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PRODUCTION OF BIOETHANOL FROM AN AGRO WASTE, PROCESSED *ARECA CATECHU* WATER

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ABSTRACT

Bioethanol is a promising potential renewable energy to petroleum-derived transportation fuel. Ethanol can be produced from a variety of biological sources. Lignocellulosic biomass has been suggested as the most promising alternative for the traditional starch feedstock. Areca nut processed agro waste water is an attractive biomass for bioethanol production due to carbohydrate contents. The biochemical tests of Areca nut agro processed water showed the presence of more of cellulose in the raw material. Saccharification was better When the enzymes, cellulase and amylase were used for hydrolysis, than acid hydrolysis (23.5 mg/ml Glu). The bioethanol production revealed that SSF (Simultaneous Saccharification and fermentation) (11.6 g/L ethanol) process is better as compared to SHF (Separate Hydrolysis and Fermentation) process (9.25 g/L ethanol) in bioethanol production. First time we are showing that the Areca nut processed agro waste water is an attractive biomass for bioethanol production due to its higher carbohydrate contents.

KEY WORDS: ***Areca catechu*, Bioethanol, Hydrolysis, Fermentation, Enzymes, Saccharification.**

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INTRODUCTION

Increase on world's energy demand and the progressive depletion of oil reserves motivate the search for alternative energy resources, especially for those derived from renewable materials such as biomass[1]. Global concern about climate change and the consequent need to diminish greenhouse gases emissions have encouraged the use of bioethanol as a gasoline replacement or additive[2]. Bioethanol may also be used as raw material for the production of different chemicals, thus driving a full renewable chemical industry. Bioethanol is produced from the fermentation of sugars either sucrose (e.g. sugarcane, sugar beet) or starch (e.g. corn, wheat) or be a lignocellulosicmaterial (e.g. sugarcane bagasse, wood and straw). India is amongst rapidly expanding large economy, facing a formidable challenge to meet its energy needs to support its growing population. India needs to generate two - to three fold more energy than the current output[3]. It is must for countries like India to invest in to renewable energy options. At present, sugar and starch based raw materials and cereal grains are used for the production of bioethanol. In India, population already reached a billion and thus food security is a national priority and hence India cannot afford to use cereal grains for ethanol production as is commonly done in other biofuel promoting countries in Europe and USA. So, the available sources are plant biomass which is an abundant and renewable source of energy-rich carbohydrates which can be efficiently converted by microbes into biofuels of which, bioethanol is widely produced on an industrial scale today[4]. Ethanol can be produced from a variety of feedstocks, but lignocellulosic biomass has been suggested as the most promising alternative for the traditional starch feedstock. Developing biofuel such as ethanol from renewable biomass materials such as arecanut husk waste has been reported in many literatures as strategic, environmental, and societal benefits. Bioethanol production requires three major processes for the conversion of polysaccharides into ethanol through pretreatment, saccharification and fermentation. The first-step is thermal acid pretreatment which alter the structural integrity of biomass and increase the enzymatic accessibility prior to enzymatic hydrolysis. Enzymatic hydrolysis is second process to hydrolyze the cellulose to oligomeric and monosaccharide as well as decrease the viscosity of hydrolysates, which enables easier pumping and stirring[5]. The enzymatic hydrolysis are currently has monomeric sugar yields of 75~80% and improvements are still projected to 85~95% [6]. After the pretreatment and the enzymatic hydrolysis, fermentation was carried out to produce ethanol by yeasts[7]. In the fermentation step, hexoses and pentoses are converted to ethanol. Distillation is the step required to separate the ethanol from the fermentation broth[8]. When two processes, enzymatic hydrolysis and fermentation, were separately performed, it is referred to the separate hydrolysis and fermentation (SHF). However, each process can be simultaneously carried out, which is referred to simultaneous saccharification and fermentation (SSF) process. The areca nut (*Areca catechu* L.) is a tropical fruit, which is also called betel-nut and is widely distributed in different parts of the world [9, 10]. It is cultivated in India covering an area of about 2.6 lakh ha with an annual production of 3.13 lakh tonnes. The Arecanut contains water (30%), protein (5%), fat (3%), carbohydrates (47%) and total alkaloids (arecoline) (0.2-0.7%). Areca nut husk, which constitutes about 60-80% of the fruit, is a solid residue generated as waste of which a large quantity is disposed off. Areca processed agro waste water is an attractive biomass for bioethanol production due to carbohydrate contents. In the present study, cost effective means of producing ethanol from processed areca water by pretreatment followed by saccharification carried out by of *Aspergillus niger* and fermentation of the released sugars to ethanol, using a yeast strain *Saccharomyces cerevisiae* has been carried out. The study of bioethanol production process was carried out by both SHF and SSF methods.

MATERIALS AND METHODS

(i) Raw material

Areca nut was collected from the local farmer in Bangalore rural district, Karnataka, India. Collected material was stored in a laboratory condition until further usage. The husking of fruits was carried to remove embryos. Embryos were cut into four parts and boiled in water for 10-20 min. The processed water was filtered, autoclaved and stored in refrigerator for further use. The raw material Areca nut was a regular produce around the Bangalore and available in plenty. The farmer never mind to give freely for research purpose.

(ii) Qualitative analysis of agro processed waste water

Qualitative analysis was carried out for the presence of different sugars by a series of biochemical tests for reducing and non-reducing sugars, monosaccharide and disaccharides, and for aldoses and ketoses.

(iii) Fungal cultures maintenance

The fungal culture used for microbiological pretreatment was *Aspergillus niger* was maintained on Potato dextrose agar (PDA).

(iv) Production and extraction of amylase and cellulase

Autoclaved substrate was utilized for enzyme production using *Aspergillu niger.* Inoculation was done with 1 x 10^8 spores. The composition of media was (g/L): KCl 2; KH_2PO_4, 1.0; $MgSO_47H_2O$, 0.05; $FeSO_47H_2O$, 0.02; D.H_2O 1lt. Flasks were inoculated and maintained at 30 ^{0}C for 7days on rotary shaker with 120rpm speed. Crude enzyme was extracted from the flasks after centrifugation at 10,000 rpm for 15min. The supernatant obtained was analyzed for enzyme activities like cellulase and amylase. Enzymes obtained were utilized for carrying out saccharification and saccharified hydrolysate utilized for ethanol production[11].

(v) Hydrolysis

Substrate, areca nut processed agro waste water, was subjected to acid treatment using 2% of 10N H_2SO_4 for 5h at room temperature and later adjusted to pH 6.0

using 1N NaOH. Samples were filtered and stored at 4^oC for the determination of reducing sugar content by 3,5-dinitrosalicyclic acid (DNS) method[12].

(vi) Enzymatic hydrolysis

The pretreated acid slurry performed with sulfuric acid adjusted to pH 6.0 was treated with commercial enzymes such as amylase and cellulase purchased from Sigma Aldrich company and crude enzymes (amylase and cellulase) extracted from *Aspergillus niger* respectively, in the ratio of 1:10. The reaction mixture was incubated on a rotary shaker at room temperature at120 rpm for 5hours for saccharification process. Reducing sugar content was determined by the DNS method[12].

(vii) Analytical methods

The reducing sugar of the acid hydrolysate was estimated by Dinitrosalicylic acid (DNS) method[10]. The 0.5ml aliquot of extract was pipetted into test tubes. The volume was equalized to 3ml with water in all the test tubes. Then 3ml of DNS reagent was added, mixed and kept in boiling water bath for five minutes. The reaction was stopped by taking out the test tubes and they were cooled and the absorption was taken at 540 nm under UV-visible spectrophotometer. The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose[13]. Viscosity was measured by viscometer at 30^oC.

(viii) Amylase assay

Amylase activity was measured by DNS method[10]. Amount of reducing sugars liberated from 1% of 1ml soluble starch solution was determined by dissolving in 0.5 ml of 50mM phosphate buffer, pH 7.2 mixed with 0.5ml of enzyme. The mixture was incubated for 10 min at 30°C for the enzyme substrate reaction to occur and the reaction was stopped by adding DNS solution. The samples were boiled for five min in boiling water bath, cooled in water for colour stabilization, and the optical density (OD) was measured at 540nm using spectrophotometer against a suitable blank. The amylase activity was determined using a calibration curve for glucose (standard glucose 5mg/ml)[14].

(ix) Cellulase assay

Crude cellulase activity was determined by the Carboxy methyl cellulose method. Reaction mixture comprised of 0.5 ml 1% carboxyl methyl cellulose in 0.5 ml of 0.05M citrate buffer at pH 4.8 and 0.5ml culture filtrate in test tubes. Mixture was incubated at 50 °C for 15min. After incubation, 1ml DNS reagent was added to stop the reaction. The reaction mixture is further boiled for 5min in boiling water bath and shifted to cold water bath. Absorbance was measured at 540nm in spectrophotometer. Amount of reducing sugar was calculated using standard curve of glucose[15, 16].

(x) Fermentation

(x a) Separate hydrolysis and fermentation (SHF)

SHF was carried out in 250ml flask with 100ml of saccharified hydrolysate supplemented with 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$ and 1.0 g/L yeast extract. The next process of fermentation was carried out by the inoculation of one percent (g/ml) of dry yeast, *S. cereviceae.* Potassium disulphite, an antiseptic, was added to the medium to prevent contamination with harmful microorganisms. Bioethanol formed was estimated after 5 days of fermentation[17].

(xb)Simultaneous saccharification and fermentation (SSF)

SSF was carried out in 250ml flask with 100ml of hydrolysate. Additional nutrients were also added to the medium; 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$ and 1.0 g/L yeast extract along with Potassium disulphite. Fermentation was initiated by the adding enzyme mixtures together with a one percent (g/ml) of dry yeast, *S. cerevisiae* suspension. The flasks were incubated at 32^0c under static anaerobic conditions. A CO_2 outlet was bent was inserted into the hole of rubber cork stopper and was immersed into a glass tube filled with $Ca(OH)_2$ solution. Bioethanol formed was estimated after 5days of fermentation[17].

(xi) Distillation of Bioethanol and Bioethanol Assay Bioethanol assay by specify gravity bottle method

After 5days, the sample was filtered using Whatman Filter Paper to separate the ethanol from the residues. The specific gravity of the sample was determined by Specific gravity bottle method. Specific gravity of the sample was determined by finding the ratio of the sample and the weight of the distilled water at the room temperature 28 ^0C in a specific gravity bottle. Initially the weight of the empty specific gravity bottle was determined followed by the weight of the bottle with the sample and later weight of the bottle with distilled water[18]. The specific gravity of alcohol was then calculated using the formula. The percentage of alcohol in the given sample to specific gravity at temperature was find out using Association of analytical chemist method (AOAC 1980).

$$\frac{W2 - W1}{W3 - W1} \times \text{Density of water at Specific Gravity } (0.9971 \text{ g/cm}^3)$$

W1- weight of empty specific gravity bottle
W2- weight of bottle with sample
W3- weight of bottle with distilled water

(xi a) Potassium Dichromate Method

Alcohol standard was prepared by taking different aliquots of standard 99.9% ethanol (0.1-1 ml) and volume was made up to 5ml with distilled water. Then 1ml of Potassium dichromate reagent (0.1g/ml) was added. All the test tubes were kept in ice water and 4

ml of conc. H_2SO_4 was added to each tube gently through the walls. The OD was measured at 660 nm and standard graph was plotted to obtain the concentrations of unknown samples[14].

(xii) Gas chromatography analysis

The ethanol so produced by SSF and SHF methods was analyzed for its presence. To authenticate the qualitative of Bioethanol, Gas Chromatography (GC) was performed.

RESULTS AND DISCUSSION

Qualitative test of Areca nut processed Agro waste water for carbohydrates

Areca nut processed agro waste water is an attractive biomass for bioethanol production due to its carbohydrate contents. In the present study, cost effective means of producing ethanol from processed areca nut waste water were carried out. The processed water which was filtered, autoclaved and stored in refrigerator was used for qualitative analysis tests for the presences of different sugars both, reducing and non-reducing sugars. The results are presented in the Table 1. The results revealed the presence of reducing sugars in the areca nut processed agro waste water. There is absence of pentose sugar (Bial's test negative). Though the polysaccharide starch is absent, other polysaccharide such as cellulose is present. There is presence of ketose sugar, which must be fructose (Seliwanoff's test). Over all, there is monosaccharide reducing sugars in the processed water in addition to cellulose which makes it necessary for hydrolysis treatment of the water.

Carbohydrate Hydrolysis

Substrate requires pretreatment in order to make the carbohydrate accessible to enzymes. The pretreatment of raw substrate with sulfuric acid increases the enzymatic digestibility of biomass, giving the higher sugar yield than that without sulfuric acid treatment[17]. The results of reducing sugar yield (g/L) by 2 % H_2So_4 concentrations at room temperature for 5hrs was given in table 2. The glucose concentration was less in the raw substrate (processed waste water) (16.5 mg/ml) as compared to all other samples after acid hydrolysis and enzyme hydrolysis (Fig 1). After treatment of raw material with acid and enzymes, the concentration of reducing sugar (glucose) was found to be more due to hydrolysis of polysaccharide (cellulose) into its monomers. When the pure cellulase and amylase enzymes were used for hydrolysis, the saccharification was better than acid hydrolysis and pure enzyme hydrolysis is most efficient method of all. This reveals the necessity of hydrolysis of the raw material with various chemicals and enzymes for increasing the reducing sugar content.

Bioethanol production

After analysis of capability of the carbohydrates present in the areca nut processed agro waste water, the potential of the hydrolysates for fermentive bioethanol production was studied. Fermentation was carried out using both by Separate hydrolysis and fermentation (SHF) and Simultaneous saccharification and fermentation (SSF) methods. After 5 days of fermentation, the sample was filtered using Whatman Filter Paper to separate the ethanol from the residue and bioethanol formed was estimated by Specific gravity bottle method and Potassium di chromate method. The results were showed in fig 2. Both the methods confirm the presence of ethanol by quantitative method and concentration of alcohol were found to near to each other. To authenticate the qualitative properties of Bioethanol, Gas Chromatography (GC) was performed and sample result is shown in Fig 3. The highest alcohol (11.6 g/L) was produced in SSF method after the hydrolysate was enzyme treated as compared to SHF method (9.25g/L) (Fig 2). There is a rapid reduction in the reducing sugar content after fermentation in SSF method. During the same period, a rapid increase of ethanol concentration was also observed. However, some amount of reducing sugar was still remained at the end of the SSF process. This indicates that non-fermentable sugars presence and it was not utilized by yeast *S. cerevisiae* during fermentation. With crude enzyme treatment of the hydrolysate, little less amount of alcohol is produced by same method of fermentation (9.25 g/L). Similar pattern of reducing sugar concentration and increased ethanol production was also observed in the case of SHF method of fermentation. But in this method, the extent of carbohydrate conversion into reducing sugar was lower than that obtained in the SSF process. This might be due to the difference in process temperatures during enzymatic hydrolysis and fermentation. The alcohol concentration of 8.4 g/L was produced from *Moringa olifera* pod husk has been reported[19]. And also there is not much difference in the production level of alcohol by this method after enzymatic hydrolysis by pure enzymes as compared to crude enzymes. This reveals that the simultaneous hydrolysis and fermentation (SSF) method of bioethanol production is more efficient in producing alcohol in this case than the separate hydrolysis and fermentation (SHF) method of bioethanol production. Similar kind of results, the SSF method of alcohol is superior over the SHF method, have been reported for bioethanol production using different materials like empty fruit bunch of palm oil[20], wheat straw[21] and corn stover[22].

Table 1
Qualitative analysis of Substrate

Sl No	Test tube	Conc. Of Glucose (mg/ml)
A. Before fermentation		
1	Areca nut processed waste water	16.5
2	Acid treatment	19.5
3	Pure Enzyme treatment	23.5
4	Crude Enzyme treatment	22.75
B. After fermentation		
1	SHF (Pure Enzyme)	9.25
2	SHF (Crude Enzyme)	9.20
3	SSF (Pure Enzyme)	11.60
4	SSF (Crude Enzyme)	9.25

Table 2
Concentration of Glucose as estimated by DNS method in different components

Sl. No	Test	Results
1	Molisch	+
2	Fehlings	+
3	Bials	-
4	Seliwanoffs	+
5	Barfords	+
6	Starch	-
7	Cellulose	+

Table 3
Amylase and Cellulase activity of purified and crude enzyme

	Purified	Crude enzyme
Amylase	0.033 IU	0.032 IU
Cellulase	0.036 IU	0.033 IU

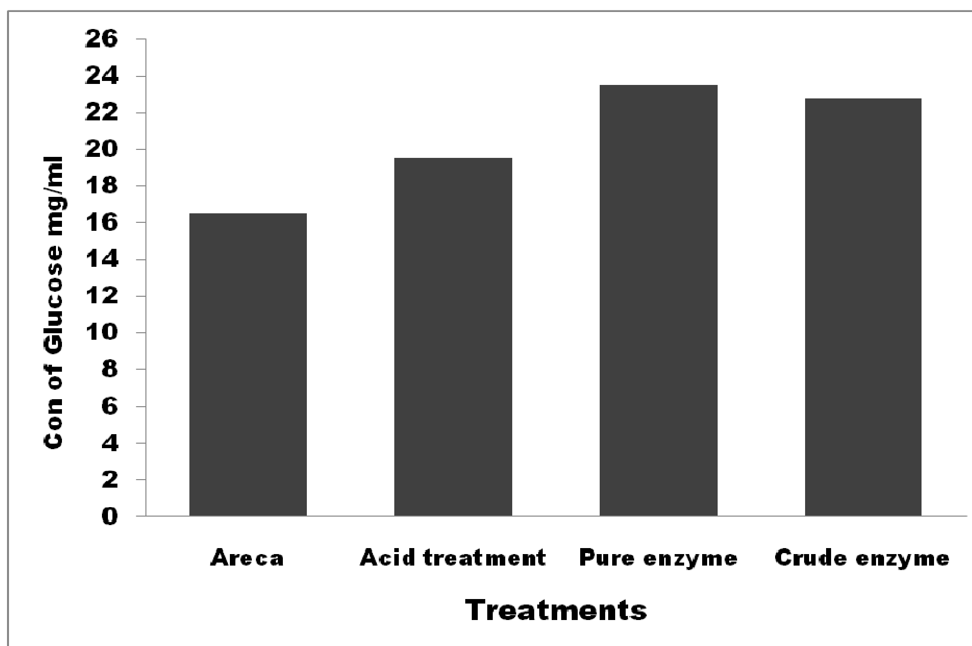

Figure 1
Concentration of reducing sugar (Glucose) (mg/ml) after Acid and Enzyme hydrolysis

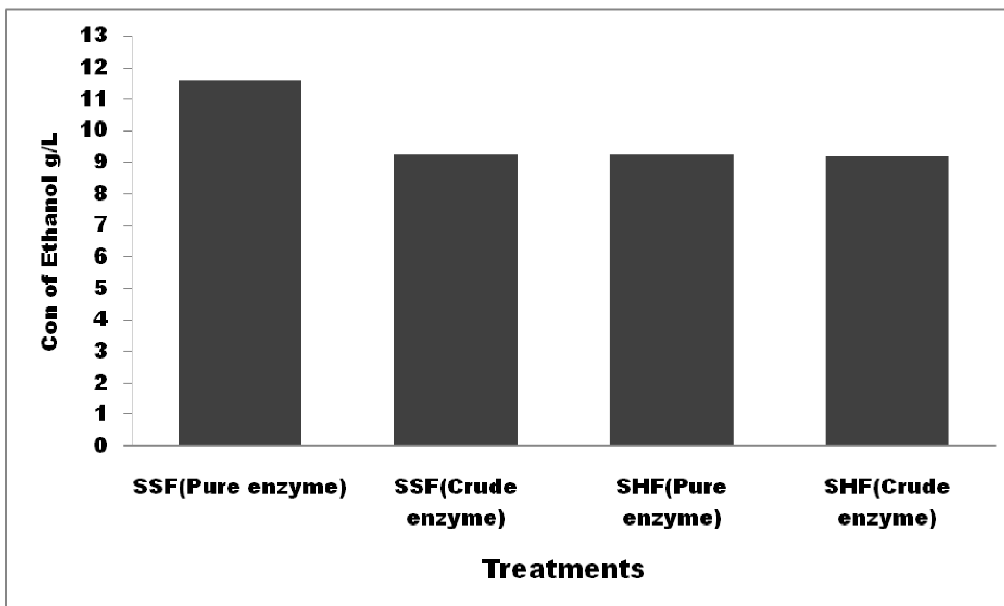

Figure 2
Quantity of Ethanol produced (g/l) with different fermentation methods (SSF& SHF)

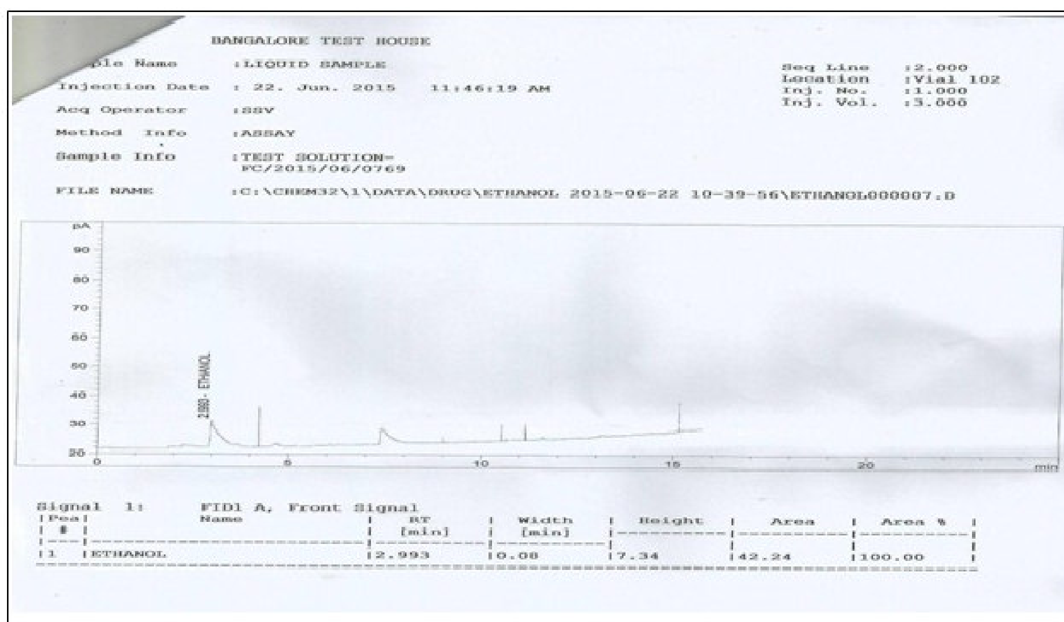

Figure 3
GC analysis of ethanol

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CONCLUSION

First time we are showing that the Areca nut processed agro waste water is an attractive biomass for bioethanol production due to its higher carbohydrate contents. When the enzymes, cellulase and amylase enzymes were used for hydrolysis, the saccharification was better than acid hydrolysis and pure enzyme hydrolysis is most efficient method of all. The study of bioethanol production by SSF and SHF process revealed that SSF (11.6 g/L ethanol) process is better as compared to SHF (9.25 g/L ethanol) in both carbohydrate utilization and also amount of bioethanol produced (Fig 2). Yet an improvement of saccharification step is required for optimization of parameter conditions like pH, temperature, time and combination of enzymes for higher ethanol production.

CONFLICT OF INTEREST
Conflict of interest declared none.

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