Effectiveness of *Trichoderma viride* and *Phanerochaete chrysosporium* NCIM 1197 Pre-treatment for Second Generation Ethanol Production from Areca Nut (*Areca catechu* L.) Husk by Separate Hydrolysis and Fermentation (SHF) Process

Naveen Kumar Kudure Jayanna[1]*, Thippeswamy Basaiah[1] and Pradeepa Krishnappa[2]
1. Department of P.G. Studies and Research in Microbiology, Bioscience Complex, Kuvempu University, Jnana Sahyadri, Shankaraghatta - 577 451, Shivamogga District, Karnataka, INDIA
2. Department of P.G. Studies and Research in Biotechnology, Sahyadri Science College, Shivamogga District, Karnataka, INDIA
*naviekj@gmail.com

Abstract
Production of ethanol using cellulosic material as feedstock is crucial for sustainable fuel ethanol production. However, a production process based on cellulosic biomass involves several energy and cost intensive steps like storage of biomass, pretreatment, hydrolysis and fermentation. Areca nut husk waste is most abundant renewable energy source that may be considered as potential feedstock for ethanol production by microbial fermentation. The areca nut husk waste was initially subjected to microbiological pretreatment for obtaining reducing sugars. Cellulolytic fungi are more effective for enzymatic hydrolysis.

In fungal treatment, increase the reducing sugar concentration for maximum ethanol production. Trichoderma viride and Phanerochaete chrysosporium NCIM 1197 (TV+PC) are more effective fungi for biological treatment. Maximum ethanol production by Saccharomyces cerevisiae NCIM 3095, Pichia stipitis NCIM 3498, Candida shehatae NCIM 3500 and Zymomonas mobilis NCIM 2915 is obtained in all the parameters like temperature, pH and inoculum concentration. Hence, fungal pretreatment by cellulolytic fungi was more effective for ethanol production. Areca nut husk was revealed as a suitable substrate for ethanol production.

Keywords: Areca nut husk, Bioethanol, *Phanerochaete chrysosporium* NCIM 1197, *Trichoderma viride*, *Zymomonas mobilis* NCIM 2915.

Introduction

In order to reduce dependence on fossil fuels and thus alleviate associated economic and environmental concerns, biofuels derived from renewable and domestic sources have received extensive interest for displacement of fossil transportation fuels in many countries[1-3]. Lignocellulosic biomass, mostly from agricultural and forestry sources, is rich in carbohydrates (55–75% dry basis) and widely available, thus providing attractive feedstock's for ethanol production[4,5]. To maximally utilize carbohydrates in the biomass, a pretreatment process is needed to overcome the biomass recalcitrance and subsequently improve its accessibility to hydrolytic enzymes[6-9].

Thermal/chemical pretreatment methods have been regarded as the current leading pretreatment technologies; however, they usually need expensive corrosion resistant reactors, processing large volumes of the waste stream, extensive washing of treated solids and detoxification of compounds inhibitory to ethanol-fermenting microorganisms. Thus, pretreatment still remains one of the most costly steps in cellulosic ethanol production and is a significant barrier to its commercialization[7,10].

Biological pretreatment, as a safe and environmental friendly method for lignin removal from lignocellulose, is attracting extensive interests[11-14]. White-rot fungi are the most promising microorganisms used for biological pretreatment because of their abilities to selectively degradation of lignin[15-17]. Presently, lots of studies on pretreatments with various white-rot fungi have been reported. Many white-rot fungi were applied to pretreatment of wheat straw for enzymatic hydrolysis and it was found that about 30% of cellulose was converted to glucose

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%. 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[18,19]. Areca nut popularly known as betel nut or supari is one of the most important plantation crops in Shivamogga district[20].

The area under areca nut cultivation has increased more rapidly in Shimoga district as compared with 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 tons[18,19]. The areca nut husk fibers are predominantly

composed of cellulose and varying proportions of hemicelluloses, lignin, pectin and protopection. The total hemicellulose content varies with the development and maturity, the mature husk is containing less hemicellulose than the immature ones. The lignin content proportionately increases with the development until maturity[21].

The availability of areca husk waste is very high in Shivamogga district due its area and high productivity in this region[18,19]. Areca nut husk is most abundant renewable energy source that may be considered as potential feed stock for ethanol production by microbial fermentation[22].

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 separate hydrolysis and fermentation (SHF) Process.

Material and Methods

Collection of areca nut husk: Areca nut husk was collected from the Shivamogga region, Karnataka State, India. The sample was brought to the laboratory and was maintained at room temperature for microbiological study.

Physical Pretreatment (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) (Figure 1)[20-23].

Selection of fungal species: The more efficient cellulolytic fungal specie such as *Trichoderma viride* 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 and *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 pH to 7.0–7.5). These yeast cultures and bacteria were procured from the National Chemical Laboratory (NCL), Pune, India[24-27].

Inoculum preparation: For inoculum preparation, yeast cultures were 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[28].

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 contents were determined [(26)].

(a) **(b)** **(c)**

Figure 1: A. Areca nut palm (plant), B. Areca nut husk and C. Milled areca nut husk

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 isolate was 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 separately.

Step 1- Saccharification: Raw material was taken in Mandle's medium and sterilized. Each flask was inoculated with different fungal isolates. These flaks 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 [(29)].

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 was 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 inoculums 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 [(30)].

Analytical Methods

Determination of reducing sugar: The glucose concentration was determined by dinitrosalicylic acid (DNS) method as described by Miller,[31] using glucose as a standard. The aliquots of extract were pipetted 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 sulfuric 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 pipetted into 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 were 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[26,32,33].

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.[34]

Statistical analysis: All the results were statistically analyzed using SPSS software 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 is 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 %) (Table 1).

Table 1
Initial chemical composition of areca nut husk

S.N.	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
5	Cellulose (%)	46.0 ± 1.53
6	Hemi-cellulose (%)	40.3 ± 0.23
7	Lignin (%)	21.0 ± 0.19

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

Selection of fungal species: More efficient cellulolytic fungi *Trichoderma viride* and lignin degrading fungi *Phanerochaete chrysoprorium* NCIM 1197 were selected for microbiological treatment. Before fermentation, raw material was treated with specific fungal species to increase the maximum sugar yield. After fungal treatment, obtained 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 *Trichoderma viride* and *P. chrysoprorium* NCIM 1197 on the raw material. The optimum temperature was between 25 °C to 30 °C for the isolates (Table 2) (Figure 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 *T. viride* and *P. chrysoprorium* NCIM 1197 on the raw material (Table 3) (Figure 3).

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

Temperature (°C)	Reducing Sugar yield (mg/g)		Non-Reducing Sugar Yield (mg/g)		Total Sugar Yield (mg/g)	
	TV	PC	TV	PC	TV	PC
20	20.16 ± 0.98	32.0 ± 3.21	2.0 ± 0.07	10.5 ± 0.54	22.16 ± 4.0	42.5 ± 2.31
25	28.0 ± 1.41	44.32 ± 2.35	5.0 ± 0.11	12.6 ± 0.62	33.0 ± 3.81	56.92 ± 2.45
30	38.0 ± 1.50	54.0 ± 4.10	8.11 ± 0.36	15.0 ± 1.0	36.11± 3.30	69.0 ± 3.12
35	36.0 ± 2.36	41.85 ± 3.38	7.04 ± 0.39	14.0 ± 0.94	43.0 ± 2.81	55.85 ± 3.42
40	36.45 ± 2.80	40.0 ± 3.83	7.0 ± 0.40	13.2 ± 0.81	43.45 ± 4.83	53.2 ± 2.45

Note: Results are mean ± S.E. of three replicates (*n*=3). **TV**– *Trichoderma viride*, **PC**– *Phanerochaete chrysosporium* NCIM 1197

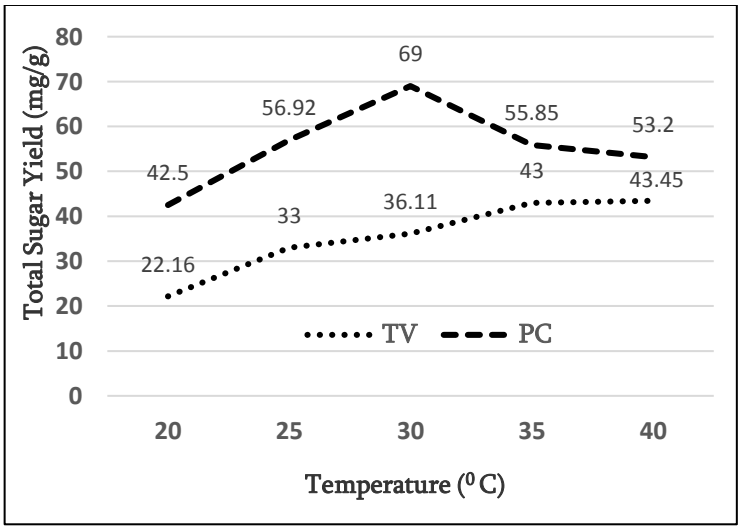

Figure 2: Graphical representation of effect of temperature on fungal treatment of areca nut husk
Note: TN- Trichoderma viride, PC- Phanerochaete chrysoprorium NCIM 1197

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

pH	Reducing Sugar Yield (mg/g)		Non-Reducing Sugar Yield (mg/g)		Total Sugar Yield (mg/g)	
	TV	PC	TV	PC	TV	PC
4	26.0 ± 3.64	29.0 ± 4.21	4.0 ± 0.07	9.0 ± 0.10	30.0 ± 1.40	38.0 ± 3.33
5	33.0 ± 3.45	35.31 ± 4.12	6.6 ± 0.06	10.0 ± 0.14	39.6 ± 1.63	45.31 ± 2.12
6	40.24 ± 4.78	52.0 ± 5.10	12.0 ± 0.21	16.5 ± 0.29	52.24 ± 2.45	68.5 ±1.42
7	39.0 ± 2.85	42.0 ± 5.0	9.4 ± 0.18	14.0 ± 0.34	48.4 ± 2.91	56.0 ± 2.96
8	38.12 ± 3.10	36.0 ± 3.69	9.0 ± 0.17	12.0 ± 0.41	47.12 ± 3.20	48.0 ± 3.45

Note: Results are mean ± S.E. of three replicates (*n*=3). **TV**– *Trichoderma viride*, **PC**– *Phanerochaete chrysosporium* NCIM 1197

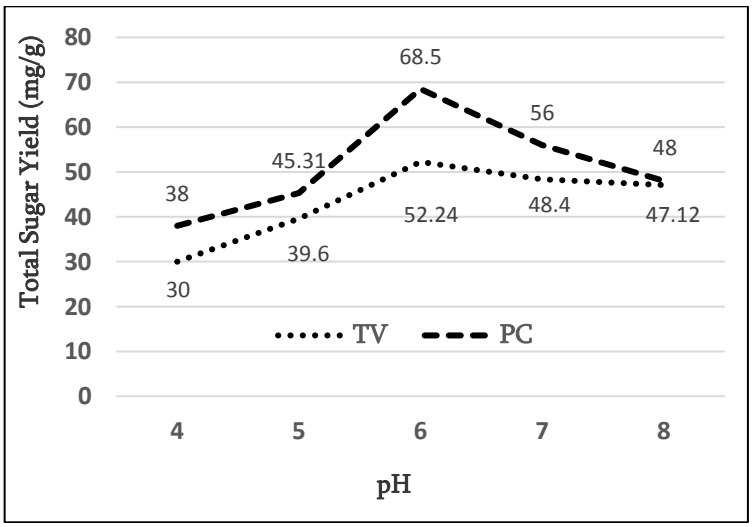

Figure 3: Graphical representation of effect of pH on fungal treatment of areca nut husk
Note: TN- *Trichoderma viride*, PC- *Phanerochaete chrysoprorium* NCIM 1197

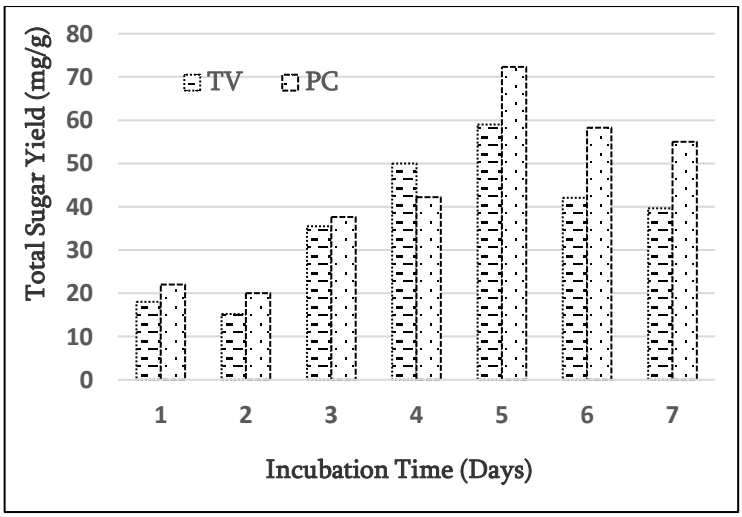

Figure 4: Graphical representation of effect of incubation time on fungal treatment of areca nut husk
Note: TN- *Trichoderma viride*, PC- *Phanerochaete chrysoprorium* NCIM 1197

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) (Figure 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 *T. viride* + *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) (Figure 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 pH 6 (Table 6) (Figure 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 TV+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) (Figure 7).

Discussion

The major component of lignocellulosic materials is cellulose followed by hemicellulose and lignin[35,36]. Cellulose and hemicelluloses are macromolecules constructed from different sugars whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors[37-39]. The composition and proportions of these compounds vary between plants. Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1, 4 glycosidic bonds forming the dimer cellobiose. Cellulose usually is present as a crystalline form and a small amount of non organized cellulose chains forms amorphous cellulose. In the latter conformation, cellulose is more susceptible to enzymatic degradation[10,40,41].

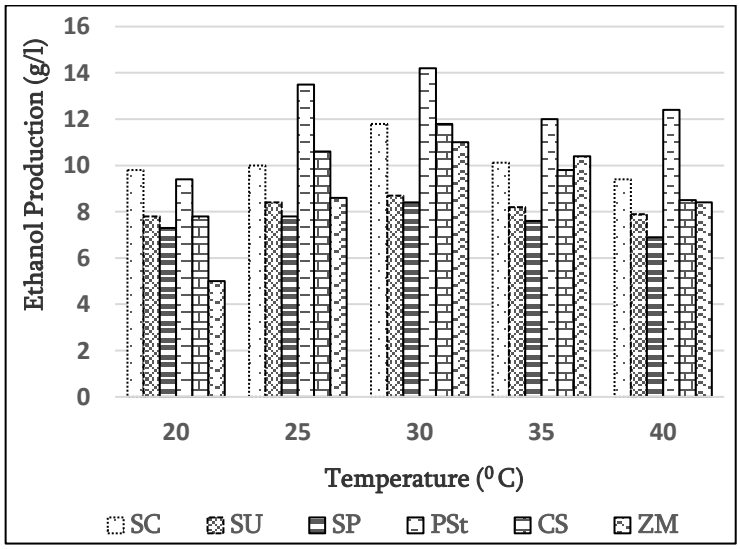

Figure 5: Graphical representation of effect of temperature on fermentation used for *T. viride* + *P. chrysosporium* NCIM 1197

Note: SC–Saccharomyces cerevisiae NCIM 3095, SU–Saccharomyces uvarum NCIM 3455, SP–*Schizosaccharomyces pombe* NCIM 3457, PSt–*Pichia stipitis* NCIM 3498, CS–*Candida shehatae* NCIM 3500 and ZM-*Zymomonas mobilis* NCIM 2915

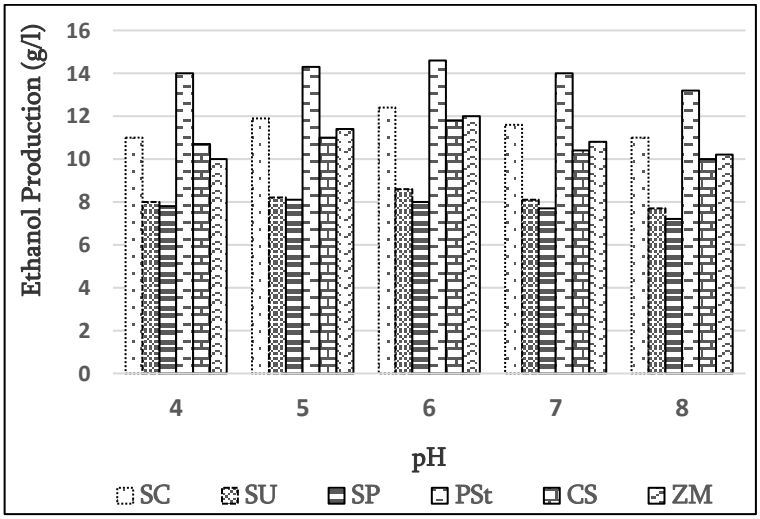

Figure 6: Graphical representation of effect of pH on fermentation used for *T. viride* + *P. chrysosporium* NCIM 1197

Note: SC–*Saccharomyces cerevisiae* NCIM 3095, SU–*Saccharomyces uvarum* NCIM 3455, SP–*Schizosaccharomyces pombe* NCIM 3457, PSt–*Pichia stipitis* NCIM 3498, CS–*Candida shehatae* NCIM 3500 and ZM-*Zymomonas mobilis* NCIM 2915

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)		Total Sugar Yield (mg/g)	
	TV	PC	TV	PC	TV	PC
1	14.0 ± 0.94	17.0 ± 1.12	4.0 ± 0.06	5.0 ± 0.09	18.0 ±0.21	22.0 ± 0.24
2	11.0±0.85	13.0±1.13	4.2±0.08	7.0±0.08	15.2 ±0.24	20.0 ±0.29
3	29.2±2.10	29.4±2.23	6.3±0.09	8.2±0.05	35.5 ±3.8	37.6 ±1.5
4	42.0±2.31	32.2±2.65	8.0±0.10	10.0±0.14	50.0 ±2.8	42.2 ±2.5
5	50.0±5.63	58.0±6.10	9.0±0.09	14.3±0.28	59.0 ±1.6	72.3 ±2.4
6	38.0±4.15	47.3±5.84	4.1±0.08	11.0±0.32	42.1 ±3.3	58.3 ±1.3
7	36.4±4.10	45.0±4.89	3.2±0.06	10.0±0.09	39.6 ±3.1	55.0 ±3.0

Note: Results are mean ± S.E. of three replicates (*n*=3).
TV– *Trichoderma viride*, **PC**– *Phanerochaete chrysosporium* NCIM 1197

Table 5
Effect of temperature on fermentation for *T. viride* + *P. chrysosporium* NCIM 1197

Temperature (°C)	Ethanol yield (g/L)					
	S. cerevisiae NCIM 3095	*S. uvarum* NCIM 3455	*S. pombe* NCIM 3457	*P. stipitis* NCIM 3498	*C. shehatae* NCIM 3500	*Z. mobilis* NCIM 2915
20	9.8 ± 1.20	7.8 ± 0.54	7.3 ± 0.52	9.4 ± 0.08	7.8 ± 0.09	5.0 ± 0.07
25	10 ± 1.30	8.4 ± 0.56	7.8 ± 0.41	13.5 ± 0.64	10.6 ± 0.18	8.6 ± 0.09
30	11.8 ± 0.94	8.7 ± 0.35	8.4 ± 0.40	14.2 ± 1.32	11.8 ± 0.34	11.0 ± 0.47
35	10.12 ± 1.40	8.2 ± 0.47	7.6 ± 0.23	12.0 ± 0.94	9.8 ± 0.45	10.4 ± 0.35
40	9.4 ± 0.87	7.9 ± 0.68	6.9 ± 0.09	12.4 ± 0.92	8.5 ± 0.64	8.4 ± 0.41

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

Table 6
Effect of pH on fermentation for *T. viride* + *P. chrysosporium* NCIM 1197

pH	Ethanol yield (g/L)					
	S. cerevisiae NCIM 3095	*S. uvarum* NCIM 3455	*S. pombe* NCIM 3457	*P. stipitis* NCIM 3498	*C. shehatae* NCIM 3500	*Z. mobilis* NCIM 2915
4	11.0 ± 1.10	8.0 ± 0.78	7.8 ± 0.14	14.0 ± 1.14	10.7 ± 0.87	10.0 ± 0.94
5	11.9 ± 1.0	8.2 ± 0.54	8.1 ± 0.23	14.3 ± 0.98	11.0 ± 0.84	11.4 ± 1.0
6	12.4 ± 0.94	8.6 ± 0.36	8.0 ± 0.65	14.6 ± 1.11	11.8 ± 0.96	12.0 ± 1.20
7	11.6 ± 0.65	8.1 ± 0.47	7.7 ± 0.87	14.0 ± 1.0	10.4 ± 0.74	10.8 ± 0.87
8	11.0 ± 0.68	7.7 ± 0.25	7.2 ± 0.09	13.2 ± 0.91	10.0 ± 0.64	10.2 ± 0.83

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

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

Inoculum Concentration (%)	Ethanol yield (g/L)					
	S. cerevisiae NCIM 3095	*S. uvarum* NCIM 3455	*S. pombe* NCIM 3457	*P. stipitis* NCIM 3498	*C. shehatae* NCIM 3500	*Z. mobilis* NCIM 2915
1	10.4 ± 0.45	8.4 ± 0.24	7.4 ± 0.09	13.4 ± 0.45	10.8 ± 0.25	10.2 ± 0.16
2	10.8 ± 0.44	8.9 ± 0.29	8.0 ± 0.15	13.9 ± 0.53	10.9 ± 0.34	11.4 ± 0.28
3	11.4 ± 0.36	9.9 ± 0.34	8.6 ± 0.21	14.3 ± 0.54	11.8 ± 0.26	12.4 ± 0.19
4	11.0 ± 0.38	9.6 ± 0.35	8.0 ± 0.26	14.0 ± 0.64	10.2 ± 0.33	11.0 ± 0.34
5	10.6 ± 0.29	9.0 ± 0.28	7.4 ± 0.33	13.5 ± 0.61	9.4 ± 0.22	10.6 ± 0.37

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

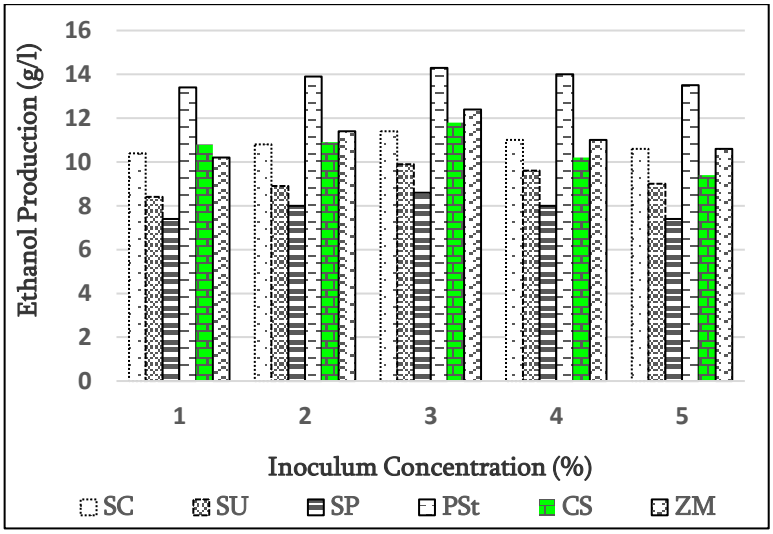

Figure 7: Graphical representation of effect of inoculum concentration on fermentation used for *T. viride* + *P. chrysosporium* NCIM 1197
Note: SC–*Saccharomyces cerevisiae* NCIM 3095, SU–*Saccharomyces uvarum* NCIM 3455, SP–*Schizosaccharomyces pombe* NCIM 3457, PSt–*Pichia stipitis* NCIM 3498, CS–*Candida shehatae* NCIM 3500 and ZM-*Zymomonas mobilis* NCIM 2915

In the present study, we have to use more efficient cellulolytic fungi *Trichoderma viride* and lignin degrading fungi *P. chrysoprorium* NCIM 1197 was selected for microbiological treatment. Hatakka[42] proposed a biological pretreatment of wheat straw using white rot fungi instead of thermo chemical pretreatment. Even though he was able to produce high ethanol yield, the requirement for sterile incubation of biomass for 5 weeks proves the technique non-economic and practically impossible. Hatakka also tested a semi bio-chemical treatment with a reduced time of biological treatment and subsequent thermo chemical pretreatment. But some sugars released by the initial biological treatment were converted to inhibitors in the later chemical treatment.

The organisms predominantly responsible for lignocelluloses degradation are fungi and the most rapid degraders in this group are basidiomycetes[43,44]. The ability to degrade lignocelluloses efficiently is thought to be associated with a mycelial growth habit that allows the fungus to transport scarce nutrients such as nitrogen and iron to a distance into the nutrient-poor lignocellulosic substrate that constitutes its carbon source[45]. The fungal degradation occurs exocellular either in association with the outer cell envelope layer or extracellular because of the insolubility of lignin, cellulose and hemicelluloses[46].

Fungi have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases that are responsible for polysaccharide degradation and a unique oxidative and extracellular ligninolytic system which degrades lignin and opens phenyl rings. Several microorganisms, mainly fungi have been isolated and identified as lignocellulolytic organisms[47]. In the present work, 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 *T. viride* + *P. chrysoprorium* NCIM 1197 (TV+PC) on areca nut husk.

Extracellular enzymes produced during fungal pretreatment of corn stover were extracted and assayed to determine the activities of oxidative and hydrolytic enzymes. No LiP was detected during the corn stover decay process[47], which is consistent with the observations of Ruttimann-Johnson et al[48] and Rajakumar et al[49] that *C. subvermispora* lacks LiP activity in either liquid culture or solid culture. MnP and laccase were two major lignin degradation enzymes detected in the solid culture on corn stover[47].

The main drawback that is limiting the industrial use of this kind of pretreatment in lignocellulosic ethanol production is represented by the very low process rate[50]. The fact that some of the carbohydrate fraction is consumed by the microorganism could also be a disadvantage[51,52].

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. Fungal pre-treatment, however, has not received much attention in the past probably due to the substantial loss in cellulose and hemicellulose during the pre-treatment step and the very slow rate of hydrolysis reaction leading to long pre-treatment time, both of which tend to reduce the overall yield.

Conclusion

Compared to current leading thermal or chemical pretreatment processes, fungal pretreatment with white rot fungi is an environmental friendly and energy-efficient

process. In the present work, fungal treatment of areca nut husk was found to be the best method of saccharification. Consortium of fungi was more effective (*Trichoderma viride* with *Phanerocheate chrysoprorium* NCIM 1197). White rot fungi with a high selectivity of lignin degradation over cellulose loss are important for fungal pretreatment. Complete decontamination may not be necessary since white rot fungi can survive in contamination and actively act on degradation. Fungal pretreatment prior to mild physical and chemical pretreatment has shown synergism on the improvement of cellulose digestibility with advantages similar to that of the biopulping process. Fungal pretreatment can modify the cell walls before evident degradation takes place; the required pretreatment severity of thermo-chemical pretreatment can be substantially reduced. In the present study, *Pichia stipitis* NCIM 3498 was found to give better ethanol yield after fermentation from areca nut husk followed by *Candida shehatae* NCIM 3500 and *Zymomonas mobilis* NCIM 2915.

Acknowledgement

The authors gratefully acknowledge to the Department of Microbiology, Kuvempu University, Shivamogga, Karnataka for providing laboratory facilities.

References

1. Krishna S.H. and Chowdary GV., Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic biomass, *J. Agric. Food Chem*., **48**, 1971–1976 **(2000)**

2. Itoh H., Wada M., Honda Y., Kuwahara M. and Watanabe T., Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi, *J. Biotechnol*., **103**, 273–280 **(2003)**

3. Kovacs K., Szakacs G. and Zacchi G., Comparative enzymatic hydrolysis of pretreated spruce by supernatants, whole fermentation broths and washed mycelia of Trichoderma reesei and Trichoderma atroviride, *Bioresour. Technol*., **100**, 1350–1357 **(2009)**

4. Nazarpour F., Abdullah D.K., Abdullah N. and Zamiri R., Evaluation of biological pretreatment of rubber wood with white rot fungi for enzymatic hydrolysis, *Materials*, **6**, 2059–2073 **(2013)**

5. Chacha N., Dyrset N. and Mtui G., Optimization of independent variables affecting bioethanol production using *Escherichia coli* KO11, *Int. J. Environ. Bioener*., **11**, 98–112 **(2016)**

6. Abe J., Bergman F.W., Obata K. and Hikuri S., Production of raw starch digesting amylase by *Aspergillus* K-27, *Appl. Microbiol. Biotechnol*., **27**, 447–450 **(1988)**

7. Mosier N. et al, Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresour. Technol*., **96**, 673–686 **(2005)**

8. Ahmed S., Bashir A., Saleem H., Saadia M. and Jamil A., Production and purification of cellulose degrading enzymes from a filamentous fungus *Trichoderma harzianum*, *Pak. J. Bot.*, **41**, 1411–1419 **(2009)**

9. Adesina F.C. and Onilude A.A., Isolation, identification and screening of xylanase and glucanase-producing microfungi from degrading wood in Nigeria, *Afr. J. Agric. Res.*, **8**, 4414–4421 **(2013)**

10. Wan C. and Li Y., Fungal pretreatment of lignocellulosic biomass, *Biotechnol. Adv*., **30**, 1447–1457 **(2012)**

11. Akin D.E., Sethuraman A., Morrison W.H., Martin S.A. and Eriksson K.E., Microbial delignification with white-rot fungi improves forage digestibility, *Appl. Environ. Microbiol*., **59**, 4274–4282 **(1993)**

12. Lee S.M. and Koo M.Y., Pilot-scale production of cellulose using *Trichoderma reesei* Rut C-30 in fed-batch mode, *J. Microbiol. Biotechnol.*, **11**, 229–233 **(2001)**

13. Keller F.A., Hamilton J.E. and Nguyen Q.A., Microbial pretreatment of biomass potential for reducing severity of thermo-chemical biomass pretreatment, *Appl. Biochem. Biotechnol*., **105**, 27–41 **(2003)**

14. Taniguchi M., Suzuki H., Watanabe D., Sakai K., Hoshino K. and Tanaka T., Evaluation of pretreatment with *P. ostreatus* for enzyme hydrolysis of rice straw, *J. Biosci. Bioeng*., **100**, 637–643 **(2005)**

15. Coll M.P., Abalos J.M.F., Villanueva J.R., Santamaria R. and Perez P., Purification and characterization of phenoloxidase (laccase) from the lignin degrading basidomycete, strain PM1, *Appl. Environ. Microbiol*., **59**, 2607–2613 **(1993)**

16. Karthikeyan S., Ramasamy K. and Prabhakaran J., Conversion of industrial solid wastes to value of lignocellulosics: microbial, chemical and enzymatic aspects of fungal attack of lignin, *Brain Res. Rev*., **8**, 195–204 **(2005)**

17. Dashtban M., Schraft H. and Qin W., Fungal Bioconversion of Lignocellulosic Residues; opportunities and perspectives, *Int. J. Biol. Sci*., **5**, 578–595 **(2009)**

18. Ramappa B.T., Economics of areca nut cultivation in Karnataka a case study of Shivamogga District, *J. Agric. Vet. Sci*., **3**, 50–59 **(2013)**

19. Ramappa B.T. and Manjunatha M.S., Cost cultivation of areca nut non-traditional region of Karnataka -an analysis, *Int. J. Pharm. Sci. Invent*., **2**, 25–31 **(2013)**

20. Narayanamurthy G., Ramachandra Y.L., Rai S.P., Manohara Y.N. and Kavitha B.T., Areca husk: An inexpensive substrate for citric acid production by *Aspergillus niger* under solid state fermentation, *J. Biotechnol*., **7**, 99–102 **(2008)**

21. Rajan A. et al, Biosoftening of areca nut fiber for value added products, *Biochem. Eng. J*., **25**, 237–242 **(2005)**

22. Prasad S., Singh A. and Joshi H.C., Ethanol as an alternative fuel from agricultural, industrial and urban residues, *J. Resour. Conser. Recycl*., **50**, 1–39 **(2007)**

23. Naveenkumar K.J. et al, Effect of pretreatment for delignification and sugar recovery for bioethanol production from Areca nut husk waste, *Int. J. Current Res*., **4**, 18–24 **(2012a)**

24. Amin G. and Doelle H.W., Production of high ethanol concentrations from glucose using Zymomonas mobilis entrapped in a vertical rotating immobilized cell reactor, *Enzyme Microbial. Technol.,* **12**, 443–446 **(1990)**

25. Gurav M.S. and Geeta G.S., Effectiveness of fungal pretreatment of agro residues on ethanol production by yeasts and *Zymomonas mobilis*, *Kar. J. Agric. Sci.,* **20**, 301–304 **(2007)**

26. Patel S.J., Studies on ethanol production from bioconverted agricultural residues, Ph.D. Thesis, Kuvempu University, Shivamogga, 1–300 **(2008)**

27. Naveenkumar K.J., Thippeswamy B., Banakar S.P., Thirumalesh B.V. and Krishnappa M., Effectiveness of fungal pretreatment of areca nut (Areca catechu L.) husk waste on bioethanol production by yeasts and *Zymomonas mobilis* NCIM 2915, *Int. J. Recent. Sci. Res*., **3**, 400–406 **(2012b)**

28. Ciani M. and Ferraro L., Combined use of immobilized Candida stellata cells and *Saccharomyces cerevisiae* to improve the quality of wines, *J. Appl. Microbiol*., **85**, 247–254 **(1998)**

29. Sun Y. and Cheng J., Hydrolysis of lignocellulosic materials for ethanol production: a review, *Bioresour. Technol*., **83**, 1–11 **(2002)**

30. Oyeleke S.B. and Jibrin N.M., Production of bioethanol from guinea cornhusk and millet husk, *Afr. J. Microbiol. Res.*, **3**, 147–152 **(2009)**

31. Miller G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugars, *Anal. Chem.,* **31**, 426–428 **(1959)**

32. Sadasivam M. and Manickam A., Text book of Biochemical methods, New age international Limited Publishers, 1–60 **(1996)**

33. Agbor V.B., Cicek N., Sparling R., Berlin A. and Levin D.B., Biomass pretreatment: Fundamentals toward application, *Biotechnol. Adv*., **29**, 675–685 **(2011)**

34. Caputi A., Ueda J.M. and Brown T., Spectrophotometric determination of chromic complex formed during oxidation of alcohol, *Am. J. Enol. Vitic*., **19**, 160–165 **(1968)**

35. Acuna-Arguelles M.E., Gutierrez-Rojas M., Viniegra-Gonzalez G. and Favela-Torres E., Effect of water activity on exopectinase production by *Aspergillus niger* CH4 on solid-state fermentation, *Biotechnol. Lett*., **16**, 23–28 **(1994)**

36. Acharya P.B., Acharya D.K. and Modi H.A., Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate, *Afr. J. Biotechnol*., **7**, 4147–4152 **(2008)**

37. Damisa D., Ameh J. and Umoh V.J., Effect of chemical pretreatment of some lignocellulosic wastes on the recovery of cellulase from *Aspergillus niger* AH3 mutant, *Afr. J. Biotechnol*., **7**, 2444–2450 **(2008)**

38. Akhter N.M., Morshed A., Uddin A., Begum F., Sultan T. and Azad A.K., Production of pectinase by *Aspergillus niger* cultured in solid state media, *Int. J. Biosci*., **1**, 33–42 **(2011)**

39. Abinaya S.T., Deepa P. and Nithya T., Screening, optimization and separation of ligninolytic enzyme by Polyporaceae group fungi, *Int. J. Sci. Innov. Disc*., **3**, 434–445 **(2013)**

40. Gautam S.P., Bundela P.S., Pandey A.K., Awasthi M.K. and Sarsaiya S., Isolation, identification and cultural optimization of indigenous fungal isolates as a potential bioconversion agent of municipal solid waste, *Annals of Environ. Sci*., **5**, 23–34 **(2011)**

41. Bakri Y., Jawhar M. and Arabi M.I.E., Xylanase production by *Fusarium solani* in solid state fermentation, *Res. Biotechnol*., **4**, 31–37 **(2013)**

42. Hatakka A.I., Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose, *Appl. Microbiol. Biotechnol*., **18**, 350–357 **(1983)**

43. Rabinovich M.L., Vasilchenko and Bolobova A.V., Fungal decomposition of natural aromatic structures and xenobiotics: a review, *Appl. Biochem. Microbiol*., **40**, 1–17 **(2004)**

44. Baldrian P. and Snajdr J., Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes, *Enzyme* and *Microbial Technol.,* **39**, 1023–1029 **(2006)**

45. Banu A.R., Devi M.K., Gnanaprabhal G.R., Pradeep B.V. and Palaniswamy M., Production and characterization of pectinase enzyme from *Penicillium chrysogenum*, *Indian J. Sci. Technol*., **3**, 377–381 **(2010)**

46. Cassanco A.M., Aguillas T.B.A. and Aguilar G., Physiological comparison between pectinase producing mutants of *Aspergillus niger* adopted either to solid state fermentation or submerged fermentation, *Enzyme* and *Microbial Technol.*, **21**, 26–27 **(1997)**

47. Sanchez C., Lignocellulosic residues: Biodegradation and bioconversion by fungi, *Biotechnol. Adv*., **27**, 185–194 **(2009)**

48. Ruttimann-Johnson C., Salas L., Vicuna R. and Kirk T.K., Extracellular enzyme production and synthetic lignin mineralization by *Ceriporiopsis subvermispora*, *Appl. Environ. Microbiol*., **59**, 1792–1797 **(1993)**

49. Rajakumar S. et al, Lip-like genes in *Phanerochaete sordida* and *Ceriporiopsis subvermispora*, white rot fungi with no detectable lignin peroxidase activity, *Appl. Environ. Microbiol*., **62**, 2660–2663 **(1996)**

50. Chiaramonti D., Prussi M., Ferrero S., Oriani L., Ottonello P., Torre P. and Cherchi F., Review of pretreatment processes for lignocellulosic ethanol production and development of an innovative method, *Biomass and Bioenergy*, **46**, 25–35 **(2012)**

51. Agblevor F.A., Cundiff J.S. and Mingle W., Storage and Characterization of cotton gin waste for ethanol production, *Resour. Conserv. Recy*., **46**, 198–216 **(2006)**

52. Myat L. and Gi-hyung R., Pretreatments and factors affecting saccharification and fermentation for lignocellulosic ethanol production, *Cell Chem. Technol*., **50**, 177–188 **(2016)**.

(Received 07th March 2020, accepted 10th May 2020)