ISSN 2320-1355

JOURNAL OF GLOBAL BIOSCIENCES

Volume 3, Number 7, 2014

Website: www.mutagens.co.in
E-mail: submit@mutagens.co.in
researchsubmission@hotmail.com

Journal of Global Biosciences
ISSN 2320-1355
Volume 3, Number 7, 2014, pp. 1076-1083
Website: www.mutagens.co.in
E-mail: submit@mutagens.co.in
researchsubmission@hotmail.com

Research Paper

EFFECT OF *CANDIDA SHEHATAE* IN THE CONVERSION OF LIGNOCELLULOSIC ETHANOL

Shubhaneel Neogi[1], Apurba Dey[2], Swati Ghosh[3], Amit Ganguly[1] and Pradip K. Chatterjee[1]

[1]CSIR Central Mechanical Engineering Research Institute,
Durgapur – 713209, India
[2]National Institute of Technology,
Durgapur - 713209, West Bengal, India
[3]Institute of Engineering and Industrial Technology,
Durgapur – 713212, India

Abstract

Parthenium hysterophorus L., a highly toxic invasive weed, having 21.01% hemicellulose, 13.9% lignin & 27.8% cellulose is found to be an excellent resource for biomass-fuel. Conversion of lignocellulose to monosugar was catalyzed under 5% concentrated sulphuric acid loading at 121 ^{o}C and 15 lb pressure to achieve a maximum yield of 114.13 mg/g of total reducing sugar & 60.33 mg/g of xylose content. *Candida shehatae*, was utilized to reduce this total sugar and xylose content to ethanol by fermentation process. The entire fermentation process was carried out up to 72 h, where maximum ethanol yield was observed up to 20.12 mg/g at 51 h by utilizing around 83% of total reducing sugar & 63% of xylose content.

Key words: Biofuel, ethanol, green energy, *Parthenium hysterophorus*, fermentation, candida.

INTRODUCTION

A countries growth & power to lead future always can be measured from how much it is independent on its energy resources, besides its soil & water resources. Seeing the rapid growth of third world countries like India, china to become soldier to soldier competitor with first world countries like US on every parameter, it can be an obvious & definite prediction that the energy consumption is going to rise drastically. But the limit of natural oil resources & dependency to Arabic countries for crude oil & disturbed politics account a dark spot to the actual future of this country's growth. Hence the necessity to find a strong & potential alternative source becomes obvious day by day.

The use of cellulose and hemicellulose, a component of biomass can be converted into monosaccharaides through chemical & biochemical ways[1]. Microbial fermentation of monosaccharide can lead to generation of green fuel, ethanol and other by-products. Also comparing the acceptability to alternative energy resources, like solar, wind and other natural renewable resources, it is been a very clear for effective transportation system, liquid fuel would be the bests choice amongst all. Ethanol can be blended with petrol or can be used as neat.

Globally the biomass resources almost contributing 14% of global energy, of which 38% of energy is produced & consumed by developing countries[2]. Biomass, adding less total carbon di-

oxide value to atmosphere, can be successfully utilized on present technological advancement on power generation and can also reduce the use and cost of natural fuel resources, if blended with them[3].

In the recent technological advancement on generation of biofuel, the extensive focus is moving to utilize waste materials as a source, rather valuable forest greenery, as it costs to depletion severely worldwide, which also became a prime concern on bio-geographical environmental chemistry in respect to global warming. So a technological approach was conducted to evaluate new approach to biofuel generation using a commonly found toxic weed with almost no commercial value till date, *Parthenium hysterophorus* L., available worldwide and widely in India after 1956.

Parthenium hysterophorus Linn. As shown in Fig. 1, a plant from Asteraceae family[4], is native to the American tropics, known as congress weed, carrot weed, star weed, feverfew, white top, chatak chandani, bitter weed, ramphool, garghas[5] in India, was biochemically evaluated. The presence of 27.8% cellulose, 21.01% hemicellulose, and 13.9% lignin was found on each gram of dried parthenium biomass showing a promising resource for biofuel raw material substituent. An extensive biochemical evaluation on each gram of biomass revealed the presence of 12.6% moisture content, 61.9% volatile matter, 3.1% ash content, 22.4% fixed carbon, & a gross calorific value of 4278 Kcal/kg. Also chemical analysis of *Parthenium hysterophorus* revealed the presence of certain sesquiterpene-lactone compounds like parthenin, a highly toxic compound. Also the presence of other allelochemicals are hysterin, ambrosin, flavonoids such as quercelagetin 3, 7-dimethyl ether ($C_{17}H_{14}O_8$), and fumaric acid ($C_4H_4O_4$), p-hydroxy benzoin (HOC_6H_4COOH) and vanillic acid ($C_8H_8O_4$), caffeic acid ($C_9H_8O_4$), p-courmaric acid ($C_9H_8O_3$),p-anisic acid ($C_8H_8O_3$),cholorogenic acid ($C_{16}H_{18}O_9$),feluric acid ($C_4H_4O_4$), beta-sitosterol ($C_{29}H_{50}O$)[6]. Due to high regenerating capacity and presence of potential toxins, this plant lacks interspecies competitiveness and thus became highly invasive. Therefore as technological approaches geared for preventing the spread of, or to eradicate parthenium, simultaneously a dedicated effort pushed on the potential and constrains of using parthenium for variety of applications[7]. The possibility of converting parthenium biomass to ethanol is currently elaborated in India[8] and is currently under technological developmental stage.

Fig. 1. Image of P. *hysterophorus* L. grown on wasteland beside CSIR-CMERI entrance gate II. (25° 31' 60N, 88° 7' 60E)

Therefore, the present study focuses on biochemical evaluation of parthenium substrate as a raw material for future fuel and applying a simple and reliable process of two sequential steps; acid hydrolysis, to make the lignocellulose accessible to the microorganisms and fermentation of the converted sugar by *candida shehatae* to ferment it to ethanol. Utilization of sugars in terms of pentose (xylose) and hexose (glucose) to produce ethanol was evaluated by

spectrophotometry. The total initial reducing sugar 114.13 mg/gm & xylose 60.33 mg/ gm was reduced to 15.73 mg/gm & 23.14 mg/gm to produce maximum ethanol of 20.12 mg/g at 51 hour.

MATERIALS AND METHODS

Feed-stock preparation:

The feedstock, parthenium plant was collected from the nearby areas surrounding CSIR-CMERI laboratory (25° 31' 60N, 88° 7' 60E) to perform the entire test procedure. The fresh plants were washed of thoroughly with tap water to remove earthy clogs. Then the chopped mass is dried in hot air oven at 106°C for 6 hours. The dried mass was then grounded to approx. 3-5 mm ranging size and stored in a dry airtight container for further work.

Preparation of feed hydrolysate:

10 gm of dried biomass was mixed with 100 ml of 5% concentrated H_2SO_4 in a conical flask. The acid catalysed breakdown of hemicellulose to monomeric sugar was carried out on 15 lb pressure at 121°C for 15 minute active heat application in autoclave device. Then the hydrolysate was cooled down to room temperature and aseptically filtered through Whatman no.1 to remove solid of residue unhydrolysed material. The filtrate was then quantitatively evaluated for the available reducing sugar and xylose content.

Detoxification:

The filtrate was over-limed in boiling condition up to pH 10 with calcium hydroxide and sulphite to synergize fermentation ability and to reduce furfural, phenolic, other acid derivatives, heavy metals, etc. Finally the pH was neutralized and adjusted to 5.6 for the next step.

Inoculum preparation:

Candida shehatae (NCIM 3479) was cultured on nutrient media (microscopy of cell provided in fig. 2 & 3 with binocular microscope.) of following composition: D-xylose – 50 gm, malt extract- 3 gm, peptone- 5 gm, pH- 5.6, for 18 hours at 30°C in a shaker incubator shacked at 120 rpm. The media was centrifuged at 10000 rpm for 10 min and pellets were collected.

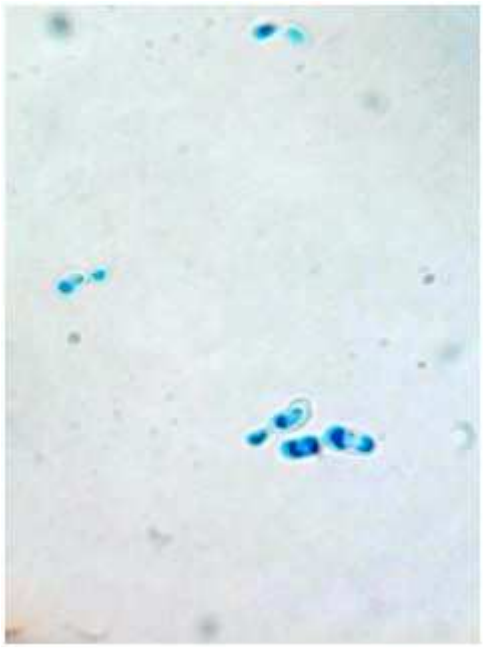

Fig. 2: *C.shehatae* picture taken at 1000 D zoom on Magnus Binocular Microscope with 1.3 MP camera with background staining with Indian ink.

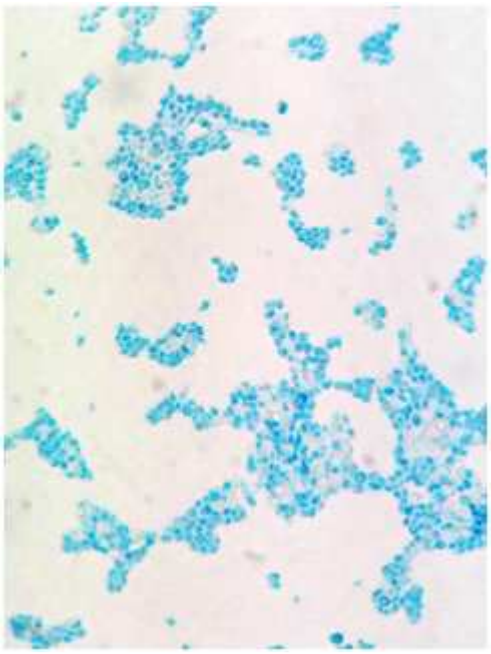

Fig. 3: *C.shehatae* picture taken at 400 D zoom on Magnus Binocular Microscope with 1.3 MP camera with background staining with Indian ink

Fermentation media preparation:
Neutralized hydrolysate was supplemented with 0.5 gm NH_4Cl, 2 gm KH_2PO_4, 0.5 gm $MgSO_4$, $7H_2O$, 0.1 gm $CaCl_2$, $2H_2O$, 0.01 gm $FeCl_3$, 2 H_2O, 0.001 gm $ZnSO_4$, 7 H_2O, 1.5 gm yeast extract per 1 liter volume. Centrifuged pellets were inoculated in this medium and incubated at 120 rpm at 30°C in shaker incubator.

Estimation of total reducing sugar in the biomass:
The reducing sugars were estimated by 3, 5 di-nitrosalicylic acid reagent solution by spectrophotometry using glucose standard solution of 10 mg/ml.

Composition of DNSA solution:
DNSA – 10.6 gm.
Rochelle salt- 306 gm
Phenol- 7.6 ml.
Sodium meta-bisulphate- 8.3 gm
NaOH- 19.8 gm
Distilled water- 1416 ml.
300 µl of DNSA solution was mixed with 200 µl of sample and heated for 5 minute at 90°C in a water bath then cooled down to room temperature and diluted with 5 ml distilled water. The final solution was checked at 540 nm in spectrophotometer against standard graph.

Estimation of xylose in the biomass:
Xylose, a major pentose sugar present in the biomass reacts with pholoroglucinol in acidic medium to impart purple blue colored xylose-pholoroglucinol complex, a highly photosensitive compound. The presence of xylose in the solution was measured at 554 nm against a standard graph in spectrophotometer.

D-xylose preparation:
0.05 gm of xylose was mixed with 10 ml. of benzoic acid to make 10 g/L solution.

Benzoic acid solution:
0.2 gm Of benzoic acid was thoroughly mixed with 100 ml distilled water preheated at 60 °C then filtered.

$ZnSO_4$ solution preparation:
10gm of $ZnSO_4$ was added with 100 ml of distilled water.

NaOH solution preparation:
100 ml distilled water was used to dissolve 2 gm. of NaOH. Xylose content was measured by mixing 200 µl sample with 300µlNaOH, 500 µl concentrated HCl and 5 ml pholoroglucinol solution and heated to 90 ^{o}C for 5 minutes and measured at 554 nm at spectrophotometer.

Ethanol estimation:
300 µl sample was mixed with 3 ml of acid dichromate solution and left for 30 min at room temperature then was evaluated by spectrophotometer at 590 nm against standard graph.

Acid-dichromate solution:
7.5 gm Of potassium dichromate was measured and dissolved in 5M H_2SO_4 and the final mixture volume was adjusted to 250 ml.

Fermentation:
The biochemical composition of parthenium was the biggest challenge for this study because of the presence of parthenin toxin, a potential Map kinase inhibitor[9] resisting microbial growth. An initial conformational study was conducted with neutralized treated parthenium hydrolysate to observe whether promotion of microbial growth was possible or not which has shown positive result in broth system.

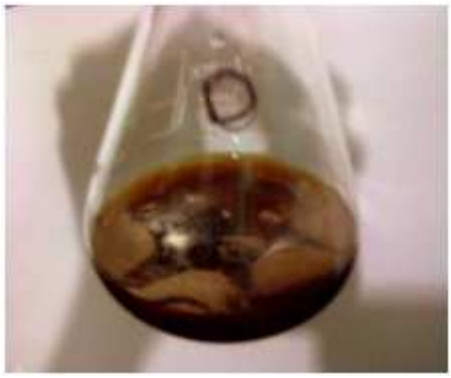

Fig 3. Cell growth capability on neutralized hydrolysed biomass

The fermentation of parthenium hydrolysate was carried out in a closed glass vessel provided with swan necked tapped outlet, to minimize aeration and outside contamination. The hydrolysate was inoculated with 10 mg of *candida shehatae* biomass per 200 ml and mixed well with sterile magnetic stirrer. The initial cell concentration in hydrolysate was measured at 600 nm for 10 mg/ml inoculum load was 0.047 mg/ml. The fermentation bottle was then incubated at shaker incubator for 72 hours at 30±0.5 ^{o}C and pH 5.6±0.2. On each 3 hour from initial inoculation the fermentation broth was collected aseptically to estimate the utilization of hexoses and pentose in order to promote growth and production of ethanol as a secondary metabolite.

RESULTS AND DISCUSSION
The maximized pre-treatment procedure was obtained from previous researches on acid catalysed hydrolysis of parthenium biomass[10], and revaluated for total reducing sugar & xylose content at the same treatment condition. Application of 15 lb pressure on 121^{o}C for 15 minute yielded 114.1 mg/g of total reducing sugar and 60.3 mg/g of xylose content.
Candida shehatae, a slow growing single celled, ovular fungal species from Ascomycota family, was utilized to reduce this total sugar & xylose content to ethanol by fermentation process. The above maintained pure culture strain was primarily evaluated for parthenin tolerance and MAP kinase activity resistance by sub culturing it on synthetic broth and agar media containing hydrolysate as a source of carbon. Prominent growth, as shown in Fig. 4, was observed ason both the media confirming utilization possibility of the strain. Microbial growth pattern was finally observed for a long incubation period of 72 hours to find out utilization phenomena of total reducing sugar and xylose for production of secondary metabolite, ethanol. The fermentation on optimized *P. hysterophorus* hydrolysate containing additional micronutrients shows that the culture reached into stationary phase within 30th hour utilizing maximum

amount of hexose. Utilization of residue pentose maximized after 42 hours leading to increase in production of ethanol up to 51 hour before entering into death phase due to cytotoxicity of ethanol and limitation of available nutrient resources.

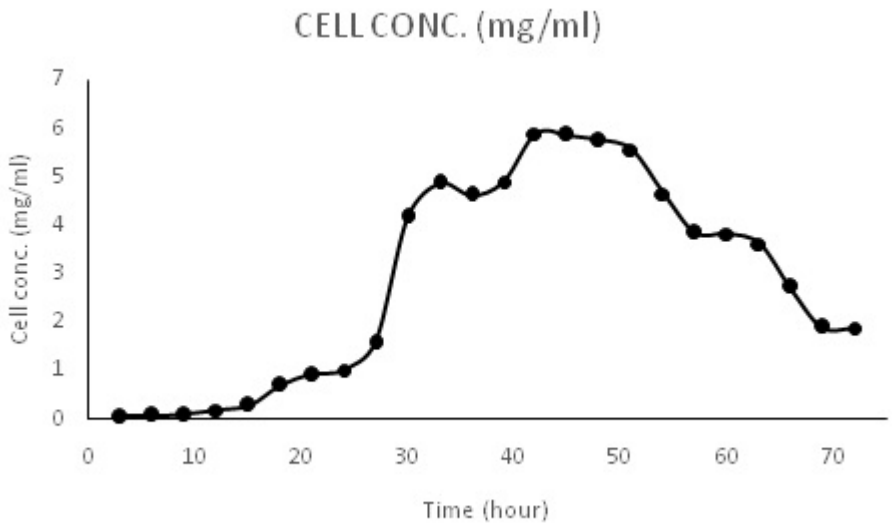

Fig 4. Cell growth pattern of *candida shehatae* on parthenium hydrolysate

The co-relationship of pentose & hexose utilization in production of ethanol from per gram of biomass is established below in Fig. 5. Maximum ethanol of 20.12 mg/g. of biomass was obtained at 51 hour over utilization of 83 % of total sugar and 63 % of xylose content.

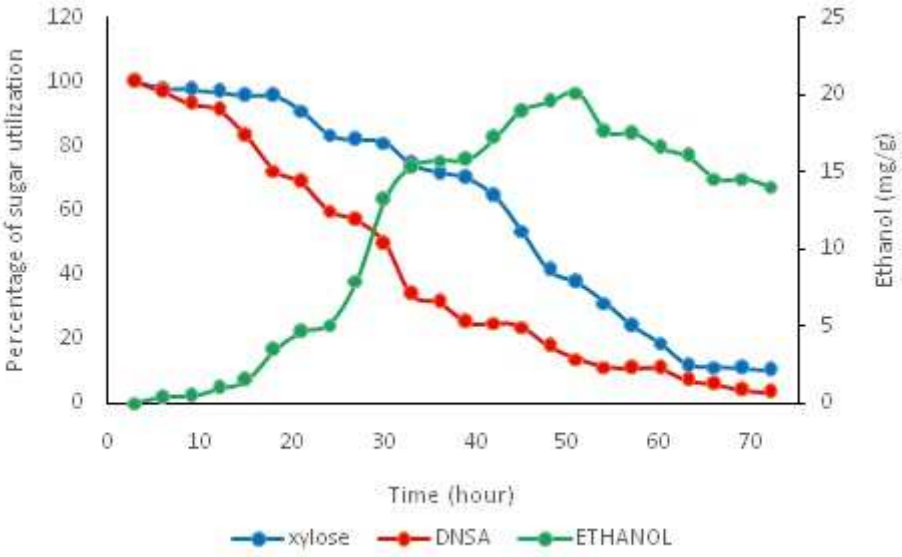

Fig 5. Percent of sugar utilization in respect to ethanol production

The ethanol production was maximum by conversion of 114.1 mg/g of total reducing sugar and 60.3 mg/g of xylose content as obtained through acid hydrolysis of *P hysterophorus* L. The sugar contents was utilized to convert into ethanol through growth process of *candida shehatae* up to 72 hour, which was studied on liquid growth system. The relationship of cell density, production of ethanol is established in with utilization of hexoses & pentose's sugar and is shown in Fig.6.

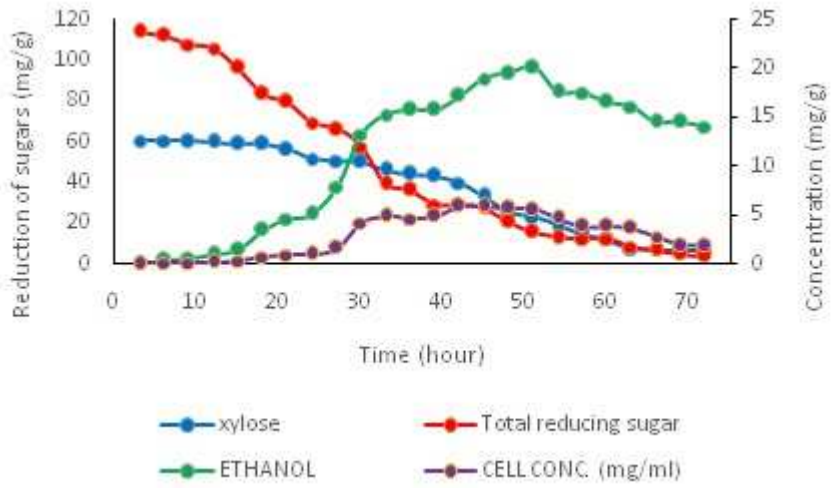

Fig 6. Relationship of cell density, production of ethanol with utilization of hexoses & pentose's

CONCLUSION

Parthenium hysterophorus, a highly toxic weed from *astraceae* family with excessive growth rate & high adaptability on harsh environmental condition, is a very good option for zero cost resources for biofuel. The present study conducted in CSIR-CMERI laboratory on the conversion of lignocellulosic sugar to ethanol using *candida shehatae* at around 30 ^{o}C and a pH of 5.6 reveals a maximum yield of 20.12 mg /g of ethanol utilizing 83% of total sugar & 63% of xylose content. The conducted study also suggests possibility of utilization of parthenium biomass for future study to optimizing it for large scale industrial utilization.

ACKNOWLEDGMENTS

Authors wish to thank Director, CSIR Central Mechanical engineering Research Institute, Durgapur for his constant support, encouragement & permission to publish this paper.

REFERENCES

1 Y. Chuan-min , Y. Yong-jie , R. Zheng-wei, L Ting-chen and C. Jian-qin, The Chinese Journal of Process Engineering. Vol.4 No.1, Feb.2004.

2 A. Grubler, N. Nakicenovic, The Dynamic Evolution of Methane Technologies, in Lee T.H, H.R. Linden,D.A. Dryefus and T. Vasko, (eds.), The MethaneAge, Kluwer Academic Publishers, Dordrecht. 1988.

3 B. Patel and B. Gami. Biomass Characterization and its Use as Solid Fuel for Combustion. Iranica Journal of Energy & Environment 3 (2): 123-128, 2012, ISSN 2079-2115, DOI: 10.5829/idosi.ijee.2012.03.02.0071, 2012

4 Bailey L.H. 1960. Manual of cultivated plants, Macmillan, New York.

5 P. Oudhia, and R.S. Tripathi. Allelopathic effects of *Parthenium hysterophorus* L. on kodo, mustard and problematic weeds. p. 136-139. In: Proc. First Int. Conference on Parthenium Management, (Vol-II). UAS, Dharwad. 1998a.

6 H Lata, V.K. Garg and R.K.Gupta. Removal of a basic dye from aqueous solution by adsorption using *Parthenium hysterophorus*: an agricultural waste. Dyes Pigment. 74:653–658. doi: 10.1016/j.dyepig.2006.04.007. 2007.

7 Shubhaneel N, Swati Ghosh, S Haldar, A Ganguly and P K Chatterjee. Acid catalyzed auto-hydrolysis of *Parthenium hysterophorus* L. for production of xylose for lignocellulosic ethanol. International Journal of Emerging Technology and Advanced Engineering, Website: www.ijetae.com, ISSN 2250-2459, Volume 3, Special Issue 3: ICERTSD, Feb 2013, pages 167-172. 2013

8 S. Ghosh, S Haldar, N. Shubhaneel, A Ganguly and P. K Chatterjee, Kinetic Study of the Acid Hydrolysis of *Parthenium hysterophorus* L. for xylose yield in the Production of

Lignocellulosic ethanol., IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), ISSN: 2278-3008. Volume 3, Issue 3, PP 35-41, www.iosrjournals.org. Sep-Oct. 2012.

9 D Hwang, N.H. Fischer, B.C. Jang, H. Tak , J.K. Kim and W. Lee, Inhibition of the expression of inducible yclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with the inhibition of MAP kinases, Biochem Biophys Res Commun, 226:810-8, 1996.

10 P Singh, S. Saha, N. Shubhaneel, S. Ghosh, A. Ganguly, S. Haldar and P. K. Chatterjee, Dilute acid hydrolysis of *Parthenium hysterophorus* L. for the production of ethanol using Pichia stipites, International Journal of Energy and Power (IJEP) Volume 2 Issue 4, November, Pages 88-93, 2013.