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Bioethanol production from grass (*Ischaemum Santapaui* Bor.) by enzymatic hydrolysis

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Abstract
Many countries are searching and developing renewable energy sources including the production of bioethanol. Carbohydrate rich plant materials are mostly used for production of bioethanol. In this investigation, enzymatically hydrolysed slurry of weed *Ischaemum satapaui* biomass was used as medium for ethanol fermentation. The *Ischaemum satapaui* biomass was *enzymatically hydrolysed* by *Trichoderma reesei.* Using DNSA method, 7.92% reducing sugars were estimated from hydrolyzed plant slurry. Plant slurry was fermented by inoculating *Saccharomyces cerevisiae* for conversion of sugars into ethanol. 33.44% ethanol produced from biomass was estimated by potassium dichromate method.

Keywords: Bioethanol, enzymatic hydrolysis, Fermentation, *Ischaemum satapaui, Saccharomyces cerevisiae.*

Introduction

Energy is one of the important factors to global prosperity & continued growth of the global economy has increased the energy consumption which in turn badly affected the climate change. Many countries are developing renewable energy, including the production of bio-fuels. Bio-fuel is a fuel that is produced over a short time span from biomass rather than by the very natural processes involved in the formation of fossil fuels, such as oil. Bio-fuel can be produced from plants or from agricultural, domestic or industrial bio-waste. Bio-fuels are mostly used for transportation, heating & for producing electricity. They are regarded as a renewable energy source.

Bio-ethanol is one of the common types of bio-fuel, which is a form of renewable energy that can be produced from agricultural feed-stocks. It is a type of alcohol that is obtained from different types of plants rich in cellulose. It can be made from very common crops such as sugarcane, sugar beet, potato or some grains such as corn. It is frequently used as motor fuel or as an additive in gasoline and is an option for more 'renewable' energy. Now a days, research is going all around the world for the production of bio-ethanol.

In India, the bioethanol industry is primarily based on sugarcane, which is the main raw material used for the production of bioethanol. In recent years, the Indian government has implemented several policies to promote the bioethanol industry. These include tax incentives for farmers and companies that produce bioethanol, subsidies for the production of biofuels, and mandatory blending of ethanol with gasoline. These policies have helped to increase the production of bioethanol in India and have attracted investment from both domestic and foreign companies.

Ischaemum santapaui Bor. Belongs to the family Gramineae (Poaceae). It is an annual plant and grows primarily in the seasonally dry tropical region. The plant is native to India, particularly in the state of Maharashtra and widely distributed in Bombay, Nashik, Pune, Raigad, Ratnagiri, Sindhudurg and Thane.The plant is pedicellate spikelet with pedicel half as long as the sessile spikelet. The flowering period starts from September-November (Sharma *et al.*,1996) [2].

The selected *Ischaemum* plant is found in abundance after rainy season and it is rich in carbohydrate known as cellulose. Cellulose makes up the most of a plant cell wall. It is commonly eaten by grazing livestock. It is incredibly powerful plant with a wide range of benefits for the ecosystem and plays critical role in soil stabilization, habitat creation, nutrient cycling, water regulation and erosion control.

The fungus *Trichoderma* plays a crucial role in bioethanol production. *Trichoderma* species like *Trichoderma reesei* produce hydrolytic enzymes that break down lignocellulose into fermentable sugars. *Trichoderma* can be co-cultured with yeast like *Saccharomyces cerevisiae* for bioethanol production from lignocellulosic biomass. *Saccharomyces cerevisiae* is a robust organism that can withstand various environmental conditions and efficiently convert a wide range of sugars into ethanol, making it a popular choice for bioethanol production. *Saccharomyces cerevisiae* can tolerate high ethanol concentrations, allowing for high ethanol yields.

Many researchers have been working on bioethanol for over a century with significant advancements in the past (Adesanya *et al*., 2008 [3]; Zabochnicka and Slawik, 2010 [4]; Abideen *et al*., 2011 [5]; Wang *et al*., 2012 [6]; Kumar *et al*., 2013 [7]; Bharti and Chauhan, 2016 [8]; Jayus *et al*., 2016 [9]; Kenechi *et al.*, 2016 [10]; Stanley *et al*., 2017 [11]; Zheng *et al*., 2019 [12]; Yingjie *et al*., 2019; Lugani *et al*., 2020 [14]; Mulyana *et al* [15]., 2020; Arefin *et al*., 2020 [16]; Bangalore and Siva, 2021 [17]; Vasic *et al*., 2021 [18]; Mueansichai *et al*., 2022 [19]; Zhang *et al*., 2023, etc.) [20] but the references related to work on production of bioethanol from grass (*Ischaemum santapaui*) were not seen. So, it was decided to undertake the present investigation entitled "Bioethanol Production from Grass (*Ischaemum santapaui*) by Enzymatic Hydrolysis" during the academic year 2023-24.

Materials and methods

1. Sample collection and pre-treatment

Ischaemum santapaui plants were collected from grass lands and crop field near to Vidyanagar and adjoining area of Jalgaon, Tal-Dapoli. The collected plant was identified correctly with local floras (Cook, 1958 [1]; Sharma *et al.*, 1996) [2] and authenticated from plant taxonomist. Plant was

cut into pieces of 2-3 cm and dried for 4 days, subsequently oven dried at 70°C for 6 hours and then ground into fine powder using high speed blender. (Nitesh Kumar *et.al.*, 2013) [7]

2. Formation of Slurry & Liquefaction: -

50 gm of dry powder were mixed with 200 ml distilled water. Whole mixture was heat at 90°C for 45 minutes in hot water bath to liquefy. (Nitesh Kumar *et.al.*, 2013) [7]

3. Inoculum preparation

Trichoderma reesei and *Saccharomyces cerevisiae* were grown on petriplate containing PDA. Isolated colony were inoculated in conical flask containing 50 ml of 3% PDA for preparing spore suspension.

Trichoderma reesei was kept at room temperature for 36 hours. Saccharomyces cerevisiae was kept on incubator shaker at 37 °C for 18 hours prior to inoculation. (Nitesh Kumar *et.al.*, 2013) [7]

4. Enzymatic hydrolysis

To convert cellulose into simple monomeric carbohydrate 6ml of *T. reesei* spore suspension were inoculated in 200 ml of slurry and kept for 4 days in an incubator shaker at 100 r.p.m at 37°C for continuous shaking. (Nitesh Kumar *et.al.*, 2013) [7]

5. Estimation of reducing sugar

Liquefied slurry was transferred into centrifuge tube and centrifuged at 10,000 r.p.m for 10 min. Supernatant were transferred to fresh tube and reducing sugars were estimated by DNSA reagent (Dinitro salicylic acid) as described by Miller (1959).

DNSA reagent is prepared by dissolving 1g of dinitro salicylic acid, 200 mg crystalline phenol and 50 mg of sodium sulphite in 100 ml of 1% NaOH. After addition of DNSA reagent to standard solution and test solution whole reaction mixture was incubated in boiling water bath for 15 minutes followed by addition of 40 % of Rochelle salt in solution to stabilize colour of reaction mixture. Subsequently observance was measured at 510 nm. The whole set of experiment was performed in triplicate. (Nitesh Kumar *et.al.*, 2013) [7]

6. Fermentation

Fresh Saccharomyces *cerevisiae* culture was inoculated into liquefied slurry. Conical flask was sealed completely to induce an anaerobic condition for efficient fermentation. Monomeric sugars unit were converted into bioethanol by action of enzyme produced by *Saccharomyces cerevisiae*. (Nitesh Kumar *et.al.*, 2013) [7]

7. Ethanol estimation

Amount of ethanol produced by fermentation was estimated by potassium dichromate method. Initially 10ml of liquor was centrifuged at 10,000 r.p.m for 30 min at 4°C. 1gm of Potassium dichromate was dissolved in 100ml of 5M sulfuric acid solution.

Prepared chromic acid reagent was added to standard as well as teat sample. Kept in water bath at 90° C for 10 min. Add 40% Rochelle salt in solution. Absorbance was measured at 600 nm. (Nitesh Kumar *et.al.*, 2013) [7]

Observations

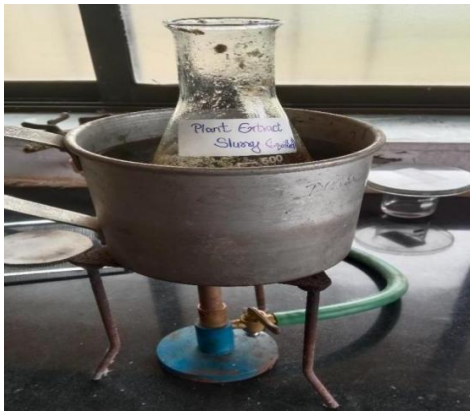
- **Sample collection and pre-treatment**

Plant was collected from the grasslands located near Vidyanagar and adjoining area of Jalgaon, Tal-Dapoli, Dist-Ratnagiri. The collected plant was identified using local floras and authenticated from taxonomist. Plant was cut into pieces of 2-3 cm and dried for 4-5 days. Oven dried at 70°C for 6 hours then ground into powder using a blender.

Collected *Ischaemum santapaui* plant and after drying converted into powdered form

Formation of Slurry & Liquefaction

50 gm of dry powder were mixed with 200 ml distilled water and was heated at 90°C for 45 minutes in hot water bath to liquefy.



Preparation of slurry

Preparation of inoculum

Trichoderma reesei and *Saccharomyces cerevisiae* were grown on petriplate containing PDA. Isolated colony were inoculated in conical flask containing 50 ml of 3% PDA for preparing spore suspension. *Trichoderma reesei* was kept at room temperature for 36 hours and *Saccharomyces cerevisiae* was kept on incubator shaker at 37 °C for 18 hours prior to inoculation.

Preparation of *Trichoderma reesei* and *Saccharomyces cerevisiae* inoculum.

Enzymatic hydrolysis

6ml of *T. reesei* spore suspension were inoculated in slurry and kept for 4 days in a shaker at 100 r.p.m at 37°C for continuous shaking.

Enzymatic hydrolysis process

Estimation of reducing sugar by DNSA reagent

Liquefied slurry was centrifuged at 10,000 r.p.m for 10 minutes. DNSA reagent is then added to standard solution and test solution and was incubated in boiling water bath for 15 min. It is followed by addition of 40 % of Rochelle salt. It was measured at 510 nm.

Table 1: Reducing Sugar Estimation by DNSA Method

Sr.no.	Concentration (µg/ml)	Volume (µl)	Water (ml)	DNSA (ml)	Rochelle salt (ml)	O.D at 510 nm
1	000	0.00	3.000	3.0	1.00	0.00
2	200	600	2.400	3.0	1.00	0.03
3	400	1200	1.800	3.0	1.00	0.06
4	600	1800	1.200	3.0	1.00	0.09
5	800	2400	0.600	3.0	1.00	0.12
6	1000	3000	0.000	3.0	1.00	0.18
7	Test sample	10	2.900	3.0	1.00	0.03

Ethanol estimation by potassium dichromate method

Initially 10ml of liquor was centrifuged at 10,000 r.p.m for 30 min at 4°C. Prepared chromic acid reagent was added to standard as well as test sample and kept in water bath at 90° C for 10 min. After add 40% Rochelle salt in solution. Absorbance was measured at 600 nm.

Table 2: Potassium Dichromate Method for Estimation of Bioethanol.

Sr.No.	Ethanol (ml)	Distilled water (ml)	Chromic acid (ml)	Rochelle salt (ml)	O.D at 600 nm
1.	0.00	10.0	3	1	0
2.	0.20	9.80	3	1	0.05
3.	0.40	9.60	3	1	0.11
4.	0.60	9.40	3	1	0.16
5.	0.80	9.20	3	1	0.21
6.	1.00	9.00	3	1	0.25
7	Test sample (10 ml)	0.00	3	1	0.88

Results and discussion

The dried powered biomass of *Ischaemum santapaui* was enzymatically hydrolysed by inoculating *Trichoderma reesei*. It was estimated that, **7.92%** reducing sugar were produced from enzymatically hydrolysed biomass of *Ischaemum santapaui*. The enzymatically hydrolysed slurry of *Ischaemum santapaui* biomass was directly used as media for ethanol fermentation. *Saccharomyces cerevisiae* was inoculated in hydrolysed slurry to carry out fermentation. The bioethanol produced by fermentation was estimated by potassium dichromate method which was 33.44%.

Most of the countries are using crops like corn, sugarcane, potato, etc to produce bioethanol. But in many countries like India, China and other countries which has high population density, it is not possible to use crop plant or potato for fuel resources because chances of facing food crisis will increase. In these situations, weed plants like *Ischaemum santapaui* can be good alternative sources for ethanol production.

Conclusion

Fuel crisis is biggest problem in the world. It motivates researchers to find out a new and low-cost source for ethanol production. In this study, *Ischaemum santapaui* was used for ethanol production. During this study it was found that, this *Ischaemum santapaui* weed can be used commercially for large scale production of bioethanol.

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