Research in Biotechnology, 6(6): 17-30, 2015 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

Acid and enzyme hydrolysis to convert pretreated areca nut (*areca catechu* l.) husk into glucose for bioethanol production by yeasts and *Zymomonas mobilis* NCIM 2915

Naveen Kumar K. J[1], Thippeswamy B[1*] and Krishnappa M[2]

[1]Department of P.G. Studies and Research in Microbiology, [2]Department of P.G. Studies and Research in Applied Botany, Bioscience Complex, Kuvempu University, Jnana Sahyadri, Shankaraghatta-577 451, Shivamogga district, Karnataka, India
[*]Corresponding Author Email: thippeswamyb272@yahoo.in

Production of renewable fuels, especially bio-ethanol from lignocellulosic biomass, holds remarkable potential to meet the current energy demand as well as to mitigate greenhouse gas emissions for a sustainable environment. Determining optimal pretreatment techniques for fermentation is essential for the success of lignocellulosic energy production process. The study involved the acid pretreatment and use of laccase enzyme to degrade the complex lignocellulosic biomass to simple sugars. Sugars so formed in turn are converted to ethanol by employing suitable yeast strains and bacterium *Zymomonas mobilis*. Different fermentation process like separate hydrolysis and fermentation process (SHF) and simultaneous saccharification and fermentation process (SSF) have been evaluated for the biethanol production. In separate hydrolysis and fermentation process, the higher ethanol production was in *Zymomonas mobilis* (44.97±3.21 g/L) and *Schizosaccharomyces pombe* (42.60±3.0 g/L), average ethanol production in *Saccharomyces cerevisiae* (33.13±1.96 g/L) and very low ethanol production in *Candida shehatae* (25.24±2.30 g/L). In simultaneous saccharification and fermentation process, the higher ethanol production was in *Zymomonas mobilis* (47.34±3.22 g/L) and *Saccharomyces uvarum* (44.18±2.67 g/L), average ethanol production in *Pichia stipitis* (34.71±1.89 g/L), and very low ethanol production in *Saccharomyces cerevisiae* (26.82±2.63 g/L) was monitored after the fermentation process. Structural changes of areca nut husk before and after acid pretreatment were further investigated through Scanning electron microscopy (SEM) and Fourier transformed infrared spectroscopy (FTIR). Hence, acid and enzymatic pre-treatment is more effective for ethanol production. Areca nut husk was revealed as a suitable substrate for ethanol production.

Key words: Areca nut husk, Bioethanol, Laccase, Yeasts, *Zymomonas mobilis*

Ethanol is an oxygenated fuel with high octane value like that of petroleum fuels. Ethanol is known to run combustion engines at higher compression ratios and thus provides superior performance. The blending of ethanol into petroleum-based automobile fuels can significantly decrease petroleum use and release of greenhouse gas emissions. Further, ethanol can be a safer alternative to the common additive, methyl tertiary butyl

ether (MTBE), in gasoline. Thus, ethanol can be a substitute to mitigate the problems associated with the rising energy demands across the world as well as a way to reduce green house gas emissions to an extent of 85% (Joshi *et al*., 2011).

Ethanol may be produced either from petroleum products or from biomass. Today, most of the ethanol produced comes from renewable resources. Although currently most of the ethanol produced from renewable resources comes from sugarcane and starchy grains, significant efforts are being made to produce ethanol from lignocellulosic biomass (almost 50% of all biomass in the biosphere) such as agriculture residues (Bothast and Saha, 1997). The technological advances in recent years are promising to produce ethanol at low cost from lignocellulosic biomass.

Some of the important reasons for the pretreatment step are to (i) break the lignin-hemicellulose-pectin complex, (ii) disrupt /loosen-up the crystalline structure of cellulose and (iii) increase the porosity of the biomass. These changes in lignocellulosic materials make it easier for enzymatic saccharification (hydrolysis), results in higher fermentable sugars levels and will have a significant impact on the overall process (Mosier *et al*., 2005b; Sun and Cheng, 2007; Yang and Wyman, 2008).

Areca nut (*Areca catechu* L.) is one of the most important commercial crops in India. India ranks first in areca nut production in the world. In India the cultivation of areca nut is mostly confined to Karnataka, Kerala and Assam in terms of total area under cultivation and production is around 83 percent. The area under areca nut is around 4 lakh hectares with a production of around 4.78 lakh tons in India. Karnataka stands first both in terms of area and production followed by Kerala and Assam. In Karnataka, around 2.15 lakh hectares are under areca nut cultivation (Ramappa and Manjunatha, 2013; Ramappa, 2013). Areca nut popularly known as betel nut or supari is one of the most important plantation crop in Shivamogga district (Narayanamurthy *et al*., 2008). The area under areca nut cultivation has increased more rapidly in Shimoga district as compared to Dakshina Kannada and Uttara Kannada districts, Karnataka state, India. The area under Shivamogga district areca nut is 94, 077.50 hectares with a production of around 52,781 Metric tonns (Ramappa and Manjunatha, 2013; Ramappa, 2013). The areca nut husk fibers are predominantly composed of cellulose and varying proportions of hemicelluloses, lignin, pectin and proto-pection. The total hemicellulose content varies with the development and maturity, the mature husk containing less hemicellulose than the immature ones. The lignin content proportionately increases with the develop-ment until maturity (Rajan *et al*., 2005).

The availability of areca husk waste is very high in Shivamogga district due its area and high productivity in this region. Areca nut husk is most abundant renewable energy source that may be considered as potential feed stock for ethanol production by microbial fermentation. There is no report available on ethanol production from areca nut husk.

The major objective of the present investigation was to evaluate the effect of acid and enzymatic pretreatment on areca nut husk for improved yield of reducing sugar and bioethanol production by yeasts and bacterium *Zymomonas mobilis*. The process was carried out in two methods like separate hydrolysis and fermentation process (SHF) and simultaneous saccharification and fermentation process (SSF). FTIR and SEM were used as analytical tools for qualitative determination of the structural changes in areca nut husk after acid pretreatment.

Materials and Methods

Study area

Shivamogga is a district in the Karnataka state of India. A major part of Shivamogga district

lies in the Malnad region of the Western Ghats. Shimoga lies between the latitudes 13°27' and 14°39' N and between the longitudes 74°38' and 76°04' E at a mean altitude of 640 meters above sea level. As the district lies in the tropical region, rainy season occurs from June to October. The average annual temperature of Shimoga District is around 26 °C. The average temperature has increased substantially over the years. In some regions of the district; the day temperature can reach 40 °C during summer. This has led to water crisis and other problems (Figure 1).

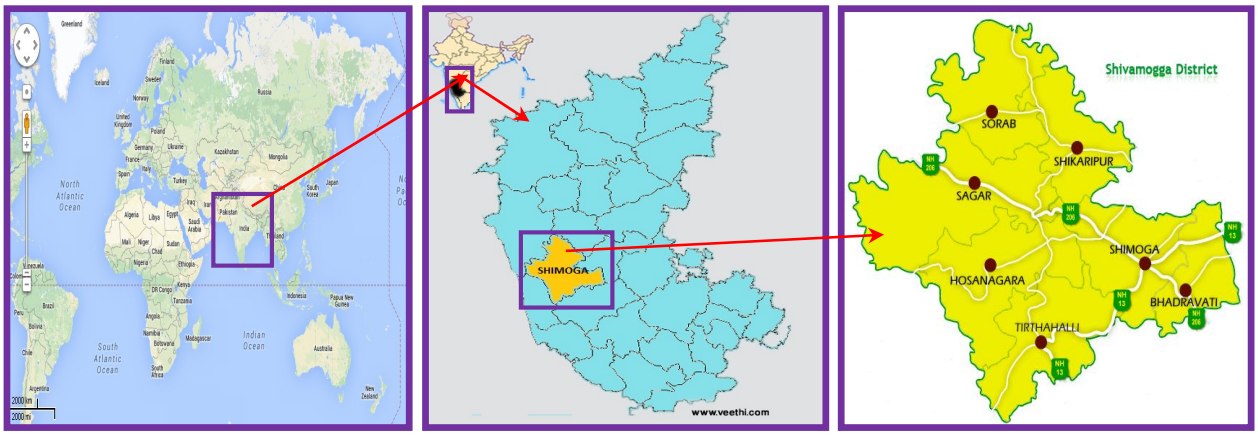


Figure 1: Study location and map of Shivamogga district, Karnataka state, India

Figure 2: A. Areca nut palm (plant), **B.** Areca nut husk and **C.** Areca nut husk fine sized powder (0.28±0.01mm).

Collection of areca nut husk

Areca nut husk was collected from the Shivamogga region, Karnataka state, India during 2010-2014. The sample was brought to the laboratory and was maintained at room temperature for microbiological study.

Milling of areca nut husk

The areca nut husk sample was sun dried for 24 hours in order to remove the moisture content present. And later the Areca nut husk was kept in hot air oven 80 °C for 24-48hr. Then, the Areca nut husk was completely air dried and the areca nut husk was poured to the milling machine for hammer milling, where the milling was done in order to cut the areca nut husk into small pieces. Later areca nut husk was sewed using sewing machine having larger pore size and the bigger particles were obtained after sewing, bigger size particles were again allowed for the milling. So that the particles of small size having 3mm diameter have been obtained, later, the obtained areca nut husk was again

subjected to flour milling machine. The areca nut husk was finely powdered and the powder was sewed using a sewing machine, in order to obtain the fine sized particles (0.28 ± 0.01 mm) (Figure 2) (Narayanamurthy *et al*., 2008; Ballesteros *et al*., 2010; Naveenkumar *et al*., 2012a).

Laccase enzyme

The laccase enzyme was obtained from Sigma Aldrich with product number 51639–1G, off-white powder derived from culture of *Trametes versicolor*. Laccase preparation had >10 units activity per milligram (mg) and to maintain the temperature -4^0 C in refrigerator for further study.

Selection of yeast cultures and bacteria

The standard yeast strains used for the fermentation process were *Saccharomyces cerevisiae* NCIM 3095, *Candida shehatae* NCIM 3500, *Saccharomyces uvarum* NCIM 3455, *Pichia stipitis* NCIM 3498, *Schizosaccharomyces pombe* NCIM 3457 maintained on MGYP medium (Composition of MGYP medium: Malt extract 3g, Glucose 10g, Yeast extract 3g, Peptone 5g, Agar 20g, distilled water 1000 mL, Adjust p^H to 6.4-6.8) and bacterium *Zymomonas mobilis* NCIM 2915 was maintained on nutrient agar with 2% glucose (Composition of nutrient agar: Beef extract 10g, Sodium chloride 5g, Peptone 10g, Glucose 20g, Agar 20g, distilled water 1000 ml, Adjust p^H to 7.0–7.5). These yeast cultures and bacteria were procured from the National Chemical Laboratory (NCL), Pune India (Amin and Doelle, 1990; Gurav and Geeta, 2007; Patel, 2008; Naveenkumar *et al*., 2012b).

Inoculum preparation

For inoculum preparation, yeast cultures was grown in YPD broth (Composition of YPD broth: Yeast extract 10g, Peptone 10g, Glucose 50g and Distilled water 1000mL) and bacterium *Zymomonas mobilis* NCIM 2915 was grown in nutrient broth with 2% glucose (Composition of nutrient broth: Beef extract 10g, Sodium chloride 5g, Peptone 10g, Glucose 20g and Distilled water 1000mL) at 30 °C in a rotary shaker (150 rpm) for 72 hours, harvested by centrifugation, washed three times with sterile distilled water and suspended in sterile water to get 1x10^6 cells per mL (Ciani and Ferraro, 1998).

Acid hydrolysis

In acid hydrolysis pre-treatment, 100 gram of the raw material was weighed in to two litter conical flasks and 1000 mL of 2N sulphuric acid was added to the conical flask. The flasks were covered with aluminium foil and heated for six hours on boiling water bath. The flask was allowed to cool and filtered. The pH was adjusted to 4.5 with 0.4 M Sodium hydroxide. The total reducing and non reducing sugar content of pre-treated raw materials was estimated. This filtrate was used for further enzymatic saccharification (Patel, 2008).

Separate Hydrolysis and Fermentation

Step-1: Enzymatic Saccharification

Separate hydrolysis and fermentation (SHF) was performed with media and it was sterilized (SHF media composition: Yeast extract 5g, Ammonium sulfate 7.5g, Dipotassium hydrogen phosphate 3.5g, Magnesium Sulfate heptahydrate 0.75g, Calcium chloride dehydrate 1g, Substrate 1g, distilled water 1000ml). Then, 1 mL of cellulase enzyme was added to each flask aseptically with filtrate (acid pretreated) and incubated at 50 °C for 4–6 hours. Then, the media was filtered using Whatman No. 1 filter paper. The filtrate was used for further studies.

Step-2: Fermentation

Culture filtrate obtained from the above procedure were further inoculated with each of the organisms as mentioned above individually (3%) and allowed for fermentation for 72 hours. After fermentation, the sample was recovered by distillation unit for spectrophotometric analysis was used for determining ethanol concentration (Patel,

2008; Sun and Cheng, 2002; Moniruzzaman *et al*., 2013).

Simultaneous Saccharification and Fermentation

Simultaneous saccharification and fermentation (SSF) was performed with media and it was sterilized (SSF media composition: Yeast extract 5g, Ammonium sulfate 7.5g, Dipotassium hydrogen phosphate 3.5g, Magnesium Sulfate heptahydrate 0.75g, Calcium chloride dehydrate 1g, Substrate 1g, distilled water 1000ml). Then, 1 ml of cellulase enzyme was added to each flask aseptically with filtrate. All the Simultaneous Saccharification and Fermentation experiments were performed at 30 °C. The organism's inoculum was added after two days individually and incubated further for three days. Then, the media was filtered using Whatman No. 1 filter paper. After fermentation, the sample was recovered by distillation unit for spectrophotometric analysis ethanol concentration (Patel, 2008).

Ethanol recovery by distillation process

The fermented broth was dispensed into round bottom flask and fixed to a distillation column attached in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 78 °C was used to heat the round bottomed flask containing the fermented broth. When the vapors enter the condenser, condenser will cool the vapors and 10 to 20 mL of distillate was collected in a test tube and immediately plugged in order to avoid escaping of alcohol (Oyeleke and Jibrin, 2009).

Analytical Methods

Determination of reducing sugar

The reducing sugars were estimated by Dinitrosalicylic acid method. The aliquots of extract were pipette out from 0.5 to 3 mL in test tubes the volume was equalized to 3 ml with water in all the tubes. Then 3 mL of DNS reagent was added, mixed and heated for 5 min. on a boiling water bath. After the colour has developed, 1 mL of 40% Rochelle salt solution was added and mixed. The tubes were cooled under running tap water and the absorption was read at 510 nm. The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose (Sadasivam and Manickam, 1996).

Determination of Non-reducing Sugar

Non-reducing sugars present in the extracts were hydrolyzed with sulphuric acid to reducing sugars. Then the total reducing sugars were estimated by DNS method. About 100 mg of the sample was taken and the sugars were extracted with 80 % alcohol (hot) twice (5mL each time). The supernatant was collected and evaporated on water bath. Ten ml of distilled water was added to dissolve the sugars. One ml of extract was pipette in to a test tube and 1ml of 1N H_2SO_4 was added. The mixture was hydrolyzed by heating at 49 °C for 30 min. and then 1 or 2 drops of methyl red indicator was added. The contents were neutralized by adding 1N NaoH drop wise from a pipette. Appropriate reagent blanks were maintained. Then total non-reducing sugar was estimated by DNS method (Sadasivam and Manickam, 1996; Agblevor *et al*., 2006; Patel, 2008).

Determination of ethanol concentration

The amount of ethanol content was estimated by spectrophotometric method (JENWAY–6305, UV–VIS Spectrophotometer) as described by Caputi *et al*., (1968).

Scanning Electron Microscopy (SEM) analysis

Microscopy analysis was conducted using a scanning electron microscope to determine the morphological structure changes in the treated and untreated sample of areca nut husk. Scanning electron microscopy (SEM) analysis was performed with a FEI Quanta

200 operated at 20 Kv. The dried samples were coated (gold/palladium) with a SC7 640 Suto/Manual High Resolution Sputter Coater (Kuila et al., 2011; Dhabhai et al., 2013).

Fourier Transformation Infrared (FT-IR) analysis

FT-IR analysis was performed in both the untreated and pre-treated samples. NICOLET 380 FTIR from Thermo Fisher Scientific, France, which gives transmittance spectra in the IR range 4,000 to 400 nm, was used for analysis. Solid sample for analysis was mixed with Potassium Bromide (which does not have any absorbance in IR range) as a supporting compound and grinded well. This powder was put in the die and applied the pressure of about 10 tons. This gives circular pellet which was put in the IR holder and run (Kuila *et al*., 2011; Dhabhai *et al*., 2013).

Statistical analysis

All the results were statistically analyzed using SPSS soft ware to determine the mean of three replicates and its standard error value from independent experiments.

Results

Initial composition of areca nut husk

The initial composition of areca nut husk was as follows, particle size (0.28 ± 0.01 mm), reducing sugar (3.40±0.12 mg/g), non-reducing sugar (0.91±0.03 mg/g), cellulose (28.4 ± 0.85 %), hemicelluloses (8.30 ± 0.15 %), and lignin (8.0 ± 0.45 %) (Table 1).

Table 1: Initial chemical composition of areca nut husk

Sl. No.	Parameters	Areca nut husk
1	Particle size (mm)	0.28 ± 0.01
2	Reducing sugars (mg/g)	3.40±0.12
3	Non-reducing sugars (mg/g)	0.91±0.03
4	Total sugars (mg/g)	4.79±0.14
5	Cellulose (%)	28.4 ± 0.85
6	Hemi-cellulose (%)	8.30 ± 0.15
7	Lignin (%)	8.0 ± 0.45

Note: Results are mean ± S.E of three replicates (n=3)

Effect of acid pretreatment of areca nut husk

The reducing (110.0 ± 6.54 mg/g) and non reducing sugar (44.54 ± 2.98 mg/g) yield was maximum compared to initial composition of areca nut husk in the acid treatment of 2N H_2SO_4, 100 °C and 6 hours reaction time (Figure 4).

Separate Hydrolysis and Fermentation (SHF)

The concentration of reducing and non-reducing sugar of the samples, before and after fermentation was estimated. Before fermentation, the concentration of reducing sugar was 128.24±3.98 mg/g and non-reducing sugar was 50.0±2.11 mg/g of the samples. After fermentation, the concentration of residual reducing and non-reducing sugar of the fermented samples treated with different yeast cultures were as follows, *Saccharomyces cerevisiae* (10.2±0.65, 6.4±0.64 mg/g), *Candida shehatae* (12.6±0.84, 5.0±0.47 mg/g), *Saccharomyces uvarum* (11.0±1.0, 3.8±0.28 mg/g), *Pichia stipitis* (15.0±1.10, 6.0±0.39 mg/g), *Schizosaccharomyces pombe* (8.41±0.92, 4.4±0.24 mg/g) and bacterium *Zymomonas mobilis* (13.0±1.23, 4.6±0.36 mg/g). The percentage of sugar used in the fermented samples was estimated (Table 2).

The production of ethanol was estimated in the fermented samples by separate hydrolysis and fermentation (SHF) process treated with laccase enzyme and

different yeast cultures were as follows, *Saccharomyces cerevisiae* (33.13±1.96 g/L), *Candida shehatae* (25.24±2.30 g/L), *Saccharomyces uvarum* (31.56±2.45 g/L), *Pichia stipitis* (29.98±1.98 g/L), *Schizosaccharomyces pombe* (42.60±3.0 g/L) and bacterium *Zymomonas mobilis* (44.97±3.21 g/L) (Figure 3).

Table-2. Production of reducing and non-reducing sugar (mg/g) from areca nut husk using acid and enzymatic pretreatment (laccase) during separate hydrolysis and fermentation process (SHF).

Sl. No.	Name of the organisms	Reducing sugar				Non-reducing sugar			
		Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)	Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)
1	*S. cerevisiae* NCIM 3095	128.24±3.98	10.0±0.63	118.24±3.01	92.20±3.21	50.0±2.11	6.0±0.32	44.0±1.11	88.0±3.33
2	*C. shehatae* NCIM 3500	128.24±3.98	10.6±0.82	117.64±2.96	91.73±3.41	50.0±2.11	5.3±0.25	44.7±1.36	89.4±3.20
3	*S. uvarum* NCIM 3455	128.24±3.98	12.8±1.2	115.44±3.0	90.01±2.89	50.0±2.11	4.2±0.19	45.8±2.3	91.6±2.98
4	*P. stipitis* NCIM 3498	128.24±3.98	8.0±0.97	120.24±2.89	93.76±3.14	50.0±2.11	6.3±0.39	43.7±2.0	87.4±2.87
5	*S. pombe* NCIM 3457	128.24±3.98	14.3±1.4	113.94±3.21	88.84±2.56	50.0±2.11	4.5±0.28	45.5±1.68	91.0±3.84
6	*Z. mobilis* NCIM 2915	128.24±3.98	6.80±0.64	121.44±2.65	94.69±3.65	50.0±2.11	4.0±0.27	46.0±2.10	92.0±3.24

Note: Results are mean ± S.E of three replicates (n=3) ; **(1)** *Saccharomyces cerevisiae*, **(2)** *Candida shehatae*, **(3)** *Saccharomyces uvarum*, **(4)** *Pichia stipitis*, **(5)** *Schizosaccharomyces pombe* and **(6)** *Zymomonas mobilis*

Simultaneous Saccharification and Fermentation (SSF)

The concentration of reducing and non-reducing sugar of the samples, before and after fermentation was estimated. Before fermentation, the concentration of reducing sugar was 128.24±3.98 mg/g and non-reducing sugar was 50.0±2.11 mg/g in the samples. After fermentation, the concentration of residual reducing and non-reducing sugar of the fermented samples treated with different yeast cultures were as follows, *Saccharomyces cerevisiae* (10.0±0.63, 6.0±0.32 mg/g), *Candida shehatae* (10.6±0.82, 5.3±0.25 mg/g), *Saccharomyces uvarum* (12.8±1.2, 4.2±0.19 mg/g), *Pichia stipitis* (8.0±0.97, 6.3±0.39 mg/g), *Schizosaccharomyces pombe* (14.3±1.4, 4.5±0.28 mg/g) and bacterium *Zymomonas mobilis* (6.80±0.64, 4.0±0.27 mg/g). The percentage of sugar used in the fermented samples was estimated (Table 3).

The concentration of ethanol was estimated in the fermented samples by simultaneous saccharification and fermentation (SSF) process treated with cellulase enzyme and different yeast cultures were as follows, *Saccharomyces cerevisiae* (26.82±2.63 g/L), *Candida shehatae* (33.92±2.11 g/L), *Saccharomyces uvarum* (44.18±2.67 g/L), *Pichia stipitis* (34.71±1.89 g/L), *Schizo-saccharomyces pombe* (33.92±2.0 g/L) and bacterium *Zymomonas mobilis* (47.34±3.22 g/L) (Figure 3).

Scanning Electron Microscopy (SEM) analysis

SEM imaging of untreated and pretreated areca nut husk samples was carried out; the images are presented in Figure 4 (a) and (b). The untreated sample revealed ordered and compact structure. After acid pretreatment these ordered structures were destroyed and it's easy for enzymatic saccharification of areca nut husk. This result was due to removal of lignin and hemicelluloses.

Table-3. Production of reducing and non-reducing sugar (mg/g) from areca nut husk using acid and enzymatic pretreatment (laccase) during in simultaneous saccharification and fermentation (SSF).

Sl. No.	Name of the organisms	Reducing sugar				Non-reducing sugar			
		Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)	Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)
1	*S. cerevisiae* NCIM 3095	128.24±3.98	10.2±0.65	118.04±2.98	92.04±3.64	50.0±2.11	6.4±0.64	43.6±1.21	87.2±2.64
2	*C. shehatae* NCIM 3500	128.24±3.98	12.6±0.84	115.64±3.21	90.17±2.65	50.0±2.11	5.0±0.47	45.0±1.32	90.0±2.89
3	*S. uvarum* NCIM 3455	128.24±3.98	11.0±1.0	117.24±3.0	91.42±3.12	50.0±2.11	3.8±0.28	46.2±2.0	92.4±2.63
4	*P. stipitis* NCIM 3498	128.24±3.98	15.0±1.10	113.24±1.98	88.30±2.87	50.0±2.11	6.0±0.39	44.0±1.78	88.0±1.98
5	*S. pombe* NCIM 3457	128.24±3.98	8.41±0.92	119.83±2.87	93.44±2.55	50.0±2.11	4.4±0.24	45.6±1.55	91.2±3.01
6	*Z. mobilis* NCIM 2915	128.24±3.98	13.0±1.23	115.24±2.65	89.86±2.11	50.0±2.11	4.6±0.36	45.4±2.21	90.8±3.0

Note: Results are mean ± S.E of three replicates (n=3); **(1)** *Saccharomyces cerevisiae*, **(2)** *Candida shehatae*, **(3)** *Saccharomyces uvarum*, **(4)** *Pichia stipitis*, **(5)** *Schizosaccharomyces pombe* and **(6)** *Zymomonas mobilis*

FT-IR analysis of untreated and pretreated areca nut husk for qualitative estimation of constituents

FT-IR is an analytical tool that helps in the qualitative estimation of biomass constituents. In the present work, FT-IR spectra of pre-treated and untreated samples were analysed. FT-IR spectra clearly show the effect of pre-treatment. FT-IR spectra of untreated sample showed the broad peak at 3402 cm^{-1} is attributed to -N-H symmetric and asymmetric stretching in carboxylic acids and amines group, 2915 cm^{-1} is attributed to -C-H stretching in alkanes and alkyls group, 2367 cm^{-1} is attributed to -H-C=O stretching in aldehydes group and 734 cm^{-1} is attributed to -C-H bending in aromatic compounds (Figure 5). The FT-IR spectra of acid treated areca nut husk sample-'B' showed the broad peak 3408 cm^{-1} (N-H symmetric and asymmetric stretching in amides), 2926 cm^{-1} (C-H stretching in alkanes and alkyls), 1742 cm^{-1}(C=O stretching in ketones), 1052 cm^{-1} (C-O Stretching in alcohols), 887 cm^{-1} (=C-H bending in alkenes), 695 cm^{-1} (C-H bending in aromatic compounds) and 580 cm^{-1} (C-Br stretching in alkyl halides) (Figure 6).

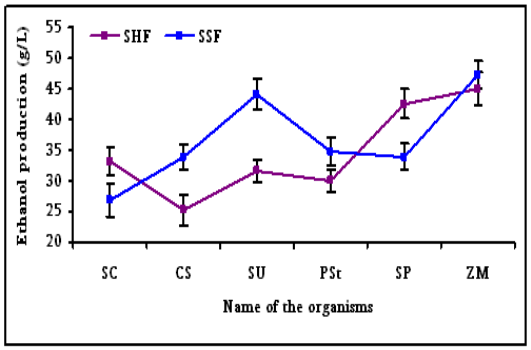
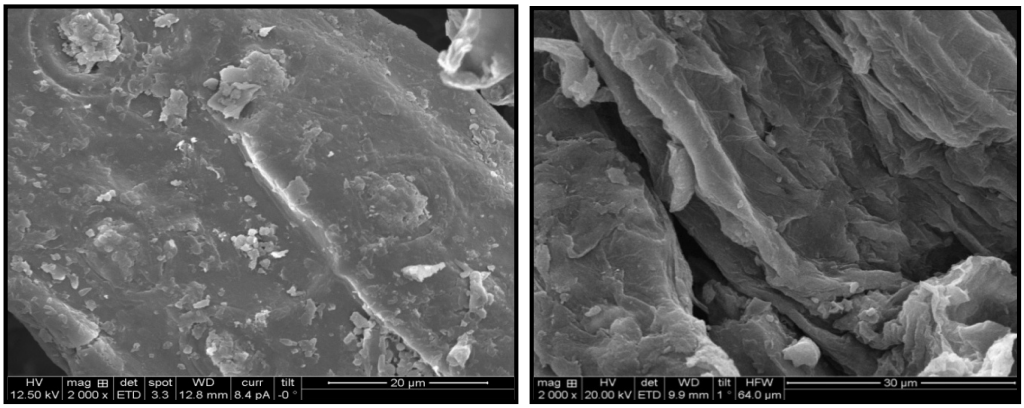


Figure 3. Production of ethanol (g/L) from areca nut husk using acid and enzymatic pretreatment (laccase) in SHF and SSF. Vertical bar indicates the standard error value of three replicates (n=3). **SC**–*Saccharomyces cerevisiae* NCIM 3095, **CS**–*Candida shehatae* NCIM 3500, **SU**–*Saccharomyces uvarum* NCIM 3455, **PSt**–*Pichia stipitis* NCIM 3498, **SP**–*Schizosaccharomyces pombe* NCIM 3457 and **ZM**–*Zymomonas mobilis* NCIM 2915

Discussion

Bioethanol from lignocellulosic biomass can be utilized for clean and renewable energy production. Pre-treatment of lignocellulosic biomass is a costly step (Lynd *et al*., 1996), but is essential for high ethanol yields on a commercial level (Mosier *et al*., 2005a). Efficient pre-treatment can affect downstream process costs by reducing the use

of enzymes or fermentation time (Lynd *et al*., 1996). Previous studies, reported that acid pre-treatment was better than alkaline pre-treatment in removing lignin from commercial sugarcane residues such as leaf and bagasse (Dawson and Boopathy, 2007, 2008).



(a) **(b)**

Figure 4: (a) SEM image of untreated areca nut husk; **(b)** SEM image of treated raw material: exposed cellulosic fibres and large pores of biomass pretreated at 100^0 C, 2N H_2SO_4 acid concentration and 6 hours reaction time.

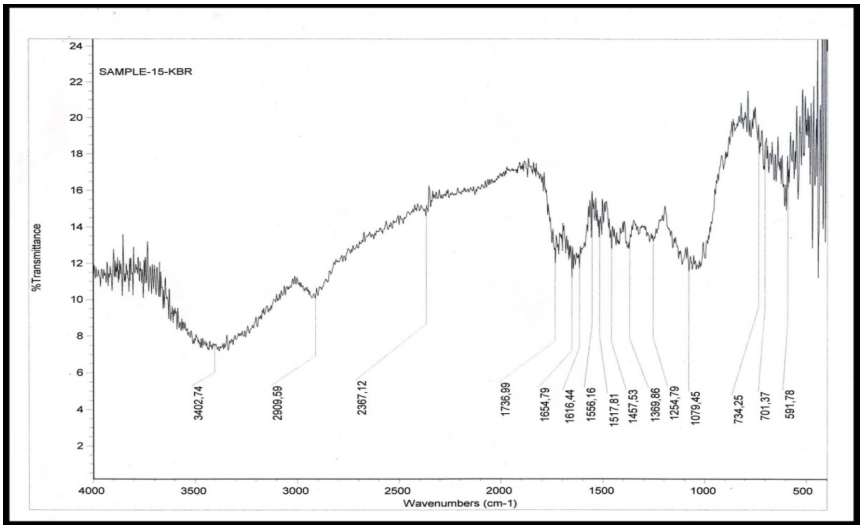


Figure 5: Fourier transform infrared spectra of untreated areca nut husk

In the current study, acid pre-treatment was chosen as the best pre-treatment to release cellulose and hemicellulose from lignin in the biomass of areca nut husk. The concentration of 2N H_2SO_4 was used for the pre-treatment of areca nut husk (sample-A) to yield maximum reducing sugar (110.0 ± 6.54 mg/g) and non-reducing sugar (44.54 ± 2.98 mg/g). The acid pre-treated biomass was fermented with enzymatic saccharification using laccase to increase the maximum reducing (128.24±3.98 mg/g) and non-reducing sugar (50.0±2.11 mg/g) yield in SHF and SSF methods.

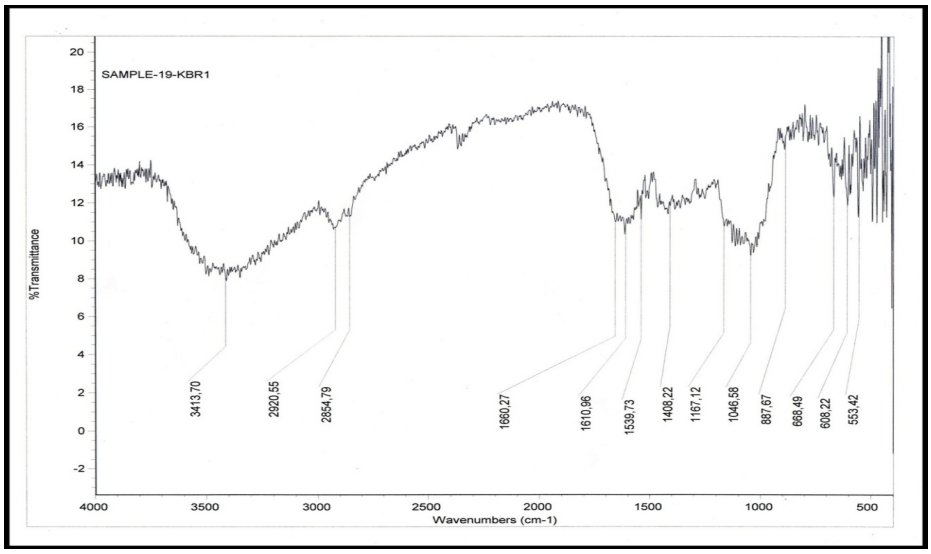


Figure 6: Fourier transform infrared spectra of acid (2N H_2SO_4) treated areca nut husk

The acid pre-treatment and enzymatic hydrolysis and effective microbial strains were used to enhance the maximum sugar and ethanol production from areca nut husk. We observed that the sugars utilization efficiency was high in the SSF compared to SHF process. In SSF process, higher sugar utilization (121.44±2.65 mg/g) was observed and percentage of 92.0±3.24 sugar in *Z. mobilis* NCIM 2915. In SHF process, higher sugar utilization (117.24±3.0 mg/g) was observed and percentage of 92.4±2.63 sugar in *Saccharomyces uvarum* NCIM 3455 was observed after fermentation period which was treated with laccase enzyme.

Kuila *et al*., (2011) reported that the optimum enzymatic delignification (88.79 %) was achieved after 8 h of incubation. After delignification the substrate was further treated with the mixture of carbohydratases for appropriate saccharification. The enzyme treated substrate yielded maximum reducing sugar (713.33 mg/g dry substrate) after 9 h of saccharification. Using conventional yeast strain, 9.63 g/L bioethanol was produced from saccharified samples of *Lantana camara*. Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment, mainly because the lignin in plant cell walls forms a barrier against enzymatic attack (Sewalt *et al*., 1997; Dawson and Boopathy, 2008). An ideal pretreatment would reduce the lignin content and crystallinity of the cellulose and increase the surface area (Krishna and Chowdary, 2000; Lalitha and Sivaraj, 2011).

In the present study, we observed that in sample treated with laccase enzyme, maximum ethanol production was shown by *Z. mobilis* NCIM 2915 (44.97±3.21 g/L), and low ethanol production was shown by *C. shehatae* NCIM 3500 (25.24±2.30 g/L) in SHF method. Garcia *et al*., (2014) reported that H_2SO_4 pretreatment is potential for improving the enzymatic hydrolysis of cellulose. In pretreatment condition the effect of temperature (110–150 °C), H_2SO_4 concentration (0.5–2.5%) and time (15-45 min) on the formation of sugars and on the enzymatic conversion of cellulose was observed. Cellulose conversions above 80% were achieved both in the separated enzymatic hydrolysis and in the SSF of the pretreated *Jatropha curcas* shells.

Sindhu *et al*., (2014) reported that Bamboo (BM) were used as a feed stock for the

production of bioethanol after dilute acid pretreatment and enzymatic saccharification. Dilute H_2SO_4 was selected and the effectiveness of pretreatment was evaluated by enzymatic saccharification. Under optimized pretreatment conditions, 0.319 g/g of reducing sugar was produced. Fermentation of the enzymatic hydrolysed liquid from the pretreated biomass using *S. cerevisiae* showed bioethanol yield of 1.76% (v/v) with an efficiency of 41.69% in SHF process.

In SSF method, we observed that in sample treated with laccase enzyme, maximum ethanol production was shown by *Z. mobilis* NCIM 2915 (47.34±3.22 g/L), and lowest ethanol production was shown by *S. cerevisiae* NCIM 3095 (26.82±2.63 g/L). Chen and Wang, (2010) reported that SSF is an important process strategy for bioethanol production where the enzyme hydrolysis and fermentation are run in the same vessel. Therefore, compared to SHF, the requirement for enzyme is lower and the bioethanol yield is higher in SSF.

Acid pretreatment of lignocellulosic biomass for ethanol production has attracted extensive attention as to technology improvement and process simulation. Acid-based pre-treatment/hydrolysis processes for the conversion of lignocellulosic materials to ethanol was carried out by Taherzadeh and Karimi, (2007), highlighting the dependence of acid-based hydrolysis efficiency on properties of the substrate, acidity, and rate of decomposition of the biomass components during the process.

The major impact of acid pretreatment is to solubilize hemicellulose to form xylose, with little or high lignin removal for batch or flow-through reactors, respectively (Mosier *et al*., 2005a; Yang and Wyman 2004; George *et al*., 2010). With this commonly-agreed mechanism, predicting the change of hemicellulose content in lignocellulosic biomass becomes the priority for model development. Although dilute acid pretreatment has insignificant influence on the dissolution of cellulose (Taherzadeh and Karimi 2007; Yat *et al*., 2008; Redding *et al*. 2011), cellulose saccharification with pretreatment conditions and biomass physical or chemical properties in model development favours the process design of subsequent enzymatic hydrolysis.

Initially, the most apparent effect of the milling pre-treatment apart from a size change from coarse in to fine particles. In fermentation the smaller particle size would enhance both the hydrolysis and fermentation rates this would increase ethanol production efficiency eventually. Moreover, acid pre-treatment, enzymatic hydrolysis, different types of yeast strains and bacterium *Z. mobilis* used in fermentation would play a very important role in the conversion of the waste biomass into fuel ethanol by fermentation.

Only few yeast strains and bacterium *Z. mobilis* were used to evaluate the ethanol production and still needs an effective method for improvement of strains to produce ethanol production. The energy sector has played a crucial role in the context of the global economy as well as the socio economic development. Ethanol contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate emission that create health hazard to living beings. The present study was undertaken to utilize the areca nut husk waste for bioethanol production by microbial fermentation.

Conclusion

Biomass structural characteristics play a crucial role in the production of fermentable sugars during acid and enzymatic pretreatment for bioethanol production. Saccharification of acid and enzymatic pretreated sample resulted in higher reducing sugar yield (128.24±3.98 mg/g) in both SHF and SSF methods. Laccase enzyme as potential for the hydrolysis and saccharification of lignocellulosic material in order to obtain fermentable sugars that can be

converted into second generation ethanol by fermenting effective yeast strains and bacterium *Zymomonas mobilis*. FTIR and SEM study also revealed the effectiveness of acid pretreatment for efficient saccharification and fermentation of areca nut husk. Hence, acid and enzymatic pretreatment is more effective for ethanol production. The superiority of ethanol yield and productivity was very high in both the methods such as simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Hence areca nut husk was revealed as a suitable substrate for ethanol production.

Acknowledgement

The authors gratefully acknowledge the Department of Microbiology for providing laboratory facilities and also grateful to other backward class and minority cell, Kuvempu University for their inspiring help and providing financial support to carry out the research work.

References

Agblevor FA, Cundif JS, Mingle W. 2006. Storage and Characterization of cotton gin waste for ethanol production. Journal of Resources Conservation and Recycling, 46: 198–216.

Amin G, Doelle HW. 1990. Production of high ethanol concentrations from glucose using *Zymomonas mobilis* entrapped in a vertical rotating immobilized cell reactor. Enzyme and Microbial Technology, 12: 443–446.

Ballesteros M, Saez F, Ballesteros I, Manzanares P, Negro MJ, Martinez JM, Castaneda R, Dominguez JMO. 2010. Ethanol production from the organic fraction obtained after thermal pretreatment of municipal solid waste. Applied Biochemistry and Biotechnology, 161: 423–431.

Bothast RJ, Saha BC. 1997. Ethanol production from agricultural biomass substrates. Advances in Applied Microbiology, 44: 261-286.

Caputi A, Ueda JM, Brown T. 1968. Spectrophotometric determination of chromic complex formed during oxidation of alcohol. American Journal of Ethanol Viticulture, 19: 160–165.

Ciani, M, Ferraro, L. 1998. Combined use of immobilized Candida stellata cells and *Saccharomyces cerevisiae* to improve the quality of wines. Journal of Applied Microbiology, 85: 247–254.

Dawson L, Boopathy R. 2007. Use of post-harvest sugarcane residue for ethanol production. Bioresource Technology, 98: 1695–1699.

Dawson L, Boopathy R. 2008. Cellulosic ethanol production from sugarcane bagasse without enzymatic saccharification. BioResource, 3: 452–460.

Dhabhai R, Chaurasia SP, Dalai AK. 2013. Effect of pretreatment conditions on structural characteristics of wheat straw. Chemical Engineering Communications, 200: 1251–1259.

Garcia A, Cara C, Moya M, Rapado J, Puls J, Castro E, Martin C. 2014. Dilute sulphuric acid pretreatment and enzymatic hydrolysis of *Jatropha curcas* fruit shells for ethanol production. Industrial Crops and Products, 53: 148–153.

George N, Yang Y, Wang Z, Shivappa RS, Tungate K. 2010. Suitability of canola residue for cellulosic ethanol production. Energy Fuels, 24: 4454–4458.

Gurav MS, Geeta GS. 2007. Effectiveness of fungal pretreatment of agro residues on ethanol production by yeasts and *Zymomonas mobilis*. Karnataka Journal of Agricultural Sciences, 20: 301–304.

Joshi B, Bhatt MR, Sharma D, Joshi J, Malla R, Sreerama L. 2011. Lignocellulosic ethanol production: Current practices and recent developments. Biotechnology and Molecular Biology Review, 6:172-182.

Krishna SH, Chowdary GV. 2000. Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic

biomass. Journal of Agricultural and Food Chemistry, 48: 1971–1976.

Kuila A, Mukhopadhyay M, Tuli DK, Banerjee R. 2011. Production of ethanol from lignocellulosics: An enzymatic venture. Experimental and Clinical Sciences, 10: 85–96.

Lalitha G, Sivaraj, R. 2011. Use of fruit biomass peel residue for ethanol production. International Journal of Pharma and Bio Sciences, 2: 15–23.

Lynd LR, Elander RT, Wyman CE. 1996. Likely features and costs of mature biomass ethanol technology. Applied Biochemistry and Biotechnology, 57: 741–761.

Moniruzzaman M, Zahangir MA, Sujan SMA, Hossain M, Jamal MS. 2013. Enzymatic Saccharification of Bagasse: Effects of Different Pre-treatment Methods. International journal of Renewable Energy Research, 3: 230–234.

Mosier N, Hendrickson R, Ho N, Sedlak M, Ladisch M. 2005b. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresource Technology, 96: 1986-1993.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. 2005a. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 96: 673–686.

Narayanamurthy G, Ramachandra YL, Rai SP, Manohara YN, Kavitha BT. 2008. Areca husk: An inexpensive substrate for citric acid production by *Aspergillus niger* under solid state fermentation. Journal of Biotechnology, 7:99–102.

Naveenkumar KJ, Thippeswamy B, Banakar SP, Thirumalesh BV, Krishnappa M. 2012b. Effectiveness of fungal pretreatment of areca nut (*Areca catechu* L.) husk waste on bioethanol production by yeasts and *Zymomonas mobilis*. International Journal of Recent Scientific Research, 3: 400–406.

Naveenkumar KJ, Thippeswamy B, Thirumalesh BV, Banakar SP. 2012a. Effect of pretreatment for delignification and sugar recovery for bioethanol production from Areca nut husk waste. International Journal of Current Research, 4: 18–24.

Oyeleke SB, Jibrin NM. 2009. Production of bioethanol from guinea cornhusk and millet husk. African Journal of Microbiology Research, 3: 147–152.

Patel SJ. 2008. Studies on ethanol production from bioconverted agricultural residues. Ph.D Thesis, Kuvempu University, Shivamogga, India, 1–237.

Rajan A, Kurup JP, Abraham TE. 2005. Biosoftening of areca nut fiber for value added products. Biochemical Engineering Journal, 25: 237–242.

Ramappa BT, Manjunatha MS. 2013. Cost cultivation of areca nut non-traditional region of Karnataka -An analysis. International Journal of Pharmaceutical Science Invention, 2: 25–31.

Ramappa BT. 2013. Economics of areca nut cultivation in Karnataka, a case study of Shivamogga District. Journal of Agriculture and Veterinary Science, 3: 50–59.

Redding AP, Wang Z, Keshwani DR, Cheng JJ. 2011. High temperature dilutes acid pretreatment of coastal Bermuda grass for enzymatic hydrolysis. Bioresource Technology, 102: 1415–1424.

Sadasivam M, Manickam A. 1996. Biochemical methods, New age international Limited Publishers, New Delhi, 1–235.

Sewalt VJH, Glasser WG, Beauchemin KA. 1997. Lignin impact on fiber degradation: Reversal of inhibition on enzymatic hydrolysis by chemical modification of lignin and by additives. Journal of Agricultural and Food Chemistry, 45: 1823–1828.

Sindhu R, Kuttiraja M, Binod P, Sukumaran RK, Pandey A. 2014. Bioethanol production from dilute acid pretreated Indian bamboovariety (*Dendrocalamus* sp.) by separate hydrolysis and fermentation. Industrial Crops and Products, 52: 169–176.

Sun FB, Cheng HZ. 2007. Evaluation of enzymatic hydrolysis of wheat straw pretreated by atmospheric glycerol autocatalysis. Journal of Chemical Technology and Biotechnology, 82: 1039-1044.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology, 83: 1–11.

Taherzadeh MJ, Karimi K. 2007. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review. BioResources, 2: 472–499.

Yang B, Wyman CE. 2004. Effect of xylan and lignin removal by batch and flow through pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnology and Bioengineering, 86: 88–95.

Yang B, Wyman CE. 2008. Pretreatment: The key to unlocking lowcost cellulosic ethanol. Biofuels, Bioproducts and Biorefining, 2: 26-40.

Yat SC, Berger A, Shonnard DR. 2008. Kinetic characterization for dilute sulfuric acid hydrolysis of timber varieties and switch grass. Bioresource Technology, 99: 3855–3863.