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BIOETHANOL PRODUCTION FROM BIOMASSES OF FRESH WATER BLOOMS - SPIROGYRA SP. AND EICHHORNIA CRASSIPES BY SACCHAROMYCES CEREVISIAE

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1. ABSTRACT
In this study, we explored efficiency of *Saccharomyces cerevisiae* the fermentative microflora for biological pretreatment of *Spirigyra* sp., and *Eichhornia crassipes* to fermentable sugar and fermentation of both glucose and xylose to ethanol to increase ethanol yield. The technique herein provides cost effective process to get economically important product from it and more or less helps in lowering plant. The isolated fungi appear as unicellular, large spherical individual cells and taken crystal violet and appeared purple in colour. The fungal cells taken up lactophenol cotton blue stain and appeared blue colour spherical to oval structure.Some of the cells also showed bud formation. Gas formation observed in media inoculated yeast. No gas formation observed in control tube. Colour of the media also turned from red to yellow in colour.

KEYWORDS: Algal bloom, bioethanol, fungus and fermentation.

1. INTRODUCTION
Ethanol is also known as grain alcohol. It can be made from barley and wheat or from cellulosic biomass such as wood, paper pulp or agriculture wastes. Large quantities of ethanol are used as solvent and chemical feed stock in various industries. Most of the industrial ethanol is currently produced from the catalytic conversion of ethylene. Bioethanol is being considered as a potential liquid fuel due to limited amount of natural resources.

Major water bloomers in India are *Eichhorniacrassipes* (water hyacinth), and *Spirogyra* sp. So the present study investigates on bioethanol production from these water blooming organism.

An interest on the potential use of microalgae to produce biofuels through other biomass energy conversion technologies such as anaerobic digestion, thermochemical technologies and anaerobic fermentation growing up and are referred as advanced biofuel technologies or Third Generation Biomass. The fermentation of carbohydrate present in algae biomass, to ethanol is achieved by *Saccharomyces cerevisiae*.

Algae *Spirogyra*sp., is a very common in relatively eutrophic water, developing slimy filamentous green masses. In spring spirogyra grown under water, but when there is enough sunlight. Algae are considered to be most important source for the production of clean and renewable energy. But very easily available biomass grows as filamentous masses of *Spirogyra* sp.,come to the surface and become visible as slimy green mates. Spirogyra can reproduce sexually and asexually. In vegetative reproduction fragmentation takes place, and Spirogyra simply under goes the intercalary mitosis to form new filaments.

Eichhornia crassipes has become a persistent and expensive aquatic problem damaging the environment, irrigation system and crops. and on the other hand to reduce these problems much attention is focused on the potential use of water hyacinth for variety of applications such as its use for ethanol production and hydrogen production.

2. OBJECTIVES
- Collection of plant and algal blooms
- Mass cultivation of algae in laboratory
- Harvesting of algal biomass and collection of weed plant
- Preparation of substrate
- Cultivation of yeast
- Fermentation of substrate
- Estimation of sugar
- Estimation of alcohol

3. MATERIALS AND METHODS

4. 1.ISOLATION OF ALGAE

4. 1.1 Collection of sample

Algae showing profound in pond water were collected in a sterile container and transport immediately. The algal sample was stored at 4^0 until further use.

About 100ml of Bolds Basal medium was prepared. The collected algal sample was allowed to grow in the medium for a period of 20 days at room temperature in the presence of sunlight (20 lux).

4. 2. IDENTIFICATION OF ALGAE

4. 2. 1. Wet mount method

After established growth of algae a small volume of culture was transferred on a clean glass slide to identify the specimen under microscope by wet mount method. One drop of water was placed on the slide and then specimen of algal culture was added and mixed well. Then cover slip was placed over the specimen and observed under microscope.

4. 3. MASS CULTIVATION OF ALGAE

In 1000ml of bolds and basal medium was mass prepared for cultivation of algae .for algal broth was aseptically and about 10ml of inoculums from transferred in to 100ml of medium incubated at 25^0 for 20 days.

4. 4. ISOLATION & CULTURING OF YEAST

4. 4.1 Collection of spoiled fruit

Spoilage fruits was collected from fruit shop and ground in a mixer grinder gently.Then the inoculum was transferred in to peptone water.

4. 4. 2 Cultivation on Sabouraud Dextrose agar

In order to isolate the yeast 100ml of Sabouraud dextrose agar was prepared and sterilized in autoclave at 121^0C for 15 minutes and poured in to sterile petridishes. After solidification one loopful of sample from the peptone water was and inoculated at room temperature for 2 -3 days.

4. 4.3. Identification of isolate

Different identification techniques like simple staining, lacto phenol cotton blue, germ tube method and carbohydrate fermentation tests were carried out.

4. 5. PREPARATION OF SUBSTRATE FROM ALGAE

4.5. 1. Collection Of Algae

The algae was cultured in mass was separated from the broth by centrifugation of cultured broth at 5000rpm for10minutes. Well grown algal mass collected at the bottom of the test tube.It was transferred in to a separate test tube and alga mass pooled.

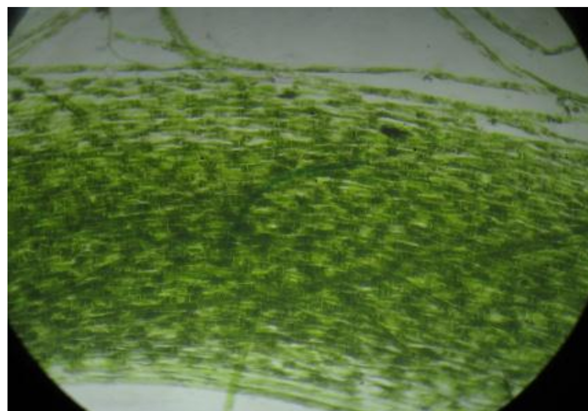

Fig: Mass cultured algal sample

4. 5. 2. Hydrolysis Of Algae

The pellet obtained after centrifugation was hydrolyzed with 1ml of dilute 0.70% H_2SO_4 and was heated at 105^0C for 6 hrs. Then the sample was neutralized by adding Ba_2CO_3. Sample was again centrifuged at 5000 rpm for 10 minutes. Then sample was allowed to be evaporated in water bath. Filtration process carried out to filter the extract.

4. 6. PRETREATMENT (SACHHARIFICATION) OF *Eichhornia crassipes*

Eichhornia crassipes plant were collected from natural pond in Palakkad Dt, Kerala, India. Washed thoroughly several times with tap water to remove dirt on it; then plants were chopped into small pieces, dried in sunlight and blended into fine powder and stored in airtight container at room temperature for further assay.

4. 7. FORMATION OF SLURRY AND LIQUEFACTION

50 grams of dry weed powder were mixed with 200 ml of distilled water. Whole mixture was heated at 90°C for 45 min in hot water bath to liquefy.

4. 8. PRODUCTION OF ETHANOL FROM *E. crassipes* BY FERMENTATION

After saccharification process respective flasks were inoculated with *Sachharomyces cerevisae* inoculum and incubated at 27°C for 15 days anaerobically. Ethanol concentration from all flasks were determined by dichromate assay (GO.ogawa Masami *et al.,* 2008).

4. 9. PRODUCTION OF ALCOHOL ALGAL SLURRY

About 10 ml of the isolated yeast (*Saccharomyces cerevisiae*) is added to the 100 ml of the filtrate taken after centrifugation of the hydrolysis of algae and incubated at room temperature for 5 days.

4. 10. CONFIRMATION OF ALCOHOL PRODUCTION

Different confirmatory tests like litmus test, iodoform test and ester test were performed to confirm the production of alcohol.

4. 11. ESTIMATION OF REDUCING SUGAR

Liquefied slurry was transferred in to centrifuge tube and centrifuged at 1000rpm for 10 minutes. Supernatant were transferred to fresh tube and reducing sugars were estimated by DNSA reagent (Di-nitro-salicylic acid).

4. 12. STANDARDIZATION OF GLUCOSE

Seven clean test tubes and were taken glucose stock solution was added in to each test tubes in the volume of 0µl, 600µl, 1200µl, 1800µl, 2400µl, 3000µl respectively. In 7th test tube 10µl sample was taken for assay. Water was added in to each test tube as 3ml, 2.4ml, 1.8ml, 1.2ml, 0.6ml, 0ml, 2.9mlrespectively. One ml of DNSA reagent was added in to each test tube. The tubes were kept in water bath for 15 minutes. Rochelle salt 1ml to each test tube.OD value for each test tube measured at 510nm.

4. 13. ESTIMATION OF ALCOHOL

Amount of ethanol produced by fermentation was estimated by potassium dichromate method. Initially 10ml of liquor was centrifuged at 1000rpm for 30 minutes at 4^{0} C. Seven clean test tubes were taken and ethanol was added in to each test tubes was taken as 00 ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.00 ml respectively. In 7th test tube 10 ml of sample was taken. Water added to each test tube to make up the volume to 10 ml as 10 ml, 9.8ml, 9.6ml, 9.4ml, 9.2ml, 9ml,and 0ml respectively. 3ml of chromic acid solution was transferred each test tube. Tubes were kept in water bath for 15 minutes. Rochelle salt 1ml was added to each test tube. OD value measured at 600 nm.

5. RESULT

5. 1. WET MOUNT METHOD

The observation of algal mass indicated the pure growth of green filamentous algae. Individual trichome with the spiral chloroplast with nucleus was observed. From the structure of chloroplast the purified algal biomass was confirmed as *Spirogyra* sp.

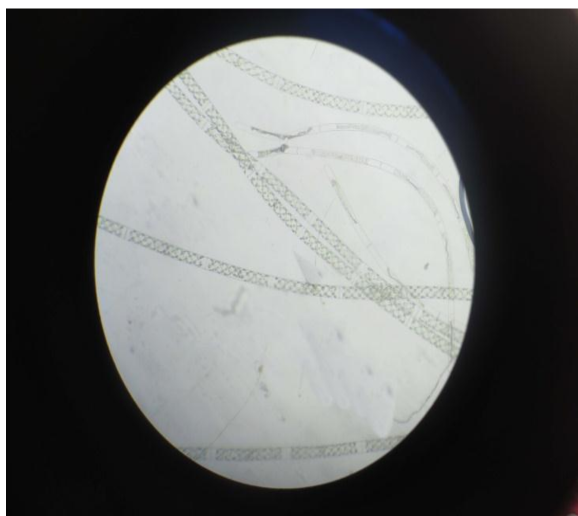

Fig- 1. Microscopic observation of collected algal (*Spirogyra* sp.) specimen.

Fig 2: Collected water weed – *Eichhornia crasippes.*

5. 2. MICROSCPIC OBSERVATION OF ISOLATED YEAST

5. 2. 1. Simple Staining

The isolated fungi appear as unicellular, large spherical individual cells and taken crystal violet and appeared purple in colour.

4. 2. 2. Lactophenol Cotton Blue Wet Mount

The fungal cells taken up lactophenol cotton blue stain and appeared blue colour spherical to oval structure. Some of the cells also showed bud formation.

5. 3. CARBOHYDRATE FERMENTATION TEST

Gas formation observed in media inoculated yeast. No gas formation observed in control tube .Colour of the media also turned from red to yellow in colour. Glucose and sucrose were fermented by yeast culture. Confirmed by accumulation of gas in the Durham's tube, and absence of gas formation and colour change in sucrose and mannose containg medium.

5. 4. RECOVERY OF EXTRACT FROM CULTURE BROTH

Dilute acid hydrolysis was carried out within 100 to 105^{0}C for six hours in water bath by adding 0.70% diluteH_2SO_4 in algae sample. After this treatment followed by evaporation and filtration 4.6 gms of carbohydrate were extracted from 50 ml of algae sample.

5. 5. CONFIRMATION TEST OF ETHANOL PRODUCTION

5. 5. 1. Litmus Test

After fermentation of the extract, it was qualitatively evaluated for the production of ethanol. Alcoholproduction confirmed by change of litmus paper from Blue to red. It indicated the presence of ethanol in the sample.

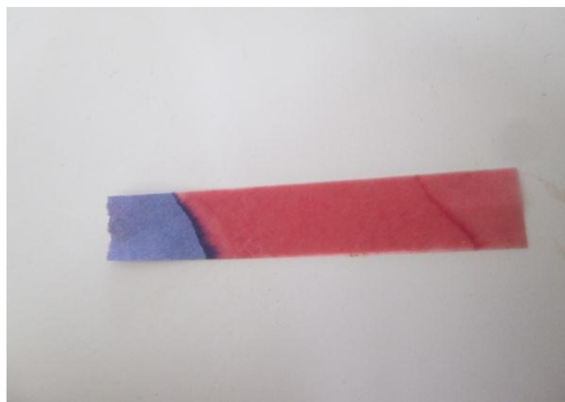

Fig 4: Confirmation of ethanol production by litmus test.

5. 5. 2. Idoform Test

In iodoform test, a yellow colourprecipitation was formed at the bottom of the test tube after the addition of iodine indicated the production of alcohol.

Fig- 5: A brown colour precipitate formed in iodoform test.

5. 5. 3. Ester Test

Fruity smell indicated presence of ethanol.

5. 6. ALGAL BIOMASS

The algal biomass was inoculated with yeast culture and incubated for 15 days under closed environment at 28°C. At odd days of incubation an aliquots was collected and estimated for the presence of remaining sugar and production of alcohol. The sugar concentration was decreasing from initial of 250 mg / ml to 70 mg/ml and at the same time alcohol production wasincreasing from 0.47 ml to 1 ml / 10 ml of the extract which was relatively increasing in volume. Still one third of the sugar is remaining there is enough potential is there to produce more amount of ethanol from remaining sugar. May take another 5 days to completely utilize whole sugar present in the extract into ethanol.

Table 1: Table showing production of alcohol from algal extract.

S. No	Days of incubation	OD at 510nm	OD at 600nm	Concentration of sugar (µg/ml)	Volume of ethanol (ml)
1	1	1.63	0.26	250000	0.47
2	3	1.21	0.32	260000	0.58
3	5	0.82	0.38	160000	0.64
4	7	0.73	0.41	130000	0.7
5	9	0.55	0.43	90000	0.73
6	11	0.41	0.46	70000	0.79
7	13	0.39	0.60	70000	1.00

5. 7. WATER PLANT EXTRACT

The *Eichhornia crassipes* biomass extract was inoculated with yeast culture and incubated for 15 days under closed environment at 28°C. At odd days of incubation an aliquots was sample collected and estimated for the presence of remaining sugar and production of alcohol. The sugar concentration was decreasing from initial concentration of 323 mg / ml to 115 mg/ml and at the same time alcohol production was increasing from 0.60 ml to 1.8 ml / 10 ml of the extract which was relatively increasing in volume. Still one third of the sugar is remaining there is enough potential is there to produce more amount of ethanol from remaining sugar. About 60% of sugars converted to ethanol that accounts for 20% of the total sample volume.

Table 2: Table showing production of alcohol from *E. crassipes* biomass extract

S. No	Days of incubation	OD at 510nm	OD at 600nm	Concentration of sugar (µg/ml)	Volume of ethanol in ml
1	1	1.82	0.34	323000	0.60
2	3	1.65	0.44	260000	0.73
3	5	0.93	0.49	190000	0.82
4	7	0.85	0.52	175000	0.85
5	9	0.72	0.55	127000	0.88
6	11	0.70	0.61	125000	1.1
7	13	0.65	0.67	115000	1.8

6. DISCUSSION AND SUMMARY

Recently, due to shortage in natural resources like petrochemical and natural gas Scientist have been trying to develop new renewable energy resources. Nowadays bioethanol is used as alternative to petrochemical, due to this reason many researchers have tried to find out various sources for production of Bioethanol, biomass is one of the resources for bioethanol.

Microalgal and water weed plant biomass composition can be manipulated by applying various stress cultivation conditions. Under stress conditions, such as nutrient starvation or high light intensity, some microalgal and water weed species accumulate carbohydrates in their biomass, which can amount to a significantly high level. *Eichhornia crassipes* is a water weed contains less amount of lignin compared to other terrestrial plants. In the present study the potential of bioethanol production using carbohydrate-enriched biomass of the cyanobacterium *Spirogira* sp. and from the water weed plant *Eichhornia crassipes*were studied.

The isolated fungi appear as unicellular, large spherical individual cells and taken crystal violet and appeared purple in colour. The fungal cells taken up lactophenol cotton blue stain and appeared blue colour spherical to oval structure.Some of the cells also showed bud formation. Gas formation observed in media inoculated yeast. No gas formation observed in control tube. Colour of the media also turned from red to yellow in colour. Glucose and sucrose were fermented by yeast culture. Confirmed by accumulation of gas in the Durham's tube, and absence of gas formation and colour change in sucrose and mannose containg medium.

In conclusion we explored efficiency of *Saccharomyces cerevisiae* the fermentative microflora for biological pretreatment of *Spirigyra* sp., and *Eichhornia crassipes* to fermentable sugar and fermentation of both glucose and xylose to ethanol to increase ethanol yield. As previously mentioned water hyacinth is one of the expensive aquatic weed which is creating problems to aquatic and terrestrial systems; for this much emphasis is given to control its growth, but most of the time all measures are ineffective due to pernicious invasive growth. The technique herein provides cost effective process to get economically important product from it and more or less helps in lowering plant.

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