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RESEARCH ARTICLE

CELLULYTIC ALTERNATIVE FUEL PRODUCTION USING *AGAVE* AS A MODEL PLANT

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ABSTRACT

Fuel belongs to the non-renewable energy resources, which is depleting in rapid amount. Thus throughout world every country is investing huge time and money. There is always a thirst to identify an alternative source for fuel production. In this current research an attempt is taken to produce cellulytic bioethanol production by *Saccahromyces cerevisiae* using Agave plant as the source of the substrate for bioethanol production. The bioethanol production was confirmed by the reaction with potassium dichromate. The bioprocessing of the ethanol production was carried out as static and shaking conditions. The bioethanol production was found to be maximum in shaking conditions. Further research must be carried out to have a complete understanding about the bioprocessing involved in the the bioethanol production at commercial level.

Key words: Fuel, Non-renewable energy resources Cellulytic Bioethanol, Saccharomyces cerevisiae, Agave americana var americana, Agave Americana var marginata

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INTRODUCTION

BIOFUELS: A bio fuel is a fuel that is produced through contemporary biological processes, such as agriculture and anaerobic digestion rather than a fuel produced by geological processes such as those involved in the formation of fossil fuels, such as coal and petroleum, from prehistoric biological matter. Bio fuels can be derived directly from plants, or indirectly from agricultural, commercial, domestic, and/or industrial wastes (International Energy Agency, "Biofuels for Transport"-An International Perspective- 2004). Renewable bio fuels generally involve contemporary carbon fixation such as those that occur in plants or microalgae through the process of photosynthesis. Other renewable bio fuels are made through the use or conversion of biomass (referring to recently living organisms, most often referring to plants or plant-derived materials). This biomass can be converted to convenient energy containing substances in three different ways: thermal conversion, chemical conversion, and biochemical conversion. This biomass conversion can result in fuel in solid liquid or gas form. This new biomass can also be used directly for bio fuels (Durante, D and Miltenberger, M., 2004).

BIOETHANOL: Bioethanol is an alcohol made by fermentation, mostly from carbohydrates produced in sugar or starch crops such as corn, sugarcane, or sweet sorghum. Cellulosic biomass, derived from non-food sources, such as trees and grasses, is also being developed as a feedstock for ethanol production.

Ethanol can be used as a fuel for vehicles in its pure form, but it is usually used as a gasoline additive to increase octane and improve vehicle emissions. Bio ethanol is widely used in the USA and in Brazil. Current plant design does not provide for converting the lignin portion of plant raw materials to fuel components by fermentation (Brinkman *et al.*, 2005).

Advantages of Bioethanol

- Ethanol burns more cleanly (more complete combustion)
- The use of ethanol-blended fuels such as E85 (85% ethanol and 15% gasoline) can reduce the net emissions of greenhouse gases by as much as 37.1%, which is a significant amount.
- **Positive energy balance** – Depending to the type of raw stock it can vary from 1.24 to **8**. The output of energy during the production is more than the input.
- You can use any plant for production of bio ethanol; it only has to contain sugar and starch. The best choice is sugar cane, but you can also use potatoes, barley, wheat etc.
- It is carbon neutral i.e. the carbon dioxide released in the bio ethanol production process is the same amount as the one the crops previously absorbed during photosynthesis.
- Ethanol-blended fuel as E10 (10% ethanol and 90% gasoline) reduces greenhouse gases by up to 3.9% (Jensen, K.H., and Thyo, A., 2007).

- The net effect of ethanol use results in an overall decrease in ozone formation, an important environmental issue. (The emissions produced by burning ethanol are less reactive with sunlight than those produced by burning gasoline, which results in a lower potential for forming the damaging zone.
- Ethanol is considered a renewable energy resource because it is primarily the result of conversion of the sun's energy into usable energy. Creation of ethanol starts with photosynthesis, which causes feedstock, such as sugar cane, to grow. These particular feed stocks are processed into ethanol (**Fichera, J., and Kueter, J., 2006).**
- It benefits energy security as it shifts the need for some foreign-produced oil to domestically-produced energy sources. Countries that do not have access to crude oil resources can grow crops for energy use and gain some economic freedom (**Reed, D.D, 2007).**
- It reduces the amount of high octane additives.
- The fuel spills are more easily biodegradable or diluted to non toxic concentrations (http://www.buzzle.com/ articles/advantages-and-disadvantages-of-biofuels.html).

Agave: They are succulents with a large rosette of thick, fleshy leaves, each ending generally in a sharp point and with a spiny margin; the stout stem is usually short, the leaves apparently springing from the root. Along with plants from the related genus Yucca, various *Agave* species are popular ornamental plants (Irish and Mary, 2000).

Each rosette is monocarpic and grows slowly to flower only once. During flowering, a tall stem or "mast" grows from the center of the leaf rosette and bears a large number of short, tubular flowers. After development of fruit, the original plant dies, but suckers are frequently produced from the base of the stem, which become new plants (Gentry and Howard, 1982**).** It is a common misconception that agaves are cacti. They are not related to cacti, nor are they closely related to aloe whose leaves are similar in appearance. *Agave* species are used as food plants by the larvae of some *Lepidoptera* (butterfly and moth) species, including *Batrachedra striolata* which has been recorded on *A. shawii*.

Adaptations: The agave root system, consisting of a network of shallow rhizomes, is designed to help the agave efficiently capture moisture from rain, condensation and dew.

Taxonomy: Agave was divided into two subgenera; Agave and Littaea Mark W, *et.al.,* 2009.

- Kingdom: Plantae
- Clade: Angiosperms
- Order: Asparagales
- Family: Asparagaceae
- Subfamily: Agavoideae
- Genus: Agave.L

(**Sara v.Good Avila *et.al.*, 2006**)

Commonly grown species

The most commonly grown species agave around the world

- *Agave americana*
- *Agave angustifolia*
- *Agave tequilana*
- *Agave attenuata*
- *Agave parviflora*
- *Agave murpheyi*
- *Agave wilmoriniana*
- *Agave palmeri*
- *Agave parryi*
- *Agave victoriae reginae*

Common species of agave available in India

- *Agave sisalana*
- *Agave Americana*
- *Agave augustifolia*
- *Agave lurida*

(William, B., and Peter, C., 2002)

***Agave americana*: Tamil name: Aanai katraalai**

One of the most familiar species is *Agave americana*, a native of tropical America. Common names include century plant, *maguey* (in Mexico), or American aloe (it is not, however, closely related to the genus Aloe). The name "century plant" refers to the long time the plant takes to flower. The number of years before flowering occurs depends on the vigor of the individual plant, the richness of the soil, and the climate; during these years the plant is storing in its fleshy leaves the nourishment required for the effort of flowering. (www.missouribotanicalgarden.com). *Agave americana*, century plant, was introduced into Europe about the middle of the 16th century, and is now widely cultivated as an ornamental, as it is in the Americas. In the variegated forms, the leaf has a white or yellow marginal or central stripe. As the leaves unfold from the center of the rosette, the impression of the marginal spines is conspicuous on the still erect younger leaves.

The plants require protection from frost. They mature very slowly and die after flowering, but are easily propagated by the offsets from the base of the stem. Blue *A. americana* occurs in abundance in the Karoo, and arid highland regions of South Africa. Introduced by the British settlers in 1820, the plant was originally cultivated and used as emergency feed for livestock. Today it is used mainly for the production of syrup and sugar

Biological Applications

The juice made from the agave plant contains estrogen like isoflavonoid, alkaloids, coumarin and vitamins B1, B2, C, D and K, and provitamin A.

- *Agave Americana* has antiseptic wound healing and anti inflammatory properties.
- Traditionally it was used internally to treat ulcers, stomach inflammation, tuberculosis, jaundice and other liver diseases, syphilis, menstrual problems.
- A poultice made from the root and leaves of agave plant is often used to treat toothache.

- *Agave Americana* was used as a herbal remedy for weak digestion, intestinal gas and constipation
- The juice of agave plant has antibacterial properties.

- It is a source of hecogenin, a compound used in the production of many steroidal drugs.
- Also used to make alcoholic beverages such as tequila, pulque, mescal.
- The flower and base leaves can be roasted and consumed.
- *Agave sisalana* is used to make woven mats and paper.
- Extract of leaves or roots is used to make soap.
- Agave nectar is a sweetener derived from the sap of agave is used as an alternative for sugar (http://www.herbal-supplement-resource.com/agave-americana.html):

MATERIAL AND METHODOLOGY

Sample Collection: In this current research two plant samples: *Agave americana var americana and Agave americana marginata* were used*, e*ach of the plant samples were collected from the Hindustan University campus (Padur, Chennai), 10 g of plant leaf was taken at initial steps such as estimation of total carbohydrates, types of carbohydrates, total protein estimation.

Biomolecule Analysis of *Agave*

Carbohydrate estimation: The estimation of carbohydrates present in both the plant samples was done following the standard protocol of Benedicts Test.

Protein estimation: The protein content was determined by the method of Lowry *et al.* (1951).

Phenyl hydrazine test: This test is performed for the identification of sugars to detect reducing sugars by osazone formation.

Test for ketose sugar: The test was performed using the standard protocol of Seliwanoff's test to distinguish between aldose and ketose sugars.

Test for aldehyde or ketone : The test was done following the protocol of Tollen's test to determine the presence of an aldehyde or alpha hydroxy ketone functional groups.

Test for galactose: The test was performed using the methodology of Mucic test.

Microscopic examination of yeast: The microscopic examination of yeast was done using Gram's staining.

Biochemical characterization of yeast

Starch hydrolysis test: The test was performed to determine if organism was producing the amylase, which breaks down starch into glucose following the standard protocol of starch hydrolysis.

Carbohydrate utilization test: This test is performed to check if the organism is utilizing the sugar present in the broth and producing carbon dioxide, following the standard carbon utilization test methodology.

Strain improvisation: For the bioprocessing technique the yeast cells have to be adapted to utilize the carbohydrates present in the plant leaf as the sole source of carbohydrates. Thus there is a need for the improvisation of the strain to make them to be adapted to utilize the plant based carbohydrate and convert it into the necessary product, 50 ml of OF broth was prepared and sterilized, it was inoculated with 1 ml of *Saccharomyces cerevisiae*, the same procedure was repeated for 3 days.

Cell Counting: Cell counting is performed to count the number of cells present in the desired amount of sample and to check if the substrate used is supporting cell growth.

Analysis of plant pigments and of broth in the yeast cells

1 ml of OF broth, Of broth inoculated plant sample (each) and normal plant samples (each) were taken in 5 eppendorf tubes, the tubes were subjected for centrifugation at 1500rpm for 15 minutes, pellet was collected, the pellets were suspended in 1 ml of trichloroacetic acid, the pellets were homogenized, a pinch of magnesium sulfate was added, the samples were then transferred to 5 different test tubes (5 ml) for each sample and 1 ml of chloroform was added, then the tubes were incubated for 15 minutes, the upper layers formed in the tubes were transferred to eppendorf tubes, then it was used to spot for thin layer chromatography.

Thin layer chromatography: The standard protocol of thin layer chromatography was followed.

Preliminary test for ethanol: 4 Test tubes were taken, to the first tube plant sample (green) and to the second (green and yellow) plant sample was added, to the third tube 1 ml of distilled water was added and to the fourth tube 1 ml of ethanol was added, a pinch of potassium dichromate was added to all the tubes, then 500 µl of sulfuric acid was added to all the tubes, the tubes were kept in a water bath and the color change was observed.

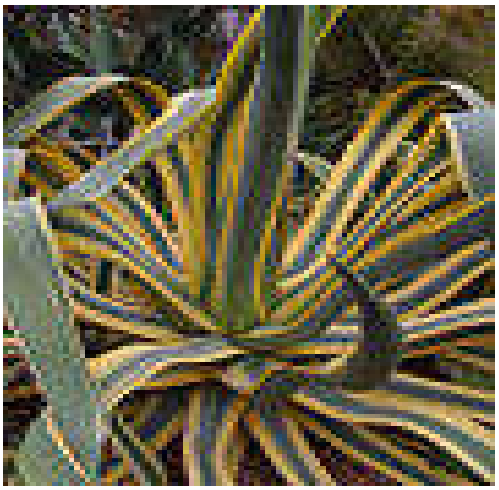
Setup For Bioprocess

- **Estimation of pH:** The pH of OF broth and both the plant sample was estimated using a pH meter.
- **Designing of medium:** 250 ml of each plant sample was prepared by crushing 125 g of each plant leaf with 240 ml PBS, the plant samples were inoculated with 10 ml of each OF broth inoculated plant sample, each batch was kept for static culture and shaking culture.
- **Distillation:** The standard protocol for distillation was performed using a distillation setup.
- **Ethanol estimation:** The ethanol present in each plant sample was estimated using the potassium dichromate method by finally measuring the OD values at 578nm.

RESULTS AND DISCUSSION

Sample Collection: Plant sample *Agave americana var americana* and *Agave americana marginata* were collected from Hindustan University campus (Padur, Chennai) and was used for carbohydrate and protein estimation, and checked for the presence of different types of carbohydrates (Figure 1).

(A)*Agave americana var Americana*

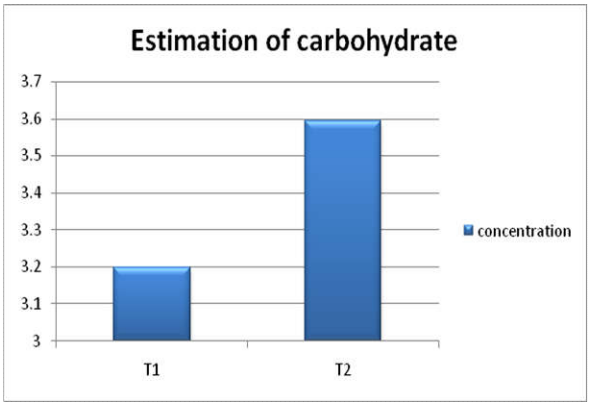


(B) *Agave americana marginata*

Figure 1: *AGAVE SPP.*

Table 1 : Carbohydrate Estimation

STANDARD AND SAMPLES	CONCENTRATION
S1	0.094
S2	0.109
S3	0.227
S4	0.238
S5	0.265
T1	0.339
T2	0.381



Graph 1. Estimate of Carbohydrate

Table 2. Estimation of Protein

STANDARD AND SAMPLES	CONCENTRATION
S1	0.352
S2	0.704
S3	0.769
S4	1.048
S5	1.152
T1	0.043
T2	0.142

Table 3. Estimation of ethanol on 5th day of inoculum

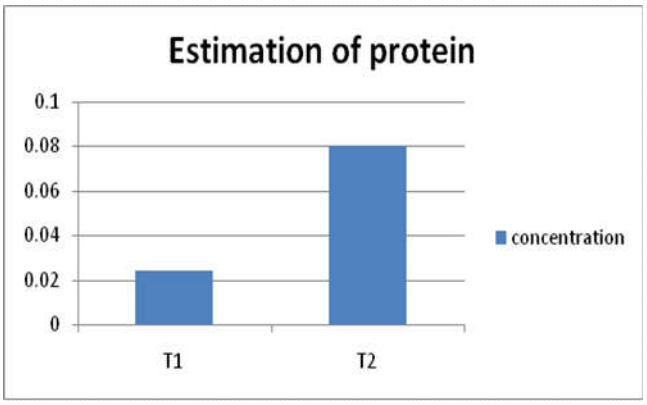
STANDARD AND SAMPLES	CONCENTRATION
S1	3.107
S2	3
S3	1.645
S4	1.262
GT1(Shaking)	1.914
GT2(Shaking)	1.716
GYT1(Shaking)	2.630
GYT2(Shaking)	2.414
GT3(Static)	2.504
GT4(Static)	2.413
GYT3(Static)	1.146
GYT4(Static)	1.018

Table 4. Estimation of ethanol on 10th day of innoculum

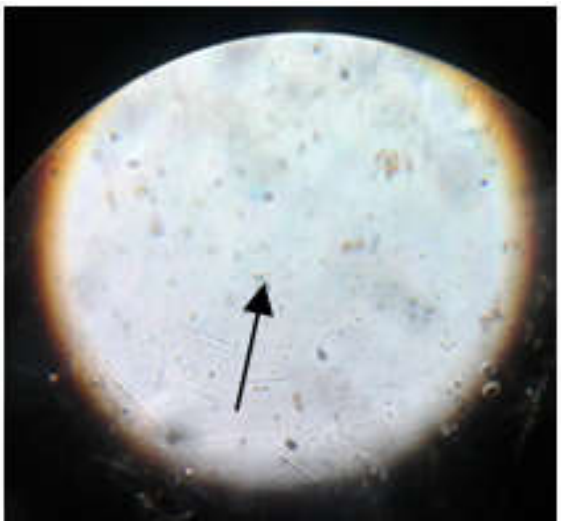
STANDARD AND SAMPLES	CONCENTRATION
S1	3.107
S2	3
S3	1.645
S4	1.262
GT1(Shaking)	1.317
GT2(Shaking)	1.028
GYT1(Shaking)	2.412
GYT2(Shaking)	2.283
GT3(Static)	2.034
GT4(Static)	2.012
GYT3(Static)	1.382
GYT4(Static)	0.981

Table 5. Estimation of ethanol on 20th day of innoculum

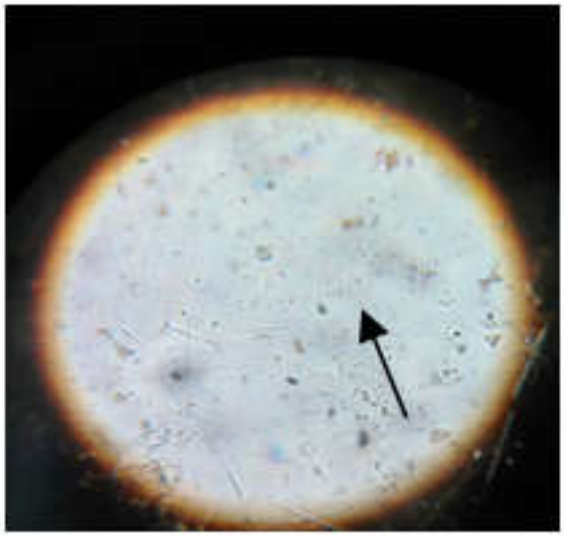
STANDARD AND SAMPLES	CONCENTRATION
S1	3.107
S2	3
S3	1.645
S4	1.262
GT1(Shaking)	1.413
GT2(Shaking)	1.192
GYT1(Shaking)	2.324
GYT2(Shaking)	2.291
GT3(Static)	2.041
GT4(Static)	2.161
GYT3(Static)	1.417
GYT4(Static)	1.021



Graph 2. Estimation of Protein



(A) ***Agave americana var americana***



(B) ***Agave americana marginata***

Figure 2 .Osazone crystals of fructose

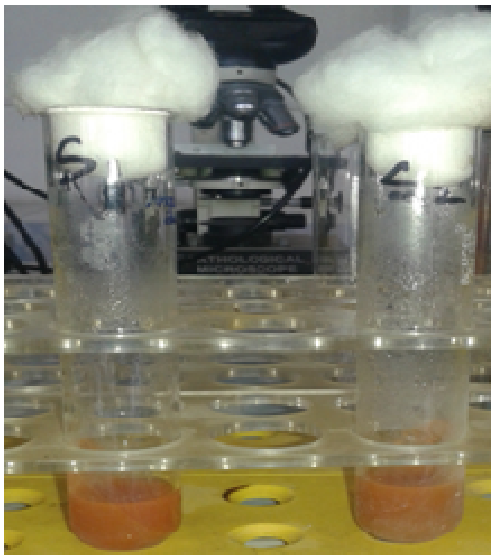
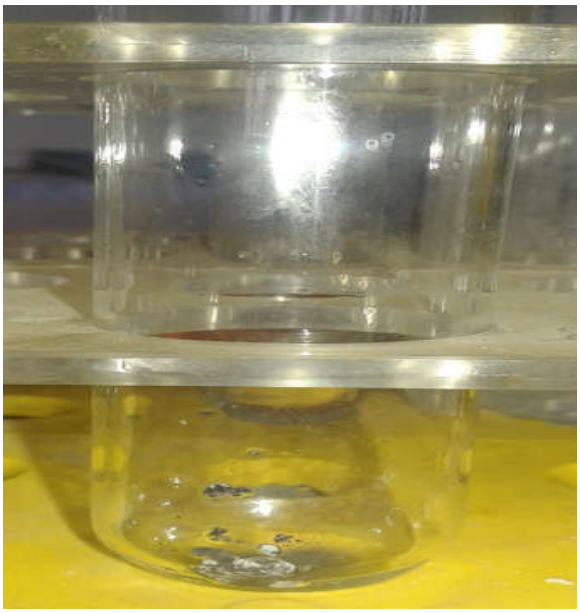


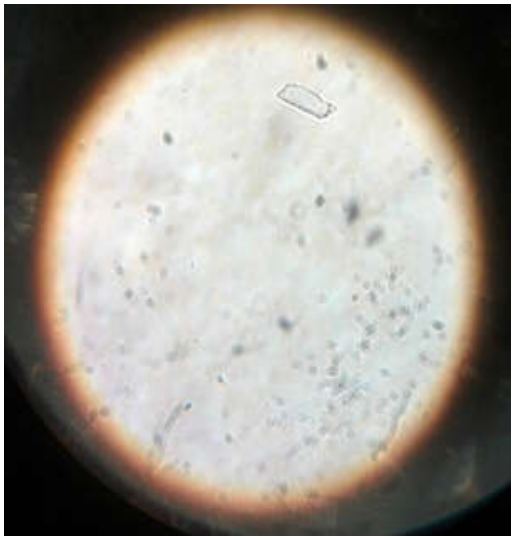
Figure 3. Positive for the presence of fructose by the formation of cherry red color



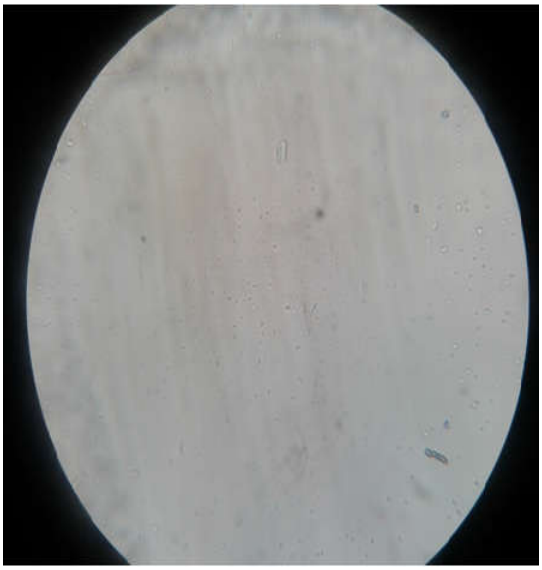
(A) ***Agave americana var americana***

(B) ***Agave americana marginata***

Figure 4. Positive test resulting in the formation of silver mirror



(A)*Agave americana var americana*



(B) *Agave americana marginata*

Figure 5. Positive for the presence of galactose in the form of broken glass structure

Figure 6. Carbon dioxide production

Figure 7 . Potassium permanganate treated TLC slides to confirm the presence of plant pigments

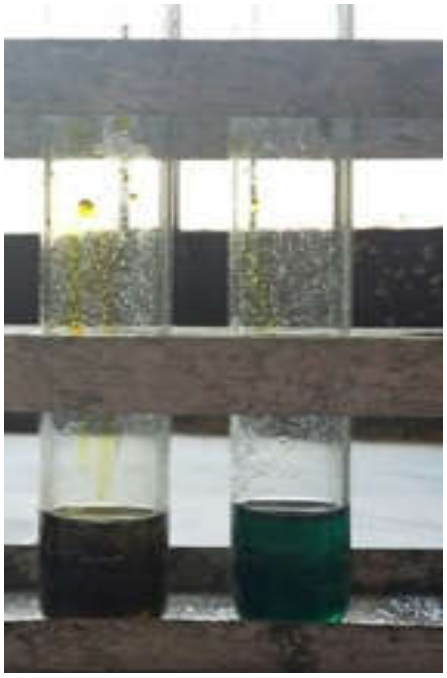


Figure 8. Positive result due to formation of green color

Figure 9. Distillation set up

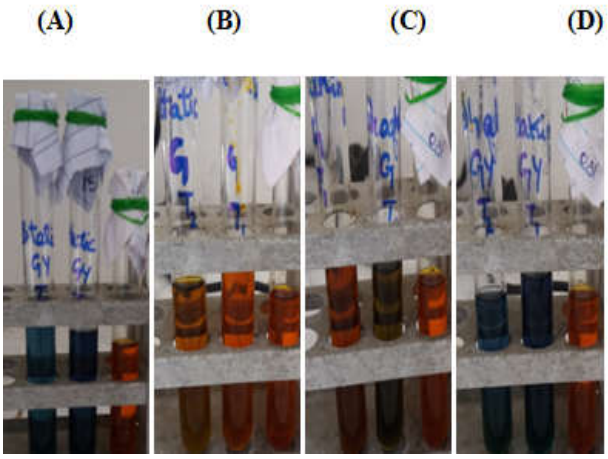
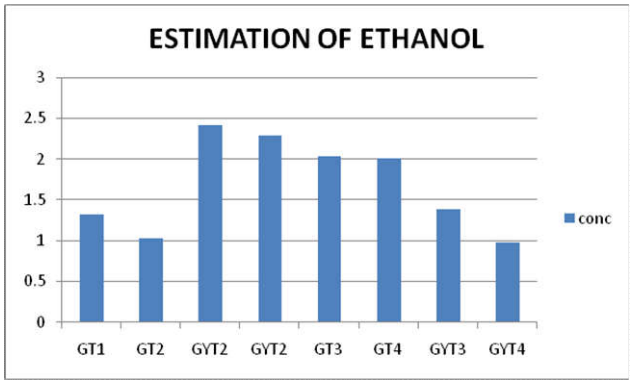


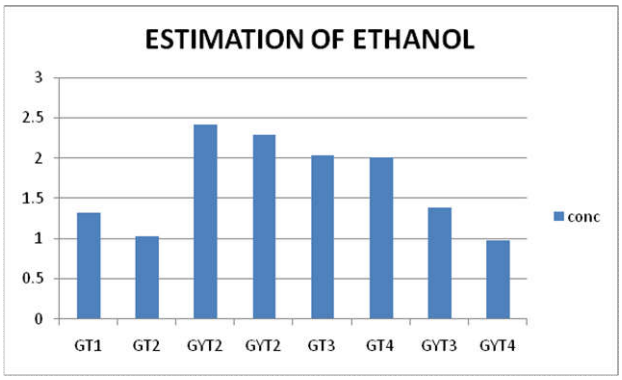
Figure 10. Estimation of ethanol in both static and shaking cultures of *Agave americana var americana* and *Agave americana marginata.*

Biomolecule analysis of *agave americana* var *americana and agave americana mariginata*

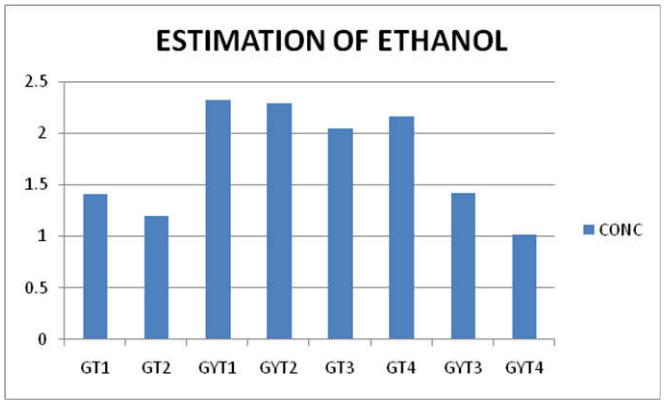
Carbohydrate Estimation: The estimation of carbohydrate was done following the protocol of Benedict's test and the samples optical density was checked at 735nm (Table 1 and Graph 1).



Graph 3A. Estimation of ethanol on 5th day of inoculums



Graph 3B. Estimation of ethanol on 10th day of inoculums



Graph 3C. Graph Estimation of ethanol on 20th day of inoculum

Protein Estimation: The estimation of protein was performed following the protocol of Lowry's method and the samples optical density was checked at 660 nm (**Table 2 and Graph 2**).

Phenyl Hydrazine Test: This test was performed to identify the type of carbohydrate present in the plant sample and presence of small needle bunch (fructose) crystals and long slender broken glass (galactose) crystals were observed under microscope at 40X for both the plant samples (**Figure 2**).

Seliwanoff's test: This test was performed to confirm the presence of fructose which was confirmed by the formation of cherry red color (**Figure 3**).

Tollen's test: Positive Tollen's test will show the formation of silver mirror which was observed in both the plant samples.Silver mirror formation was observed in both the plant samples (Figure 4).

Mucic Test: This test is performed to confirm the presence of galactose. Presence of galactose in the appearance of small broken glass was observed in both the plant sample (**Figure 5**).

Microscopic examination of yeast

Gram's staining: Gram's staining procedure was followed and yeast cells were observed under 40X microscope.

Biochemical characterisation of yeast starch hydrolysis: Starch hydrolysis was performed to determine if Saccharomyces cerevisiae was producing amylase which will break down starch into glucose. Zone formation was observed which confirms the breakdown of starch into glucose. The zone was confirmed using iodine solution which makes the zone visible where starch was hydrolyzed into glucose.

Carbohydrate Utilisation Test: The Durham's tube was floating in the test tube containing yeast inoculated OF broth which confirms the production of carbon dioxide by the yeast cells**.** Hence yeast cells were utilizing the carbohydrate (fructose) from the OF broth and producing carbon dioxide (Figure 6).

Strain improvisation: Bacterial strains were improved as auxotroph mutants in order to increase the production of bioethanol by *Saccharomyces cerevisiae*.

Cell Counting: Cell counting was performed to make sure if substrate used is supporting cell growth. It was performed using a haemocytometer and the cells were counted on the diagonal chambers.

Of broth: Cells present in the diagonal chambers were 45 and 29

Calculation
45+29/2=74/2=**37**

The total number of yeast cells present in 10µl of OF broth was 37

AGAVE AMERICANA* VAR *AMERICANA

Cells present in the diagonal chambers were 20 and 26/µl

CALCULATION

20+26/2=46/2=**23**

The total number of yeast cells present in 10µl of plant sample was 23/µl

AGAVE AMERICANA MARGINATA

Cells present in the diagonal chambers were 45 and 29

CALCULATION

89+103/2=192/2=**96**

The total number of yeast cells present in 10µl of plant sample was 37/µl.

Analysis of plant pigment and of broth by the yeast cells: TLC was performed using Silica gel as stationary phase and chloroform: isopropanol : water as mobile phase (**Figure 7**) in order to confirm the uptake of the substrate by the yeast cells TLC technique was performed. It was designed based upon the appearance of yeast cells during microscope observation. The yeast cells grown in OF broth did not generate any positive result for TLC but the cells grown in plant extract medium generated positive spots for the plant pigment which was further confirmed by potassium permanganate treatment.

Preliminary test for ethanol: The preliminary test for ethanol was done using potassium dichromate and sulfuric acid. The presence of ethanol will give green color which was observed in both the plant samples (Figure 8).

BIOPROCESS

ANALYSIS OF pH: The pH of *Saccharomyces cerevisiae* inoculated OF broth and both plant samples was estimated using a ph meter. The ph was found to be of acidic ph which confirms the production of acid by the organism.

DISTILLATION: The whole distillation set up was arranged and the plant sample was heated at 63ºC which is the boiling point of ethanol. The vapors formed were cooled down by the water jacket connected outside the condenser and the vapors were collected in a receiving flask. Temperature factor is a critical factor as increase in temperature will lead to the condensation of water also (Figure 9).

ESTIMATION OF ETHANOL: Presence of ethanol will give a green colored reaction when treated with potassium dichromate and sulfuric acid. Hence both the plant samples were showing a positive result towards production of ethanol. Shaking culture was producing more ethanol compared to the static culture and *Agave americana marginata* was giving more yield than *Agave americana var Americana* (Figure 10, Tables 3 - 5 and Graph 3A-C).

Conclusion

From the present investigation, it is clear that *Saccharomyces cervisiae* helps in the production of cellulytic bioethanol production using *Agave americana* and *Agave marginata* as the source of substrate. The production of bioethanol was carried out in both static and shaking conditions, out of which shaking conditions gave maximum production. The confirmatory tests for various biomolecules present in Agave provides a strong basic information for further studies of this plant species as a potent source for bioethanol production.

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