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Production of Bioethanol using Water Hyacinth, an Aquatic Weed, as a Substrate

Research Article

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Abstract

Water Hyacinth is a one of the aquatic plants which causes serious concern on aquatic environment. All efforts to control the growth and spread of this weed have failed and hence concept of "Eradication through utilization" is being attempted. The purpose of present work is to study the production of ethanol from water hyacinth, used as substrate, using isolated microbial strain. The cellulase enzyme producing microbial strains were isolated and the potent cellulase producer strain is used to digest water hyacinth biomass, for the production of ethanol. The main objective is to reduce production cost of the ethanol by using Water Hyacinth as a raw material and explore its feasibility for commercial scale utilization.

Keywords: Bio-ethanol; Water hyacinth; Cellulase; Aquatic environment

Introduction

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Bio ethanol is being considered as a potential liquid fuel due to the limited amount of natural resources. Cellulose biomass is also being investigated as a potential substrate for bioethanol production [1]. Especially bioethanol produced from non-food lignocellulosic waste products as wood chips and straw or non-food crops as willow could be an environmentally-friendly alternative [2,3]. Lignocellulose consists of three major constituents: cellulose, hemicelluloses and lignin [4]. The water hyacinth (*Eichhornia crassipes*) is a native plant of Brazil but has been naturalized in many tropical/temperate countries. The water hyacinth plant is a free-floating aquatic plant has spread to more than 50 countries on five continents [5]. The plant tolerates extremes in water level fluctuations, seasonal variations in flow velocity, nutrient availability, pH, temperature and toxic substances [6]. It can even grow at salinity levels up to 0.24% as was shown in Indonesia. Extremely high growth rates of up to 100-140 ton dry material Ha-1year-1 were reported, depending on the location and time of the year. The coverage of waterways by water hyacinth has created various problems. Examples are the destruction of ecosystems, irrigation problems and an increase in mosquito populations [7]. These negative effects therefore, on one hand, attempts have been geared towards the use of biological, chemical and mechanical approaches for preventing the spread of, or eradication of, water hyacinth [8,9]. Aquatic plants have many advantages such as growing on and in bodies of water without competing against most grains and vegetables for arable land; they are also used for water purification to extract nutrients and heavy metals. These studies indicate that water hyacinth is a promising plant for ethanol production [9]. The purpose of present work is to study the production of ethanol from water hyacinth, used as substrate, using isolated microbial strain. The cellulase enzyme producing microbial strains were isolated and the potent cellulase producer strain is used to digest water hyacinth biomass, for the production of ethanol. The main objective is to reduce production cost of the ethanol by using Water Hyacinth as a raw material and explore its feasibility for commercial scale utilization [10-14].

Objective

To study the feasibility of water hyacinth as energy crop and to isolate potential cellulase strain of microorganism for the conversion of Lignocellulosic biomass into ethanol at lower cost with effective technologies by "Eradication through utilization"

Materials and Method

Stage 1: Isolation and Screening Of Cellulase Producing Microorganism

Collection and Enrichment of sample: For isolation of cellulase producing microorganisms various sample were collected. Samples were collected from cattle dung, composite soil, the wood-wastes near by the vicinity of Bhagwan Mahavir Education Foundation, Bharthana, Surat, Gujarat, India. Samples were collected in the airtight sterile plastic container with the help of sterile spatula and transfer immediately to laboratory. The collected samples were enriched by substrate induction method by using Bushnell Hass (BH) media supplemented with carboxy methyl cellulose (CMC). Composition of BH agar medium gl^{-1}: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; (NH_4) NO3, 1.0; $FeCl_3$, 0.05. After 15 days, 5 ml of sample was removed from the enriched flask and added it to sterile BH medium supplemented with CMC. Such three transfers were made.

Isolation and primary screening of Cellulase producing microorganism: After third transfer; 0.5 ml of culture was spread on the BH agar medium plate. Plates were incubated at 30 °C for 48 h. After 48hr, growth was observed and plates were labeled as master plates, replica of those master plates were made on BH agar medium, replica plates were incubated at 30 °C for 48 h. Master plates were flooded with an aqueous solution of 1% Congo red and shaken at 50 rpm for 15 min in shaking incubator. The Congo red solution was then poured off; plates were further flooded with 1 M NaCl solution and shaken again at 50 rpm for 15 min. Unstained areas surrounding colonies of microorganism indicate the CMC has been broken Down. Diameter of unstained area is measured, based on the diameter potent strains of microorganisms were selected. Pure culture of that organism was obtained from the respective replica plates. Pure cultures were preserved on BH medium agar slants supplemented with CMC.

Isolated cellulolytic bacteria were characterized on the base of Gram reaction, morphology and biochemical characteristics.

Stage 2: Production Of Cellulase Enzyme

Fresh water hyacinth was collected from fresh water pond, Bharthana, Surat, Gujarat. Leaves of Water Hyacinth were chopped 2-3 cm and dried in hot air oven at 70 °C for 48hr. Then dried material was pulverized and sieved through 80-mesh sieve, the under-size material was used for the substrate for production of cellulase enzyme.

100 ml Bushnell Hass (BH) media supplemented with 10% water hyacinth was taken in 250 ml Earlene Mayer flask, the initial pH of the medium was adjusted to 6 before being autoclaved at 121°C for 15min.

Inoculum was prepared from preserved BH medium agar slant by activation of culture for 24hrs in BH medium broth supplemented with CMC. 5 ml of inoculum was added in respective flask of production medium. Inoculated flasks were kept on rotary shaker (100 rpm) at 30 °C.

Enzyme assay: After the interval of every 24 h, samples were removed from flask. CMC activity was determined at 50°C by filter paper assay using filter paper as a substrate. A reactive mixture contains substrate in 0.05 M citrate buffer (pH 4.8) and 1.0ml of culture supernatant. The mixture was incubated at 50 °C for 30 min. The reducing sugar released was measured using 3, 5-dinitrosalicyclic acid (DNSA). Control was prepared with 5min boiled enzyme. One unit of enzyme activity was expressed as the above assay condition by using glucose as a standard curve.

Stage 3: Ethanol Production

Cellulose in water hyacinth is hydrolysed to reducing sugars which are then subjected to fermentation by yeast to yield alcohol. *Saccharomyces cerevisiae* was used for the production of ethanol. Glucose yeast extract agar medium was used to maintain *S. cerevisiae*. The production medium from previous stage was autoclaved at 121°C for 20 min and inoculated with yeast. The samples were incubated at room temperature for 3 days. The alcohol content of the reaction mixture was estimated by dichromate oxidation method.

For the estimation of ethanol by dichromate method ,we can take 1ml filtrate and then make final volume 5ml with distill water and then add 10ml potassium dichromate method (same way prepare blank but instead of filtrate take distill water) and put flask in dark for 30min and then after incubation take out flasks and then add 4ml 20% KI solution then immediately titrate against 0.1N Sodium thiosulphate ($Na_2S_2O_3$) till pale yellow colour appear after that add 2-3 drops of 1%Starch as indicator and again titrate against Sodium thiosulphate ($Na_2S_2O_3$) till blue colour disappear and note the amount of Sodium thiosulphate($Na_2S_2O_3$) required to bring colour changes, then using blank, calculate (B-E) and using standard graph find out the concentration of our filtrate.

Results

From the enriched sample, five cellulase producing strains were isolated.

Determination of cellulase activity of Isolates

For the screening and isolation of potential strain which have maximum activity of degradation of cellulose we can used carboxy methyl cellulase(CMC) as subtract from this we can observed maximum activity through observing Zone of clearance which summarized in Figure 1.

Characterization of isolates collected from samples

After collection of sample from different areas, we characterized potential isolates summarized in Table 1.

Cellulase activity in water hyacinth by FPA method

After hydrolysis of cellulosic biomass, yield mixture of sugar, which content determined by Filter paper assay, as summarized in Table 2.

Ethanol estimation by dichromate method

A significant amount of ethanol was produced by *S.cerevisiae* from the sugar obtained by the saccharification of water hyacinth substrate (Table 2 and Table 3). Yeast grow well in the pH range

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Figure 1: Determination of cellulase activity of Isolates.

Table 1: Characterization of isolates having maximum celluolytic activity.

ISOLATES	COLONY CHARCTERISTIC	MORPHOLOGICAL FEATURES	GRAM REACTION
SN1	Pink moist colonies	Rod shaped	Gram positive

Table 2: Cellulase activity in water hyacinth by FPA method.

Days	pH	Biomass (gm %)	Cellulolytic activity (gm %)
1	6.5	1.4	1.4
2	7.2	2.2	2.5
3	8.0	3.1	3.1
4	8.6	4.2	3.8
5	8.8	4.4	4.6

Table 3: Alcohol fermentation from fermentable sugars obtained by hydrolysis of Water hyacinth.

Days	pH	Biomass (gm%)	Reducing Sugar(gm%)	Consumed Sugar(gm%)	Ethanol production(gm%)
1	6.5	1.7	12	0.0	0.6
2	5.2	2.9	10.45	1.55	1.7
3	4.5	3.8	8.32	3.68	2.9
4	3.6	4.6	6.45	5.55	3.7
5	3.2	5.8	4.43	7.57	4.2

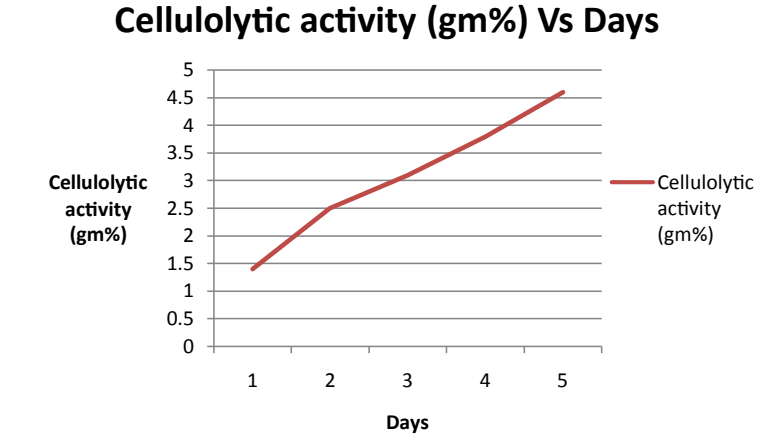

Figure 2: Cellulolytic activity (gm %).

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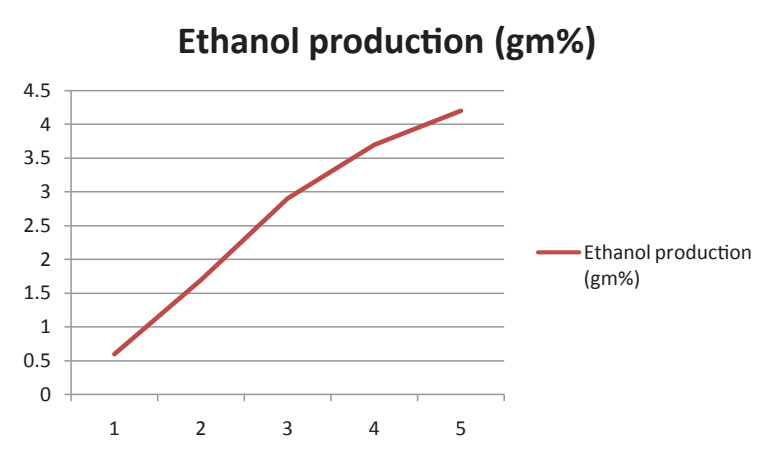
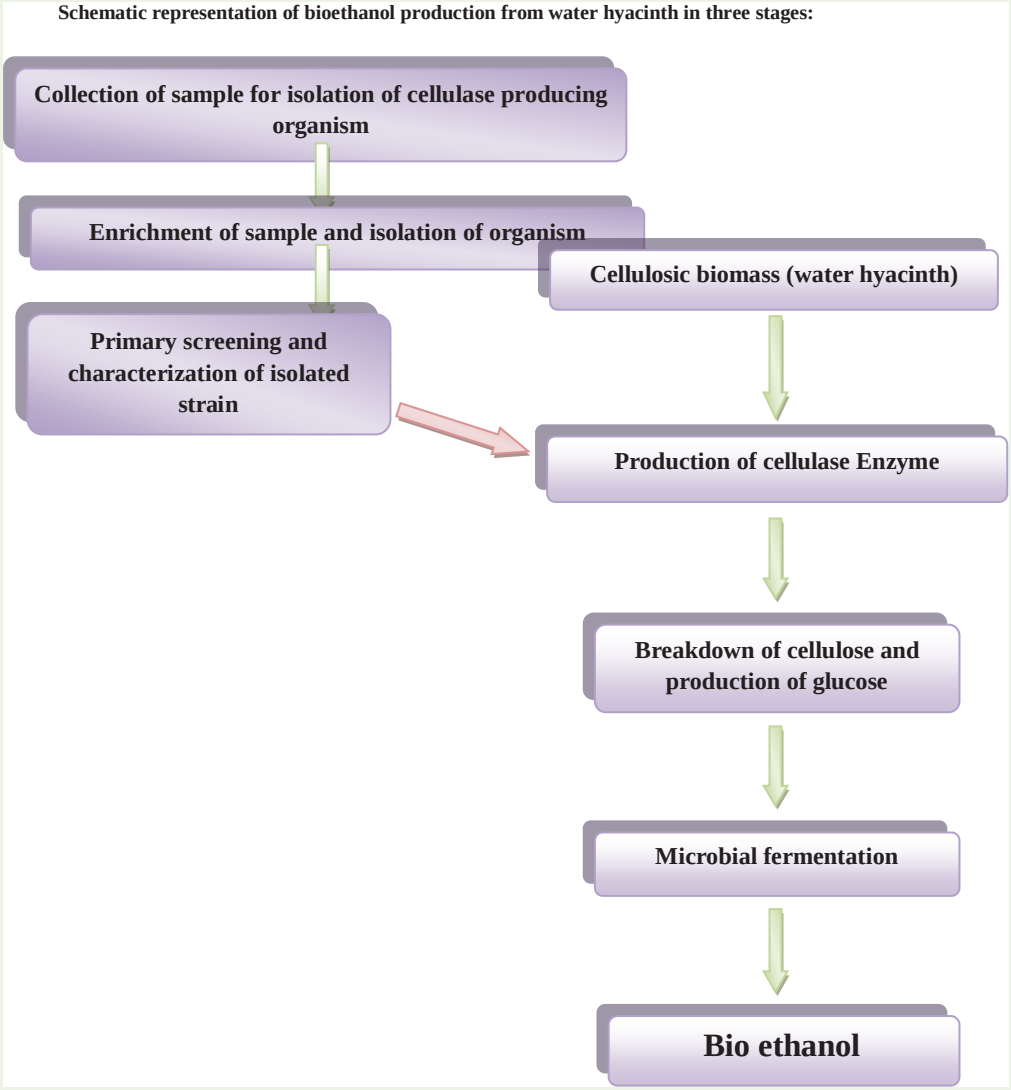


Figure 3: Ethanol production.



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3.5 – 5.0. Alcohol yield was lower below pH 3 and optimal at pH 4. Maximum alcohol production was found to be at a temperature of 29oC, summarized as Table 3.

Conclusion

In conclusion, we explored a high feasibility of using an appropriate technology (Acid hydrolysis and yeast fermentation) for the bioconversion from water hyacinth to ethanol. And by using protein isolated strain we can get maximum ethanol production from saccharification of water hyacinth. As previously mentioned, the water hyacinth is one of the worst weeds that causing the major problem to the global aquatic or terrestrial particularly in the tropics. Although control managements have been widely applied to keep the plant at a low level using herbicides and mechanical removal, in most of the cases, it remains ineffective due to the pernicious invasive growing of the aquatic hyacinth. The technique here in, more or less helps lowering the plant while provides the simple and low cost process that suit to the developing countries.

Acknowledgement

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