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BIOCHEMICAL CONVERSION OF ACID-PRETREATED WATER HYACINTH (*Eichhornia Crassipes*) TO ALCOHOL USING *Pichia Stipitis* NCIM3497

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ABSTRACT:

Cleaning and removal of water hyacinth from lakes and various historical places government spends lacks of rupees per year. High rate of propagation and easy availability, water-hyacinth could be used as a renewable carbon source for alcohol (fuel ethanol) production. Water-hyacinth's (*Eichhornia crassipes*) hemicellulose acid hydrolysate has been utilized as a substrate for alcohol production using *Pichia stipitis* NCIM3497. Acid hydrolysis were carried out by using (1% v/v) sulfuric acid. Perhydrolysate was detoxified, boiled and overlimed up to pH 10.0 with NaOH to produce acid hydrolysate. Acid hydrolysate had higher fermentability than perhydrolysate. Freshly prepared acid hydrolysate was directly used in fermentation broth as substrate. Fermentation was carried out for 40 hrs. Total reducing sugars were 51.3 gm/lit and at the end of 40 hrs the fermentation it was 9.2 gm/l. About 82.06% of the available sugars were utilized within 40 hrs. At the end of fermentation, alcohol was estimated by of K2Cr2O7 method, which is 19.2 gm/lit and an alcohol yield of 0.45 gm/gm sugar utilized. Considering the cost various feed stock, use of water- hyacinth which is freely available in large amount as a substrate, offers an opportunity to reduce the cost of fuel alcohol production.

Keywords: Pichia stipitis; Water hyacinth; Alcohol; hemicellulose acid hydrolysate**.**

[I] INTRODUCTION

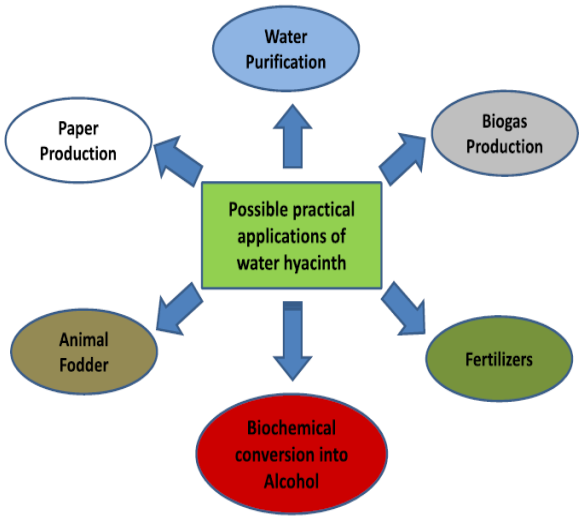
This part should be in Water hyacinth is an invasive species, which invades fresh water habitats and is listed along with some of the worst weeds [1]. Three types of free floating aquatic weeds found in the Rankala lake (Kolhapur, India (MH)) were water-hyacinth (*Eichhornia crassipes*) **[Figure-1]**, water lettuce (*Pistia stratiotes*) and the water carpet, azolla (*Azolla azolla*) were the major culprits for reducing healthy environment of the lake. However,

calculated total cost of manual eradication of submergent and emergent aquatic plants as well as weeds and floating aquatic plants and KMC (Kolhapur Municipal Corporation) spending cost for cleaning and removal of water hyacinth in the lake body and along the lake periphery is 62.7 lacks/ year by removing about 6000 MT of wet hyacinth mass (as per data gathered from KMC). Water hyacinth is low in lignin content (3.50%) and hemicellulose (48.70%), cellulose (18.20%), crude protein (13.30%). It was also found by calculation about 300 MT dry biomass of water hyacinth was available after each year after cleaning the Rankala lake, Kolhapur, India (MH).
There are number of possible application uses for the water hyacinth **[Figure-2]**, some which have been developed and others which are still in their infancy or remain as ideas only. It can be used in paper production and also in grease- proof paper manufacture [2].

Fig: 1. Water-hyacinth (*Eichhornia crassipes*)

The mixture of cowdung and water hyacinth slurry has proven to produce more biogas than when used alone [3]. Also, it contains high nitrogen level; Water hyacinth can be used on the land either as a green manure or as compost. In Malaysia fresh water hyacinth is cooked with rice bran and fishmeal and mixed with copra meal as feed for pigs, ducks and pond fish.

Fig: 2. Possible applications of water hyacinth.



Water hyacinth has low lignin, which means the cellulose and hemicellulose are more easily converted to fermentable sugar thus resulting in enormous amount of utilizable biomass for the biofuel industry. As aquatic plants do not compete with land resources used in arable food crop cultivation and thus are an incentive factor when it comes to biofuel production [4]. However, there are no exact figures available for bio-alcohol production from water hyacinth [5]. The energy crisis of the 1970s renewed interest in alcohol production for fuels and chemicals. Ethanol is used in vehicles either as a sole fuel or blended with gasoline. As an oxygenated compound, ethanol provides additional oxygen in combustion, and hence obtains better combustion efficiency. Since the completeness of combustion is increased by the present of oxygenated fuels, the emission of carbon monoxide is reduced by 32.5% while the emission of hydrocarbons is decreased by 14.5%. Pentose utilizing yeast strain like *Pichia stipitis* can be use for production of alcohol from D-Xylose.
In this study, acid hydrolysis carried by sulphuric acid and hydrolysate detoxified by overliming with NaOH. Resulting C5 and C6 sugar solution directly used as substrate for fermentation for

alcohol production by *Pichia stipitis* NCIM3497 under batch conditions.

[II] MATERIALS AND METHODS

2.1. Microorganisms and media

Pichia stipitis NCIM3497 used in this study was grown and maintained at 30 +/-0.2 and 4 ℃, respectively, on agar slants **[Figure-3]** containing agar media composition is given in **[Table-1].**

Fig: 3. *Pichia stipitis* NCIM3497 was grown and maintained on agar slants.

The culture of above yeast was obtained from National Collection of Industrial Microorganism (NCIM), Pune, India (MH).

Constituents	gm/l
D-Xylose	20
Yeast extract	3
Malt extract	3
Peptone	5
Agar	20
pH	5.0+/-0.2

Table: 1. Media composition for *Pichia stipitis* NCIM3497 growth and maintenance.

2.2. Seed culture preparation

The composition of medium used for inoculum preparation **[Table-2].**

The media were sterilized by autoclaving at 120°C for 15 min. D-Xylose and D-glucose were autoclaved separately at 110°C for 10 min. After sterilization and cooling, the solutions were mixed to form a complete medium prior to inoculation. To prepare the inoculum, a 250 ml Erlenmeyer flask containing 50 ml medium was inoculated from a fresh agar slant, and incubated at 30 +/-0.2°C an a rotary shaker at 250 rpm. The cells were grown for 20 hrs.

Constituents	gm/l
D-Xylose	50
Yeast extract	3
Malt extract	3
Peptone	5
D-Glucose	5
pH	5.0+/-0.2

Table: 2. Inoculum preparation media composition for *Pichia stipitis* NCIM3497.

2.3. Inoculum preparation

The 5 ml of this culture was transferred to 100 ml medium of the same composition in 500 ml Erlenmeyer flasks. The culture was grown again for 20 hrs under conditions similar to those described above and the broth was centrifuged at 10,000×g for 10 min. The cell pellet was washed and suspended in 100 ml sterile distilled water.

2.4. Substrate preparation

Fresh water-hyacinth plants with long stem were collected from Rankala Lake, Kolhapur. Collected water hyacinth washed to remove adhering dirt and chopped in small pieces, dried in sunlight **[Figure-4]**, and powdered in pulp forming machinery.

Fig: 4. Dried water-hyacinth plant with long stem were collected from Rankala Lake, Kolhapur.

The average composition of water-hyacinth is summarized in **[Table-3].**

Constituents	% of wet weight
Total Solids	5.0-7.6
Moisture	92.8-95
Volatile solids (as % of TSs)	4.2-6.1
Organic component	(% TSs)
Hemicellulose	48.7
Cellulose	18.2
Lignin	3.5
Crude Protein	13.3

Table: 3. Average composition of water-hyacinth [5].

2.5. Acid hydrolysate preparation

Hemicellulose acid hydrolysate was prepared by refluxing the dried powder with 10 volumes of (1% v/v) sulfuric acid for a period of 8 hrs, in a glass lined reactor, stirred at 250 rpm. Pre-hydrolysate was filtered to remove the unhydrolysed residue, and washed with warm water (60°C). The filtrate and washings were pooled together.

2.6. Detoxification of hemicellulose acid hydrolysate

By heating: Hemicellulose acid hydrolysate (2 lit) was heated to 100°C, held at that temperature for 15 min to remove or reduce the concentration of volatile components. Any loss in volume during boiling was replaced with heated distilled water.

Overliming with NaOH: Prehydrolysate was then overlimed with solid NaOH up to pH 10.0, filtered to remove insolubles and then reacidified to pH 6.0, with 1 N sulfuric acid. The filtrate was concentrated under vacuum at 25°C to achieve (5–6% w/v) of xylose concentration.

Storing at -10°C: The resulting solution was stored at −10°C for further use as substrate. The concentrated hemicellulose acid hydrolysate used for fermentation studies having higher percentage of D-Xylose, also contains other components like D-Glucose, and D-Galactose.

2.7. Alcohol fermentation

Prepared inoculum of *Pichia stipitis* used for inoculation of 2 lit concentrated hemicellulose acid hydrolysate supplemented with the defined media ingredients **[Table-4]** [5]. Fermentations were carried out in a shaker flask (working volume 5 lit). Fermenter (shaker flask) and the media were sterilized by autoclaving at 120 °C for 20 min. The pH was maintained at 6.0+/-0.4, with 1 N HCl and 1 N NaOH. The fermentation temperature was kept at a constant value of 30+/-0.4°C by a temperature control incubator. The broth was kept under agitation at 250 rpm, for 40 hrs.

Components	gm/l Acid Hydrolysate
Yeast extract	1
(NH4)2HPO4	2
MgSO4·7H2O	0.25
(NH4)2SO4	1
Trace element solution	1 ml/l

Table: 4. Fermentation medium supplement composition [5]. The trace element solution contained (gm/l): CuSO4.H2O,2.5; FeCl3.6H2O,2.7; MnSO4.H2O,1.7; Na2Mo2O4.2H2O,2.42;ZnSO4.7H2O,2.87;CaCl 2.6H2O,2.4; Medium pH 6.0+/-0.2.

2.8. Analytical methods

Bial test a colorimetric method was carried for analyzing pentose (D-xylose) concentration in that acid hydrolysate. In this analysis 10 ml of acid hydrolyaste sample is treated with 3 ml of solution A (150 mg orcinol + 50 ml HCl) and then it was heated up to boiling to complete the reaction which gives blue-green color after 10 min. incubation period and determine the optical density 510 nm using spectrophotometer[6]. Total reducing sugars (TRS) were estimated by dinitrosalicylic acid (DNSA) method of [7]. For alcohol analysis $K_2Cr_2O_7$ and sulphuric acid method [8] were used, which is a spectrophotometric assay in which standard plot

of optical density versus concentration of pure alcohol were plotted. In this analysis 1 ml of alcohol sample is treated with 4 ml of $K_2Cr_2O_7$ and then add 1ml of concentrated sulphuric acid to complete the reaction which gives brownish green color after 30 min. incubation period and determine the optical density 600 nm using spectrophotometer. Then calculate percentage of alcohol by using this standard plot.

[III] RESULTS

D-Xylose concentration in acid hyrolysate were estimated by Bial test, in which optical density versus concentration of standard D-Xylose standard plot prepared and calculated D- Xylose extraction from water hyacinth which is 39.2 gm/lit of acid hydrolysate. Before inoculation total reducing sugars were 51.3 gm/lit and at the end of 40 hrs fermentation it was 9.2 gm/l. About 82.06% of the available sugars were utilized within 40 hrs. At the end of fermentation, alcohol was estimated by of $K_2Cr_2O_7$ method at 600 nm, which is 19.2 gm/lit and alcohol yield was 0.45 gm/gm sugar utilized.

[IV] DISCUSSION

In case of cost calculation, KMC spending lacks for cleaning the Rankala Lake each year, producing 300 MT of dry biomass. There is large scope for using this waste biomass for alcohol/ alcohol production. Alcohol production from waste biomass could reduce cost of cleaning the lake and also producing waste to energy. Various biomass Pretreatment methods are available, in case of dilute acid hydrolysis need to operate at high temperature and also giving low sugar yield. This method can lead to production of some toxic product possible reduction in alcohol yield. *Pichia stipitis* is suitable for high pentose sugar substrate, but giving lower alcohol yield in comparison to *Saccharomyces cerevisiae* or *Zymomonas mobilis*. *Saccharomyces cerevisiae* or *Zymomonas mobilis* utilize C6 sugars or sucrose highly efficiently but their inability to utilize C5 sugars make them inappropriate candidates for bio-refineries [9].

[V] CONCLUSION

Increase demand of alcohol and brewery products ultimately require high amount of corn and other grains, so they are not available in proper proportion for the daily food , it increases the cost of food products , so non useful plant like water hyacinth can be better source for alcohol production. It has confronted with the oil crisis as well as many parts in the world and is seeking for other challenging energy source. Alcohol, an environmentally friendly fuel, which can be produced from various renewable biological waste materials like water hyacinth, can be a solution for an agricultural country like India.

This study proved water hyacinth has a potential, renewable and low cost biomass for alcohol production on a commercial scale. Present cost effectiveness of respective process at commercial scale need to be standardized, and then water hyacinth biomass could be a better substrate source for alcohol production.

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