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Synergistic Biofuel Production, Refractometric Monitoring, and Trypsin Digestion Studies from *Clitoria ternatea, Cymbopogon citrates*, and *Saccharomyces cerevisiae*

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Abstract

The rising demand for renewable and green energy sources has encouraged researchers to seek sustainable alternatives to fossil fuels. In the present study, an investigation was conducted on the synergistic production of biofuel from plant materials of Clitoria ternatea and Cymbopogon citratus with microbial fermentation by Saccharomyces cerevisiae. The study also evaluated the refractometric monitoring and trypsin digestion activity for the understanding of biochemical changes during biofuel production. The oil was obtained from the seeds of Clitoria ternatea and the dried leaves of Cymbopogon citratus using cold pressing machine. The extracted oil was further processed through transesterification with methanol, petroleum ether, and sodium hydroxide to prepare biofuel. The prepared biofuel mixture was added with yeast for fermentation studies. Changes in soluble compounds and fermentation activity were monitored by refractometric analysis for three consecutive days. The results showed that the samples with combined plant extracts and yeast have higher °Brix values, indicating increased biochemical activity and better fermentation potential. The highest refractometric readings were obtained in the mixture of biofuel with both plant extracts and yeast from all samples, indicating efficient utilization of the substrate and synergistic interaction between plant biomass and microbial fermentation. In the trypsin digestion studies with the X-ray film assay, partial inhibition of proteolytic activity was observed in the plant extracts, which suggested the presence of bioactive compounds with probable trypsin inhibitory properties. Results of experiments indicate that synergistic interactions of Clitoria ternatea, Cymbopogon citratus and Saccharomyces cerevisiae can improve the efficiency of biofuel production. The work demonstrates the potential of plant biomass and microbial systems as sustainable resources for renewable energy production and provides a simple refractometric approach for monitoring biofuel synthesis.

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Keywords: Biofuel production, bioethanol, *saccharomyces cerevisiae*, microbial fermentation, fermentation technology, plant-based biofuel

INTRODUCTION

The global increase in energy demand and the rapid depletion of fossil fuel reserves have created an urgent need for sustainable, renewable energy alternatives [1–6]. Conventional fossil fuels contribute significantly to environmental pollution, greenhouse gas emissions, and climate change, thereby threatening ecological balance and human health [7]. As a result, scientists and researchers across the globe have focused on developing renewable energy systems capable of reducing environmental impact while meeting industrial and household energy demand.

Recent advances in biofuel research have focused on synergistic production systems that integrate plant biomass, enzymatic reactions, and microbial fermentation [4]. By combining the metabolic capability of microorganisms with nutrient-rich plant-derived substrates, these systems enhance substrate utilization and boost total biofuel production. In a synergistic system, plant biomass supplies carbohydrates, lipids, proteins, and secondary metabolites, while microorganisms metabolize these substrates to produce bioethanol or biodiesel [8].

Clitoria ternatea, commonly known as butterfly pea, is a nutritionally and pharmacologically important plant rich in proteins, carbohydrates, flavonoids, anthocyanins, and phenolic compounds [9]. The seeds of *Clitoria ternatea* contain fermentable biomolecules that can support microbial growth and the fermentation process. The plant's seeds contain carbohydrates and oils, making it a possible candidate for biodiesel and bioethanol production (Figures 1 & 2).

Figure 1. *Clitoria ternatea* (Butterfly pea).

Figure 2. *Cymbopogon citratus* (Lemon grass).

Cymbopogon citratus (Lemon grass) is an aromatic grass widely known for its essential oils and lignocellulosic biomass composition [5]. The plant contains cellulose and hemicellulose, which may be hydrolyzed into fermentable sugar suitable for microbial fermentation (Tables 1 & 2) [10].

Table 1. Taxonomic classification of *Clitoria ternatea* (Butterfly pea).

Kingdom	**Plantae**
Division	Magnoliophyta.
Class	Magnoliopsida.
Order	Fabales.
Family	Fabaceae.
Genus	*Clitoria.*
Species	*ternatea.*

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Table 2. Taxonomic classification of *Cymbopogon citratus* (Lemon grass).

Kingdom	**Plantae**
Division	Magnoliophyta.
Class	Liliopsida.
Order	Poales.
Family	Poaceae.
Genus	*Cymbopogon*.
Species:	*citratus*.

Ethanol production from lignocellulosic biomass of *Clitoria ternatea* and *Cymbopogon citratus* may thus provide a nutrient-rich substrate mixture capable of increasing biofuel production [11]. Analytical monitoring by refractometer is equally important in determining the efficiency of biofuel production [12]. Refractometry is a rapid and non-destructive analytical technique that measures the refractive index of the solutions, which changes according to the concentration of dissolved solids such as sugar and alcohols. During fermentation, the refractive index changes as sugar is converted into ethanol, making refractometric monitoring useful for tracking the fermentation process and estimating substrate utilization. Another important aspect of this study is trypsin digestion analysis. Trypsin is a proteolytic enzyme capable of hydrolyzing peptide bonds in proteins [7, 10]. Evaluating trypsin inhibition helps determine the biochemical interactions occurring between plant-derived compounds and enzymatic systems.

The present study aimed to investigate synergistic biofuel production using *Clitoria ternatea*, *Cymbopogon citratus*, and *Saccharomyces cerevisiae*. The study also evaluated refractometric changes during fermentation and analyzed trypsin digestion activity using an X-ray film assay [13].

MATERIALS AND METHODS

In the present study, Synergistic biofuel or Synergy Biofuels production integrates several biological processes to maximize energy yield. Synergy Biofuels particularly create synergies in the production of renewable biofuels by integrating the utilization of biomass like *Clitoria ternatea* plant seeds, *Cymbopogon citratus* (Lemon grass) dry leaves, and *Saccharomyces cerevisiae* (Table 3).

Table 3. List of the materials required during production.

S. N.	**Materials required**	**Quantity**
1	*Clitoria ternatea* plant seeds	Approximately 50 g.
2	*Cymbopogon citratus* (Lemon grass) dry leaves	Approximately 10 g.
3	Mixture of *Clitoria ternatea* seeds and Lemon grass leaves	Approximately 50 g.
4	Methanol	8 ml.
5	Petroleum ether	7 ml.
6	Sodium hydroxide (NaOH) pellets	4 pellets.
7	*Saccharomyces cerevisiae* (yeast)	Approximately 1–2 g.
8	Centrifuge tubes	5 tubes.
9	Oil pressing machine	1 unit.

Collection of Plant Materials

Fresh seeds of *Clitoria ternatea* and fresh leaves of *Cymbopogon citratus* (Lemon grass) were collected locally. The plant materials collected were washed carefully to remove dust, soil particles, and unwanted impurities. Healthy and mature seeds of *Clitoria ternatea* were selected for the study. Fresh Lemon grass leaves were washed with distilled water before further processing.

Preparation of Plant Biomass

The collected leaves of Lemon grass were chopped into small pieces with the help of a sterile blade to increase the surface area for drying and extraction. The sliced leaves were spread on clean trays and dried in the sun for about 24 hours to reduce the moisture content. The dried plant material was kept in sterile containers until it was used (Figure 3).

Figure 3. Plant samples: (A) Mature seeds of *Clitoria ternatea*, (B) fresh leaves of *Cymbopogon citratus.*

Oil Extraction from Plant Materials

Oil extraction was carried out with an oil pressing machine at a temperature of 160–210°C. Various plant samples were processed separately and in combination to evaluate oil yield and synergistic effects. A moderate quantity of oil was obtained from nearly 50 g of *Clitoria ternatea* seeds for extraction. Similarly, the dried biomass of *Cymbopogon citratus* was processed separately. To study the effect of mixed plant substrates on extraction efficiency, a combined mixture of *Clitoria ternatea* seeds and dried Lemon grass biomass was also subjected to oil extraction. The separated oils were collected in sterile centrifuge tubes and stored for biofuel synthesis.

Preparation for Biofuel

The biofuel was prepared by transesterification. The extracted plant oil was transferred into a centrifuge tube (about 2 ml). It was mixed with 8 ml of methanol and 7 ml of petrol. The transesterification reaction was catalyzed using sodium hydroxide (NaOH) pellets. The mixture was shaken well to ensure proper mixing and conversion of the plant oils into the components of the biofuel. The prepared samples of the biofuel were stored for further experimental analysis under controlled laboratory conditions.

Fermentation Using *Saccharomyces cerevisiae*

To determine the fermentation process by microbes, a small volume of *Saccharomyces cerevisiae* yeast suspension was mixed with a small fraction of the prepared biofuel samples. The mixture was then shaken to achieve an even distribution of the yeast cells. This was followed by fermentation by keeping the mixture under appropriate conditions to facilitate microbial metabolism.

Refractometric Analysis

Digital refractometry was performed to determine the refractive index and °Brix value of the prepared samples. Refractometer readings were taken over three consecutive days to assess changes in soluble substances during fermentation and biofuel production. Changes in the °Brix values were indicative of biochemical reactions taking place.

Trypsin Digestion and X-Ray Film Assay

Experiments on trypsin digestion were done using the X-ray film assay technique. The X-ray film has a layer of gelatin that serves as a substrate for proteolytic enzymes like trypsin. Experimental samples of various types were applied onto the X-ray film and left for a particular time. The X-ray film was then washed with distilled water to observe the breakdown of the gelatin layer. If there is partial or complete digestion of the gelatin layer, then it indicates the presence of a protease enzyme, whereas if there is no digestion, it means there are inhibitors of trypsin in the sample.

RESULT AND DISCUSSION

Oil Extraction

Around 50g of the seeds of the *Clitoria ternatea* plant gave roughly 3ml of oil, whereas the oil extracted from Lemon grass was only in a small amount. The mixture of these plants together gave around 8ml of oil (Tables 4 & 5) (Figure 4).

Table 4. Yield of oil.

Sample	Oil yield/50 gm	%Oil yield
Clitoria ternatea seeds	3 ml	6%
Lemon grass leaves	Trace amount	1% approx.
Combined plant mixture	8 ml	16%

Table 5. Combustion levels of biofuels.

Item	Sample	Observation
A	Plant oil	Moderate combustion.
B	Plant oil biofuel	High flammability.
C	Biofuel + *Saccharomyces cerevisiae*	Strong combustion.

Figure 4. Samples of oils and biofuels in centrifuges tubes.

Note

- Sample 1 is *Clitoria ternatia* seeds oil is 3 ml.
- Sample 2 is the oil of mix *Clitoria ternatia* seeds + Lemon grass leaves.
- Sample 3 is Lemon grass oil.
- Sample 4 is mix of 2 ml combine oil of *Clitoria ternatia* seeds + Lemon grass + 8 ml methanol + 7 ml petroleum ether and 1 pellets NaOH.
- Sample 5 (Synergy Biofuels) is mixed of oil of *Clitoria ternatia* seeds + Lemon grass + 8 ml methanol + 7 ml petroleum ether and 1 pellets NaOH + *saccharomyces cerevisiae* fermented.

Biofuel Production

Oil was efficiently made into biofuel with the use of methanol, petroleum ether, and sodium hydroxide with fermentation. All biofuels were found to be flammable upon ignition (Table 5) (Figure 5).

(A) Plant oil.

(B) Plant oil biofuel

(C) Combined plant mixture.

Figure 5. Combustion levels of biofuels.

Note

- Plant oil – *Clitoria ternatea* seeds + *Clitoria ternatea* seeds.
- Plant oil biofuel – *Clitoria ternatea* seeds + *Clitoria ternatea* seeds + methanol, petroleum ether, and sodium hydroxide.
- Biofuel + *Saccharomyces cerevisiae* – *Clitoria ternatea* seeds + *Clitoria ternatea* seeds+ methanol, petroleum ether, and sodium hydroxide.

Refractometric Analysis

The highest °Brix reading was recorded for the biofuel sample with *Saccharomyces cerevisiae*, showing improved fermentation efficiency (Table 6) (Figures 6 to 9).

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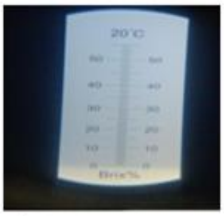
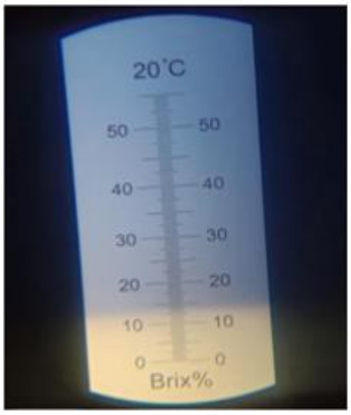
Table 6. Yield of oil.

Sample	**Day 1**	**Day 2**	**Day 3**
Clitoria ternatea seed oil	0	0	0
Lemon grass oil	0	1	0
Combined plant oil	15	0	1
Combined biofuel	30	10	15
Biofuel + Yeast	1	25	25



Sample 1: *Clitoria ternatia* seeds oil.

Sample 2: *Clitoria ternatia* seeds oil + Lemon grass.

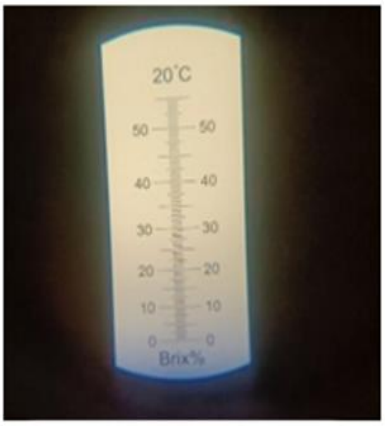


Sample 3: Lemon grass oil.

Sample 4: *Clitoria ternatia* seeds oil + Lemon grass biofuel.

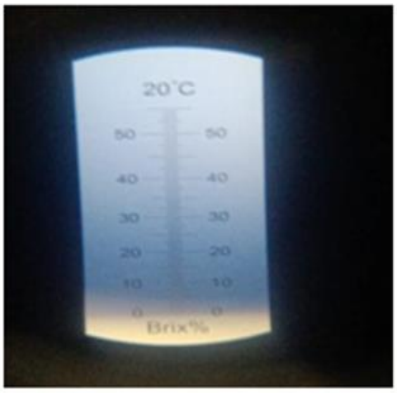
Sample 5: *Clitoria ternatia* seeds oil + Lemon grass + *Saccharomyces cerevisiae* biofuel.

Figure 6. Refractometry analysis (Day 1).

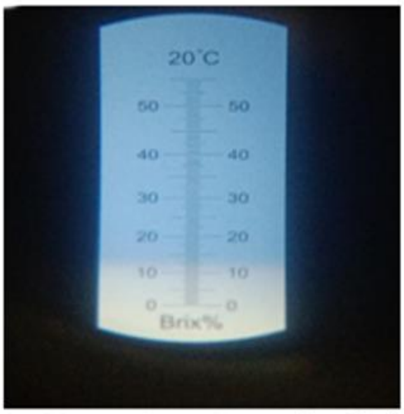


Sample 1: *Clitoria ternatia* seeds oil.

Sample 2: *Clitoria ternatia* seeds oil + Lemon grass.



Sample 3: Lemon grass oil.



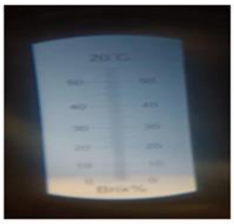
Sample 4: *Clitoria ternatia* seeds oil + Lemon grass biofuel.

Sample 5: *Clitoria ternatia* seeds oil + Lemon grass + *Saccharomyces cerevisiae* biofuel.

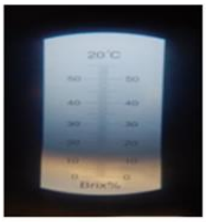
Figure 7. Refractometry analysis (Day 2).

Sample 1: *Clitoria ternatia* seeds oil.

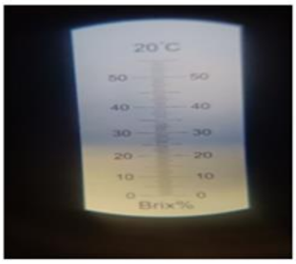
Sample 2: *Clitoria ternatia* seeds oil + Lemon grass.



Sample 3: Lemon grass oil.



Sample 4: *Clitoria ternatia* seeds oil + Lemon grass biofuel.



Sample 5: *Clitoria ternatia* seeds oil + Lemon grass + *Saccharomyces cerevisiae* biofuel

Figure 8. Refractometry analysis (Day 3).

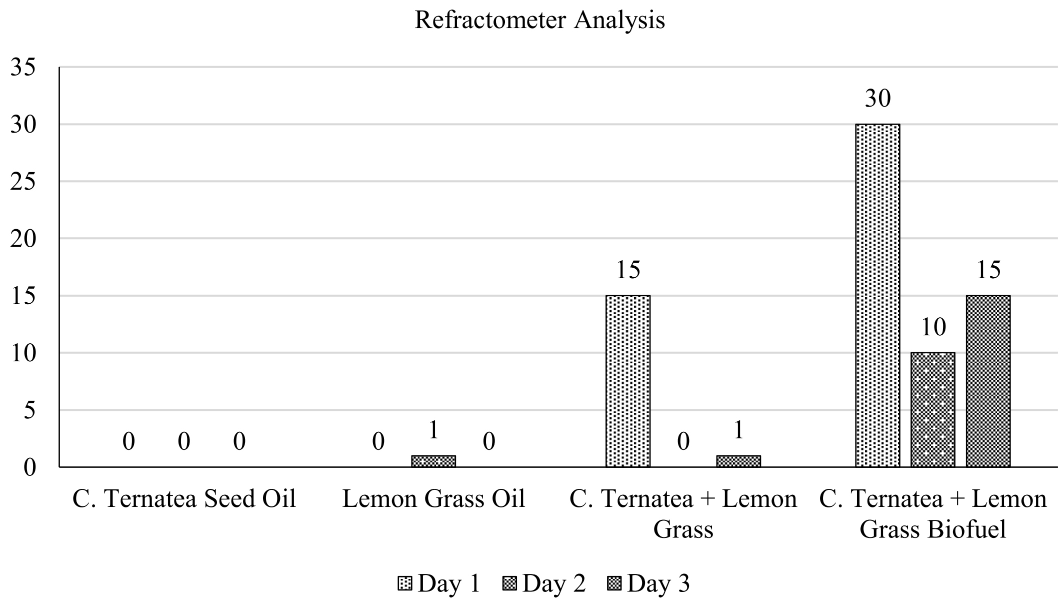


Figure 9. Comparative analysis of refractometry on day 1, 2 and 3.

Trypsin Digestion

The data suggest the presence of trypsin inhibitory agents in the plant samples (Table 7) (Figures 10 & 11).

Table 7. Combustion levels of biofuels.

Sample	**Result**
Clitoria ternatia seeds oil	No digestion.
Oil of mix *Clitoria ternatia* seeds + Lemon grass	Partial digestion.
Lemon grass oil	Very low digestion.
2 ml combine oil of *Clitoria ternatia* seeds + Lemon grass + 8 ml methanol + 7 ml petroleum ether and 1 pellets NaOH	Moderate digestion.
Oil of *Clitoria ternatia* seeds + Lemon grass + 8 ml methanol + 7 ml petroleum ether and 1 pellets NaOH + *saccharomyces cerevisiae* fermented	Partial inhibition with enzyme activity.

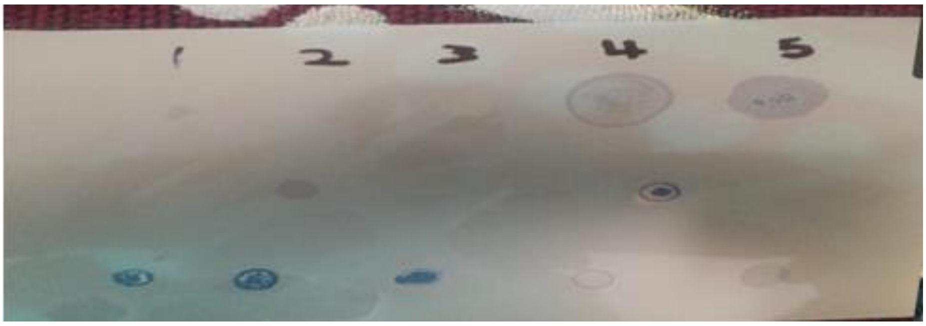
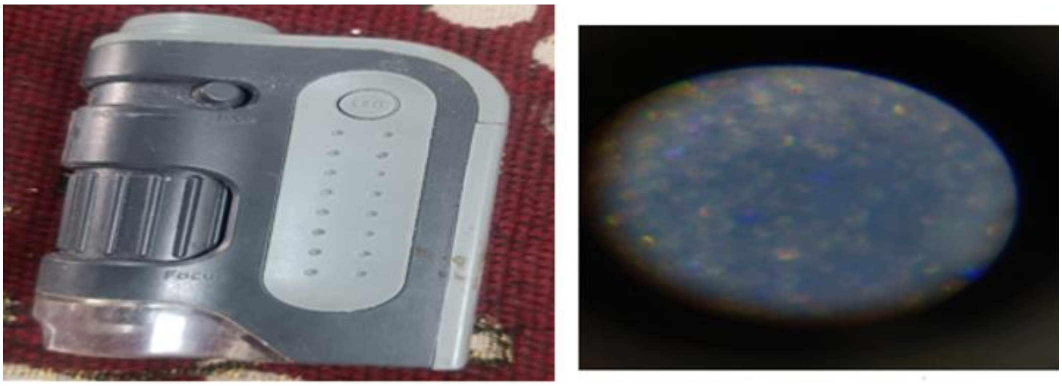


Figure 10. X-ray film analysis.



(A) Microscope. (B) Image of Trypsin + Saccharomyces.

Figure 11. Microscopic image of trypsin + Synergy Biofuels.

Synergistic Effect on Biofuel Production

Combining *Clitoria ternatea,* Cymbopogon citratus, and *Saccharomyces cerevisiae* proved to be effective in boosting oil extraction rate, fermenting action, and biofuel quality. Yeast–biofuel exhibited the best °Brix value, improved combustion efficiency, and the highest level of biochemical activities (Table 8).

Table 8. Performance of biofuel.

Parameter	**Best performing sample**
Highest Oil Yield	Combined plant biomass.
Highest °Brix Value	Biofuel + Yeast.
Best Combustion Efficiency	Biofuel + Yeast.
Maximum Fermentation Activity	Biofuel + Yeast.
Highest Enzymatic Interaction	Biofuel + Yeast.

DISCUSSION

The current study found that synergistic interactions between *Clitoria ternatea, Cymbopogon citratus*, and *Saccharomyces cerevisiae* can increase biofuel efficiency. The combined plant biomass yielded more oil than individual plant samples, showing complementary biochemical and structural interactions between the two plant components.

Previous research has shown that plant biomass high in lipids can boost microbial fermentation efficiency and biofuel production [2]. The current findings support these observations by demonstrating enhanced refractometric values in solutions containing microbial fermentation.

The high °Brix values found in yeast-containing samples imply higher quantities of dissolved solids and vigorous fermentation [8]. Similar findings have been observed in fermentation-based biofuel research, where microbial metabolism plays a substantial role in ethanol production, substrate conversion, and increased metabolic activity. The persistent refractometric values obtained in fermented samples point to successful microbial adaptability, constant metabolic activity, and consistent fermentation dynamics during biofuel generation.

The trypsin digestion assay demonstrated the presence of protease inhibitory substances in plant extracts [11]. Many legumes and medicinal plants include protease inhibitors, which prevent enzymatic protein digestion. *Clitoria ternatea* contains phytochemicals, such as flavonoids, phenolic compounds, and cyclotides, which may contribute to its inhibitory effect.

The study also highlights the value of refractometric measurement as a quick, cost-effective, and practical monitoring approach for fermentation-based biofuel systems. In comparison to modern analytical approaches, refractometry is an efficient method for monitoring biochemical changes, dissolved solids, and fermentation dynamics during biofuel production.

Although the study found synergistic interactions between plant biomass and microbial fermentation systems, some limitations remain. The trials were carried out on a laboratory scale, and no extensive physicochemical analysis of the created biofuel was performed. Future research should incorporate advanced analytical techniques – such as gas chromatography, calorific value determination, viscosity analysis, and fermentation condition optimization – to improve large-scale industrial applicability and fuel efficiency.

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CONCLUSION

The present investigation demonstrated the successful production of synergistic biofuel using *Clitoria ternatea, Cymbopogon citratus,* and *Saccharomyces cerevisiae.* The combined plant produced higher oil yields than individual plant samples, indicating enhanced extraction efficiency through synergistic interaction. Refractometric monitoring revealed that microbial fermentation significantly increased dissolved solid concentration, particularly in yeast-containing samples. The highest °Brix values were consistently observed in fermented biofuel samples, suggesting enhanced biochemical activity and improved fermentation efficiency. Trypsin digestion studies demonstrated partial inhibition of proteolytic activity, likely due to bioactive phytochemicals present in the plant extracts. These findings highlight the dual biochemical role of the plant substrate in promoting fermentation while simultaneously influencing enzymatic activity. Overall, the study supports the potential of integrated plant–microbial systems for sustainable biofuel production. The results also indicate that refractometric monitoring and enzymatic assays can serve as useful analytical tools in evaluating fermentation efficiency and biochemical interactions during biofuel synthesis.

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