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Isolation of Potential Microorganisms Capable of Producing a High Amount of Bio-ethanol for Converting Plant Waste into Wealth

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ABSTRACT

The declining oil and gas reserves globally underscore the urgency for expanded research into alternative energy sources. Bio-ethanol emerges as a prominent renewable fuel in transportation. Utilizing microorganisms to convert sugar containing waste into ethanol presents a promising avenue due to its speed and cost-effectiveness. This study aimed at isolating and identifing ethanol producing yeasts from over ripened fruits from the local market of Lakshmangarh, Rajasthan. Various biochemical tests were conducted for microbial identification of potential yeast strains isolated from over ripened plums, oranges, and grapes as substrates. The study explored the use of lignocellulosic waste, specifically rice straw avoiding competition with first generation alternatives. Chemical pre-treatment with 4% NaOH (1:10) was employed to reduce recalcitrant nature of lignin. Enzymatic hydrolysis using cellulase alpha-amylase and amyloglucosidase in 2:1:1 yielded 0.65 g/l of sugar, which was then fermented by isolates at 27°C for seven days. This process led to a significant increase in bio-ethanol production, achieving a yield of 16%.

Key words: Rice straw, renewable feed stocks, lignocellulosic biomass, fermentation, bio-ethanol

INTRODUCTION

In 2030, it is anticipated that 8.5 billion people will inhabit the earth due to ongoing population growth (Novia *et al*., 2025). Fossil fuels stand as one of the primary energy sources, leading to an escalation in energy consumption and, consequently, increased usage. The idea of generating energy from renewable sources has become increasingly important worldwide (Liu *et al*., 2021). The emphasis is on creating a sustainable bio-fuel that responsibly uses natural resources while protecting the environment. Bio-ethanol is a promising bio fuel because only renewable energy is utilized in its manufacturing process (Tse *et al*., 2021). Since no additional carbon dioxide is released into the atmosphere, Bio-ethanol serves as a beneficial energy source for the environment (Broda *et al*., 2022). Bio-ethanol can be derived from various generations of feed stocks, categorized as first generation, second generation or third generation. First generation feed stocks encompass food crops such as corn, wheat and soybeans (Susmozas *et al*., 2020), but have raised concerns regarding food security and land use competition. Second generation feed stocks, exemplified by lignocellulosic biomass, offer a promising alternative. Lignocellulosic biomass comprises non-food plant materials like agricultural residues, forest residues and dedicated energy crops, thus mitigating concerns associated with first generation feed stocks (Branco *et al*., 2019; Beluhan *et al*., 2023). Furthermore, third generation feed stocks, such as algae present another avenue for bio-fuel production.

Regarding second generation bio-ethanol production, efficient conversion of biomass to ethanol requires development of microorganisms capable of fermenting a wide range of carbohydrates and tolerating high concentrations of ethanol. Varieties of yeast demonstrating significant resilience to both salt and ethanol are especially valued in this procedure, with their exceptional attributes highly sought after (Elhalis, 2024). The yeast organisms like *Saccharomyces cerevisiae*, *Pichiastipitis*, *Saccharomyces carlbergensis* and *Candida tropicalis*, as well as specific bacteria such as *Zymomonasmobilis* and *Clostridium thermocellum*

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are utilized in bio-ethanol production because they can ferment hexose sugars (Nieves *et al*., 2019). Yeasts have been essential in alcohol production for millennia, especially in the wine and beer sectors. Their ability to yield high ethanol levels, maintain productivity and withstand elevated ethanol concentrations helps in cutting costs in distillation processes (Mohd Azhar *et al*., 2017). Microorganisms like *S. cerevisiae* are extensively employed in ethanol fermentation (Ozdingis and Kocar, 2017; Ruchala *et al*., 2020). This yeast is also used in commercial ethanol production because it tolerates a broad pH range making the process more resistant to infection.

There are several phases involved in the production of bio-ethanol, including pre-treatment, hydrolysis, fermentation and distillation. Pre-treatment affects the entire process and its costs, making it a crucial step in the production of bio-ethanol. The main objectives of pre-treatment are to breakdown the lignin and disrupt the crystalline nature of cellulose. The pre-treatment method can be divided into three main groups: Physical (steam explosion, milling); chemical (acid or alkali) and biological pre-treatment (by microorganisms). Pre-treatment is a cost consuming step and approximately 20% of the total cost is caused by it. Bringing down the pre-treatment cost is the key point in the commercialization of cellulosic bio-ethanol due to its high cost (Anu *et al*. 2020; Shukla *et al*., 2023). Enzymatic hydrolysis is a pivotal process in the conversion of cellulose into glucose, utilizing a diverse array of enzymes. These enzymes play a crucial role in breaking down the complex cellulose molecules into simpler glucose units through hydrolysis. Each enzyme involved in this process exhibits specialized functions, collectively facilitating the efficient breakdown of cellulose chains into glucose molecules. This enzymatic conversion is vital in bio-fuel production, as glucose serves as a primary precursor for the synthesis of bio-ethanol and other valuable products (Vasic *et al*., 2021). Using readily available and cost-effective materials for producing cellulosic ethanol could reduce dependence on petroleum.

In view of the above, a cheap and renewable source of substrate in the form of rice straw was used for its conversion into bio-ethanol. The objective of this research was to isolate and identify microorganisms with the capacity to efficiently generate large quantities of bio-ethanol from such abundantly available lignocellulosic waste, thereby promoting sustainable development. While selecting the micro-organisms for bio-ethanol production, the type of sugar utilized by these microbes was kept into the mind, so that mix culture was used to utilize variety of sugars available and thereby maximizing the yield of bio-ethanol from rice straw.

MATERIALS AND METHODS

Over ripened plums, oranges and grapes sourced from the Lakshmangarh local market underwent experimentation at the Department of Biosciences, Mody University of Science and Technology, Sikar. Rice straw (RS) from diverse locations, including Karnal (29.6857° N, 76.9905° E), Kurukshetra (30.1738° N, 76.9600° E) and Manipur (24.8170° N, 93.9368° E), was collected, cut into 3-4 mm pieces, milled and processed into fine powder. The YEPDA agar medium, comprising yeast extract, peptone, D-glucose and chloramphenicol was utilized for isolating microorganisms from over ripened fruits (Nasir *et al*., 2017; Dharaneesh *et al*., 2024). Various methods were employed for isolation and identification, including morphological and biochemical analyses. Morphological characteristics, such as colony colour, shape and budding cell arrangement were observed under a microscope at 100X magnification. Biochemical identification involved conducting fermentation tests on carbohydrates like glucose, galactose, lactose and fructose using Durham tubes and YEPD broth with phenol red. The change in colour from red to yellow in the fermentation media and the formation of CO_2 gas in the Durham tube indicated fermentation activity (Moremi *et al*., 2020). The pre-treatment of rice straw (RS) involved chemical treatment with acids (HCl) and alkali (NaOH). The fine ground substrate underwent a 30-min heat treatment at 121°C while immersed in a 4% NaOH solution, with a liquid to solid ratio of 1:10 (w/v). Subsequently, to neutralize the alkaline effect, the material was treated with hydrochloric acid (HCl). Enzymatic hydrolysis was conducted employing enzymes including cellulase, alpha-amylase and amyloglucosidase (0:1:1, 1:1:1, 2:1:1). Following

this, the mixture underwent incubation for 96 h at 150 rpm in a rotary shaker incubator. The subsequent identification of reducing sugars formed in the hydrolysate was accomplished using the DNS approach (Zhang *et al.,* 2019; Theresa *et al.,* 2024). Isolated microorganisms' inoculums were introduced into treated substrate under optimized enzyme conditions. Fermentation was conducted at 27°C for a week until characteristic alcoholic odour developed. Ethanol yield was quantified employing the potassium dichromate method. Purification of bio-ethanol was ensured via distillation, incurring significant costs due to repetitive vaporization and condensation steps. Following distillation, ethanol underwent analysis through combustion, Jones oxidation and tri iodomethane tests.

RESULTS AND DISCUSSION

A total of nine isolates were obtained from samples of plum, orange and grape fruits, with the majority of colonies exhibiting a creamy to white appearance. From these nine isolates, three were chosen and observed to be in an actively budding stage (Fig. 1). Specifically, isolates P_1, P_2 and P_3 were derived from plum, orange and grape samples, respectively. Morphological identification revealed spherical shapes, cream-coloured colonies and budding-like structures observed in P_1, P_2 and P_3 isolates as shown in Fig. 2 (Bitew *et al.,* 2023). Table 1 shows that all three isolates were capable of producing hydrogen peroxide gas during the catalase test. All three isolates produced a red colour during the methyl red

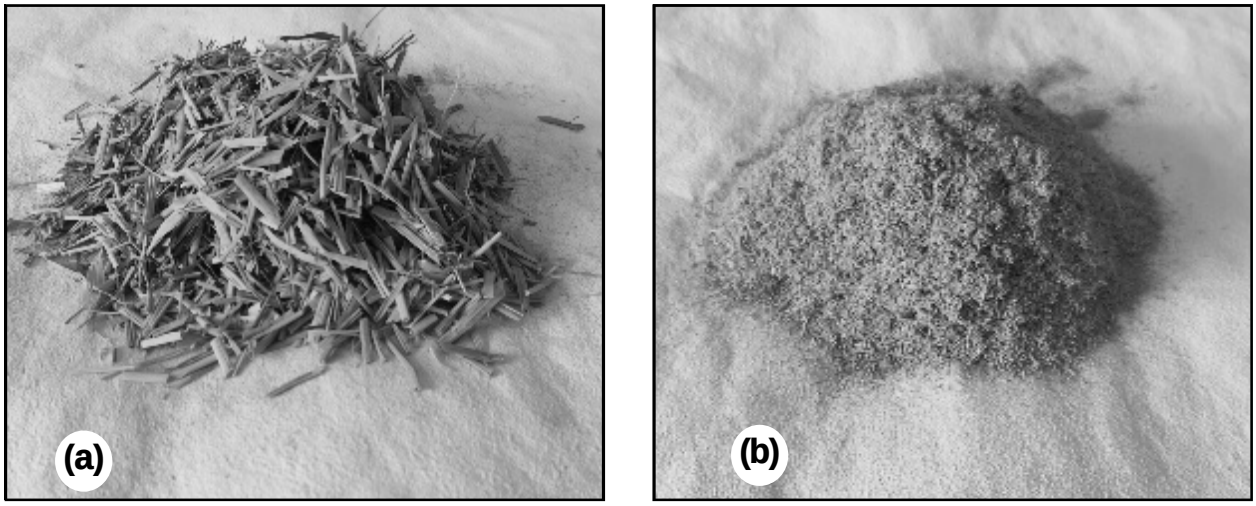

Fig. 1. (a) Chipped rice straw and (b) Powdered rice straw.

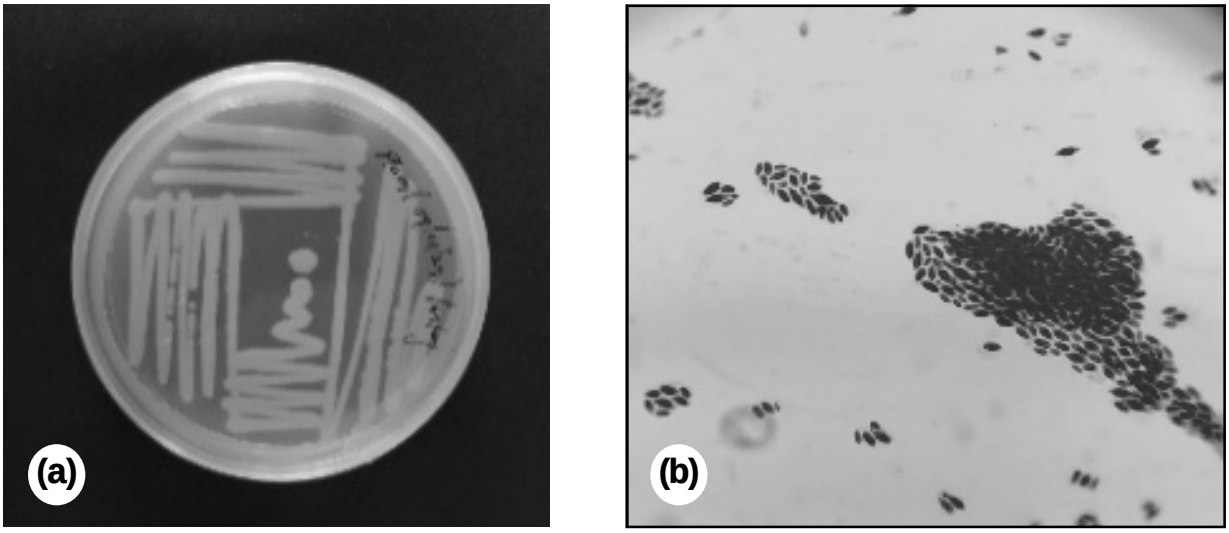

Fig. 2. Morphological identification of isolates: (a) Growth of isolates on YEPD media and (b) Budding structure observation under 100x microscopic resolution.

Table 1. Biochemical tests of isolates P_1, P_2 and P_3

Strains	Catalase	Indole	Methyl red	Voges-Proskauer	Amylase
P_1	Positive (White froth)	Negative (No red colour)	Positive (Red colour)	Negative (No red colour)	Negative (Blue colour)
P_2	Positive (White froth)	Negative (No red colour)	Positive (Red colour)	Negative (No red colour)	Negative (Blue colour)
P_3	Positive (White froth)	Negative (No red colour)	Positive (Red colour)	Negative (No red colour)	Negative (Blue colour)

test, and all isolates responded negatively to the Indole, VP and Amylase tests. Table 2 illustrates that the majority of the chosen isolates possess the capability to ferment different sugars.

Alkali treatment was the most effective pre-treatment technique to break down the recalcitrant nature of biomass. 4% NaOH alkali treatment was the most effective pre-treatment technique to break down the recalcitrant nature of biomass. The FTIR data demonstrated that lignin underwent degradation after treatment with 4% NaOH, which was evident from FTIR data of untreated rice straw sample (Fig. 3a) which differs from 4% NaOH treated rice straw (Fig. 3b). The lignin peak values in treated rice straw, which ranged from 1542 to 1484/cm were absent, whereas these peaks were present in untreated rice straw. In this investigation, glucose was used as the reference sugar. The optimal quantity of reducing sugar was provided to the cellulase, alpha-amylase and amyloglucosidase enzymes at 2:1:1 concentration. In this experiment, pre-treated rice straw hydrolyzate yielded 0.65 g/l of reducing sugar. Rice straw that was not treated

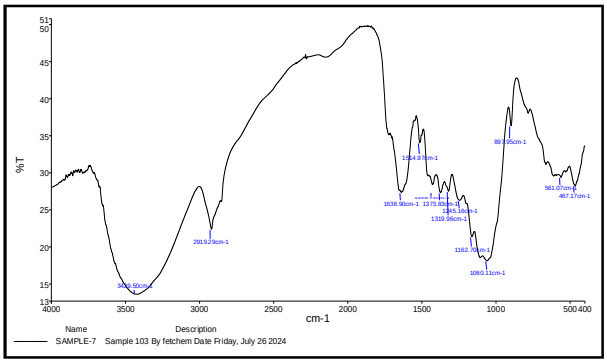

Fig. 3. (a) FTIR analysis of untreated rice straw sample.

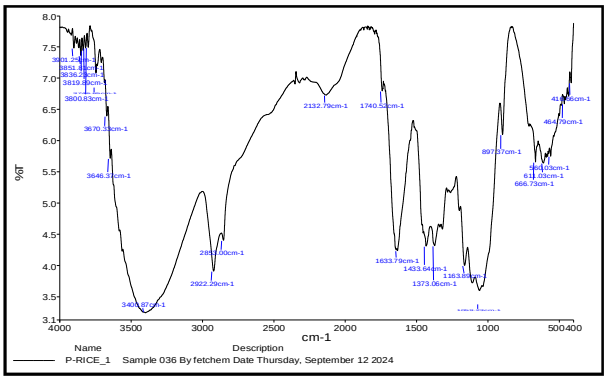

Fig. 3. (b) FTIR analysis of 4% NaOH treated rice straw sample.

Table 2. Fermentation studies of different carbohydrates by isolates P_1, P_2 and P_3

Isolate	Carbohydrate	Before fermentation (colour)	After fermentation (colour), Gas production
P_1	Glucose	Red	+ (Yellow), Gas production
	Galactose	Red	+ (Yellow), Gas production
	Sucrose	Red	+ (Yellow), Gas production
	Lactose	Red	+ (Yellow), Gas production
	Fructose	Red	+ (Yellow), Gas production
P_2	Glucose	Red	+ (Yellow), Gas production
	Galactose	Red	+ (Yellow), No gas production
	Sucrose	Red	+ (Yellow), Gas production
	Lactose	Red	+ (Yellow), Gas production
	Fructose	Red	+ (Red), No gas production
P_3	Glucose	Red	+ (Yellow), Gas production
	Galactose	Red	+ (Yellow), Gas production
	Sucrose	Red	+ (Yellow), Gas production
	Lactose	Red	+ (Yellow), Gas production
	Fructose	Red	+ (Red), No gas production

Table 3. Potassium dichromate method for ethanol estimation

Alcohol conc. (v/v) (%)	Ethanol (µl)	Dist. H_2O (µl)	Total vol. (ml)	Chromic acid reagent (ml)	Dist. H_2O (ml)
0	0	1000	1	25	24
2	20	980	1	25	24
4	40	960	1	25	24
6	60	940	1	25	24
8	80	920	1	25	24
10	100	900	1	25	24
12	120	880	1	25	24
14	140	860	1	25	24
16	160	840	1	25	24
18	180	820	1	25	24
20	200	800	1	25	24
22	220	780	1	25	24

Table 4. Ethanol estimation tests for different isolates P_1, P_2 and P_3

Isolates	Combustion test	Jones oxidation test	Triiodomethane test
P_1	+ (Blue colour)	+ (Green to blue colour)	+ (Yellow colour)
P_2	+ (Blue colour)	+ (Green to blue colour)	+ (Yellow colour)
P_3	+ (Blue colour)	+ (Green to blue colour)	+ (Yellow colour)

yielded no or negligible sugars, demonstrating the importance of pre-treatment in the conversion of biomass to sugars. After fermentation, the ethanol retrieved from isolates P_1, P_2 and P_3 was subjected to quantitative evaluation for bio-ethanol yield using the potassium dichromate method (Table 2 and Fig. 1). These specified conditions led to the production of 16% ethanol. Tables 3 and 4 show that all isolates produced ethanol. Nasir *et al.* (2017) reported yeast for bio-ethanol production from pineapple and orange. Cutzu and Bardi (2017) reported bio-ethanol production from agricultural waste.

The primary importance of this research endeavour is the identification of innovative yeast strains tailored for ethanol production on an industrial scale. Research indicates that yeasts are frequently present in sugar rich samples, including leaves, flowers, fruits, grains and similar materials. In our current investigation, yeast isolates were discerned through detailed examinations of morphology, colony traits and biochemical attributes.

CONCLUSION

The isolation and identification of prospective microorganisms capable of producing large amounts of bio-ethanol represented a significant leap in sustainable bio-fuel technologies. Following thorough morphological and biochemical screening and selection, isolates P_1, P_2 and P_3 proved their ability to efficiently ferment substrates to ethanol. These isolates produced good results in terms of bio-ethanol production and yield improvement.

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