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Production And Characterization Of Ethanol Derived From Enzymatic Hydrolysis Of Water Hyacinth Biomass Using *Saccharomyces Cerevisiae*

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ABSTRACT:

The water hyacinth is a major aquatic plant concern. Since all attempts to limit this plant's growth and spread failed, "Eradication through utilization" is being sought. Current research uses Baker's Yeast to generate ethanol from water hyacinth. Baker's Yeast digests water hyacinth material to make ethanol. The main goal is to reduce the cost of manufacturing ethanol from water hyacinth and determine if it is commercially viable. This study found that water hyacinth is the most abundant aquatic plant for ethanol generation. This study investigates using Baker's Yeast to produce bioethanol from water hyacinth. The current study's microbial inoculants showed bioethanol production potential. This work makes water hyacinths more valuable and can be removed from all waterways.

Keywords: Production, Characterization, Ethanol, Enzymatic Hydrolysis, Water Hyacinth, Biomass and Baker's Yeast.

INTRODUCTION:

The consumption of fossil fuels has raised widespread concern regarding the environment and global economy. Excessive utilization of fossil fuels is a major factor in the occurrence of global warming and the elevation of atmospheric carbon dioxide concentrations. Governments worldwide have established policies that promote biomass as a future energy source in order to minimize their reliance on fossil fuels and meet the carbon dioxide reduction objectives of the Kyoto Protocol. Given these circumstances, it is imperative that we shift towards the utilization of bioethanol as a renewable and environmentally-friendly energy alternative. Recent research has emphasized the utilization of non-edible biomass, such as lignocelluloses,

celluloses, and marine phytoplankton, as raw materials. This approach deviates from the utilization of first-generation biomass, such as starch and sugar biomass (1, 2).

The operation of large-scale facilities for the production of cellulosic ethanol is hindered by several barriers, including the expensive transportation costs of feedstock, substantial capital investment, and the requirement for technical competence. However, using agricultural leftovers to produce ethanol can partially alleviate this problem. The aquatic plant known as water hyacinth (*Eichhornia crassipes*), which is considered a weed, was initially identified in West Bengal in the early 1890s. Currently, it is widespread over India, except for Kashmir, the dry western area of Rajasthan, and the wild northern regions. The proliferation of this tropical plant in large water bodies leads to a decrease in biodiversity, obstruction of rivers and drainage systems, modification of water chemistry, depletion of dissolved oxygen, and environmental deterioration. According to Ganguly et al. (2012), the plant has the ability to endure changes in water level, variations in flow velocity, nutrient availability, and pH that occur over different seasons (3).

The use of water hyacinth as the substrate for bioethanol production presents several benefits. According to Poddar et al. (1991) and Gressel (2008), water hyacinth has a high concentration of cellulose and hemicellulose, while the amount of lignin is very low. The lignocellulose can be enzymatically transformed into fermentable sugar more efficiently, leading to a significant amount of biomass that can be used for bioethanol production. Moreover, this plant is aquatic, hence eliminating any competition with food crops for cultivable land. Commercial cultivation of this plant is highly beneficial since it has an extremely fast growth rate of 60-100 tonnes per hectare per year. However, it is essential to decompose and separate lignin and hemicellulose from the cellulosic biomass, as cellulose components are commonly surrounded by these substances. Therefore, it is necessary to use the correct method of preparing the biomass in order to speed up the process of breaking down cellulose and converting it into ethanol (4, 5).

MATERIALS AND METHOD:

Chemicals and Reagents:

- **For pretreatment:**

Sulphuric acid, Hydrochloric acid, Sodium hydroxide, Potassium hydroxide, Distilled water.

- **For enzymatic hydrolysis**

Pretreated and Dried Water Hyacinth Biomass, Accellerase® 1500 Enzyme, Citrate Buffer (50 mM, pH 4.8).

- **For sugar estimation:**

Dinitrosalysilic acid (DNSA), Distilled water, D glucose.

- **For inoculum preparation:**

Yeast *Saccharomyces cerevisiae* (commercially available Baker's yeast), Yeast extract, Peptone, Dextrose, Urea, Magnesium sulphate.

- **For ethanol determination by potassium dichromate reagent:**

Potassium dichromate, Distilled water, Anhydrous ethanol.

- **For ethanol quantification by gas chromatography:**

Distilled water hyacinth ethanol as sample, Ethanol Standard Solutions (for calibration curve), Internal Standard Solution (e.g., n-propanol).

Harvesting of Water hyacinth:

Water Hyacinth plants were harvested from river Gadhi, Panvel region of Maharashtra, gathered at various periods from 3 different locations and examined.

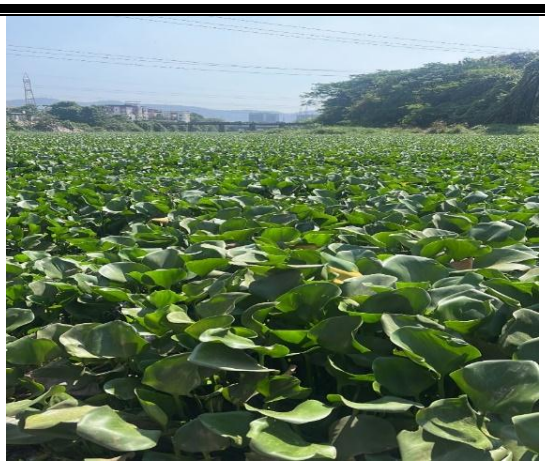

Figure 1 Fresh water hyacinth plants at river Gadhi, Panvel-Maharashtra.

Preparation of Water Hyacinth Biomass:

It is critical to wash the harvested water hyacinth plant well under running water to remove any dirt or other impurities. Then, after chopping it into little pieces, let it out in the sun for 7 or 8 hours to dry off. Using a hot air oven, the dried sample was heated to 50°C for two hours. The sample was carefully dried before being ground with a motor and pestle. It was then filtered through a 1.0 mm screen and kept in sealed plastic containers at room temperature until needed (6).

Figure 2 Harvested, washed, chopped, dried and powdered water hyacinth

Extraction of Extractives:

The dried water hyacinth biomass weighed 2.5 grammes and was placed within a cellulose thimble. One hundred fifty milliliters of acetone were used as the solvent in the Soxhlet extraction method. For a 4-hour run, the boiling and rising phases were set at 70°C for 25 minutes each. The sample was first allowed to dry naturally at ambient temperature and then dried in a convection oven at 105°C to get a consistent weight. The extractives content was determined by calculating the weight differential (%w/w) between the biomass with and without extractives (7).

Chemical Pretreatment:

Pretreatment is a vital step in the process of converting biomass into high-value products such as sugar and ethanol. To address the resistance of lignocellulosic biomass and promote its decomposition into cellulose, hemicellulose, and lignin, several methods of pretreatment and hydrolysis are used to convert the biomass into sugar that may be fermented. Pretreatment of biomass is conducted to separate the lignin from the cellulose and hemicellulose components present in its complicated structure. This allows the enzymes to reach the sugars in the holocellulose fraction, which can

subsequently be hydrolyzed to create ethanol. Chemical pretreatment of biomass involves breaking down the structure of the biomass by interacting with the intra- and interpolymer connections of its main organic components. The water hyacinth powder underwent pretreatment using water, acid, and alkali to facilitate the breakdown of lignocellulosic biomass into fermentable sugars. To achieve the best possible outcomes, we prepared all solutions and conducted exams in three sets of three (8).

Enzymatic hydrolysis of Chemical pretreated water hyacinth Biomass:

The commercial enzyme Accelerate 1500, used for enzymatic hydrolysis to convert cellulose and hemicellulose into fermentable sugars, consisted of cellulose, xylanase, and beta-glucosidase. The polysaccharides in the water hyacinth biomass undergo enzymatic hydrolysis utilizing the Accellerase 1500 enzyme blend, resulting in the breakdown of complex carbohydrates into simpler sugars. Measure precisely 0.5 grammes of the prepared powdered water hyacinth biomass. Make a solution of 50 milliliters of citrate buffer with a concentration of 50 Millimolar and a pH of 4.8. The Erlenmeyer flask should be supplemented with 250 mL of the measured biomass. In addition, pour the 50 mL of citrate buffer into the vial and then fill it with the biomass. Dispense 0.5 mL of the Accellerase® 1500 enzyme preparation into the bottle. Insert the flask into an oscillating incubator programmed to rotate at a speed of 150 revolutions per minute and maintain a temperature of 50°C. Collect samples at specified intervals (e.g., 0, 12, 24, 48, and 72 hours) throughout the 72-hour reaction period. On each occasion, withdraw a 2 mL sample from the reaction mixture. To immediately halt the enzymatic action, subject the sample to boiling for a duration of five minutes. To separate the liquid from any solid residue, subject the sample to centrifugation at a speed of 10,000 revolutions per minute for a duration of ten minutes. The amount of reducing sugar in the supernatant was determined (9).

Estimation of sugars by DNSA Method:

DNSA (3, 5 dinitrosalicylic acid) was used to estimate reducing sugar in untreated, chemically pretreated, and enzymatically hydrolyzed water hyacinth hydrolysate. The alkaline 3,5-dinitrosalicylic acid solution combines with reducing sugars to form 3-amino-5-nitrosalicylic acid. Reducing sugars have free aldehyde or ketone groups. An aldehyde or ketone group lowers 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid when reducing sugar combines with it. This reaction reduces DNSA and oxidizes reducing sugar. Yellow to orange-red when DNSA is reduced. Sample lowering sugar concentration determines colour

intensity. A spectrophotometer measures the absorbance of the coloured solution at 540 nm. A standard curve is created using known decreasing sugar concentrations. By comparing absorbance to the standard curve, the unknown sample's reducing sugar content is calculated (10).

Fermentation of water hyacinth hydrolysate:

Using microbes, water hyacinth hydrolysate may be fermented into ethanol, butanol, or other biofuels and chemicals by converting the sugars in the hydrolysate. A crucial and widely utilised bacterium in fermentation processes is Saccharomyces cerevisiae, more often known as baker's yeast (11). Baking, brewing, and biofuel manufacturing are just a few of the many industries that rely on yeast due to its ability to convert glucose, fructose, and sucrose into ethanol and carbon dioxide. The yeast cells perform fermentation through a series of biochemical reactions collectively known as glycolysis, followed by the conversion of pyruvate into ethanol and CO_2.

$$(C_6H_{12}O_6) \rightarrow 2\ (C_2H_5OH) + 2\ CO_2$$

Ethanol Production:

The water hyacinth hydrolysate, which consists of acid, alkali, and enzyme, was autoclaved at 121°C for 15 minutes to kill any bacteria that might be present before fermentation. The hydrolysates were combined with a 10 ml inoculum of S. cerevisiae, which is well-known for its high bioconversion efficiency into ethanol, in 250 ml Erlenmeyer flasks. Aeration and mixing were adequately achieved by extending the fermentation process to 72 hours at a temperature of 30 ± 0.5 °C per case, while the rotation speed was maintained at 150 rpm. From the 12th to the 72nd hour, samples were taken at 12-hour intervals throughout the fermentation process. The amount of ethanol produced and the leftover sugars in the broth were the targets of these samples. Sterile syringes and needles are used to remove samples in an aseptic manner. Make note of and double-check the fermentation solution's pH at each stage. Three separate experiments were carried out for each (12, 13). The volumetric ethanol productivity (QP) was calculated using the following equation:

$$Qp = \frac{p}{t}$$

Fermentation efficiency or yield efficiency (Ey) was calculated as per the following equation:

$$E_y = \frac{Yps \times 100}{0.511}$$

0.511 is the maximum theoretical ethanol yield per gram of glucose consumed. Ethanol yield (Yps) was also calculated as the actual ethanol produced and expressed as g ethanol per g sugar utilized (g/g).

Ethanol Estimation:

Gas chromatography (GC 2010, Shimadzu, Japan) was employed for the final product analysis. A Porapak 'Q' column and a flame ionization detector were installed. The carrier gas, nitrogen gas, was supplied at a flow rate of 30 mL/min. A temperature of 130°C was chosen for the oven. The dichromate technique was initially used to ascertain the ethanol concentrations. A temperature of 200 °C was maintained for the injector and 230 °C for the detector. The Dichromate test is used to quantify the ethanol recovered following basic distillation, and Gas Chromatography with static headspace analysis is used for analysis. Results are compared to a calibration curve using Iso-propanol as an internal standard, and the analysis is carried out at a corrected salt concentration of 0.1 mM MgSO4. Purge the distillate of any remaining water by dehydrating it and then passing it through molecular sieves (14, 15).

RESULTS AND DISCUSSION:

Harvesting of Water Hyacinth:

Freshwater hyacinth (*Eichhornia crassipes*) plants were harvested from river Gadhi, Panvel-Maharashtra, and transferred to the laboratory for preparation at different times.

Figure 3 Harvested of Water hyacinth (Eichhornia crassipes) plants

Preparation of Water Hyacinth Biomass:

Chop, clean, and rinse with tap water the fresh water hyacinth (Eichhornia crassipes) after transferring it to the lab. The sun's beams were eventually permitted to dry it off. At a temperature of 65±3ºC, the dry material was again ground and dried. The material was filtered through a sieve to get a size of 350 µm after it had dried to a consistency below 10%. Lastly, polypropylene bags were used to keep the sample.

Figure 4 Washing of water hyacinth and sun-drying process

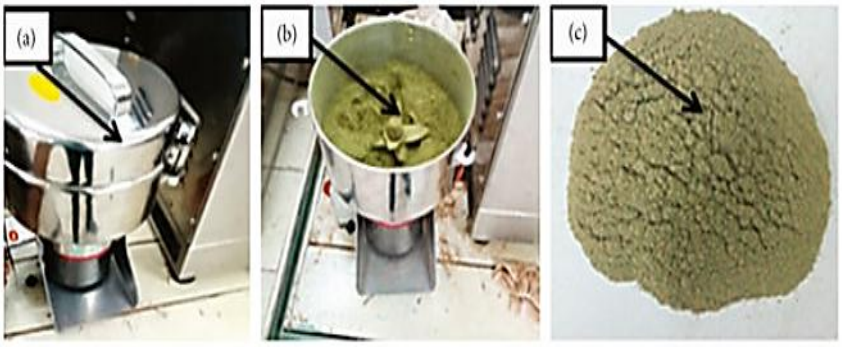

Figure 5 Grinding and sieving of dried water hyacinth

Analysis of chemical composition of water hyacinth:

The chemical composition of the desiccated powder of water hyacinth (Eichhornia crassipes) was analysed using TAPPI procedures and compared to that of previous studies (Fig 6). The dehydrated water hyacinth powder contained approximately 8.40±0.7% lignin, 32.9±0.9% hemicellulose, and 31.9±0.7% cellulose, according to an analysis. The total holocellulose content is approximately 64±1.8% composed of cellulose and hemicellulose. The water hyacinth contains cellulose and hemicellulose in the range of 17.5% to 34.00% and 18.33% to 48.7%, respectively, according to the results of other investigations. The water hyacinth contains a range of lignin content, from 1.8% to 27.36%. This suggests that the constitution of the water hyacinth is contingent upon its developmental environment.

Table 1 Chemical composition of water hyacinth from different sources

Cellulose%	Hemicellulose%	Lignin%	Reference
31	22	7	B. R. Murphy (2010)
19.5	33.4	9.27	Aruna Kumari. (2013)
25.61	18.42	9.93	Pandey A. (2010)
18	33.39	27.36	A.L. Leão (2017)
17.5	43.4	7.8	Yasser Vasseghian (2022)
35	18.3	1.8	Santhana Krishnan (2020)
18.2	48.7	3.5	Bronzato et al., (2017)
21.1	25.9	12	Das et al., (2016)
19.5	33.4	9.27	Xiaoyu Zhang (2010)
32.9±0.7	**34.9±0.9**	**8.40±0.7**	**This Study**

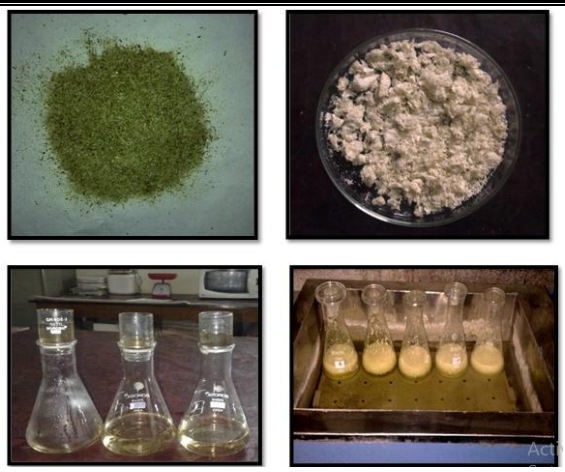

Figure 6 Determination of chemical composition of water hyacinth

Physical properties of water hyacinth:

The Physical properties of the WH powder is summarized in Table 2. The WH contained over 8.5% moisture, 11.2% ash, crude protein 6.2%, crude fibre 11.5%.

Table 2 Physical properties of water hyacinth

Components	% of dry weight
Moisture	8.5
Ash	11.2
Crude protein	6.2
Crude fibre	11.5

Mineral content of water hyacinth:

The mineral yield of the WH sample from organic extraction and by ashing method is shown in Table 3. The concentration of various mineral content Ca, Na, K, Mg, Cu and Zn of WH ranged from 0.63, 0.19, 0.40, 0.18, 0.12 and 0.37 g respectively.

Table 3 Mineral content of water hyacinth

Minerals	g/100 g dry weight
Calcium	0.63
Sodium	0.19
Potassium	0.40
Manganese	0.18
Copper	0.12
Zinc	0.37

Chemical Pretreatment Evaluation:

1. Sodium hydroxide pretreatment:

The initial substrate was pretreated with sodium hydroxide (NaOH) at values between 0.1 M and 0.4 M. Subsequently, the pretreated substrates' chemical makeup was determined. By hydrolyzing sulfuric acid at a concentration of 2.0%, the maximum amount of ethanol was produced. Table 4 shows the results of hydrolyzing sulfuric acid, while Table 5 shows the results of hydrochloric acid, both of which are used in the manufacturing of ethanol. With a

marked drop in sugar output compared to the 4% sodium hydroxide hydrolysis, the alkali hydrolysis approach proved to be economically viable.

Table 4 Effect of sodium hydroxide pretreatment on water hyacinth for ethanol production

Sodium hydroxide (v/v) (%)	1.0	2.0	3.0	4.0
Ethanol (g/L)	3.19±0.3	4.72±0.2	4.35±0.2	3.83±0.4

2.Potassium hydroxide pretreatment:

Potassium hydroxide (KOH) was used to pretreat the native substrate at concentrations ranging from 0.1 M to 0.4 M. The pretreated substrates' chemical composition was then measured. With a potassium hydroxide concentration of 4.0%, the experiment produced the maximum amount of ethanol by hydrolysis.

Table 5 Effect of potassium hydroxide pretreatment on water hyacinth for ethanol production

Potassium hydroxide (v/v) (%)	1.0	2.0	3.0	4.0
Ethanol (g/L)	5.10±0.2	5.63±0.5	5.84±0.2	6.10±0.4

3. Sulphuric acid pretreatment:

The native substrate was pretreated using sodium chlorite at concentrations ranging from 1% (w/v) to 4% (w/v), and the chemical composition of the pretreated substrates was determined. Based on the data obtained, it was found that the hydrolysis of sulphuric acid with a concentration of 2.0% resulted in the maximum yield of ethanol.

Table 6 Effect of sulphuric acid pretreatment on water hyacinth for ethanol production

Sulphuric acid (v/v) (%)	1.0	2.0	3.0	4.0
Ethanol (g/L)	10.05±0.2	14.80±0.3	11.76±0.3	12.33±0.1

4. Calcium hydroxide pretreatment:

Calcium hydroxide was employed to pretreat the native substrate at concentrations ranging from 0.1 M to 0.4 M. The chemical composition of the pretreated substrates was subsequently analysed. The results indicated that the highest quantity of ethanol was produced during hydrolysis with 4.0% hydrochloric acid.

Table 7 Effect of hydrochloric acid pretreatment on water hyacinth for ethanol production

Hydrochloric acid (v/v) (%)	1.0	2.0	3.0	4.0
Ethanol (g/L)	8.30±0.2	9.25±0.5	11.35±0.2	11.90±0.3

Figure 7 illustrates the impact of pre-treatment with dilute acid and alkali (specifically sulfuric hydrochloric acid hydrolysis, sodium hydroxide, and potassium hydroxide) on the generation of ethanol.

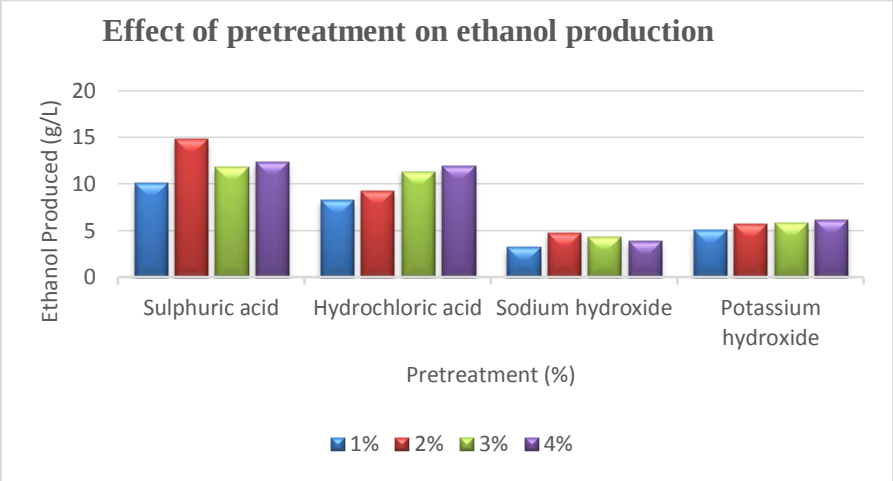

Figure 7 Effect of acid and alkali pretreatment on water hyacinth biomass for ethanol production

Estimation of reducing sugar by DNSA method:

The reagent undergoes a colour transformation from yellow to either orange or red, depending on the quantity of reducing sugar that is present. The DNSA test is capable of detecting glucose concentrations ranging from 0.5mM (0.09% glucose w/v) to 40mM (0.72% glucose w/v).

Effect of Enzyme (Accelerase 1500) on sugar release:

The biomass that was left after the filtration process was treated with a commercial enzyme (Accelerase 1500) to break down cellulose and hemicellulose using enzymatic hydrolysis. The residual biomass of water hyacinth was dried and exposed to different doses of the enzyme Accelerase 1500 for 48 hours at a temperature of 37°C, following pretreatment with 2% H2SO4 and 4% KOH.

Table 8 Effect of enzyme biomass pretreated with 2% sulphuric acid.

Enzyme (µ/L)	10	20	30	40	50	60	70
Sugar Yield (mg/g)	122	148	215	246	265	285	272

Table 9 Effect of enzyme on biomass pretreated with 4% potassium hydroxide.

Enzyme (µ/L)	10	20	30	40	50	60	70
Sugar Yield (mg/g)	54	66	122	136	148	144	132

In comparison to a 4% potassium hydroxide pretreatment (148 mg/g) and a lesser enzyme concentration of 50 u/L, the efficiency of sugar release from water hyacinth biomass is considerably improved by the use of a 2% sulphuric acid pretreatment and a higher enzyme concentration of 60 u/L.

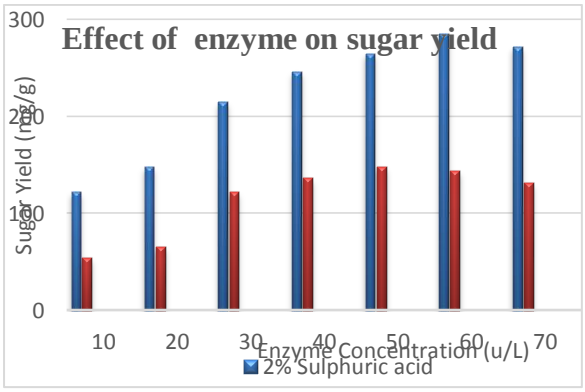

Figure 8 Effect of enzyme on pretreated biomass for sugar release.

Figure 8 demonstrates the effect of different doses of cellulase enzyme on the amount of reducing sugar released from water hyacinth biomass after being treated with 2% sulfuric acid and 4% potassium hydroxide. The findings indicate that utilizing a 2% sulphuric acid pretreatment along with a higher concentration of cellulase enzyme (60 u/g) leads to a more efficient extraction of sugar from water hyacinth biomass, as opposed to employing potassium dichromate pretreatment with a lower cellulase concentration (50 u/L).

Fermentation of water Hyacinth Hydrolysate:

1. Parametric optimization studies:

The optimal pH, temperature, and other variables for baker's yeast ethanol generation from water hyacinth biomass have been examined. The findings are in Table 10.

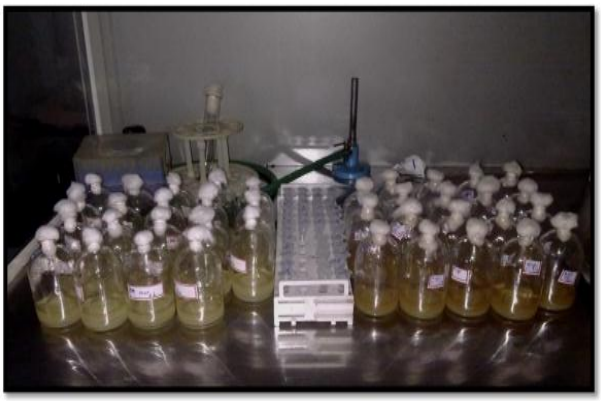

Figure 9 Parametric optimization studies by baker's yeast (S. cerevisiae)

Table 10 Parametric optimization studies for ethanol production by S. cerevisiae

Sr. No	Parameters	Optimum level	Ethanol yield g/g
1	pH	5.5	0.3±0.013
2	Temperature	30	0.3±0.012
3	Agitation	150rpm	0.36±0.015
4	Initial glucose concentration	2%	0.38±0.0162
5	Inoculum size	15%	0.4±0.014
5	Time	48h	0.4±0.012

2. Effect of pH on ethanol production:

The study investigated the influence of initial pH levels ranging from 3.5 to 7.5 on the production of ethanol by baker's yeast using water hyacinth biomass. Either 1N HCl or 1N NaOH was employed as one of the two solutions to adjust the pH. The yeast was cultured at a temperature of 30 °C with an agitation rate of 150 rpm. The greatest ethanol yield of 0.3±0.013 (g/g) was attained, as seen in Figure 10.

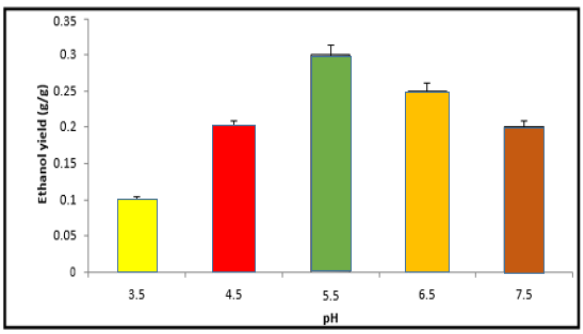

Figure 10 Effect of pH on ethanol production

3. Effect of temperature on ethanol production:

An investigation was conducted to determine the impact of temperature on ethanol production within the range of 20-40°C. The findings are displayed in Figure 11. The temperature at which the highest ethanol yield (0.3±0.012g/g) was obtained was determined to be 30°C. As the temperature climbed beyond 30°C, the ethanol yield also increased. However, additional increases in temperature resulted in a drop in ethanol yield.

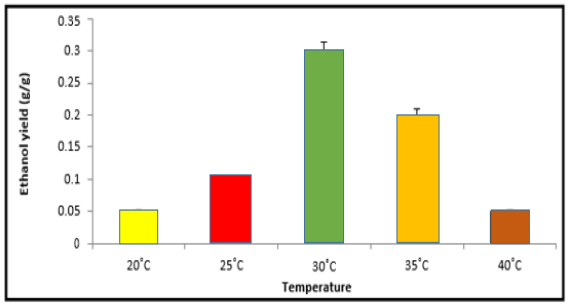

Figure 11 Effect of temperature on ethanol production

3.1Effect of agitation on ethanol production:

The ethanol production method utilised both stationary and oscillating conditions. The level of agitation was optimized by controlling the speed of agitation within the range of 100 to 250 rpm. When comparing the effects of agitation at 150 rpm with the quiescent mode, it was seen that the former

resulted in a larger output of ethanol, namely 0.36±0.015 g/g (Fig 12).

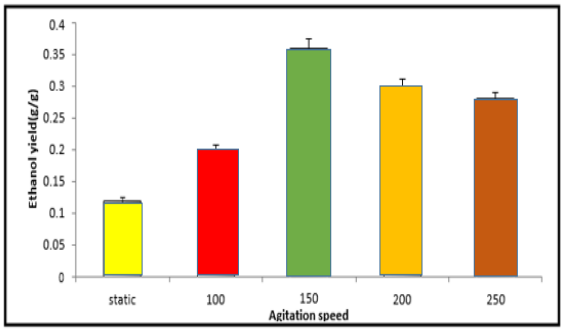

Figure 12 Effect of agitation speed on ethanol production

3.2 Effect of different inoculum size on ethanol production by S. cerevisiae:

An investigation was conducted to find the optimal inoculum size for maximum ethanol production by studying the impacts of different inoculum sizes. The ethanol production shown a steady rise from 5 to 15 when the capacity was expanded; however, it subsequently declined. An ethanol yield of 0.4±0.014 g/g was achieved at an inoculum size of 15%. (Figure 13)

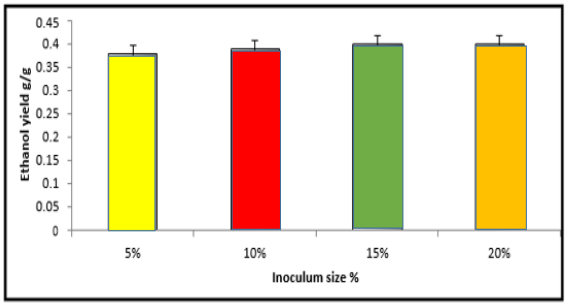

Figure 13 Effect of different inoculum size on ethanol production

3.3 Effect of fermentation time on ethanol production:

We studied the effect of fermentation duration on ethanol output to determine the best period. Initial ethanol output increased steadily over 48 hours. As fermentation continued, ethanol production declined. In 48 hours, ethanol production peaked at 0.4±0.012 g/g, as shown in Figure 14.

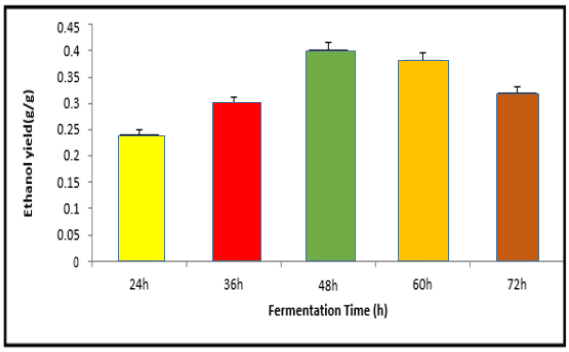

Figure 14 Effect of fermentation time on ethanol production

4. Estimation of Ethanol by Gas Chromatography:

The gas chromatogram provides evidence of ethanol generation by the S. cerevisiae strain from water hyacinth biomass, with a retention period of 2.65 minutes. Figures 15 and 16 indicate the presence of 99.8% standard ethanol.

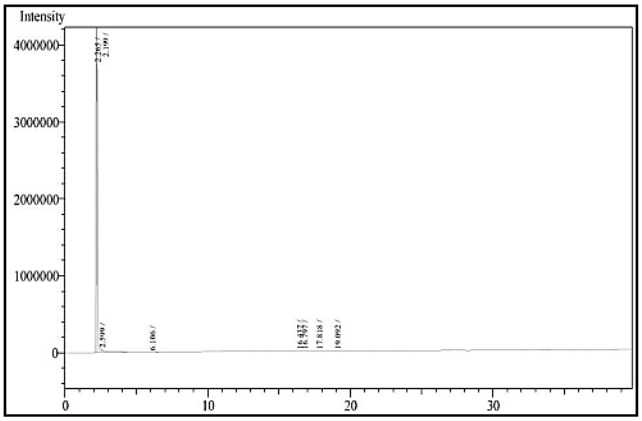

Figure 15 Gas chromatogram from distilled ethanol fermented by S. cerevisiae

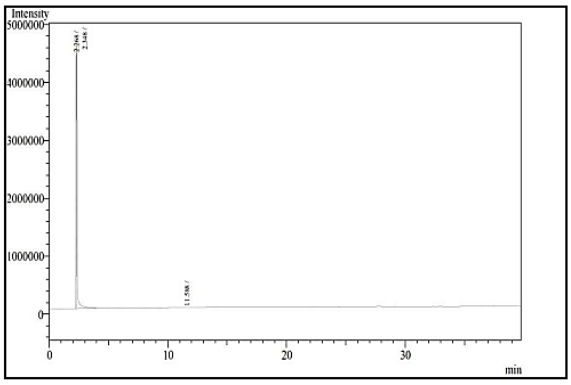

Figure 16 Gas chromatogram of standard ethanol

SUMMARY AND CONCLUSION:

This article provides a comprehensive analysis and categorization of the various potential applications of water hyacinth. Research suggests that incorporating water hyacinth co-compost into soil can enhance its hydro-physical and chemical characteristics. This is particularly accurate for soils that lack fertility. Plants in a state of active growth also derive advantages from the nutrients it supplies. Nevertheless, if the water hyacinth utilised for composting originated from uncontaminated water sources, the levels of heavy metals would be significantly lower compared to other organic fertilizers available in the market. Water hyacinth has the capacity to effectively eliminate various contaminants by absorbing and incorporating heavy metals and harmful chemical compounds (16, 17). Water hyacinth stands out due to its exceptional capacity for fast growth and high biomass production. This technology is highly successful in removing contaminants from both industrial and domestic waste effluents. Phytoremediation utilizes plants' natural capacity to detoxify themselves, hence reducing the amount of heavy metals in their native environment. Water hyacinth can serve as a biological solution for water pollution. The rhizomes and roots of water hyacinth have the ability to effectively remove heavy metals from water and artificial wetlands through filtration. Therefore, it is advisable to refrain from using contaminated roots and rhizomes for the purpose of producing organic fertilizer by co-composting or for feeding animals. The process of dry ashing the polluted water hyacinth has been demonstrated to effectively extract and recover heavy metals, as evidenced by the findings obtained (18–20).

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