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Enzymatic hydrolysis of weed plant Agave tequilana for the production of bio-ethanol

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ABSTRACT

Burning of fossil fuels has not only led to health problems but also have a great impact on our environment like acid rain, climate change, global warming. And it is accepted by 2070, the current globe will be running out of fossil fuel. Our work was conducted on weed plant Agave tequilana, wherein microbial fermentation was carried out on pretreated biomass with 0.8% of NaOH and 98% H_2SO_4 by using standard cultures of Aspergillus niger (ATCC 16404)and Saccharomyces cerevisiae (NCIM 3622). The percentage of Bio-Ethanol yield was found to be 45.55%.

Keywords— *Bio-ethanol, Enzymatic hydrolysis, Agave tequilana, Aspergillus niger*

1. INTRODUCTION

Increased in the usage of non renewable petroleum products like petrol, diesel due to the increased population and standard of living has resulted in its depletion. Burning of fossil fuels causes increased in the emission of green house gases not only cause diseases like Asthma, Respiratory infections, Cancer, Cardiovascular disease, stroke. According to the WHO, Every year around 4.2 million deaths are caused due to the exposure of green house gases [15]. The increased concentration of green house gases especially CO_2 has caused increased in average planet temperature by 1.62°F since the late 19th century [16]. Beginning of the Industrial revolution, the acidity of surface ocean water has increased by about 30% due to the increased emission of CO_2 to the atmosphere which is absorbed into the ocean [17]. Taking all these into considerations, various countries started to produce biofuel from various food sources such as corn, wheat, potato, sugarcane and bagasse. In this work, we have selected weed plant named *Agave tequilana*, which is perennial belonging to the genus monocot, primarily known as succulent and form large rosettes of strong, fleshy leaves. It is found growing in Mexico, United States and South East Asia. Can grow upto 30ft with hardy thorny tip leaves and was used for the production of alcoholic beverages anciently forming a viable, inexpensive, renewable source of lignocellulosic material. The temperature conditions for growth is minimum of 10°C and the optimum of 26°C with pH conditions of soil and it can also tolerate drought[1].

It is rich in carbohydrate and dietary fibre. The physicochemical composition of *Agave tequilana* on dry matter basis contains a high amount of total dietary fibre (38.40%), total sugars (45.83%), and protein (35.33%), with a relatively low content in ash (5.94%) and lipid (2.03%) [12].

Agave species can grow well in semi-arid marginal agricultural lands around the world. Selected Agave species are used largely for alcoholic beverage production in Mexico. There are expanding research efforts to use the plentiful residues (bagasse) for ethanol production as the beverage manufacturing process only uses the juice from the central core of mature plants. Here, we investigate the potential of over a dozen Agave species, including three from cold semi-arid regions of the United States, to produce biofuels using the whole plant.

Results: Ethanol was readily produced by Saccharomyces cerevisiae from hydrolysate of ten whole Agaves with the use of a proper blend of biomass-degrading enzymes including inulinase that overcomes inhibition of most of the species tested. As an example, US grown Agave neomexicana produced 119 ± 11 mg ethanol/g biomass. Unlike yeast fermentations, Clostridium beijerinckii produced n-butanol plus acetone from all species tested. Butyric acid, a precursor of n-butanol, was also present due to incomplete conversion during the screening process. Since Agave contains high levels of free and polyfructose which are readily destroyed by acidic pretreatment, a two-step procedure was developed to depolymerize polyfructose while maintaining its fermentability. The hydrolysate from before and after dilute acid processing was used in C. beijerinckii fermentations with selected Agave species with A. neomexicana producing 144 ± 4 mg fermentation products/g biomass.

2. MATERIALS AND METHODS

2.1 Sample Collection

Agave tequilana plant was collected from the forest, Bangalore [Figure 1]. The plant was washed with running tap water for 3-4 times to remove mud and other dirt particles. Then they were cut into pieces of 2-3 cm and sun dried for 6 days, subsequently, oven dried at 70°C for 6 hours [Figure 2] and then it was grinded into a fine powder using high speed blender and the sample was sieved by using 0.95-1mm pore size.

Fig. 1: Collection of *Agave tequilana*

Fig. 2: Sample after drying and heating

2.2 Pretreatment

20g of the grinded sample was treated with 300ml of 0.8% (0.2M) of NaOH solution for 6 hrs. Then immediately the sample was filtered and adjusted the pH to 7 by adding 98% H_2SO_4. Then the sample was kept in a hot air oven for 48 hrs at 70°C.

2.3 Media Preparation

2.3.1 *Aspergillus niger*: Standard culture of *Aspergillus niger* (ATCC 16404) was collected and it was cultured on Petri dish containing SDA media. Cells from a single colony were isolated and were added in 3% of SCDM broth of 50ml. Finally, the spore suspension was kept at room temperature for 36 hrs.

2.3.2 *Saccharomyces cerevisiae:* Standard culture of *Saccharomyces cerevisiae* (NCIM 3622) was collected and it was cultured on Petri dish containing SDA media. Cells from a single colony were isolated and added in 3% of SCDM broth of 50ml. Finally, the spore suspension was incubated at 37°C for 18 hrs prior to inoculation.

2.4 Sample Preparation

10g of weed powder was added in 50ml of distilled water, pH of the slurry was adjusted at 5.3 (by adding 1% NaOH or 1% H_2SO_4). 4ml of *Aspergillus niger* broth was added to the slurry and was kept in incubator shaker of 120 rpm at 35°C for 4 days.

2.5 Estimation of Reducing Sugar

The slurry was centrifuged at 5000 rpm for 12 mins. And the supernatant was transferred to a fresh tube and reducing sugar was estimated by DNSA (Dinitrosalicylic acid) reagent described by Miller (1959).

2.5.1 DNSA reagent preparation: 1g of dinitrosalicylic acid is dissolved in 50ml distilled water. To this solution add 30g of Sodium potassium tartrate tetrahydrate in small lots; the solution turns milky yellow in colour. Then add 2N NaOH which turns to transparent orange yellow colour. The final volume is made to 100ml with distilled water. The samples were added accordingly to the Table 1, and the tubes were kept in boiling water bath for 15mins, after adding DNSA reagent. Finally, 40% of Rochelle's salt was added to stabilise the reaction and OD was measured at 520nm (Table 1). The whole set of experiment was performed 3 times.

Table 1: Estimation of reducing sugar by DNSA method

S. no.	Concentration of glucose (µg/ml)	Volume of glucose sol. (µl)	Volume of dist. Water (ml)	DNSA reagent (ml)	40% Rochelle's salt (ml)	OD at 520nm
1	0.00	0.00	3	3	1	0.00
2	200	600	2.4	3	1	0.300
3	400	1200	1.8	3	1	0.610
4	600	1800	1.2	3	1	0.920
5	800	2400	0.6	3	1	1.240
6	1000	3000	0.00	3	1	1.500
7	Test sample	10	2.990	3	1	1.225

2.6 Fermentation

A liquefied slurry containing *Aspergillus niger* along with the sample kept for 4 days in incubator shaker was taken, 4ml of *Saccharomyces cerevisiae* broth was added to the slurry and pH was maintained at 4.8. The slurry was kept in a dark room for 5 days to maintain anaerobic conditions.

2.6.1 Estimation of Bioethanol: After 5 days, the slurry was centrifuged at 5000 rpm for 15 mins and the supernatant was collected in a fresh tube. Distillation of supernatant was carried out at 78°C, evaporating Ethanol which was collected in a beaker (figure 3). The density of the distillate was measured, for the confirmation of Ethanol. Finally, the volume of Bioethanol and Supernatant was measured (table 2) to determine the yield.

Fig. 3: Bio-Ethanol produced from *Agave tequilana*

Table 2: Estimating the volume of Bioethanol produced

S. no.	The initial volume of supernatant (ml)	Volume of Bioethanol produced (ml)
1	45	24.5
2	48.3	27.7
3	46.2	25.7

3. RESULTS AND DISCUSSIONS

Currently, due the huge impact of greenhouse gases on both health and environment and its depletion caused a various scientist to find an alternative source for it which should not only be abundant and renewable but it shouldn't also cause any damage to health as well as environment. Nowadays, biofuel is being produced by various methods such as acid pretreatment method, submerged fermentation and surface fermentation by using various strains of microorganisms including bacteria, fungi in order to decrease the cost of production and increasing the yield. In this study, weed plant *Agave tequilana* were selected, containing 45.83% of sugar of total biomass. The inoculation of *Aspergillus niger* (ATCC 16404) with weed sample caused the hydrolysis of polysaccharide to monomeric sugar due to cellulase enzyme. Result for the estimation of sugar in the solution revealed that 17.67 % of total biomass was converted into simple sugar by enzymatic hydrolysis.

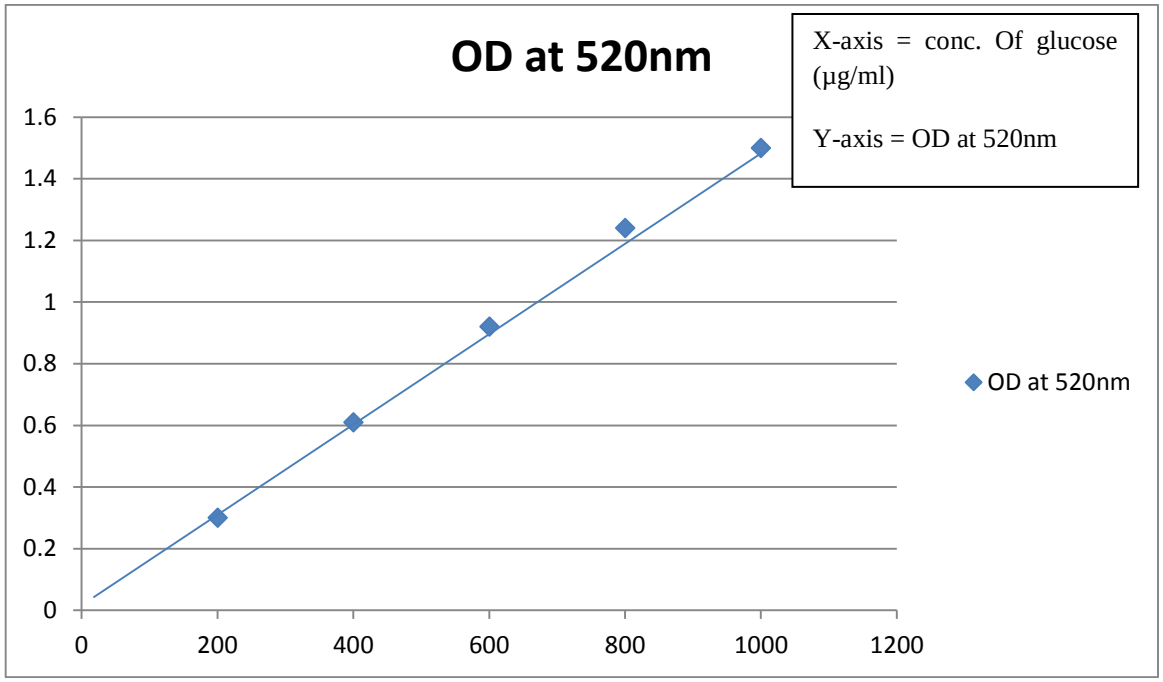

Fig. 4: Concentration of Glucose v/s Optical Density at 520nm

Saccharomyces cerevisiae was added to the hydrolyzed solution containing simple sugar and was kept for 5 days under anaerobic conditions. The supernatant obtained after centrifugation was distilled at 78°C. And the volume of Bio-Ethanol and Supernatant was measured. The yield of Bioethanol produced was found to be

$$Percentage\ of\ Bioethanol\ yield\ = \frac{Volume\ of\ bioethanol\ (ml)}{Volume\ of\ supernatant\ (ml)}$$

$$Percentage\ of\ Bioethanol\ yield\ = 45.55\%$$

Since the production of 1st generation biofuel is an inconvenient method as it involves the usage of various food sources thereby, creating a demand for the scientist to go for 2nd generation biofuel which utilizes non-food crop plants having high lignocellulosic material. In such situations weed plant like *Agave tequilana* can be used as a good substitute for petroleum products like petrol, diesel.

4. CONCLUSION

Burning of fossil fuel causes a great impact on the environment, actuated most of the researchers to discover an alternate source of fuel. In this study, *Agave tequilana* was used as a substrate for the production of bioethanol and this study revealed that sufficient amount of biofuel can be produced from this plant, thereby utilizing a waste into a useful product and reducing the cost of production. Production of Bioethanol from *Agave Tequilana* provides many employment opportunities, thereby improving economic development. Similarly, there are various other biomasses which are waste but may have high sugar content in it. Since the research in this field is not properly developed; farther probing is required for increasing the yield of Bioethanol production.

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