International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Bioethanol Production by Immobilized Enterobacter Cloacae Using Different Matrices

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Abstract

Biofuels can be produced through the bioconversion of lignocellulosic substrates, which are derived from sustainable and renewable resources. One such biofuel, bioethanol, stands out as a viable alternative for transportation fuel, offering a sustainable approach to address the challenges posed by fossil fuels. The present study aimed to investigate the immobilization of *Enterobacter cloacae* cells for bioethanol production from rice husk hydrolysates. For this purpose, the biocompatible carriers such as Calcium alginate and activated charcoal beads were used for immobilization. The parameters of bioethanol fermentation, such as the incubation period in different carriers and the choice of a convenient carrier for efficient ethanol production were studied. The maximum bioethanol production of 10.8% was obtained in the 24 hour of the incubation period, with *Enterobacter cloacae* immobilized in Ca-alginate using a droplet method. However, after the second fermentation cycle, Calcium alginate beads got degraded and resulted in lower bioethanol production. The *Enterobacter cloacae* immobilized on activated charcoal also showed better production at 48 hour of 9.15% as compared to free cells (8.03%).

Keywords: Agro-waste; Bioethanol; Ca−alginate; Enterobacter cloacae; Immobilization

Introduction

The overuse and combustion of fossil fuels are severely impacting the environment by releasing carbon dioxide and other harmful gases, thus intensifying the greenhouse effect, global warming, and climate change. The transportation sector alone accounts for almost 40% of all fossil fuel usage, highlighting the urgent need to minimize CO2 emissions through advanced technology and reduce reliance on fossil fuels (Chacón-Navarrete et al., 2021). One of the most promising sustainable fuels that have gained significant attention worldwide is biofuels, as they are renewable, environmentally friendly, and do not interrupt the balance of the environment. Biofuels possess low carbon and sulfur emissions, which make them cheap and could eventually displace energy sources generated from petroleum. The usual natural process of fossil fuel generation takes hundreds or thousands of years, whereas biofuels are manufactured from biomass in a very short period of time. Biofuels from lignocellulosic waste are produced as a substitute for renewable sources of energy (Beliya et al., 2013; Takano and Hoshino, 2018). Among all the biofuels, bioethanol can be used in the transport sector mixed with gasoline or as an octane enhancer as ETBE (ethyl tertiary butyl ether, with 45% ethanol by volume and 55% isobutylene). Bioethanol can directly be used in vehicles as it has similarities with conventionally used fuels, with some modifications in the engine. Bioethanol and gasoline (5–10% by volume) can be used without modifying the vehicle engine. (Bušić et al. 2018).

To enhance the efficiency of bioethanol production from lignocellulosic agro-wastes, different approaches have been introduced nowadays so that they can achieve economically competitive status. In this instance, cell immobilization has increased bioethanol output and decreased expenses in bioreactors by lowering inhibition brought on by high substrate and product concentrations.

Numerous studies have described the process of immobilizing microorganisms in different matrices to enhance the fermentation process and produce bioethanol more cheaply. These systems have shown potential by producing more ethanol yield than free cells. Cell immobilization provides strong support due to its high biological compatibility, affordability, easy availability, and simple preparation. Compared to free cells, immobilized cells offer advantages such as increased cell density, easier separation from the reaction medium, continuous operation without downstream transport, reduced lag phase, improved substrate conversion, diminished product inhibition, shortened reaction times, and controlled cell replication (Duarte et al. 2013). The most commonly applied techniques for immobilizing cells and enzymes are adsorption, entrapment, covalent binding, and cross-linking. Calcium alginate gel entrapment is the most promising immobilization technique, offering several advantages as a support. This technique has many advantages, such as biocompatibility, low cost, easy availability, and a simple preparation method.

The purpose of this study was to convert rice husk, an inexpensive, environmentally valuable agricultural waste product, into second-generation biofuel (bioethanol) by studying the effect of immobilization on different matrices. In this work, the fermentation of sugar to produce bioethanol was carried out by free *Enterobacter cloacae* cells as well as its immobilization in calcium alginate and activated charcoal matrix and their effect on the incubation period was also evaluated.

Materials and Methods

Agricultural waste

Rice husk, an underutilized substrate, was used for bioethanol production. It was collected from local rice mills in Raipur (C.G.), India. After collection, it underwent air drying, grinding, and milling before being stored for future use.

Microorganism

Enterobacter cloacae, a gram-negative bacterium, was used for immobilization and was isolated from the rice husk itself to improve the bioethanol efficiency. This bacterial culture was provided by the School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur (C.G.), India. The bacterium was maintained in nutrient agar medium for further use.

Effect of Immobilization on Bioethanol Production

For immobilization, calcium alginate and activated charcoal were used to investigate their effects on bioethanol production.

Immobilization of bacterial cells using calcium alginate beads

For the bead immobilization, sodium alginate (4%, w/v) was dissolved in 500 mL of water, followed by adding a suspension of Enterobacter cloacae in a beaker (1:1). This solution was mildly shaken using a magnetic stirrer at 400 °C. A 2% CaCl2 solution was prepared in a separate beaker. The bacterial cells and sodium alginate mixture were added drop-wise to an ice-cold CaCl2 solution using a syringe. The beads were left for 1 hour in this solution to harden before use. After hardening, the beads were washed with distilled water for future use (Fig. 1) (Duarte et al. 2013).

Immobilization of bacterial cells using activated charcoal beads

10 g of activated charcoal was taken and sterilized in a hot air oven at 450 °C for 180 minutes. After this, 10 mL of pure bacterial slurry obtained by centrifuging the culture broth was mixed with activated charcoal and left at room temperature for 24–48 hours. After the incubation period, the charcoal was washed with distilled water and left to dry at room temperature (Al-Hilo et al., 2007).

The dried activated charcoal powder with bacterial broth was mixed with the 3% (w/v) sodium alginate solution and added drop-wise in ice-cold CaCl2 (2%) using a syringe. The beads were left for 1 hour in this solution to harden before use. After hardening, the beads were washed with distilled water for future use (Fig. 2).

Fig. 1: Sodium-alginate beads

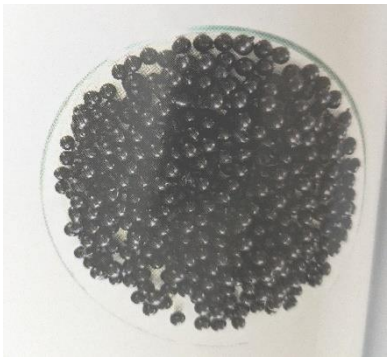

Fig. 2: Activated charcoal beads

Batch fermentation experiments using the immobilized cells

30 g of calcium alginate beads were added to a flask containing a nutrient-rich fermentation medium for maximum bioethanol production. All steps before fermentation were carried out under sterile conditions. The same procedure was carried out for the charcoal beads. The flasks were sealed with a cotton plug and placed in an orbital shaker for 24 hour to 72 hour to study the effect of incubation at 37°C at 100 rpm. After a respective incubation period, the filtered beads were washed with sterile water and added to a fresh fermentation medium. This method was repeated 3 times for the successive fermentation cycles to study the bioethanol production.

Storage of immobilized yeast cells

Bacterial cells, immobilized for experimentation, were securely sealed in Falcon tubes using parafilm strips and stored at 4°C. To evaluate viability and culture load, these immobilized bacterial cells utilized in fermentation were carefully extracted, cleansed with autoclaved distilled water, and precisely preserved at 4°C.

Estimation of bioethanol

The specific gravity method was preferred for quantitative estimation. In this procedure, the ratio of the liquid's density to the density of water is measured at a specific temperature. 100 mL of distilled water was combined with 90 mL of distilled substrate. Additionally, this combination was placed in a 25 mL specific gravity bottle.

The amount of bioethanol can be calculated by using the formula (Pharmacopoeia of India, 1985).

$$\rho(t°C) = \frac{w3 - w1}{w2 - w1} * density\ of\ water\ at\ t°C$$

Were,

W_1 = weight of empty bottle

W_2 = weight of empty bottle and distilled water

W_3 = weight of empty bottle and fermented sample

Statistical analysis of data

The data obtained from the experiment were represented as mean and standard error values. The analysis of the data was done by one-way analysis of variance (ANOVA) with mean values having significant differences, determined and measured with the Duncan multiple range test using Statistical Package for Social Science Research (SPSS) version 16.

Results and Discussions

Effect of bacterial cell immobilization in different matrices

(a) Calcium alginate

Bacterial cells were immobilized within calcium alginate beads, demonstrating reusability for up to three cycles. The cells were immobilized and incubated for 24 hours and 48 hours to study the effect of incubation. The best results were obtained with a second cycle of 24-hour incubation, yielding bioethanol of 10.8% (Fig. 3). This was higher than the yield from free cells. The second cycle of the 48-hour incubation period also exhibited high bioethanol production at 9.68% compared to free cells. The initial lack of production growth in the first cycle is attributed to cell stress induced by immobilization, possibly entering an adaptation phase. The subsequent rise in production in later cycles suggests improved cell adaptation or proliferation within the beads, as theorized by Duarte et al. (2013). However, challenges arose during extraction, leading to bead breakage and potential cell leakage, reducing the concentration of beads utilized in each cycle.

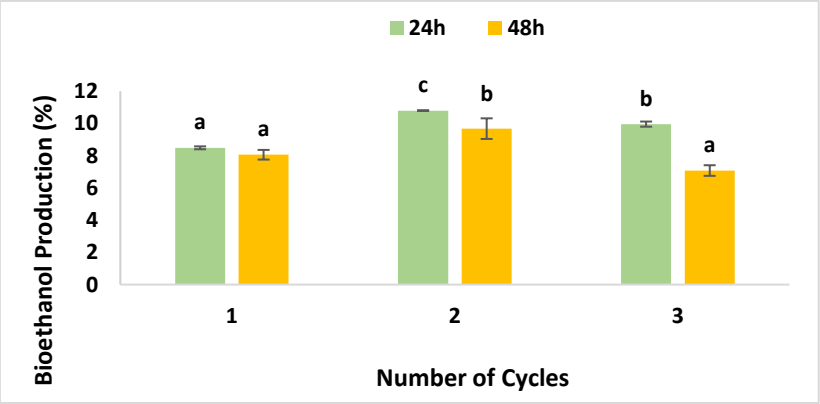

Fig. 3: Bioethanol production with immobilization of *Enterobacter cloacae* in sodium alginate beads

(b) Activated charcoal

Enterobacter cloacae was adsorbed on an activated charcoal surface and entrapped in calcium alginate beads, demonstrating reusability for up to three cycles. The best results were achieved with a first-cycle 48-hour incubation period, yielding bioethanol of 9.15% (Fig. 4). This amount was higher than the yield obtained from free bacterial cells. The production of bioethanol increased in the first cycle. In the second cycle, production does not increase as expected, possibly due to the cell being stationary and undergoing stress due to immobilization. The subsequent release of activated charcoal from the reused beads contributed to a significant improvement in both sugar utilization and bioethanol production, as predicted by Jones et al. (2011).

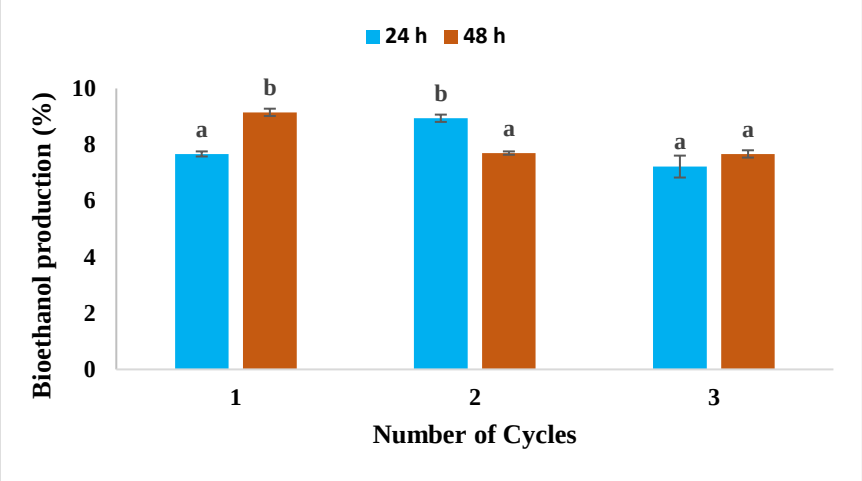

Fig. 4: Bioethanol production with activated charcoal beads

Conclusions

Rice husk as a feedstock to produce bioethanol could provide a solution to waste management issues, help maintain an eco-friendly environment, be cost-effective, and help minimize economic loss. In this study, the efficiency of the bacteria was assessed through immobilization in various matrices. Upon comparing sodium alginate and activated charcoal matrices for immobilization, it was found that sodium alginate exhibited better production of bioethanol. Immobilization proves better yield as well as reusable advantage for improved fermentation process.

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