STUDIES ON BIOETHANOL PRODUCTION FROM AGRICULTURAL WASTE BY SUBMERGED FERMENTATION

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***Abstract*- This study involves an analysis into Bio-ethanol production from agricultural wastes. Agricultural wastes like bagasse, rice straw waste, wheat straw waste, vegetable wastes etc. were used to produce ethanol by different *Saccharomyces cerevisiae* strains. Initially the wastes were subjected to a pretreatment process using acid hydrolysis to remove lignin which is not acted upon by cellulosic enzymes. Ethanolic fermentation was done using *Saccharomyces cerevisiae* for 8 days and the ethanol yield and total reducing sugar were determined. From the results it was seen that the Rice straw waste fermentation media gave maximum percentage Bioethanol yield of 11.38% and 18.84% when two different strains of indigenous *Saccharomyces cerevisiae were used for fermentation*. These findings show very clearly that Bioethanol can be produced from the agricultural wastes by a cost effective and efficient method.**

INTRODUCTION

India produces a large amount of Agricultural wastes with approx. 350–990 Mt/y. After China, India is the world's second-largest producer of agricultural waste. India produces around 130 million tonnes of paddy straw waste, out of which half is used as fodder and the other half is discarded. Another harmful aspect of Paddy waste is the rice residue burning (parali) practise in the north western regions of India which creates substantial air pollution and raises public health concerns (Shyamsundar et al., 2019). Improper disposal of crop residue leads to generation of greenhouse gasses (GHGs) like carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4), which poses a threat to the human and the natural environment (Shyamsunder et al, 2019).

Thus, Agricultural waste to economically useful product development is an important issue to be taken up at all levels. Fossil fuel usage and leading to air pollution, greenhouse gas emission is another major problem. Bioethanol has been identified highly used biofuel worldwide since it significantly contributes to the reduction of crude oil consumption and decrease in environmental pollution. The main reasons for the enhanced development of bioethanol are its use as a favorable and near carbon-neutral renewable fuel, thus reducing CO2 emissions and associated climate change; its use as octane enhancer in unleaded gasoline; and its use as oxygenated fuel-mix for a cleaner combustion of gasoline, hence reducing tailpipe pollutant emissions and improving the ambient air quality.

Bioethanol can be produced from various types of feedstock such as sucrose, starch, lignocellulosic and algal biomass through fermentation process by microorganisms. Compared to other types of microorganisms, yeasts especially Saccharomyces cerevisiae is the common microbes employed in ethanol production due to its high ethanol productivity, high ethanol tolerance and ability of fermenting a wide range of sugars. The common processes involves in ethanol production are pretreatment, hydrolysis and fermentation. Production of bioethanol during fermentation depends on several factors such as temperature, sugar concentration, pH, fermentation time, agitation rate, and inoculum size. (Martin et al 2007, Naik et al 2020).

The pretreatment leads to effective saccharification and release of free sugars which can be then used by different yeast species to metabolize sugars to ethanol. (Zabed et al., 2019, Vasik et al, 2021)

The Indian government has set targets of 10 percent bioethanol blending of petrol by 2022 and to raise it to 20 per cent by 2030 under the ethanol blending programme to curb carbon emissions and reduce India's dependence on imported crude oil. At present only 5% blending of ethanol is being done. 1st generation (1G) and 2nd Generation (2G) bioethanol plants are set to play a key role in making bioethanol available for blending but face challenges in attracting investments from the private sector.

1G bioethanol plants utilize sugarcane juice and molasses, byproducts in the production of sugar, as raw material, while 2G plants utilize surplus biomass and agricultural waste to produce bioethanol. Sugar mills, which are the key domestic suppliers of bio-ethanol to OMCs, were only able to supply 1.9 billion liters of bio-ethanol to OMCs equating to 57.6 per cent of the total demand of 3.3 billion liters. The constraints with using Agricultural waste or Lignocellulosic waste is that, the price of obtaining agricultural waste required for the production of bio-ethanol at 2G plants is currently too

high for it to be viable for private investors in the country. Thus, small investors and entrepreneurs need to take up the issue of setting up fermentation units near the rural areas where lignocellulosic wastes are available so as to make the production profitable and the government should be ready to support these plants.

MATERIALS AND METHODS

Sample collection:

Sugarcane bagasse, Rice stalk and wheat stalks were collected and processed. The agriculture waste was collected, dried at 60^0C for 1-2 days and then was ground to powder and stored for further use.

Pretreatment of the agricultural waste products:

The powdered waste material was then treated with acid for hydrolysis. Acid pretreatment was done by dissolving 50g of each substrate into 500ml of 0.5N H_2SO_4 using a 500ml conical flask. The hydrolysis was continued for 24 hours and then the next day the pH was adjusted to 6.5 by 1% NAOH solution. After the pH adjustment the mixture then autoclaved. The autoclaving at 1210C leads to further hydrolysis of the complex sugars into simpler sugars. Acid hydrolysis was done to achieve delignification. The removal of lignin was necessary for cellulose to become readily available for the enzymes produced by the yeast to convert the glucose to ethanol. The filtrate obtained from the acid hydrolysis and heat pretreatment was used to determine the reducing sugar contents of each of the agricultural waste.(Zhao et al 2020)

Microbial source an Inoculum (yeast) development for fermentation process :

The yeast Saccharomyces cerevisiae was obtained from a bread bakery and brewery in and around Noida. The yeast inoculum was prepared as described by **Vasik et al, 2021, Anu et al 2020**. Two grams (2g) of dry brewer's yeast obtained from the bakery and brewery was grown on Potato dextrose agar (PDA) plate at 30^oC for 48hrs to activate the yeast and check for contaminants. A loopful of the yeast colony was transferred from the agar plate into 100ml of the 5% PDB broth and incubated at room temperature on a shaker at 130rpm for 48 hrs. 10 ml of the broth was centrifuged at 3000rpm for 5min. The supernatant was decanted and the pellet was resuspended in 10ml of sterile distilled water twice, centrifuged and the supernatant decanted. The pellet was resuspended in saline and was used as the inoculum for the fermentation media.

Alcoholic fermentation process:

The hydrolysed agricultural waste media was then inoculated with the yeast pellets obtained above and was kept in shaker to production of alcohol. The sugar and alcohol content in the fermentation media was estimated daily for 3-4 days.

Determination of ethanol production:

Ethanol production was analysed by 2 methods. The first, Dichromate method and second by using a gas chromatography. (Zhao et al 2020)

Determination of alcohol by Dichromate method:

Qualitative estimation of ethanol using biochemical method was done by potassium dichromate method. Most of the chemical oxidation methods are based on the complete oxidation of ethanol by dichromate in the presence of sulphuric acid with the formation of acetic acid. Ten ml of each sample was pipette in a glass tube. The media was centrifuged for 10 minutes at 3000 rpm and the supernatant was taken for all the further testing. For the alcohol determination by Dichromate method the supernatant was mixed with Dichromate solution and kept at 50 0C for 5 minutes and then Optical density was taken using a spectrophotometer. A standard curve was initially prepared using pure ethanol.

Determination of the alcohol content by Gas chromatography:

The supernatant from each sample was also used for ethanol estimation using a Gas chromatography. A Nucon gas chromatograph was used to estimate the ethanol at each sample. The GC uses a Flame ionizing detector for detection of the ethanol produced.

Determination of Reducing sugar by Dinitrosalisylate method:

3,5-Dinitrosalicylic acid is an aromatic compound which reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, that absorbs light strongly at 540 nm. Initially a standard curve is made to estimate the OD and the free sugars. Then the supernatant from the fermentation broth is used to measure the sugar present by the DNS method.

RESULTS AND DISCUSSION

ISOLATION OF YEAST

Dry yeast or *Saccharomyces cerevisiae* powder was collected from local bakery and brewery. The yeast culture obtained were then maintained as purified slants. The Yeast was observed under 40X magnification using methylene blue dye (simple staining) The yeast cells are seen as oval elongated cells.

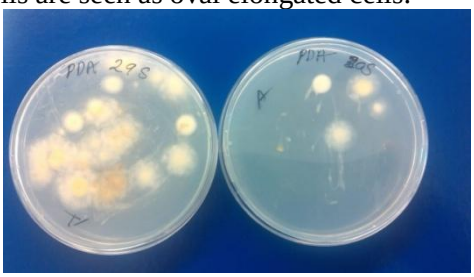

Fig: 1 - PDA Plates with yeast samples

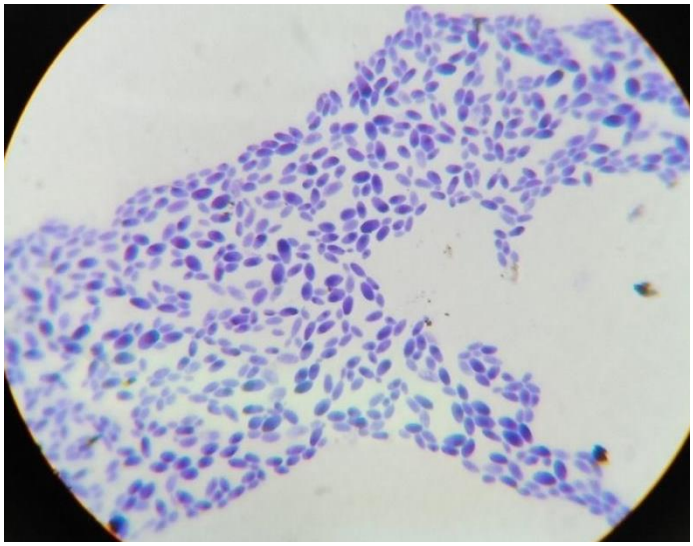

Fig :- 2 Staining and observation of Yeast Strain -1 at 40X

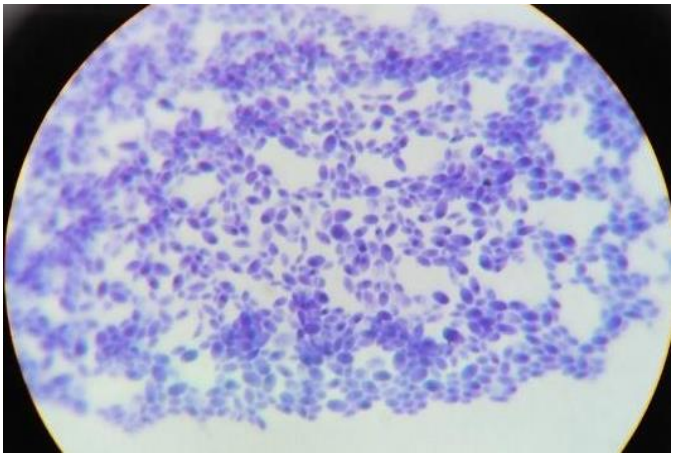

Fig :- 3 Staining and observing Yeast strain- 2 at 40 x

Quantitative estimation of Reducing sugar:

The estimation of reducing sugars in the fermentation broth was done using the DNS method which involved an initial standard curve with known sugars and then the sugar concentrations of the fermentation broth was measured at different days.(Braide et al,2016)

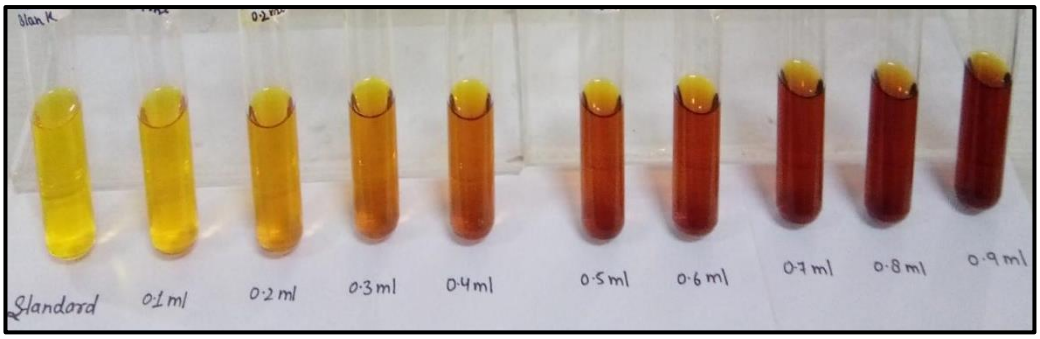

Fig:3 - DNS test Result of the Standard sugar

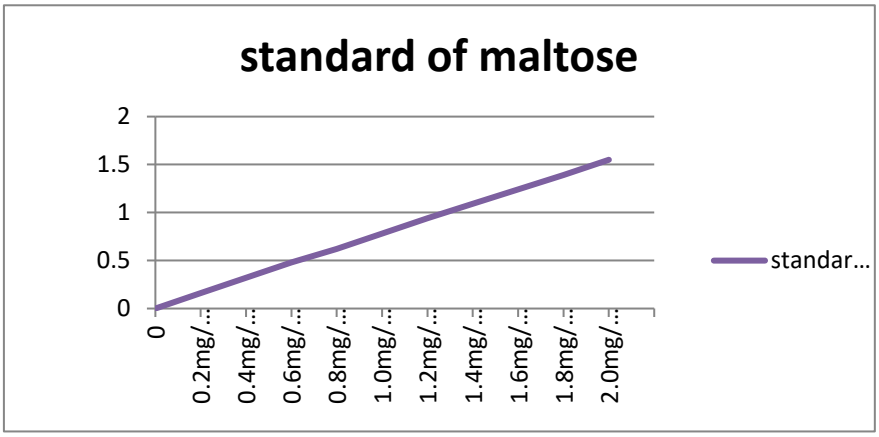

Fig: 4 - Standard Curve of sugar at 540nm

4 Flasks, of which 2 containing hydrolysed Rice straw waste and 2 flasks containing wheat straw waste were inoculated with Yeast strain 1 and 2. 10% inoculum was added to the fermentation broths and the reduction in total sugar was observed by adding DNS solution and boiling at 60^0C. Then, the Optical density at 540nm is noted to estimated the sugars. Optical density is noted from day 0 to day 4 which is as follows:-

S.NO.	Type of media and days after inoculation	YEAST STRAIN 1		YEAST STRAIN 2	
		OPTICAL DENSITY	CONCENTRATION OF MALTOSE (mg/ml)	OPTICAL DENSITY	CONCENTRATION OF MALTOSE (mg/ml)
1.	Rice straw waste media	1.30	16.66	1.52	19.48
	DAY 0				
	DAY 2	1.22	15.64	1.52	19.48
	DAY 4	0.45	5.77	1.15	14.74
	DAY 6	0.39	4.99	1.00	12.82
	DAY 8	0.31	3.97	0.85	10.89
2.	Wheat straw waste media				
	DAY 0	1.39	17.81	1.52	19.48
	DAY 2	1.29	16.53	1.40	17.95
	DAY 4	0.53	6.8	1.28	16.4
	DAY 6	0.45	5.76	0.69	6.10
	DAY 8	0.36	4.61	0.60	7.69

TABLE 1. Optical Density & concentration of reducing sugar estimated at different days in different media by DNS Test .

ESTIMATION OF ETHANOL PRODUCED –
The produced ethanol was estimated by two methods-

1. Potassium dichromate method- This method is a preliminary method which gives percentage of Bioethanol produced by Biochemical method.(Anu et al, 2020)

STRAINS	**YEAST - 1 (Rice straw waste media)**		**YEAST – 2 (Rice straw waste media)**		**YEAST - 1 (Wheat straw waste media)**		**YEAST- 2 (Wheat straw waste media)**	
	OD	% ETHANOL	OD	% ETHANOL	OD	% ETHANOL	OD	% ETHANOL
DAY 0	0.08	1.54	0.06	1.15	0.18	3.47	0.13	2.5
DAY 2	0.43	**8.3**	0.50	**9.62**	0.40	7.7	0.46	**8.85**
DAY 4	0.46	**8.85**	0.53	**10.2**	0.46	**8.85**	0.43	**8.3**
DAY 6	0.16	3.07	0.14	2.69	0.20	3.85	0.04	0.77
DAY 8	0.20	3.85	0.26	5.0	0.19	3.65	0.26	5

Table : 2 Production of Ethanol (in %) Using Different Agricultural waste

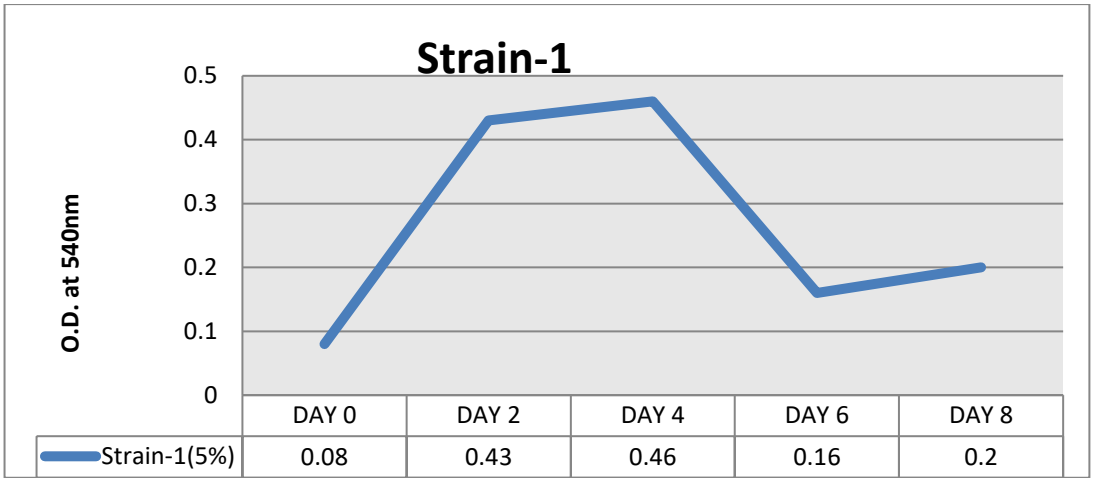

Fig:5 - Bioethanol produced by Strain-1 inoculated in Rice straw waste media (estimated by Dichromate method)

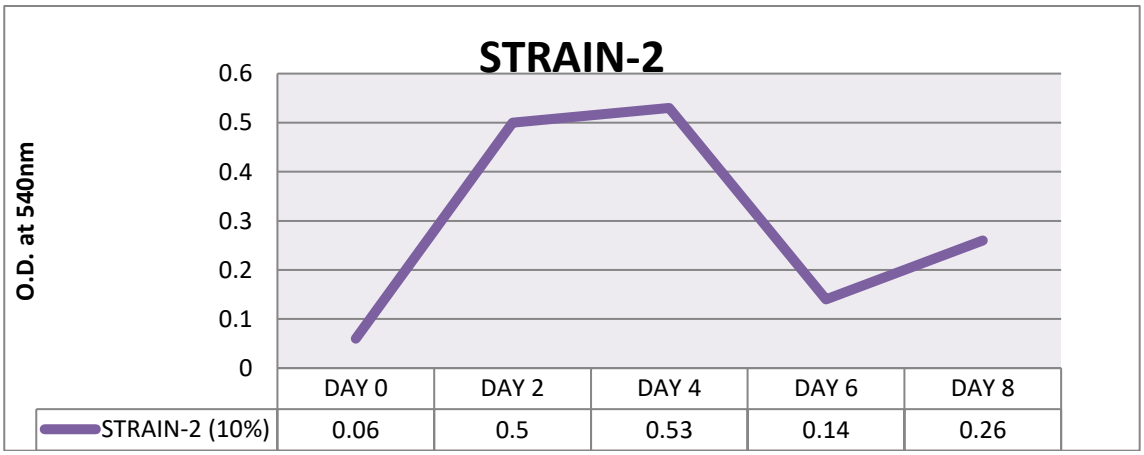

Fig: - 6 Bioethanol produced by Strain-2 inoculated in Rice straw waste media (estimated by Dichromate method)

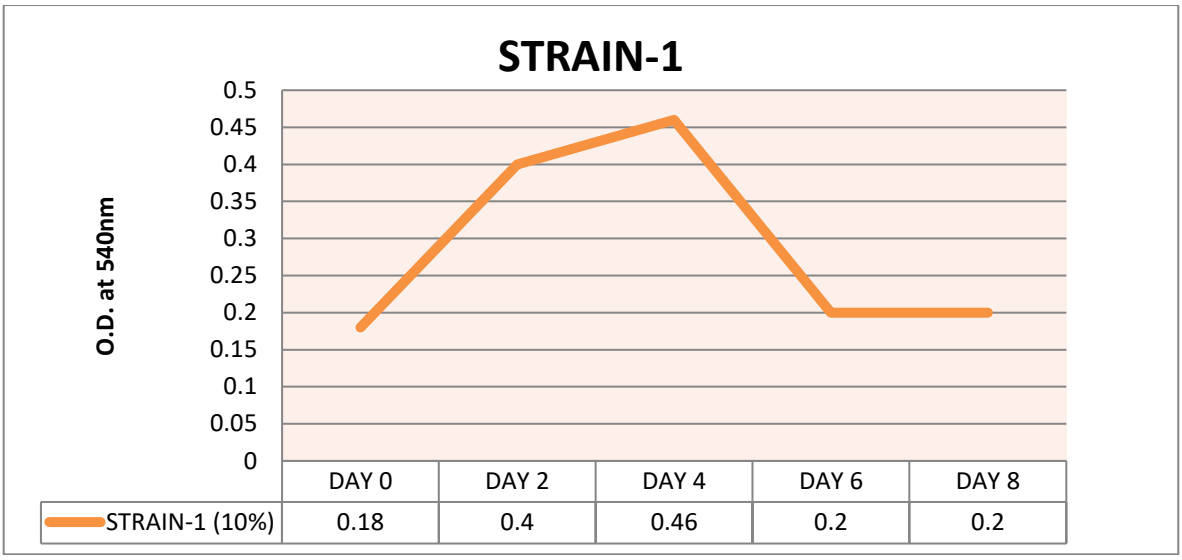

Fig: - 7 Bioethanol produced by Strain-1 inoculated in Wheat straw waste media (estimated by Dichromate method)

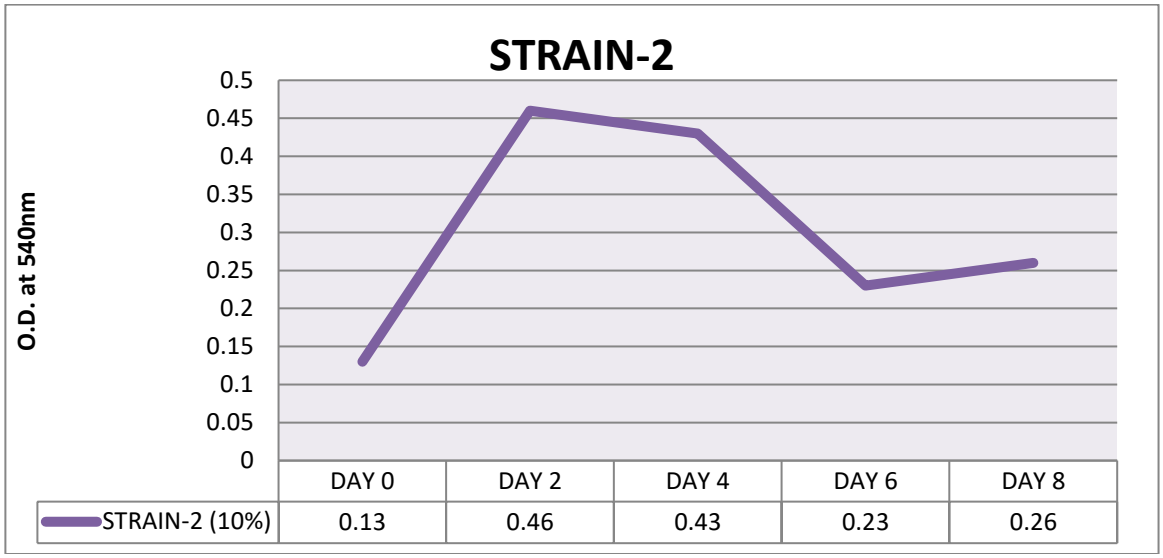

Fig:-8 Bioethanol produced by Strain-2 inoculated in Wheat straw waste media (estimated by Dichromate method)

Gas Chromatography estimation of Bioethanol produced-

Gas chromatography was used to estimate and detect the amount of bioethanol produced for 3-4 days. It was found that the highest amount of ethanol was produced on the 3rd day in most of the cases. The concentration of ethanol produced is shown as peak area and after the total amount of ethanol is calculated in percentage. Relevant GC peaks and tables are shown to clarify the actual production as well as related ethanol production utilizing the different Agricultural wastes.(Braide eta al 2016)

Formula For Calculation Of Percentage Of Ethanol Produced:

$$g/l = \text{Percentage of Injected Ethanol Standard (100\%)} \times \frac{\text{Area of sample}}{\text{Area of Standard (5374164)}}$$

The Gas Chromatographic peaks and areas of the Ethanol produced after submerged batch fermentation when the yeast culture was inoculated in different concentrations. The two yeast cultures gave the Bioethanol yields as given in table below.

Strains	**Yeast Strain 1 (Rice waste)**		**Yeast Strain 2 (Rice waste)**		**Yeast Strain 1 (Wheat waste)**		**Yeast Strain 2(Wheat waste)**	
	Area of ethanol peak	**Ethanol Conc. (%)**	**Area of ethanol peak**	**Ethanol Conc. (%)**	**Area of ethanol peak**	**Ethanol Conc. (%)**	**Area of ethanol peak**	**Ethanol Conc. (%)**

DAY 2	279366	5.19	8613	0.16	105796	1.97	623338	11.6
DAY 4	363822	6.77	1013106	**18.84**	125097	2.32	745587	**13.87**
DAY 6	611478	**11.38**	673521	**12.52**	583707	**10.86**	383228	7.12
DAY 8	166663	3.1	578745	10.76	104103	1.94	142450	2.65

Table : 3 Estimation of Bio-Ethanol produced by Gas Chromatography using different agricultural wastes and Yeast strains.

The present study was carried to estimate and study the production of bioethanol from agricultural wastes. The Yeasts strains used were 2 different *Saccharomyces cerevisiae* strains obtained as dry yeasts from local Bakery and Brewery in Noida.

Rice stalk waste and Wheat stalk waste were used as substrates for conversion to Bioethanol. The released sugars were fermented for 2 to 8 days to produce Bioethanol and estimation of Bioethanol was done after every 48 hours. Preliminary ethanol estimation was done by potassium dichromate method and later confirmatory estimation was by using gas chromatography. The different yeast strains produced different levels of Bioethanol when different wastes were used as substrates.

The agricultural waste powder was hydrolyzed by acid and used as a substrate for Bioethanol production. The results of fermentation showed that *Saccharomyces cerevisiae Strain-2,* when inoculated in Rice straw waste media produced a maximum 18.8 %Bioethanol on the fourth day while it produced a concentration of 13.87 % in wheat straw media. *Saccharomyces cerevisiae Strain-2* showed highest Bioethanol production on the 4th day in both the fermentation media. The maximum sugar utilization was also observed on the 4th and 6th day. Thus, the results of the present study clearly shows that 2nd generation fuels can be used to produce Bioethanol efficiently.

Another similar study used yeast species like *Candida shehatae*, *Pichia (Scheffersomyces) stipitis, and Pachysolentannophilus*, metabolize xylose to ethanol. Enzymatic hydrolysis and fermentation can be performed in a number of ways: by separate saccharification and fermentation, simultaneous saccharification and fermentation or consolidated bioprocessing. (Vasic et al 2021). Balat & Balat 2014, have utilized switch grass waste for Bioethanol production and have finalized a cost effective procedure .

CONCLUSION

It can be concluded that agricultural waste materials such as Rice straw waste and Wheat straw waste have the capability to undergo acid and enzymatic hydrolysis and fermentation to produce bioethanol. Agricultural wastes are rich in cellulose thus can be used to produce cellulosic ethanol. Ligno-cellulosic wastes consist of high amount of glucose which can be converted to bioethanol. Use of Bioethanol as a as a mixture in Petrol can reduce greenhouse gas emission thus reduces air pollution.

Acknowledgements

Thank you for the guidance of Dr. Tripti Bhatnagar from Codon Biotech in the development of this research paper.

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