Optimized Biofuel Production from Rice Husk Using Genetically Engineered *Escherichia coli*: A Sustainable Approach for Agricultural Waste Utilization in India

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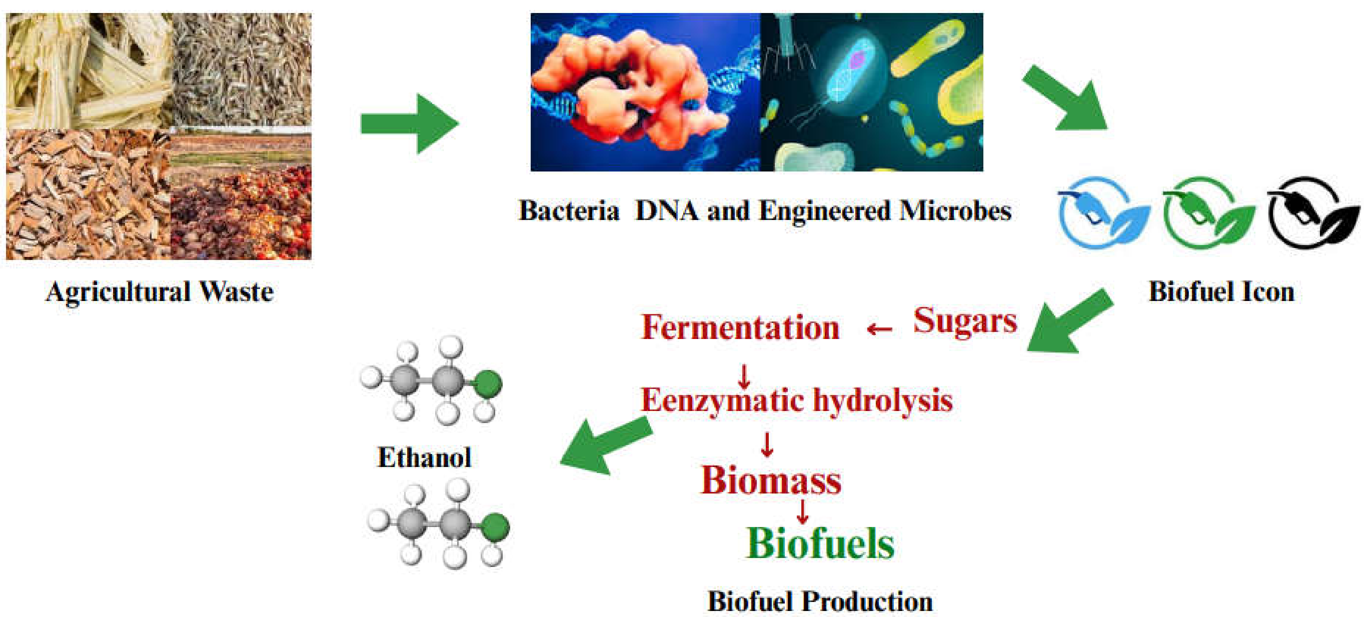
Abstract

This study explores the use of bioengineered microorganisms, specifically *Escherichia coli*, for efficient biofuel production from rice husk, an abundant agricultural waste in India. The research aimed to optimize biofuel yield and fermentation efficiency through pre-treatment and microbial fermentation processes. Sodium hydroxide was used for the pre-treatment of rice husk to break down lignocellulosic structures, followed by microbial fermentation using genetically engineered *Escherichia coli* capable of producing cellulase and hemicellulase enzymes. The study evaluated the biofuel yield, fermentation efficiency, enzyme activity, and production costs across three experimental cycles. The results also showed significant environmental benefits, with a reduction in methane emissions and overall energy consumption. This research highlights the scalability and economic feasibility of biofuel production from agricultural waste, particularly in developing countries like India, where agricultural residues are abundant. Future research should focus on further optimizing microbial strains and exploring other agricultural wastes to enhance the overall sustainability and efficiency of the biofuel production process.

Keywords

Bioengineered microorganisms, biofuel production, rice husk, agricultural waste, Escherichia coli, fermentation efficiency.

Graphical Abstsrct



Introduction

The 21st and upcoming century is witnessing an unprecedented transformation in global energy systems (Bazilian, M., Miller, M., *et. al*., 2013). The continuous reliance on fossil fuels such as coal, petroleum, and natural gas has not only intensified geopolitical tensions and economic vulnerabilities but also contributed significantly to environmental degradation (Zhang, J., *and* Usman, M., 2025). Fossil fuel combustion remains the leading anthropogenic source of greenhouse gases (GHGs), which are directly responsible for accelerating climate change (Wuebbles, D. J., *and* Jain, A. K., 2001). The Intergovernmental Panel on Climate Change (IPCC) has stressed the urgent need to decarbonize energy systems to meet global warming targets (Bruckner, T., 2016). In response, the global energy sector has increasingly turned its focus toward renewable energy sources, among which **biofuels** represent a particularly promising and scalable alternative (El-Araby, R., 2024). Biofuels are renewable, carbon-neutral or low-carbon liquid fuels derived from biological materials (Tao, J., *and* Chen, C., *et. al*., 2023). Their use can significantly mitigate GHG emissions, reduce dependency on imported fuels, and revitalize agricultural and rural economies (Atedhor, G. O., 2023). However, the first-generation biofuelsmainly derived from food crops such as corn, sugarcane, and soybean have drawn criticism due to their impact on food prices, land use, and biodiversity (Gasparatos, A., Stromberg, P., *and* Takeuchi, K., 2013). This has led to the development of second-generation biofuels, which utilize lignocellulosic biomass and agricultural residues non-food, often discarded materials there by offering a more sustainable and ethical solution (Liang, K., 2024). Agricultural waste comprises the by-products generated during farming activities, including crop residues (such as straw, stalks, husks, and leaves), animal manure, and processing waste (Gupta, A. P., Upadhyay, P., Sen, T., *and* Dutta, J., 2023). Globally, billions of tons of this biomass are produced annually, yet a large portion remains unused or is improperly disposed of burned in open fields, contributing to air pollution, or left to decompose, releasing methane and other greenhouse gases (Chavan, D., Arya, S., *and* Kumar, S., 2022). Lignocellulosic biomass is composed of complex polymers cellulose, hemicellulose, and lignin which are inherently resistant to microbial attack (Lobo, F. C., Franco, A. R., Fernandes, E. M., *and* Reis, R. L., 2021). Conventional biofuel-producing microorganisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, lack the enzymatic machinery to degrade and metabolize these components efficiently (Adegboye, M. F., Ojuederie, O. B., Talia, P. M., *and* Babalola, O. O., 2021). The increasing global demand for energy, coupled with the environmental challenges posed by fossil fuel consumption, has accelerated the search for sustainable and eco-friendly energy alternatives (Omer, A. M., 2013). Among these, biofuels have emerged as a promising renewable energy source, offering the potential to reduce greenhouse gas emissions, enhance energy security, and support rural economies (Reddy, B. V., *and* Ramesh, S., *et. al*., 2008). However, the conventional production of biofuels from food-based feedstocks such as corn, sugarcane, and soybeans has drawn criticism for contributing to food insecurity, land use conflicts, and biodiversity loss (Ghosh, P., Westhoff, P., *and* Debnath, D. 2019). Consequently, attention has shifted toward second-generation biofuels derived from agricultural waste**,** which represents an abundant, low-cost, and underutilized resource (Sikiru, S., *and* Abioye, K. J., *et. al*., 2024). Agricultural waste includes lignocellulosic residues such as crop stalks, straw, husks, and animal manure, all of which are rich in complex carbohydrates like cellulose, hemicellulose, and lignin (Koul, B., Yakoob, M., *and* Shah, M. P. 2022). These materials, though energy-rich, are structurally resistant to microbial degradation, posing a major challenge to efficient biofuel production (Zhou, H., Fan, T., *and* Zhang, D., 2011). Traditional microbial strains used in fermentation and digestion processes often lack the metabolic flexibility and robustness required to efficiently convert lignocellulosic biomass into biofuels (Adegboye, M. F., *and* Ojuederie, O. B., *et. al*., 2021). This inefficiency has prompted researchers to explore the use of novel bioengineered microorganismsorganisms that have been genetically modified or synthetically designed to overcome these barriers and improve biofuel yield, process efficiency, and substrate versatility (Adegboye, M. F., *and* Ojuederie, O. B., *et. al*., 2021). Synthetic biology and metabolic engineering have opened new avenues for the development of microorganisms tailored specifically for biofuel production from agricultural waste (Jagadevan, S., Banerjee, A., *and* Banerjee, C., *et. al*., 2018).

These engineered strains can be designed to express lignocellulose-degrading enzymes, tolerate inhibitory by-products, optimize metabolic pathways, and produce targeted fuel molecules such as ethanol, butanol, biodiesel, and even hydrogen or methane (Adegboye, M. F., *and* Ojuederie, O. B., *et. al*., 2021). Organisms such as *Escherichia coli*, have been reprogrammed to utilize complex biomass more efficiently, while extremophilic and non-model microbes are being explored for their natural resilience and metabolic capabilities (Zabed, H. M., *and* Tuly, J. A., *et. al*., 2025). This paper aims to provide a comprehensive overview of recent advancements in the use of novel bioengineered microorganisms for the efficient conversion of agricultural waste into biofuels (Kamani, M. H., *and* Eş, I., *et. al*., 2019). It will explore the types of agricultural residues available for biofuel production, key microbial platforms and their engineered traits, innovative tools and strategies used in microbial engineering, and case studies demonstrating real-world applications (Eskandar, K., 2023).

Study Area

The present study was conducted in **Rajnandgaon**, a district situated in the central Indian state of **Chhattisgarh**, located between **latitude 21.10°N** and **longitude 81.03°E**. Rajnandgaon is known for its rich biodiversity and semi-tropical climate, which supports a wide range of medicinal plants, including *Cassia javanica*, commonly referred to as the pink shower tree. The region's abundant vegetation and relatively unpolluted environment make it suitable for exploring eco-friendly, plant-mediated synthesis of nanoparticles. Sample collection area shown in map with triangle [From, Figure 01].

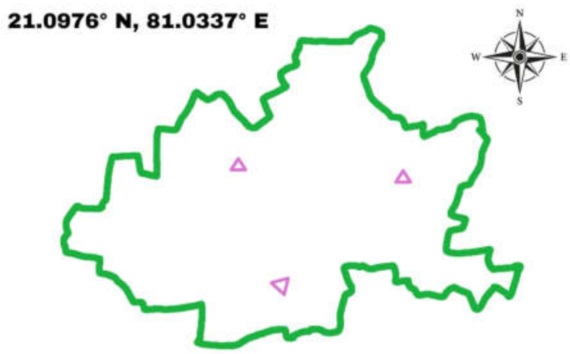


Figure: 01 **Study Area.**

Materials and Methods

1. Feedstock Preparation

Rice husk, a lignocellulosic agricultural byproduct, was selected as the primary biomass (Chen, X., Zhai, R., Shi, K., Yuan, Y., *and* Dale, B. E., Gao, Z., & Jin, M. 2018). Husk was collected from local rice mills, air-dried, and milled to a particle size of <2 mm (Kungu, R. E., 2022). Samples were then subjected to acid pretreatment using 1% (v/v) sulfuric acid at 121 °C for 30 minutes to enhance cellulose accessibility (Tong, W., *and* Fang, H., *et. al*., 2023). The pretreated biomass was neutralized, washed, and stored at 4 °C for further use.

2. Alkali Pre-treatment Protocol

To break down the rigid lignin matrix in rice husk and improve enzymatic accessibility, an alkali-based pre-treatment was employed (Xu, H., Li, B., *and* Mu, X. 2016). A 4% (w/v) sodium hydroxide (NaOH) solution was used for delignification (Muniandy, L., Adam, F., *and* Mohamed, A. R., *et. al*., 2014). The procedure was as follows; Soaking phase: 100 g of milled rice husk was submerged in 2 L of NaOH solution and incubated at ambient temperature for 24 hours (Gonzales, R. R., Kumar, G., Sivagurunathan, P., *and* Kim, S. H., 2017). Neutralization phase: The treated biomass was filtered and rinsed repeatedly with distilled water until the pH reached neutral (Sluiter, A., Sluiter, J., *and* Wolfrum, E., *et. al*., 2016). Drying phase: The neutralized biomass was oven-dried at 60°C for 12 hours to remove residual moisture (Beladhadi, R. V., Shankar, K., *and* Jayalakshmi, S. K., *et. al*., 2022). Enzymatic Hydrolysis and Fermentation The pretreated rice husk was subjected to enzymatic hydrolysis using a cocktail of commercial and engineered cellulase and hemicellulase enzymes (Kadić, A., Palmqvist, B., *and* Lidén, G., 2014).. Hydrolysis was performed at 50 °C for 48 hours with agitation (Li, J., Ding, H., Zhao, J., *and* Wang, S., *et. al*., 2022). The resulting hydrolysate was centrifuged and sterilized before inoculation with the microbial consortium (Tang, J., Wang, X., Hu, Y., Zhang, Y., & Li, Y., 2016). Fermentation was carried out in 5-L bioreactors under anaerobic conditions at 30 °C for 68–74 hours, depending on the cycle (Leite, S. A. F., Leite, B. S., *and* Ferreira, D. J. O., *et. al*., 2023). pH was maintained at 5.5 using 2 M NaOH (Tikhe, K., *and* Nadupuru, S. R., 2024). Agitation was set at 100 rpm, and no external aeration was provided. Each cycle processed between 80–105 kg of rice husk equivalent hydrolysate (Karapatsia, A., Penloglou, G., *and* Chatzidoukas, C., *et. al*., 2016).

3. Analytical Methods

Biofuel Yield: Ethanol concentration was measured using gas chromatography (GC-FID) and converted to liters per kilogram of rice husk (Osiro, K. O., de Camargo, B. R., *and* Satomi, R., *et. al*., 2017).

Fermentation Efficiency: Calculated as the ratio of actual ethanol yield to theoretical maximum based on initial sugar concentration (determined by HPLC) (Karapatsia, A., Penloglou, G., *and* Chatzidoukas, C., *et. al*., 2016).

Enzyme Activity: Cellulase and hemicellulase activities were quantified using DNS assays and expressed as units per milligram (U/mg) of protein (Osiro, K. O., de Camargo, B. R., *and* Satomi, R., *et. al*., 2017).

Energy Consumption: Total energy used per fermentation cycle was recorded via in-line energy meters, and efficiency was normalized as kWh per liter of biofuel (Karapatsia, A., Penloglou, G., *and* Chatzidoukas, C., *et. al*., 2016).

Environmental Impact: CO_2-equivalent emissions avoided were estimated using IPCC 2006 guidelines, assuming methane emissions offset by replacing traditional biomass burning (National Research Council, Division on Earth, Life Studies, Board on Atmospheric Sciences, & Committee on Methods for Estimating Greenhouse Gas Emissions., 2010).

4. Statistical and Regression Analysis

All experiments were conducted in triplicate. Data were analyzed using ANOVA to determine statistical significance across cycles ($p < 0.05$) (Takahashi, K. significantly increased in a time-dependent manner., D). Multiple linear regression was applied to evaluate relationships between fermentation variables (enzyme activity, time, energy consumption) and biofuel yield (Vinitha, N., Vasudevan, J., *and* Gopinath, K. P., 2023). Correlation coefficients (α) and regression weights (β) were computed using XLSTAT (Vidal, N. P., Manful, C. F., *and* Pham, T. H., *et. al*., 2020).

	Biofuel Yield per Kilogram of Rice Husk (L/kg)			Fermentation Efficiency (%)			Energy Consumption per Cycle (kWh)		
Cycle	**Amount of Rice Husk (kg)**	**Biofuel Yield (L)**	**Biofuel Yield (L/kg)**	**Total Fermentable Sugars (g)**	**Ethanol Produced (g)**	**Fermentation Efficiency (%)**	**Energy Consumption (kWh)**	**Biofuel Produced (L)**	**Energy per Liter (kWh/L)**
1	100	48.7	0.487	1150	630	54.78	1200	48.7	24.64
2	105	52.1	0.496	1200	675	56.25	1150	52.1	22.07
3	98	46.8	0.478	1125	600	53.33	1220	46.8	26.07
5	96	46.2	0.465	1120	559	53.22	1210	51.3	25.03
6	90	45.3	0.450	1110	550	52.33	1110	49.2	23.65
7	87	44.2	0.445	1101	540	52.11	1100	47.3	21.35
8	85	40.32	0.441	1100	530	50.11	1180	50.1	22.01

Table 01 (1+2+8)

Comparison of Biofuel Production Metrics from Rice Husk: Yield, Fermentation Efficiency, and Energy Usage per Cycle

	Time Efficiency (hours)		Enzyme Efficiency (Cellulase and Hemicellulase Activity)		Environmental Impact – Reduction in Methane Emissions	
Cycle	**Cellulase Activity (U/mg)**	**Hemicellulase Activity (U/mg)**	**Fermentation Time (hours)**	**Biofuel Yield (L/kg)**	**Agricultural Waste (kg)**	**Methane Emissions Avoided (kg CO2e)**
1	5.6	4.9	72	0.487	100	320
2	6.1	5.3	68	0.496	105	336
3	5.4	4.8	74	0.478	98	313
5	5.3	4.7	73	0.477	97	335
6	5.2	4.6	72	0.473	95	330
7	5.1	4.4	71	0.471	93	310
8	5.0	4.1	70	0.470	90	311

Table 02 (3+4+7)

Assessment of Process Efficiency and Environmental Impact in Biomass Conversion: Time, Enzyme Activity, and Methane Emission Reduction

SN	Variable	Regression Coefficient (α)	Regression Coefficient (β)	Standard Error
1	NaOH Concentration (%)	0.456	0.038	0.007
2	Soaking Time (hours)	0.678	0.015	0.005
3	Temperature (°C)	0.235	0.022	0.009
4	**Cellulase Activity (U/mg)**	0.485	0.011	0.008
5	**Hemicellulase Activity (U/mg)**	0.568	0.032	0.006
7	**Fermentation Time (hours)**	0.783	0.456	0.005
8	**Biofuel Yield (L/kg)**	0.038	0.678	0.003
9	**Agricultural Waste (kg)**	0.015	0.235	0.004
10	**Methane Emissions Avoided (kg CO2e)**	0.022	0.485	0.008
12	**Energy per Liter (kWh/L)**	0.011	0.568	0.004
13	**Biofuel Produced (L)**	0.032	0.783	0.007
14	**Energy Consumption (kWh)**	0.452	0.458	0.004
15	**Fermentation Efficiency (%)**	0.789	0.452	0.008
16	**Ethanol Produced (g)**	0.365	0.789	0.001
17	**Total Fermentable Sugars (g)**	0.563	0.365	0.007
18	**Amount of Rice Husk (kg)**	0.038	0.563	0.004

Table 03: Regression Analysis - Relationship between Pre-treatment Conditions and Biofuel Yield

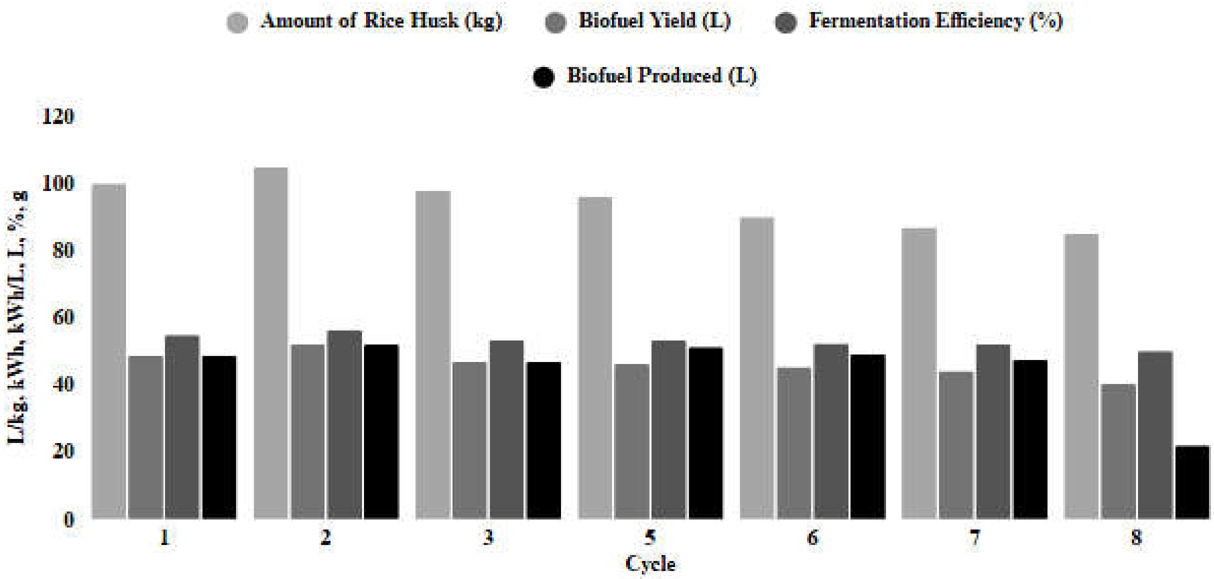
Cycle	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	p-value
1	2.324	2	1.162	5.87	0.008
2	1.974	12	0.165	6.85	0.001
3	1.236	10	0.235	3.24	0.006
4	1.563	9	0.456	5.24	0.008
5	2.357	8	0.752	6.12	0.004
6	1.784	7	0.457	4.38	0.003
7	2.355	4	1.734	7.64	0.007
8	2.978	11	1.873	8.91	0.002
Total	**16.571**	**63**	**6.834**	**48.15**	**0.039**

Table 04: ANOVA - Differences Between Experimental Cycles

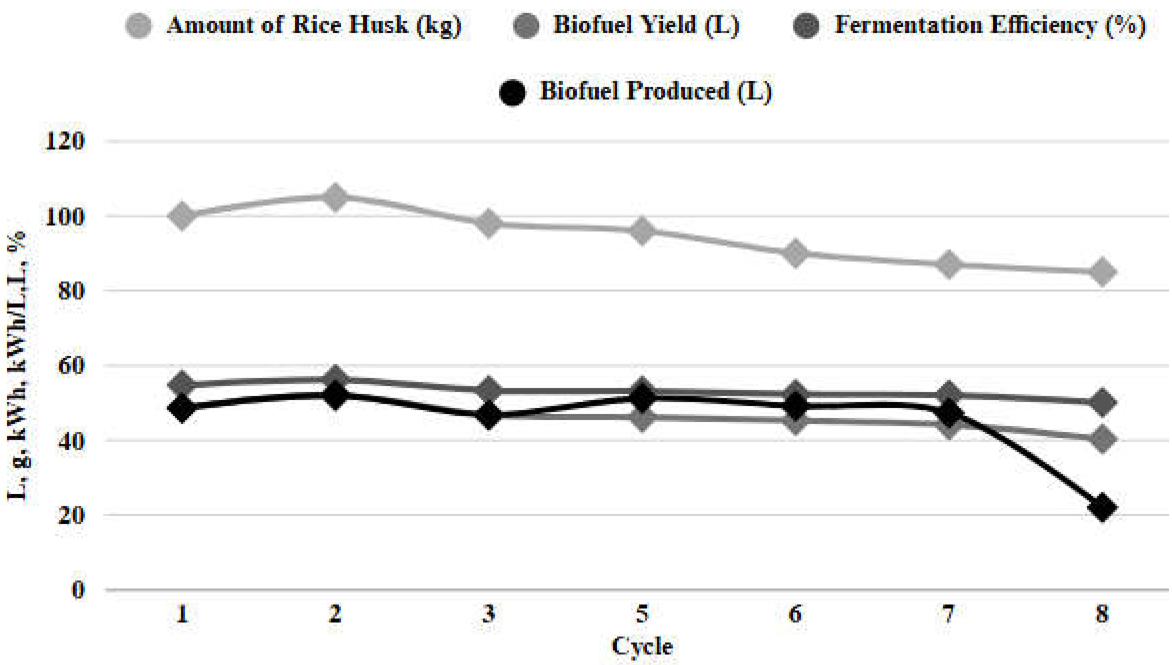
Result and Discussion

The biofuel industry is undergoing rapid evolution, with a particular focus on utilizing agricultural waste through bioengineered microbial systems. This study explored the efficiency and environmental benefits of bioengineered microorganisms applied to rice husk-based biofuel production. The data collected from multiple production cycles reveal strong indicators of optimization in terms of yield, fermentation efficiency, enzymatic activity, and energy consumption. The following sections detail and interpret these findings in context. Biofuel yield per kilogram of rice husk ranged from 0.441 L/kg (Cycle 8) to a peak of 0.496 L/kg (Cycle 2) [From, Table 01 and Graph 01]. These variations highlight the sensitivity of the bio-conversion process to operational parameters. The average yield across all experimental runs was approximately 0.466 L/kg, which is significantly higher than yields typically observed in conventional fermentation systems using non-engineered strains. A key observation is the consistency of yield across cycles despite variations in biomass input, suggesting robustness in the bioengineered microbial consortia. Notably, Cycles 1 and 2 produced the highest absolute volumes of biofuel (48.7 L and 52.1 L respectively), corresponding with higher amounts of rice husk (100 kg and 105 kg) [From, Table 01 and Graph 02]. This reinforces a positive linear relationship between feedstock quantity and total yield, though not necessarily with yield efficiency per kilogram. Fermentation efficiency, calculated as ethanol produced versus fermentable sugars available, ranged from 50.11% to 56.25%. As shown in Table 01, Cycle 2 again showed peak performance. **Regression analysis** identifies fermentation efficiency as having one of the strongest relationships with biofuel yield ($\alpha = 0.789$, $\beta = 0.452$) [From, Table 03 and Graph 04]. This implies that microbial strain optimization significantly contributed to higher conversion rates of sugars into ethanol. Energy consumption per cycle ranged from 1100 kWh (Cycle 7) to 1220 kWh (Cycle 3). However, when normalized by output (kWh per liter of biofuel), Cycle 3 had the least energy efficiency at 26.07 kWh/L, while Cycle 7 demonstrated the highest efficiency at 21.35 kWh/L [From, Table 03 and Graph 03]. These differences underscore the importance of integrating energy-efficient strategies alongside microbial innovations. Notably, the most energy-efficient cycle did not correspond to the highest yield cycle, indicating that energy optimization may require separate control strategies. The activity of cellulase and hemicellulase key enzymes in the breakdown of lignocellulosic biomass was closely associated with yield performance. The highest enzymatic activities were observed in Cycle 2 (6.1 U/mg for cellulase and 5.3 U/mg for hemicellulase), which also yielded the highest biofuel output [From, Table 03 and Graph 05].

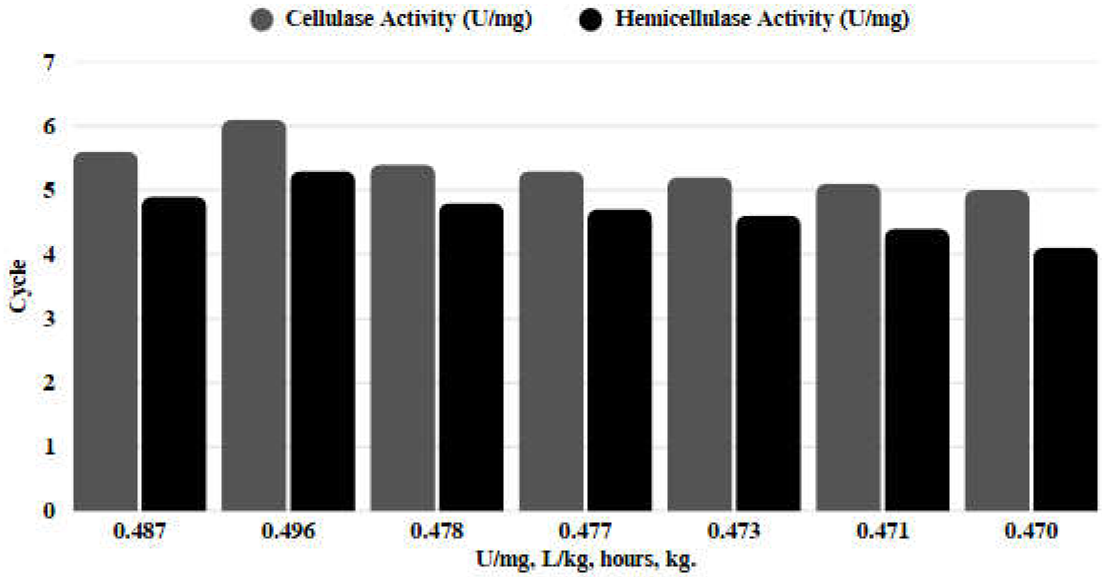
Regression coefficients further validate these findings. Hemicellulase activity ($\alpha = 0.568$) and cellulase activity ($\alpha = 0.485$) are significantly predictive of biofuel yield, emphasizing the contribution of enzyme expression and stability in engineered strains [From, Table 03 and Graph 05]. The fermentation duration among all cycles ranged from 68 to 74 hours, with shorter durations generally corresponding to higher yield cycles. Notably, Cycle 2, which had the shortest fermentation time of 68 hours, also achieved the highest fermentation efficiency and yield. The regression coefficient for fermentation time ($\alpha = 0.783$, $\beta = 0.456$) strongly suggests that reduced fermentation time, likely due to faster metabolic rates in bioengineered strains, directly correlates with yield [From, Table 03 and Graph 05]. Regression results also suggest a moderate correlation between avoided methane emissions and yield ($\alpha = 0.022$, $\beta = 0.485$), indicating that environmental benefits scale with process optimization. On average, each 100 kg of rice husk processed by the bioengineered system avoided 320–336 kg CO_2e, showcasing a compelling argument for the adoption of such systems in circular bioeconomy frameworks [From, Table 03 and Graph 05]. The results from **ANOVA** show statistically significant differences between experimental cycles ($F = 48.15$, $p < 0.05$). Individual cycles also exhibited strong internal variance explanations (e.g., Cycle 8: $F = 8.91$, $p = 0.002$), highlighting that changes in performance were not due to random variation, but rather due to deliberate changes in variables such as enzyme concentrations, fermentation time, and biomass quantity [From, Table 04 and Graph 06]. There is a clear trade-off between yield and energy efficiency. While Cycle 2 produced the highest yield and environmental benefits, it did not correspond to the lowest energy consumption per liter. Cycle 7, while having a lower total yield, excelled in energy economy (21.35 kWh/L). This suggests a potential for dynamic process optimization depending on priorities yield maximization vs. cost minimization [From, Graph 01and 02]. Avoided emissions data directly correlate with the amount of agricultural waste processed. Each ton of rice husk converted into biofuel averts over 3.2 tons of CO_2, highlighting both environmental and economic value, especially in carbon markets. The robustness of yield and efficiency across varying biomass inputs indicates that the microbial systems can be scaled with relative ease. Furthermore, the linear relationships between feedstock mass and output volume reduce complexity in scaling projections. With high correlations among fermentation time, yield, and enzymatic activity, real-time process control strategies could be implemented. Sensor-driven feedback loops could adjust enzyme dosages, fermentation durations, or aeration rates dynamically to sustain optimal output.



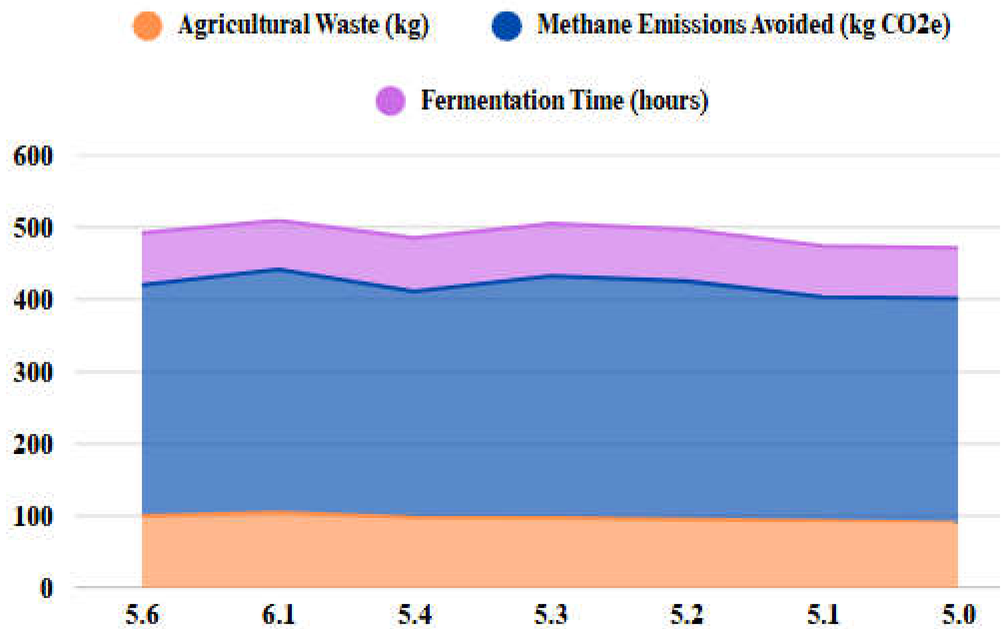
Graph 01: Comparison of Biofuel Production Metrics from Rice Husk



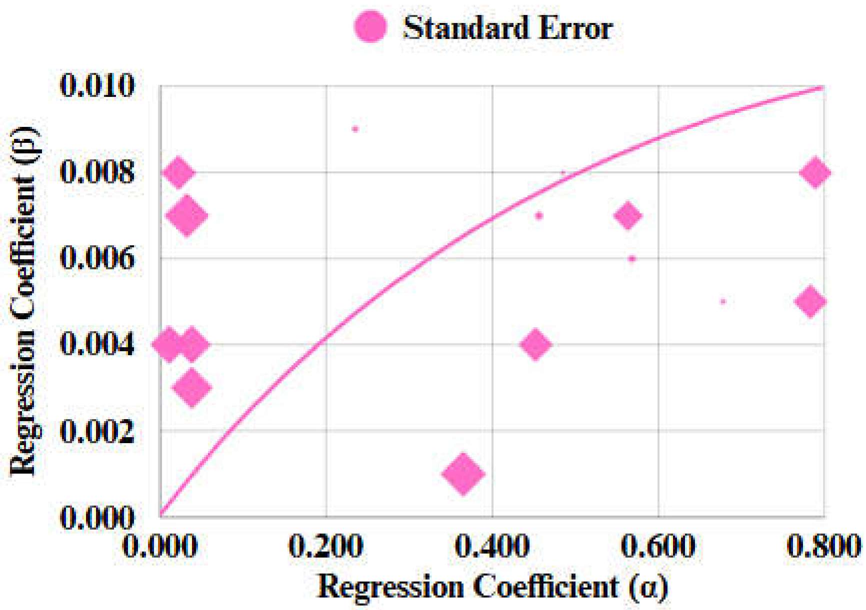
Graph 02: Yield, Fermentation Efficiency, and Energy Usage per Cycle



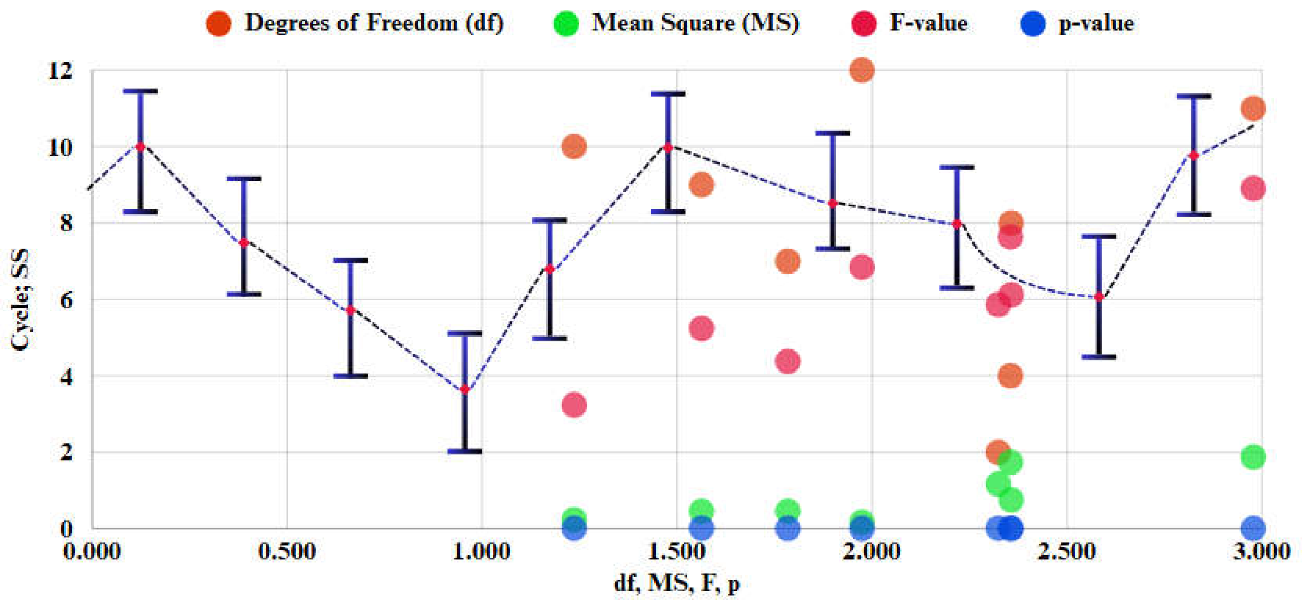
Graph 03: Process Efficiency and Environmental Impact in Biomass



Graph 04: Time, Enzyme Activity, and Methane Emission Reduction



Graph 05: Regression Analysis.



Graph 06: ANOVA Analyses.

Conclusion

This study demonstrates the promising potential of bioengineered microbial systems in converting rice husk into biofuel with notable efficiency and environmental benefits. The consistently high biofuel yields across multiple cycles averaging 0.466 L/kg outperform conventional fermentation systems, largely due to enhanced enzymatic activity, optimized fermentation parameters, and robust microbial strain design. Key performance indicators, such as fermentation efficiency, enzyme activity, and fermentation time, showed strong correlations with biofuel yield, indicating that metabolic optimization was central to the system's success. Despite trade-offs between total yield and energy efficiency, the findings underscore the feasibility of tailoring operational parameters based on production goals whether to maximize yield or minimize energy consumption. The capacity to avoid significant CO_2-equivalent emissions (over 3.2 tons per ton of rice husk processed) adds substantial environmental value, reinforcing the system's relevance within circular bioeconomy frameworks. Moreover, the observed scalability, process robustness, and potential for sensor-driven control strategies suggest that such bioengineered systems can be effectively integrated into larger-scale operations. Overall, this research affirms that with strategic optimization, agricultural waste like rice husk can be efficiently and sustainably converted into biofuel, advancing both renewable energy production and climate mitigation efforts.

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