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Hydrolysis of *Eicchornia crassipes* and *Egeria densa* for Ethanol Production by Yeasts Isolated from Colombian Lake Fúquene

P. Martínez-Nieto, M. Vanegas-Hoyos, M. Zapata-Pineda, J. Robles-Camargo

***Abstract*—**The aquatic plants are a promising renewable energy resource. Lake Fúquene polluting macrophytes, water hyacinth (*Eichhornia crassipes* C. Mart.) and Brazilian elodea (*Egeria densa* Planch.), were saccharifiedby different treatments and fermented to ethanol by native yeasts. Among the tested chemical and biological methods for the saccharification, *Pleurotus ostreatus* at 10% (m/v) was chosen as the best pre-treatment in both macrophytes (P<0.01). Subsequently 49 yeasts were isolated from Lake Fúquene and nine strains were selected, which presented the highest precipitates characteristic of ethanol in the iodoform test. The fermentations from water hyacinth and Brazilian elodea hydrolysates using these yeasts produced ethanol at a rate between 0.38 to 0.80$gL^{-1}h^{-1}$ and 0.15 to 0.27$gL^{-1}h^{-1}$ respectively. The ethanol presence was confirmed by gas chromatography–mass spectrometry. The nine yeasts chosen were preliminarily identified as belonging to the genera *Candida* spp., *Brettanomyces* sp. and *Hansenula* spp.

***Keywords*—**Bio-ethanol, Chemical hydrolysis, Invasive aquatic macrophytes, Native yeasts fermenting, *P. ostreatus.*

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

I. Introduction

THE biofuels production from lignocellulosic biomass reduces greenhouse gases emissions, are inexpensive and abundant and do not involve ethical concerns associated with the use of agricultural crops. The conversion of lignocellulosic feedstock to ethanol is achieved by pre-treatment to allow rapid and efficient hydrolysis of cell wall carbohydrates to simple sugars that can be fermented to ethanol by microbial action. However, many lignocellulosic wastes require several sequential steps to the breakdown and hydrolysis of structural carbohydrates to fermentable sugars which increases costs and limits the industrial scale-up in developing countries [1]-[5].

In lignocellulosic feedstocks, promising for the 21st century biofuels industry, are algae and invasive aquatic plants [4], [7]. Recently, it was found that water hyacinth, a floating aquatic plant regarded as one of the world's most damaging invasive species, could be sustainably managed in their natural ecosystems and used as bioenergy feedstock, because it has a high primary productivity rate, it is widely available and does not compete with agricultural crops, culture density is not limited by the light availability as it is in the case of algae and plant cell wall contains low lignin content that means the cellulose and hemicellulose are more readily converted to fermentable sugars. Ethanol production from water hyacinth can be done by simple hydrolysis methods followed by fermentation with specific microorganisms [4]-[10]. Based on those results and water hyacinth life cycle study which suggests that this plant can be processed with similar costs for other feedstocks used for biofuels production, Wilkie and Evans [4] invite to continue conducting studies with other invasive aquatic plants within environmental control programs in affected aquatic ecosystems.

The Colombian Lake Fúquene watershed, a strategic ecosystem providing goods and services to the regional economy, presents a serious infestation of introduced aquatic plants, water hyacinth (WH) and Brazilian elodea (BE), causing displacement of native species and flooding episodes by reducing the lake's storage capacity, preventing drainage, sailing and fishing [11], [12].These plants once removed mechanically from the lake could be used in biofuels production, taking into account nearly 1324ha are infested with aquatic plants out of 3156ha total lake area [13] and have also concentrations of lignin, cellulose and hemicellulose from 4.6 to 5.5%, 13.9 to 20.9% and 11.4 to 18%, respectively (unpublished data).

Therefore, the present study objective was the saccharification of WH and BE, using different hydrolytic methods for the subsequent fermentation to ethanol by native yeasts as an alternatively sustainable removal at generate benefits for the Lake Fúquene ecology and the local economy.

II. Methodology

A. Feedstock

Two hundred fifty (250) kg of WH and BE manually harvested from Lake Fúquene were cut and sun-dried up to moisture content between 40 to 60%. The total sugars and reducing sugars concentrations in dry basis were 19.3% and 5.8% for WH and of 19.7% and 4.1% for BE, respectively

B. Pre-Treatments Performed on Feedstock and Saccharification

1. Acid Hydrolysis

The hydrolysis was carried out with dilute sulfuric acid following the methodology proposed by Masami et al. [6] with

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International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

some modifications. Treatments were sulfuric acid solutions to 0.5%, 1% and 2% (m/v), liquefied or filtered macrophytes suspensions in distilled water (1:9 m/v) and two sterilization times 30 and 60 minutes. The sulfuric acid concentrations and macrophytes suspensions were mixed at 1:1 v/v. The experimental units were neutralized by addition of sodium hydroxide 10% (m/ v).

2. Acid Hydrolysis with Detoxification

This hydrolysis was carried out with aquatic macrophytes washed, cut and dried at 105°C for 24 hours, following the methodology proposed by Nigam [14].

3. Oxidative Method with Peracetic Acid

A solution of 1:1 v/v acetic anhydride and 35% hydrogen peroxide and 10g aquatic macrophytes were used for peracetic acid pre-treatment according to Abraham y Kurup [15].

4. Alkaline Hydrolysis

Two methods were carried out by alkaline hydrolys is using sodium hydroxide (NaOH) at 5and10% m/v. The first procedure was based on the methodology proposed by Eshtiaghi et al. [16] with some modifications, where aquatic plants suspensions were mixed with NaOH 5% (m/v) at 1:1v/v, and sterilized for 30 and 60 minutes in an autoclave. The other method was performed using a 1:10 (m/v) ratio of aquatic macrophyte: NaOH at 10%, following the methodology of Abraham and Kurup [15] for tapioca (*Manihot esculenta* Crantz) leaf.

5. Biological Hydrolysis.

45mL of liquid medium with water hyacinth or Brazilian elodea at 10% were inoculated with 0.9g, 2.7g or 4.5g *P. ostreatus* O-1 spwan (PO-1), obtained from Colombian Champinfung Company. This procedure was performed in triplicate for each concentration and the incubation was done at room temperature for thirteen days.

6. Sugars Estimation and Saccharification

The reducing sugar estimation was done using the DNS (3.5 dinitrosalicylic acid) method [17]. In the biological hydrolysis this were measured at 5, 7, 10, 11, 12 and 13 days of incubation, whereas in the other pre-treatments were performed to complete the procedure.

The extent of hydrolysis in the different pre-treatments was determined by Saccharification percentage, using the following formula: 100 x (Reducing sugar concentration obtained/Potential sugar concentration in the pretreated substrate) [18].

7. Experimental Design, Statistical Analysis and Better Pre-Treatment Selection.

All pre-treatments were distributed in completely randomized designs with three replicates per treatment. The data were treated statistically by factorial analysis of variance (Acid, alkaline and biological hydrolysis) at $P<0.01$ and Duncan's multiple-range test, to determine significant differences between the means. Based on these results the best treatments were selected in each of the three methods.

The final selection of the best pre-treatment was done by comparing all the methods by one-way analysis of variance ($P<0.01$) and Duncan's multiple-range test. The best pretreatment was used for the ethanol production detected by dichromate oxidation method.

For the interpretation of the results, Saccharification percentages were transformed using the formula $Y= 2 x + 0.5$, where, Y are the transformed values, and x are the percentages data obtained in the experiment, to reduce the coefficient of variation and detect significant differences [19].

C. Isolation of Ethanol-Fermenting Yeasts from the Lake Fúquene Watershed

1. Sampling.

5 points (F1-F5) were chosen within the Lake Fúquene in the Ubateprovince (Cundinamarca, Colombia) and 3 water samples were collected at each point, for a total of 15 samples. In F4 and F5, they were collected by triplicate the aquatic plants, water hyacinth and Brazilian elodea, respectively. Additionally, triplicate samples were taken from forest soil (F6) and compost produced from WH and BE wastes (F7), in Guatancuy sector belonging to Fúquene municipality (Cundinamarca, Colombia) (Fig. 1).

2. Culture

Water, forest soil and compost samples were inoculated in different liquid media as molasses at 10%, soybean meal (3%) with malt extract (0.3%), rice at 50%, water hyacinth at 10%, Brazilian elodea at 10% and in the commercial medium, yeast extract-glucose-chloramphenicol (YGC). The yeasts isolated and purified in the different media were inoculated on solid media with WH and BE as sole carbon source and the strains forming colonies were selected and tested for their ability to produce ethanol.

3. Selection of Ethanol-Fermenting Yeasts.

Qualitative ethanol production by the yeasts that grew on water hyacinth and Brazilian elodea as sole carbon source was determined by iodoform test following the methodology proposed by Bruno and Svoronos [20] in culture media, YGC, WH at 10% and BE at 10%. The ethanol presence was observed by the appearance of alaminate yellow precipitate corresponding to the iodoform formation. To establish differences in the precipitates formation with interfering substances, controls of ethanol, methanol, butanol, isobutanol, ethyl acetate, glycerol, amyl alcohol, acetone, acetaldehyde, isopropyl alcohol and phenol were prepared. The proportions used for the controls were 1:1, 1:9, 1:99v/v in sterile culture medium. For the interpretation of results, ethanol:culture medium (1:1v/v) corresponded to +++, 1:9 v/v (++) and 1:99 v/v (+).

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

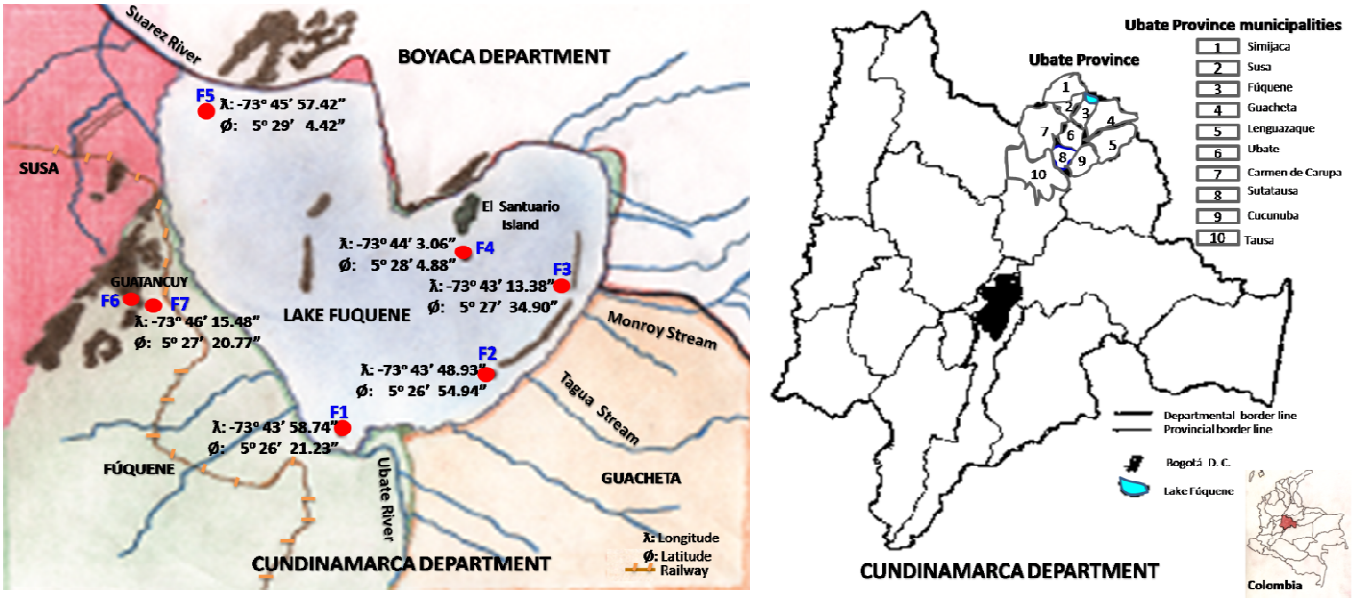

Fig. 1 Geographical location of sampling sites in Lake Fúquene (Susa and Fúquene municipalities) and Guatancuy sector (Fúquene municipality), belonging to Ubate province Note the location of Lake Fúquene in Susa and Fúquene municipalities belonging to Ubate province in the Cundinamarca department, Colombia

D. Ethanol Production by Selected Yeast Strains

1. Fermenting Strains

Yeasts in culture media with water hyacinth and Brazilian elodea at 10% presenting three crosses in the iodoform test were used for ethanol quantification by dichromate oxidation method, at a concentration of 10^6 cellsml^{-1}.

2. Fermentation Media

Four liquid culture media were used for experimental assays, two media with water hyacinth or Brazilian elodea hydrolysates obtained by the method had the highest saccharification according to the statistical analysis, and the other two liquid media containing water hyacinth or Brazilian elodea at 10%. The reducing sugars were estimated after culture media sterilization by the DNS method [17].

3. Fermentation of Biomass Hydrolysates to Ethanol

The fermentation process was carried out using two completely random designs for water hyacinth and Brazilian elodea, using as treatment the liquid media inoculated with the yeasts chosen from the test iodoform and their respective controls without inoculation, with three replicates per treatment. The batch ethanol fermentation was carried out at 15° without agitation or aeration during an incubation period of 48 hours in 100mL flasks with 48mL working volume.

4. Measuring Parameters in the Fermentation Process

In all treatments reducing sugars consumption by DNS method [17], ethanol production by dichromate oxidation method a 440nm [1], [21] and number of yeast cells by Neubauer's chamber to 24 and 48 hours after yeast inoculation were determined.

The product (ethanol) yield coefficient (Yp/s), volumetric productivity ((Qp) and the fermentation efficiency (n) were calculated according to Nigam [14], Borzani [22], Castaño and Mejia [23] and Asyraf et al. [24], using the following formulas:

$$Yp/_{S} = \frac{\text{Ethanol, g L} - 1}{\text{Reducing sugar, g L} - 1}$$

$$\text{Qp} = \frac{\text{Ethanol, g L} - 1}{\text{Time (h)}} = \frac{\text{Ethanol, g L} - 1}{(\text{Reducing sugar, g L} - 1)\text{x } 0.511} \times 100$$

5. Statistical Analysis

The data were treated statistically by factorial analysis of variance at $P<0.01$ and Duncan's multiple-range test, to determine significant differences between the means. According to statistical analysis best ethanol-fermenting yeasts were chosen in each experimental design in order to perform gas chromatography–mass spectrometry to confirm the presence of ethanol.

E. Gas Chromatography–Mass Spectrometry

Yeasts selected by dichromate oxidation method determinations were performed by gas chromatography–mass spectrometry in order to verify the presence of ethanol because potassium dichromate may react with other products fermentation [25], [26]. For gas chromatography–mass spectrometry was used the Agilent Technologer 6850 Series III with HP5M Sapolar column of 30 meters, using helium as carrier gas and injector temperature of 250°.

F. Preliminary Identification of Selected Ethanol-Fermenting Yeasts

The nine selected yeasts were identified by physiological and morphological standard methods as recommended by Barnett et al. [27] and Winn et al. [28]. Yeasts definitive identification was performed in the clinical laboratory of the

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Hospital Universitario San Ignacioby MicroScan® Rapid Yeast Identification (RYI) panel of DadeBehring.

III. Results and Discussion

A. Pre-Treatments Performed on Feedstock and Saccharification

1. Acid Hydrolysis

In water hyacinth the best reducing sugars release was obtained in the treatment with water hyacinth liquefied suspensions +0.5% sulfuric acid and autoclave sterilization by 60' (P=7.5x10^{-28}); whereas with Brazilian elodea the highest saccharification percentage was obtained with the liquefied suspensions + sulfuric acid at 1% and autoclave sterilization by 60'(P=9.4x10^{-30}) (Fig. 2). The highest reducing sugars concentrations in the water hyacinth and Brazilian elodea hydrolysates were on 13.4 g L^{-1} and 9.5 g L^{-1} in average respectively. Nigam [14] also observed release of 85% sugars from water hyacinth, but with sulfuric acid at 1% v/v before over-liming with calcium hydroxide. Masami et al. [6] found a more efficient hydrolysis with 1% (v/v) sulfuric acid at 121° by 60' in an autoclave, when they tested different sulfuric acid concentrations (0.5-4%) for water hyacinth hydrolysis, as Hossain et al. [29] with a reducing sugar content of 18g L^{-1}. Manivannan et al. [5] observed that water hyacinth saccharification with dilute acid produced a sugars mixture, mainly xylose and its concentration was uploaded from 6 to 10 times when they increased the sulfuric acid concentration from 1% to 10%. There are no published results on Brazilian elodea hydrolysis.

2. Acid Hydrolysis with Detoxification

Saccharification percentages of 37.41% and 65.68% were obtained in water hyacinth and Brazilian elodea, respectively. By comparing this technique results with the acid hydrolysis, reducing sugars production was lower, possibly because the material was not sterilized by autoclave (121°) but it was boiled and according to Redding et al. [30] an increase in temperature increases the production of reducing sugars, reducing this way the acid concentration used in the pretreatment and the process time. Although the detoxification presented loss of different fermentable sugars from 3 to 10% using $Ca(OH)_2$ for over-liming, this procedure is important because it removes or reduces the concentrations of acetic and tannic acids in the hydrolyzates improving the fermentation to ethanol. Therefore it is recommended that the increase of pH above 10 during over-liming be made in very short periods [14], [31].

3. Oxidative Method with Peracetic Acid

The reducing sugars release from water hyacinth and Brazilian elodea were averaged 27.68% and 51.39%, respectively. Abraham and Kurup [15] used on water hyacinth these pre-treatment and microbial cellulases, obtaining saccharification percentages from 19 to 43%. Combined pre-treatments included sodium chlorite, peracetic acid and cellulases allowed a 90% water hyacinth saccharification [32].

4. Alkaline Hydrolysis

Most saccharification was obtained with 10% NaOH in water hyacinth and Brazilian elodea with average values of 85.6% (P=1.21x10^{-8}) and 49% (P= 5.26x10^{-7}) respectively (Fig. 3). A water hyacinth treatment with NaOH at 121° and 60psia for 1h converted 60% of the cellulose to fermentable sugars [33]. Pre-treated with NaOH 1% and crude enzyme induced by *Aspergillus oryzae* showed saccharification rates ranged from 1.41 to 9.03% on sugarcane bagasse, sawdust and water hyacinth[34]. Contrary to this research that obtained with 5% NaOH rates between 9.7 to 22.3% saccharification from water hyacinth, Eshtiaghi et al. [16] observed the highest sugar content released of 0.63% w/w with NaOH concentrations up to 5%. The sugar content was increased to 22.9% when enzymes used as additional treatment. Ahn et al. [35] obtained the highest sugars release from water hyacinth with alkaline-oxidative pre-treatment (7% w/v NaOH and hydrogen peroxide at 2% w/v) and commercial cellulases.

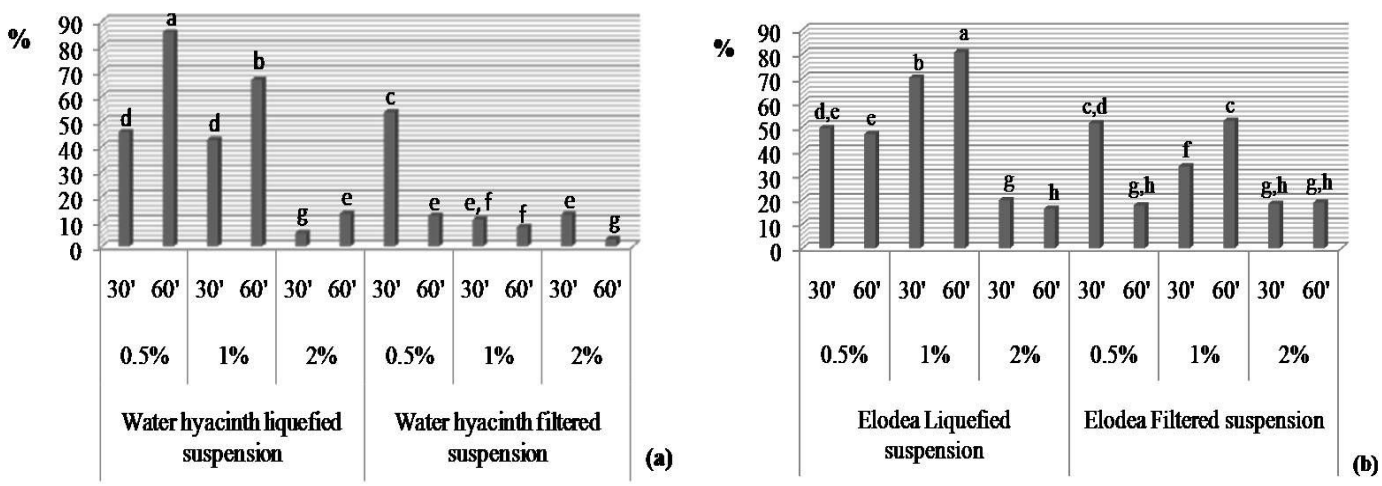

Fig. 2 Saccharification of water hyacinth (a) and Brazilian Elodea (b) treated with different concentrations sulfuric acid and sterilization times. The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test (P< 0.01)

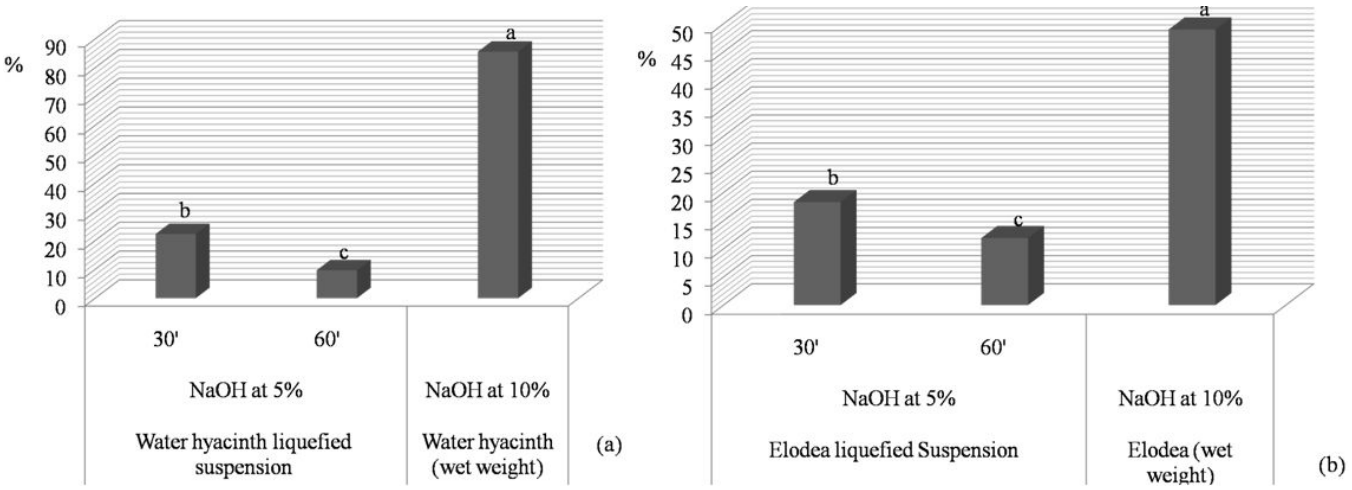

Fig. 3 Saccharification of water hyacinth (a) and Brazilian Elodea (b) by alkaline pretreatment with NaOH The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test (P< 0.01).

TABLE I
SACCHARIFICATION OBTAINED FROM WATER HYACINTH AND BRAZILIAN ELODEA BY BIOLOGICAL PRETREATMENT USING *P. OSTREATUS* AT DIFFERENT CONCENTRATIONS

Aquatic macrophytes	PO-1 (g)	Incubation (days)					
		5	7	10	11	12	13
Water hyacinth P=2.7 x 10^{-25}	**0.9**	41.20 g	44.31 g	45.91 g	45.49 g	43.68 g	42.46 g
	2.7	43.14 g	82.70 c, d	83.62 c, d	77.69 d, e, f	73.35 e, f	69.99 f
	4.5	49.95 g	96.80 a, b	99.82 a	89.34 b, c	85.22 c, d	81.01 c, d, e
Brazilian elodea P=4.4 x 10^{-18}	**0.9**	37.39 e	48.49 d	50.01 d	48.32 d	44.36 d, e	43.25 d, e
	2.7	43.13 d, e	63.58 b, c	70.34 a, b	69.02 a, b, c	63.78 b, c	60.57 c
	4.5	49.23 d	72.84 a	75.61 a	75.53 a	63.91 b, c	60.90 c

The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test (P< 0.01).

5. Biological Hydrolysis

The use of *P. ostreatus* O-1 (PO-1) for water hyacinth treatment showed saccharification rates ranged from 4.1 to 99.8%, being the best treatments (P = 2.7 x 10^{-25}) culture media, those inoculated with 4.5 g of PO-1 and incubated for 7 and 10 days with 96.8% and 99.8% of saccharification, respectively (Table I). In Brazilian elodea, culture media inoculated with 4.5 g and 2.7 g of PO-1 and incubated for 7, 10 and 11 days were the best treatments (P = 4.4 x 10^{-18}) with saccharification percentages from 69 to 75.6%. In both macrophytes the culture media inoculated with 4.5 g of PO-1 and incubated for 10 days showed the higher averages (Table I).

Similar data was obtained by Mena-Espino et al. [36], who used a *P. ostreatus* crude enzymes extract of 10 incubation days and obtained higher saccharification banana residues compared with other treatments. From 9 to 13 incubation days, the authors found high cellulase activity with a maximum peak at 10 days, which explains the sugars release. Abraham and Kurup [15] obtained a 27.5% saccharification with a mixture of *P. florida* crude enzyme extract of 12 h and water hyacinth pre-treated with peracetic acid.

6. Better Pre-Treatment Selection

The treatments comparison selected by statistical analysis of acid, alkaline and biological hydrolysis with the results of the acid hydrolysis with detoxification and oxidative method with peracetic acid from water hyacinth showed significant differences (P=5.17x 10^{-10}) in the saccharification, being the best pre-treatments 4.5g of PO-1 with incubation periods of 7 and 10 days (Table II). In Brazilian elodea, pre-treatments showing increased sugars release were the biological hydrolysis with 4.5g of PO-1 at 10 and 11 incubation days and acid hydrolysis with 1% sulfuric acid and autoclave sterilization by 60' (P= 1.19 x 10^{-11}) (Table III).

TABLE II
STATISTICAL ANALYSIS OF PRE-TREATMENTS EVALUATED IN WATER HYACINTH

Pretreatment	Saccharification (%)
Acid hydrolysis (0.5% H2SO4 and 60' sterilization)	85.65 b
Alkaline hydrolysis (10 % NaOH)	85.59 b
Acid hydrolysis with detoxification	37.41 c
Oxidative method with peracetic acid	27.68 c
Biological hydrolysis (4.5 g of PO-1 and 7 incubation days)	96.80 a, b
Biological hydrolysis (4.5 g of PO-1 and 10 incubation days)	99.82 a

P= 5.17 x 10^{-10}. The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test (P< 0.01).

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

TABLE III
STATISTICAL ANALYSIS OF PRE-TREATMENTS EVALUATED IN BRAZILIAN ELODEA

Pretreatment	Saccharification (%)
Acid hydrolysis (1% H2SO4 and 60' sterilization)	80.69 a
Alkaline hydrolysis (10 % NaOH)	48.99 e
Acid hydrolysis with detoxification	65.58 d
Oxidative method with peracetic acid	51.39 e
Biological hydrolysis (2,7 g of PO-1, and 10 incubation days)	70.34 b, c, d
Biological hydrolysis (2,7 g of PO-1, and 11 incubation days)	69.02 c, d
Biological hydrolysis (4.5 g of PO-1 and 7 incubation days)	72.84 b, c
Biological hydrolysis (4.5 g of PO-1 and 10 incubation days)	75.61 a, b
Biological hydrolysis (4.5 g of PO-1 and 11 incubation days)	75.53 a, b

$P = 1.19 \times 10^{-11}$. The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test ($P < 0.01$).

Only pre-treatment using 4.5g of PO-1 with 10 incubation days had the highest saccharification percentage from both macrophytes, occupying the first and second average in water hyacinth and Brazilian elodea (Tables II and III), therefore this was chosen as plant biomass pre-treatment for ethanol production testing with yeasts isolated from Lake Fúquene. Another criterion was considered to choose the biological process from Brazilian elodea is the knowledge that they are cheaper, require low energy and are more eco-friendly than chemical methods, nonetheless they need longer residence time [2], [10].

B. Isolation of Ethanol-Fermenting Yeasts from the Lake Fúquene Watershed

From ninety-five yeast that were isolated in the sampling, 49 strains grew in water hyacinth and Brazilian elodea as sole carbon source. The iodoform test on the 49 yeast strains showed that 27 strains didn't produced alcohol in both macrophytes. Added to these strains, 6 additional strains were negative for ethanol in Brazilian elodea. The yeast strains that showed the highest ethanol precipitated (+++) from water hyacinth were coded as L1, LT, L16, L23, LR, L30 and L45. In Brazilian elodea, the strains showed ethanol precipitated between 2 and 3 crosses were L1, LT, L17, L23, L23a and L30. These strains were chosen for testing ethanol production by dichromate oxidation method. Fig. 4 shows two different reactions to iodoform test by yeasts that grew on media containing water hyacinth at 10%.

Masami et al. [6] isolated 624 yeast strains from 28 hydrospheres in Japan and only 13 strains grew on water hyacinth hydrolysate agar plates. These yeasts produced ethanol from 0.9 to 2.17gL^{-1} in water hyacinth hydrolysates. While in two lakes in Bangladesh 16 yeasts were initially isolated chosen for their ability to anaerobic sugars utilization and only three strains were selected as producers of ethanol from water hyacinth and Azolla (*Azolla pinnata*) hydrolysates [29].

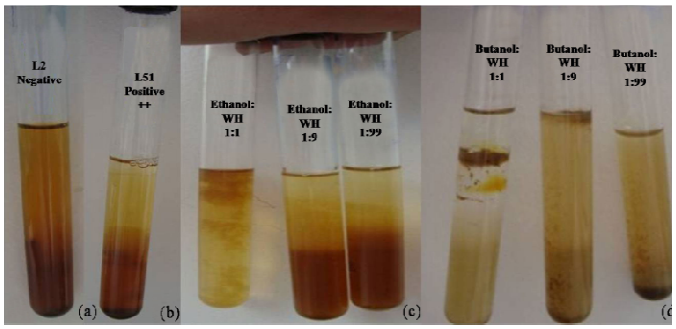

Fig. 4 Qualitative ethanol determination in medium with water hyacinth at 10% as measured by Iodoform test (a) Negative Iodoform test (b) Positive Iodoform test (+ +) (c) Ethanol Control (d) Butanol Control

C. Ethanol Production by Selected Yeast Strains

All yeasts produced ethanol from hydrolysates of both macrophytes as unhydrolyzed biomass. The ethanol production was higher with water hyacinth than Brazilian elodea, with a ethanol final mass in the aqueous phase (Me) from 0.07 to 1.84 g, volumetric productivity (Qp) from 0.38 to 0.80g L^{-1} h^{-1}, product yield coefficient (Yp/s) from 0.05 to 0.29g g^{-1} and fermentation efficiency (n) from 9.4 to 55.8% (Table IV). The fermentation of water hyacinth hydrolyzate using L30 strain was the best treatment according to Duncan's Multiple Range Test (Table IV). Ahn et al. [35] found a maximum Qp of 0.77g L^{-1} h^{-1} similar to that obtained in this study (0.8g L^{-1} h^{-1}) by using *S. cerevisiae* to ferment water hyacinth with an alkaline- oxidative pretreatment and enzymatic saccharification with commercial cellulases. Yp/s ranged from 0.19 to 0.25g g-1 with Qp from 0.010 to 0.22g L^{-1} h^{-1}, it is lower than the values found in this investigation, were observed in water hyacinth hydrolyzate by chemical methods (H_2SO_4 or NaOH) and fermented by different yeasts [5], [14], [37], [38]. Higher ethanol productions than those found in this investigation with Yp/s of 0.32 g g^{-1} and 0.42 g g^{-1} have been observed by Hossain et al. [29] by fermenting water hyacinth hydrolyzate with 1% sulfuric acid using *S. cerevisiae* and *Kluyveromyces marxianus*, and Kumar et al. [31] when working with acid hydrolyzate of water hyacinth fermented by *Pichia stipitis*. Nigam [14] observed that the fermentability of water hyacinth acid hydrolysate was increased by boiling and overliming with calcium hydroxide in combination with sodium sulfite. Yp/s of 0.19g g^{-1} and Qp of 0.018g L^{-1} h^{-1} with the acid hydrolysate was increased to Yp/s of 0.35g g^{-1} and Qp of 0.18g L^{-1} h^{-1}.Furthermore, Satyanagalakshmi et al. [9] reported maximum efficiency (59.3%) higher than that obtained in this study (55.8%) with water hyacinth hydrolyzed with sulfuric acid at 4% (w/v) and enzymatic saccharification with commercial cellulases.

The results variability in the ethanol production from water hyacinth harvested from various water sources can be explained by differences in vegetal biomass chemical composition, physical, chemical and/or biological pre-treatments employed for saccharification, the yeast species used and its concentration, physicochemical parameters such

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

as temperature, aeration, pH and fermentation time [5],[14], [16], [21], [22], [31], [37].

TABLE IV
ETHANOL PRODUCTION AND FERMENTATION PARAMETERS PRESENTED BY THE 9 YEASTS STRAINS GREW IN MEDIA WITH WATER HYACINTH HYDROLYSATE AND UNTREATED BIOMASS

Yeast	Culture medium	Me (g) P=2.17 x 10^{-36}	Yp/s (g g^{-1}) P=2.48 x 10^{-26}	Qp (g L^{-1} h^{-1}) P=2.17 x 10^{-36}	Ƞ (%) P=2.48 x 10^{-26}
L1	WH Hydrolysate	1.20	0.19	0.52	37.6
	WH (untreated)	0.07	0.05	0.03	9.4
LT	WH Hydrolysate	1.18	0.19	0.51	37.4
	WH (untreated)	0.17	0.11	0.07	21.9
L16	WH Hydrolysate	0.87	0.25	0.38	49.7
	WH (untreated)	0.39	0.14	0.17	26.8
L23	WH Hydrolysate	1.01	0.16	0.44	31.5
	WH (untreated)	0.14 g	0.09	0.06	18.4
LR	WH Hydrolysate	0.94	0.19	0.41	37.5
	WH (untreated)	0.14 g	0.09	0.06	17.3
L30	WH Hydrolysate	1.84	0.29	0.80	55.8
	WH (untreated)	0.16	0.10	0.07	20.5
L45	WH Hydrolysate	0.91	0.14	0.39	28.3
	WH (untreated)	0.19 g	0.12	0.08	23.7

Me: ethanol final mass in the aqueous phase. Y p/s: Product (ethanol) yield coefficient g/g total reducing sugars. Qp: Volumetric productivity. □ %: fermentation efficiency.

The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test (P < 0.01).

TABLE V
FERMENTATION PARAMETERS PRESENTED BY YEASTS GREW ON BRAZILIAN ELODEA HYDROLYSATES AND UNTREATED BIOMASS OVER A PERIOD OF 48 HOURS

Yeast	Culture medium	Me (g) P=9.2 x 10^{-29}	Yp/s (g g^{-1}) P=8.6 x 10^{-22}	Qp (g L^{-1} h^{-1}) P=9.2 x 10^{-29}	n (%) P=4.6 x 10^{-17}
L1	BE Hydrolysate	0.37 f	0.11 c, d	0.16 f	21,0 c
	BE (untreated)	0.13 i	0.05 f	0.06 i	10.1 e
LT	BE Hydrolysate	0.50 c	0.15 b	0.22 c	28.5 b
	BE (untreated)	0.13 i	0.05 f	0.06 i	10.1 e
L17	BE Hydrolysate	0.56 b	0.16 a	0.24 b	32.2 a
	BE (untreated)	0.18 h	0.07 e	0.08 h	13.9 d
L23	BE Hydrolysate	0.42 d	0.12 c	0.18 d	23.2 c
	BE (untreated)	0.15 i	0.06 e, f	0.07 i	11.7 d, e
L23A	BE Hydrolysate	0.63 a	0.17 a	0.27 a	34.0 a
	BE (untreated)	0.40 e	0.11 c	0.17 e	22.6 c
L30	BE Hydrolysate	0.34 g	0.10 d	0.15 g	19.6 c
	BE (untreated)	0.15 i	0.06 e, f	0.07 i	11.7 d, e

Me: ethanol final mass in the aqueous phase. Y p/s: Product (ethanol) yield coefficient g/g total reducing sugars. Qp: Volumetric productivity. n %: fermentation efficiency

The data represent averages of three replicates. Values with different letters show significant difference as determined by Duncan's Multiple Range Test (P < 0.01).

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

The yeasts from Brazilian Elodea produced 0.13 to 0.63g of ethanol final mass in the aqueous phase, the Qp ranged from 0.06 to 0.27 g L^{-1} h^{-1}, Yp/s between 0.05 -0.17g g^{-1} and n ranged 10.1 to 34% (Table V). L23a strain had the highest ethanol final mass in the aqueous phase and productivity on Brazilian elodea hydrolysates. This yeast and L17 strain obtained the best results as to fermentation parameters, Yp/s and n (Table V). There are no published results on ethanol production from Brazilian elodea. Most studies on ethanol production in aquatic plants have focused on water hyacinth, although there are reports on water lettuce (*Pistia stratiotes* L.) and water velvet (*Azolla pinnata* R. Br.) with maximum yields of 0.16g g^{-1} and 0.20g g^{-1} respectively [29], [39]. Some authors claim that aquatic plants in the future will be the next promising renewable energy resource to replace feedstock currently used because they have a higher primary productivity, not compete with agricultural crops for land and inputs, have low lignin concentrations and its use in the case of invasive aquatic macrophytes represent a return of energy to the efforts made for its removal in control programs [4], [7], [39].

The culture media fermented by L30 and L23a strains showed the highest Me values from the aquatic macrophytes, water hyacinth and Brazilian elodea, showing significant differences with other treatments; for that reason these yeasts were selected to confirm the ethanol presence by gas chromatography–mass spectrometry to be a highly selective and sensitive method[26].

D. Gas Chromatography–Mass Spectrometry

The chromatograms confirm the ethanol production, with a retention time of 1.48 minutes, by L30 and L23a strains from the aquatic macrophytes (hydrolysed and untreated biomass) and also show the presence of other substances such as carbon dioxide, butanol, propanol and ethyl acetate (Fig. 5) that can be produced during alcoholic fermentation and found in different distilled liquors [25], [40]. Carbon dioxide and butanol were produced in both macrophytes, while propanol was presented in water hyacinth and ethyl acetate in Brazilian elodea (Fig. 5). An important finding is the butanol presence in both macrophytes due to the current interest in the production of this alcohol by fermentation as a direct replacement of gasoline or fuel additive. For some authors, the butanol is superior to ethanol as a fuel additive because it has more energy content, 22% oxygen, lower volatility, less hygroscopic and less corrosive [41], [42].

The volatile compounds found can react with potassium dichromate [25], therefore it is recommended the ethanol extraction from yeast culture broth with non-alcoholic solvents before using potassium dichromate for the determination of this compound as Hyun-Beom et al. [26] when using Tri-n-butyl phosphate (non-alcoholic solvent) obtained similar results to gas chromatography.

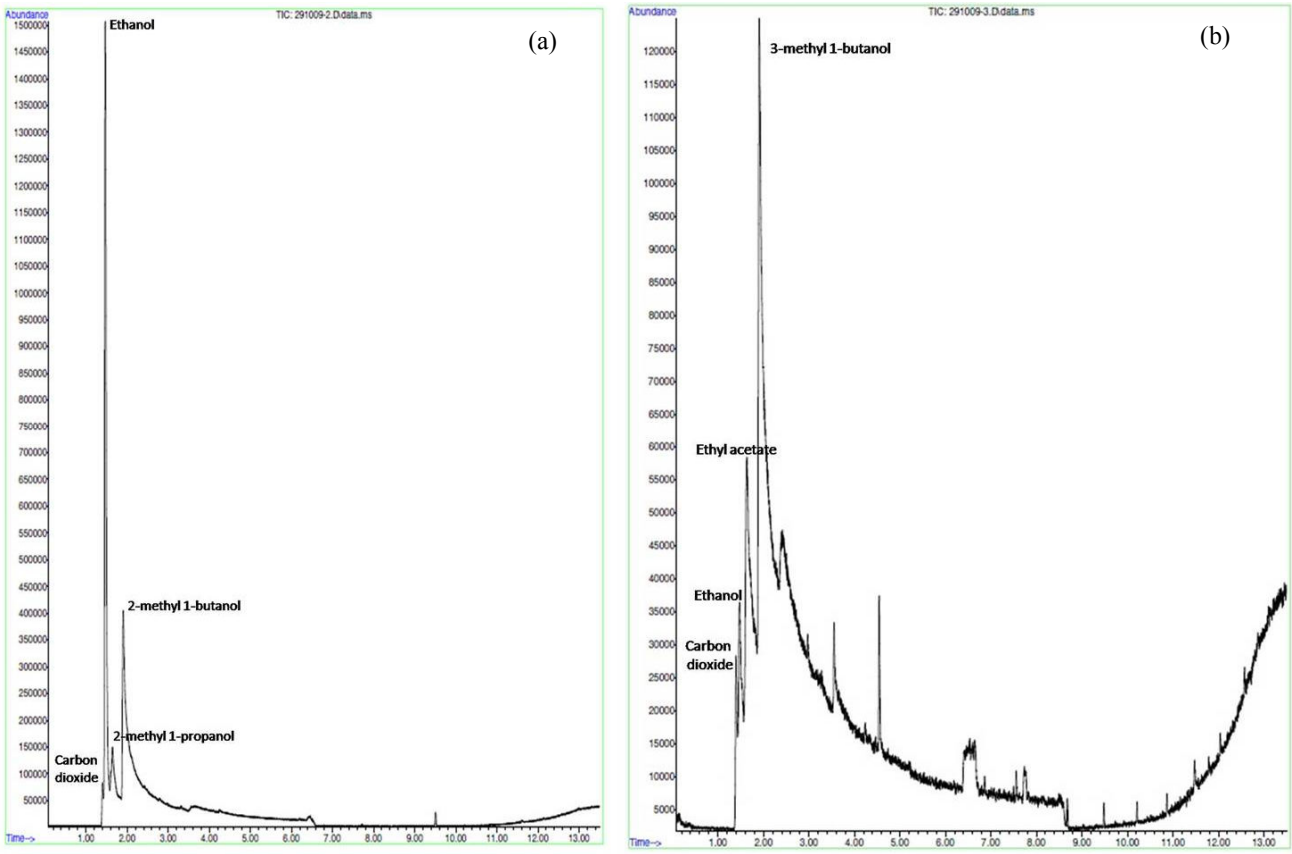

Fig. 5 Chromatograms from liquid media containing water hyacinth hydrolysate and untreated Brazilian elodea fermented by strain L30 (a) and L23a (b), respectively

International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311

E. Preliminary Identification of Selected Ethanol-Fermenting Yeasts

The nine selected yeasts were close to the genera *Candida* spp., *Brettanomyces* sp. (L1 strain) and *Hansenula* spp. L30 and L23a strains that produced the greatest ethanol amount on the culture media with macrophytes hydrolysates are close to *C. albicans* and *C. lusitaniae* respectively. Other selected yeasts were preliminarily identified as belonging to *Hansenula* spp. (L16 and L17 strains), *H. polymorpha* (L45 strain), *C. parasilopsis* (L23 and LR strains) y *C. lusitaniae* (LT strain). Candida spp. have been used to produce ethanol from water hyacinth hydrolysates [1], [5], [6], [38]. *H. polymorpha*has been reported recently as a producer of ethanol from glycerol [43].

IV. Conclusions

From the biological hydrolysis with *P. ostreatus* of Lake Fúquene invasive aquatic plants, biofuels were produced such as ethanol and butanol using yeasts isolated from this water body which has a remarkable importance to regional economy for the Colombian departments of Cundinamarca and Boyacá. The use of water hyacinth from Lake Fúquene as bio-energetic feedstock could be competitive given the results obtained in the ethanol production and that mechanical removal of this macrophyte costs about $43 per dry ton (unpublished data). Beside, this aquatic plant doesn't need inputs nor changes to land use as agricultural crops currently used in Colombia for bio-ethanol production does.

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International Science Index, Environmental and Ecological Engineering Vol:7, No:1, 2013 waset.org/Publication/17311