ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Low cost pretreatment of lignocellulosic waste by white rot fungi for ethanol production using *Saccharomyces cerevisiae*

Thamilmaraiselvi B, Steffi PF*, Sathammaipriya N, Sangeetha K

Department of Microbiology, Cauvery College for Women, Annamalai Nagar, Trichy – 18, Tamil Nadu, India

Article History:

Received on: 20.11.2017
Revised on: 27.01.2018
Accepted on: 06.02.2018

Keywords:

Saccharomyces cerevisiae
Lignocellulosic
Ethanol
FTIR

ABSTRACT

Saccharomyces cerevisiae is a species of yeast. It has been instrumental in winemaking, baking, and brewing since ancient times. The present study was performed to produce lignin degrading enzymes to degrade lignocellulosic substrates and to produce ethanol using *Saccharomyces cerevisiae* by performing FTIR method. The yeast culture *Saccharomyces cerevisiae* was isolated and screened for the production of lignolytic enzymes. Then it was pretreated to produce lignocellulosic substrates. Lignocellulosic materials are considered the most abundant renewable resource available for the production of ethanol by FTIR method. The present study concluded the utilization of lignocellulosic biomass for ethanol production in future.

* Corresponding Author

Name: Steffi PF
Phone: +91- 9600977465
Email: steffi.titu@gmail.com

ISSN: 0975-7538

Peer review under the responsibility of Editor
DOI: https://doi.org/10.26452/ijrps.v9i1.1152

Production and Hosted by

IJRPS | https://ijrps.com

INTRODUCTION

Saccharomyces is a genus of fungi that includes many species of yeasts (Azbarova *et al.,* 2017). It has been instrumental in winemaking, baking, and brewing since ancient times. It is believed to have been originally isolated from the skin of grapes (Vincent *et al.,* 2017). It is one of the most intensively studied eukaryotic model organism in molecular and cell biology. *S. cerevisiae* cells are round to ovoid, 5–10 µm in diameter (Barraza *et al.,* 2017). It reproduces by a division process known as budding. *S.cerevisiae* is currently the only yeast cell known to have Berkeley bodies present, which are involved in particular secretory pathways (Izah *et al.,* 2017). Antibodies against *S. cerevisiae* are found in 60–70% of patients with Crohn's disease and 10–15% of patients with ulcerative colitis (Baek *et al.,* 2017). The pre- sent study were focused to isolate a *fungal sp* for the production of lignin degrading enzymes to degrade lignocellulosic substrates and to produce ethanol using *Saccharomyces cerevisiae* (Hull *et al.,* 2017). Recently, a new round of interest in bio energy and bio mass has been initiated with the recognition that global crude oil reserves are finite and its depletion is occurring much faster than previously predicted (Nielsen *et al.,* 2017).

The available technology suggests that bio mass containing lignocellulosic materials represents an abundant and inexpensive source of sugars that can be microbiologically or enzymatically converted into industrial products (Gao *et al.,* 2017). Production of ethanol from lignocellulosic bio mass contains three major process, including pretreatment, hydrolysis and fermentation (Hammer and Avalos 2017). The main enzymes of fungi taking part in lignin degradation are Phenol oxidases (Laccase), Lignin peroxidase and Manganese –Peroxidase (Santos *et al.,* 2017). All three enzymes can oxidize phenolic compounds, thereby creating phenoxy radicals, while non-phenolic compounds are oxidized via cation radicals (Celaj *et al.,* 2017). These enzymes are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates, but also in the degradation of various xenobiotic compounds including dyes (De

La Torre *et al.,* 2017). In recent years, the ligninolytic system of fungi with respect to enzymatic potential for the bioremediation of persistent pollutants has been extensively studied (Chua *et al.,* 2017). Out of which cellulose and hemicelluloses can serve as an alternative raw material for its bioconversion to ethanol (Jansen *et al.,* 2017). Sugars formed after scarification of biomass are converted to alcohol through a process called fermentation (Cadete and Rosa 2017). *Saccharomyces cerevisiae* plays a major role in applied research due to its outstanding capacity to produce ethanol and carbon dioxide from sugars with high productivity, titer and yield (Lopes *et al.,* 2017).

MATERIALS AND METHODS

Isolation of fungi: Fungi showing good growth on decayed wood samples were collected in and around Karur District in a polythene bag. Fungi were isolated by means of direct touch method. The fungi were inoculated on Ampillicin incorporated potato dextrose agar plates and incubated at room temperature for 7 days. The isolated fungi were maintained on potato dextrose agar plates at 20˚C.

Screening of Fungi for lignolytic activity: The isolated fungi were screened for the production of lignolytic enzymes. The isolates were inoculated aseptically onto the pre-solidified potato dextrose agar medium containing 0.05% guaiacol and incubated at room temperature for 7 days. Lignolytic positive organisms developed colored zone.

Collection of lignocellulosic wastes: The lignocellulosic substrates (Paddy straw, Sugar cane Bagasse and Sorghum Leaves) were collected in and around Trichy district. The collected lignocellulosic substrates were shade dried to remove the moisture content. The substrates were then milled to a size of 1mm and stored in an air tight container.

Substrate pretreatment: 25 g of milled dry substrates particle size (1mm) were weighed into conical flask containing 75 ml of distilled water. The fresh weight of the substrates was determined and the flasks were autoclaved. The bottles were inoculated with two agar plugs (diameter 9 mm) of fungi mycelia in triplicates and incubated for 30 days at 30 ± 2˚C at constant moisture level. The controls were put in an oven at 80˚C ± 2˚C for 48hrs to determine the initial dry weight of the substrates. Harvested samples were also put in the oven for 48 hrs at 100˚C ±2˚C to remove the moisture content.

Analysis of lignin and cellulose content: A mixture containing 1 g of dried sample and 150 ml of aqua dest was heated in a water bath at a temperature of 90 – 100˚C for 1 h. The mixture was filtered and the residue was washed with hot water (300 ml). The residue was dried in the oven until the weight was constant. The residue was mixed with 150 ml of 1N H_2SO_4 and heated in the water bath at 90 – 100˚C for 1 h. The mixture was filtered and washed with 300 ml of aqua dest and then the residue was dried. The dried residue was soaked with 10 ml of 72 % H_2SO_4 at room temperature for 4 h. After that, 150 ml of 1 N H_2SO_4 was added into the mixture and refluxed in the water bath for 1h. The solid was washed with 400 ml of aqua dest, heated in the oven at 105˚C and weighed. Finally the solid was heated until it become ash and weighed. The percentage of cellulose and lignin was calculated.

Estimation of reducing sugars: 1 ml of diluted sample was taken in a test tube to which 3ml of DNS reagent was added. The tubes were boiled in a boiling water bath for 15 minutes. 1 ml of Rochelle salt was added to these test tubes and tubes were cooled to room temperature and used for measuring optical density at 575 nm. A standard curve of glucose was prepared by using various concentration of glucose in distilled water.

Fourier Transformed Infrared (FTIR) studies: FTIR studies of untreated and treated lignocellulosic substrates were carried out at St.Joseph's College, Trichy. Pure cellulose was used as a reference material to compare the treated and untreated substrates.

Fermentation: The yeast culture *Saccharomyces cerevisiae* was subcultured and maintained on YEPD agar at 4˚C. Starter media and production media was prepared. 50gms of treated wastes (sugarcane bagasse, sorghum leaves, paddy straw) were weighed and added to 1 liter of distilled water. To this, 5 gms of diammonium phosphate and 1 gm of yeast extract were added and sterilized. 10 ml of the inoculum from the production media were added and kept in a shaker for 94 hrs.

Estimation of ethanol: 10 ml of fermented medium was centrifuged at 10,000 rpm for 10 min at room temperature. To 1 ml of the supernatant, 24 ml of distilled water was added. It was then poured into the round bottomed distillation flask and distilled.15 ml of the distillate was collected in a volumetric flask containing 25 ml of 3.4 % chromic acid. This was then made up to 50 ml. The condensate was heated at 80˚C for 15 min in a water bath and cooled. Absorbance was read at 580 nm using chromic acid and distilled water (1:1) as blank. Different concentration of ethanol (1 -10 %) was prepared and the above procedure was followed.

RESULTS AND DISCUSSION

Isolation of Fungi: The fungal colonies showed good growth on Ampillicin incorporated PDA plates. White colored, short hairy growths were

observed on the next day of incubation and carpet like colonies were observed on the seventh day refer Figure 1.

Figure 1: Isolation of fungi from decayed wood

Screening of the isolates for the lignolytic activity: The fungal colonies were screened for the development of brown colour in the PDA plates incorporated with 0.05% guaiacol. On third day of incubation, brown color was observed and the maximum color development was observed on the seventh day. This clearly indicates that the fungal isolates are capable of producing the laccase enzyme that is involved in lignin degradation. Guaiacol is an indicator, which reported to be used for laccase production. Reddish brown colour was observed due to the oxidative polymerization of guaiacol in the presence of extracellular fungal laccase.

Analysis of lignin and cellulose content: The lignin content of treated paddy straw, sugarcane bagasse and sorghum leaves were found to be 13%, 12% and 4% respectively. Untreated paddy straw, sugarcane bagasse and sorghum leaves showed 15%, 14% and 8% lignin content respectively. Maximum delignification was observed in sorghum leaves treated with fungi at a reduction rate of 3 %, followed by 2 % reduction rate was observed in sugarcane bagasse and paddy straw compared with untreated substrates. The cellulose content of treated paddy straw, sugarcane bagasse and sorghum leaves were 22%, 28% and 25% respectively. Untreated paddy straw, sugarcane bagasse and sorghum leaves showed 25%, 31% and 28% cellulose content respectively. The values were presented in the Table 1 and Table 2.

The reduction rate of cellulose was found to be 3 % in all the substrates compared with untreated lignocellulosic substrates.

Estimation of reducing sugars: All the samples including treated and untreated showed color change when incubated with DNS reagent in water bath. The intensity of red color was found to be more in treated lignocellulosic substrates when compared with the untreated lignocellulosic substrates. This clearly indicates the presence of hydrolytic enzymes in the fungal isolate. The amount of sugars liberated in treated sugarcane bagasse, sorghum leaves and paddy straw were 470 µg/ ml, 405 µg/ ml and 365 µg/ ml respectively. The untreated sugarcane bagasse, sorghum leaves and paddy straw were 330 µg/ ml, 380 µg/ ml, and 305 µg/ ml respectively**.** The standard values of glucose were presented in Table 3 and the optical density of the treated and untreated substrates were presented in Table 4.

Table 1: Biologically treated lignocellulosic substrates

Lignocellulosic Substrate	a (g)	b (g)	c (g)	d (g)	e (g)	% of Lignin	% of cellulose
Paddy straw	1	0.67	0.40	0.18	0.05	13	22
Sugarcane bagasse	1	0.72	0.47	0.14	0.02	12	28
Sorghum leaves	1	0.74	0.35	0.10	0.06	4	25

Table 2: Untreated lignocellulosic substrates

Lignocellulosic Substrate	a (g)	b (g)	c (g)	d (g)	e (g)	% of Lignin	% of cellulose
Paddy straw	1	0.75	0.58	0.30	0.15	15	25
Sugarcane bagasse	1	0.80	0.65	0.34	0.20	14	31
Sorghum leaves	1	0.70	0.47	0.17	0.10	7	28

Table 3: Standard value of glucose solution

Volume of Standard Solution (ml)	Concentration (µg)	Volume of distilled water (ml)	Volume of DNS reagent (ml)	Optical density at 575 nm
0.1	100	0.9	3	0.40
0.2	200	0.8	3	0.78
0.3	300	0.7	3	1.20
0.4	400	0.6	3	1.58
0.5	500	0.5	3	2.00
Blank	--	1.0	3	0.00

Table 4: Estimation of reducing sugars in treated and untreated lignocellulosic substrates

Lignocellulosic substrates	Volume of the sample (ml)	Volume of DNS reagent (ml)	Optical density at 575 nm
Treated sugarcane bagasse	1.0	3	1.73
Untreated sugarcane bagasse	1.0	3	1.25
Treated paddy straw	1.0	3	1.45
Untreated paddy straw	1.0	3	1.28
Treated sorghum leaves	1.0	3	1.60
Untreated sorghum leaves	1.0	3	1.45

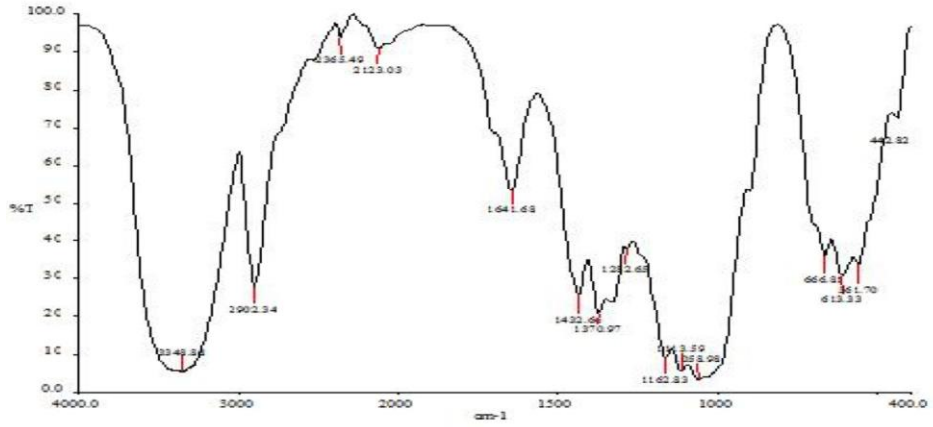

Figure 2: FTIR analysis of standard cellulose

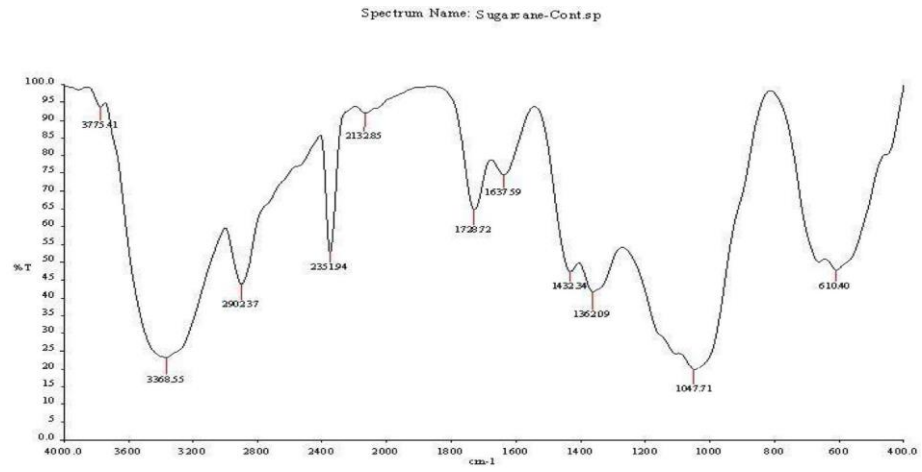

Figure 3: FTIR spectral studies of untreated sugarcane bagasse

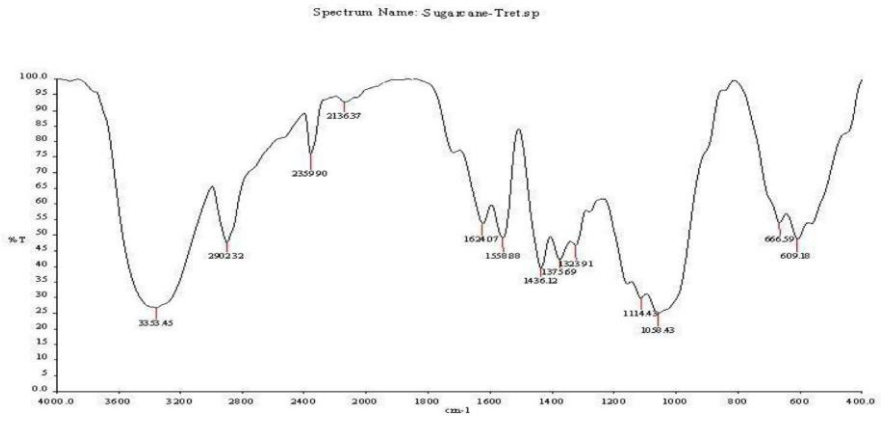

Figure 4: FTIR spectral studies of treated sugarcane bagasse

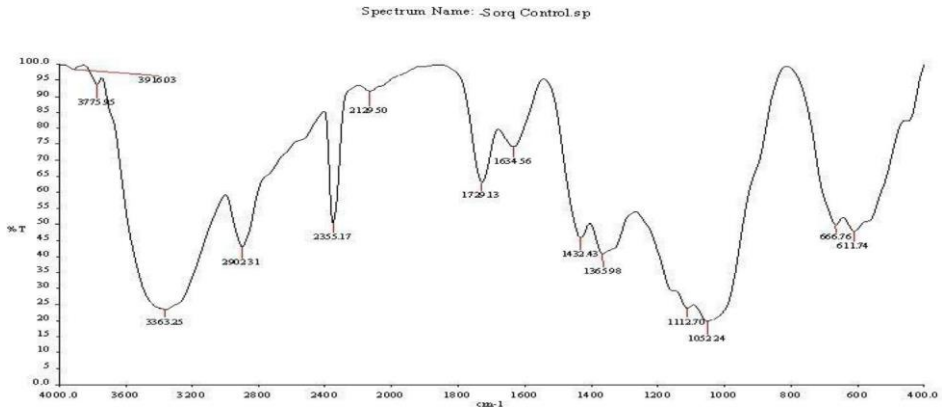

Figure 5: FTIR spectral studies of untreated sorghum leaves

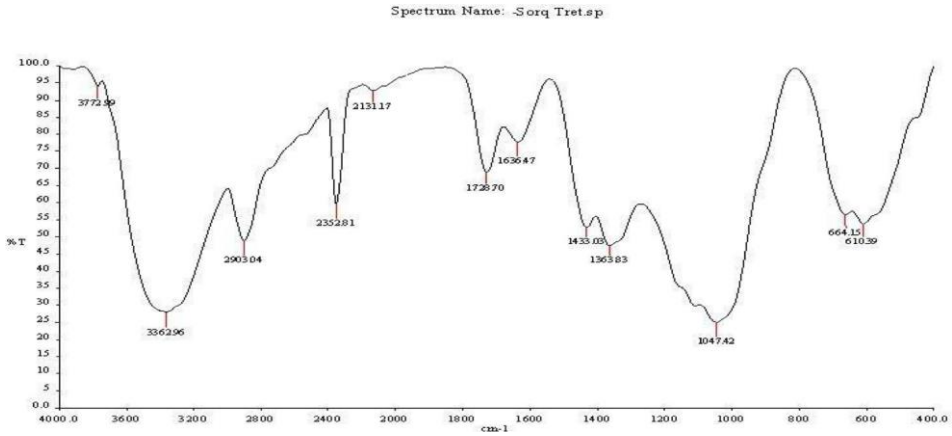

Figure 6: FTIR spectral studies of treated sorghum leaves

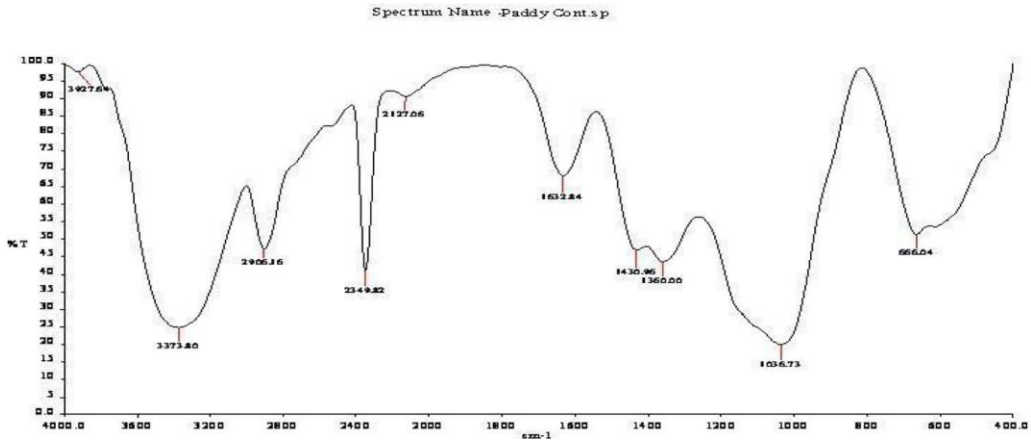

Figure 7: FTIR spectral studies of untreated paddy straw

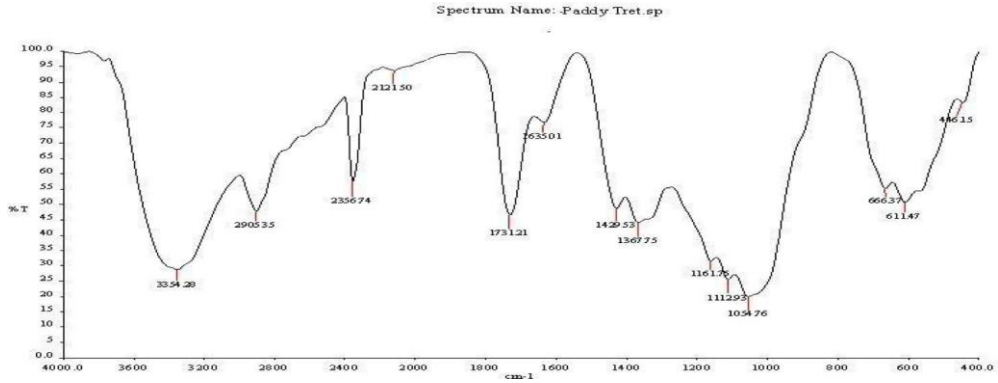

Figure 8: FTIR spectral studies of treated paddy straw

Table 5: Ethanol production in biologically treated lignocellulosic substrates

Treated lignocellulosic substrates	Optical density at 540 nm
Paddy straw	0.176
Sugarcane bagasse.	0.89
Sorghum leaves	0.45

FTIR spectra of sugarcane bagasse: All the absorption peaks located from 3500 to 3000 cm^{-1} are of higher wave number than pure cellulose. The intensities of these stretching vibrations decreased after biological treatment. The peak located at 2360 cm-1 shown in Figure 3 corresponds to the stretching of C = NH^{+} groups present in the peptides and nucleotides suggesting that the hydrogen bonds of lignin are partially digested.

The absorption peak at 1729 cm^{-1} corresponds to the stretching of C =O groups. It indicates the release of monosaccharides. This frequency is found in the untreated substrates. The disappearance of monosaccharide units may be utilized by the fungi for their own growth. The peak at 1637 cm^{-1} shown in Figure 4 is due to the stretching of CO – NH bond attributed to the peptides and quinoid vibration in the GTP. The intensity of these stretching vibrations increased after biological treatment.

The peak at 1362 cm^{-1} corresponds to the stretching of CO – NH and C = C bond. There is an increased stretching of vibrations observed after biological treatment. The peaks ranging from 1400 – 1200 cm^{-1} may be assigned to the lignin moiety and also to the stretching vibrations of nitrite, nitrate or phosphate groups that may be present in the nucleic acids. The intensity was found to be increased in biologically treated when compared with untreated substrates. The peak located at 1042 cm^{-1} is assigned to C- O-C stretching mode. The intensity of these stretching vibrations increased after the treated. The peaks 668 and 609 cm^{-1} found in the treated substrates, represents the breakdown of cellulose to glucose.

FTIR spectra of sorghum leaves: All the absorption peaks located from 3500 – 3000 cm^{-1} are of higher wave number than pure cellulose. FTIR spectra of sorghum leaves were shown Figure 5 and Figure 6.

Peak ranges from 3500 – 3000 cm^{-1} corresponds to the presence of OH and NH group. The peaks at 1443 – 1225 cm^{-1} are assigned to phosphate and amide bending vibration. The peaks at 1729 cm^{-1} to C= O. Only minor changes were observed after treatment.

FTIR spectra of paddy straw: The peak ranging from 3000- 2500 cm^{-1} corresponds to CH vibrations. FTIR spectra of paddy straw were shown in Figure 7 and Figure 8. The absorption peak at 1631 cm^{-1} is assigned to the stretching of CO- NH in peptides and quinoid vibrations in nucleotides. During pretreatment, hydrolysis of protein present in the substrate may lead to the release of peptides.

The absorption peaks to 1430 and 1368 cm^{-1} are assigned to the stretching of phosphate and CO – NH bending vibrations. The peak at 1054 cm^{-1} corresponds to C= C, C=N stretching.

Ethanol estimation: The optical density of treated lignocellulosic substrates was shown in Table 5. The yield of the ethanol in sugarcane bagasse, sorghum leaves and paddy straw were found to be 40 ml, 12 ml and 5 ml per 1000 ml of the fermented samples.

CONCLUSION

Based on the above FTIR analysis, the pretreatment (biological) showed various structural changes including release of GTP, decrease of monosaccharides, liberation of simple sugars and deformation of lignin structure. The maximum ethanol production was observed in sugarcane bagasse due to the increased concentration of sugars than the other two substrates. Theoretical ethanol yields from sugar and starch (g ethanol/ g substrate) are higher than from lignocellulose, these conventional sources are uneconomical on the basis of food security, insufficient for worldwide bioethanol production. In that aspect, agricultural lignocellulosic wastes are renewable, less costly and abundantly available in nature and don't demand separate land, water, and energy requirements. For economically feasible bioethanol production, several hindrances are to be overcome.

REFERENCES

Azbarova AV, Galkina KV, Sorokin MI, Severin FF, Knorre DA. 2017. The contribution of Saccharomyces cerevisiae replicative age to the variations in the levels of Trx2p, Pdr5p, Can1p and Idh isoforms. Sci Rep 7:13220.

Baek SH, Kwon EY, Bae SJ, Cho BR, Kim SY, Hahn JS. 2017. Improvement of d-Lactic Acid Production in Saccharomyces cerevisiae Under Acidic Conditions by Evolutionary and Rational Metabolic Engineering. Biotechnol J 12.

Barraza CE, Solari CA, Marcovich I, Kershaw C, Galello F, Rossi S, Ashe MP, Portela P. 2017. The role of PKA in the translational response to heat

stress in Saccharomyces cerevisiae. PLoS One 12:e0185416.

Cadete RM, Rosa CA. 2017. The yeasts of the genus Spathaspora: potential candidates for second-generation biofuel production. Yeast.

Celaj A, Schlecht U, Smith JD, Xu W, Suresh S, Miranda M, Aparicio AM, Proctor M, Davis RW, Roth FP, St Onge RP. 2017. Quantitative analysis of protein interaction network dynamics in yeast. Mol Syst Biol 13:934.

Chua JY, Lu Y, Liu SQ. 2017. Biotransformation of soy whey into soy alcoholic beverage by four commercial strains of Saccharomyces cerevisiae. Int J Food Microbiol 262:14-22.

De La Torre M, Martin-Sampedro R, Fillat U, Eugenio ME, Blanquez A, Hernandez M, Arias ME, Ibarra D. 2017. Comparison of the efficiency of bacterial and fungal laccases in delignification and detoxification of steam-pretreated lignocellulosic biomass for bioethanol production. J Ind Microbiol Biotechnol 44:1561-1573.

Gao J, Wang B, Han X, Tian C. 2017. [Genome-wide screening of predicted sugar transporters in Neurospora crassa and the application in hexose fermentation by Saccharomyces cerevisiae]. Sheng Wu Gong Cheng Xue Bao 33:79-89.

Hammer SK, Avalos JL. 2017. Uncovering the role of branched-chain amino acid transaminases in Saccharomyces cerevisiae isobutanol biosynthesis. Metab Eng.

Hull CM, Warrilow AGS, Rolley NJ, Price CL, Donnison IS, Kelly DE, Kelly SL. 2017. Co-production of 11alpha-hydroxyprogesterone and ethanol using recombinant yeast expressing fungal steroid hydroxylases. Biotechnol Biofuels 10:226.

Izah SC, Bassey SE, Ohimain EI. 2017. Changes in the Treatment of Some Physico-Chemical Properties of Cassava Mill Effluents Using Saccharomyces cerevisiae. Toxics 5.

Jansen MLA, Bracher JM, Papapetridis I, Verhoeven MD, de BH, de Waal PP, van Maris AJA, Klaassen P, Pronk JT. 2017. Saccharomyces cerevisiae strains for second-generation ethanol production: from academic exploration to industrial implementation. FEMS Yeast Res 17.

Lopes DD, Rosa CA, Hector RE, Dien BS, Mertens JA, Ayub MAZ. 2017. Influence of genetic background of engineered xylose-fermenting industrial Saccharomyces cerevisiae strains for ethanol production from lignocellulosic hydrolysates. J Ind Microbiol Biotechnol 44:1575-1588.

Nielsen JC, Senne de Oliveira LF, Rasmussen TG, Thykaer J, Workman CT, Basso TO. 2017. Industrial antifoam agents impair ethanol fermentation and induce stress responses in yeast cells. Appl Microbiol Biotechnol.

Santos ELI, Rostro-Alanis M, Parra-Saldivar R, Alvarez AJ. 2017. A novel method for bioethanol production using immobilized yeast cells in calcium-alginate films and hybrid composite pervaporation membrane. Bioresour Technol 247:165-173.

Vincent NG, Charette JM, Baserga SJ. 2017. The SSU processome interactome in Saccharomyces cerevisiae reveals potential new protein subcomplexes. RNA.