

Original Research Article

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Bioethanol Fermentation from Fungal Pretreated Lignocellulosic Areca nut (*Areca catechu* L.) Husk using Yeasts and *Zymomonas mobilis* NCIM 2915

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ABSTRACT

Keywords

Areca nut husk, *Aspergillus flavus*, Bioethanol, *Phanerochaete chrysosporium* NCIM 1197, Yeasts, *Zymomonas mobilis* NCIM 2915.

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Bioethanol produced from renewable biomass has received considerable attention in current years. Ethanol as a gasoline fuel additive as well as transportation fuel helps to alleviate global warming and environmental pollution. This paper reports a preliminary study on the microbial pretreatment (*Aspergillus flavus*, *Phanerochaete chrysosporium* NCIM 1197 and co-culture) and fermentation of the areca nut husk. A combination of different fungi obtained from screening was used for pretreatment and, yeasts and bacterium *Zymomonas mobilis* NCIM 2915 was used for carrying out fermentation. *Zymomonas mobilis* NCIM 2915 was showed maximum ethanol production after fermentation from areca nut husk as followed by *Pichia stipitis* NCIM 3498, *Saccharomyces cerevisiae* NCIM 3095 and *Candida shehatae* NCIM 3500. Hence, fungal pretreatment by cellulolytic fungi was more effective for ethanol production. Areca nut husk was revealed as a suitable substrate for ethanol production.

Introduction

Ethanol is a widely accepted and currently used fuel alternative or fuel blend of microbial origin. But currently 90% of all fuel ethanol is derived from sugar or starch based raw materials (Hamelinck *et al.*, 2005; Patel *et al.*, 2007), which can cause competition with food. Therefore it is important to develop an ethanol production technique from non food materials like agricultural residues and forest remains, mainly known as lignocellulosic material.

But the lignocellulosic materials are not readily fermentable, since the fermentation yeast cannot convert the complex polysaccharides into ethanol (Hamelinck *et al.*, 2005; Sun and Cheng, 2007). For this reason a suitable conversion step has to be applied to break the complex structure of the polysaccharide into much simpler structures (softening) for the easy access of the saccharification enzymes where the enzymes convert the polysaccharides into fermentable

sugars (Hendriks and Zeeman, 2009; Moniruzzaman *et al.*, 2013).

Areca nut (*Areca catechu* L.) is one of the most important commercial crops in India. 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 (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 (Ramappa and Manjunatha, 2013; Ramappa, 2013). The areca nut husk fibers are predominantly composed of cellulose and varying proportions of hemicelluloses, lignin, pectin and protopectin. 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 development 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 fungal pretreatment on areca nut husk for improved yield of reducing sugar and bioethanol production by yeasts and bacterium *Zymomonas mobilis* NCIM 2915.

The process was carried out by submerged fermentation.

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).

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 (1mm) (Narayanamurthy *et al.*, 2008; Kumar *et al.*, 2012a).

Selection of fungal species

The more efficient cellulolytic fungal species such as *Aspergillus flavus* was isolated from naturally contaminated areca nut husk waste. *Phanerochaete chrysosporium* NCIM 1197 was procured from National Chemical Laboratory (NCL), Pune, India. The cultures were maintained on Potato Dextrose Agar slants and stored at 4°C.

Selection of yeast cultures and bacteria

The standard yeast strains and bacteria 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 (Gurav and Geeta, 2007; Kumar *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 1x10⁶ cells per mL (Ciani and Ferraro, 1998).

Determination of the effect of fungal treatment

About 10g/lit of each residue was suspended in Mandle's medium and sterilized. Each flask was inoculated with individual fungal isolates. These flasks were incubated at room temperature for five days on an incubator shaker (150rpm). After five days of incubation, mycelium was separated by filtration. The filtrate was centrifuged and supernatant was collected for further studies. The reducing and non-reducing sugar content was determined (Patel, 2008).

Optimization of conditions for fungal treatment

Fungal isolates were cultivated on areca nut husk and optimization of conditions for maximum reducing sugar yield by each isolate was recorded.

Incubation temperature

To find out the optimum temperature for saccharification, each isolates were cultivated in basal salt medium containing 1g of substrate. Incubation was carried out at different temperature (20 to 40 °C) in an incubator shaker (150 rpm). Determination of reducing and non-reducing sugar in the culture filtrate was done by DNS method.

Medium pH value

The effect of the pH value was investigated by cultivation of the isolate in media which were adjusted to different pH values from 4 to 8. Media were inoculated and incubated at 30°C in an incubator shaker (150 rpm).

Determination of reducing and non-reducing sugar in the culture filtrate was done by DNS method.

Incubation time

Fungal isolates were cultivated on raw material under the optimum conditions of pH and temperature. Every 24 hours sample was taken and reducing sugar, non-reducing sugar content was estimated by DNS method.

Separate Hydrolysis and Fermentation (SHF)

The substrates were initially hydrolyzed by the action of cellulolytic enzymes (fungal treatment) for saccharification. After complete hydrolysis, the fermentation was conducted in separate.

Step-1: Saccharification

Raw material was taken in Mandel's medium and sterilized. Each flask was inoculated with different fungal isolates. These flasks were incubated at 30 °C for 5 days in an incubator shaker (150 rpm). After five days mycelium was separated by filtration.

Step-2: Fermentation

Culture filtrate from fungal treatment was further inoculated with each of the yeasts and bacteria separately (3 % inoculum) and allowed for fermentation for 72 hours. After fermentation, the sample was recovered by distillation unit for spectrophotometric analysis of ethanol concentration (Sun and Cheng, 2002).

Optimization of conditions for fermentation

Fermentation conditions were optimized for the highest reducing sugar yield treatment.

Incubation temperature

To optimize the temperature for fermentation, incubation was carried out at different temperature (20 to 40 °C) in an incubator shaker (150 rpm).

Medium pH value

The effect of the pH value investigated by carrying out the fermentation in media which were adjusted to different pH values 4 to 8.

Inoculum concentration

The culture filtrate was inoculated with different amount of inoculum concentration (1 to 5 %) and fermentation was carried out to find the optimum inoculum concentration for better ethanol yield.

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 the alcohol (Oyeleke and Jibrin, 2009).

Analytical Methods

Determination of reducing sugar

The glucose concentration was determined by DNS method, as described by Miller, (1959) and Sadasivam and Manickam, (1996) using glucose as a standard. 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.

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 H₂SO₄ 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).

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 and Discussion

Initial composition of areca nut husk

The initial composition of areca nut husk as follows, particle size (0.64 ± 0.01 mm), reducing sugar (2.91 ± 0.01 mg/g), non-reducing sugar (0.30 ± 0.001 mg/g), total sugar (3.21 ± 0.11 mg/g), cellulose (46.0 ± 1.53 %), hemicelluloses (40.3 ± 0.23 %), and lignin (21.0 ± 0.19 %) was estimated (Table 1).

Selection of fungal species

More efficient cellulolytic fungi *Aspergillus flavus* and lignin degrading fungi *P. chrysosporium* NCIM 1197 was selected for microbiological treatment. Before fermentation, raw material was treated with specific fungal species to increases the maximum sugar yield. After fungal treatment, obtained the filtrate was further subjected to fermentation using yeast strains and bacterium *Z. mobilis* NCIM 2915.

Optimization of the culture conditions for fungal treatment

Incubation temperature

The fungal isolates were grown in the cultivation medium with raw material and incubated at various temperatures (5 °C to 50 °C) to define the optimal temperature for growth and saccharification. The fungal isolates were able to grow and yield reducing sugar in a broad range of incubation temperatures from 5 °C to 50 °C.

The maximum reducing sugar yield temperature was between 25 °C to 30 °C for the isolates *A. flavus* and *P. chrysoprorium* NCIM 1197 on the raw material. The optimum temperature was between 25 °C to 30 °C for the isolates (Table 2).

Medium pH value

The fungal isolates were grown on the media with raw material and pH adjusted to different values from 4 to 8. The isolates have shown the reducing sugar yield in wide range of pH from 4 to 8. The maximum reducing sugar yield was observed at pH 6 for the isolate of *A. flavus* and *P. chrysoprorium* NCIM 1197 on the raw material (Table 3).

Incubation time

There was increase in the reducing sugar after two days until five days of incubation. But, from sixth day of incubation there was a decrease in the reducing sugar in all the fungal species. Therefore, the optimum incubation time for saccharification of raw material was considered as 5th day (Table 4).

Optimization of conditions for fermentation (SHF)

Incubation temperature

Ethanol yield was observed at range of 20 to 40 °C. For *S. cerevisiae* NCIM 3095, *P. stipitis* NCIM 3498, *C. shehatae* NCIM 3500 and *Z. mobilis* NCIM 2915, 30 °C was optimum, with the treatment of *A. flavus* + *P. chrysoprorium* NCIM 1197 (AF+PC) on areca nut husk, similar yield was observed at both 25 °C and 30 °C. Overall 30 °C was found as the optimum temperature for fermentation (Table 5).

Medium pH value

Fermentation was carried out at a range of 4-8 pH. In AF+PC treatment, for *S. cerevisiae* NCIM 3095, *P. stipitis* NCIM 3498, *C. shehatae* NCIM 3500 and *Z. mobilis* NCIM 2915 on areca nut husk showed maximum ethanol yield at 5 and 6 pH. Overall maximum ethanol yield was observed in 6 pH (Table 6).

Inoculum concentration

Fermentation with inoculum concentrations ranging from 1-5 % was conducted. In each case yield was similar with 2 and 3 % inoculum. But with AF+PC treatment, for *S. cerevisiae* NCIM 3095, *P. stipitis* NCIM 3498, *C. shehatae* NCIM 3500, *Z. mobilis* NCIM 2915 on areca nut husk showed maximum ethanol yield at 2 % and 3 % inoculum. Overall maximum ethanol yield was observed with 3 % inoculum (Table 7).

Microorganisms will treat the lignocelluloses biomass and enhance enzymatic hydrolysis. The microorganisms usually apply use to degrade lignin and hemicelluloses but very little part of cellulose, since cellulose is more resistance to microorganism than other parts of lignocelluloses biomass. The microbial treatment of office paper with *Sphingomonas paucimobilis* and *Bacillus circulans*, and found that treatment with the combined strains improved the enzymatic hydrolysis. White-rot fungi are the most effective Basidiomycetes for biological pretreatment of lignocellulosic materials. However the rate of hydrolysis in most biological pretreatment processes is very low and the most ligninsolubilising microorganisms also solubilise or consume cellulose (Idi and Mohamad, 2011).

In the present study, more efficient cellulolytic fungi *Aspergillus flavus* and lignin degrading fungi *P. chrysoprorium* NCIM 1197 was selected for microbiological treatment. After fungal treatment, obtained the filtrate was further subjected to fermentation using yeast strains and bacterium *Z. mobilis* NCIM 2915. Singh *et al.*, (2008) evaluated eight bioagents, including fungi and bacteria, for their pretreatment effects on sugarcane trash. The maximum drop in C/N ratio of 61% was observed using *Aspergillus terreus*, followed by those obtained using *Cellulomonas uda* (52%) and *Trichoderma reesei* and *Zymomonas mobilis* (49%). The C/N ratio is important for biomass pretreatment, because degradation of lignocellulosic material depends on the material's C/N ratio.

In the current study, the maximum reducing sugar yield temperature was between 25 °C to 30 °C for the isolates *A. flavus* and *P. chrysoprorium* NCIM 1197 on the raw material. The optimum temperature was between 25 °C to 30 °C for the isolates. Wichern *et al.*, (2004) reported that fungi have a higher C/N ratio of 30:1 as compared to 10:1 for the bacteria; hence, fungi are more capable of degrading any lignocellulosic material, as their dependency

on nitrogen is comparatively lower.

Biological pretreatment in combination with other pretreatment technologies has also been studied. Itoh *et al.*, (2003) reported production of ethanol by simultaneous saccharification and fermentation (SSF) from beech wood chips after bio-organosolvation pretreatments by ethanolysis and white-rot fungi, *Ceriporiopsis subVermispora*, *Dichomitus squalens*, *Pleurotus ostreatus*, and *Coriolus versicolor*. The purpose of biotreatments with wood-rot fungi was to reduce the energy input required for the separation of wood components by ethanolysis.

Keller *et al.*, (2003) reported that the microbial pretreatment of biomass. Typical pretreatment requires high-energy (steam and electricity) and corrosion-resistant, high-pressure reactors. A review of the literature suggests that fungal pretreatment could potentially lower the severity requirements of acid, temperature and time. These reductions in severity are also expected to result in less biomass degradation and consequently lower inhibitor concentrations compared to conventional thermochemical pretreatment.

Table.1 Initial chemical composition of areca nut husk

Sl. No.	Parameters	Areca nut husk
1	Particle size (mm)	0.64 ± 0.01
2	Reducing sugars (mg/g)	2.91±0.01
3	Non-reducing sugars (mg/g)	0.30±0.001
4	Total sugars (mg/g)	3.21±0.11
13	Cellulose (%)	46.0 ± 1.53
14	Hemi-cellulose (%)	40.3 ± 0.23
15	Lignin (%)	21.0 ± 0.19

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

Table.2 Effect of temperature on fungal treatment of areca nut husk

Temperature	Reducing sugar yield (mg/g)		Non-reducing sugar yield (mg/g)	
	AF	PC	AF	PC
20	28.78±1.0	32.0±3.21	7.0±0.24	10.5±0.54
25	35.25±1.21	44.32±2.35	9.21±0.25	12.6±0.62
30	33.0±1.32	54.0±4.10	8.0±0.31	15.0±1.0
35	30.36±2.10	41.85±3.38	7.51±0.17	14.0±0.94
40	23.0±1.90	40.0±3.83	4.0±0.08	13.2±0.81

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

AF- *Aspergillus flavus*, PC- *Phanerochaete chrysosporium* NCIM 1197

Table.3 Effect of pH on fungal treatment of areca nut husk

pH	Reducing sugar yield (mg/g)		Non-reducing sugar yield (mg/g)	
	AF	PC	AF	PC
4	29.0±3.87	29.0±4.21	6.0±0.08	9.0±0.10
5	34.14±4.23	35.31±4.12	8.0±0.12	10.0±0.14
6	36.6±4.10	52.0±5.10	10.12±0.24	16.5±0.29
7	35.0±3.10	42.0±5.0	9.0±0.28	14.0±0.34
8	34.0±2.20	36.0±3.69	8.0±0.30	12.0±0.41

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

AF- *Aspergillus flavus*, PC- *Phanerochaete chrysosporium* NCIM 1197

Table.4 Effect of incubation time on fungal treatment of areca nut husk

Time in days	Reducing sugar yield (mg/g)		Non-reducing sugar yield (mg/g)	
	AF	PC	AF	PC
1	15.0±1.0	17.0±1.12	4.0±0.04	5.0±0.09
2	12.0±0.98	13.0±1.13	2.2±0.01	7.0±0.08
3	27.0±2.27	29.4±2.23	8.0±0.09	8.2±0.05
4	31.0±2.63	32.2±2.65	10.0±0.13	10.0±0.14
5	40.0±4.10	58.0±6.10	15.2±0.21	14.3±0.28
6	36.0±3.63	47.3±5.84	14.1±0.32	11.0±0.32
7	31.2±3.51	45.0±4.89	13.0±0.14	10.0±0.09

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

AF- *Aspergillus flavus*, PC- *Phanerochaete chrysosporium* NCIM 1197

Table.5 Effect of temperature on fermentation for *A. flavus* + *P. chrysosporium* NCIM 1197

Temperature	Ethanol yield (g/L)					
	<i>S. cerevisiae</i> NCIM 3095	<i>S. uvarum</i> NCIM 3455	<i>S.pombe</i> NCIM 3457	<i>P. stipitis</i> NCIM 3498	<i>C. shehatae</i> NCIM 3500	<i>Z. mobilis</i> NCIM 2915
20	8.6±0.46	3.4±0.08	4.0±0.03	9.6±0.18	8.5±0.14	8.3±0.38
25	9.4±0.56	4.2±0.09	4.3±0.02	10.0±0.45	8.7±0.21	8.7±0.420
30	9.6±0.23	4.6±0.08	4.8±0.08	11.0±0.36	8.8±0.36	8.9±0.51
35	9.2±0.98	4.0±0.06	3.8±0.05	9.4±0.039	8.5±0.39	7.5±0.50
40	8.2±0.84	3.5±0.04	3.4±0.09	9.0±0.41	8.1±0.41	7.1±0.44

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

Saccharomyces cerevisiae NCIM 3095, *Saccharomyces uvarum* NCIM 3455, *Schizosaccharomyces pombe* NCIM 3457, *Pichia stipitis* NCIM 3498, *Candida shehatae* NCIM 3500 and *Zymomonas mobilis* NCIM 2915.

Table.6 Effect of pH on fermentation for *A. flavus* + *P. chrysosporium* NCIM 1197

pH	Ethanol yield (g/L)					
	<i>S. cerevisiae</i> NCIM 3095	<i>S. uvarum</i> NCIM 3455	<i>S. pombe</i> NCIM 3457	<i>P. stipitis</i> NCIM 3498	<i>C. shehatae</i> NCIM 3500	<i>Z. mobilis</i> NCIM 2915
4	9.1±0.42	4.3±0.02	4.0±0.03	10.3±1.0	8.9±0.09	8.0±0.14
5	9.3±0.65	5.0±0.03	4.6±0.04	10.8±0.91	9.4±0.14	8.6±0.19
6	9.8±0.47	4.3±0.06	5.4±0.07	11.2±0.84	9.0±0.28	8.8±0.25
7	8.7±0.87	4.0±0.01	4.4±0.02	10.5±0.74	8.6±0.63	8.3±0.31
8	8.4±0.09	3.8±0.02	3.9±0.01	9.8±0.23	8.4±0.08	7.8±0.21

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

Saccharomyces cerevisiae NCIM 3095, *Saccharomyces uvarum* NCIM 3455, *Schizosaccharomyces pombe* NCIM 3457, *Pichia stipitis* NCIM 3498, *Candida shehatae* NCIM 3500 and *Zymomonas mobilis* NCIM 2915.

Table.7 Effect of inoculum concentration on fermentation for *A. flavus* + *P. chrysosporium* NCIM 1197

Inoculum concentration	Ethanol yield (g/L)					
	<i>S. cerevisiae</i> NCIM 3095	<i>S. uvarum</i> NCIM 3455	<i>S. pombe</i> NCIM 3457	<i>P. stipitis</i> NCIM 3498	<i>C. shehatae</i> NCIM 3500	<i>Z. mobilis</i> NCIM 2915
1%	8.6±0.33	4.3±0.08	4.0±0.01	10.0±0.44	8.8±0.32	8.1±0.65
2%	9.0±0.41	4.6±0.07	5.4±0.03	10.6±0.56	9.0±0.54	8.7±0.45
3%	9.7±0.51	4.9±0.05	6.0±0.04	11.8±0.58	10.8±0.69	8.9±0.87
4%	9.1±0.21	4.0±0.09	5.8±0.08	9.4±0.64	9.4±0.25	7.4±0.56
5%	9.0±0.11	3.8±0.008	4.2±0.01	8.0±0.39	9.1±0.69	7.0±0.47

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

Saccharomyces cerevisiae NCIM 3095, *Saccharomyces uvarum* NCIM 3455, *Schizosaccharomyces pombe* NCIM 3457, *Pichia stipitis* NCIM 3498, *Candida shehatae* NCIM 3500 and *Zymomonas mobilis* NCIM 2915.

Fig.1 Study location and map of Shivamogga District, Karnataka State, India

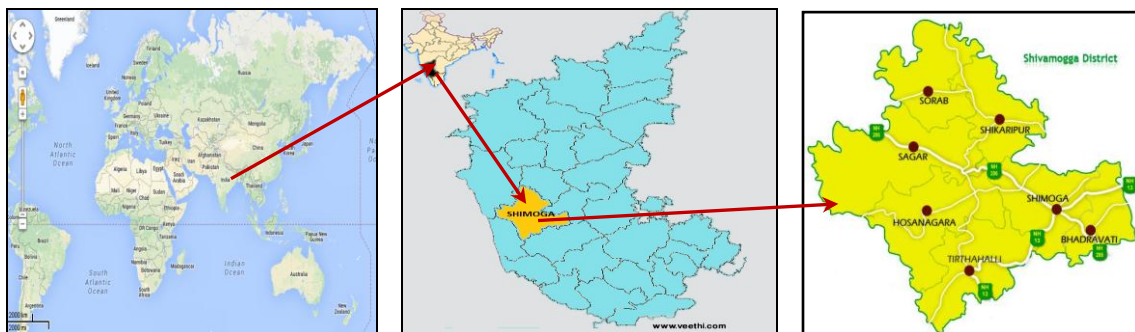


Fig.2 A. Areca nut palm (plant), B. Areca nut husk and C. Milled areca nut husk (0.64mm)



Furthermore, potential advantages of fungal pretreatment of agricultural residues, such as corn stover, are suggested by its effectiveness in improving the cellulose digestibility of many types of forage fiber and agricultural wastes.

In the present study, overall maximum ethanol yield was observed with 3 % inoculums. Suhardi *et al.*, (2013) reported that the process of converting lignocellulosic biomass to ethanol involves pretreatment to disrupt the complex of lignin, cellulose, and hemicellulose, freeing cellulose and hemicellulose for enzymatic saccharification and fermentation. Determining optimal pretreatment techniques for fermentation is essential for the success of lignocellulosic energy production process.

Fungal pre-treatment of lignocellulosic materials is an important biological pre-treatment method in which microorganisms are used for selective degradation of lignin and hemicelluloses. It is a safe, environmentally friendly and less energy intensive method compared to other pre-treatment methods.

In conclusion, as highest reducing sugar yield was seen in *Aspergillus flavus* and *Phanerochaete chrysosporium* NCIM 1197 treated with areca nut husk, the ethanol yield was also observed to be the highest in areca nut husk. Overall yield of ethanol was high with the areca nut husk, best fungal treatment with *Aspergillus flavus* and *Phanerochaete chrysosporium* NCIM 1197. The fungal treatment was found to be

effective for conversion of cellulosic material for ethanol production.

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