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ORIGINAL ARTICLE

Bioethanol production from Municipal Sewage sludge by Yeast strain NRRI *Schefferomyces stiptis* Y-7124

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ABSTRACT
The present study deals with the bioethanol production from sewage sludge in conventional study on factors affecting the enzyme production using Trichoderma. reesei showed the maximum enzyme activity at pH 5.4, 35℃ for 7 days. In the present study, Schefferomyces stiptis showed high ethanol production 0.96%. The enzyme activity cellulase 13.75 IU/mL and xylanase 30.50 IU/mL was observed during the enzymatic hydrolysis of the sewage sludge. The FTIR and XRD analysis showed very clear structural and functional group changes that occurred during the T. reesei mediated enzymatic hydrolysis. Upon comparing the chromatogram of distilled ethanol and standard ethanol, it showed same retention time and also showed the presence of other compounds in the ethyl acetate group. In the present study, yeast S.stiptis yielded maximum ethanol This study was useful to identify the xylose fermenting yeasts for the conversion of hemicelluloses along with celluloses for better ethanol production.
Key words: Lignocellulosic, Bioethanol, enzymes and yeast

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INTRODUCTION

Bioethanol (C_2H_5OH) is mainly from feedstock or non-feedstock sources [1]. Thus, a review of bioethanol production from Municipal sewage waste biomass is currently needed to be researched extensively in order to decipher environmental and energy issues.

According to Gourmont *et al*., ,2013, Brazil consumed 12,500 million liters of bioethanol fuel and exported 2,500 million liters at an average price of 0.21 US$ (21 cents)/liter. The US produced 12,900 million liters and the EU Member States produced 500 liters. Asian countries such as China, Korea, Japan, India and Bioethanol (C2H5OH) or ethyl alcohol is an alcohol conformation that recently has emerged as a renewable bio-energy, biodegradable clear-colorless liquid, eco-friendly potential fuel to power automotive engines, as well as a potential petrol substitute for road transport vehicles [2]. Bioethanol is synthesized from alcoholic fermentation of sucrose or simple sugars of diverse types of biomass, either others started to import bioethanol from Brazil in early 2005 and successful applications of bioethanol encouraged them to produce bioethanol, due to direct association with cost effect on raw materials, environment-friendly characteristics and fuel- blending purposes. In 2010, an Italian company Mossi & Ghisolfi constructed a high scaled- up bioethanol plant with 200,000 ton/year production capacity. Meanwhile, Japan, Korea, India, and Germany also generated both pilot and large scale bioethanol plants [1,3]

In developed and developing countries municipal wastes have become a severe problem during the last century [4]. The shrinking of landfill capacity resulted in rising of landfill costs which is mainly from the municipal waste. Because of the above concern the municipal sewage sludge is used a source for the production of bioethanol. Due to the shrinking landfill capacity, the tighter environmental control exists on their sitting operation, construction, and of the unwillingness of communities to have new landfill sites nearby. The tighter environmental regulations are responsible for the premature closure of existing landfills and higher costs for constructing new ones [5]. Among the various components the municipal solid

waste consists of food waste, wood, leaf, garden or yard trimmings, rubber, textile, leather, metals (ferrous metals or Nonferrous metals), glass and major of paper and paper boards. About 35% to 40% by weight of the municipal solid waste is made of thepaper.

Generally, the yeast attack on hexose sugars, but carbohydrates containing pentose subunits could also be digested by specific yeast into ethanol [6]. Along with the production of industrial ethanol research studies are also focused upon the upgrading the bioconversion process of ethanol production. Ethanol as a fuel has promising prospects. For biofuel purpose 85-95% alcohol is needed; the distilled out product contains approximately 95% ethanol. However further treatment may lead to 99% pure ethanol.

For the optimal production of ethanol from municipal sewage waste are obtained from enzymatic saccharification, maximum amount of sugar release and conversion of all fermentable sugars into ethanol is desired [7]. However, the lack of efficient microorganisms to ferment a variety of sugars released by the hydrolysis of municipal sewage sludge materials is one of the major factors limiting the complete utilization of the municipal sewage sludge for bioethanol production [8]. Morever, the most commonly used glucose fermenting yeast strain *Saccharomyces cerevisiae* has a limiting in fermenting sugar monomers [9]. Previous studies have been exhibited promising results on co-fermentation and with the co-culture process with *Pichia stiptis* and *S. cerevisiae* [10,11] *Zymomonas mobilis* and *P. stiptis* [11,12] *Candida shehatae* and *S. cerevasiae* [13]. In this study the municipal sewage sludge is converted into bioethanol by the enzymatic hydrolysis by *Trichoderma ressei* and followed by the fermentation of yeast *schefferomyces stiptis.*

Since large quantities of sewage sludge and agro wastes are available in the environment, vegetable growing fields and wastages during transportation, their disposal is a problem. Therefore, an attempt is made to process sewage waste into ethanol.

MATERIAL AND METHODS

Collection of sewage and substrate preparation:

The municipal sewage sludge was collected from the sewage discharge of Hindusthan college of arts and science in Coimbatore city, Tamil Nadu, India

Physico-chemical properties sewagesludge

Determination of total solids:

The total solids of the sewage sludge were determined by taking the 5g of the solids in the filtered disk and allowed to dried in the oven for 1 hour and they allowed to the desiccator to get cooled in 30 minutes. The sludge was allowed to wash in distilled water and they are again dried at 105°C for 1 hour

$Tss = a\text{-}b \div c \times 10^6$

A=weight of the disk+solid (g) b=weight of the empty disk c=volume of the sample used(ml)

Determination of moisture

The moisture content of the sewage sludge was determined by the method described in AOAC (1990) [14]. Five gram of fresh WH sample was taken in an aluminum dish and placed in a hot air oven at 67ºC and dried for 24 hours. The following formula was used to calculate the percentage of moisture.

$$\text{Moisture (\%)} = \frac{[\text{Weight of original sample} - \text{Weight of oven dried sample (g)}]}{[\text{Weight of original sample (g)}] \times 100}$$

Estimation of cellulose

Three mL acetic/nitric reagent was added to a known amount (0.5 g or 1 g) of the sludge sample in a test tube, mixed in a vortex mixer and placed in the boiling water bath at 100°C for 30 minutes. Cooled and then centrifuged the contents for 15–20 minutes. Discarded the supernatant and washed the residue with distilled water. Ten mL of 67% sulphuric acid was added and allowed it to stand for 1 hour. One mL of the above solution was diluted to 100 mL. To 1 mL of this diluted solution, 10 mL of anthrone reagent was added and mixed well. Then the tubes were heated in the boiling water-bath for 10 minutes and measured the colour at 630 nm. Set a blank with anthrone reagent and distilled water. Take 100 mg cellulose in a test tube and proceed from sulphuric acid addition for standard. Instead of just taking 1 mL of the diluted solution took a series of volumes (say 0.4–2 mL corresponding to 40–200 µg of cellulose). The standard graph was plotted and the amount of cellulose was calculated in the sample [15].

Estimation of hemicellulose

Added 1 g of the powdered sludge sample in a refluxing flask 10 mL of cold neutral detergent solution was added. 2 mL of decahydronaphthalene and 0.5 g sodium sulphite was added to the solution and heated, refluxed for 60 minutes. The contents were filtered through sintered glass crucible (G-2) by suction and washed with hot water and acetone. The residue was transferred to a crucible, dried at 100°C for 8 hours. The crucible was cooled in desiccators and weighed [16].

Hemicellulose = Neutral Detergent Fibre (NDF) – Acid Detergent Fibre (ADF)

Determination of the BOD and COD of the sample:

Biochemical oxygen demand is the amount of dissolved oxygen needed by the aerobically biological organism to break down organic material present in the given water sample at certain temperature over a specific time period.

Chemical oxygen demand is the total measurement of all chemicals in the water that can be oxidized. Total organic carbon is the measurement of organic carbons.

Enzyme activity

Cellulase activity

Carboxy Methyl Cellulose (CMC) in PCB buffer was used in CMCase activity. About 500µL of 2% CMC and crude enzyme (culture supernatant) was added together and boiled to 50°C for 30 minutes in water bath. This reaction was terminated by adding 3 mL of DNSA reagent and boiled in water bath for 5 minutes. Cooled to the room temperature 20 mL of distilled water was added. A control was kept with the reagent without adding enzyme and absorbance was measured at 540 nm (Hitachi U2910 Spectrophotometer, Japan). One IU of activity was expressed as the amount of enzyme required to release 1µmol of product/min under assay conditions [17].

Xylanase activity

Birch wood xylan in acetate buffer was used in xylanase enzyme activity. About 300 µL of 1% xylan and crude enzyme (culture supernatant) was added together, 400 µL of water was added to make up the volume up to 1000 µL and boiled to 50°C for 30 minutes in water bath. This reaction was terminated by adding 1 mL DNSA reagent and kept in boiling water bath for 10 minutes for colour development. A control was kept with the reagent without adding enzyme and absorbance was measured at 540 nm (Hitachi U-2910 Spectrophotometer, Japan). One IU of activity was expressed as the amount of enzyme required to release 1µmol of product/min under assay conditions [17].

Estimation of reducing sugars

Dinitrosalicylic acid (DNSA) method was used to estimate the total reducing sugars [18]. Three mL of DNSA reagent was added to 3 mL of hydrolyzed sample in a test tube. The mixture was heated at 90°C for 15 minutes to develop the red brown colour. To this 1 mL of 40% Potassium tartrate (Rochelle salt) solution were added to stabilize the colour. It was then cooled to room temperature and the absorbance was measured at 520 nm (Hitachi U-2910 Spectrophotometer, Japan).

Determination of microbial ability for glucose and xylose fermentation

Microorganisms ability for glucose and xylose fermentation was determined using minimal basal media where the carbon source was 5% glucose and xylose with $(NH_4)_2SO_4$ 0.26%, KH_2PO_4 0.272%, $MgSO_4$ 0.05% and $CaCl_2$ 0.05% and the pH adjusted to 7.2 in Durham fermentation tube. The pre and post xylose content were analyzed using phloroglucinol assay, 10 mL media was equally distributed in test tubes with Durham tubes and sterilized at 121°C for 15 minutes. Microorganisms were inoculated and incubated at 28±2°C for 3 days. After 3 days CO_2 accumulations in Durham tube was observed and ethanol production was analyzed using dichromate assay.

Estimation of ethanol [19,20]

Potassium dichromate assay method was followed to estimate the ethanol concentration in fermentation media. Add 34 g of $K_2Cr_2O_7$ in 500 mL distilled water and 325 mL concentrated H_2SO_4 slowly added to the flask in an ice bucket. Added 7.5 mL of distillate with 12.5 mL of $K_2Cr_2O_7$ solution and made up the volume to 25 mL with distilled water then kept at 60°C for 30 minutes and absorbance was measured at 600 nm (Hitachi U-2910 Spectrophotometer, Japan).

Estimation of xylose [21,22**]**

At first 0.5 g of Phloroglucinol was mixed with 100 mL of glacial acetic acid and 10 mL of concentrated HCL to form a coloured reagent. 200 µL of sample was mixed with 5 mL of coloured reagent and heated at 100°C for 4 minutes and cooled down to room temperature and absorbance was measured at 540 nm (Hitachi U-2910 Spectrophotometer, Japan).

Ethanol quantification

At first the ethanol concentrations were determined by dichromate assay and the final distillate after incubation time was analyzed using gas chromatography (GC- 2010, Shimadzu, Japan) equipped with a flame ionization detector and a column of Porapak 'Q' using N_2 as carrier gas at a flow rate of 30 mL min-1 at an oven temperature of 130° C. The injector and detector temperature were kept at 200 and 230°C respectively.

Fourier transmission infrared analysis

Structural and functional group changes in water hyacinth constituents during pretreatment were analyzed by FTIR (Shimadzu Spectrometer, Japan). Raw and pretreated biomass (3-4% *w/w*) were thoroughly mixed with dry powdered spectroscopic grade KBr and the mixture was pressed with 10,000 psi into a transparent pellet. The spectra were obtained at 4 cm^{-1} resolution accumulating 25 scans per spectrum over the wave number range 4000 – 400 cm^{-1}.

X-ray diffraction

Crystallographic structure was analyzed by X-ray diffraction peak height method of raw and pretreated samples using XRD – 6000 (Shimadzu diffractometer, Japan). Diffraction patterns were recorded by using Cu-Kα radiation at 40 kv and 30 mA and grade range between 10 to 30˚ with a step size of 0.03˚. Cellulose crystallinity was calculated using the pragmatic equation (Segal et al. 1959).

$$Crl = [(I_{020} - I_{am})/I_{020}]x100$$

Where I_{020} is the intensity for the crystalline portion of cellulose whereas I_{am} is the amorphous portion and I_{020} is intensity diffraction at 20˚.

RESULTS AND DISCUSSION

Chemical composition of substrate:

The chemical composition of the sewage sludge was determined in the table 6.1. The sewage sludge contains the total solids, moisture content of 4.3%, cellulose of 40.17%, 20% of hemicellulose was obtained in this study (Table 1)

Table 1: Chemical composition of the substrate

Components	% of dry weight
Total solids	9,57,00
Moisture	4.3
Cellulose	30.45
Hemicellulose	25.24

Enzyme activity

Cellulase activity

The cellulase activity for the substrate was determined in the alternative days and the OD values of the enzyme was given in the table 2 and shown in Fig.1.

Table 2: Cellulase Enzyme activity by *T. ressei*

S.no	Days of incubation	Cellulase Activity IU/ml
1.	1st day	1.3111
2.	3rd day	1.1095
3.	5th day	1.2579
4.	7th day	1.9819

Xylanase activity

The xylanase activity for the substrate was determined in the alternative days and the OD values of the enzyme was given in the table 3 and shown in Fig.1.

Table 3: Xylanase Enzyme activity by *T. ressei*

S.no	Days of incubation	Xylanase activity IU/ml
1.	1st day	1.2675
2.	3rd day	2.8917
3.	5th day	3.9599
4.	7th day	4.3869

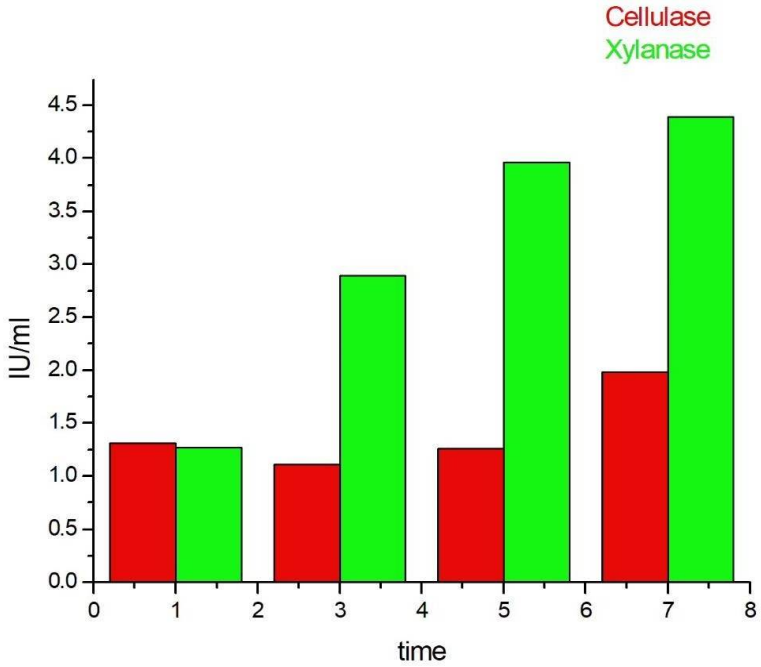

Figure 1: Enzyme activity of Cellulase and Xylanase by *T. ressei*

Estimation of the Reducing sugars

The total sugars and the reducing sugars of the substrate was determined before the enzymatic hydrolysis and compared with them for the value of the reducing sugars for the yield of the ethanol production. The value of the sugars was given in the table 4 and shown in Fig.2.

Table 4: Reducing Sugar content

s.no	Sample	Mg/ml
1.	Sewage sludge(before hydrolysis)	4.000
2.	Sewage sludge(reducing sugars)	2.654
3.	Total sugars(fermentation by yeast)	1.457
4.	Reducing sugars	0.365

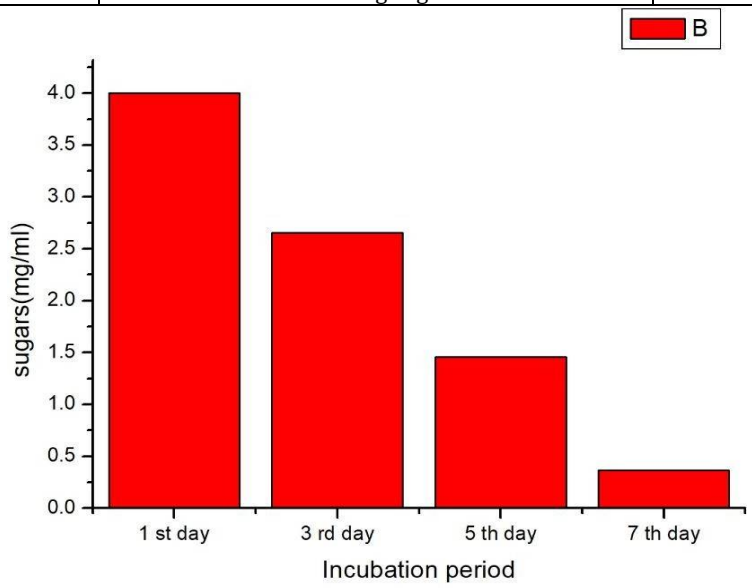

Figure 2 : Reducing sugar content

Estimation of xylose

The xylose was estimated by the addition of the bial's reagent which shows the presence of the blue or green coloured formation in the substrate which shows the presence of the xylose in the sample. It was given in the table 5 and shown in Fig.3

Table 5 : Xylose estimation

S.no	Sample	Xylose g/L
1.	1st day	**3.801**
2	3rd day	**3.032**
3.	5th day	**2.847**
4.	7th day	**0.527**

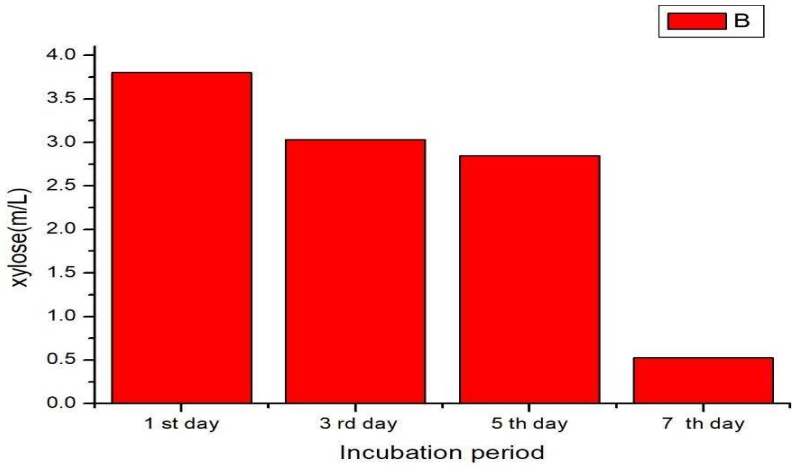

Figure 3: Xylose estimation

Estimation of ethanol

After the distillation process the ethanol produced was allowed for the chromic acid method. The f0.0ormation of the dark green colour indicates the presence of the ethanol was given in the table 6.

Table 6: Estimation of ethanol by chromic acid method

S.no	Sample	g/L	
1.	**Sewage sludge**	**0.0978**	**1.984**

FTIR analysis

Chemical changes in the lignin skeleton of sewage sludge biomass constituents during *T. reesei* mediated bio pretreatment were analyzed by FTIR shown in figure 4.

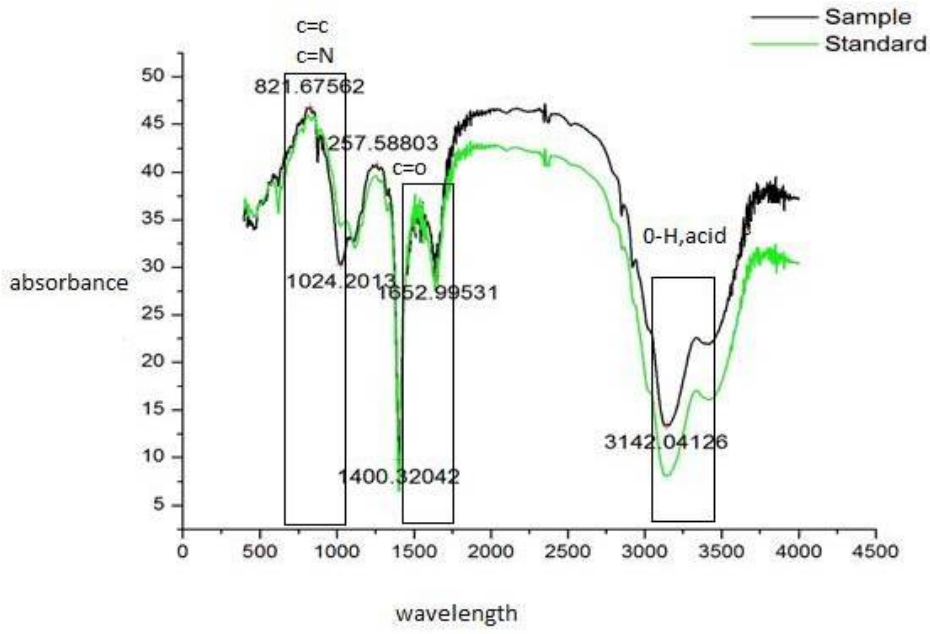

Figure 4 : Chemical changes in T. reesei pretreated sewage sludge by FTIR analysis

XRD analysis

The XRD data illustrates further significant differences in the patterns in *T. reesei* bio pretreated sewage sludge biomass as shown in Figure 5. Initially cellulose I characteristic diffraction pattern at two theta equal to 14.9°, 17.1° and 22.8° were observed in raw sewage sludge. The crystallinity index of raw sludge was 43.17%, which decreased to 41.03% after *T.reesei* bio pretreatment.

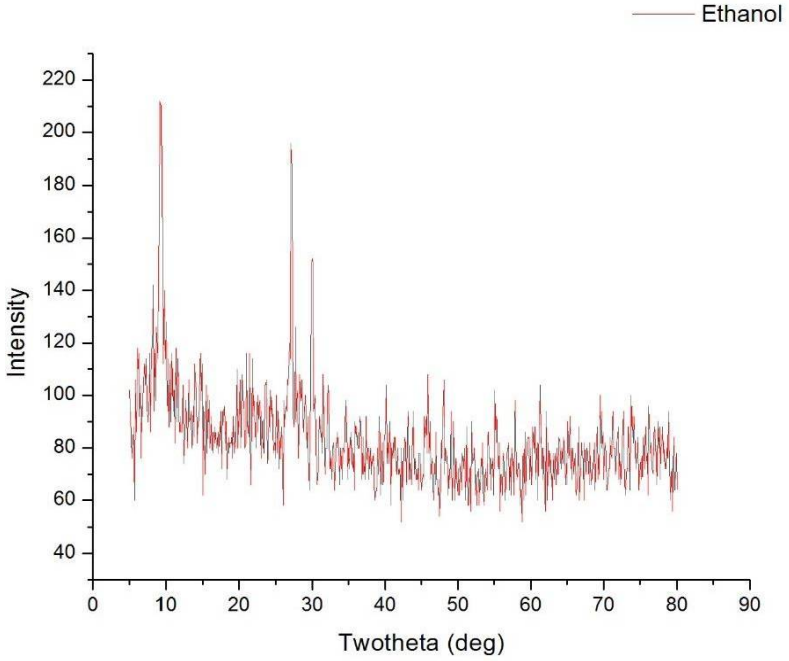

Figure 5 : X ray diffraction analysis of Native and *T. reesei* pretreated sewage sludge for cellulose crystallinity

Gas chromatography

The gas chromatogram confirms the ethanol production with a retention time of2.65 minutes by *S.stiptis* strain from the sewage sludge biomass and 99.8% standard ethanol are shown in Figure 6.

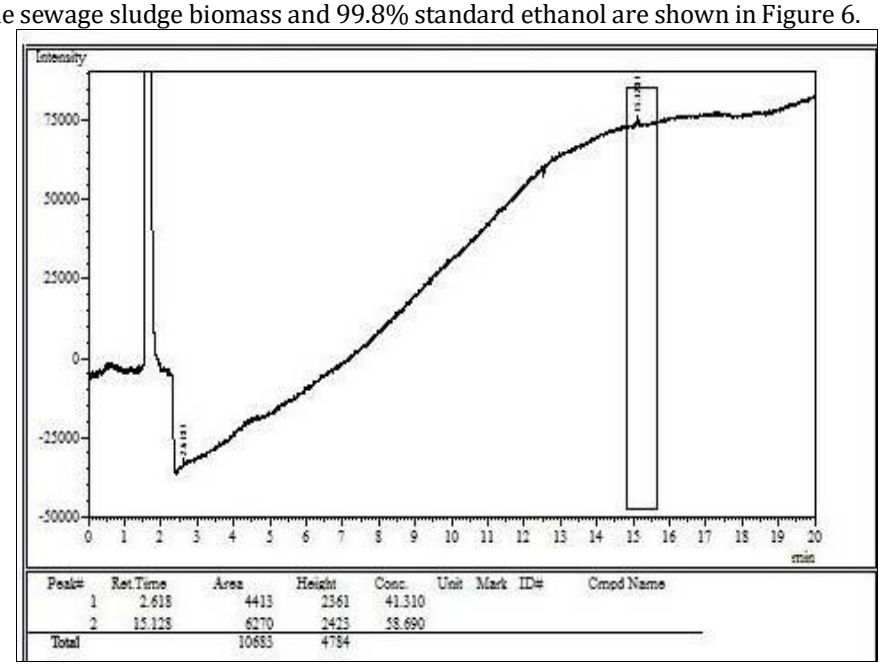

Figure 6: Gas chromatogram analysis of Standard and yeast produced ethanol

SUMMARY AND CONCLUSION

It is observed that sewage sludge contained high cellulose 30.45%, hemicellulose 25.24% and low lignin content of 12.18% which makes the sludge suitable for bioethanol production. On the other hand, the

presence of trace minerals like zinc, copper and manganese have been reported to enrich the growth of fermenting yeasts which results in the enhanced ethanol yield. In this study, *T. reesei* showed high enzyme activity when compared with *S.stiptis* in the medium supplemented with cellulose and xylose.

In conventional study on factors affecting the enzyme production using *T. reesei* showed the maximum enzyme activity at pH 5.4, 35°C for 7 days. In the present study, *S.stiptis* showed high ethanol production 0.96% among the other yeasts. It also showed thermal tolerance up to 50°C, 1.0 M osmotic tolerance and 10% exogenous ethanol tolerance.

The enzyme activity cellulase 13.75 IU/mL and xylanase 30.50 IU/mL was observed during the enzymatic hydrolysis of the WH. The FTIR and XRD analysis showed very clear structural and functional group changes that occurred during the *T. reesei* mediated enzymatic hydrolysis. Upon comparing the chromatogram of distilled ethanol and standard ethanol, it showed same retention time and also showed the presence of other compounds in the ethyl acetate group. In the present study, yeast *S.stiptis* yielded maximum ethanol. This study was useful to identify the xylose fermenting yeasts for the conversion of hemicelluloses along with celluloses for better ethanol production. Consolidated bioprocessing (CBP) represents the combination of cellulase and xylanase production, lignocellulosic hydrolysis and reducing sugar fermentation into a single step is a promising technology for cost effective lignocellulosic bioethanol production.

Most effort should be devoted to engineering of yeast for CBP because yeast has many properties, including high tolerance, yield and ethanol productivity which is sturdiness in industrial fermentation by naturally or by genetic engineering tools.

The results from the investigation showed that sewage sludge can be used as an alternative substrate for ethanol production, in comparison to energy-rich food crops, if pretreated suitably prior to fermentation by some low-cost energy sources such as solar energy. Further research is however warranted to examine the economic viability of the process. This study may lead to focus on improving methodologies for lignocellulosic bioconversion and can help in turn what is considered an environmental waste into a resource.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

REFERENCES

1. Govumoni, S.P., Koti, S., Kothagouni, S.Y., Venkateshwar, S., Linga, V.R. (2013). Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production. Carbohyd Polym, 91, 646–650.
2. Hossain, N., Jalil, R., (2015). Sugar and Bioethanol Production from Oil Palm Trunk (OPT). Asia Pacific J En and Envi, 2(2), 89-99.
3. Frances chin, M. Sudro, T. Ingram, I. Smirnova, G. Brunner, A. Bertucco. (2011). Conversion of rye straw into fuel and xylitol; a technical and economic assessment based on experimental data Chem Eng Res. Des, 89, 631-640.
4. Olsson, L.: (2007). Editor Biofuels. Advances in Biochemical Engineering/Biotechnology, vol. 108, p. 41. Springer, Berlin
5. Alya Limayem, Steven C.Ricke. (2012). Progress in Energy and Combustion Science. Progressing Energy and combustion science, 38, 449-467.
6. Ohgren, K., Rudolf, A., Galbe, M., Zacchi, G., (2006). Fuel ethanol production from steam- pretreated corn stover using SSF at higher dry matter content. Biomass Bioenergy. 30, 863-869.
7. Talebnia, F., D. Karakashev and I. Angelidaki, (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresour Technol, 101: 4744-4753.
8. Zaldivar, J., J. Nielsen, and L. Olsson. (2001). Mini-review fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. App Mcrobiol Biotechnol, 56,17-34.
9. Lin,Y. and Tanka,S. (2006). Ethanol fermentation from biomass resources: Current state and prospects. Appl. Microbiol. Biotechnol, 69, 627-642.
10. Grootjen DRJ, Meijlink LHHM, Lans RGJM van der, Luyben KOAM (1990). Cofermentation of glucose and xylose with immobilized *Pichia stipitis* and *Saccharomyces cerevisiae*. Enzyme Microb Technol, 12,860–864.
11. Taniguchi M,Yuasa S, Fujisawa H, Naruse I, Saga S, Mishina M, Yagi T. (1997) Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron. 19,519–530.

12. Laplace JM, Delgenes JP, Moletta R, Navarro JM (1993). Cofermentation of glucose and xylose to ethanol by a respiratory deficient mutant of *Saccharomyces cerevisiae* cocultivated with a xylose fermenting yeast. J Ferment Bioeng, 75(3),207–21.
13. Lebeau,T, Jouenne,T and Junter,G.A. (2009). Long-term incomplete xylose fermentation, after glucose exhaustion, with *Candida shehatae* co-immobilized with *Saccharomyces cerevisiae*. Microbiological Research,162(3),211-218.
14. A.O.A.C. (1990). Official Methods of Analysis. 15th Edition, Association of Official Analytical Chemist, Washington DC.
15. Norman, A G.,and Jenkins, S. H. (1933). A new method for the determination of cellulose, based upon observations on the removal of lignin and other encrusting materials. Biochem. Jour. 27,818-831.
16. O'dwyer, M.H. (1923). The hemicelluloses. III. The hemicellulose of American white oak. Biochem J, 17(4–5), 501–509
17. Ghose, T.K. (1987). Measurement of cellulase activities. Pure and Applied Chemistry, 59, 257268. doi:10.1351/pac1987590202Gnansounou, E., Dauriat, A., 2005. Ethanol Fuel from Biomass: A Review. Journal of Scientific and Industrial Research, 64, 809-821
18. Miller, G.L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Journal of Analytical Chemistry, 31, 426-428.
19. Bennett,C.(1971).Spectrophotometricacid dichromate method for the determination of ethyl alcohol. Am J Med Technol.37:217-20.
20. Pilone,GJ.(1985).Determination of ethanol in wine by titrimetric and Spectrophotometric dichromate methods: collaborative study. J Assoc Off Anal Chem, 68,188-190.
21. Eberts, T. J., R.H.B. Sample, M. R. Glick, and G. H. Ellis, (1979). A Simplified, colorimetric micromethod for xylose in serumor urine, with phloroglucinol. Clin, Chem, 25,1440–1443
22. Johnson, S.L., Bliss, M., Mayersohn, M., Conrad, K.A. (1984). Phloroglucinol-based colorimetry of xylose in plasma and urine compared with a specific gas chromatographic procedure. Clin Chem 30, 1571–1574.