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Biodegradation of Lignocellulosic Waste by Enriched Environmental Microbial Sources and Sequential Fermentation by *Saccharomyces Cerevisiae*

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Abstract

The increasing global demand for energy and the rapid depletion of fossil fuels have led to the search for alternative energy sources. Bioethanol is one option that can be produced from lignocellulose materials, which involves their biodegradation and fermentation. This study investigated the degradation of three lignocellulose waste materials by enriched microbial communities from three environmental sources and their sequential fermentation by *Saccharomyces cerevisiae*. The NaOH-pretreated lignocellulose substrates in a basal medium were inoculated with enriched microbial suspensions and incubated at room temperature. The extent of biodegradation was determined after 5 days of incubation by the gravimetric method. The bioethanol content was estimated by the solvent extraction & dichromate oxidation method after incubating another 3 days with *S. cerevisiae*. Both the extent of biodegradation and ethanol yield differed significantly among the different lignocellulose-microbial source combinations ($p<0.05$). The highest biodegradation (80.95%) was found in grass clippings by cow dung microbial consortium, while the lowest degradation was in palmyra by compost consortium (19.46%). The ethanol yield ranged from 1.24 to 1.87% (V/V) substrate, which was comparable to reported values from similar studies, but it did not correlate with the extent of lignocellulose degradation ($r = 0.0378$ and $P = 0.923$). This could be either because microbial consortia, though they are good degraders they are poor saccharifiers, or the fermentation would have been limited by broth conditions. Microbial consortia with high lignocellulose-degrading potential may be formulated from natural microbial sources, but their contribution to ethanol production needs further studies.

Keywords: Biodegradation, bioethanol, lignocellulosic waste materials, *Saccharomyces cerevisiae*

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Introduction

Energy consumption has increased steadily over the last century as the world population has grown and due to rapid global industrialisation and motorisation. The cost of crude oil, coal, and natural gas is increasing from time to time due to the imminent shortage of fossil fuels. The global fossil fuel reserves are expected to be depleted in the next 40–50 years due to a rapid increase in the consumption rate. Most significantly, using fossil fuels leads to greenhouse gas emissions and global warming, resulting in climate change, rising sea levels, biodiversity loss, and urban pollution. Hence, the need for alternative sources of energy is on the rise. Fossil fuels may be replaced with ecologically benign renewable energy sources such as biofuels (Rodionova *et al*., 2017). Bioethanol, biodiesel, and biogas are the most used renewable energy sources among biofuels. Among them, ethanol is mostly used in the transportation sector. Bioethanol is a non-petroleum-based, carbon-neutral liquid transportation fuel that can be produced from various lignocellulosic substrates. Bioethanol can be produced from a variety of renewable materials rich in carbohydrates, including lignocellulosic biomass (Broda *et al*., 2022). However, the use of non-edible lignocelluloses is favored over, using food crops as feedstock for producing bioethanol and value-added products due to food insecurity caused by using food crops (Achinas and Euverink, 2016). Lignocellulose biomass is considered a renewable, abundant, and economical alternative to fossil resources, and it has been recognised as a promising resource for the production of bioethanol due to its higher cellulose concentration.

One of the hurdles in the efficient utilisation of lignocellulose is its recalcitrant structure, which is resistant to degradation. There is no natural microorganism possessing all the desired properties to be utilized efficiently in bioethanol production at the required high yields and titers. When compared to monoculture approaches, the use of microbial consortia is considered an effective and sustainable way of promoting lignocellulose degradation. Microbial communities found in nature can be utilised in such processes since synergies may exist that can result in effective lignocellulose biodegradation and increased bioethanol production. Enriched microbial communities, obtained from environmental samples through selective processes, can effectively contribute to lignocellulose degradation (Puentes and Falcao, 2018). While the long-term objective of this study was to develop efficient lignocellulose biomass-degrading microbial consortia, the specific objectives were to compare the lignocellulose biodegradation efficiency of microbes from natural environmental sources and their sequential fermentation into ethanol by *Saccharomyces cerevisiae*.

Materials and Methods

Sampling of Lignocellulose waste material and environmental microbes

Lignocellulose waste materials, i.e., factory refuse tea obtained from a tea factory in Ratnapura, palmyra husk collected from Jaffna, and grass clippings from the premises of the Faculty of Applied Sciences, Southeastern University of Sri Lanka, Sammanthurai. The environmental microbial sources, cow dung and compost, were obtained from Sammanthurai, and coir retting water from a coir pit in Kurunegala, Sri Lanka.

Pretreatment of lignocellulosic materials (LM)

The LMs were air-dried, ground into powder, and passed through a 2 mm sieve. A slightly modified method of pretreatment using sodium hydroxide was used here (Wunna *et al*., 2017). Hundred grams each of the powdered LM was pretreated separately in 1 L of 2% sodium hydroxide (w/v) for 12 hours at room

temperature, oven dried at 60 ℃ to a constant weight, and stored in sealed, sterilized glass bottles at room temperature until use.

Enrichment of lignocellulose-degrading microbes

Microbial suspensions from cow dung and compost were prepared by mixing 1 g each of fresh material in 10 mL of sterile distilled water. The coir retting water was used directly. These were enriched for lignocellulose-degrading microbes separately in 100 mL of basal media supplemented with 1 g of each pretreated LM and pH adjusted to 7. The basal media used consisted of 5 g peptone powder and 3 g yeast extract powder per liter of distilled water. The 100 mL media in 250 mL flasks were inoculated with 10 mL each from the three microbial suspensions separately and incubated for five days at room temperature in static conditions. The second and third enrichments were done in the same manner by inoculating fresh respective media from the respective previous enrichment culture (Mallik *et al*., 2015). The third enrichment culture was used to study the degradation of the respective LM.

Determination of lignocellulose degradation and sequential fermentation

The basal medium containing 1% (w/v) LM was inoculated with 10 mL each from the respective third enrichment cultures of the respective microbial source (MS) separately and incubated for five days at 30 °C under static conditions with occasional shaking. The uninoculated medium was used as the control. Eight replicates were maintained for each microbial LS combination. The suspensions of four replicates were filtered using a Buchner funnel and the residue was suspended in 100 mL of glacial acetic acid reagent, heated at 100 ℃ for 30 minutes, and refiltered using a Buchner funnel. The residue was washed three times with 100 mL of distilled water each time, dried at 80 ℃ and weighed (Mallik *et al*., 2015). The degradation ratio was calculated using the following equation;

$$Degradation\ ratio = \frac{Mt - Mr}{Mt} x\ 100$$

Here, Mt represents the total weight of the LM before degradation, and Mr represents the weight of the residual substrates after degradation. The remaining four replicates were inoculated with 1 g each of *S. cerevisiae* (Baker's yeast) and incubated for 3 days at room temperature (~30 °C) under static conditions for bioethanol production.

Ethanol estimation

The bioethanol concentration was estimated using the solvent extraction and dichromate oxidation method (Miah *et al*., 2017). The fermented experimental samples were centrifuged at 14,000 rpm for 10 minutes, and the supernatant was collected. The dichromate reagent was prepared by dissolving 1 g of fresh potassium dichromate in 100 mL of 5 M sulfuric acid solution. Tri-n-butyl phosphate [density = 0.975 to 0.976, Solubility in water = 0.028% (w/v] was used to extract ethanol in an aqueous solution. To prepare the sample for bioethanol estimation, 1 mL of tri-n-butyl phosphate and 1 mL of experimental samples were mixed in a 2 mL Eppendorf tube and vortexed for 10 minutes. The reaction mixture was left until it separated into two phases. From the upper phase, a 500 µL solution was transferred to a new 2 mL Eppendorf tube. Then, 500 µL of dichromate reagent was added, and the tube was vortexed vigorously for 10 minutes and left for separation into two phases. A 100 µL aliquot of the lower phase was transferred to the cuvette, diluted 10 times with experimental samples, and the optical density was measured at 595 nm using a UV spectrophotometer. The bioethanol content of the unknown sample was estimated from the ethanol standard curve prepared by 1% - 5% ethanol samples.

Statistical Analysis

All data were tested for normality (Shapiro-Wilk normality test). Two-way ANOVA and mean comparison by Tukey post hoc test (p = 0.05) were performed for biodegradation ratio and bioethanol yield using R software (Version 4.4.2).

Results

Biodegradation of lignocellulosic materials

All three LMs were degraded by all three MSs to different extents. The grass clippings were the highest degraded by all three MSs, ranging from 49.73 to 80.95%. The highest was about 66.7% higher compared to tea refuse and 195% higher compared to palmyra. Thus, the grass clippings were the lowest recalcitrant LM and the palmyra the highest, which showed the lowest degradation ranging from 19.46 to 27.44%. Tea refuse by all three MSs and grass clippings with compost and coir-retting water showed a medium level of degradation, ranging from 40.33 to 51.16% (Figure 1). The degradation ratios of a particular LM by the three MSs were significantly different. Among the three MSs, the cow dung showed the highest degradation of all three LMs. This was 80.95% for grass clippings, which was about 60% higher compared to the other two MSs. The degradation ratios by coir retting water and compost of a particular LM did not differ significantly.

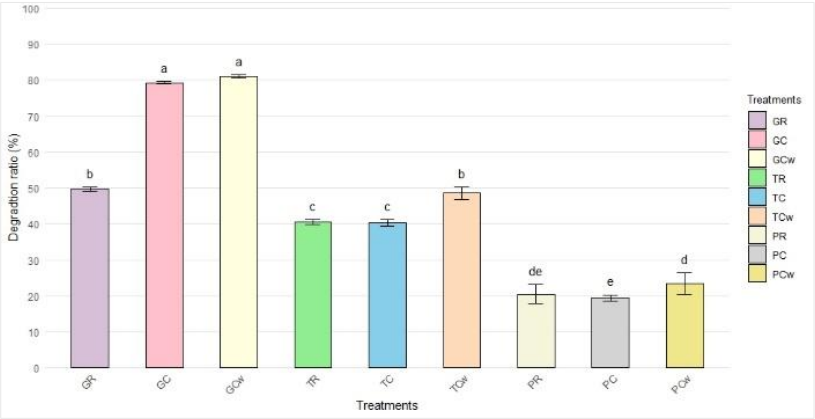

Figure 1: *Degradation ratios of different lignocellulosic materials by different enriched microbial sources. T: tea refuse, G: grass clippings, P: palmyra husk, Cw: cow dung consortium, R: retting water consortium and C: compost consortium. Values shown are means of triplicates and that do not share the same letter are significantly different (p<0.05).*

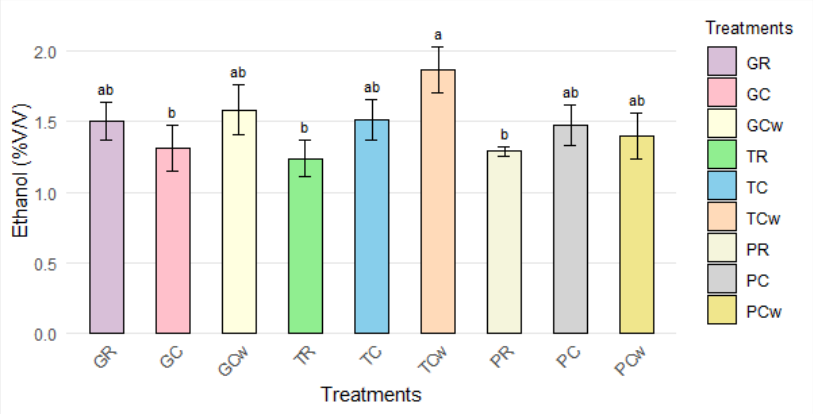

Figure 2: *Ethanol yield from sequential fermentation by S. cerevisiae of different lignocellulosic materials degraded by different enriched microbial sources.*

Bioethanol yield

The ethanol yields produced from the three LMs treated with different MSs are illustrated in Figure 2. All LM-MS combinations yielded bioethanol by sequential fermentation, ranging from 1.24 to 1.87% (V/V). The highest ethanol production (1.81% v/v) was observed in the tea refuse with cow dung (TCw), which was significantly greater than treatments such as TR, GC, and PR. Other treatments, including GR, GCw, TC, PC, and PCw, showed moderate yields and did not significantly differ from the highest-yielding treatment. However, the ethanol yield did not correspond to the lignocellulosic biomass degradation in this study ($r = 0.0378$ and $P = 0.923$). The ethanol yield in grass clippings and tea refuse, despite high degradations, was similar to the yield from the lowest degraded palmyra.

Statistical analysis using a two-way ANOVA revealed that ethanol production was significantly affected by the type of microbial source ($p = 0.0217$), but not by the lignocellulosic material alone ($p = 0.2812$). A marginally significant interaction between lignocellulosic material and microbial source ($p = 0.0523$) suggests that the impact of microbial source on ethanol yield may vary depending on the specific substrate. Overall, cow dung was the most effective microbial source for enhancing ethanol production across different lignocellulosic materials.

Discussion

The difference in degradation ratios is due to its inherent recalcitrance, which refers to the resistance of cellulosic biomass to enzymes, microorganisms, and/or chemicals. Lignocellulose biomass includes varying amounts of cellulose, hemicellulose, and lignin depending on the type of biomass. Lignin is the main factor influencing recalcitrance, with its amount, position, and type (Kumar *et al*., 2016). Due to the highly recalcitrant nature of lignocelluloses, the bioconversion of cellulose to hemicellulose to fermentable sugars is a rate-limiting step (Singh *et al*., 2017). The pretreatment procedure affects degradation, such as longer pretreatment times and higher temperatures, which improve delignification efficiency (Wi *et al*., 2015). Grasses with low lignin content are advantageous, but different pretreatment techniques may result in significant differences in susceptibility and thus variations in degradation ratios. We used the NaOH pretreatment for all three LMs due to its advantages, including high removal of lignin (Wunna *et al*., 2017), and as there is no single pretreatment technology usable for the multiple biomass conversion (Wi *et al*.,

2015). It must be tailored to the specific chemical and structural composition of the specific LM (Mosier *et al*., 2005). Higher degradations would have been achieved in our study if LM-specific pretreatments could be used.

Regarding microbial sources, the degradation ratios of a particular LM by the three microbial sources were significantly different. This could be because the species compositions of LM-degrading microbial consortia developed from the different microbial sources are different. Among the three microbial sources, the cow dung showed the highest degradation of all three LMs, which could be because of the inherent potential of bowel microbes to act on grass, as it is a cattle feed. The higher degradation ratios of palmyra and tea refuse by cow dung compared to the other two microbial sources indicate that the cow dung harbors a higher diversity of microbes that leads to the development of microbial consortia, enabling the degradation of LMs of different chemical compositions. The degradation ratios by coir retting water and compost of a particular LM did not differ significantly, indicating some similarity in the microbial consortia developing under anaerobic conditions from the two sources. Obviously, the original microbial composition of compost would have been different from that of anaerobic coir retting water since the compost used in this study was aerobic. Possibly, the maintained anaerobic conditions have favoured LM LM-degrading consortium similar to such a community developed from coir retting water. Thus, testing these microbial sources on different LMs, either alone or in combination, and under different conditions (aerobic and anaerobic) may yield high-potential lignocellulose-degrading microbial consortia. Testing these microbial sources in combination may yield a microbial consortium with higher diversity. Diversity begets higher-order properties, thus leading to the production of self-regulating, mutually reinforcing, functionally stable, and robust microbial communities to produce a desirable resource (Konopka *et al*., 2015).

The ethanol yield in grass clippings and tea refuse, despite high degradations, was similar to the yield from the lowest degraded palmyra. This indicates that even though the microbial consortia developed from microbial sources were good LM degraders, they were not good saccharifiers. Another reason could be that the consortia developed are good saccharifiers, but the subsequent fermentation would have been inhibited or affected by contaminants, or the degradation gives products other than fermentable sugars. A series of inhibitors, such as furans, carboxylic acids, and phenolic compounds, was produced during the pretreatment. The furan derivatives limit yeast glycolysis and ethanol production, making fermentation processes less efficient, and small fragments of lignin might be inhibitory or toxic to yeast. Contamination, especially by lactic acid bacteria, which compete with fermentable sugars and nutrients, and create inhibitory byproducts such as lactic acid. Antibiotics are often used in fuel ethanol plants to control bacterial contamination during fermentation. In case the microbial consortia developed are not good saccharifiers, they apparently have yielded various compounds other than fermentative sugars, and these intermediates would be potential candidates to produce various other value-added chemicals. Even though lignocellulose biomass is mostly used for bioenergy, in recent years, considerable attention has been given to it as a source to produce high-value chemicals and thus a significant contribution to the 'circular bioeconomy'. The concept of circular bioeconomy is based solely on the use of natural sources and enables greater environmental sustainability compared to the use of fossil resources. In addition, most of the LMs obtained from agricultural waste are not utilized due to a lack of awareness of their biotechnological importance. Use of LM to obtain various products would also be a sustainable option for agricultural waste management (Yadav *et al*., 2021). Further studies will reveal the significance of these microbial consortia in the production of bioethanol and other high-value chemicals and may pave the way for the integration of both. Such integration of various conversion techniques on a single platform under a biorefinery approach is gaining interest worldwide (Devi *et al*., 2022).

Conclusion

Microbes from natural environments may be formulated into effective microbial consortia capable of degrading various lignocellulosic waste materials. Their efficiency seems to be limited by the pretreatment of the LM, and hence, identifying LM-specific pretreatment techniques is critical. The sequential fermentation by *S. cerevisiae* apparently is limited due to the consortia developed are not efficient saccharifiers, or the fermentation is inhibited by the physicochemical conditions in the broth. However, this effort is meaningful, as the products of LM degradation beyond fermentable sugars could contribute to the circular bioeconomy and thus sustainable development. Further studies could improve the bioethanol yield and production of high-value chemicals from the degradative products of microbial consortia.

References

Achinas, S., & Euverink, G. J. W. (2016). Consolidated briefing of biochemical ethanol production from lignocellulosic biomass. *Electronic Journal of Biotechnology*, *23*, 44-53. https://doi.org/10.1016/j.ejbt.2016.07.006

Broda, M., Yelle, D. J., & Serwańska, K. (2022). Bioethanol production from lignocellulosic biomass—challenges and solutions. *Molecules*, *27*(24), 8717. https://doi.org/10.3390/molecules27248717

Devi, A., Bajar, S., Kour, H., Kothari, R., Pant, D., & Singh, A. (2022). Lignocellulosic biomass valorization for bioethanol production: a circular bioeconomy approach. *Bioenergy Research*, *15*(4), 1820-1841.

Konopka, A., Lindemann, S., & Fredrickson, J. (2015). Dynamics in microbial communities: unraveling mechanisms to identify principles. *The ISME journal*, *9*(7), 1488-1495. https://doi.org/10.1038/ismej.2014.251

Kumar, R., Tabatabaei, M., Karimi, K., & Sárvári Horváth, I. (2016). Recent updates on lignocellulosic biomass derived ethanol-A review. *Biofuel Research Journal*, *3*(1), 347-356. 10.18331/BRJ2016.3.1.4

Malik, S., Sharma, S., Sharma, J., & Mandhan, R. P. (2015). Bioprocessing of crop residues using fibrolytic enzymes and *Flavobacterium bolustinum* for enriching animal feed. *International Journal of Biotechnology for Wellness Industries*, *4*(1), 12

Miah, R., Siddiqa, A., Tuli, J. F., Barman, N. K., Dey, S. K., Adnan, N., ... & Talukder, A. A. (2017). Inexpensive procedure for measurement of ethanol: Application to bioethanol production process. *Advances in Microbiology*, *7*(11), 743.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource technology*, *96*(6), 673-686. https://doi.org/10.1016/j.biortech.2004.06.025

Puentes-Téllez, P. E., & Falcao Salles, J. (2018). Construction of effective minimal active microbial consortia for lignocellulose degradation. *Microbial ecology*, *76*, 419-429.

Rodionova, M. V., Poudyal, R. S., Tiwari, I., Voloshin, R. A., Zharmukhamedov, S. K., Nam, H. G., ... & Allakhverdiev, S. I. (2017). Biofuel production: challenges and opportunities. *International Journal of Hydrogen Energy*, *42*(12), 8450-8461. https://doi.org/10.1016/j.ijhydene.2016.11.125

Singh, N., Mathur, A. S., Tuli, D. K., Gupta, R. P., Barrow, C. J., & Puri, M. (2017). Cellulosic ethanol production via consolidated bioprocessing by a novel thermophilic anaerobic bacterium isolated from a Himalayan hot spring. *Biotechnology for biofuels*, *10*, 1-18.

Wi, S. G., Cho, E. J., Lee, D. S., Lee, S. J., Lee, Y. J., & Bae, H. J. (2015). Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials. *Biotechnology for biofuels*, *8*, 1-11

Wunna, K., Nakasaki, K., Auresenia, J. L., Abella, L. C., & Gaspillo, P. A. D. (2017). Effect of alkali pretreatment on removal of lignin from sugarcane bagasse. *Chemical Engineering Transactions*, *56*, 1831-1836. 10.3303/CET1756306

Yadav, N., Nain, L., & Khare, S. K. (2021). One-pot production of lactic acid from rice straw pretreated with ionic liquid. *Bioresource Technology*, *323*, 124563. https://doi.org/10.1016/j.biortech.2020.124563