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Optimized fermentation strategies for efficient bioethanol production

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ABSTRACT

Energy is essential for economic growth and sustainable development. The conventional finite exhaustible resources such as petroleum, natural gas, and coal dominate the global energy supply but are limited and geographically dispersed. India has turned to sustainable alternatives like ethanol blending to reduce dependency on imported oil. Bioethanol production leverages lignocellulosic biomass through enzymatic saccharification, fermentation, and pre-treatment processes. This study investigates bioethanol production using a wild *Escherichia coli* strain isolated from the Coum River, employing a fed-batch fermentation method to optimize yield and efficiency. Samples of cellulose-rich compost and Coum River water were collected to isolate microbial strains for fermentation trials. *E. coli* isolates were purified using nutrient agar and characterized microscopically and biochemically. Fermentation was carried out in controlled batches and continuous reactors, using various nutrient combinations. Key process parameters like pH, temperature, and RPM were optimized using Response Surface Methodology (RSM) to maximize ethanol yield. The study extensively analyzed ethanol production using various microbial strains and fermentation conditions. Batch operation with *E. coli* FBWHR and sugar maple hydrolysate revealed optimal ethanol production (20.38 g/L) at 50% sugar concentration, with sequential sugar utilization. Levoglucosan fermentation by *E. coli* KO11 achieved a 40% theoretical yield, prompting comparative studies with *Zymomonas mobilis*. Continuous operation with *E. coli* FBR5 using wheat straw hydrolysate (WSH) produced stable ethanol yields (~19.2 g/L), improved by desalting. Salt tolerance (up to 40 g/L NaCl) and xylose tolerance (250 g/L) were noted. Regression analysis highlighted key factors like sugar utilization, ethanol yield, and cell mass influencing productivity. This study highlights the critical role of substrate optimization, inhibitor removal, and strain engineering in enhancing ethanol productivity under varying fermentation conditions, aligning with prior findings on microbial robustness and process efficiency.

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Introduction

Energy is essential for promoting economic expansion and attaining sustainable development, irrespective of a nation's development. Non-renewable resources, including natural gas, coal, and petroleum, meet most of the world's energy needs. Since energy was created over thousands of years, these resources cannot be renewed

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quickly enough to allow for sustainable consumption. They are also geographically dispersed, which forces many countries to purchase crude oil, especially from the Middle East, to meet their energy needs. At almost 4% of worldwide energy usage, India is the fifth-biggest energy consumer, ranking behind the United States, China, Russia, and Japan (Niphadkar et al., 2018; Singh et al., 2017).

India has also seen significant growth in ethanol (EtOH) production, driven by its focus on reducing dependency on imported oil and meeting energy needs sustainably. India generated over 3.3 billion litres of EtOH in the 2020–21 supply year, mostly for use in gasoline blends. By 2022, EtOH and gasoline were blended at a rate of about 10%, with intentions to expand to 20% by 2025. India imported about 2 million barrels of crude oil in 2021 as a result of EtOH generation (Pattnaik et al., 2024).

Energy crops such as switchgrass and miscanthus, along with processing byproducts like rice hulls and maize fiber, and agricultural residues including corn stover, wheat straw, barley straw, rice straw, and sugarcane bagasse, represent cost-effective feedstocks for gasoline-ethanol production. Enzymatic saccharification, fermentation, product recovery, and feedstock pre-treatment are the four main steps during the conversion of lignocellulosic (LC) biomass into EtOH. Pre-treatment is a crucial step in preparing LC biomass for enzymatic hydrolysis. After pre-treatment and saccharification, a mixture of fermentable sugars including glucose, xylose, arabinose, and galactose is produced, which is essential for supporting microbial fermentation (Hiloidhari et al., 2019; Nahak et al., 2022).

The metabolic process of fermentation, which occurs in bacteria, yeast, and oxygen-depleted muscle cells, transforms carbohydrates into acids, gases, or alcohols. Fermentation also refers to zymology, the large-scale cultivation of microbes for producing goods like food additives, enzymes, and antibiotics. In low oxygen conditions, fermentation replaces the electron transport chain (ETC) as the primary source of ATP by converting pyruvate and NADH into NAD+ and other byproducts to sustain glycolysis (Mani et al., 2018). Obligate anaerobes rely entirely on fermentation, whereas oxidative phosphorylation is more effective under oxygen-rich conditions.

Since the Neolithic period, fermentation has been used to preserve foods like yoghurt, kimchi, and pickles, and to produce alcoholic beverages like wine and beer. Common fermentation products include EtOH, CO2, lactic acid, and hydrogen gas, with yeast playing a key role in EtOH and CO2 production for beverages (Ross et al., 2002). Industrial fermentation involves the controlled use of bacteria and fungi to produce useful products for food and pharmaceutical industries, including EtOH, citric acid, and acetic acid. Factors such as temperature, pH, oxygen levels, microbial strains, and enzymes significantly impact fermentation yield. Genetically engineered microbes are increasingly used to produce enzymes like invertase, lipase, and rennet. Fermentation processes are categorized by their outputs, including biomass (viable cells), extracellular metabolites (chemicals), intracellular components (proteins or enzymes), or substrate conversion to useful products (Formenti et al., 2014; Ross et al., 2002).

Biological processes are optimized in sterile, controlled environments using specialized vessels called bioreactors. These vessels regulate nutrient flow, oxygen levels, and metabolic byproducts to create a biomechanical and biochemical environment for cell growth. Unlike conventional chemical reactors, bioreactors require precise control to address the sensitivity of living organisms, which are prone to contamination and mutation. Bioreactors vary in size, from small laboratory fermenters to large industrial systems. To maintain process stability, key parameters such as temperature, dissolved oxygen, pH, gas flow, agitation speed, and foam control must be monitored and adjusted. Based on operation mode, bioreactors can be batch, fed-batch, or continuous systems (Kaur et al., 2021).

Batch reactors use sterilized culture media and microbes, allowing reactions to proceed over a set period with aeration for aerobic cultures, and products are collected at the end. Continuous reactors maintain steady nutrient flow and product removal, ensuring constant conditions. Fed-batch reactors combine batch and continuous techniques by gradually adding nutrients during the process to enhance yields. Specialized bioreactors include plug-flow reactors, which allow unidirectional flow for uniform reaction times but present challenges with heat and temperature control; bubble column reactors, which use gas bubbles for efficient heat and mass transfer with minimal maintenance; and packed-bed reactors, where fluids flow through immobilized biocatalysts but may face poor oxygen transport and temperature control issues (Singh et al., 2014; Spier et al., 2011).

Advances in metabolic engineering have enhanced n-butanol production in *E. coli* and other hosts by utilizing NADH accumulation and NADH-dependent CoA reductases. Similarly, the Ehrlich pathway, naturally present in yeast, catabolizes branched amino acids to produce higher alcohols or fusel alcohols (Lee and Trinh, 2019). Enzymes like 2-keto acid decarboxylase and alcohol dehydrogenase have been introduced into *E. coli*,

C. glutamicum, and cyanobacteria to boost alcohol production. In cell-free systems, isobutanol production has reached near-commercial yields. Fusel alcohols are enzymatically esterified with acyl-CoAs to produce esters for fuels, flavors, perfumes, and solvents. Ethanol and isobutanol lactate esters engineered in *E. coli* serve as eco-friendly solvents (Keasling et al., 2021; Rodriguez et al., 2014).

Additionally, *Rhodococcus opacus* produces high levels of free fatty acids (FFAs) and long-chain hydrocarbons, advancing bio-based sustainable oleochemical industries (Lee and Trinh, 2019). *Yarrowia lipolytica* has been engineered to boost long-chain methyl ketone production by optimizing peroxisomal β-oxidation pathways, facilitating β-KCoA accumulation. Integrating heterologous pathways and targeting enzymes to peroxisomes maximizes production efficiency, with methyl ketone yields significantly influenced by oxygen availability (Hanko et al., 2018).

This study aimed to explore bioethanol production from biomass using a wild *E. coli* strain isolated from the Cooum River. A minimal nutrient broth, supplemented with essential ingredients, was used to support microbial growth and ethanol production. The fermentation process was optimized using a Design of Experiments (DOE) approach and Response Surface Methodology (RSM) to identify the best combination of ingredients for higher ethanol yield. Fermentation trials were conducted in a pilot-scale fermenter using a fed-batch method, with particular attention to how fermenter design and process conditions could improve efficiency and stability. The study highlights the potential of using naturally occurring microbial strains and process optimization for sustainable bioethanol production.

Materials and Methods

Sample collection

The cellulose-rich plant compost heaps, both wet and dry, were gathered from the Western Ghats region. These samples were selected due to their high cellulose and organic content, which qualifies them for the synthesis of bioethanol. To isolate a wild strain of *E. coli*, water samples were taken from the Cooum River's banks, particularly from upstream areas. To guarantee access to a variety of microbial populations that could be able to convert biomass effectively, these riverside samples were chosen. The goal of the sampling strategy was to supply fermentation trials with raw materials and microbial isolates.

Bacterial isolation

The samples were primarily diluted in H_2O. After the samples were diluted, 0.1 ml of the solution was applied to nutrient agar plates. The samples were grown on soybean-casein digest media. The plates were incubated at 30 °C for a period of 2–3 days. Following incubation, bacterial colonies were found and chosen for additional examination. Continuous subculturing was carried out to purify colonies. Then, purified isolates were grown on MacConkey Agar plates. The appearance of the colonies was noted, and the arrangement of the cells was evaluated under a microscope (Crecchio et al., 2004).

Inoculum and culture conditions

One isolated colony from the sample was transferred into 3 mL of liquid medium and incubated overnight at 37 °C to generate the inoculum. Subsequently, 250 mL Erlenmeyer flasks containing 50 mL of liquid medium were inoculated with 0.2 ml aliquots of the inoculum. These were incubated at 37 °C and 200 rpm in a rotary shaker. The remaining mixtures were incubated at 30 °C for 2–4 hours to enhance the concentration of bacteria, which were initially present in low quantities. Quantity of 10, 25, and 50 µL aliquots of these enhanced mixes were plated on nutritional agar and cultured at 30 °C for 2–3 days. The color and shape of the colonies were used to distinguish between the bacterial isolates. The discovered pure culture of the isolated colonies was stored in 0.9% biological saline. The morphology, nutritional qualities, and biochemical, physiological, and molecular characteristics of bacterial cultures were used to characterize them. Gram staining, non-spore formation, and organic compounds were the techniques used first to identify the *E. coli* isolates. Following confirmation of the Gram stain results, the bioMérieux VITEK® 2 Compact system was used to further identify the same strain.

Fermentation process for the EtOH yield

The EtOH obtained was executed by using batch and continuous process. In 500 mL pH-controlled fleakers with a 350 mL working volume, batch fermentation tests were carried out at 35 °C and pH 6.5. The setup used 4 M NaOH instead of KOH to adjust the pH. The fleaker caps were fitted with ports for a pH probe, CO_2 vent, sampling needle, and addition of base. No oxygen exclusion procedures were used, and mixing at 100 rpm was guaranteed using a magnetic stirrer was positioned beneath a water bath. For continuous culture (CC), a

500 mL fleaker fermenter with feed and output ports, automatic pH control, and a working volume of 240 mL was used operating in a partially anaerobic condition. To minimize the need for 4 M NaOH additions to maintain a pH of 6.5, the substrate was prepared with 5 g/L yeast extract and 10 g/L tryptone. The pH was then adjusted to 7.5 using 10 M NaOH. The volume of the inoculum was 10% (v/v), and the medium was filter-sterilized. Following 16 hours of batch fermentation, a peristaltic pump was used to continuously feed filter-sterilized substrate at a predetermined rate while concurrently removing and collecting the effluent under sterile conditions. Continuous operation lasted for 16–105 days, with duplicate experiments run in parallel to ensure reproducibility and comparison (Saha and Cotta, 2011). Table 1 (supplementary) summarizes the media combinations employed in the experimental conditions.

Results and Discussion

This is the simplest mode of operation wherein substrates, culture medium, inoculum, and nutrients are provided at the beginning of the process and left to run for a predetermined time. The batch mode of operation is regarded as efficient, as it enables complete sterilization. A multivessel system makes it easy to manage feedstock and does not require laborious skills. The most important characteristic is flexibility in choosing various product specifications. Yet, we cannot neglect the disadvantages, such as low productivity and the substrate inhibition phenomenon observed at higher concentrations of substrates. Also, it is labor-intensive and involves higher production costs.

In the *E. coli* FBWHR strain, ethanol (EtOH) production in batch culture has been reported (Yang and Shijie, 2015). The *E. coli* FBWHR strain was selected due to its well-characterized genome, high plasmid uptake efficiency, robust growth, stress resistance, and extensive use in synthetic biology and biofuel studies. This strain is adapted to hot-water extracts from sugar maple wood, which predominantly consist of xylose, along with small amounts of galactose, mannose, rhamnose, arabinose, and glucose. Biomass and ethanol production were monitored at sugar concentrations of 30% (v/v), 40% (v/v), 50% (v/v), and 60% (v/v). Table 2 (supplementary) shows the phases of batch culture ethanol production at various sugar concentrations.

With an increased glucose concentration (60 g/L), fermentation improved along with ethanol accumulation. Another notable strain developed for ethanol production is *E. coli* FBR5, which is engineered to metabolize both pentose and hexose sugars to ethanol and other metabolites at higher yields. In this study, the effect of xylose concentration and the inhibitory effects of salts and ethanol on both cell growth and ethanol production were observed. The microbe was initially acclimatized to 100 g/L xylose, then subjected to concentrations ranging from 50 to 250 g/L. To understand salt inhibition, NaCl was provided within a concentration range of 0 to 40 g/L.

The increase in sugar concentration showed a marginal increase in ethanol production. The lack of change at higher sugar levels likely stems from saturation, inhibition, or other limiting factors. Adjusting pH, temperature, and other culture conditions helped improve metabolic efficiency. Notably, *E. coli* FBWHR exhibited a selective sugar usage pattern: during the lag phase, it consumed glucose first, followed by galactose (rapidly), then arabinose, rhamnose, and mannose, with xylose being utilized last. At a sugar concentration of 50% (v/v), biomass production was maximum (2.469 g/L at 99 hours), although ethanol production decreased.

A cost-effective and abundant substrate for ethanol production is lignocellulosic material obtained from agriculture. The initial step involves depolymerizing monosaccharides, traditionally using enzymes or acids, which increases process time and requires mass production of those agents. A cheaper alternative is pyrolysis of lignocellulosic material to produce levoglucosan. Levoglucosan or its derivatives can be obtained at 22–33% yield from biomass via pyrolysis, though this yield is significantly affected by salt concentration.

Various studies have examined microbial utilization of levoglucosan for ethanol production. One such study developed *E. coli* strain KO11 + lgk kinase. Even with lgk kinase, the strain produced only 0.24 g ethanol per gram of levoglucosan, approximately 40% of the theoretical yield, and did not fully utilize levoglucosan. Further optimization processes were undertaken, including different pyrolysis and detoxification methods, along with identifying the presence of inhibitors.

In this experiment, a growth comparison was conducted between *Zymomonas mobilis* ATCC 11020 and ethanologenic *E. coli* ATCC 1117 strains. The *E. coli* ATCC 1117 strain demonstrated a preference for the pyrolysate sugar medium and was subjected to batch fermentation. Glucose concentrations were adjusted to 20 g/L, 40 g/L, and 60 g/L. Figure 1 (supplementary) shows the results for 20 g/L and 40 g/L glucose concentrations, while figure 2 (supplementary) illustrates observations at 40 g/L glucose in hydrolysate broth.

In this study, rapid growth was observed at a glucose concentration of 20 g/L, and the observations at 40 g/L were used to construct the kinetic model. Additionally, the model was validated using observations made at glucose concentrations of 20 g/L and 60 g/L. It is obvious from tables 3 and 4 (supplementary) that product inhibition was also studied by adding EtOH in the concentration range of 0 to 50 g/L. At first, a control experiment was conducted at pH 6.5 along with other nutrient supplies. At this pH, fermentation was sluggish, possibly due to pH, and acidic byproduct accumulation (lactic acid, succinic acid, acetic acid and formic acid). Further, the observation of fermentation runs monitored for EtOH against residual sugar. Similarly, substrate inhibition (xylose tolerance) was also studied and is shown in Fig. 5 (supplementary). Further, added EtOH and xylose utilization was shown in Fig. 6 (supplementary).

Optimization of fermentation parameters

Using statistical analysis tools, a relationship is drawn between the parameters used for optimization. The factors are time (X) vs. OD (Y) and the following results were obtained. Table 5 (supplementary) highlights the duration of fermentation and the optical density (OD) obtained for microbial growth cycle.

Continuous operation

The vessel containing viable cells is continuously fed with substrates, nutrients, and culture medium with simultaneous removal of products and utilized medium. The major advantage of this mode of operation is that it has higher productivity, a smaller working medium volume, and relatively lower investment costs. However, there is a high chance of product contamination. The work done by Badal C. Saha and Michael A. Cotta (2011) on *Escherichia coli* strain FBR5 to produce EtOH using Wheat straw hydrolysate highlights the factors and conditions to be considered while conducting continuous fermentation. The substrate is pretreated by alkaline peroxide and enzymatically saccharified to Wheat straw hydrolysate (WSH). The role of salts is also studied, so desalted WSH is also prepared. After growing the inoculum in batch condition for 16 hours the culture is subjected to continuous fermentation. In this study, sugar concentrations and dilution rates varied. Table 6 (supplementary) represents the data on the duration of fermentation, dilution rates, total sugar utilized, and their respective observations (EtOH produced-productivity, succinic acid, and biomass produced). It gives us a brief idea that glucose is completely exhausted by *E. coli* FBR5; making it utilize the sugars completely is a challenge and majorly it is xylose that remains unutilized. EtOH production is stable; it is average the same in both 1x and 0.5x sugar concentrations. It is succinic acid production that shows an increasing trend even though sugar concentration is halved.

To understand the role of the salts in EtOH production WSH is desalted and supplied as substrate.
This observation makes it clear that there is an improvement in EtOH production (almost a 9.6% increase), though there is no big change in residual sugars. But when the feedstock is 0.5x then succinic acid production is reduced with a 3-5% increase in EtOH production. Apart from these, 75% of xylose has been utilized.

Scatter plot:

A scatter plot is used to study the relationship between the two variables i.e. Time and Optical density. A graph is plotted for time on the x-axis and optical density on the y-axis. Figure 7 (supplementary) shows the optical density vs time plot graph.
Upon reviewing the trend, it is observed that both the variables (Time and Optical density) exhibit a strong relationship, and it was explored further.

Regression

Linear regression technique is applied to study the type of relationship between the two variables Time and optical density concerning the parameters. While applying the regression analysis, 24 hours was the time point chosen because the decrease in the trend was seen after 24 hours and it was not considered for the study.

Recipe 1:

As shown in figure. 8 (supplementary) the regression plot of Time vs Optical Density at $y = 0.1859$ and r-squared at 0.721. Further, it is noted that the two factors have a positive relation, and the r-square reveals that 72% of the data fits the regression model.

Recipe 2

The two factors in the model represented in figure 9 (supplementary) has a positive relation and the R-square reveals 80% of the data fits the regression model and illustrates the regression plot of Time vs Optical Density at $y = 0.204$ and r-squared at 0.806.

Recipe 3

In model 3 the two factors have a positive relation and r-square reveals 93% of data fits the regression

model. Figure 10 (supplementary) illustrate Time vs Optical Density where y = 0.262 and r-squared at 0.937.

Recipe 4

In this model, the two factors exhibit a positive relation and R-square reveals that 81% of the data fits the regression model. Figure 11 (supplementary) illustrates Time vs Optical Density where y = 0.275 and r-squared at 0.810.

Recipe 5

Even in this model, the two factors have a positive relation, and the R-square reveals 77% of the data fit the regression model. Figure 12 (supplementary) illustrates Time vs Optical Density where y = 0.296 and r-squared at 0.777.

Amongst figures 7–12 (supplementary), the regression analysis exhibited by figure 10, recipe 3 showed a maximum co-relation as it has a very strong linear relationship concerning time and OD. This will be used for repeatability studies.

Co-relations

From table 7 (supplementary), it is evident that all the selected parameters are significantly correlated, as also shown in table 8 (supplementary). This table indicates that total sugar utilized, ethanol yield consumed, ethanol yield available, and cell mass are all correlated.

To further confirm the potential impact, a hierarchical regression analysis was performed. Table 9 (supplementary) demonstrates that all the selected parameters show significant correlation. Additionally, tables 10 and 11 (supplementary) present the significant parameters along with predictions from steps 3 and 4 for the optimized parameters.

Based on the above tables, the productivity of bioethanol is calculated using the following equation:
$Y = y = -0.260 - 0.009x1 + 1.418x2 + 1.137x3 + 0.356x4$ (where x1 – Total sugar utilized, x2 – EtOH yield consumed, x3 – EtOH yield available, x4 – cell mass).

The results obtained from the hierarchical regression show a 17.5% variance in ethanol productivity, which can be predicted by the total sugar utilized. The hierarchical regression variance results are presented in table 12 (supplementary). Based on these results, table 13 (supplementary) further confirms that Experimental Design 3, with its significantly correlated parameters, is the most effective for optimizing bioethanol production.

RSM analysis

Response surface design

From this plot in supplementary figures, we can conclude that the R^2 value indicates a reasonably good fit, and the data regression model is applicable. There is clear goodness of fit, and the residual plots do not show any major violations. Based on the above analysis, for biomass concentration, glucose, magnesium sulphate, and temperature, the variation is less than 0.05, indicating that the chosen factors are significant. This observation is supported by the graphs shown in figure 13, consistent with the results presented in figure 11 (Tabassum et al., 2018).

Main effect plot

Figure 14 (supplementary) demonstrates that biomass concentration, glucose, magnesium sulfate, and temperature have significant correlations (Asad et al., 2021).

Contour Plot

Figure 15 (supplementary) shows that the highest optical density (OD) is reached when both biomass concentration and glucose levels are low, while magnesium sulfate and temperature are held steady at 255g and 34.25°C, respectively (Balakrishna et al., 2021).

Similarly, Figure 16 (supplementary) indicates that the maximum OD occurs at higher levels of magnesium sulfate and temperature, with biomass concentration and glucose fixed at 87.5g and 550g, respectively (Bhaskara Rao et al., 2013).

Surface plot

The data in figure 17 (supplementary) reveals that increasing biomass concentration from 50 to 100 to 150 causes a decline in optimal optical density. A comparable trend is observed with changes in nutrient concentration. These findings indicate that lower glucose and biomass concentrations lead to higher optical density when magnesium sulfate and temperature are fixed at 255g and 34.25°C, respectively (Ojha et al., 2020; Wee et al., 2011).

According to Figure 18 (supplementary), increasing magnesium sulfate concentration from 0 to 450 enhances the optimal optical density. Temperature variations between 32 and 34°C exhibit a similar effect. This suggests that higher magnesium sulfate levels combined with elevated temperatures result in increased optical density. The maximum OD was recorded at magnesium sulfate 450g and temperature 34°C, while biomass and glucose concentrations were maintained at 87.5g and 550g, respectively (Lahiri et al., 2021).

Optimization plot

Figure 19 (supplementary) shows that the optimal optical density of 2.0 can be achieved with the following conditions: biomass concentration at 25, glucose at 100, magnesium sulfate at 15, and temperature around 31.77 °C (Kizhakedathil et al., 2018; Naili et al., 2016).

The experimental models and contour plots demonstrate a strong correlation between these factors and the optical density response. Keeping biomass concentration, glucose, magnesium sulfate, and temperature at lower levels helps reach the desired optical density. On the other hand, higher levels of these factors lead to increased optical density, which is not desirable.

Conclusion

This study demonstrates the efficiency of an isolated wild strain of *Escherichia coli* (NCBI accession# PRJNA1138183, locus tag prefix: AB3Z12) under fermentation conditions optimized for ethanol (EtOH) production, providing insights into key factors influencing yield and productivity. Batch fermentation results using *E. coli* tested the effects of biomass, glucose concentration, pH, NaCl, xylose, and added EtOH, revealing significant parameters that influence EtOH production. Similar studies confirmed that sugar maple hydrolysate showed optimal EtOH production at 50% sugar concentration, with sequential utilization of glucose and xylose. This pattern highlights the substrate-specific metabolic preferences of *E. coli*, consistent with previous findings reporting preferential glucose utilization in mixed sugar substrates (Choi et al., 2016).

Levoglucosan conversion to EtOH by *E. coli* KO11 with lgk kinase, achieving 40% of the theoretical yield, underscores the challenges of processing inhibitory substrates. Comparative studies with *Zymomonas mobilis* suggest potential for strain improvement to enhance yields, corroborating similar observations in strain engineering (Abbate et al., 2023). Continuous fermentation using *E. coli* FBR5 with wheat straw hydrolysate showed stable EtOH production (~19.2 g/L), with improved yields upon desalting of the hydrolysate. These results align with studies emphasizing the importance of inhibitor removal to enhance fermentation performance (Parawira and Tekere, 2011). Salt tolerance tests indicated robust growth up to 40 g/L NaCl, with EtOH productivity peaking at controlled pH and high xylose concentrations, consistent with tolerance thresholds observed in industrial strains (Fuchino and Bruheim, 2020).

Regression analysis further confirmed significant predictors of EtOH productivity, including total sugar utilization, EtOH yield, and cell mass. The high R^2 values obtained (up to 93%) reflect a strong relationship between these factors and productivity, supporting findings from statistical optimization studies (Mihajlovski et al., 2021). Hierarchical regression showed that 17.5% of the variance in EtOH production was predicted by total sugar utilized, and 17.4% by cell mass. Additionally, response surface methodology reconfirmed the vital role of these predictive parameters in EtOH production. The study indicates that biomass, glucose concentration, magnesium sulfate, and temperature are significant factors for optimizing EtOH production.

Collectively, these results emphasize the importance of substrate optimization, strain improvement, and process control in advancing bioethanol production. Therefore, future work should include experimental knockout of specific genes to enhance ethanol tolerance and productivity in engineered strains.

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Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

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