cocktail and fermenting fungus. Bioresources and Bioprocessing, 5(1):1- 16.

3. Binod, P., Sindhu, R., Singhania, R.R., Vikram, S., Devi, L., Nagalakshmi, S., Kurien, N., Sukumaran, R.K. and Pandey, A. (2010). Bioethanol production from rice straw: An overview. Bioresource Technology, 101(13): 4767–4774.

4. Kshirsagar, S.D., Waghmare,P.R., Lonic, P.C., Patila, S.A., Govindwar, S.P. (2015). Dilute acid pretreatment of rice straw, structural characterization and optimization of enzymatic hydrolysis conditions by response surface methodology, RSC Adv., 5(58): 46525–46533.

5. Sindhu, R., Kuttiraja, M., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A. (2011). Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. Bioresource Technology, 102(23): 10915–10921.

6. Alfenore, S., Jouve, C.M., Guillouet, S.E., Uribelarrea, J.L., Goma, G.,L. Benbadis, L. (2002). Improving ethanol production and viability of Saccharomyces cerevisiae by a vitamin feeding strategy during fed-batch process. Appl Microbiol Biotechnol , 60: 67–72.

7. Smerilli, M., Neureiter, M., Wurz, S., Haas, C., Frühauf, S., Fuchs, W. (2015). Direct fermentation of potato starch and potato residues to lactic acid by Geobacillus stearothermophilus under non-sterile conditions, Journal of Chemical Technology & Biotechnology, 90(4): 648–657.

8. Adesanya,O.A., Oluyemi,K.A., Joel, J.S., Adesanya, R.A., Ofusori, D.A., Bankole, M.A. and Babalola, G.B. (2008). Ethanol Production By Saccharomyces Cerevisiae From Cassava Peel Hydrolysate . The Internet Journal of Microbiology, 5(1): 1-7.

9. Thapa, S., Rajani, S., Tirewal, A., Sharma, S.,Arjun; K.C., Yuvraj (2015). Isolation of yeast from soil and different food samples and its characterization based on fermentation. Nepal Journal of Biotechnology, 3(1): 29.

10. Tsunatu1, D.Y., Atiku, K. G., Samuel, T. T., Hamidu, B. I. and Dahutu, D. I. (2017). Production of bioethanol from rice straw using yeast extracts peptone dextrose. Nigerian Journal of Technology (NIJOTECH), 36 (1): 296 – 301.

11. Selvaraj, C. and Vasan, P.T. (2017). Pretreatment of cellulosic waste materials using Acid and Alkali to enhance bio-ethanol production . International Journal of Academic Research and Development, 2(5): 191-196.

12. Abraham, M. and Kurup, G. M. (1996). Bioconversion of tapioca (Manihot esculenta) waste and water hyacinth (Eichhornia crassipes)—Influence of various physico-chemical factors. Journal of Fermentation and Bioengineering, 82(3): 259–263.

13. Aneja, K.R.(2001). Experiments in Microbiology Plant Pathology Tissue Culture and Mushroom Production Technology, (3rd edition). New Age International (P) Limited Publishers. pp. 264-266 .

14. Cheesbrough M. (2006). District Laboratory Practice in Tropical Countries, (2nd Edition), Cambridge University Press, Cambridge, UK, ISBN-13: 9781139449298.

15. Oyeleke, S.B. and Manga, B.S. (2008). Essential of laboratory practical's in microbiology. First Edition, Tobest Publishers S.W. 225 Hospital Road Minna, Niger State. Pp. 28, 33, 36, 51, & 58.

16. Ono, B.I, Ishii, N., Fujino, S. and Aoyama,I. (1991). Role of Hydrosulfide ions (HS^-) in Methylmercury Resistance in Saccharomyces cerevisiae. Appl. Envir. Micro., 57: 3183-3186.

17. Taherzadeh, M.J., and Karimi, K. (2008). Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. International Journal of Molecular Sciences, 9(9): 1621–1651.

18. Oberoi, H. S., Babbar, N., Sandhu, S. K., Dhaliwal, S. S., Kaur, U., Chadha, B. S., & Bhargav, V. K. (2011). Ethanol production from alkali-treated rice straw via simultaneous saccharification and fermentation using newly isolated thermotolerant Pichia kudriavzevii HOP-1. Journal of Industrial Microbiology & Biotechnology, 39(4): 557–566.

19. Talebnia, F., Karakashev, D. and Angelidaki, I. (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresource Technology, 101(13): 4744–4753.

20. Ortega, N., Busto, M.D. and Mateos, M.P. (2001). Kinetics of cellulose saccharification by Trichoderma reesei cellulases. International Biodeterioration & Biodegradation, 47(1): 7–14.