E- ISSN No: 2454-4639

The Indian Journal of Basic and Applied Research
Volume 4 |Issue 2 | 2019

Utilization of paddy straw extract as cellulose substrate for the production of bioethanol using cellulolytic *A.niger*

Dr.H.Vajiha Banu [1] *Dr.A.Raja* [2] *and Ayisha Chithika N* [3]
Department of Microbiology, Jamal Mohamed College (Autonomous), Tirichirappalli, Tamilnadu, India
Email: vajihabanumicro@gmail.com

ABSTRACT

Conversion of lignocellulosic waste to sugars is important application of microbial cellulases. In the present study, one cellulose degrading fungi isolated from litre composted soil and were screened for cellulase and amylase production. Effect of paddy straw substrate on growth of fungal colonies was evaluated for the isolate *Aspergillus* sp. FS1. The isolate produced 0.92 exogluconase after 72 h incubation at 37°C in optimized medium composed of paddy extract and 0.54 IU for cellulose substrate (C-source), on mineral salt solution having pH 5.Bioethanol production from pretreated paddy extract with 1 % cellulose conversion was high because of fungi was able to utilize the pentoses form as has been confirmed by FTIR. Results indicate that agricultural waste can be efficiently used for bioethanol production achieved by submerged fermentation using agro waste by *A.niger*.

KEYWORDS: Bio ethanol, agro waste, *A.niger*, Cellulase

INTRODUCTION

Agricultural wastes are defined as the residues from the growing and processing of raw agricultural products such as fruits, vegetables, meat, poultry, dairy products, and crops. They are the non-product outputs of production and processing of agricultural products that may contain material that can benefit man but whose economic values are less than the cost of collection, transportation, and processing for beneficial use. Their composition will depend on the system and type of agricultural activities and they can be in the form of liquids, slurries, or solids. There is likely to be a significant increase in agricultural wastes globally if developing countries continue to intensify farming systems. It is estimated that about 998 million tonnes of agricultural waste is produced yearly (Aiduan et al., 2007). Rice straw is usually considered a residue or even a waste material although it may be used as mulching material, feed, compost substrate, cattle house flooring, as well as for straw handicraft and combustion (Kiran et al., 2000). But, conversely to other similar materials, the use of rice straw for feeding or for combustion for energy production may cause problems due to its high silica content and thus most of this material is left unused in the fields. An alternative for rice straw valorisation can be the production of oligosaccharides within the bio refinery framework along with the production of biofuels.

Rice straw has several characteristics that make it a potential feedstock for fuel ethanol production. It has high cellulose and hemicelluloses content that can be readily hydrolyzed into fermentable sugars. In terms of chemical composition, the straw predominantly contains cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%) (Garrote et al., 2002). Cellulose is the earth's most abundant natural biopolymer. It is present as the basic structural component of plants, compositionally making up 30-35% of the total biomass. In order to exploit and make use of this cellulose, the presence of cellulolytic enzymes, produced mainly by microbial strains, is necessary for converting it into simpler units. Microorganisms such as fungi and bacteria are the main contributory organisms for the production of cellulase enzyme. Fungi have been found to exhibit efficient cellulase activities, but there is an increasing interest in the production of cellulase through bacteria. This is attributed to their faster growth rates (Shaikh et al., 2013).

Cellulose can be converted to fermentable sugars (saccharification) by many bacteria and fungi. Microorganisms use the released sugars for their growth by producing extracellular enzyme cellulase, that catalyze the process of hydrolysis. Fungi such as *Aspergillus sp., Fusarium sp., Penicillium sp., Trichoderma sp., Chaetomium sp*., have been reported to be efficient cellulase producers . Cellulose is a linear polymer composed of D-glucose subunits linked by β-1, 4 glycosidic bonds forming the dimer cellobiose. These form long chains linked together by hydrogen bonds and van der Waals forces. Cellulose present in crystalline form and a small amount of non organized cellulose chains forms amorphous cellulose (Perez *et al*, 2002) . Cellulases are a group of enzymes composed of three major components: endo-glucanases, exoglucanases and β-glucosidase (Anwar *et al* 2014).They are used in the detergents, and other industries for production of biofuels. Ethanol as most important alcohol can be produced by converting the sugar content of any starchy material into alcohol with the evolution of carbon dioxide (CO_2) under controlled environmental conditions (Ye Sun and Jiayang Cheng, 2002). The process is an anaerobic fermentation in accordance with embden-meyerhoff pathway (EMP) catalysed by enzymes produced by bacteria and fungi.

The fermentation process is essentially the same process used to make alcoholic beverages. Bio ethanol is a liquid bio-fuel alternative to fossil fuel. Bio-ethanol fermentation is by far the largest scale microbial process (Devi and Shankar, 2009). Its production from renewable biomass has received considerable attention in recent years. It is presently the most abundant bio-fuel for automobile transportation. In addition to being renewable, ethanol has a major advantage in that it can be easily blended with gasoline. Ethanol produced from agricultural waste using separate hydrolysis and fermentation also had problems as the higher concentration of reducing sugars inhibited the growth of yeast (Zhang *et al*., 2010).

MATERIALS AND METHODS

Sample Collection:
Soil sample was collected aseptically from the Jamal Mohammed College, garden around composted litre zone .

Isolation of fungi from soil:
Serial dilution was done for the collected soil sample. Sabourauds Dextrose Agar (SDA) plates were prepared. For pouring the media in the sample plates, pour plate method was used. By using Micropippets 1ml of sample solution was taken from the desired test tubes step by step and dropped on the petridish. SDA medium was taken and poured on the petridish containing sample. Allow the plates to solidify for few minutes. After solidification incubated the plates in room temperature for 4-5 days.

Screening of cellulose degrading fungi procedure:
The soil samples were collected and serially diluted with sterile distilled water. Isolation of fungal strain was carried by performing serial dilution method and pour plate technique on SDA plates. The fungal isolates was grown in agar plates containing 1% CMC (Carboxy Methyl Cellulose). After fungal isolates were grown and plates was flooded with aqueous solution of 1% congo red for 15 minutes at room temperature. Then the plates were thoroughly washed with 1N NaCl for counter staining the petriplate.

E- ISSN No: 2454-4639
The Indian Journal of Basic and Applied Research
Volume 4 |Issue 2 | 2019

Primary screening of amylase :
The cellulase producing fungi selected isolates were individually subjected for primary screening to determine their amylase production capability using starch agar medium. Each fungal isolate was streaked on to starch agar plates and incubated for 24h at 37°C. After incubation, the plates were over layered with iodine solution (0.3% iodine and 1% KI). Amylase positive fungal strains were identified and recorded based on the clear zone formation around the fungal growth.

Identification of Cellulase producing Fungi
The morphological characteristics of the fungi such as surface, color, border and spores were carefully observed and recorded.

Extraction of cellulose from paddy:
Paddy straws were collected from agricultural land, Vayalur, Trichy. Paddy straws were chopped into small pieces. Then the chopped paddy straws were added into the 0.1N HCl and soaked for 30 minutes. Then the soaked paddy straws were added into conical flask containing 250ml of distilled water and boiled until extract of the paddy straw were collected. Then these extract were used for estimation of reducing sugar by DNS method

Estimation of reducing sugar by DNS method:
50ml of 2N Sodium Hydroxide (2N NAOH) were prepared. 2.5g of Dinitro salisilic acid (DNSA) were weighted and dissolved in 50ml of 2N Sodium Hydroxide (2N NAOH) with the help of magnetic stirrer. 75g of Sodium Potassium Tartarate were weighted and dissolved in 150ml of distilled water. Sodium Potassium Tartarate solution was poured in the DNSA and NaOH solution and made the volume up to 250 ml. Then stored it in a brown bottle. Eight test tubes were taken and labelled them as Blank and 1-7. Dilution of glucose standars (S1, S2, S3, S4, S5) were prepared. 1ml of DNSA reagent was added to the all the 8 tubes and mixed well. These tubes were placed in boiling water both for 15 minutes. Then recorded the absorbance with a spectrophotometer at 540nm. Then the absorbance (OD) of blank was taken and maked at zero. The OD of all the tubes were taken (NO 1-7). The cuvettes were washed each time after taking OD.

Cellulase Production:
The cellulose degrading fungi was cultured on production media composed of MgSO4 0.25 g, KH2PO4 0.5 g, $MnSo_4$0.001, $FeCl_3$ 0.001g/L and 1% of cellulose and paddy extract used as substrate. The culture were grown at optimum temperature at pH 5, and 120 rpm. After 48 h of incubation period, the cultures broth was taken and OD was recorded at 600 nm. Followed by 72 h incubation culture filtrate were centrifuged for 10 min at 8000 rpm and at 4º C. The supernatant were collected and protein was isolated by ammonium sulphate precipitation and purified by cellulose membrane dialysis.

Cellulase Enzyme Activity Assay:
The cellulase activity was determined by measuring the amount of reducing sugar. The enzyme activity was determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology (Ghose et al. 1987). 0.5 mL of supernatant was added to 0.5 mL of 2% CMC in 0.05 mL sodium acetate buffer (pH 4.8). The mixture was incubated at optimum temperature for 1 h. The reaction was terminated by adding 3 mL of 3,5- dinitrosalicylic acid (DNS) reagent to 1 mL of reaction mixture . The mixture were boiled in water bath then cooled by ice. The colour intensity was measured at OD 540 nm. The reducing sugars were estimated spectro photometrically with 3,5-dinitrosalicylic acid using glucose standard curve previously prepared. The enzymatic activity of cellulase defined in international units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µ mol reducing sugars (measured as glucose) per mL per minute.

Production of bio ethanol
The fungal inoculum was prepared in minimal media broth. A loopful of cellulose degrading fungi was inoculated and incubated at 30 ºC on incubator for about 5 days. The initial fungal count in fermentation sample was $8x10^8$ cfu/mL. (Patel *et al.*, 2007). To initiate the saccharification process, the cellulose degrading fungal grown in basal medium with 1% of husk extract. Fermentation carried out at optimum fermentation conditions with shaking at 120 rpm for 48 and 72h. The culture was centrifuged; the supernatant was resterilized by 0.2 µm filter for the growth of *fungal mat.* The total reducing sugar was estimated before and after the fermentation process by DNS method.

Ethanol Assay:
Ethanol production was estimated by potassium dichromate method. The acid dichromate solution was prepared by dissolving 33.76 gm of K2Cr2O7 in 400 ml distilled water and 325 ml concentrated sulphuric acid, cooled and then volume was made up to 1 litter with distilled water. Calibration curve was prepared using 5% ethanol in different dilution, making final volume 1 ml to which 2 ml of K_2Cr2O_7 (1N) was added and mixed. This solution was boiled for 10 minutes in a water bath. 2 ml of 2 N NaOH was added and mixed after cooling and absorbance was taken at 600 nm in spectrophotometer.

FTIR spectrum analysis
FTIR relies on the fact that the most molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. Crude paddy extract and fermented extract was subjected to FTIR to find out the degradation of cellulose.

RESULTS AND DISCUSSION
Isolation and identification of fungi:
Nearly 6 X 10^3 fungal colonies were isolated and majority of colonies were Black and followed by green and white cottony .Three different isolates were tested for cellulase activity and isolate FS1was found to be positive (plate 1). Micrscopic observation indicates the presence short, smooth walled and have conical shaped terminal vesicle which support a single row of phialides on the upper two third of the vesicle indicates isolate belongs to of *Aspergillus* sp, Conidial heads are green echinulate columnal and uniseriate. Conidia are produced in basipetal succession forming long chains, globuse, black and rough walled filamentous, branching hyphae that generally lack cross-walls and sporangiospores are produced inside a spherical structure (Ghosh et al., 2012).

Cellulose and amylase producer:
The three isolates were subjected to qualitative analysis of cellulase and amylase and results were given in table 1. In this study out of three one fungal isolate FS1isolates was found to be positive on cellulase and amylase producing fungi able to grow on CMC media indicating their cellulolytic capability but only on isolate identified as *A.niger also* found to be amylase producer.

Effect of substrate on enzyme production: In this study, cellulolytic ability of *A.niger* cellulose degrading fungi was checked in a liquid medium. Among the two different tested substrate the paddy extract

The Indian Journal of Basic and Applied Research
Volume 4 |Issue 2 | 2019
E- ISSN No: 2454-4639

(plate 1 b-c). The reducing sugar estimation showed 0.14g of for cellulose and 0.18g paddy extract.Growth of fungal was higher in paddy extract than cellulose (plate 2) and fungal isolate gave highest cellulase yield of and 0.92 IU/ml after 72 hours of incubation on paddy extract and 0.054 IU/ml under cellulose as raw material. The use of various C-sources on the basis of which the substrates were selected determine the yield of bioethanol. Immanuel et al. (2007) have reported use of coir waste and saw dust as substrates for cellulase production by submerged fermentation.

Table 1: Qualitative Enzyme Screening Plate Assay

Strain code	Colony morphology	Cellulase	Amylase	Genera
FS1	Light Green to black	Positive	Positive	*A.niger*
FS2	Dark Green	Positive	Negative	*Penicillium sp*
FS3	White cottony	Negative	Negative	*Rhizopus sp*

Ethanol production:

The total reducing sugars of 1% paddy extract was initially 0.18g/L and it was found to be increased to 0.43g/L and 0.53 g/L respectively on 48 and 72h fermentation. An ethanol yield of 12.6g/l was obtained followed by 72 h incubation. For the ethanol production, simultaneous saccharification and co-fermentation (SSCF) technique have been used with cellulase and amylase. Rice straw consists of three main components, cellulose, hemicellulose and lignin. Cellulose and hemicellulose are first converted to fermentable sugars, which then are fermented to produce ethanol. The fermentable sugars include glucose, xylose, arabinose, galactose, and mannose. Hydrolysis of cellulose and hemicellulose to generate these sugars can be carried out by using either acids or enzymes (Yonah, K et al., 2008).

Paddy straw pulp recovered from lignocellulosic pulping was analyzed using FTIR to determine the changes in chemical structure during degradation. Figure 1 and 2 reveals FTIR spectrum of crude extract sample and fermented broth. Small absorption features due to the impurities are evident throughout the spectrum. Due to the large number of species present, the bands are overlapped.

The infrared spectra were used to determine changes in the structure of cellulose, hemicellulose, and lignin of paddy straw after fermentation. The FT-IR spectra of paddy straw wit band at 899.95 cm-1 is the characteristic of the glycosidic bond β-(1→4) cellulose. The range between 1200 cm-1 and 1100 cm-1 is region of hemicellulose and cellulose, which attained a maximum value around 1035 cm-1 due to C-O stretching. The region between 3800 cm-1 and 3000 cm-1 indicates the crystalline structure of cellulose. This range covers the sum of the vibration of valence bands of the hydrogen bond of the O-H group and the bands of infra-molecular and intermolecular hydrogen bonds (Chandel et al., 2014). figure 2 shows the region of 1247 cm-1 indicating the removal of hemicellulose. It can be seen that the region 1734 cm-1 is affected after DES pretreatment which denotes the decrease in hemicellulose content. However, the region 1630 cm. In addition, region between 2918 cm-1 and 2851 cm-1 is affected during fermentation.

Table 2: Estimation of reducing sugar by DNS method

Samples	**Concentration**	**OD**
Standard 1	**0.2**	**0.24**
Standard 2	0.4	0.48
Standard 3	0.6	0.6
Standard 4	0.8	0.9
Standard 5	1	1.2
CONCENTRATION OF SUGAR IN SAMPLE		
Cellulose	**0.14**	**0.008**
Crude extract	**0.18**	**0.16**
48 h	**0.42**	**0.48**
72 h	**0.54**	**0.60**

Plate 1: Screening of cellulolytic fungi and paddy straw extract

Plate 2: Growth on cellulose and husk extract

E- ISSN No: 2454-4639

The Indian Journal of Basic and Applied Research

Volume 4 |Issue 2 | 2019

Figure 1. FTIR Spectrum of crude paddy extracts broth.

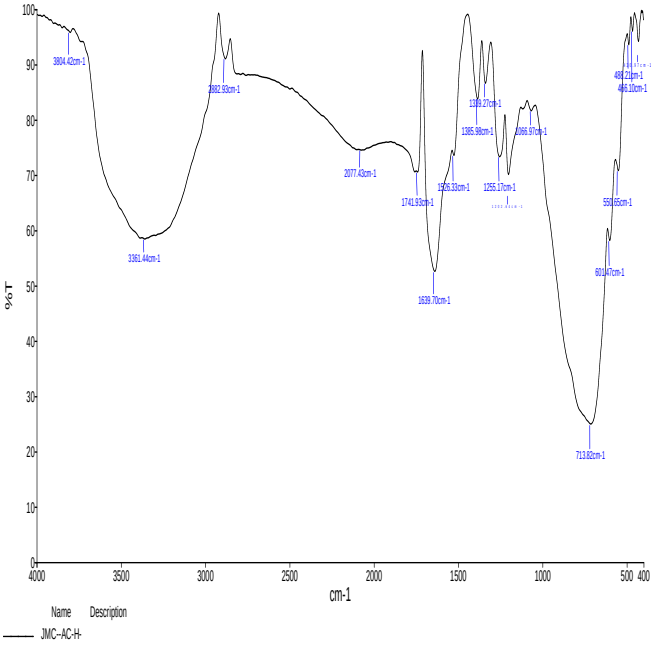
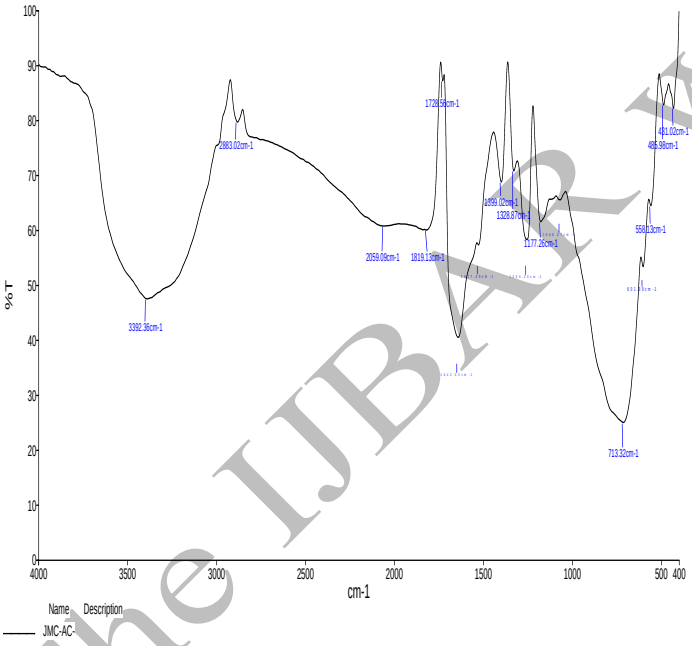


Figure 2. FTIR Spectrum of fermented paddy extract



REFERENCES

Aiduan, Li., Blanca Antizar-Ladislao and Majeda Khraisheh., (2007). Bioconversion of municipal solid waste to glucose for bio-ethanol production. Bioprocess and Biosystems Engineering. 30(3):189-196.

Anubhuti Gupta., Jay Prakash Verma., (2015). Sustainable bio-ethanol production from agro-residues: A review. Renewable and Sustainable Energy Reviews. 41: 550-567.

Anwar Z, Gulfraz M, and Irshad M., (2014). Agro industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. Journal of radiation research and applied sciences. 7(2):163-73.

Chundakkadu K., (1999). Production of bacterial cellulases by solid state bioprocessing of banana wastes. Bioresource Technology. 69(3):231-239.

Devi, R.N. and S. Shankar., (2008). Bioconversion of cellulose into fermentable sugars by *Saccharomyces cerevisiae* cells for the production of ethanol using cellulolytic fungi isolated from soil. The Internet Journal of Microbiol. 7(2).

Doi, R.H., (2008). Cellulase of mesophilic microbes: cellulosome and non – cellulosome producers. Ann. NY. Acad. Sci. 1125: 267–279.\

Garrote, G., Dominguez, H., Parajo, J.C., (2002). Auto hydrolysis of corncob: study of non-isothermal operation for xylooligosaccharide production. Journal of Food Engineering, 52(3): 211–218.

Ghosh U, Subhashini P, Dilipan E, Raja S, Thangaradjou T, Kannan L., (2012). Isolation and characterization of phosphate-solubilizing bacteria from seagrassrhizosphere soil. J Ocean Univ China. 11(1):86–92.

Immanuel G, Bhagavath C, Iyappa Raj P, Esakkiraj P, Palavesam A., (2007). Production and partial purification of cellulase by *Aspergillus niger* and *Aspergillus fumigatus* fermented in coir waste and saw dust. Internet journal of Microbiology. 3(1):1-11.

Kiran L Kadam, Alan Jacobson. W, Loyd H Forrest., (2000). Rice straw as a lignocellulosic resource: Collection, processing, transportation, and environmental aspects. Biomass and Bioenergy.18(5):369-389.

Perez J, Munoz-Dorado J, de la Rubia TD, Martinez J., (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. International Microbiology. 5(2):53-63.

Shaikh, N. M., Patel, A. A., Mehta, S.A and Patel N., (2013). Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulase production. Univ. J. E. Res. Technol. 3(1): 39-49.

Wenshuai Chen, Haipeng Yu, Yixing Liu, Peng Chen, Mingxin Zhang, Yunfei Hai., (2011). Individualization of cellulose nanofibers from wood using high-intensity ultrasonication combined with chemical pretreatments, Carbohydrate polymers. 83(4): 1804-1811.

Wong Y.C and Sanggari V., (2014). Bioethanol Production from Sugarcane Bagasse using Fermentation Process. Oriental journal of chemistry. 30(2): 507-513.

Yang B, Wyman CE., (2008). Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioproducts and Biorefining. 2(1):26-40.

Ye Sun and Jiayang Cheng., (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. 83(1):1-11.

Yonah K. Turinayo, Fred Kalanzi, Jude M. Mudoma, Peter Kiwuso, Godwin M. Asiimwe, John F. O. Esegu, Paul Balitta, Christine Mwanja., (2015). Physiochemical Characterization of Jatropha curcas Linn Oil for Biodiesel Production in Nebbi and Mokono Districts in Uganda. Journal of Sustainable Bioenergy Systems. 5(3).

Yi Zheng, Zhongli Pan and Ruihong Zhang., (2009). Overview of biomass pretreatment for cellulosic ethanol production. Int J Agric & Biol Eng. 2(3): 51-68.

Zhang, M.J., Wang, F., Su, R.X., Qi, W. and He, Z.M., (2010). Ethanol production from high dry matter corncob using fed-batch simultaneous saccharification and fermentation after combined pretreatment.Bioresour Technol. 101(13): 4959–4964.