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Suitability of crude glycerol as a substrate for biobutanol production

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Abstract. Glycerol is a natural polyol formed as a major by-product during biodiesel production. The use of glycerol, which is uniquely utilised by *Clostridium pasteurianum*, for butanol production is highly promising but requires a thorough understanding and optimisation of the process. This study aimed to determine the composition of crude glycerol and evaluate its suitability as a substrate for butanol accumulation by *Clostridium* sp. UCM B-7570. During the research, a chromatographic method was used to determine the composition of crude glycerol and the solvent content in the culture liquid. An experimental approach was employed according to a developed scheme, incorporating microbiological methods (microorganism cultivation), biotechnological methods (strain cultivation under conditions resembling industrial settings, investigation of the solvent accumulation), and statistical methods for the mathematical processing of research results. A detailed study of the composition of different crude glycerol fractions showed that the initial glycerol layer contained a relatively low proportion of glycerol itself. The identified components accounted for more than half of the mass of the glycerol layer (51.6%). It was shown that the glycerol layer was found to contain only up to 20% glycerol and approximately 17% methanol, which is an inhibitor of microbial growth and development. It was determined that the highest butanol accumulation (9 g/L) occurred at a crude glycerol concentration of 35 g/L, while culture development was inhibited at 45 g/L. During the initial phase of fedbatch cultivation of *Clostridium* sp. UCM B-7570, butanol accumulation remained unchanged. However, the subsequent fermentation of crude glycerol led to a twofold reduction in solvent by accumulation, ultimately resulting in complete inhibition of production by the eighth period, possibly due to the presence of methyl esters in the medium. To enhance butanol production technology, the use of sorbents such as activated carbon during fermentation is recommended. This study provides practical insights for biotechnologists and

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the demonstrated ability of *Clostridium* sp. UCM B-7570 to ferment crude glycerol for butanol production presents numerous research opportunities. These findings contribute to improving the feasibility of biobutanol production and advancing biomass-based industrial processes as viable alternatives to petroleum-derived products

Keywords: biofuel; waste; producer strains; fermentation; *Clostridium*

Introduction

In the modern context of increasing demand for alternative energy sources, special attention is paid to bioresources as a promising foundation for renewable fuel production. The development of biological, ecological, and biotechnological approaches to the extraction and rational use of bioresources is becoming crucial, given the need to preserve ecosystems and support sustainable natural resource use. The utilisation of industrial by-products, such as crude glycerol, for biofuel synthesis, exemplifies an innovative technology that combines biotechnology, ecology, and economic efficiency, addressing pollution reduction while generating additional resources.

Biobutanol, produced through the fermentation of glycerol, is an environmentally friendly biofuel capable of competing with traditional fuels. The use of *Clostridium* sp. cultures in biobutanol production not only enables efficient recycling of industrial by-products but also results in an energy-efficient product with minimal environmental impact. Evaluating the suitability of crude glycerol as a substrate for biobutanol synthesis is highly relevant, as it reveals the potential of this by-product from biodiesel production as a viable biofuel feedstock, as noted in the study by T. Attarbachi *et al.* (2023). Research in this area expands knowledge of bioresource processing and contributes to improving their utilisation efficiency.

In the process of biodiesel production from renewable biomass, glycerol is generated in significant amounts as a by-product of the transesterification of vegetable oils, as highlighted by Z. Pirzadi & F. Meshkani (2022). T. Attarbachi *et al.* (2024) observed that with the expansion of biodiesel production, the cost and availability of glycerol have noticeably changed due to variations in output across typical biodiesel and ethanol production processes. According to Y. Bansod *et al.* (2024), this crude glycerol is generally regarded as waste. A key challenge in using crude glycerol for industrial fermentation is the inhibitory effect caused by impurities, as noted by S. Mehariya *et al.* (2023). After transesterification, glycerol typically contains varying concentrations of methanol (a residue from the methylation process in biodiesel production), sulphate or chloride salts, residual free fatty acids, fatty acid methyl esters, and soap – by-products of the hydroxide catalyst used in transesterification, as described by M. Tomatis *et al.* (2024). One approach to mitigating this inhibitory effect, as demonstrated by Y. Wang *et al.* (2024), is the purification of crude glycerol, though this significantly increases substrate cost.

Butanol production from glycerol derived from biodiesel aims not only to utilise it as waste but also to enable large-scale chemical production for use in the chemical industry. J. Kazimierowicz *et al.* (2024) reviewed the biosynthesis of liquid fuels and other value-added products from waste glycerol, emphasising its potential as a feedstock for biobutanol and other biofuels. They highlighted technological advancements and identified future research needs to enhance glycerol conversion efficiency, contributing to a more sustainable circular economy. M.H. Moklis *et al.* (2022) investigated methods for upgrading crude glycerol into high-value products. Their study explored approaches to improve the economic feasibility of glycerol conversion, including fermentation and catalytic upgrading techniques.

They suggested that process optimisation could enhance biobutanol yields and other bio-based chemicals, positioning glycerol as a viable alternative to petrochemical products while lowering production costs and promoting sustainability in the chemical industry.

Studies such as P. Arbter *et al.* (2021) have demonstrated the potential of using crude glycerol as a substrate for fermentative butanol production by *Clostridium pasteurianum*. However, the extremely slow growth of the culture and the high toxicity of unrefined glycerol have limited its direct industrial application. Although experiments conducted by Y. Liu *et al.* (2022) showed promising results for butanol production from crude glycerol, impurities – including methanol, ash, free fatty acids, and triglycerides – likely interfered with solvent production. This was particularly evident with residual methanol and sodium or potassium salts, which are known to inhibit cell growth, as highlighted by S. Silva *et al.* (2023). M. Elsayed *et al.* (2024) emphasised that high yield and productivity are essential requirements for developing an industrial process for butanol production based on crude glycerol obtained from biodiesel.

The aim of this study was to evaluate the composition of crude glycerol from industrial biodiesel production to determine possible nutrient limitations and the influence of crude glycerol concentration on butanol accumulation by *Clostridium* sp. UCM B-7570.

Materials and Methods

The butanol-producing strain *Clostridium* sp. UCM B-7570, from the "Collection of Strains of Microorganisms and Plant Lines for Agricultural and Industrial Biotechnology" (Tigunova *et al.*, 2023) at the Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine (IFBG), was used for this study. Determination of residual methanol in crude glycerol (Pharma, Belgium) was performed by gas chromatography using an Agilent 7890A gas chromatograph (USA) with a flame ionisation detector, following the international standard ASTM D7716-11a (2020). Free glycerol content was determined according to the international standard SN/T 3344-2012 (2013), while the overall composition of crude glycerol was analysed using the international standard SN/T 2995-2011 (2011).

To determine butanol accumulation using glycerol, the following medium composition (g/L) was used**:** crude glycerol (10.0-20.0), yeast extract – 1.0, $(NH_4)_2SO_4$ – 0.6, $(NH_4)_2HPO_4$ – 1.6; pH 6.5. The medium was sterilised for 30 minutes under a pressure of 1 atm. Cultivation of the butanol-producing strain *Clostridium* sp. UCM B-7570 was carried out in flasks containing liquid medium or on Petri dishes in a Crystal anaerostat (Germany) at a temperature of 35°C in a nitrogen atmosphere. After 120 hours of fermentation, the cells were pelleted using a Labofuge 400R ultracentrifuge (Germany) for 30 minutes at 13,000 rpm. Fermentation was conducted in 100 mL flasks containing 60 mL of medium. The flasks were weighed and incubated at 35°C.

Ethanol, butanol, and acetone were determined in the culture liquid using a gas chromatograph with a flame ionisation detector and a 2.4 m×3 mm column packed with Chromsorb Carbovax 6000 (Merck, Germany). The column temperature was 80 ± 5°C, while the evaporator temperature was 140 ± 10°C. The ratio of nitrogen, hydrogen, and air flows was 1:1:10. To determine the concentration of butanol in the culture liquid, a calibration curve was constructed using known butanol concentrations in distilled water. All experiments were performed in triplicate. Statistical analysis of the experimental data was conducted using Microsoft Excel 12.0, applying Student's t-test. A difference between the two mean values was considered significant at $P < 0.05$.

Results

The use of crude glycerol plays a key role in the efficient use of bioresources by promoting the recycling and valorisation of a by-product from biodiesel production. Crude glycerol, which accounts for 10% of the total volume of biodiesel produced, has traditionally been considered an unwanted

byproduct. However, with the growing focus on sustainability and resource optimisation, its conversion into valuable biofuels and chemicals has emerged as a practical solution for improving the overall efficiency of bioresource utilisation.

By using crude glycerol as a substrate for microbial fermentation, it can be converted into highvalue products, such as butanol – a renewable alternative to petroleum-derived fuels and chemicals. This not only reduces dependence on finite non-renewable resources but also contributes to the development of a more sustainable chemical industry. The process of converting crude glycerol into biofuels and other value-added products helps address environmental concerns by reducing glycerol waste, which, if left untreated, can contribute to pollution and environmental degradation. Moreover, crude glycerol serves as an ideal feedstock for various biotechnological processes due to its high organic content, which can be metabolised by microorganisms to produce biofuels, bioplastics, and other bio-based chemicals. By integrating crude glycerol into industrial applications, biodiesel producers can generate additional income streams, making the overall biodiesel production process more economically viable. This also allows for a circular production approach, where waste by-products are reintegrated into the value chain, reducing waste and optimising resource use.

The use of crude glycerol as a renewable resource also aligns with the broader goal of developing a circular economy, where waste from one process is used as an input for another. In this context, the efficient utilisation of crude glycerol reduces the environmental burden of waste disposal, lowers the carbon footprint associated with fossil fuel consumption, and encourages the use of renewable bioresources. Furthermore, it supports innovation in green technologies that foster sustainable industrial practices, promoting the responsible use of natural resources and enhancing the overall sustainability of the bioenergy sector.

Overall, the transformation of crude glycerol into valuable products not only enhances the economic sustainability of biodiesel production but also supports a broader environmental strategy by reducing waste, lowering emissions, and promoting the use of renewable bioresources in various industrial applications. By maximising the potential of crude glycerol, a more sustainable future can be achieved, where the efficient use of bioresources serves as a cornerstone of both economic development and environmental stewardship.

The composition of different crude glycerol fractions was studied in detail using chromatographic analysis. The initial glycerol layer contained a relatively low amount of glycerol itself (Table 1). In total, the identified components comprised more than half of the mass of the glycerol layer (51.6%). The remaining 48.4% may include products of incomplete transformation of triglycerides – mono- and diglycerides, alkali metal salts of fatty acids (soap), alkaline transesterification catalysts that migrated to the glycerol layer, water, or non-lipid components of the original oil. If the original oil contained a significant amount of phosphatides, then the products of their incomplete transesterification with methanol-diacylated phosphatides (esters of phosphoric acid with glycerol and choline/ethanolamine/inositol, etc.) – could also be present in the glycerol composition. It was determined that the glycerol layer from methanol transesterification contained a significant amount of an alkaline catalyst; however, the specific type of catalyst used remains unknown.

Table 1. *Component content of the initial glycerol layer*

Component	Content, %
Glycerol	21.9
Methanol	17.1
Fatty acid methyl esters	12.6

Source: developed by the author

Sodium or potassium hydroxides, as well as sodium or potassium methylates, are most commonly used (Meng *et al.*, 2023). Titration with hydrochloric acid indicated that 100 g of the glycerol layer contained 0.072 mol of monovalent alkali metal. The alkali metal may be present in its original catalyst form, partially converted into soaps, or bound in alkali metal glycerate. This amount corresponds to: 2.81 g K / 4.04 g KOH / 5.19 g CH_3OK / 7.07 C_3H_7OK or 1.66 g Na / 2.88 g NaOH / 4.04 g CH_3ONa / 5.92 g C_3H_7ONa. If the alkaline catalyst is calculated as soap, its amount in the glycerol layer is equivalent to 23.1% potassium oleate or 21.9% sodium oleate. Additionally, during the distillation of methanol, several unidentified light impurities, likely hydrocarbons, were removed, producing several small peaks on the chromatograms of the obtained methanol-water mixture. In total, 1.2-1.5% of the glycerol layer mass consisted of unidentified impurities that passed into the distillate.

Distillation of methanol from the glycerol layer of methanol transesterification was complicated by the presence of an alkaline catalyst and all the components required for the progression of individual stages in the three-stage equilibrium process of triglyceride transesterification. In addition, the presence of alkali and water in the system facilitated the saponification of both glycerides and esters, while heating significantly accelerated the process. The chemical equilibrium of each stage in the transesterification process of triglycerides can be represented by the following reaction schemes:

Stage I:

$$TG + MeOH \xrightarrow{kat} DG + FAME$$

Stage II:

$$DG + MeOH \xrightarrow{kat} MG + FAME$$

Stage III:

$$MG + MeOH \xrightarrow{kat} {}^{W}G + FAME$$

In the given scheme, *TG*, *DG*, and *MG* are triglyceride, diglyceride, and monoglyceride, respectively; *G* is the glycerol, *MeOH* is the methanol, and *FAME* is the fatty acid methyl ester.

During storage of the glycerol layer, the reaction equilibrium stabilised at a certain level, corresponding to a constant concentration of individual components. The removal of methanol from the system shifted the equilibrium towards the formation of the starting substances – methanol and glycerides – due to the reverse transesterification of methyl esters with free glycerol. Consequently, these esters were converted into glycerides, leading to the additional formation of methanol. Since the transesterification reaction exhibits a weak exothermic effect at each stage, heating the mixture during methanol distillation further shifted the chemical equilibrium towards the formation of glycerides and methanol. Reverse transesterification of 100 g of methyl oleate required 31.1 g of glycerol, yielding 120.3 g of monoolein (monoglyceride) and 10.8 g of methanol. Additionally, saponification resulted in the formation of free glycerol, producing 25.8 g of glycerol and 90 g of potassium oleate from 100 g of monooleate. Methyl esters also underwent saponification, leading to the release of additional methanol – from 100 g of monooleate, 108.1 g of potassium oleate and 10.8 g of methanol were generated.

Initially, an attempt was made to extract methanol from the glycerol layer by distillation under atmospheric pressure without neutralising the catalyst. However, the complete extraction of methanol was hindered by intense foaming of the mixture, caused by the simultaneous presence of a significant amount of glycerides and soaps. The distillation process was halted when excessive foam formation made it impossible to continue. As a result, a fraction was collected with a distillation temperature range of 70-78°C, which exceeds the boiling point of pure methanol (65°C). During condensation, the methanol-rich distillate exhibited a tendency to separate into two distinct layers: a transparent upper layer and a cloudy white lower layer. After settling, the distillate became uniformly transparent and homogeneous. The overall material balance for distillation is presented in Table 2.

Table 2. *Material balance of methanol distillation from a glycerol layer without neutralisation*

Loaded, % wt.		Obtained, % wt.	
Initial glycerol layer	100	Distillation (70-78˚C)	20.24
		Cubic residue	79.76

Source: developed by the author

It was not possible to provide a fully detailed material balance for individual components due to the absence of comprehensive quantitative data on the mixture's composition, the potential for complex transformations during distillation, and the semi-quantitative nature of glycerol content determination. The composition of the identified components in the original glycerol layers was provided earlier. The distillate contained 63.4% methanol, 5.9% unidentified organic compounds – which appeared on chromatograms as numerous peaks of unidentified components, possibly of hydrocarbon origin – and 30% water. The cubic residue contained 15.9% glycerol, 3.6% methanol, 4% fatty acid methyl esters, and the remainder consisted of glycerides and soaps.

During distillation, the concentration of methanol in the cubic residue decreased significantly, but complete extraction was not achieved. The concentration of free glycerol also declined, with its absolute amount decreasing even more noticeably, while the concentration of fatty acid esters dropped even sharply. This suggests the occurrence of reverse transesterification of esters with glycerol. Saponification of esters and glycerides likely also took place, which should have resulted in the additional formation of methanol and glycerol. The total mass of methanol in both the cubic residue and the distillate was found to be slightly lower than in the original glycerol layer. This discrepancy suggests that the concentration of methanol in the distillate may be somewhat underestimated. To prevent foaming during the distillation of transesterification products containing significant amounts of glycerides and soaps, these compounds should be decomposed using a strong acid. Therefore, prior to the next distillation, the initial glycerol layer was neutralised with phosphoric acid (as an aqueous solution with (H_3PO_4) = 85% by mass). The acid was added in the amount required to bind the alkali metal present in the glycerol layer, either through neutralisation of alkali/alcohol or breakdown of soaps, forming dihydrogen phosphate. Potassium and sodium dihydrogen phosphates are insoluble in transesterification products. After the addition of acid, the consistency of the glycerol layer became heterogeneous, with the formation of a solid phase. No foaming occurred during distillation, and methanol was extracted quantitatively. The boiling range of the volatile fraction was 72.5-100.3°C.

The cubic residue, after settling, separated into two distinct layers: a lower "salt" layer, where most of the phosphates accumulated, and an upper "salt-free" layer (though a small quantity of salts remained in the upper layer). The overall material balance for distillation with neutralisation is provided in Table 3.

Table 3. *Material balance of methanol distillation from a glycerol layer with neutralisation*

Loaded, % wt.		Obtained, % wt.	
Initial glycerol layer	92.12	Distillation (70-78˚C)	28.97
Phosphate acid (85% by weight	7.88	Cubic residue Upper layer Lower layer	70.76 41.81 28.25
		Losses	0.97

Source: developed by the author

The distillate contained 52.8% methanol, 4.6% unidentified organic compounds (which appeared on chromatograms as numerous peaks of unidentified components, possibly of hydrocarbon origin), and 40% water. The upper "salt-free" layer of the cubic residue contained 28.7% fatty acid methyl esters, trace amounts of glycerol (no more than 0.2%), and excess methanol (only in trace amounts). Chromatograms also displayed additional peaks of unidentified components, the concentration of which did not exceed 2-3%. The remaining composition included glycerides, phosphoric acid esters, and a smaller fraction of potassium/sodium acid phosphates. The lower "salt" layer of the cubic residue contained 44.7% glycerol, 1.9% fatty acid methyl esters, and no detectable methanol (only trace amounts, likely in dispersed particles). The remaining composition consisted of glycerides, phosphate esters, and the bulk of potassium/sodium acid salts.

During distillation, methanol was completely removed. The total mass of methanol in both the cubic residue and the distillate was approximately equal to that in the original glycerol layer, although it was slightly lower. This discrepancy suggests that the concentration of methanol in the distillate may be somewhat underestimated. The mass of glycerol after distillation decreased significantly, likely due to reverse transesterification via an acid-catalysed mechanism involving acid phosphates. All free glycerol migrated to the lower "salt" layer, while the methyl esters remained in the upper "salt-free" layer. It can be inferred that the majority of bound glycerol (in the form of mono- or possibly triglycerides) was concentrated in the upper "salt-free" layer. The study demonstrated that the glycerol layer contained a relatively low amount of glycerol up to 20%, and approximately 17% methanol. Distillation without neutralising the glycerol layer with phosphoric acid enabled the complete extraction of methanol. However, this process also led to the formation of acid phosphates of potassium or sodium. To investigate butanol accumulation by *Clostridium* sp. UCM B-7570, experiments were conducted using crude glycerol at various concentrations (Table 4).

Table 4. *Accumulation of solvents at different crude glycerol concentrations*

Concentration crude-glycerol, g/L	Butanol, g/L	Ethanol, g/L
10	6.23 ± 0.39	0.18 ± 0.01
15	6.72 ± 0.07	0.34 ± 0.01
20	7.92 ± 0.22	0.36 ± 0.01
25	8.28 ± 0.39	0.59 ± 0.05
30	8.87 ± 0.4	0.68 ± 0.05
35	9.0 ± 0.08	1.2 ± 0.04
40	8.5 ± 0.03	0.7 ± 0.05
45	7.9 ± 0.22	0.3 ± 0.05

Source: developed by the author

Using crude glycerol at concentrations ranging from 10 to 45 g/L, as shown in the results presented in Table 4, bioconversion to butanol occurred. The highest butanol accumulation (9 g/L) was observed at a crude glycerol concentration of 35 g/L, while culture inhibition occurred at 45 g/L. To increase butanol accumulation at lower crude glycerol concentrations, *Clostridium* sp. UCM B7570 was cultivated using a fed-batch system. The results are presented in Table 5.

Table 5. *Accumulation of solvents using fed-batch culture*

Fed-batch period	Butanol, g/L	Ethanol, g/L
0	9 ± 0.01	0.36 ± 0.01
1	8.8 ± 0.05	0.22 ± 0.05
2	4.5 ± 0.39	0.23 ± 0.03
3	4.3 ± 0.05	0.05 ± 0.01
4	4.2 ± 0.04	0.02 ± 0.01
5	1.2 ± 0.04	0.01 ± 0.01
6	0.5 ± 0.05	-

Source: developed by the author

During cultivation, butanol accumulation in the culture liquid remained unchanged during the initial period of extraction and medium replenishment. However, from the second to the fourth period, butanol accumulation decreased twofold. With continued use of the negative replenishment method, butanol accumulation decreased eightfold and eventually ceased by the sixth period.

Discussion

Glycerol purification has been identified as an expensive and energy-intensive process, as concluded by T. Arofai *et al.* (2024). In their study, they investigated methods for separating residual salts from glycerol pitch, a by-product of refined glycerol production. This process was found to be crucial for reducing both the cost and energy consumption associated with glycerol purification, which has been a major obstacle to its widespread industrial application.

Similarly, M. Oliveira *et al.* (2022) highlighted potential advancements in crude glycerol purification through computational modelling. Their research focused on optimising purification steps to make glycerol purification more economically viable. By enhancing process efficiency, their study indicated that the cost of producing high-quality glycerol could be significantly reduced, addressing the challenges posed by traditional, energy-intensive methods.

N. Armylisas *et al.* (2023) demonstrated that crude glycerol, a by-product of biodiesel production, could be effectively utilised in microbial synthesis. Their research showed that crude glycerol could serve as a substrate for biobutanol production, thereby reducing production costs and increasing the profitability of biofuel manufacturing. This approach emphasised the potential of repurposing glycerol waste into a valuable resource for industrial applications, thus enhancing economic sustainability.

Furthermore, M. Boro *et al.* (2022) and M. Zhao *et al.* (2023) explored the broader use of crude glycerol in biotechnological processes, focusing on its role as a cost-effective substrate for biofuel synthesis. Their studies revealed that crude glycerol not only reduced the cost of microbial synthesis but also contributed to the economic sustainability of biofuel production. By repurposing glycerol waste from biodiesel production, these studies demonstrated how glycerol could support the development of more environmentally friendly energy sources.

Crude glycerol poses a significant financial and environmental challenge in terms of global disposal. E. Almeida *et al.* (2023) emphasised that for the sustainable use of this resource, biotechnology must be prioritised. A. Al-Haimi *et al.* (2024) noted that glycerol, or propyne-1,2,3-triol, is a by-product in the production of fatty acid methyl esters (biodiesel). It is also traditionally formed as a by-product of oleochemical and soap production. Depending on the intended application, glycerol obtained during the transesterification of oils is purified to varying degrees: crude (30-80% pure), technical-grade distilled

(50-86%), and glycerol for use as a chemical reagent or in cosmetics and pharmaceuticals (99.0-99.9%). After transesterification and methanol removal, the crude glycerol contains various impurities, is heavily coloured, and has low commercial value as a commodity. In small-scale production, it is often discarded, contributing to environmental pollution. Given these challenges, industrial biotechnology plays a crucial role in identifying microbial strains capable of metabolising waste, including crude glycerol, as highlighted by Y. Wang *et al.* (2024). Modern biotechnology presents significant opportunities for utilising glycerol, traditionally regarded as a by-product of numerous production processes. The authors M.U. Faruk *et al.* (2023) determined that to facilitate bioconversion, it is first necessary to analyse the composition of residues from biodiesel production, as they may contain substances that inhibit microbial growth and development. The use of waste substrates introduces stress factors due to the accumulation of toxic agents in the fermentation medium, in addition to the issue of variable substrate quality even within the production process. One approach to mitigating this inhibitory effect is the purification of crude glycerol, which negatively impacts the cost of the substrate, as described in the research by V. Boas & F. Mendes (2022). A more promising strategy involves the mutation or adaptation of wild-type strains to crude glycerol, as reported for *Clostridium pasteurianum*, by I. Kushkevych (2023). R.J. Humphreys *et al.* (2023) noted that instability and culture degeneration, leading to the loss of solvent production capability, has been a significant challenge in the study of solvent production through glucose fermentation. On the other hand, D. Karayannis *et al.* (2024) investigated the adaptation of strains to a specific type of crude glycerol, noting that even well-adapted strains may still experience toxicity from different substrate batches. Similar site-directed mutagenesis techniques could be applied to generate mutants with improved stability or tolerance to impurities such as methanol. However, until a stable culture is developed, it will be difficult to determine the extent to which parameters such as pH, substrate concentration, and impurities influence solvent production.

This study aimed to determine the maximum concentrations of pure and crude glycerol that support the growth and efficient metabolism of *Clostridium sp.* UCM B-7570. Since glycerol is an osmotically active substance that significantly affects the osmotic potential of the fermentation medium, it can restrict the production capacity of microorganisms, as demonstrated by D.T. Jones (2024). *Clostridium sp*. UCM B-7570 has been shown to grow on crude glycerol and produce butanol. This is the first report on cell growth and solvent production by the domestically isolated strain *Clostridium sp*. UCM B-7570 using crude glycerol as the sole carbon source, in comparison with global studies by other researchers. These findings align with the study of P. Ponsetto *et al.* (2024) and indicate that butanol production via glycerol fermentation is a promising alternative for biobutanol production that warrants further investigation. Several articles (Koh *et al.*, 2024; Sim *et al.*, 2024) have reported the growth and product formation of other *Clostridium* species using crude glycerol as the sole substrate. The results of this study demonstrate that *Clostridium sp.* UCM B-7570 can grow on crude glycerol derived from biodiesel at concentrations up to 35 g/L, with the highest butanol production reaching 9 g/L. Although these experiments yielded promising results for butanol production from crude glycerol, impurities such as methanol and salts may interfere with solvent production, as emphasised by M.U. Faruk *et al.* (2023). Y. Gan *et al.* (2023) noted that while several *Clostridium* species can metabolise methanol (producing acetate), *C. pasteurianum* has not been identified as one of the species capable of converting this compound. Although the potential for producing butanol from crude glycerol derived from biodiesel is promising, further research is required

to fully understand and optimise the process. As D.T. Jones (2024) previously highlighted, culture stability is crucial and may be improved by developing mutant strains. Additionally, it is important to identify and quantify key parameters of the nutrient medium, such as pH, nutrient concentration and trace metal levels, which significantly influence solvent formation and product distribution. Additional research efforts should evaluate growth and solvent production using purified glycerol with added methanol to determine whether the presence of methanol (or other impurities) affects cell growth, solvent production, and long-term culture stability, as recommended by Y. Gan *et al*. (2023). At the very least, further studies should be conducted to confirm whether these initial butanol yield figures can be maintained over extended periods and with higher concentrations of crude glycerol (and, consequently, higher impurity levels). Considering that the use of crude glycerol would eliminate the need for pretreatment, the associated cost savings may compensate for the slightly lower yield. Additionally, *C. pasteurianum* does not produce acetone, which should simplify the separation and purification of butanol. Unlike *C. acetobutylicum* and *C. beijerinckii*, *C. pasteurianum* also produces significant amounts of ethanol, as demonstrated by D.T. Jones (2024). Although ethanol is far from an ideal fuel, it can be used alongside butanol for blending with petroleum fuels. Finally, A. Tyszak & L. Rehmann (2024) demonstrated that carbon recovery during glycerol fermentation by *C. pasteurianum* ranges from 75% to 90%, compared with less than 60% during sugar fermentation. As a result, less carbon is lost through CO_2 production. Industrial-scale utilisation of crude glycerol could open new prospects in the biofuel industry and reduce production waste, thereby having a positive impact on the environment and promoting the rational use of bioresources. Integrating microbiological butanol production into biodiesel production could lead to lower raw material costs. D.T. Jones (2024) noted that in sugar-based fermentation processes, raw material costs typically account for 60%-80% of total production costs and are one of the most significant factors influencing the cost of butanol production.

The use of crude glycerol enhances the rational use of bioresources by transforming a byproduct of biodiesel production into valuable bio-based products. Biodiesel production generates large quantities of crude glycerol as a waste product, which often remains underutilised. However, by repurposing this by-product for the production of high-value chemicals, biofuels, and other value-added products, both the environmental impact and overall inefficiencies in biodiesel production can be mitigated.

Conclusions

These results demonstrate that crude glycerol derived from biodiesel is a promising, low-cost, renewable feedstock for butanol production. Since crude glycerol contains impurities, including salts and methanol, the methanol concentration in these samples varied significantly. The average methanol concentration in the fermentation broth samples was 1.78 ± 2.98 g/L. No direct correlation was observed between methanol concentration and glycerol concentration (i.e., samples with the highest crude glycerol concentrations did not necessarily contain the highest methanol levels). The fermentation of glycerol by the related organism *Clostridium pasteurianum* can facilitate the reoxidation of excess NADH produced during glycerol catabolism. The results indicate that *Clostridium* sp. is capable of growing and maintaining viability on crude glycerol obtained from biodiesel at a rate comparable to that observed during growth on purified glycerol. Furthermore, *Clostridium* sp. can utilise crude glycerol for solvent production, primarily yielding significant amounts of butanol. Different concentrations of crude glycerol (an unrefined by-product of biodiesel production) were used to investigate butanol accumulation. It was shown that at low crude glycerol concentrations, conversion to butanol

is feasible. However, as crude glycerol concentrations increased, culture growth and development were inhibited, possibly due to the presence of methyl esters in the medium. Given that the use of crude glycerol eliminates the need for pretreatment, the associated cost savings could offset the slightly lower yield. This study clearly demonstrated that crude glycerol obtained from biodiesel production can be fermented at high concentrations and with rates relevant to industrial applications. The findings offer significant potential for biofuel production technology using crude glycerol, which supports the rational use of bioresources, reduces production waste, and contributes to environmental improvement.

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Conflict of Interest

None.

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Придатність гліцерину-сирцю, як субстрату для біобутанолу

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Анотація. Гліцерин – природний поліол, який утворюється як основний побічний продукт під час виробництва біодизеля. Використання гліцерину, яке притаманне лише для *Clostridium pasteurianum*, для отримання бутанолу є багатообіцяючим та має високий потенціал, однак потребує розуміння та оптимізації процесу. Метою дослідження було визначити склад гліцерину-сирцю та оцінити його придатність як субстрату для накопичення бутанолу культурою *Clostridium* sp. UCM B-7570. Під час проведення досліджень було застосовано низку методів: хроматографічний – для аналізу складу сирого гліцерину та вмісту розчинників у культуральній рідині; мікробіологічний – для культивування мікроорганізмів; біотехнологічні методи – для вирощування штаму в умовах, наближених до промислових, та дослідження накопичення розчинників; статистичний – для математичної обробки результатів. Детальне вивчення складу різних фракцій гліцерину-сирцю показало, що вихідний гліцерновий шар містив досить мало власне гліцерину. Продемонстровано, що ідентифіковані компоненти складали у сумі більше половини маси гліцеровинового шару (51,6 %). Показано, що гліцериновий шар містив досить незначну кількість власне гліцирину до 20 % та близько 17 % метанолу, який є інгібітором росту та розвитку мікроорганізмів. Визначено, що найбільше накопичення бутанолу (9 г/л) було при концентрації сирого гліцерину в середовищі 35 г/л, а пригнічення розвитку культури – при концентрації 45 г/л. Здійснено культивування *Clostridium* sp. UCM B-7570 від'ємно-доливним методом та визначено, що накопичення бутанолу протягом першого періоду не змінювалось. Послідуюча ферментація гліцерину-сирцу знижувала акомулювання розчинника у два рази до повного інгібування продукцію у восьмому періоді, що можливо пов'язано з вмістом метилових ефірів у середовищі. Для удосконалення технології накопичення бутанолу потрібно використовувати сорбенти під час ферментацію, наприклад активоване вугілля. Матеріали статті становлять практичну цінність для фахівців-біотехнологів, а показана властивість *Clostridium* sp. UCM B-7570 до вироблення бутанолу шляхом бродіння сирого гліцерину відкриває безліч потенційних дослідницьких можливостей, які можуть підвищити життєздатність виробництва біобутанолу та сприяти розвитку промислових процесів на основі біомаси, як альтернативи нафтопродуктам

Ключові слова: біопаливо; відходи; штами-продуценти; ферментація; *Clostridium*