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Innovative Approaches to Bioethanol Production: Utilizing Olive Oil Wastewater, Milk Whey, and Sugarcane Molasses through Enzymatic Hydrolysis and Yeast Immobilization

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ABSTRACT

This work describes a new method for fermentative ethanol production using a triple waste substrate mixture of olive oil wastewater (OOWW), milk whey (MW), and sugarcane molasses (SCM). Enzymatic hydrolysis was performed using a commercial enzyme complex, Natuzyme, at concentrations of 0.25%, 0.5%, and 0.75%. Fermentation was performed at 30 °C, pH 5.5, and 150 rpm using immobilized cells of *Saccharomyces cerevisiae* (Sc) previously isolated from OOWW. The ethanol yields produced by immobilized *S. cerevisiae* ranged from 16.56 g/L to a maximum of 34.56 g/L at the 0.5% enzyme concentration, demonstrating an optimal balance between hydrolytic efficiency and yeast activity. Four different fermentation formulations were prepared by varying the proportions of the waste components, resulting in different substrate compositions and fermentation outcomes. These results demonstrate the potential of valorizing heterogeneous waste streams for the sustainable production of ethanol. This study advances environmentally responsible waste management and opens a promising avenue for large-scale ethanol production using yeast immobilization techniques.

Key words: renewable biofuels, agro-industrial by-products, enzymatic bioconversion, immobilized fermentation, multi-substrate fermentation, sustainable energy

INTRODUCTION

In Algeria, various agro-food industries generate their primary products and millions of tons of by-products and residues annually. These by-products represent a significant source of energy and nutrients. For instance, milk whey (MW) from cheese production, olive oil wastewater (OOWW) from olive oil processing, and sugarcane molasses (SCM)—a residual syrup from sugar refining—are all rich in fermentable sugars and organic compounds. Although molasses is widely used in some industrial applications, a considerable portion, especially from small or semi-industrial sugar facilities, remains underutilized or discarded in regions lacking ethanol recovery systems. Consequently, SCM can be regarded as a by-product with significant valorization potential.
Moreover, national estimates indicate that Algeria produces approximately 1 to 1.5 million cubic meters of OOWW (from about 100,000–150,000 tons of olives), around 96,000 to 160,000 tons of SCM, and nearly 100,000 tons of MW each year (Bouizar et al., 2021; Djeziri et al., 2023; Tebbouche et al., 2024). These large volumes, if not properly managed, contribute to environmental pollution and represent a valuable bioethanol production resource and other bioproducts (Abu Tayeh et al., 2014; Álvarez-Cao et al., 2020; Pasotti et al., 2017; Rouam & Meziane, 2025). Their efficient utilization in fermentation processes has gained increasing interest, particularly when integrated into multi-waste co-fermentation systems.
Enzymatic hydrolysis of agro-industrial waste has attracted growing attention due to its efficiency in breaking down complex carbohydrates into fermentable sugars (Vasić et al., 2021). Although enzymatic treatment is well established for single substrates, its application in multi-waste systems remains underexplored (Cheng et al., 2020). Similarly, yeast immobilization—a technique that enhances fermentation performance by improving cell stability, ethanol tolerance, and reusability—has rarely been studied in the context of co-fermentation (de Araujo et al., 2024).

This study investigated the synergistic effects of co-processing three types of agro-industrial waste—OOWW, MW, and SCM—for bioethanol production. We focus on two main strategies: optimizing enzymatic hydrolysis using Natuzyme (a commercial multi-enzyme complex), and applying yeast immobilization using *Saccharomyces cerevisiae* cells embedded in pozzolan, a porous volcanic rock. The use of immobilized yeast aims to improve fermentation efficiency and process stability.

The main objectives of this research are to optimize enzymatic hydrolysis to increase sugar availability, assess the impact of yeast immobilization on ethanol yield in a heterogeneous waste system, and compare different substrate formulations by varying the ratios of OOWW, MW, and SCM to identify the most efficient combination.

Despite extensive research on bioethanol production from individual agro-industrial by-products, few studies have explored the combination of multiple waste streams in a single co-fermentation process. Most existing studies also rely on free-cell systems, which suffer from reduced stability, contamination risk, and lower reusability. Furthermore, the application of enzymatic hydrolysis in multi-waste systems remains largely unexplored, particularly when coupled with yeast immobilization. This study addresses these gaps by proposing an integrated approach that combines enzymatic pretreatment and immobilized yeast fermentation using a mixture of OOWW, MW, and SCM. By doing so, the study will enhance ethanol yield, improve process robustness, and promote the circular use of agro-industrial waste—a critical step toward sustainable and scalable biofuel technologies.

MATERIALS AND METHODS

Materials

Samples of agro-food by-products were collected from local agro-industries. Each sample was coded and stored at 4 °C in a dark environment at the Laboratory of Natural Bio-Resources, University of Hassiba Benbouali, Chlef, Algeria, until further analysis. The substrates used in this study were:

- Olive oil wastewater (OOWW): Sourced from the El Nakhla olive mill, located in northwestern Algeria (36°26′03″ N, 1°41′32″ E). Samples were collected during the olive harvesting period (October–December) to ensure maximum sugar content.
- Milk whey (MW): Obtained from El Saada dairy production unit, a yogurt and cheese factory in northern Algeria (35°68′63″ N, 0°34′50″ W).
- Sugarcane molasses (SCM): Collected from Berrahal sugar refinery, located in western Algeria (35°91′53″ N, 0°07′78″ E).
- Pozzolan rocks: Used as an immobilization support, collected from the ENG Pozzolan quarry in western Algeria (35°28′58″ N, −1°40′95″ S).
- Natuzyme was purchased from Safana, an animal nutrition company in eastern Algeria.

Methods

Samples Preparation

To standardize the substrate composition and offer optimal fermentation conditions, OOWW and SCM were diluted 1:10 with distilled water to reduce the inhibitory compounds present in OOWW. MW was diluted 1:5, due to its high water content, to avoid excessive dilution of fermentable sugars.

Pozzolan rocks were crushed to smaller aggregates varying from 4 to 6mm in diameter. All the samples were sterilized by autoclave at 121 °C for 15 min to eliminate contaminants before the enzymatic hydrolysis and fermentation.

Yeast Strain and Preparation of Inoculum

The yeast strain used in this study was *Saccharomyces cerevisiae Y17*, that we previously isolated from OOWW. To prepare the inoculum, the yeast was cultured on Sabouraud agar medium (40 g/L dextrose, 10 g/L peptone, 20 g/L agar) and incubated at 30 °C for 48 h. A pre-culture was prepared by inoculating selected yeast colonies in 100 mL of sterilized substrate mixture and incubated at 150 rpm for 24 h to reach the exponential growth phase.

Static Fermentation Tests

Preliminary tests were conducted to assess the feasibility of ethanol production, and optimize the experimental conditions, troubleshoot potential issues in the experimental setup. Primary fermentation tests were conducted over a 48-h' period using the Sc Y17 strain. The production of CO_2, a by-product of ethanoic fermentation, was measured to estimate the volume of ethanol produced. This was based on the stoichiometry of the fermentation equation, where one mole of glucose produces two moles of ethanol and two moles of CO_2, as described by (Kumara Behera & Varma, 2017). The volume was measured based on the displacement of the syringe piston attached to a sealed test tube. Each test was run three times to ensure the results were reliable.

Enzymatic Hydrolysis

To improve sugar availability, enzymatic hydrolysis was performed using Natuzyme from Bioproton, a commercial enzyme complex known for its broad-spectrum activity on polysaccharides with the following labeled composition: phytase, α-amylase, xylanase, β-mannanase, β–glucanase, cellulase, protease, lipase and pectinase.
Three enzyme concentrations were tested: 0.25%, 0.5%, and 0.75% (w/v), based on preliminary trials.
Enzymatic hydrolysis was conducted under a temperature of 30 °C; pH was adjusted to 5.0 (using 0.1 M HCl or NaOH) for an incubation time of 48 h with continuous stirring at 150 rpm.
The 3,5-Dinitrosalicylic Acid (DNS) method was used to measure the concentration of glucose both before and after hydrolysis (Jain et al., 2020).

Simultaneous Saccharification and Fermentation (SSF) with Immobilized Cells

Fermentation experiments were performed using batch culture in 1 L glass flasks, each containing 700 mL of substrate mixture incubated at 30 °C with continuous shaking at 150 rpm for a period of 72h of fermentation.
To maintain sterility and anaerobic conditions, flasks were equipped with one-way gas release valves and 22-micron filters to prevent contamination. Sampling was assured in a sterile zone using the sampling orifice.
Four different fermentation formulations (Table 1) were tested, adjusting the ratios of OOWW, MW, and SCM. The overall experimental procedure is summarized in Figure 1.

Table 1. Fermentation media (Mixtures) compositions.

Mixtures	*OOWW*	*MW*	*SCM*
Mix 1	33%	33%	33%
Mix 2	25%	25%	50%
Mix 3	50%	25%	25%
Mix 4	25%	50%	25%

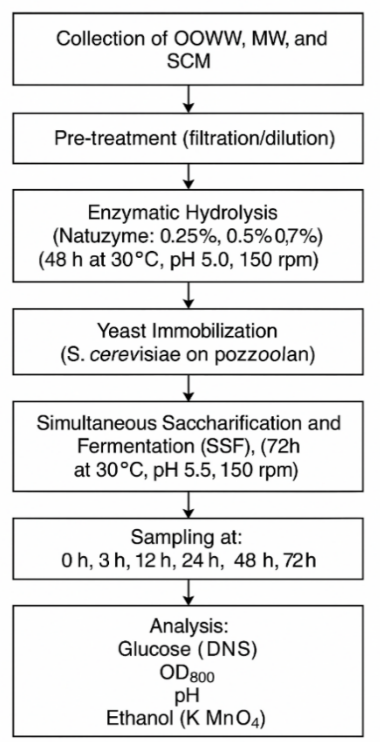


Fig. 1. Schematic representation of the experimental procedure. Three agro-industrial by-products (OOWW: olive oil wastewater, MW: milk whey, and SCM: sugarcane molasses) were pretreated and hydrolyzed enzymatically. Fermentation was carried out using immobilized *S. cerevisiae* on pozzolan. Samples were collected at regular intervals for glucose, ethanol, OD_{600}, pH, and CO_2 analysis.

Cell Immobilization

In our previous study (Ayadi et al., 2022), we developed a method for cell immobilization using pozzolan, a porous volcanic rock capable of enhancing cell attachment and retention. The pozzolan was washed and dried then autoclaved at 121 °C for 15 min.
Sterile pozzolan was placed in YPD medium (pre-cultured *S. cerevisiae* Y17) and incubated at 30 °C for 24 h to allow biofilm formation. Successful immobilization was confirmed by microscopic observation as shown in Figure 2 and viable cell counting.

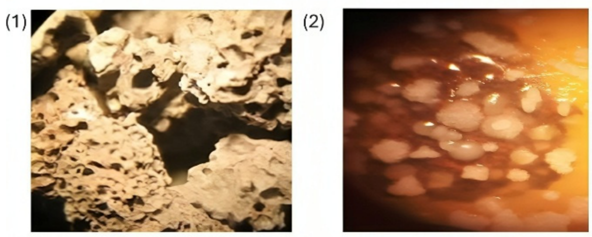


Fig. 2. Pozzolane rocks under binocular observation ×40: (**1**) before yeast immobilization, showing a porous structure, and (**2**) after immobilization, highlighting yeast clusters formation on the surface.

Analytical Methods

To monitor fermentation progress, the following key parameters were measured, the pH was measured using BANTE-210 benchtop pH meter, the optical density (OD_{600}) was measured using the Shimadzu UV-1800 coupled to a computer, (Jain et al., 2020) described the method for glucose determination using the 3,5-Dinitrosalicylic Acid (DNS) method, we used 3.5 DNS 97+ from Alfa Aesar Germany. Ethanol was separated from the fermentation broth using a rotary evaporator (Rotavapor Büchi R-100) and then its concentration was determined via Potassium permanganate titration described by (Zhang et al., 2019).

Statistical Analysis

A comprehensive statistical analysis was done using GraphPad Prism 10. To study the correlation between enzyme dosage, glucose release, and the production of biogas. This analysis was designed to study both the direct effect of enzyme dose on these parameters and the correlation between glucose concentration and biogas yield.

Linear Regression

A simple linear regression model was applied to determine the effect of enzyme dose on glucose release and biogas production for each substrate (MW, OOWW, SCM) at two-time intervals (T1: 24 h and T2: 48 h). The enzyme dose was treated as the independent variable, while glucose concentration and biogas production were treated as dependent variables in separate models.

Equation (1) describes the linear regression model that was used.

$$Y = \beta_0 + \beta_1 X + \epsilon \qquad (1)$$

The dependent variable is Y (glucose or biogas), X is the enzyme dose, β_0 is the intercept, β_1 the slope, and ε the error term. Significance was determined by R^2 and p-values ($p < 0.05$).

Also, the relationship between glucose concentration and biogas yield was investigated using a Pearson correlation analysis. Normality, homoscedasticity, and linearity assumptions were tested to ensure data validity.

This analysis pointed out how enzyme dose affects glucose availability and its production of biogas, besides interrelating both variables.

RESULTS AND DISCUSSION

Physicochemical Parameters of Co-Products

The physicochemical properties of OOWW, MW, and SCM were analyzed to assess their suitability as fermentation substrates (Table 2). The composition of these by-products influences yeast growth, enzymatic hydrolysis efficiency, and ethanol production.

Table 2. Physicochemical parameters of OOWW, MW and SCM.

Parameter	*OOWW*	*SCM*	*MW*	*Methods*
Reducing Sugars (%)	3.42	37.02	4.1	3.5 DNS Method (Jain et al., 2020)
Protein (%)	1.1	0.4	1.03	Lowry's Method (Waterborg & Matthews, 1984)
Fat (%)	3.19	0.0	0.21	(Clément, 1956)
DBO5 O_2/l ($g \cdot L^{-1}$)	11	52.4	7.3	ISO 5815-1:2019
DCO O_2/l ($g \cdot L^{-1}$)	123	102.2	14	ISO 15705:2002
pH	4.73	4.99	4.89	pH meter (BANTE-210)

OOWW

The OOWW composition observed in this study were consistent with those from previous investigations, but there were some differences. For instance, the fat content (3.19%) was slightly higher than the range reported by Esmail et al., 2013 (1–2.5%) and Djeziri et al., 2023 (1.25%), while also falling within what (Bouknana et al., 2014) reported (0.8–27.4 g/L). This can be explained by different factors such as processing of olives, seasonal changes, and geographic specificity of olive cultivars.

Secondly, the reducing sugar content was 3.42 g/L, within the range of 3.52–10.48 g/L obtained by (Bouknana et al., 2014), indicating medium availability of fermentable sugars.

The COD of OOWW was 123 g/L, higher than that obtained by (Esmail et al., 2013) and (Djeziri et al., 2023) at 104 g/L and 90.5 g/L, respectively. It was similar to (Bouknana et al., 2014) (120 g/L) but lower than (Ayadi et al., 2022) 183 g/L. The BOD_5 11 g/L was lower

than (Esmail et al., 2013) (35 g/L), (Djeziri et al., 2023) 29 g/L, and (Bouknana et al., 2014) 17–25 g/L, but comparable to (Ayadi et al., 2022) 7 g/L.
The pH of OOWW in this study was 4.73, which is slightly higher than (el Kafz et al., 2023) 4.09 but lower than 4.88 reported by (Ayadi et al., 2022).

SCM

The value of reducing sugars in SCM 37.02% is considerably low compared to 51.36% found by (Hassan et al., 2019), indicating possible dilution effects or variations in sugar extraction efficiency.
The COD (102.2 g/L) in this study was lower than (Hakika et al., 2019) 132.25 g/L, and the BOD_5 52.4 g/L was higher than what (Hakika et al., 2019) reported at 31.25 g/L. This lower value of sugars might be due to the low concentration of the SCM used in this study.
The pH of SCM 4.99 was higher than that reported by Hakika et al., 2019 at 3.8, but lower than the one obtained by Hassan et al., 2019 at 5.1.

MW

Lastly, the composition of MW in this study was compared with previous reports, where our MW contained a higher protein content 1.03%, than the (0.84%) mentioned by (Lievore et al., 2015) but lower than (Lachebi & Yelles, 2018) at 6.2%.
The fat content in this study (0.21%) was comparable to (Lievore et al., 2015)(0.08%) but much lower than (Lachebi & Yelles, 2018) (1.6%), suggesting partial skimming in our sample.
Comparing the reducing sugar content in this study (4.1%) was lower than the 6.2% reported by (Lachebi & Yelles, 2018), which may affect its fermentability unless supplemented with SCM.
The COD and BOD_5 of our MW was 14 g/L and 7.3 g/L, respectively, which were slightly higher than the values reported by (Lachebi & Yelles, 2018) COD of 11 g/L and BOD_5 of 6.4 g/L.
For the pH of MW in this study 4.89 was slightly higher than (Lievore et al., 2015) at 4.37 and (Lachebi & Yelles, 2018) at a value of 4.5.
Only glucose was measured using the DNS method, which primarily detects reducing sugars. Other carbohydrates, such as sucrose and lactose may have been present but were not individually quantified. Their contribution to ethanol production likely occurred indirectly through enzymatic hydrolysis.

Effect of Enzymatic Hydrolysis on Sugar Release and Biogas Production

Glucose Concentration before and after Enzymatic Treatment

To evaluate the efficacy of the enzymatic hydrolysis, Glucose concentration was compared at T0 (before treatment) and at T2 (after 48 h of treatment) for the different wastewaters at varying concentrations (0.25%, 0.5% and 0.75%), the results are presented in Table 3 and illustrated in Figure 3.

Table 3. Percentage increase in glucose concentration after enzymatic hydrolysis.

Waste type	Enzyme dose (%)	T0 (g/L)	T2 (g/L)	% Increase
OOWW	0.25	3.42	7.58	121.6%
	0.5	3.42	10.42	204.4%
	0.75	3.42	11.12	225.1%
SCM	0.25	27.02	61.45	127.4%
	0.5	27.02	79.24	193.2%
	0.75	27.02	86.35	219.5%
MW	0.25	8.2	17.98	119.3%
	0.5	8.2	23.84	190.7%
	0.75	8.2	26.21	219.6%

The results showed a significant increase in glucose concentration ($p < 0.05$) across all substrates with increasing enzyme doses. The R^2 values from linear regression analyses were consistently above 0.85, indicating a strong correlation between enzyme dose and glucose release.
The results showed that OOWW exhibited the highest percentage increase (up to 225.1%), which could be explained by the high content of complex sugars such as cellulose that could be hydrolyzed to simple fermentable sugars.
Both CM and MW showed a similar increase (219.5% and 219.6%, respectively), which indicates a positive enzymatic activity despite MW containing lactose.
The greatest amount of glucose was observed between the enzyme doses of 0.25% and 0.5%, where the increases were over 190%. This shows that 0.5% is the most efficient and economical for large-scale hydrolysis.

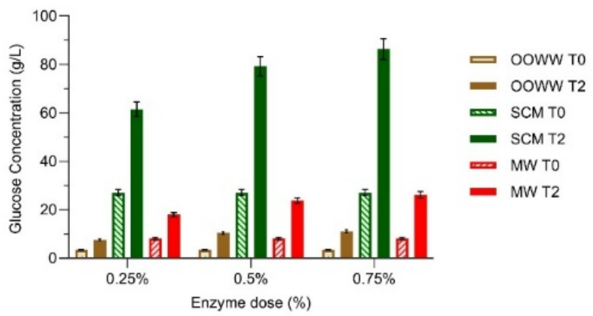


Fig. 3. Glucose release after enzymatic hydrolysis at different Natuzyme concentrations.

The ability to maintain a consistent increase of 200% across all substrates at higher enzyme doses demonstrates the efficiency of the enzymatic hydrolysis. This can be attributed to the component enzymes found in Natuzyme, each of which targets important substrate components for OOWW. Enzymes such as cellulase, xylanase, β-glucanase, and pectinase were essential in the breakdown of complex polysaccharides and structural carbohydrates, which improved the release of glucose despite inhibitory phenolic compounds (Bhardwaj et al., 2021; Nguyen et al., 2018).

For the SCM, the high percentage increase in glucose concentration is due to the action of α-amylase (breaking down residual starch) and potentially invertase (hydrolyzing sucrose into glucose and fructose), facilitating rapid sugar availability for fermentation (Manoochehri et al., 2020). Lactose in MW would be hydrolyzed into glucose and galactose in the presence of β-galactosidase (Saqib et al., 2017).

These enzymes work synergistically to optimize the breakdown of complex carbohydrates, augmenting substrate accessibility and glucose yield, which are critical for efficient bioethanol production from agro-industrial wastes.

The plateau effect observed at 0.75% enzyme dose suggests a point of substrate saturation, where further enzyme addition yields diminishing returns, indicating the necessity for enzyme dose optimization in industrial applications (Bisswanger, 2017).

Enzymatic Hydrolysis Effect on Biogas Production

To assess the impact of enzymatic hydrolysis on biogas production, biogas volumes were measured at T1 (24 h) and T2 (48 h) following the addition of different enzyme doses (0.25%, 0.5%, and 0.75%). The biogas production trends are illustrated in Figure 4.

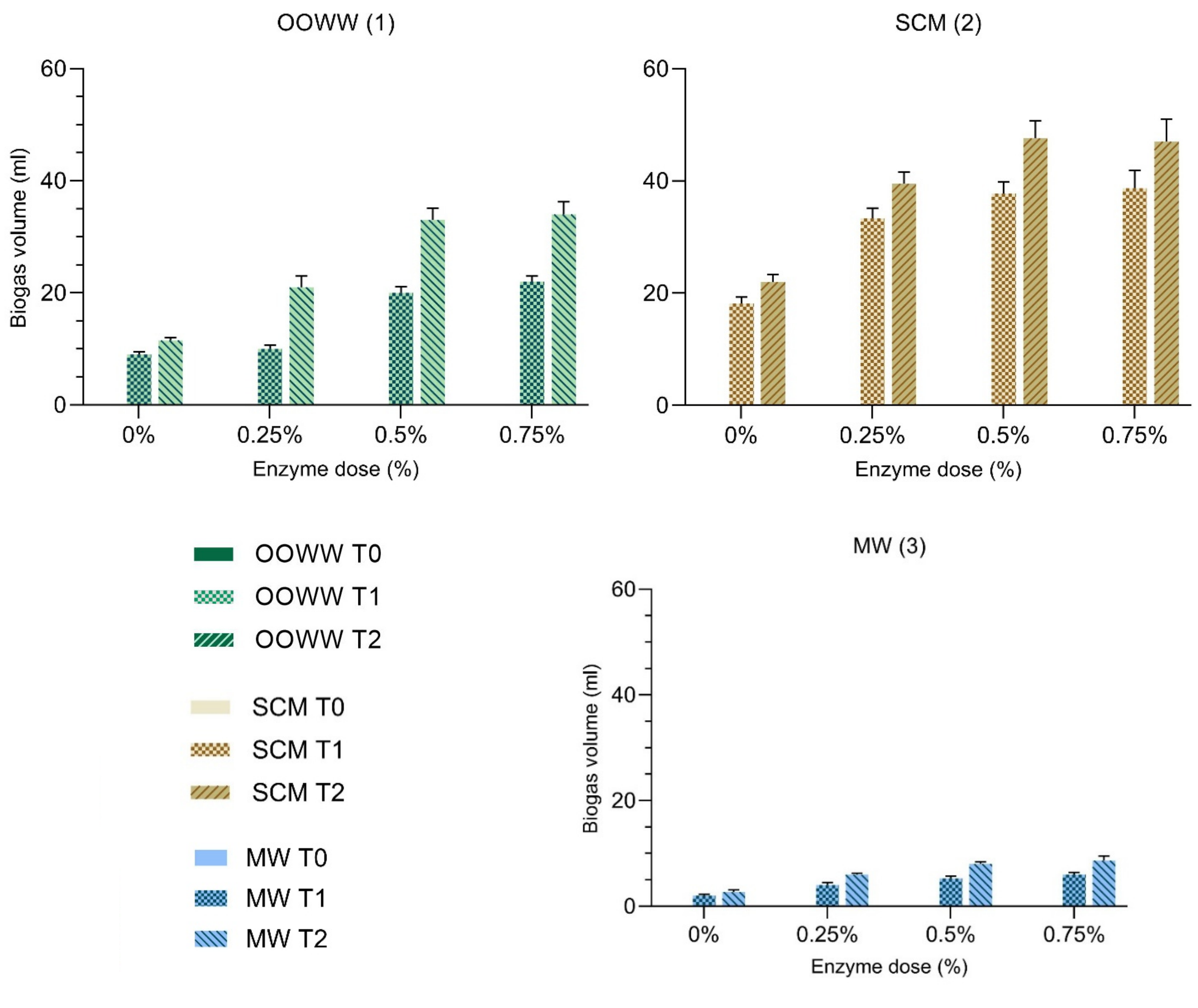


Fig. 4. Biogas production (mL) at T1 and T2 Across different enzyme doses for OOWW, SCM, and MW.

After 48 h (T2), SCM produced the most biogas, up to 47 ± 2 mL at a 0.75% enzyme dose, followed by OOWW with 34 ± 1.5 mL and MW, yielding 8.7 ± 1 mL.

SCM's higher performance can be caused by the high sugar content, promoting strong microbial activity during anaerobic digestion. While OOWW's moderate biogas yield can be justified by the presence of polyphenolic inhibitors, as explained by Calabrò et al., 2018, which may partially inhibit microbial activity despite improved sugar availability.

MW produced the least biogas, likely due to its composition rich in lactose and proteins, which are less readily converted into biogas compared to simple sugars (Kovács et al., 2013).

The highest increase in biogas production was observed between the 0.25% and 0.5% enzyme doses, particularly in SCM, where biogas yield improved by over 35%.

Comparatively, the 0% enzyme dose showed lower biogas production at both t1 and t2, indicating that the absence of the enzyme complex has a negative impact on fermentation and biogas production.

A significant increase in biogas production was observed with higher enzyme doses ($p < 0.05$). The R^2 values were greater than 0.80, proving that a strong linear relationship existed between the dose of the enzyme and the yield of biogas. Similarly, a strong correlation of glucose release with biogas production, r > 0.85, indicates the direct effect of substrate availability on microbial activity.

Although methane, hydrogen, and other gases may be produced during anaerobic digestion, only CO_2 was measured as a proxy for ethanol fermentation due to its direct stoichiometric link to glucose conversion.

Simultaneous Saccharification and Fermentation (SSF) with Immobilized Cells

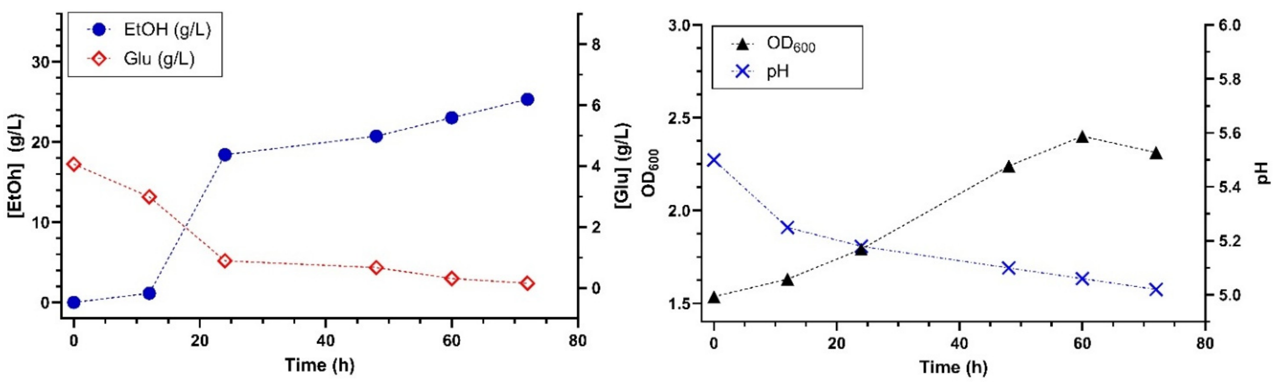
The pH of the fermentation process is critical because it directly affects enzymatic activity and microbial growth, both of which are required for optimal ethanol production (Yang et al., 2016). In this study, pH was initially adjusted to 5.5 across all fermentations.

pH

During fermentation, there was a progressive acidification of all the mixtures, which was expected since the production of organic acids, such as pyruvic acid, is a common metabolic by-product of fermentation and one of the main precursors of ethanol production (Darwin et al., 2019). For example, as shown in Figure 5, Mix 1 had its pH drop from an initial 5.5 to 5.02 at the end of 72 h. Also, Mix 2 went down to 5.05 while Mix 3 declined to 4.98 toward the end of the fermentation period. These consistent trends show active fermentations across the mixtures with the pH within a range not inhibitory to microbial activity (Mohd-Zaki et al., 2016).

Although a continuously decreasing pH indicates continuous fermentation, it also suggests that the process is under good control, preventing drastic drops that could inhibit microbial growth or enzyme activity. Keeping a stable pH close to pH of enzymes is still important to ensure maximum ethanol production, since extreme acidity could impair microbial viability and fermentation efficiency (Yusuf et al., 2023).

(1)



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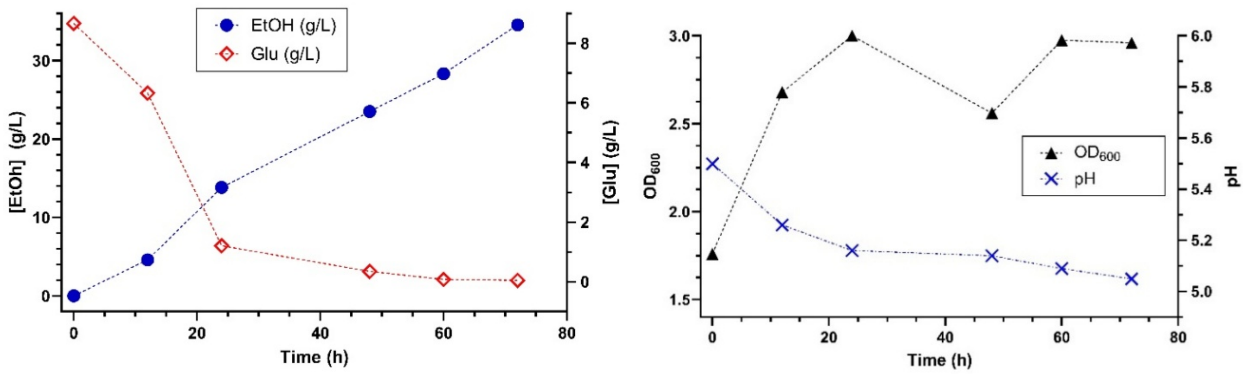

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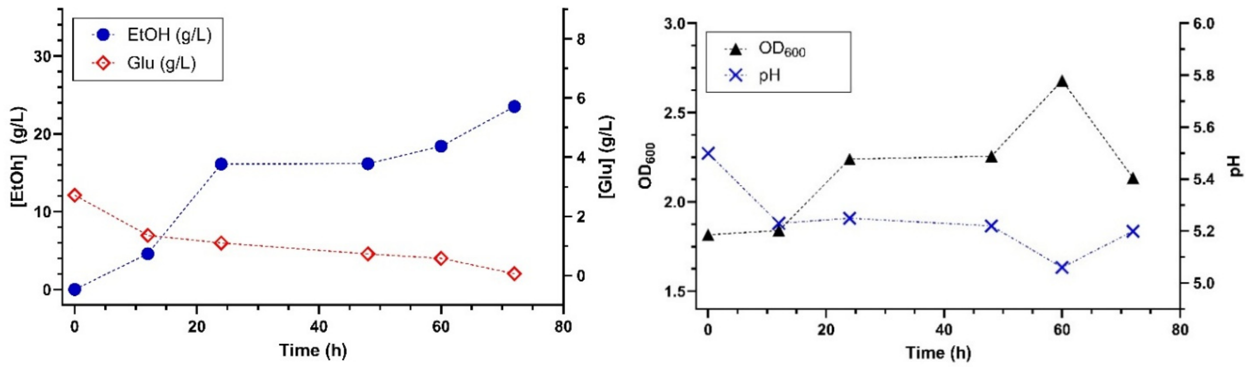

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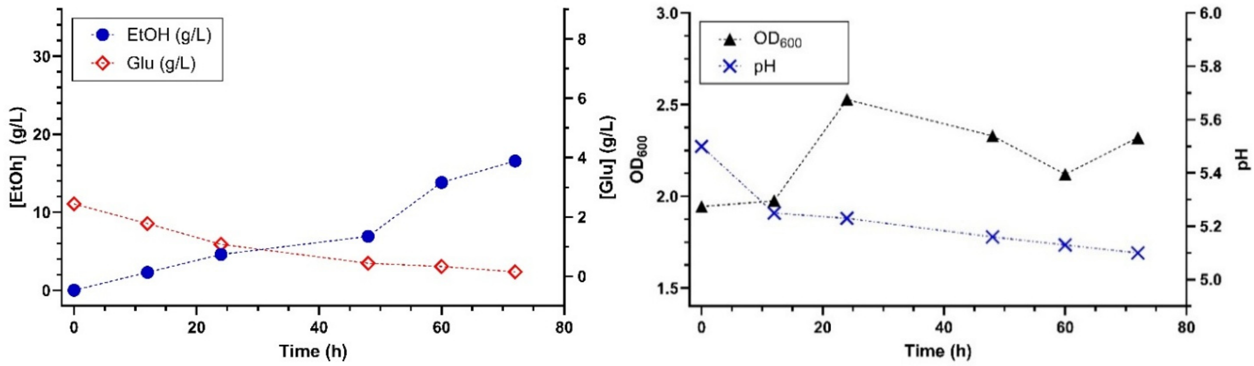

Fig. 5. Variation of ethanol concentration (g/L), glucose concentration (g/L), and optical density (OD_{600}) during fermentation of different waste mixtures. Measurements were taken over 72 h. Mix1 (**1**), Mix2 (**2**), Mix3 (**3**) and Mix4 (**4**).

Microbial Biomass

Optical density at 600 nm (OD_{600}) was an indicator used for microbial biomass in fermentation. In all fermentation mixes, a DO_{600} first started increasing and therefore reflected active microbial growth. For example, Mix 1 had an initial reading of 1.536 that peaked to 2.4 at 48 h, after which there was a slight decline in 2.312 at 72 h; it may be due to nutrient depletion, particularly glucose, or other environmental factors (Maier & Pepper, 2015).

Interestingly, Mix 3 showed a fast exponential phase at 12 h, maintaining relatively stable levels around 2.96–3.0 until the end of fermentation. This demonstrates that, in contrast to other mixes, such stability suggests longer microbial activity and most likely an efficient use of the nutrients that are available (Gonzalez & Aranda, 2023).

These differences in the pattern of optical density show the differences in dynamics for microbial growth and activity, each depending on the mixture composition. The slight decrease in DO_{600} observed after the peak in all mixtures could be attributed to a decrease in cell growth or changes in microbial population composition, possibly due to nutrient limitation (diauxic pattern) or the accumulation of inhibitory metabolites (Galdieri et al., 2010).

Glucose Consumption

Glucose concentration was one of the key parameters in this study, since it is the main carbon source for microbial fermentation (Carteni et al., 2020). All mixtures showed a gradual decrease in glucose concentration throughout the 72-h period, indicating active fermentation. In Mix 1, glucose concentration decreased from 4.06 g/L at the beginning to as low as 0.16 g/L at 72 h, showing efficient glucose utilization.

By the end of the fermentation period, Mix 2's glucose concentration had significantly decreased to 0.05 g/L from its initial higher concentration of 8.67 g/L. Mix 2's faster and more thorough glucose depletion points to a more effective fermentation process, possibly as a result of the higher initial glucose availability, which also probably helped to produce the higher ethanol yield (34.5g/L) that was noted (Chang et al., 2018).

Both Mixes 3 and 4 produced intermediate amounts of ethanol because the glucose depletion was slightly slower than in Mix 2 but faster than in Mix 1. These results evidently suggest that initial glucose concentration has a crucial role in driving the process of ethanol production, since higher glucose availability increases microbial activity and ethanol yield. However, high initial substrate concentrations may inhibit substrate utilization and/or reduce end-product yields, implying that there is an optimal glucose concentration range beyond which ethanol production efficiency may decline (Jessen & Orlygsson, 2012).

Ethanol Production

The ethanol concentration, the main point of interest, was significantly different among the mixtures. Mix 2 produced the highest ethanol concentration of 34.56 g/L after 72 h, significantly outperforming Mix 1 with 25.34 g/L and Mix 3 with 23.5 g/L. This is greater than the 14 g/L reported by (Ayadi et al., 2022), who only used immobilized cells and untreated OOWW.

Mix 2's superior performance could be explained by enzymatic treatment, which provided hydrolysis of complex sugars into fermentable sugars like glucose. Mix 2 also contained the highest SCM ratio and thus had enough and continuous substrate for ethanol production.

The order of ethanol yield across the mixtures (Mix 2 > Mix 1 > Mix 3) is consistent with the trends observed in glucose consumption and pH changes, this again confirmed that substrate availability and controlled fermentation conditions are crucial.

Mix 4 generated the least amount of ethanol (16.58 g/L) for having the lowest initial glucose concentration. This further confirms that higher initial glucose concentrations lead to greater ethanol production, if other conditions such as pH and microbial activity are adequately maintained.

This further confirms that higher initial glucose concentrations lead to greater ethanol production, provided that other conditions, such as pH and microbial activity are adequately maintained. Compared to earlier studies, the ethanol yield achieved in this work, 34.56 g/L using Mix 2 with 0.5% enzymatic dose, stands out as significantly higher. This enhanced performance can be attributed to the combined use of enzymatic hydrolysis and yeast immobilization, which together improved substrate accessibility and fermentation efficiency. Unlike conventional approaches that often rely on free yeast cells or single substrates, this study introduces a co-fermentation system that integrates three agro-industrial by-products—OOWW, MW, and SCM—while using *S. cerevisiae* immobilized on pozzolan, a natural porous material. This configuration not only increased ethanol yield but also offered operational benefits such as cell reuse, process stability, and reduced contamination risk.

A comparative overview of ethanol production across related studies is presented in Table 4. As shown, the optimized conditions in this study yielded results that are superior or comparable to those reported using synthetic sugars, treated lignocellulosic biomass, or engineered microbial strains, highlighting the potential of this strategy for scalable and sustainable bioethanol production.

As shown, our results demonstrate a competitive or even superior ethanol yield compared to existing studies, validating the effectiveness of combining enzymatic treatment, co-substrate utilization, and cell immobilization for bioethanol production. This positions our process as a promising candidate for future scale-up and industrial application.

Table 4. Comparative ethanol yields from the literature.

Study/author	Substrate(s) used	Treatment method	Fermentation mode	Ethanol yield (g/L)	Remarks
This study	OOWW + MW + SCM	Enzymatic hydrolysis + immobilized yeast	Batch SSF	34.56	Highest yield at 0.5% enzyme, Mix 2
Ayadi et al. (2022)	OOWW	Immobilized yeast, no enzyme	Batch	14.00	No enzymatic pretreatment
Duque et al. (2021)	Lignocellulosic residues	Enzymatic hydrolysis	Free-cell	25.30	Requires detoxification step
Pasotti et al. (2017)	Cheese whey	Engineered *E. coli*	Free-cell	19.70	Lactose-to-ethanol conversion
Chang et al. (2018)	Glucose	Fed-batch	Free-cell	33.20	Synthetic sugar, high control setup

CONCLUSIONS

This study demonstrates the effectiveness of simultaneous saccharification and fermentation (SSF) using immobilized Saccharomyces cerevisiae on pozzolan for bioethanol production from a combination of three agro-industrial by-products: olive oil wastewater (OOWW), sugarcane molasses (SCM), and milk whey (MW). The integration of enzymatic hydrolysis using Natuzyme significantly improved glucose availability, resulting in higher ethanol yields, with a maximum concentration of 34.56 g/L observed for Mix 2 with 0.5% enzyme concentration.

By applying immobilized yeast fermentation in a co-substrate system, this work overcomes several limitations reported in earlier studies that used single substrates or free-cell systems. Using pozzolan as a natural, cost-effective immobilization support contributed to process stability, biomass reusability, and contamination risk reduction. These combined strategies not only improved fermentation performance but also offered a scalable and sustainable solution for the valorization of agro-industrial waste.

Furthermore, the correlation between glucose consumption and ethanol yield underscores the importance of optimizing enzymatic treatment and fermentation conditions. In addition to bioethanol, the potential for residual biomass valorization through biogas production highlights the broader applicability of this integrated biorefinery concept. Overall, the findings of this study provide a strong foundation for the future development of industrial-scale processes that support circular economy principles and green energy production.

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