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"ETHANOL FUEL PRODUCTION THROUGH MICROBIAL EXTRACELLULAR ENZYMATIC HYDROLYSIS AND FERMENTATION FROM RENEWABLE AGROBASED CELLULOSIC WASTES"

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ABSTRACT

In the present work renewable agricultural cellulosic wastes groundnut hulls and rice husks were selected for production of bioethanol fuel. Two locally isolated microorganisms cellulase producing fungus *Aspergillus niger* and ethanol producing *Saccharomyces cerevisiae* were employed for saccharification and fermentation respectively. Substrates were enzymatically saccharified by using *A. niger* followed by addition of *S. cerevisiae* for fermentative production of bioethanol. Two methods of fermentation i.e. stationary and shaking were adopted. High yield of ethanol was obtained from groundnut hulls in stationary fermentation method.

KEY WORDS

Ethanol, cellulose, saccharification, Aspergillus niger, Saccharomyces cerevisiae.

INTRODCUTION

In 1925, Henry Ford quoted ethyl alcohol, ethanol as "the fuel of the future". He furthermore stated, "The fuel of the future is going to come from apples, weeds, saw-dust almost anything. There is fuel in every bit of vegetable matter that can be fermented". Today Henry Ford's futuristic vision significance can be easily understood [1].

The increasing demand for ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives has necessitated increased production of this alcohol. Ethanol production is usually accomplished by chemical synthesis of petrochemical substrates and microbial conversion of carbohydrates present in agricultural products. Owing to depleting reserves and competing industrial needs of petrochemical feedstocks, there is global emphasis in ethanol production by microbial fermentation process. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology [2].

In the current time, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of limited fossil fuel stock but also for safe and better environment. With an inevitable depletion of the world's energy supply, there has been an increasing worldwide interest in alternative sources of energy [1, 3, 4, 5, 6, 7, 8, 9 & 10]. Keeping in view all the above said advantages, biomass based fuel development technologies should rapidly gain momentum and the barriers imposed earlier should be removed for successfully attempting the production of bioethanol at the commercial level.

Ethanol represents closed carbon dioxide cycle because after burning of ethanol, the released CO_2 is recycled back into plant material because plants use CO_2 to synthesize cellulose during photosynthesis cycle[1,3]. Ethanol production process only uses energy from renewable energy sources; no net CO_2 is added to the atmosphere, making ethanol an environmentally beneficial energy source. In addition, the toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources[11]. Ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the green house gas effect [12].

As energy demand increases the global supply of fossil fuels, it causes harm to human health and contributes to the green house gas (GHG) emission. Hahn-Hagerdal [13] alarmed to the society by seeing the security of oil supply and the negative impact of the fossil fuel on the environment, particularly on GHG emissions. The reduction of GHG pollution is the main advantage of utilizing biomass conversion into ethanol[14]. Ethanol contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate emission that pose health hazard to living beings. The present study was therefore undertaken to utilize lignocellulosic biomass for bioethanol (biofuel) production. The objective of the present study was to produce ethanol as a fuel from renewable agricultural cellulosic wastes through microbial extracellular enzymatic hydrolysis and fermentation. The process was carried out in two steps saccharification and fermentation, with saccharification at 30^0C by *Aspergillus niger* and fermentation by *Saccharomyces cerevisiae* at 30^0C. For comparative studies two different methods of

fermentation were adopted: stationary and shaking.

MATERIALS & METHODS

Raw materials: In present study lignocellulosic agricultural wastes- groundnut hulls and rice husks were utilized as substrates. The solid particles of substrates with particle sizes of 2.4 x 1.0cm for groundnut hulls and 1.0 x 0.3cm for rice husks were used.

Pre-treatment of substrates: The substrates were treated chemically with 1%NaOH for a period of 2 hrs [15, 16].

Chemical analysis of Substrates: The substrates were subjected to the estimation of total sugars [17], reducing sugars [18] and cellulose content [19].

Microorganisms and culture:

Aspergillus niger:

The fungal culture *Aspergillus niger* was screened from different soil samples of local paddy and groundnut fields and identified with the help of manuals like "Dermataceous fungi" by Barnett[20], "Text book of Mycology" by Alexopolus [21] and "Handbook of soil fungi" by A. Nagamani, I K Kunwar and C. Manoharachary [22]. The fungus was cultured and maintained on Potato Dextrose agar medium at 30^0C. After optimum growth the culture was stored at 4^0C in refrigerator for further use.

Preparation of Inoculum:

For the preparation of inoculum the culture was plated on PD agar plates. The plates were incubated at 30^0C for 72 hrs until the mycelium sporulates black conidia. Inoculum was produced in 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth by transferring 2 discs from the PDA plates. The flasks were incubated for another 72 hrs at 30^0C till the mycelial mat develops. This mycelial mat was used as inoculum in further saccharification experiments.

Saccharomyces cerevisiae:

The yeast *Saccharomyces cerevisiae* was isolated from soil samples collected from vineyards rich in waste materials which include fallen and discarded grapes. Samples were collected in sterile containers and transferred to laboratory. The soil samples were suspended in sterile distilled water and allowed to settle, then the supernatant was diluted by serial-10 fold dilutions and the samples were inoculated on to sterile Yeast-extract, Peptone and Dextrose (YEPD) plates. The plates were incubated at 30^0C for 48 hrs. The grown yeast isolates were identified as *Saccharomyces cerevisiae* by studying some of the morphological, biochemical and physiological characteristics [23].

Setting up of fermentation:

The fermentative production of bioethanol was carried out in two steps- a) saccharification and b) fermentation. Two methods i.e. stationary and shaking were adopted. The chemically pre-treated substrates were used for all the experiments. In order to optimize bioethanol production the substrates were taken in three different variations in the following manner (Table-1).

Table1
Design of fermentation experiments

EXP No	RICE HUSKS	GROUNDNUT HULLS
1.	5g substrate + 100ml distilled water	5g substrate + 100ml distilled water
2.	5g substrate + 100ml distilled water + 0.5% Lactose	5g substrate + 100ml distilled water + 0.5% Lactose
3.	5g substrate + 100ml chemically defined media	5g substrate + 100ml chemically defined media

Chemically defined medium (Table-2) was used in experiment-3. All the flasks were autoclaved at 15lbs for 15 minutes.

Table – 2
Composition of chemically defined medium (%)

Component	Percentage (%)
L-Glutamic acid	0.03
NH_4No_3	0.14
KH_2PO_4	0.2
$CaCl_2$	0.03
$MgSO_4$	0.03
Proteose peptone	0.75
$FeSO_4$	0.5
$MnSO_4$	0.16
$ZnSO_4$	0.14
Tween 80	2%

Saccharification and fermentation studies were performed in 250 ml Erlenmeyer flasks in which 5 grams of substrate was taken in each flask (as presented in Table-1) and fermentation experiments were carried out.

Saccharification of substrates by Aspergillus niger:

For saccharification of substrates locally isolated fungal culture *Aspergillus niger* was employed. The chemically treated substrates were autoclaved and inoculated with sporulating mycelial mat of *Aspergillus niger*. Saccharification was carried out in stationary and shaking methods for a period of six days at 30^0C and the process was monitored every 24 hrs for total sugars released [24]. For the shaking method an orbital shaking incubator was employed and shaking was performed at 100 rpm at 30^0C temperature.

The *A. niger* was selected for saccharification as it is cellulolytic in nature and can hydrolyze cellulose present in the substrates to simple sugars. Generally this step is carried out by commercially available cellulase enzyme which is very expensive. In our study an attempt was made to design an economical process by the use of intact fungal organism as a source of cellulase enzyme instead of commercially available enzyme. As *A. niger* grows on the cellulosic substrates **it** hydrolyzes cellulose of the substrate and release simple sugars which can be fermented to produce bioethanol.

Fermentative production of bioethanol by Saccharomyces cerevisiae:

For fermentative production of bioethanol (stationary and shaking) yeast *Saccharomyces cerevisiae* was employed. After six days of saccharification mycelial mat of *Aspergillus niger* was removed under aseptic conditions and 10% of *Saccharomyces cerevisiae* culture was added to all the flasks. The process was carried out for a period of six days at 30^0C. During the fermentation process every 24 hours samples were taken for the estimation of bioethanol [25].

RESULTS

In the present study fuel ethanol was produced by saccharification and fermentation of cellulosic substrates. The abundantly and cheaply available renewable cellulosic substrates- groundnut hulls and rice husks were utilized. Before starting the saccharification and fermentation experiments total sugars, reducing sugars and cellulose content were estimated (Table-3).

Table - 3
Chemical composition of Cellulosic substrates (% w/w)

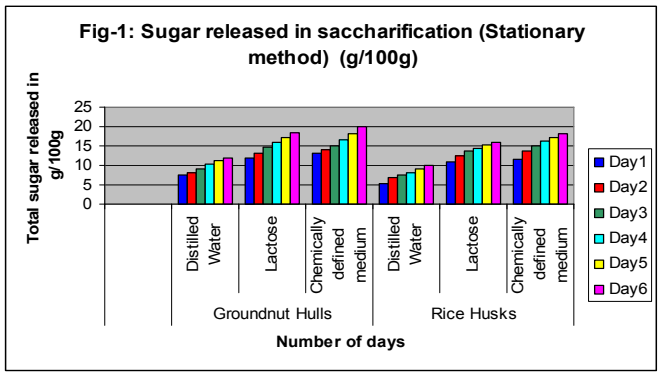
Substrates	Rice Husks	Groundnut Hulls	Analytical Method
Total Sugars	20%	25%	Spectrophotometric method of Hedge et al., 1962
Reducing Sugars	3.2%	4.5%	Spectrophotometric method of Krishnaveni et al., 1984
Cellulose	45%	65%	Spectrophotometric method of Sadasivam et al., 1992

Stationary Fermentation

Saccharification: In stationary fermentation the total sugars released increased from day 1 to day 6, highest amount of sugars released on 6th day of saccharification from all the substrates. Highest amount of sugar was released from groundnut hulls followed by rice husks (Table-4 and Figure-1).

Table – 4
Sugar released in saccharification (Stationary method)(g/100g)

Substrate	Variation	Day1	Day2	Day3	Day4	Day5	Day6
Groundnut Hulls	Distilled Water	7.5	8.2	9	10.4	11.2	12
	Lactose	12	13.2	14.8	16	17.2	18.5
	Chemically defined medium	13	14.2	15	16.5	1.8	20
Rice Husks	Distilled Water	5.2	6.8	7.4	8	9.2	10
	Lactose	11	12.4	13.9	14.4	15.2	16
	Chemically defined medium	11.5	13.8	15	16.4	17.2	18



Fermentation: In stationary fermentation increasing trend in ethanol production was observed from day 1 to day 6. The results of ethanol produced are presented in table-5 and trend is shown in figure2. Highest amount of ethanol was produced on 6th day which is in accordance with release of total sugars. Among two substrates highest amount of ethanol was obtained from groundnut hulls, followed by rice husks indicating that the efficiency of saccharifying and fermenting enzymes on these two substrates shows variations in performance.

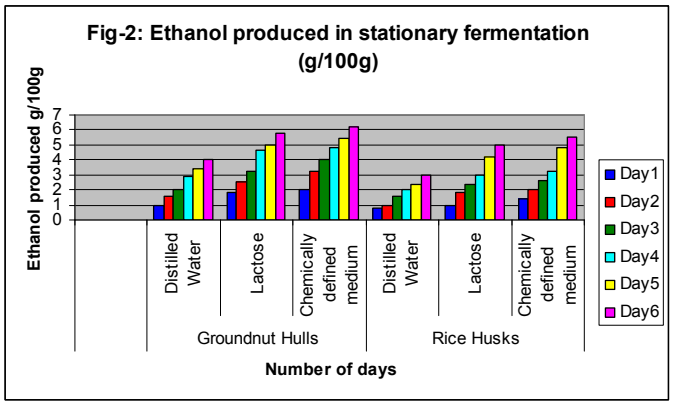
In the case of groundnut hulls high amount of ethanol was obtained from chemically defined medium + groundnut hulls combination (6.2g/100g), followed by groundnut hulls + lactose combination (5.8g/100g) and least ethanol was recorded from groundnut hulls alone (4.0g/100g).

Similar results were also obtained in saccharification and fermentation of rice husks.

Table – 5

Ethanol produced in stationary fermentation (g/100g)

Substrate	Variation	Day1	Day2	Day3	Day4	Day5	Day6
Groundnut Hulls	Distilled Water	1	1.6	2	2.9	3.4	4
	Lactose	1.8	2.5	3.2	4.6	5	5.8
	Chemically defined medium	2	3.2	4	4.8	5.4	6.2
Rice Husks	Distilled Water	0.8	1	1.6	2	2.4	3
	Lactose	1	1.8	2.4	3	4.2	5
	Chemically defined medium	1.4	2	2.6	3.2	4.8	5.5



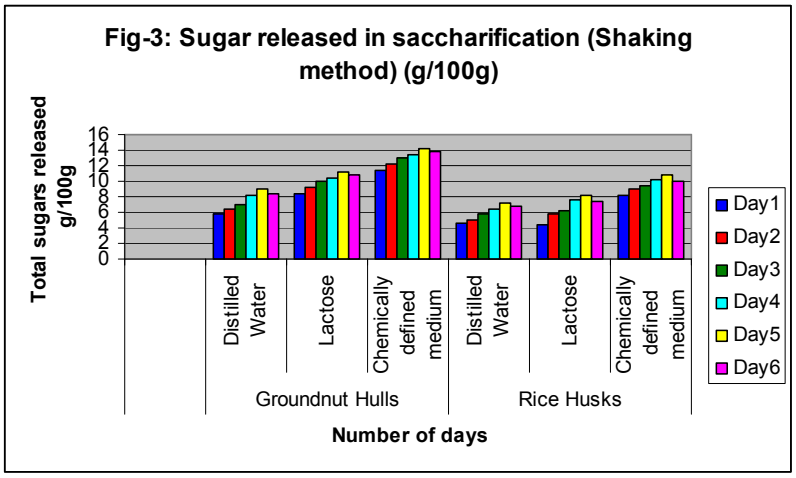
Shaking Fermentation:

Saccharification: In shaking fermentation from day 1 to day 5 steady increases in release of total sugars was observed during saccharification with highest amount of sugars released on 5th day. The highest amount of sugar released from groundnut hulls, followed by rice husks. The results of total sugars released in shaking fermentation are shown in table-6 and figure-3.

Table – 6

Sugar released in saccharification (Shaking method) (g/100g)

Substrate	Variation	Day1	Day2	Day3	Day4	Day5	Day6
Groundnut Hulls	Distilled Water	5.8	6.5	7	8.2	9	8.5
	Lactose	8.5	9.2	10	10.5	11.2	10.8
	Chemically defined medium	11.5	12.2	13	13.5	14.2	13.9
Rice Husks	Distilled Water	4.6	5	5.9	6.5	7.2	6.8
	Lactose	4.5	5.8	6.2	7.7	8.2	7.5
	Chemically defined medium	8.2	9	9.5	10.2	10.8	10



Fermentation: In shaking fermentation comparatively less yield of ethanol was obtained than the stationary method. The time course of ethanol produced and total sugar released was similar to stationary fermentation with a minor fluctuation. With increase in time of fermentation, ethanol production increased up to 5th day. On 6th day the yield of ethanol decreased indicating that the maximum amount of sugar was consumed by 5th day. The results are shown in table-7 and figure-4. The trend of ethanol production in shaking fermentation followed similar pattern to stationary fermentation-highest amount of ethanol was obtained from groundnut hulls, followed by rice husks.

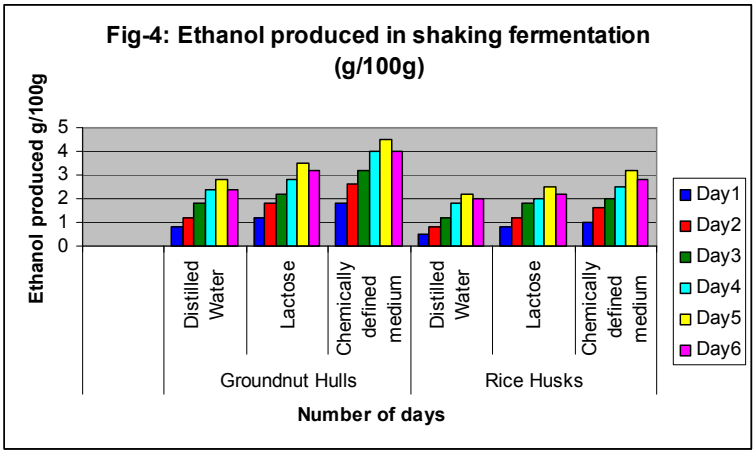
In shaking fermentation also high yield of ethanol was obtained from groundnut hulls with chemically defined medium combination

(4.0g/100g), followed by groundnut hulls + lactose combination (3.2g/100g) and least from groundnut hulls alone (2.4g/100g).

Similar results were also obtained in saccharification and fermentation of rice husks.

Table – 7
Ethanol produced in shaking fermentation (g/100g)

Substrate	Variation	Day1	Day2	Day3	Day4	Day5	Day6
Groundnut Hulls	Distilled Water	0.8	1.2	1.8	2.4	2.8	2.4
	Lactose	1.2	1.8	2.2	2.8	3.5	3.2
	Chemically defined medium	1.8	2.6	3.2	4	4.5	4
Rice Husks	Distilled Water	0.5	0.8	1.2	1.8	2.2	2
	Lactose	0.8	1.2	1.8	2	2.5	2.2
	Chemically defined medium	1	1.6	2	2.5	3.2	2.8



DISCUSSION

In the present work bioethanol production process was studied with a saccharification process by *A. niger* and fermentative production of ethanol by *S. cerevisiae*. Both the substrates were chemically treated with 1%NaOH for a period of 2 hrs before enzyme hydrolysis to improve enzyme amenability. Normally cellulosic materials can be hydrolyzed chemically or enzymatically. It was found that native biomass is extremely recalcitrant to enzyme saccharification, to improve enzyme amenability to holocellulose fraction a number of pretreatment methods have been designed [26, 27, 28]. Vaccarino et al., [29] studied the effects of SO_2, Na_2CO_3 and NaOH pretreatments on the enzymatic digestibility of grape marc. Silverstein et al., [30] studied the effectiveness of sodium hydroxide, hydrogen peroxide and ozone pretreatments on cotton stalks. Further hydrolysis using enzyme increased the release of glucose (sugar). It was found that about 95% of cellulose in pretreated bagasse pulp residue was converted to glucose by cellulase enzyme [31].

Enzymatic saccharification is the main step in biomass to bioethanol conversion.

In our study an attempt was made to use the fungal culture *A. niger* as a source of cellulase enzyme in saccharification step which hydrolyzes complex cellulosic substrates by the release of extracellular cellulase enzyme and release simple sugars. The work of Chandel et al., [1] clearly demonstrates that cost of cellulases and recovery of fermentable sugars after enzymatic saccharification are the important factors which will decide the tangible cost of biomass to ethanol process. As all these methods of saccharification adds extra cost in the production process and to the final product in our study an attempt was made to use the fungal culture *A. niger* as a source of cellulase enzyme in saccharification step which hydrolyzes complex cellulosic substrates by the release of extracellular cellulase enzyme and produce simple sugars.

Bioethanol production is a widely studied process for biofuel production. Different workers have studied various raw materials and different methods for bioethanol production but, recently it has been observed that lignocellulosic materials are focused for bioethanol production. Hence, we have selected cheaply and abundantly available agrobased wastes for bioethanol production. Cellulosic substrates were also used by Arthe et al., [32] for bioethanol production by microbial extracellular enzymatic hydrolysis and fermentation where a yield of 8.9g/l was recorded. In another report of Vaithanomsat et al., [33] bioethanol was produced from enzymatically saccharified sunflower stalks where the yield of 0.02g/100g was obtained. Enzymatically pretreated agricultural residues were also used by Seema Patel et al., [34] for ethanol production by different fungal cultures. The results obtained in our study are in correspondence with these reports.

The overall results showed that cellulolytic activity and ethanol yields are low in the flasks where substrate alone is available. Whereas in flasks where lactose and chemically defined media are present along with the substrates showed increase in cellulolytic activity and ethanol production. Maximum cellulose utilization was observed in the flasks where chemically defined media is present along with the substrate. The results indicate that additions of chemically defined media or lactose are enhancing the cellulolytic activity, the amount of cellulose metabolized and the total ethanol yield. These combinations gave higher ethanol yield than the substrate alone.

CONCLUSION

Finally we can conclude that cellulosic agro wastes particularly groundnut hulls and rice husks are potential substrates which can be exploited in industries in future for bioethanol (biofuel) production as they are cheap, abundant and more importantly renewable. Based on the results obtained it can be concluded that when crude cellulosic wastes are used as raw materials, intact microorganism such as *A.niger* can be used for saccharification of cellulose as a substitute to pure cellulase enzyme, lactose can be added as an enzyme inducer to enhance the cellulase activity, groundnut hulls can be utilized and stationary fermentation is ideal method.

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