Int.J.Curr.Microbiol.App.Sci (2015) Special Issue-2: 399-406

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 Special Issue-2 (May-2015) pp. 399-406
http://www.ijcmas.com

Original Research Article

Production of Bioethanol From *Eichhornia crassipes* (Water Hyacinth)

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A B S T R A C T

Due to rapid industrialization and growth in population, the worldwide demand of ethanol is increasing; which drives attention on use of various lignocellulosic wastes for bioethanol production. *Eichhornia crassipes* a persistent and notorious weed found in freshwater bodies creating problems to aquatic ecosystem. Present work deals with production of bioethanol by utilizing abundant and inexpensive source of a lignocellulosic waste, a aquatic weed *Eichhornia crassipes* (Water Hyacinth). Ethanol was produced in two steps; pretreatment of *Eichhornia* waste to fermentable sugar by enzymatic sachharification, and fermentation of sugars to ethanol using yeast *Sachharomyces cerevisae* and *Pichia stipitis*. Sachharification of *Eichhornia* was carried out by Mix culture of cellulose and xylan degrading bacteria isolated from 4 different sources (municipal waste, cow dung, goat excreta and pig slurry) for 3 days at 37°C. Fermentable sugar (Xylose and Glucose) were then fermented anaerobically by *Pichia stipitis* and *Sachharomyces cerevisae* respectively to ethanol. Ethanol obtained by *Sachharomyces cerevisae* from *Eichhornia* was 3.1gm% and that of pure cellulose was 2.1gm%; whereas *Pichia stipitis* produced 3.05gm% from *Eichhornia* and 1.45gm% from xylan respectively. The obtained result proves *Eichhornia* as a promising plant for bioethanol production.

Keywords

Bioethanol, Enzymatic sachharification, Cellulase, Xylanase, *Eichhornia crassipes*

Introduction

Bioethanol is being considered as a potential liquid fuel due to limited amount of natural resources (GO.ogawa Masami *et al.,* 2008) and is becoming increasingly important as a consequences of major concern for depleting oil reservoirs, rising crude oil prices in recent years, have become the driving force for developing alternative energy source. (Ashish *et al.,* 2009).

Bioethanol can be obtained from terrestrial as well as aquatic plants. Aquatic plants grow in water bodies without competing with arable lands for grains and vegetables; they are also used for bioabsorption of nutrients and heavy metal. Despite these advantages no data available on use of aquatic plants for bioethanol production except *Eichhornia crassipes*. On one hand

distribution of *Eichhornia crassipes* has become a persistent and expensive aquatic problem damaging the environment, irrigation system and crops. and on the other hand to reduce these problems much attention is focused on the potential use of water hyacinth for variety of applications such as its use for ethanol production, hydrogen production (Chartchalerm Isaarakura-Na- Ayudhya *et al.,* 2007) Cellulose and hemi-cellulose, which are the principal biodegradable carbohydrate components of the bagasse, are found together with lignin in an intense cross linked, rigid ligno-cellulose complex (Mutalik *et al.,* 2011). This ligno-cellulosic structure severely limits the biological hydrolysis of cellulose and other polymers. Therefore, it requires pretreatment prior to hydrolysis. Industrially, the pretreated material is mainly thought to be hydrolyzed and fermented in two different steps: separate hydrolysis and fermentation, or in one single step: simultaneous saccharification and fermentation. (Goshadrou *et al.,* 2001; Patel *et al.,* 2007).

Various lignocellulosic wastes such as wheat, rice straw, corncob etc are promising source for ethanol production considering its availability in both tropical as well as temperate countries and low cost (Mervate Abo-State *et al.,* 2014) These waste contain large amount of cellulose and hemicelluloses, which can be sachharified by acid or alkali or enzymatic treatment into fermentable sugar and further fermented to ethanol using microorganisms (Yadhu *et al.,* 2011). Water hyacinth has low lignin, hence cellulose and hemicelluloses are more easily converted to fermentable sugar thus resulting in enormous amount of biomass for the biofuel industry.

Therefore, the present study aims at isolating cellulose and xylan degrading bacteria from feces of animals consuming large amount of lignocellulosic residues and enzymating sachharification of cellulose and xylan (from *Eichhornia crassipes)* to glucose and xylose repectively. xylose constitutes about 45% of raw material hence *Pichia stipitis*, a pentose fermenting yeast is used in ethanol production from xylose and glucose obtained from cellulose is fermented to ethanol by *Sachharomyces cerevisae* and emphasizes on efficient utilization of total carbohydrate for ethanol production.

Materials and Methods

Chemicals reagents and microorganism

Phloroglucinol and 3,5 dinitrosalicylic acid was purchased from sigma Aldrich. Absolute ethanol, potassium dichromate were supplied from Merk. All other chemicals were of analytical grade and commercially available. *Sachharomyces cerevisae* NCIM 3523 and *Pichia stipitis* NCIM 3506 cultures were purchased from NCIM, Pune India.

Sample collection

Four samples viz municipal waste, cow dung, goat excreta and pig slurry were collected from Muncipal Corporation Area, Pune, India for isolation of cellulose and xylan degrading bacteria.

Isolation and screening of cellulose and xylan degrading bacteria

1 gm of each sample was inoculated in a 20 ml basal salt medium ($NaNO_3$ 2.5g, KH_2PO_4 2g, $MgSO_4$ 0.2g, NaCl 0.2g, $CaCl_2.6H_2O$ 0.1g in 1 litre distilled water, P^H – 6.8- 7.2) containing filter paper and xylan separately as sole source of carbon for enrichment of cellulolytic and xylanolytic bacteria respectively and were incubated for 7 days in shaker incubator at 37^oC, 100 rpm. A loopful sample from respective flask were

streaked on CMC agar (KH_2PO_4 0.5g, $MgSO_4$ 0.25g,, Cellulose powder 2g, Agar agar 15g, gelatin 2g in 1 litre distilled water, P^H – 6.8- 7.2) and Xylan agar (Xylan 5g, $NaNO_3$ 1g, K_2HPO_4 1gm, KCl 1g, $MgSO_4$ 0.5g, Yeast extract 0.5g, Agar agar 15g in 1 litre distilled water, pH 6.8–7.2) for isolation of cellulolytic and xylanolytic bacteria. Colony characteristics of isolates from both media were noted and pure culture were prepared on respective media (Ashish *et al.,* 2009; Gupta *et al.,* 2011)

Qualitative screening of cellulolytic and xylanolytic bacteria

Isolates with highest cellulolytic and xylanolytic activity were screened qualitatively by spot inoculating individual bacterial suspension on CMC and Xylan agar; plates were incubated at 37°C for 24-48 hrs. After incubation plates were flooded with Gram's iodine and diameter of zone of clearance around colony were measured; isolates which showed highest zone of clearance on respective plate were selected for saccharification of substrate *Eichhornia crassipes* (Faridha Begum *et al.,* 2013; Gupta *et al.,* 2011)

Bioethanol production from *Eichhornia crassipes* by using *Sachharomyces cerevisae* and *Pichia stipitis.*

Bioethanol from *Eichhornia crassipes* was produced in two steps:

Pretreatment (sachharification) of *Eichhornia crassipes* **-** *Eichhornia crassipes* plant were collected from natural pond in Charholi, Dist- Pune, Maharashtra, India. Washed thoroughly several times with tap water to remove dirt on it; then plants were chopped into small pieces, dried in sunlight and blended into fine powder and stored in airtight container at room temperature for further assay.

Selected cellulolytic isolates were cultured individually and in mixed culture of all isolate in basal salt medium containing 1gm% *Eichhornia crassipes* powder in separate flasks, and mixed culture were inoculated in basal salt medium containing 1gm% CMC powder. In same way xylanolytic isolates were cultured individually and in mixed culture of all isolate in basal salt medium containing 1gm%, *Eichhornia crassipes* powder in separate flasks, and mixed culture were inoculated in basal salt medium containing 1gm% xylan powder. All flasks were incubated at 37°C for 3 days. Reducing sugar produced in flask inoculated with cellulolytic bacteria were determined by dinitrisalicylic acid method using glucose as standard; xylose produced in flasks inoculated with xylanolytic bacteria were determined by phloroglucinol method using xylose as standard (Gupta *et al.,* 2011, Chartchalerm Isaarakura-Na- Ayudhya *et al.,* 2007).

Production of ethanol by fermentation process- After saccharification process respective flasks were inoculated with *Sachharomyces cerevisae* and *Pichia stipitis* inoculum and incubated at 27°C for 5 days anaerobically. Ethanol concentration from all flasks were determined by dichromate assay (GO.ogawa Masami *et al.,* 2008).

Determination of ethanol content by Dichromate assay - Acid dichromate solution (0.1M $Cr_2O_7^{-2}$ in 5M H_2SO_4) was prepared, 7.5g of potassium dichromate in dilute sulfuric acid and final volume was adjusted to 250ml with deionized water. Standard calibration curve of ethanol was prepared by taking 300µl of ethanol solution of each different concentration (ranging from 0.5g% to 4g%) into small plastic caps and placed into a beaker containing 3ml of

acid dichromate solution; beakers were tightly sealed with parafilm and kept at room temperature for 30 minutes. The absorbance was recorded at 590nm. Ethanol content in all samples after fermentation was estimated by same procedure and referring standard ethanol absorbance (Chartchalerm Isaarakura-Na- Ayudhya *et al.,* 2007).

Result and Discussion

Isolation and Qualitative screening of cellulolytic and xylanolytic bacteria

Cellulose and xylan degrading bacteria from municipal waste, cow dung, goat excreta and pig slurry were enriched in cellulose and xylan containing liquid media, and isolated on CMC agar and xylan agar respectively. Total 43 isolates were obtained out of which isolate number 1-34 have xylan degrading capacity and isolate number 35-43 have cellulose degrading capacity. Best xylan degrading isolates were screened by growing them on xylan agar and measuring respective diameter of zone of clearance; isolate no 1,2,5,6,7,8,9,10,11,20,22,23,24 showed maximum zone of clearance that was in between 1.5- 5.4cm; out of which 7,8,9,20 (source- goat excreta) were selected for sachharification of *Eichhornia crassipes* substrate (Table 1& figure 1). Among all cellulolytic bacterial isolates; isolate no 39,40 (source- Pig slurry), 42(cow dung), 43 (goat excreta) showed highest zone of clearance on CMC agar that was in between 2-4cm and were selected for sachharification of *Eichhornia crassipes* substrate (Table 2; Figure 2). Cellulose degrading bacteria from goat rumen fluid showed best cellulase activity in presence of xylose (Faridha Begum *et al.,* 2013) and Cherie J. Ziemer (2013) also isolated 573 bacterial isolates from cow feces which showed cellulose-xylan pectin degrading bacteria. We have also obtained isolate similar to them.

Table.1 Qualitative screening of xylan degrading bacteria on xylan agar

Isolate number	Source	Diameter of zone of clearance (cm)	Isolate number	Source	Diameter of zone of clearance (cm)
1	Goat excreta	4.1	15	Cow dung	2.5
2	Goat excreta	3.5	16	Cow dung	2
5	Goat excreta	3	17	Cow dung	1.5
6	Goat excreta	3	18	Cow dung	1.7
7	Cow dung	4	19	Cow dung	2.5
8	Cow dung	4	20	Cow dung	5.4
9	Cow dung	4.2	22	Cow dung	3.6
10	Cow dung	3	23	Pig slurry	3.6
11	Cow dung	3	24	Pig slurry	3.8
13	Cow dung	2			
14	Cow dung	2.3			

Table.2 Qualitative screening of cellulose degrading bacteria on CMC agar

No. Of isolate	Source	Diameter of zone of clearance (cm)
36	Soil	2
37	Soil	2
38	Pig slurry	2
39	Pig slurry	3.5
40	Pig slurry	4
42	Cow dung	3.5
43	Goat excreta	3

Table.3 Xylose content of substrate after pretreatment and ethanol obtained on fermentation by *Pichia stipitis*

Substrate	Isolates in mix culture	Xylose (mg/g of substrate)	Ethanol (g%)
E.crassipes	7,8,9,20	345	3.05
Xylan	7,8,9,20	300	1.45

Table.4 Glucose content of substrate after pretreatment and ethanol obtained on fermentation by Sachharomyces cerevisiae

Substrate	Isolates in mix culture	Glucose (mg/g of substrate)	Ethanol (g%)
E.crassipes	39,40,42,43	378	3.1
CMC	39,40,42,43	126	2.1

Figure.1 Qualitative screening of xylan degrading bacterial isolates a) Isolate 1,2 b) Isolate 5,6,7,8 c) Isolate 9,10,11,12 d) Isolate 13,14,15,16 e) Isolate 17,18,19,20 f) Isolate 22,23,24,25

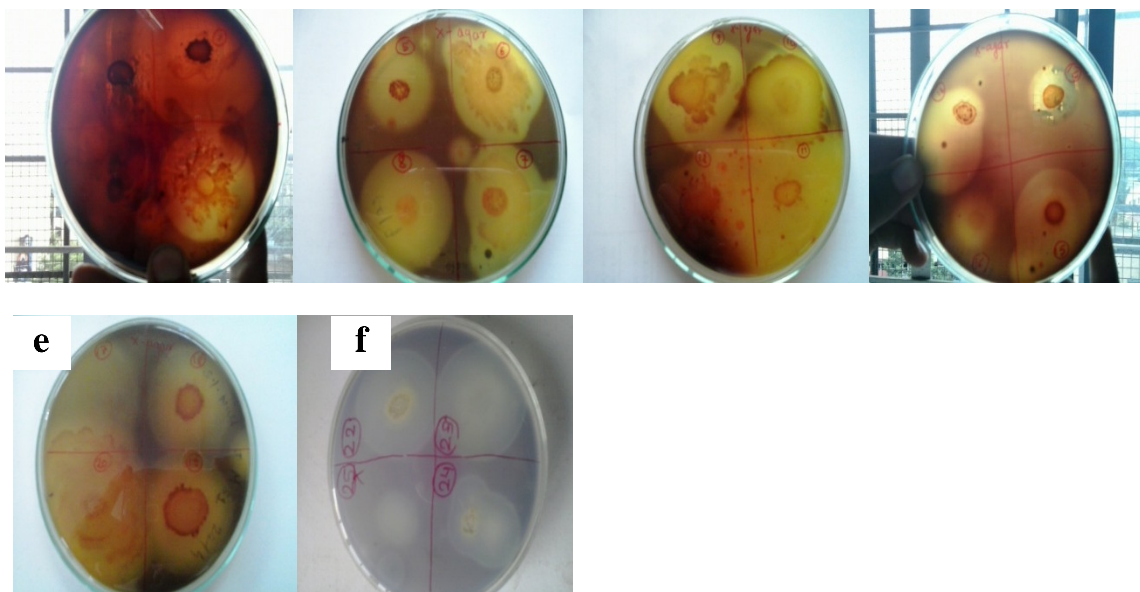


Figure.2 Qualitative screening of cellulose degrading bacterial isolates
a) Isolate 36 b) Isolate 37,38,39,40 c) Isolate 42,43

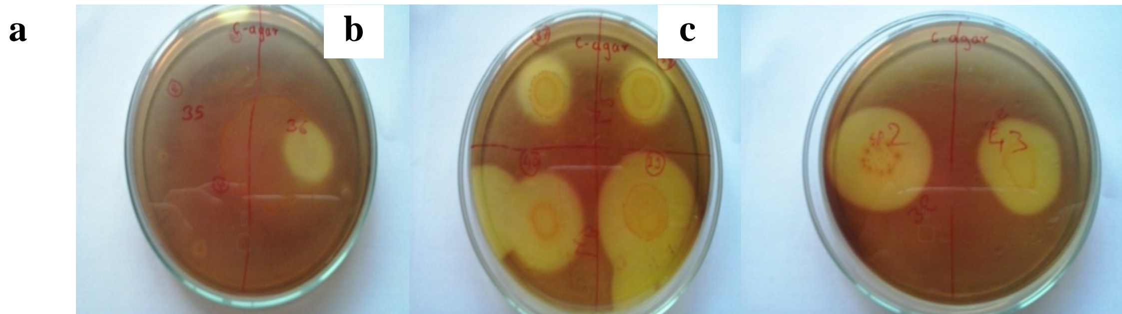


Figure.3 a) *Eichhornia crassipes* plant (Water hyacinth). b) Collection of *Eichhornia crassipes*

Figure.4 Fermentable sugar (Glucose & Xylose) produced on enzymatic
Sachharification of pure substrate and *Eichhornia crassipes*

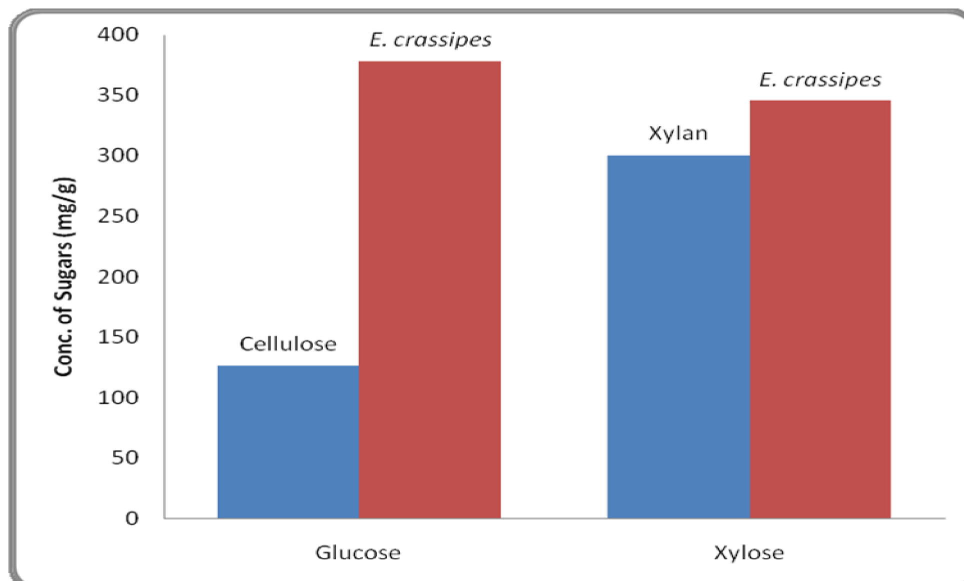
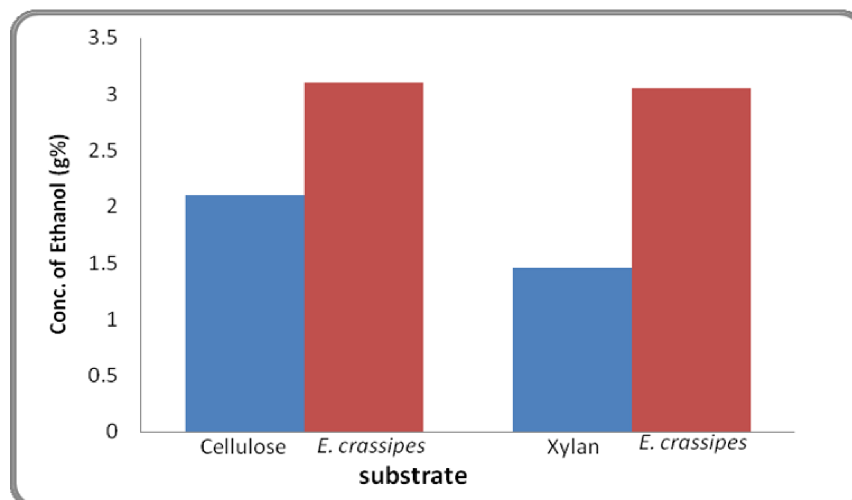


Figure.5 Bioethanol from pure substrares (Cellulose & Xylan) and *Eichhornia crassipes* by *Sachharomyces cerevisae* and *Pichia patula* respectively



Pretreatment (sachharification) of *Eichhornia crassipes* and Bioethanol production

378 mg/gm& 126 mg/gm reducing sugar and 3.1g% & 2.1g% ethanol were produced from *Eichhornia crassipes* substrate& CMC powder, respectively (Table 4; Figure 4&5). And 345 mg/gm & 300 mg/gm of xylose and 3.05g% & 1.45g% ethanol were produced from *Eichhornia crassipes* substrate& Xylan powder respectively(Table 3 Figure 4&5), which is much greater than yield (1.01g/l) obtained by Chartchalerm Isaarakura-Na- Ayudhya *et al.* (2007) from *Eichhornia crassipes* substrate & Xylan by *candida shehatae. Eichhornia crassipes* gives more sugar and ethanol than pure sources. Whereas GO.ogawa Masami *et al.,* obtained 22.4ml of ethanol/kg of dried water hyacinth in which substrate was pretreated with 1%H_2SO_4(v/v). Concentration of ethanol obtained was more compared to reducing sugar produced on sachharification that is probably because ruminant isolates are facultative (Marvin P. Bryant, 1959) therefore continuously sachharifies substrate to fermentable sugar which is fermented to ethanol by *Sachharomyces cerevisae* under anaerobic condition.

In conclusion we explored efficiency of ruminant microflora for biological pretreatment of *Eichhornia crassipes* to fermentable sugar and fermentation of both glucose and xylose to ethanol to increase ethanol yield. As previously mentioned water hyacinth is one of the expensive aquatic weed which is creating problems to aquatic and terrestrial systems; for this much emphasis is given to control its growth, but most of the time all measures are ineffective due to pernicious invasive growth. The technique herein provides cost effective process to get economically important product from it and more or less helps in lowering plant.

Acknowledgements

The authors gratefully acknowledge The Principal, Modern College of Arts, Commerce and Science, Ganeshkhind, Pune-16, India and Head, Department of Biotechnology for providing laboratory and instrument facility for this project.

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