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Faculté des Sciences de la Nature et de la Vie
Département des Sciences Biologiques
Laboratoire de Géo Environnement et Développement des Espaces

THESE de DOCTORAT

Spécialité : Biotechnologie Microbienne

Intitulée :

Production de molécules d'intérêt biotechnologique à partir de quelques coproduits agroalimentaires via immobilisation cellulaire sur supports solides

Présentée par **: Mr. ROUAM Djawad**

Le……/…..…/2025

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الجمهوريــة الجزائريــة الديمقراطيــة الشعبيــة
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DOCTORAL Thesis

Specialty: Microbial Biotechnology

Titled:

Production of Biotechnological Molecules from Agro-Industrial By-Products Using Cell Immobilization on Solid Supports

***Presented by*: Mr ROUAM Djawad**

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Résumé

La demande croissante en sources d'énergie durables et en solutions de gestion des déchets respectueuses de l'environnement a stimulé l'intérêt pour la valorisation biotechnologique des coproduits agro-industriels. Cette thèse explore des stratégies innovantes pour la production de molécules d'intérêt biotechnologique en particulier le bioéthanol et l'acide acétique par l'utilisation de systèmes microbiens immobilisés sur supports solides.

Dans un premier temps, soixante (60) souches de levures ont été isolées à partir des margines d'olive (OOWW), un substrat difficile en raison de sa forte teneur en polyphénols et de sa charge inhibitrice. Parmi ces isolats, treize (13) souches ont été identifiées comme appartenant au genre *Saccharomyces* sp. Une souche, *Saccharomyces cerevisiae* Y17, a été sélectionnée pour sa remarquable tolérance aux composés inhibiteurs et sa haute performance fermentaire, atteignant une concentration en éthanol de 11,3 g/L à partir d'OOWW non traité après 72 heures de fermentation. Les analyses comparatives ont révélé son adaptabilité supérieure par rapport aux souches commerciales testées dans des conditions identiques.

Par la suite, une approche intégrée combinant hydrolyse enzymatique et saccharification/fermentation simultanée (SSF) a été développée. L'immobilisation des cellules de *S. cerevisiae* sur des roches de pouzzolane a amélioré la stabilité du procédé et permis une production efficace d'éthanol à partir d'un mélange de margines d'olive (OOWW), de mélasse de canne à sucre (SCM) et de lactosérum (MW). L'optimisation des paramètres du procédé a conduit à une concentration maximale d'éthanol de 34,56 g/L après 72 heures, dépassant largement les résultats obtenus par fermentation conventionnelle. De fortes corrélations entre la consommation de glucose et la production d'éthanol ont souligné l'importance critique de la disponibilité du substrat et du contrôle des conditions de fermentation.

Dans un second temps, des recherches ont été menées sur l'utilisation de souches de *Bacillus*, isolées du rumen bovin, pour la production d'acide acétique à partir des margines d'olive (OMW). Au total, vingt-cinq (25) souches bactériennes ont été isolées, parmi lesquelles cinq (5) ont été sélectionnées pour une évaluation approfondie. Parmi celles-ci, la souche *Bacillus* sp. 15 a montré le rendement en acide acétique le plus élevé, atteignant 28 g/L après 108 heures de fermentation. Cette étude introduit les espèces de *Bacillus* comme de nouveaux agents pour la production d'acide acétique à partir d'OMW, élargissant ainsi le spectre des candidats microbiens pour les processus de bioconversion industrielle.

Dans l'ensemble, cette thèse démontre le potentiel de la combinaison de l'immobilisation cellulaire, de l'hydrolyse enzymatique et de l'utilisation de substrats mixtes pour la production de bioéthanol et d'acide acétique. Les résultats obtenus apportent des éléments précieux pour le développement de bioprocédés évolutifs, économiques et respectueux de l'environnement basés sur la valorisation des résidus agro-industriels.

Mots clés : Molécules d'intérêt, coproduits, immobilisation cellulaire, supports solides.

Abstract

The growing demand for sustainable energy sources and environmentally friendly waste management solutions has driven interest in the biotechnological valorization of agro-industrial by-products. This thesis explores innovative strategies for the production of biotechnologically valuable molecules specifically bioethanol and acetic acid through the use of immobilized microbial systems on solid supports.

Initially, sixty (60) yeast strains were isolated from olive oil wastewater (OOWW), a challenging substrate due to its high polyphenol content and inhibitory load. Among these isolates, thirteen (13) strains were identified as belonging to the genus *Saccharomyces* sp. One strain *Saccharomyces cerevisiae* Y17 was selected for its outstanding tolerance to inhibitory compounds and high fermentative performance, achieving an ethanol concentration of 11.3 g/L from untreated OOWW after 72 hours of fermentation. Comparative analyses revealed its superior adaptability over commercial yeast strains under identical conditions.

Subsequently, an integrated approach combining enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) was developed. Immobilization of *S. cerevisiae* cells on pozzolan rock enhanced process stability and enabled efficient ethanol production from a mixture of OOWW, sugarcane molasses (SCM), and milk whey (MW). Optimization of process parameters led to a maximum ethanol concentration of 34.56 g/L after 72 hours, significantly surpassing conventional fermentation outcomes. Strong correlations between glucose consumption and ethanol production emphasized the critical importance of substrate availability and controlled fermentation conditions.

Secondly, research was conducted on the use of *Bacillus* strains, isolated from bovine rumen, for acetic acid production from olive mill wastewater (OMW). A total of 25 bacterial strains were isolated, among which five (5) were selected for detailed evaluation. Among these, *Bacillus* sp. strain 15 exhibited the highest acetic acid yield, reaching 28 g/L after 108 hours of fermentation. This study introduces *Bacillus* species as novel agents for acetic acid production from OMW, expanding the range of microbial candidates available for industrial bioconversion processes.

Overall, this thesis demonstrates the potential of combining cell immobilization, enzymatic enhancement, and mixed-substrate strategies for the sustainable production of bioethanol and acetic acid. The findings contribute valuable insights toward the development of scalable, economical, and environmentally responsible bioprocesses based on the valorization of agro-industrial residues.

Keywords: Molecules of interest, by-products, cell immobilization, solid supports.

الملخص

تزايد الطلب على مصادر الطاقة المستدامة وحلول إدارة النفايات الصديقة للبيئة دفع إلى تعزيز الاهتمام بالتحويل البيوتكنولوجي للمخلفات الزراعية والصناعية. تستكشف هذه الأطروحة استراتيجيات مبتكرة لإنتاج جزيئات ذات أهمية بيولوجية وتقنية، بالأخص الإيثانول وحمض الأسيتيك، من خلال استخدام تثبيت خلايا ميكروبية على دعامات صلبة.

في البداية، تم عزل ستين (60) سلالة من الخمائر من مياه مخلفات الزيتون(OOWW) ، وهي مادة أولية صعبة بسبب ارتفاع محتواها من البوليفينولات ووجود مثبطات. من بين هذه السلالات، تم تحديد ثلاث عشرة (13) سلالة على أنها تنتمي إلى جنس *Saccharomyces* sp. وتم اختيار السلالة *Saccharomyces cerevisiae* Y17 بفضل قدرتها العالية على تحمل المركبات المثبطة وأدائها التخمري الممتاز، حيث حققت تركيز إيثانول بلغ 11.3 جم/لتر من OOWW غير المعالج بعد 72 ساعة من التخمير. وأظهرت التحليلات المقارنة تفوقها في التكيف مقارنة بالسلالات التجارية تحت نفس الظروف.

بعد ذلك، تم تطوير نهج متكامل يجمع بين التحلل الأنزيمي وعملية التحلل والتخمير المتزامنة .(SSF) وقد حسّنت عملية تثبيت خلايا *S. cerevisiae* على صخور البوزلان من استقرار العملية، مما سمح بإنتاج فعال للإيثانول من خليط من مياه مخلفات الزيتون (OOWW) ومولاس قصب السكر (SCM) ومصل الحليب .(MW) أدت تحسينات ظروف العملية إلى تحقيق تركيز إيثانول أقصى بلغ 34.56 جم/لتر بعد 72 ساعة، متفوقاً بشكل كبير على نتائج التخمير التقليدي. وأظهرت الارتباطات القوية بين استهلاك الجلوكوز وإنتاج الإيثانول الأهمية البالغة لتوفر المادة الأولية والسيطرة على ظروف التخمير.

في جزء آخر من البحث، تم دراسة استخدام سلالات *Bacillus* المعزولة من كرش الأبقار لإنتاج حمض الأسيتيك من مياه مخلفات الزيتون (OMW) تم عزل خمس وعشرين (25) سلالة بكتيرية، وتم اختيار خمس (5) سلالات منها لإجراء تقييم مفصل. ومن بينها، أظهرت السلالة *Bacillus* sp. 15 أعلى إنتاج لحمض الأسيتيك، حيث بلغت 28 جم/لتر بعد 108 ساعات من التخمير. تقدم هذه الدراسة أنواع *Bacillus* كعوامل جديدة لإنتاج حمض الأسيتيك منOMW ، مما يوسع نطاق الميكروبات المستخدمة في عمليات التحويل الصناعي.

بشكل عام، توضح هذه الأطروحة الإمكانيات الكبيرة للجمع بين تثبيت الخلايا، التحسين الأنزيمي، واستراتيجيات استخدام المواد الأولية المختلطة لإنتاج الإيثانول الحيوي وحمض الخليك بطريقة مستدامة. وتساهم النتائج في تقديم رؤى قيّمة نحو تطوير عمليات بيولوجية قابلة للتوسعة، اقتصادية، وصديقة للبيئة تعتمد على استغلال مخلفات الصناعات الزراعية والغذائية.

الكلمات المفتاحية :جزيئات ذات أهمية، مخلفات ثانوية، تثبيت الخلايا، دعامات صلبة.

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List of abbreviations

OOWW/OMW	: Olive Oil Wastewater
COD	: Chemical Oxygen Demand
SDGs	: Sustainable Development Goals
SCM	: Sugarcane Molasses
CWP	: Cheese Whey Permeate
BOD	: Biological Oxygen Demand
WHO	: World Health Organization
SCP	: Single-Cell Protein
FAO	: Food And Agriculture Organization
GHGs	: Greenhouse Gases
SMEs	: Small And Medium-Sized Enterprises
VOCs	: Volatile Organic Compounds
SmF	: Submerged Fermentation
SSF	: Solid-State Fermentation
MBRs	: Membrane Bioreactors
SCP	: Single-Cell Protein
LAB	: Lactic Acid Bacteria
ABE	: Acetone-Butanol-Ethanol
EPS	: Extracellular Polymeric Substances
DAC	: Dialdehyde Cellulose
DNS	: Dinitrosalicylic Acid
SDA	: Sabouraud Dextrose Agar
CO	: Carbon Dioxide
ITS	: Internal Transcribed Spacer
PCR	: Polymerase Chain Reaction
MUSCLE	: Multiple Sequence Comparison By Log-Expectation
ML	: Maximum Likelihood
K2P	: Kimura 2-Parameter
MCL	: Maximum Composite Likelihood
MW	: Milk Whey
SCM	: Sugarcane Molasses
OD	: Optical Density
PHi	: Isoelectric Point
SD	: Standard Deviation

General Introduction

General Introduction

The increasing awareness of environmental challenges has brought the sustainable management of natural resources and by-products to the forefront of global priorities. Across both developed and developing countries, addressing the environmental impacts of industrial and agricultural processes has become a key concern. Effective strategies to mitigate pollution and optimize resource utilization are now considered critical for achieving sustainable development goals (Sahoo et al., 2024).

In this context, the valorization of agro-industrial by-products has gained significant attention. This trend is driven by multiple factors, including energy shortages, the diminishing availability of raw materials, and stringent environmental regulations aimed at protecting ecosystems. By converting waste into valuable products, industries can address environmental concerns while simultaneously generating economic benefits (Singh et al., 2023).

One such underutilized by-product is olive oil wastewater (OOWW), a major pollutant generated by the olive oil extraction process. Despite its rich organic composition, which includes sugars, polyphenols, and other compounds (Cuffaro et al., 2023), its improper disposal poses a severe environmental risk. The high chemical oxygen demand (COD), acidity, and the presence of polyphenols make OOWW a pollutant that can affect soil fertility and water quality (Khdair & Abu-Rumman, 2017). However, its composition also makes it a suitable candidate for biotechnological applications, including the production of biofuels and value-added chemicals (Foti et al., 2021).

In Algeria, as one of the main olive oil producers in the world, the olive oil industry generates substantial amounts of OOWW annually, with approximately 1 to 1.5 liters produced per kilogram of processed olives (Bougherara et al., 2021a). Currently, much of this waste is discarded untreated, leading to pollution and logistical challenges for its storage. Transforming OOWW into bioethanol, biogas, or organic acids not only offers an eco-friendly solution but also addresses economic priorities by reducing dependence on imported biofuels and chemicals (Foti et al., 2021).

To enhance the efficiency of OOWW valorization, supplementation with agro-industrial by-products such as sugarcane molasses and milk whey has been explored. These supplements provide additional fermentable sugars and nutrients, improving microbial activity and boosting yields. Additionally, enzymatic treatments, such as those using the Natuzyme complex, facilitate the breakdown of complex sugars into simpler forms, further optimizing the fermentation process.

This thesis focuses on the valorization of OOWW through biotechnological processes, specifically the production of bioethanol, biogas, and acetic acid. Immobilized cell systems were employed for bioethanol production to enhance process efficiency and stability, while biogas and acetic acid production were studied using non-immobilized microbial systems. The

study aims to optimize these processes and evaluate their feasibility for scalable and sustainable applications.

To support this objective, the thesis is divided into three main chapters:

1. **Literature Review**: This chapter explores the generation, composition, and environmental impact of OOWW. It also reviews existing treatment and valorization methods, highlighting the potential of fermentation processes, supplementation strategies, enzymatic treatments, and cell immobilization technologies for converting OOWW into valuable bioproducts.
2. **Materials and Methods**: This chapter outlines the collection and characterization of OOWW samples, detailing their physicochemical and microbiological properties. It also describes the methodologies employed for fermentation experiments, enzymatic treatments, supplementation with molasses and milk whey, and the preparation of immobilized yeast systems using pozzolan rocks.
3. **Results and discussion**: The final chapter presents the findings from the experimental work conducted:
    - **Part 1** focuses on the production of bioethanol using *Saccharomyces cerevisiae* Y17 isolated from OOWW. The performance of immobilized yeast systems on pozzolan rocks is compared to free-cell fermentations, with and without enzymatic treatment and supplementation strategies (molasses and milk whey).
    - **Part 2** explores the production of biogas and acetic acid using Bacillus strains from bovine rumen in non-immobilized systems. The impact of supplementation strategies on improving yields is also analyzed.

The thesis concludes by summarizing the main findings and providing recommendations for future research, emphasizing the scalability and environmental impact of the proposed processes.

Section I:

Literature Review

Introduction

The growing awareness of environmental degradation and resource depletion has prompted a global shift toward more sustainable production systems. Agro-industrial by-products, often regarded as waste, are now increasingly recognized as valuable raw materials within the framework of a circular bioeconomy. Their valorization addresses multiple challenges, including energy insecurity, pollution mitigation, and resource recovery, while simultaneously aligning with key Sustainable Development Goals (SDGs), such as responsible consumption (SDG 12) and climate action (SDG 13) (Martins et al., 2024; Raman et al., 2024).

In this context, agro-industrial residues like olive oil wastewater (OOWW), sugarcane molasses, and milk whey stand out for their high organic content and fermentation potential. However, improper disposal of these by-products remains common, particularly in developing regions such as North Africa, where regulatory and infrastructural gaps persist (Gueboudji et al., 2022; Tebbouche et al., 2024). In Algeria—a leading olive oil producer—OOWW alone reaches 1 to 1.5 million m^3 annually and poses significant ecological threats due to its high acidity and phenolic load (Bougherara et al., 2021b; Djeziri et al., 2023).

Biotechnological innovations offer a promising alternative to traditional waste disposal methods. Microbial fermentation and enzymatic bioconversion allow for the production of value-added compounds such as composts, bioethanol, biogas, and biomolecules (such as enzymes and organic acids), from these substrates (Chauhan et al., 2024; Ning et al., 2021). Moreover, integrating strategies like co-supplementation (e.g., blending OOWW with molasses and whey), enzymatic pretreatment (e.g., with Natuzyme), and microbial cell immobilization (e.g., on pozzolane rocks) significantly improves process yields and scalability (Ayadi et al., 2022; Vasić et al., 2021).

This chapter provides a comprehensive review of the generation, composition, and valorization potential of key Algerian agro-industrial by-products. It evaluates conventional treatment methods, outlines current biotechnological applications, and highlights innovative hybrid approaches designed to overcome substrate limitations and microbial inhibition. Special emphasis is placed on the synergy of enzymatic hydrolysis, co-supplementation, and immobilized systems—an integrated strategy at the core of this study's originality.

The literature review is structured to:

- Contextualize global and regional imperatives for by-product valorization.
- Analyze the composition and biotechnological potential of OOWW, molasses, and whey.
- Review conventional and emerging treatment and conversion methods.
- Highlight the role of synergistic techniques in optimizing yields.
- Identify knowledge gaps and position this research within current scientific discourse.

I.1. Agro-Industrial By-Products

Agro-industrial by-products are organic residues resulting from the transformation of agricultural raw materials in food processing industries. These materials, while often considered waste, retain significant nutritional and chemical value that can be exploited in various biotechnological applications. Unlike inert waste, agro-industrial by-products frequently contain fermentable sugars, organic acids, proteins, lipids, and phenolic compounds. Their chemical richness makes them attractive feedstocks for microbial fermentation, enzymatic hydrolysis, and energy recovery processes such as bioethanol or biogas production (Chauhan et al., 2024; Martínez Burgos et al., 2021; Singh et al., 2023).

I.1.1. Olive mill wastewater

Olive oil wastewater (OOWW), also known as olive mill wastewater (OMW), is a significant byproduct generated during the extraction of olive oil. It is characterized by its high organic content, considerable levels of polyphenolic compounds, and acidic pH, all of which contribute to its classification as a highly polluting agro-industrial waste. However, despite its environmental challenges, OOWW presents significant opportunities for valorization through biotechnological and chemical processes. Managing and utilizing OOWW effectively is particularly critical in Algeria, where olive oil production plays a major role in agricultural and economic activities. The sustainable handling of OOWW could allow for both environmental protection and the development of valuable bioproducts, making its treatment and conversion an important research focus.

I.1.1.1. Physicochemical properties (cod, polyphenols, acidity)

The composition of OOWW depends on multiple factors, including olive variety, milling technology, and regional climatic conditions (Gharaibeh et al., 2021). However, certain physicochemical characteristics (Table 1) are common across different sources, making them key parameters for assessing wastewater treatment needs and valorization potential (Fleyfel et al., 2022).

Table 1. Physicochemical Properties of Olive Oil Wastewater (OOWW) from Different Regions and Treatment Studies

Reference	Geographic Origin	COD (g/L)	Phenolic Content (g/L)	pH	Other Notable Characteristics
(Ayadi et al., 2022)	Algeria (Ennakhla-Chlef)	183	1.72	4.88	High organic load, electrical conductivity 34 mS/cm
(Meziani et al., 2023)	Algeria (Ghardaia, Sahara)	Not specified	High (Exact concentration not provided)	4.8	Microbial analysis identified *Staphylococcus* and *Bacillus* species
(Bouharat et al., 2018)	Morocco (Ben Karrich, Tetouan)	84.5	3.79	Not provided	Continuous two-phase extraction processing
(Gueboudji et al., 2021)	Algeria (Khenchela)	Not provided	Presence of 20 identified phenolics (Gallic acid, Caffeic acid, Quercetin, Luteolin, etc.)	Not provided	Phenolic extract demonstrated strong antioxidant properties
(Bouknana et al., 2014)	Morocco (Oujda, Nador, Berkane, Taourirt, Jerada)	52-120 (depending on extraction process)	0.24-1.83 (polyphenols), 0.12-1.71 (tannins)	4.5-5.32	High conductivity (13-41 mS/cm), BOD5 (8.5-25 g O2/L), biodegradability 0.11-0.25, variation based on extraction method
(Evci et al., 2019)	Turkey (Çukurova)	83	4.8	4.7	High-pressure & temperature treatment with H2O2 removed 89.2% COD, 91.5% phenolics; antioxidant IC50 = 118 µg/mL

One of the most important parameters in OOWW is Chemical Oxygen Demand (COD), which reflects the concentration of organic matter in the wastewater (Seferlis, 2008). High COD values indicate a substantial pollutant load that contributes to environmental toxicity if left untreated (Bader et al., 2022). Studies on Algerian OOWW have reported COD levels reaching 183 g/L in wastewater collected from Ennakhla-Chlef (Ayadi et al., 2022a), a value that aligns with the significant organic pollution load observed in Mediterranean olive oil production waste. Similarly, wastewater from the GHARDAIA region in ALGERIA exhibits high COD concentrations, although exact values are not always provided (Meziani et al., 2023). Compared to similar studies in Morocco, where COD levels have been recorded as 84.5 g/L (Bouharat et al., 2018), it is evident that Algerian OOWW exhibits some of the highest organic loads, emphasizing the necessity for pretreatment prior to any reuse or valorization (Bouknana et al., 2014).

Another crucial component of OOWW is polyphenols, a diverse group of bioactive compounds that include antioxidant molecules with both beneficial and inhibitory properties (Bolat et al., 2024). While polyphenols are valuable for industries such as pharmaceuticals and cosmetics, their presence in wastewater creates challenges due to their antimicrobial nature (Muñoz-Palazon et al., 2022). Studies exploring Algerian OOWW estimate total phenolic concentrations around 1.72 g/L in Ennakhla-Chlef samples (Ayadi et al., 2022a). In contrast, other research on Moroccan olive mill wastewater has detected higher concentrations up to 3.79 g/L (Bouharat et al., 2018), highlighting variability linked to olive variety and processing techniques. Furthermore, detailed compositional analysis of Algerian OOWW from Khenchela identified at least 20 distinct phenolics, including gallic acid, caffeic acid, quercetin, and luteolin (Gueboudji et al., 2021). These compounds have recognized applications as antioxidants, anti-inflammatory agents, and antimicrobial additives, making phenolic recovery a potential route for economic valorization in addition to wastewater detoxification.

The acidity of OOWW (pH levels typically between 4.5 and 4.88 in Algerian samples) adds another layer of complexity in terms of treatment and microbial processing (Ayadi et al., 2022a; Meziani et al., 2023). Many wastewater treatment strategies, including biological treatments such as fermentation and anaerobic digestion, require near-neutral pH conditions for optimal microbial performance (Saravanan et al., 2023). The acidic nature of OOWW, largely influenced by organic acids derived from the olives themselves, complicates conventional biodegradation and often necessitates pH adjustment before processing can take place.

Pretreatment methods such as alkaline pH adjustments or electrochemical modifications are therefore recommended to enhance the feasibility of biological valorization approaches.

Taken together, these physicochemical properties indicate that OOWW poses a dual challenge: its high organic contaminant load makes direct disposal environmentally hazardous, while its rich phenolic content creates both microbial inhibition concerns and potential for high-value recovery. These factors emphasize the need for targeted treatment strategies that balance the detoxification of wastewater with the selective extraction of useful bioactive compounds.

I.1.1.2. Phenolic inhibition of microbial activity (phenolic toxicity)

One of the major roadblocks in the biological valorization of OOWW is the strong inhibitory effect of phenolic compounds on microbial activity (Calabrò et al., 2018). Many of the polyphenols found in OOWW have well-documented antimicrobial properties, which, while beneficial for some industrial applications, pose significant challenges for microbial processes employed in biofuel production, fermentation, and anaerobic digestion (Canal et al., 2019). Without adequate pretreatment, these phenolics can disrupt microbial metabolism, reducing efficiency or even halting biological conversion processes altogether.

The mechanisms of microbial inhibition caused by phenolics are diverse. Certain compounds interfere with microbial cell membranes, leading to increased permeability and leakage of intracellular contents. Others act as enzyme inhibitors, preventing essential metabolic reactions needed for microbial growth and energy production (Caroca et al., 2021). Research has particularly singled out oleuropein, caffeic acid, and protocatechuic acid as key inhibitors of anaerobic microbial activity. These compounds begin to significantly suppress microbial metabolism at concentrations exceeding 600–1000 mg/L, making untreated OOWW unsuitable for direct use in fermentation or anaerobic digestion without prior modification (Borja et al., 1996; Canal et al., 2019).

Despite the antimicrobial nature of OOWW, different microbial species exhibit varying levels of tolerance to phenolic compounds. Tyrosol, for example, is more easily metabolized at concentrations below 600 mg/L, whereas oleuropein appears to strongly inhibit methane production in anaerobic digestion at comparable levels (Borja et al., 1996; Hernandez & Edyvean, 2008). This variation highlights the importance of carefully selecting or engineering microbial cultures that can withstand or degrade toxic phenolics when developing OOWW valorization strategies. Studies on OOWW from Algeria suggest that certain bacterial strains, such as Staphylococcus aureus and Bacillus subtilis, are particularly susceptible to phenolic

toxicity, implying that indigenous microbial communities must also be considered when designing biological treatment solutions (Meziani et al., 2023).

Given these challenges, pretreatment strategies must specifically aim to reduce phenolic toxicity before OOWW can be effectively processed for fermentation-based applications. Several approaches have demonstrated success in this regard. Chemical oxidation using hydrogen peroxide under alkaline conditions has been shown to reduce polyphenol content by up to 78%, significantly improving anaerobic digestibility (Siciliano et al., 2015). Similarly, electro-Fenton oxidation has been found to remove approximately 66% of phenolics, enhancing methane production when the treated wastewater is subjected to anaerobic digestion (Bettazzi et al., 2007).

Biological degradation also holds promise, particularly through the use of phenolic-degrading microorganisms and enzymes. Research indicates that white-rot fungi and ligninolytic fungi can efficiently degrade phenolics in OOWW, reducing toxicity and improving biodegradability (Dragičević et al., 2010; Goudopoulou et al., 2010). Additionally, certain yeast species have been explored for their ability to metabolize phenolic compounds, contributing to both COD reduction and enhanced ethanol or methane production (Dragičević et al., 2010). By integrating these biological treatments with other physical or chemical strategies, it is possible to improve the fermentability of OOWW and make its microbial conversion into biofuels or other value-added products more viable.

Overcoming microbial inhibition caused by phenolic toxicity is a key step in unlocking OOWW's full potential as a substrate for bioenergy production and the recovery of high-value biomolecules. By implementing suitable pretreatment technologies, researchers can transform what is otherwise a problematic waste product into a resource for bioethanol, biogas, antioxidants, and other high-value biochemicals, contributing not only to waste minimization but also to the implementation of circular economy principles in the olive oil industry.

I.1.2. Molasses from the crystallization of sugarcane or beet

Sugarcane molasses (SCM) is a dense, viscous byproduct derived from the final stage of sugar extraction from sugarcane. It is rich in fermentable sugars, organic acids, and essential nutrients, making it a valuable raw material in multiple industries, particularly in fermentation-based applications such as ethanol production, biomaterials synthesis, and microbial bioprocesses. Due to its high sugar concentration and bioavailable nutrients, molasses is

frequently used as a substrate for microbial growth in biotechnology and industrial processes (Liu & Cheng, 2022; Zhang et al., 2021).

However, despite its desirability as a fermentation feedstock, sugarcane molasses also contains potential inhibitors, including heavy metals, excess salts, and microbial growth-controlling compounds like polyphenols or melanoidins, which can adversely affect the efficiency of biological transformations. Understanding its chemical properties and challenges related to microbial fermentation is crucial for optimizing its valorization potential (Jiranuntipon, 2008).

I.1.2.1. Physicochemical properties (sugars, acidity, and impurities)

The chemical composition of sugarcane molasses varies based on sugarcane variety (Table 2), processing conditions, and regional agricultural factors. However, several key physicochemical properties are consistently observed:

Total Sugar Content: Sugarcane molasses is particularly rich in fermentable sugars (~40–60% of total weight), making it an ideal substrate for fermentation-based bioproducts such as ethanol, citric acid, and bioplastics. The major sugar fractions in SCM include sucrose, glucose, and fructose, with sucrose typically being the predominant form. The relative composition of these sugars depends on the degree of crystallization in the sugar recovery process. The presence of non-fermentable oligosaccharides and polysaccharides may reduce fermentation efficiency if not properly managed.

Acidity and pH: Sugarcane molasses is slightly acidic due to the presence of organic acids such as acetic acid, formic acid, and oxalic acid, which can accumulate during sugarcane processing. The typical pH of molasses ranges from 4.5 to 6.5, depending on processing conditions. In fermentation-based applications, this acidity may require pH buffering or neutralization to optimize microbial efficiency.

Ash and Mineral Content: SCM contains 5–15% ash, including significant amounts of calcium, potassium, sodium, iron, and magnesium. While some of these minerals are essential micronutrients for microbial metabolism, excess salts (e.g., sodium and potassium chlorides) can lead to osmotic stress, inhibiting microbial growth and fermentation performance. Additionally, sulfates and phosphates present in molasses may affect fermentative pathways by altering intracellular pH regulation and enzymatic activities.

Nitrogen and Micronutrient Composition: Sugarcane molasses contains low levels of nitrogen (0.4–1%), which may limit microbial growth unless supplemented with ammonium salts, urea, or yeast extracts. Despite this, SCM also provides trace elements like iron, manganese, and zinc, which are important for enzymatic activities in yeast and bacterial fermentation.

Polyphenolic and Melanoidin Content: Blackstrap sugarcane molasses, in particular, contains polyphenols, tannins, and melanoidins, which may contribute to microbial inhibition (discussed in the next section). Melanoidins, formed during Maillard reactions in sugar processing, may increase molasses' antioxidant properties but also act as antimicrobial agents, requiring pre-treatment in sensitive fermentations.

Overall, SCM's high sugar concentration, rich mineral profile, and acidity make it a valuable but complex substrate for biotechnological processes. Careful management of inhibitory compounds, such as excess salts and polyphenols, is necessary for successful microbial valorization.

I.1.2.2. Role in supplementing Carbon/Nutrient deficiencies

Sugarcane molasses plays a critical role in supplementing carbon and essential nutrients in various bioprocesses, particularly in microbial fermentation, anaerobic digestion, and mixed-substrate waste valorization. Due to its high sugar content, trace minerals, and organic acids, molasses is frequently used as a co-substrate to enhance microbial growth and metabolic efficiency in fermentation-based processes where primary feedstocks lack sufficient energy sources or micronutrients (Zhang et al., 2021).

I.1.2.2.1. Carbon source for microbial metabolism

The rich carbohydrate composition of molasses (40–60% fermentable sugars) makes it an excellent carbon source for microbial metabolism. Sugars such as sucrose, glucose, and fructose are directly fermentable (Li et al., 2022), making SCM an attractive input for various industrial applications, including:

- Ethanol and Butanol Fermentation: *Saccharomyces cerevisiae* and *Clostridium spp*. utilize the fermentable sugars in molasses to produce ethanol and butanol, key biofuels for industrial applications (Gutiérrez-Rivera et al., 2015; Nikolaou and Kourkoutas, 2018; Wardani et al., 2023a).

- Lactic & Citric Acid Production: *Lactobacillus* and *Aspergillus* species can efficiently convert the hexose sugars in molasses into organic acids used in the food and pharmaceutical industries (Deme & Asfaw, 2020; Saavedra et al., 2021).

- Biogas Enhancement in Methanogenic Digestion: The high level of carbohydrates in molasses serves as a fast-acting carbon source, improving gas yield in anaerobic co-digestion with nitrogen-rich substrates, such as agricultural waste or wastewater sludge (Chen et al., 2024).

Since many organic wastes and byproducts have insufficient fermentable carbon, molasses is often used as a supplement to optimize the carbon-to-nitrogen (C/N) ratio, enhancing microbial degradation rates and preventing process imbalances.

I.1.2.2.2. Enhancing the carbon-to-nitrogen (C/N) ratio in bioprocesses

A balanced C/N ratio is essential for efficient microbial metabolism in biofermentation and biogas generation (Zhang et al., 2022). Many organic wastes (such as agricultural residues, food waste, and protein-rich substrates) have high nitrogen content but insufficient carbon, leading to microbial inhibition due to ammonia accumulation in anaerobic digestion (Salangsang et al., 2022).

- Sugarcane molasses, due to its carbon-rich profile and moderate nitrogen content (~0.4–1%), serves as an effective C/N ratio enhancer, allowing microbial consortia to efficiently degrade waste materials while stabilizing pH and ammonia toxicity (Wechgama et al., 2017).

- In anaerobic co-digestion systems, combining SCM with protein-dense byproducts (e.g., slaughterhouse waste, dairy effluents, or brewery waste) enhances microbial growth and prevents nitrogen overloading, which otherwise reduces microbial viability (Karki et al., 2021).

Optimal C/N ratios vary depending on the end-use process:

- Ethanol fermentation: Requires a C/N ratio of ~40:1, which molasses can help maintain when combined with Urea or Ammonium phosphate supplementation (Manikandan & Thangavelu, 2010).

- Methanogenic digestion: Typically functions best at C/N ratios between 20:1 and 30:1, necessitating molasses supplementation in nitrogen-rich waste mixtures (Rajlakshmi et al., 2023).

I.1.2.2.3. Mineral and micronutrient contributions

Beyond its function as a carbon source, sugarcane molasses also provides key micronutrients that enhance enzymatic activity and microbial growth, especially in industrial fermentation:

- Essential Minerals in SCM: Iron (Fe), Magnesium (Mg), Zinc (Zn), Calcium (Ca), and Potassium (K) act as cofactors for enzymatic pathways driving microbial fermentation and anaerobic bioconversion (Vicentini-Polette et al., 2024; Walker, 2004).
- Enzyme Activation: Magnesium and Zinc, for instance, are crucial for the activity of hexokinase, alcohol dehydrogenase, and phosphofructokinase, which regulate sugar metabolism in ethanol fermentation (Kounbesiou et al., 2011).
- Iron Supplementation in Anaerobic Digestion: Iron plays a vital role in electron transfer reactions during methanogenesis, improving microbial redox balances in anaerobic systems (Ugwu et al., 2020).

However, high salt concentrations (e.g., potassium and sodium chloride) in molasses require modulation to prevent microbial growth inhibition. This is particularly relevant in yeast fermentations, where excessive ionic strength reduces osmotic balance, stress tolerance, and ethanol yield (Ndiaye et al., 2024).

I.1.2.2.4. Nitrogen supplementation considerations

While molasses naturally contains some nitrogen compounds, its total nitrogen content is relatively low (~0.4–1%) compared to nitrogen-rich organic wastes (Wechgama et al., 2017). To optimize microbial metabolism in fermentation systems, nitrogen supplementation is often required, particularly in ethanol, butanol, and organic acid fermentations (Antolinez et al., 2016).

Table 2. Table: Comparative Overview of Sugarcane Molasses Physicochemical Composition from Different Studies

Reference	Geographic Origin	Total Sugar (% w/w)	COD (g/L)	pH	Total Nitrogen (% w/w)	Ash Content (% w/w)	Key Minerals (mg/L or % w/w)
(Sampaio et al., 2022)	Brazil	43–55%	90–250 g/L	4.5–6.0	0.4–1.0%	8–12%	K (1.5–3.0%), Ca (0.5–2.0%), Mg (0.3–0.6%)
(Thakare et al., 2013)	India	48–52%	120–200 g/L	4.8–6.2	0.6–0.9%	10–14%	Na (0.2–0.5%), Fe (200–500 mg/L), Zn (50–100 mg/L)
(S. Hassan et al., 2019)	Egypt	45–50%	100–180 g/L	4.7–5.8	0.5–0.8%	9–13%	Cl (0.3–1.1%), P (0.02–0.4%), S (0.1–0.6%)
(Meelom et al., 2023)	Thailand	50–60%	110–190 g/L	5.0–5.5	0.7–1.2%	7–11%	K (2.0–3.5%), Ca (0.6–1.5%), Mg (0.3–0.8%), Na (0.3–0.7%)
(Palmonari et al., 2020)	Italy	47–53%	105–190 g/L	4.5–5.8	0.4–0.9%	11–15%	P (0.08–0.5%), Fe (180–600 mg/L), Cu (20–90 mg/L)
(Samaniego-Sánchez et al., 2020)	Spain	44–50%	130–200 g/L	4.6–5.9	0.5–1.0%	10–13%	Zn (40–120 mg/L), Mn (10–60 mg/L), Ni (5–20 mg/L)

I.1.3. Cheese/Milk whey

Cheese whey, also referred to as milk whey, is the principal liquid by-product generated during the coagulation of milk in cheese production. It accounts for approximately 85–95% of the original milk volume and retains about 55% of its nutrients (Utama et al., 2017). Due to its high lactose content and the presence of soluble proteins, peptides, and micronutrients, whey has attracted interest as a fermentation feedstock in biotechnological applications. However, when untreated, it presents a significant environmental burden due to its high organic load. A thorough understanding of its composition and physicochemical characteristics is essential for optimizing its use in microbial fermentation systems.

I.1.3.1. Composition and Physicochemical Properties of Milk/Cheese Whey

Whey wastewater, a by-product of cheese manufacturing, represents a significant organic load in dairy effluents due to its complex biochemical composition. It is generated in large volumes and is considered a high-strength organic waste, primarily because of its residual lactose content, bioactive proteins, and micronutrients (Lievore et al., 2015; Maidali et al., 2024; Utama et al., 2017). As such, it poses challenges for disposal while also offering opportunities for valorization through microbial fermentation.

The primary carbon source in whey is lactose, a disaccharide sugar composed of glucose and galactose, which can serve as a substrate for fermentation by specific microorganisms (Oda & Nakamura, 2009; Sandoval-Salas et al., 2021; Tebbouche et al., 2024). However, traditional industrial yeasts like *Saccharomyces cerevisiae* lack the innate ability to metabolize lactose, requiring either genetic engineering or co-culturing with lactose-positive species such as *Kluyveromyces marxianus* (Costa et al., 2022; Ohstrom et al., 2023).

Beyond lactose, whey contains various nutrients that contribute to its value as a microbial substrate. These include essential minerals and vitamins retained in the permeate fraction following ultrafiltration for whey protein concentrate production, as observed in studies of cheese whey permeate (CWP) (Maestre et al., 2021). Such components may support microbial growth beyond basic carbon metabolism.

The high organic content of whey is reflected in its physicochemical indicators. Whey wastewater typically exhibits elevated Biological Oxygen Demand (BOD) and Chemical

Oxygen Demand (COD), signaling a substantial pollutant load and necessitating effective treatment strategies when not valorized (Maidali et al., 2024). These values contribute to the significant environmental impact associated with whey disposal, particularly at small- and medium-scale dairy operations (Utama et al., 2017).

In some cases, whey is pretreated through deproteinization or enzymatic hydrolysis to optimize its use as a fermentation feedstock (Das et al., 2017). The removal or degradation of proteins may be employed to reduce foaming or to enhance microbial accessibility to lactose. Nevertheless, the residual nitrogen, peptide, and mineral content of even deproteinized whey can continue to support microbial metabolism under appropriate conditions (Utama et al., 2017).

I.1.3.2. Synergistic Effects When Combined with OOWW and Molasses

The combination of cheese whey with other agro-industrial residues such as olive oil wastewater (OOWW) and molasses has theoretical potential to yield synergistic effects in microbial fermentation systems aimed at producing bioethanol, organic acids, and other value-added metabolites. Each of these substrates features distinct chemical profiles that may complement each other when co-fermented, enhancing both microbial growth and metabolic efficiency. Although no existing study in the reviewed literature explicitly investigated the simultaneous combination of all three substrates, individual studies involving binary mixtures or related systems provide evidence of potential synergy.

I.1.3.2.1. Complementary Substrate Profiles

Cheese whey serves as a nutrient-rich substrate, primarily valued for its lactose content but also containing minerals, peptides, and other bioavailable compounds that support microbial growth (Das et al., 2017; Domingues et al., 2010; Maestre et al., 2021; Maidali et al., 2024). Molasses, by contrast, is rich in easily fermentable sugars such as sucrose, glucose, and fructose. When combined with whey, molasses has been shown to enhance ethanol yields due to the diversity of carbon sources (Álvarez-Cao et al., 2020; Oda & Nakamura, 2009; Utama et al., 2017). For example, the inclusion of 10% molasses in whey fermentation mixtures improved ethanol production in systems using both natural and immobilized yeasts (Halema, 2014; Tesfaw, 2023; Tesfaw et al., 2021).

Olive oil wastewater (OOWW), while not addressed directly in any of the reviewed studies, presents a distinctive profile rich in organic acids and polyphenolic compounds. These phenolics can inhibit microbial growth, especially in yeasts such as *Saccharomyces cerevisiae*, unless pretreated effectively. However, cheese whey contains amino acids and peptides that may contribute to phenolic detoxification or act as protective agents for microbial cells, potentially mitigating OOWW toxicity in mixed-substrate settings.

I.1.3.2.2. Interaction with Microbial Systems

Several studies demonstrate the improved performance of microbial systems when multiple substrates are used. In particular, *Kluyveromyces marxianus* and genetically modified *Saccharomyces cerevisiae* benefit from mixed sugar sources in molasses-whey systems, which allow simultaneous or sequential use of lactose and sucrose (Domingues et al., 2010; Oda & Nakamura, 2009; Tesfaw et al., 2021). Moreover, co-cultures of *Saccharomyces cerevisiae* with *Bacillus megaterium* and *Bacillus subtilis* in whey fermentation media have been explored in at least one study (Maidali et al., 2024), resulting in enhanced ethanol yields and demonstrating tolerance of whey's complex nutrient profile. Although this co-culture system did not include OOWW or molasses, the successful use of mixed microbial species in whey broth offers a relevant model for future development.

Sequential fermentation systems are another relevant paradigm: studies involving initial ethanol production from whey by lactose-positive yeasts, followed by acetic acid fermentation using *Acetobacter spp*. and *Clostridium spp*., highlight the feasibility of staged bioconversions (Ellis et al., 2014; Maestre et al., 2021). While these studies did not employ *Bacillus* species for acetic acid conversion, the foundational model supports the possibility of integrating *Bacillus* strains capable of ethanol oxidation into broader co-culture or sequential schemes using complex substrates.

I.2. Availability in Algeria

Algeria's agro-industrial sector plays a pivotal role in its economy, contributing approximately 12% of GDP and employing over 20% of the workforce (National Office of Statistics, 2023). However, the sector's growth has generated substantial volumes of

underutilized by-products, including olive oil wastewater (OOWW), sugarcane molasses, and dairy whey as shown in Table 3. These wastes, if improperly managed, pose severe environmental and economic risks, particularly in water-scarce regions where agricultural pollution exacerbates existing resource constraints. Despite Algeria's status as a major fossil fuel exporter, its agro-industrial sector remains heavily reliant on linear production models, with limited adoption of circular economy practices. This section examines Algeria's key agro-industrial by-products, their production volumes, environmental impacts, and the systemic challenges hindering sustainable waste management. By contextualizing these issues, the chapter underscores the urgency of adopting biotechnological valorization strategies to mitigate ecological harm, diversify revenue streams, and align with global sustainability frameworks.

Algeria's agro-industrial sector produces significant volumes of organic waste, with olive oil wastewater (OOWW), sugarcane molasses, and dairy whey representing the most prominent by-products. Among these, OOWW stands out as the most environmentally hazardous due to its high pollutant load and widespread mismanagement. This section examines OOWW's production volumes, ecological risks, and emerging valorization strategies to address its environmental footprint.

Table 3. Summary of key agro-industrial by-products in Algeria, including production volumes, composition, and environmental risks.

By-Product	Annual Production in Algeria	Key Components	Environmental Challenges
Olive Oil Wastewater (OOWW)	1–1.5 million m^3 (from ~100,000–150,000 tons olive oil)	High COD (80–200 g/L), phenolics (8–24 g/L), acidity (pH 4–5)	Soil degradation, water pollution, phenolic toxicity, oxygen depletion
Sugarcane Molasses	~96,000–160,000 tons (estimated based on production)	Sucrose (40–60%), minerals, vitamins	Nutrient runoff causing eutrophication if untreated
Milk Whey	~100,000 tons/year (from dairy sector)	Lactose, proteins, calcium, phosphorus	High BOD load, risk of water contamination, underutilized nutrient source

(Sources: (Bougherara et al., 2021b; Bouizar et al., 2021; Djeziri et al., 2023; Gueboudji et al., 2022a; Smeti et al., 2019; Tebbouche et al., 2024).

I.2.1. Olive mill wastewater

As one of the world's top 10 olive oil producers, Algeria generates approximately 100,000–150,000 tons of olive oil annually, yielding 1–1.5 million cubic meters of OOWW each year (International Olive Council, 2023). Over 70% of this effluent originates in northern provinces (Figure 1) such as Tizi Ouzou, Béjaïa, and Sétif, where small-scale mills dominate production (Bougherara et al., 2021b). OOWW's hazardous properties—including a chemical oxygen demand (COD) of 80–200 g/L, acidity (pH 4–5), and phenolic compound concentrations of 8–24 g/L—pose severe risks to ecosystems (Djeziri et al., 2023).

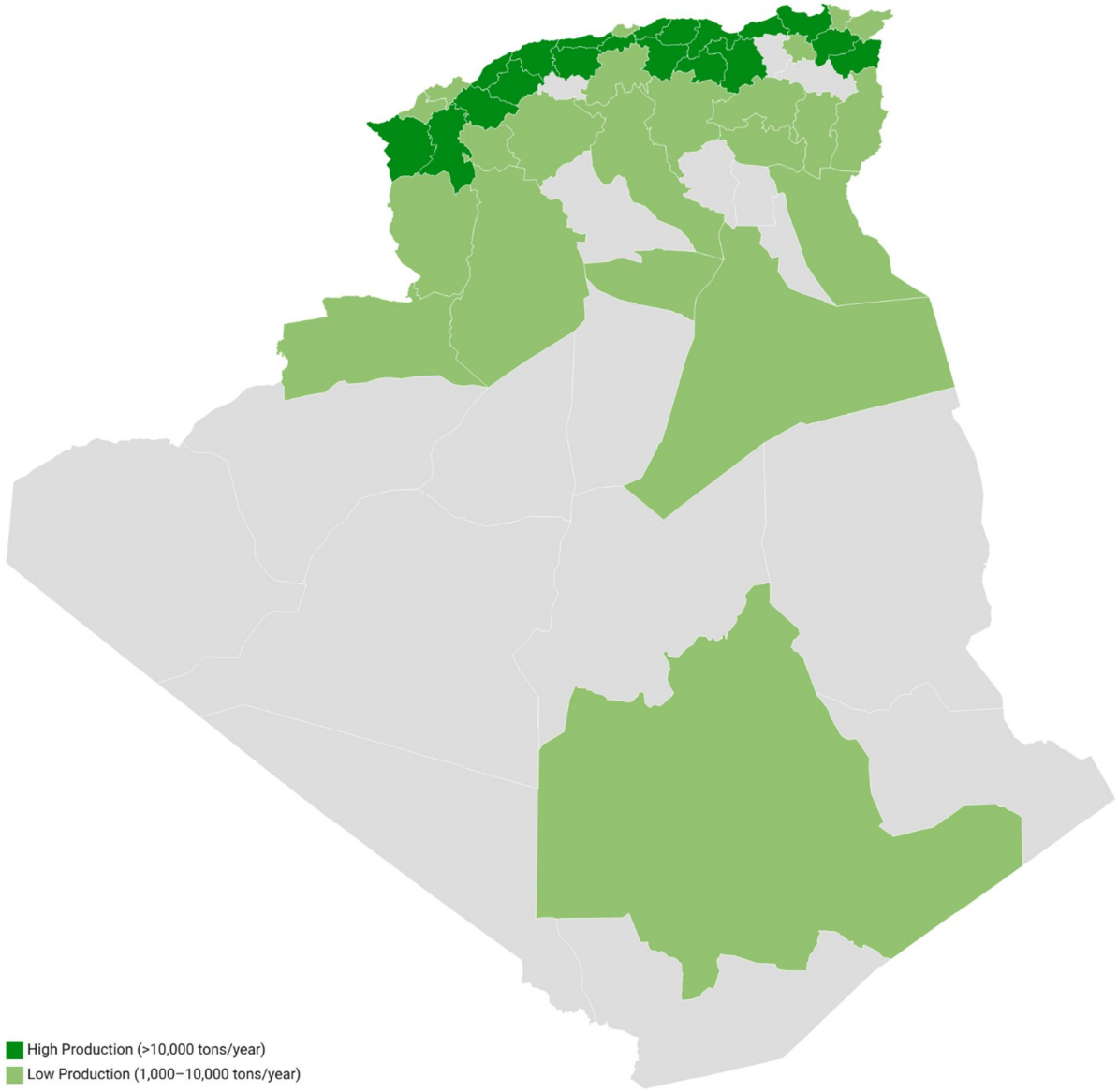


Figure 1. Map of Algeria highlighting olive oil production hubs (high/low/non-producing provinces) (Attallaoui, 2022)

The environmental impacts of untreated OOWW are multifaceted. In agricultural regions like Tlemcen, phenolic compounds have been shown to reduce soil microbial activity and crop yields, with wheat production declining by 40% in contaminated areas (Khdair & Abu-Rumman, 2017). Similarly, Studies from Mediterranean and North African river basins have shown that discharges of untreated olive oil mill wastewater can deplete dissolved oxygen levels, promote algal blooms, and lead to substantial reductions in macroinvertebrate biodiversity (e.g., reductions of up to 60% in some cases) (Smeti et al., 2019). Groundwater contamination further exacerbates water scarcity, particularly in the Mitidja Plain, where nitrate levels exceed World Health Organization (WHO) safety limits by fourfold (Khouli et al., 2021).

Current disposal practices remain largely unsustainable. Despite regulatory frameworks like Decree No. 07-149 (2007), which mandates industrial effluent treatment, an estimated 70% of OOWW is discarded untreated. Common methods include uncontrolled land spreading, which accelerates soil acidification; evaporation lagoons that concentrate pollutants in arid regions like Sétif; and illegal dumping into seasonal rivers (wadis), which disperses contaminants during rainfall (Hamli et al., 2024).

Emerging biotechnological solutions aim to mitigate these challenges. For instance, researchers have explored the potential of utilizing OOMW as substrates for bioethanol production. In one study, molasses—a byproduct of the sugar industry—was blended with crude OOMWs and fermented using *Saccharomyces cerevisiae* immobilized on delignified cellulosic material. This process achieved ethanol concentrations of up to 67.8 g/L, with daily productivity reaching 67.6 g/L/day at temperatures of 20 °C and above (Nikolaou & Kourkoutas, 2018).

Another study focused on isolating indigenous yeast strains capable of fermenting olive mill solid wastes (OMSW). Two strains, *Issatchenkia orientalis* and *Pichia galeiformis/manshurica,* were identified. While these strains efficiently utilized xylose to produce xylitol, ethanol production was not detected under the conditions tested (Abu Tayeh et al., 2014).

These findings highlight the potential of *S. cerevisiae* in bioethanol production from olive oil industry byproducts, though the efficiency varies depending on the specific substrates and fermentation conditions employed (Chang et al., 2018).

Co-digestion of OOWW with cattle manure can significantly enhance biogas production. For instance, one investigation reported that combining these substrates resulted in a 293–351% increase in methane yield compared to digesting olive mill waste alone (Rubio et al., 2019). Another study observed that co-digestion led to a 50% increase in specific methane yield. These findings highlight the potential of co-digestion strategies to improve the efficiency of biogas production from OOWW (Laabidi et al., 2023; Rubio et al., 2019). Additionally, adsorption techniques using olive pomace-derived biochar have recovered 90% of phenolic compounds for potential reuse in pharmaceuticals, showcasing OOWW's untapped value (Dib et al., 2022).

I.2.2. Sugarcane Molasses

Sugarcane molasses, a nutrient-rich by-product of Algeria's sugar industry, holds significant yet underutilized potential in both food innovation and biotechnological valorization. Annually, Algeria produces 96,000–160,000 tons of molasses alongside its 3.2 million tons of sugar, primarily in northern regions (APS, 2023). This underutilized resource holds transformative potential across biotechnology, agriculture, and food sectors, yet over 60% remains discarded, exacerbating environmental and economic inefficiencies. Below, we explore its diverse applications and challenges in the Algerian context (Chikhoune et al., 2014; Lee et al., 2023, 2023; Veana et al., 2014).

Molasses serves as a cost-effective substrate for microbial synthesis of high-value compounds. For instance, fermentation with *Aspergillus niger* produces 70–80 g/L citric acid, a critical ingredient in pharmaceuticals and food preservatives, potentially reducing Algeria's €8 million annual imports (Chikhoune et al., 2014; Deme & Asfaw, 2020). Similarly, *Bacillus subtilis* cultivated on molasses yields proteases and amylases—enzymes essential for drug formulation and industrial processes (Mihajlovski et al., 2016; Simair et al., 2017). Emerging research also highlights its role in antibiotic production, with *Bacillus spp.* synthesizing antimicrobial agents from molasses-derived sugars (Li et al., 2023).

Algerian researchers have optimized molasses-based bioethanol production using locally isolated *Saccharomyces cerevisiae* strains, achieving yields of 71.24 g ethanol/L molasses under controlled conditions (pH 5.5, 30°C) (Kechkar et al., 2024). Beyond biofuels, molasses serves as an effective substrate for cultivating yeast biomass. Notably, *Kluyveromyces marxianus* NS127 has demonstrated a biomass yield of 0.63 grams per gram of molasses, achieving a dry biomass concentration of 66.64 g/L and a protein yield of 28.37 g/L. The

extracted protein exhibits excellent solubility (62.55%) and emulsification properties (13.15 m^2/g) under neutral conditions, alongside high foaming stability (93.70–99.20%) across a broad pH range (3–11). These attributes underscore its potential as a viable alternative protein source for applications in baking and probiotic supplements (Dong et al., 2025).

Molasses' fermentable sugars facilitate the sustainable synthesis of premium flavors. For instance, solid-state fermentation (SSF) of sugarcane bagasse and sugar beet molasses using *Kluyveromyces marxianus* has been optimized to produce aroma compounds. Under specific conditions—30 °C, 25% molasses (dry basis), and a specific air flow rate of 0.11 $L\ h^{-1}\ g^{-1}$ initial total solids—this process yielded 47.6 mg of esters per gram of initial total solids, imparting a pleasant fruity odor due to the higher ester content (35%) (Martínez et al., 2017).

In agricultural trials, Molasses enhances biofertilizer efficacy by serving as a carbon-rich substrate for microbial activity and nutrient mineralization. In biotransformation trials, a blend of 12.5% molasses with poultry waste and algae, fermented by Aspergillus niger, met NF U44-551 standards and improved barley germination (Ozi et al., 2023). Similarly, molasses combined with coconut water and microbial consortia (EOM) yielded liquid fertilizer with elevated NPK levels (N: 0.09%, P: 0.04%, K: 10.5%), validated in hydroponic paddy trials (Darmawan et al., 2020). These applications demonstrate molasses' versatility in converting agro-waste into soil-enriching fertilizers, aligning with Algeria's circular agriculture goals.

Despite these opportunities, Algeria faces barriers such as phenolic inhibitors in molasses, which hinder microbial activity without enzymatic pretreatment. Policy gaps further stall progress, as the nation lacks incentives for high-value applications compared to EU Circular Economy frameworks. To address this, strategic investments in pilot plants for citric acid and vanillin production, coupled with farmer training on safe molasses use, could unlock its potential. Regulatory enforcement under Decree No. 07-149 is also critical to mandate valorization and reduce untreated disposal.

By prioritizing these strategies, Algeria can transform molasses from an environmental liability into a pillar of its sustainable bio-economy.

I.2.3. Milk/Cheese Whey

Whey, a nutrient-dense by-product of cheese manufacturing, is rich in bioactive compounds such as soluble proteins (e.g., β-lactoglobulin), lactose, vitamins (B2, B12), and minerals like calcium and phosphorus. Despite its potential as a functional food ingredient, Algeria's dairy sector struggles to harness this resource sustainably. Annual cheese production of approximately 1,540 tons results in nearly 14 million liters of whey, with major facilities like Giplait-Numidia generating 1,800 liters per production batch—discarding over 2.5 million liters annually as untreated waste (Benaissa, 2018; Bouizar et al., 2021; FAO, 2023).

The environmental toll of unchecked whey disposal is substantial. Its decomposition demands excessive oxygen, with biochemical oxygen demand (BOD) levels reaching 30,000–50,000 mg/L, depleting aquatic ecosystems and acidifying soils in regions such as Constantine. For instance, Giplait-Numidia's weekly discharge of 216,000 liters into local waterways has degraded soil fertility and aquatic biodiversity (Bouizar et al., 2021).

Several innovative strategies have been developed to valorize cheese whey, transforming it from an environmental burden into a valuable resource. Fermentation with *Kluyveromyces marxianus* converts whey lactose into bioethanol, achieving yields up to 0.62 g ethanol/g lactose (Sandoval-Salas et al., 2021), while enzymatic biocatalysis enables the production of oligosaccharides, lactic acid, and bioethanol under eco-friendly conditions (Illanes, 2011). Integrated bioprocesses also allow whey to be converted into single-cell protein (SCP), bioplastics precursors, and bioflavors, enhancing its economic viability (Addai et al., 2020). Technologies such as membrane filtration and bioreactors further improve lactose recovery, protein concentration, and effluent treatment, significantly reducing environmental impact (Pais-Chanfrau et al., 2018). Additionally, techno-economic assessments (Argenta & Scheer, 2020; El-Aidie & Khalifa, 2024) confirm the scalability of these valorization systems, and advancements in functional foods highlight whey's role in probiotic beverages and protein-enriched products (Barba, 2021).

Academic institutions have played a key role in advancing these valorization strategies. Notably, initiatives such as probiotic beverages leveraging *Lactobacillus* cultures (Bouizar et al., 2021), whey-enriched dairy alternatives like ricotta and ice cream, and partnerships with industry leaders have showcased whey's potential. A joint project between Giplait-Numidia and the GENIAAL Food Engineering Lab successfully demonstrated whey's application in

chocolate mousse production, evaluating its sensory and structural properties (Bouizar et al., 2021).

Despite these advancements, industrial adoption in Algeria remains limited. Barriers such as fragmented policies, inadequate processing infrastructure, and lack of market incentives hinder progress. Algeria's environmental regulations, including Decree No. 07-149, lack specific mandates for whey recycling, and cost-efficiency concerns often outweigh ecological considerations for dairy firms. Addressing these challenges through supportive policy frameworks, investment in infrastructure, and fostering academia-industry collaborations is essential to unlock the full potential of whey within a sustainable bioeconomy model.

To address this, policymakers must prioritize circular economy frameworks, such as subsidizing filtration technologies for lactose recovery and fostering academia-industry partnerships. Concurrently, promoting consumer awareness of whey-based products—from protein supplements to biodegradable packaging—could catalyze market demand, aligning Algeria's dairy sector with global sustainability benchmarks.

I.3. Problems related to their accumulation

The uncontrolled accumulation of agro-industrial by-products—olive oil wastewater (OOWW), sugarcane molasses, and dairy whey—has escalated into a multifaceted crisis, threatening ecosystems, economies, and public health across Algeria. Despite their inherent organic value, these materials remain underutilized due to systemic inefficiencies in waste management, technological adoption, and policy enforcement. Below, we dissect the interconnected challenges posed by their mismanagement.

I.3.1. Environmental Impact

The uncontrolled disposal of agro-industrial by-products, such as olive mill wastewater (OMW), molasses, and whey, poses significant environmental challenges. When these residues are released into the environment without adequate treatment, they can lead to soil degradation, water contamination, and air pollution.

I.3.1.1. Soil pollution

The uncontrolled disposal of agro-industrial by-products such as olive mill wastewater (OMW), molasses, and whey into the environment poses significant risks to soil health and fertility.

Olive Mill Wastewater (OMW): OMW is characterized by a high organic load and a substantial concentration of phenolic compounds, which are known for their phytotoxic and antimicrobial properties. These phenolics can inhibit soil microbial activity, leading to disruptions in nutrient cycling and a decline in soil fertility. For instance, Mekki et al. (2013) observed that the application of untreated OMW to soil resulted in a significant decrease in microbial biomass and activity, indicating a detrimental impact on the soil's biological health.

Furthermore, the accumulation of organic matter from OMW can lead to soil acidification, adversely affecting plant growth. The acidic nature of OMW, primarily due to organic acids and phenolic compounds, can lower soil pH, making it less hospitable for many plant species. Studies by (Mekki et al., 2013; Mohawesh et al., 2017) highlighted that soils treated with OMW exhibited a significant drop in pH levels, which could impair nutrient availability and uptake by plants.

Additionally, the leaching of phenolic compounds from OMW into deeper soil layers can have long-term environmental implications. Research by (El Hassani et al., 2023) demonstrated that phenolic compounds from OMW could percolate down to a depth of 1.2 meters within four months of application, potentially contaminating groundwater sources.

Molasses, a nutrient-rich by-product of sugar refining, can cause serious environmental harm when improperly disposed of on land. Studies have shown that untreated molasses wastewater can lead to the accumulation of heavy metals (e.g., cadmium and lead), soil acidification, and inhibited seed germination due to altered nutrient availability (Jiranuntipon, 2008; Li et al., 2020; Verma et al., 2011). A recent study showed that the addition of molasses affects both the physical stability of soil and the structure of microbial communities. At a 10% concentration, molasses improved soil surface hardness significantly and showed good resistance to wind erosion, although the effect on rain erosion remained limited. Microbial analysis revealed that moderate concentrations (1%) of molasses could enhance carbohydrate and energy metabolism pathways, while higher concentrations (above 5%) increased the

abundance of potentially pathogenic species, suggesting that careful dosing is essential for environmental safety (Wang et al., 2024). These findings highlight the dual potential and risks of molasses in soil management applications and the importance of optimized formulations for safe environmental use.

Whey: Whey, a by-product of cheese production, is rich in organic matter, including lactose, proteins, and fats. When disposed of in large quantities without treatment, whey can lead to soil pollution (Zandona et al., 2021). (Marwaha & Kennedy, 2007) reported that inadequate whey removal could affect the physical and chemical composition of the soil, reduce agricultural production, and release volatile organic compounds, which can then pollute the air and cause health problems. However, recent studies have explored the potential benefits of whey application in agriculture. For instance, (Akay & Sert, 2020) investigated the effects of whey application on soil biological properties and plant growth. Their findings indicated that whey application positively influenced soil microbial activity and enhanced plant growth parameters, suggesting that, when properly managed, whey can serve as a beneficial soil amendment. Nonetheless, the application rate and environmental conditions must be carefully considered to mitigate potential negative impacts.

In summary, the disposal of OMW, molasses, and whey without proper treatment can lead to soil pollution through the inhibition of microbial activity, acidification, accumulation of heavy metals, and the leaching of harmful compounds. These effects underscore the need for effective management and treatment strategies for agro-industrial by-products to protect soil health and prevent environmental degradation.

I.3.1.2. Groundwater contamination

The contamination of groundwater is a critical environmental concern associated with the uncontrolled disposal of agro-industrial by-products such as olive mill wastewater (OMW), molasses-based effluents, and dairy whey. These residues, often rich in soluble organic and inorganic compounds, can percolate through the soil profile and infiltrate aquifers, thereby altering the physicochemical quality of groundwater. This process is facilitated by leaching, especially in porous soils or during periods of high rainfall and irrigation.

According to the Food and Agriculture Organization (FAO), high concentrations of nitrates, phosphates, and dissolved organic matter in effluents can leach into groundwater,

leading to serious health and environmental consequences. One of the most documented health risks is *methemoglobinemia* ("blue baby syndrome"), which occurs primarily in infants exposed to elevated nitrate levels in drinking water (Bouselsal et al., 2025; FAO, 2020).

Beyond nitrates, research has shown that other substances such as phenolics from OMW, residual sugars and salts from molasses, and proteins and lactose from whey may also migrate into groundwater systems. Multiple studies highlighted that the application of untreated distillery effluent to agricultural soils resulted in the leaching of organic and inorganic ions, altering key groundwater parameters such as pH, electrical conductivity, and color—ultimately reducing water potability and ecosystem compatibility (Huang et al., 2024).

Moreover, although whey has been promoted as a potential soil amendment due to its nutritional profile, (Akay & Sert, 2020) caution that high application rates can cause nutrient leaching and microbial imbalances if not carefully managed. Their study found that while whey could enhance soil microbial activity and plant growth under controlled conditions, excessive accumulation of organic material increased the risk of contaminant transport into deeper soil layers, particularly in poorly drained soils.

Therefore, the unregulated application or disposal of agro-industrial by-products can significantly contribute to groundwater pollution, especially in regions lacking treatment infrastructure or environmental monitoring systems. To reduce these risks, it is essential to implement pretreatment strategies, establish application thresholds, and integrate monitoring programs that track groundwater quality near disposal or reuse sites.

I.3.1.3. Greenhouse gases emission

Furthermore, the emission of greenhouse gases (GHGs) resulting from the decomposition of agro-industrial by-products contributes significantly to climate change and atmospheric pollution. When residues such as olive mill wastewater (OMW), molasses, or whey are disposed of in open environments—such as lagoons, unlined pits, or surface land—anaerobic microbial activity rapidly sets in due to the high organic load and oxygen-limited conditions. This leads to the production of methane (CH_4) and nitrous oxide (N_2O), two of the most potent greenhouse gases (Ahmed et al., 2019; Jiranuntipon, 2008; Zandona et al., 2021).

Methane, in particular, has a global warming potential approximately 28–34 times greater than carbon dioxide (CO_2) over a 100-year timescale, while nitrous oxide is approximately 298 times more potent (López et al., 2013). These emissions occur not only from raw waste but also from partially treated or even stabilized effluents when they are not managed properly. The microbial degradation of residual sugars, lipids, proteins, and phenolic compounds in these wastes accelerates gas production, especially in warmer climates (Kharitonov et al., 2021).

In addition, liquid effluents stored in anaerobic lagoons or disposed of in poorly ventilated conditions tend to trap heat and moisture, creating ideal conditions for methanogenic archaea and denitrifying bacteria. For example, dairy whey that is rich in lactose and organic nitrogen, if left untreated, can quickly generate ammonia (NH_3) and nitrous oxide, especially when applied in high volumes to soil (Marwaha & Kennedy, 2007).

Studies have also shown that the carbon footprint of agro-industrial waste mismanagement increases significantly when no valorization strategies are implemented. For instance, composting, anaerobic digestion for biogas recovery, or converting these residues into biofuels or organic acids could significantly reduce the release of GHGs and improve environmental outcomes (Chauhan et al., 2024; Vasić et al., 2021).

Therefore, proper treatment and valorization of agro-industrial by-products are not only essential for pollution control but also play a critical role in climate change mitigation efforts. Implementing closed-loop systems such as anaerobic digesters, bioreactors, or bioethanol production plants can recover energy while reducing uncontrolled emissions to the atmosphere.

I.3.2. Economic impact

The accumulation of agro-industrial by-products presents significant economic challenges for industries, particularly concerning the treatment, storage, and disposal of these wastes (Wagh et al., 2024). For instance, the dairy industry generates substantial volumes of whey, a by-product of cheese production. Managing this by-product without appropriate treatment not only incurs high costs but also represents a missed opportunity for resource recovery (Mollea et al., 2013). Studies have highlighted that small-scale cheese producers often face considerable expenses in disposing of surplus whey, with costs averaging around USD 105 per ton, which can significantly reduce profit margins (Giulianetti de Almeida et al., 2023a).

Moreover, the underutilization of these by-products means that potential value-added products, such as biofuels, bioplastics, and animal feed, are not realized (Foti et al., 2021; Wagh et al., 2024). This under exploitation is often due to a lack of appropriate technologies or infrastructure to process these materials efficiently. For example, whey, rich in organic and nutrient content, remains a massive dairy residue worldwide, with about 42% used for low-value products or directly discharged into water streams, leading to environmental issues like eutrophication (Giulianetti de Almeida et al., 2023b).

In regions where small and medium-sized enterprises (SMEs) dominate the agro-industrial sector, the financial burden of implementing waste valorization technologies can be prohibitive (Takacs et al., 2022). This economic constraint often limits the adoption of sustainable waste management practices and contributes to the persistence of uncontrolled waste disposal. For example, molasses-based effluents, commonly generated in sugar refineries and distilleries, are characterized by high biological oxygen demand (BOD), chemical oxygen demand (COD), and dark-colored melanoidins, all of which require complex and energy-intensive treatment processes (Jiranuntipon, 2008). The treatment of distillery wastewater can account for up to 20–30% of the total operational costs of sugar-based industries, making the adoption of advanced treatment systems unaffordable for smaller operations (Mikucka & Zielińska, 2020).

In many cases, industries resort to direct land application or open dumping of molasses effluents to reduce treatment costs (Jiranuntipon, 2008). However, these practices lead to soil degradation, water contamination, and loss of biodiversity, which in turn impose hidden economic burdens on the environment and public health (FAO, 2020). Furthermore, the dark color and high organic content of molasses waste can cause aesthetic pollution and oxygen depletion in surface waters, reducing their suitability for aquatic life (Kharayat, 2012). As a result, the lack of investment in proper valorization systems not only impacts the environment but also exposes businesses to fines, regulatory penalties, or long-term operational risks.

Developing cost-effective, low-tech solutions such as anaerobic digestion, bio-composting, or co-fermentation with other agro-wastes could help bridge the gap between economic feasibility and environmental responsibility for molasses-rich effluents.

I.3.3. Health-related impact

The improper handling and accumulation of agro-industrial by-products such as olive mill wastewater (OMW), molasses effluents, and whey not only contribute to environmental degradation but also pose significant public health and sanitary risks. Due to their high organic content, these by-products create favorable conditions for the rapid proliferation of microorganisms, including pathogenic bacteria, fungi, and insects that can act as disease vectors.

One of the most common health concerns associated with organic waste accumulation is the emission of foul odors, primarily caused by the anaerobic decomposition of organic matter (Abubakar et al., 2022). These odors result from the production of volatile organic compounds (VOCs), such as ammonia, hydrogen sulfide, and volatile fatty acids, which can irritate the respiratory system and significantly reduce the quality of life for communities near disposal or storage sites (Ilyas et al., 2019). Moreover, these odors often signal underlying microbial activity that may include harmful or opportunistic pathogens (Czarnota et al., 2023).

In the case of olive mill wastewater (OMW), its high concentration of polyphenols, lipids, and organic acids makes it both chemically and biologically unstable. Studies have shown that when OMW is disposed of in open lagoons or near residential or agricultural areas, it can promote anaerobic fermentation and the growth of spoilage microbes, leading to not only bad odors but also the possible spread of fungal spores, mosquito breeding, and waterborne pathogens (Gueboudji et al., 2022a; Mekki et al., 2013). Moreover, the acidic nature and dark coloration of OMW contribute to environmental discomfort and visual pollution.

Similarly, molasses-based distillery effluents can support the proliferation of fungal pathogens and disrupt soil microbial communities. (Wang et al., 2024) reported that even low concentrations (5% v/v) of raw effluent negatively affected beneficial soil bacteria such as *Rhizobium* and *Azotobacter*, while fungal populations increased, some of which can act as plant or human pathogens. Inappropriate land application may also lead to microbial contamination of water bodies, increasing the risk of infections or allergies.

Whey, when left untreated or improperly stored, is known to attract insects and rodents due to its high lactose and protein content. These residues can serve as breeding grounds for pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, and *Clostridium* spp.,

especially under warm and moist conditions. It is also noted that stagnant whey near dairy plants may lead to air and water contamination, cross-infecting adjacent food environments and increasing the risk of foodborne illnesses (Marwaha & Kennedy, 2007; Zandona et al., 2021).

Despite the biological richness of these by-products, their reuse remains limited, primarily due to a lack of affordable treatment technologies, insufficient technical expertise, and weak regulatory enforcement in many developing countries (Vasić et al., 2021). In such contexts, open-air dumping, improper storage, or informal reuse practices further elevate the risk of disease transmission and long-term environmental health issues.

I.4. Possible valorization pathways

The sustainable management of agro-industrial by-products is increasingly viewed not only as an environmental necessity but also as a strategic opportunity for developing bio-based economies. Instead of being discarded, these residues can be transformed into valuable resources through various valorization approaches, depending on their chemical composition, treatment feasibility, and end-use applications. Biotechnology plays a central role in this transformation by enabling microbial, enzymatic, or biochemical conversions that add value while reducing waste burden.

I.4.1. Energy recovery

One of the most widely adopted valorization strategies is energy recovery, particularly through the production of biogas and bioethanol. Biogas, mainly composed of methane (CH_4) and carbon dioxide (CO_2), is produced by the anaerobic digestion of organic materials such as molasses, whey, and olive mill wastewater (OMW). These by-products are rich in fermentable sugars and volatile solids, making them ideal substrates for methanogenic microbial consortia. Studies have demonstrated that co-digestion of whey or OMW with manure or other carbon-rich wastes improves methane yields and process stability (Al Rabadi et al., 2021; Bovina et al., 2021; Laabidi et al., 2023; Rubio et al., 2019; Vasić et al., 2021).

Similarly, bioethanol production via alcoholic fermentation is a viable pathway, especially using molasses, which is already employed at an industrial scale as a sugar-rich feedstock for yeast fermentation (Wardani et al., 2023b). *Saccharomyces cerevisiae* is the most common microorganism used for this process, though co-fermentation strategies combining molasses with OMW or whey have shown improved performance by balancing nutrient profiles

and diluting fermentation inhibitors (Ayadi et al., 2022a; Halema, 2014; Nikolaou & Kourkoutas, 2018; Wardani et al., 2023b). Ethanol derived from these sources can serve both as a biofuel and as a raw material for chemical synthesis, contributing to fossil fuel substitution and carbon footprint reduction (Klein-Marcuschamer et al., 2012).

I.4.2. Agronomic valorization

Agro-industrial residues can also be reused in agriculture through composting or soil amendment after suitable pretreatment (Akay & Sert, 2020; Carmona et al., 2023). Composting transforms organic-rich by-products like olive pomace, vegetable peels, or whey sludge into stable, humus-like fertilizers that improve soil fertility and structure. OMW and molasses can be mixed with lignocellulosic waste (e.g., straw, sawdust) to enhance the carbon-to-nitrogen ratio and accelerate compost maturation (Darmawan et al., 2020; Ozi et al., 2023; Utama et al., 2017).

However, due to their high salt content, low pH, and presence of phytotoxic compounds (particularly in OMW) (Carmona et al., 2023), these by-products often require aerobic treatment, dilution, or co-composting with other biodegradable matter to reduce their environmental risks (Ozi et al., 2023). When properly stabilized, they can contribute to soil organic matter, water retention capacity, and microbial biomass, particularly in arid and semi-arid regions.

I.4.3. Industrial biotechnology

A highly promising valorization pathway involves the use of agro-industrial by-products as substrates for microbial fermentation to produce high-value bioproducts. These include:

- Organic acids (e.g., acetic, lactic, citric),
- Industrial enzymes (e.g., cellulases, amylases, lipases),
- Natural pigments (e.g., carotenoids, melanin),
- Biosurfactants, and even bioplastics.

Such products can be obtained using engineered or naturally adapted microbial strains capable of tolerating the inhibitory compounds present in these waste streams. For example, whey has been successfully used for the microbial production of lactic acid using *Lactobacillus spp.*, while molasses has served as a substrate for citric acid production using *Aspergillus niger*,

and OMW was used for acetic acid by *Bacillus licheniformis* and *Bacillus circulans* (Deme & Asfaw, 2020; Rouam & Meziane, 2025; Saavedra et al., 2021). Olive mill residues have also shown potential in producing polyphenol-rich extracts or being used in solid-state fermentation for enzyme production.

I.4.4. Animal feed

After appropriate treatment to eliminate pathogens and reduce moisture, certain agro-industrial residues may be used in livestock feeding, providing a cost-effective alternative to conventional feed ingredients. Whey, for example, is widely used in pig and calf nutrition, also it was used in its liquid form for supplementation with water on egg production and egg quality in layer chickens due to its high lactose and protein content (S. Kumar et al., 2024; Ryan & Walsh, 2016). Similarly, molasses is used as an energy supplement in ruminant diets and as a palatability enhancer in compound feeds (Mordenti et al., 2021).

Recent research has explored the inclusion of olive mill wastewater (OMW) in animal diets. A study by (Makri et al., 2020) investigated the effects of OMW-supplemented feed on lambs and found that it improved the antioxidant profile of vital organs, suggesting potential health benefits . However, caution is required, as OMW may contain polyphenols, heavy metals, or residual oils that can be toxic to animals. Therefore, detoxification and standardization procedures must be in place before inclusion in feed formulations. Research is ongoing to develop fermentation-based detoxification or adsorbent-based purification processes to render these by-products safe for feed use.

I.5. Biotechnological processes for valorization

I.5.1. Microbial fermentation

Microbial fermentation is a foundation stone of biotechnological processes, enabling the transformation of agro-industrial by-products into a spectrum of value-added compounds. This metabolic activity, carried out by microorganisms such as yeasts, bacteria, and fungi, involves the breakdown of organic substrates—including carbohydrates, proteins, and lipids—into simpler molecules. The process can occur under aerobic or anaerobic conditions, depending on the microorganism involved and the desired end product (Sadh et al., 2018).

I.5.1.1. Types of Fermentation and Their Biochemical Pathways

I.5.1.1.a. Alcoholic fermentation

Predominantly facilitated by yeasts like *Saccharomyces cerevisiae*, alcoholic fermentation converts sugars into ethanol and carbon dioxide. This anaerobic process is widely applied in bioethanol production, brewing, and winemaking, especially using molasses or co-fermentation with whey (Maicas, 2020).

Chemical Equation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_{2+}Energy \quad \text{(Eq.1)}$$

Glucose → Ethanol + Carbon dioxide + Energy

I.5.1.1.b. Lactic acid fermentation

This anaerobic process is driven by lactic acid bacteria such as *Lactobacillus* and *Streptococcus* spp., which convert sugars (like lactose in whey) into lactic acid. It is commonly used in food preservation, bioplastic precursors, and biomedicine (König & Fröhlich, 2017).

Chemical Equation:

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 + Energy \quad \text{(Eq.2)}$$

Glucose → Lactic acid + Energy

I.5.1.1.c. Methane fermentation (anaerobic digestion)

A multi-stage process used to degrade high-organic-load waste like OMW under anaerobic conditions. It is carried out by consortia of methanogenic archaea, yielding methane-rich biogas, an important renewable energy source (Lyu et al., 2018).

Simplified Equation:

$$Organic\ matter \rightarrow CH_4 + CO_2 + Other\ gases + Energy \quad \text{(Eq.3)}$$

Organic matter → Methane + Carbon dioxide + Other gases + Energy

I.5.1.1.d. Other Fermentation Types

- **Acetic acid fermentation:** *Acetobacter* spp. oxidize ethanol to acetic acid (used in vinegar production) (Gomes et al., 2018).
- **Butyric and propionic acid fermentation:** *Clostridium* and *Propionibacterium* spp. produce butyric or propionic acids from sugars or lactate (Ranaei et al., 2020).
- **ABE fermentation (Acetone–Butanol–Ethanol):** A mixed-product fermentation performed by *Clostridium acetobutylicum*, useful in biofuel production (K. Kumar et al., 2024).
- **Hydrogen fermentation:** Biohydrogen production via *Clostridium* spp. or dark fermentation of carbohydrate-rich residues (Albuquerque et al., 2024).

These additional fermentation types are of increasing interest for advanced biorefineries, particularly for the valorization of agricultural residues and the development of bio-based chemical platforms.

I.5.1.2. Operational modes and system configurations

Microbial fermentation can be adapted to various physical states of substrates, target products, and process conditions, leading to the development of two main fermentation modes: Submerged Fermentation (SmF) and Solid-State Fermentation (SSF) (Martău et al., 2021). The choice between them depends largely on substrate characteristics, moisture content, microbial tolerance, and downstream processing requirements.

I.5.1.2.a. Submerged fermentation (SmF)

Submerged fermentation involves the cultivation of microorganisms in a liquid nutrient medium, where the substrate is dissolved or suspended in water. This method is particularly well-suited for agro-industrial by-products that are naturally in liquid form or water-soluble, such as molasses, whey, and diluted olive mill wastewater (OMW) (Shah et al., 2021). Due to its fluid nature, SmF offers advantages in process control, aeration, pH adjustment, and nutrient homogeneity, making it a preferred method in industrial bioreactors and large-scale fermentation systems (Mascarin et al., 2024).

SmF is widely applied in the production of ethanol, organic acids (e.g., lactic and acetic acids), biosurfactants, and microbial enzymes (Kirimura et al., 2011). For instance, molasses-based SmF using *Saccharomyces cerevisiae* remains one of the most common methods for industrial ethanol production (Khonngam & Salakkam, 2019).

Despite its efficiency, SmF generates large volumes of effluent, and the energy cost for sterilization, agitation, and aeration can be significant, especially at industrial scales. Therefore, for high-moisture but inhibitory substrates like OMW, pre-treatment and dilution are often necessary to avoid microbial inhibition and fouling issues (Doriya et al., 2016).

I.5.1.2.b. Solid-State Fermentation (SSF)

Solid-state fermentation operates with solid substrates that possess sufficient moisture to support microbial growth but lack free-flowing water. This method is ideal for lignocellulosic materials such as olive pomace, fruit peels, bran, or agricultural residues—wastes that are often difficult to process in liquid form (Bamidele et al., 2025).

SSF is especially suitable for filamentous fungi (e.g., *Aspergillus spp.*, *Trichoderma spp.*), which thrive under low-moisture conditions and efficiently penetrate complex substrates. It has gained recognition for its use in producing industrial enzymes (e.g., cellulases, amylases), pigments, and biofertilizers, as well as in bioremediation (Boondaeng et al., 2024; Perwez & Al Asheh, 2025). Importantly, SSF mimics the natural microbial habitat, often resulting in higher yields of secondary metabolites compared to SmF (Kumar et al., 2021).

In the context of olive oil residues, SSF presents an effective route to valorize olive pomace, a solid waste rich in fibers and polyphenols. Studies have shown that co-fermentation of olive pomace with other substrates under SSF can lead to significant enzyme production and phenolic recovery (Leite et al., 2021).

While SSF offers lower water consumption and energy requirements, it faces challenges in process monitoring, scale-up, and temperature control. However, recent advances in reactor design, forced aeration, and solid immobilization are helping to overcome these limitations and improve SSF viability at commercial scale (Bamidele et al., 2025).

I.6. Bioconversion and biomolecule production

Bioconversion, also referred to as biotransformation or microbial transformation, is the biological conversion of organic materials—such as plant residues, food processing waste, or animal-derived by-products—into usable products or energy sources. This process involves the action of living microorganisms or their enzymes, which catalyze specific chemical reactions to modify or convert organic compounds into structurally related and often more valuable substances (Sivasubramanian, 2018). Unlike chemical synthesis, bioconversion typically relies on a limited number of enzymatic steps, making it highly selective, efficient, and environmentally friendly (Sarangi & Bhatia, 2022).

Microorganisms such as bacteria, yeasts, and filamentous fungi possess the natural ability to transform a wide array of substrates through specific metabolic pathways. During the bioconversion process, these microbes produce enzymes that target complex molecules—such as carbohydrates, lipids, or proteins—and convert them into simpler or functionally altered compounds (Kanimozhi et al., 2018). Although hundreds of bioconversion reactions are known, only a subset is currently exploited for the commercial production of biofuels, organic acids, enzymes, and nutraceuticals (Sarangi & Bhatia, 2022).

Agro-industrial by-products like molasses, whey, and olive mill wastewater (OMW) are particularly suitable for bioconversion due to their rich organic content and biodegradability (Kumar et al., 2022). However, to ensure efficient microbial assimilation, many of these residues require pretreatment or partial hydrolysis to make their carbon sources (e.g., sucrose, lactose, polyphenols) more accessible for microbial metabolism (Peinemann & Pleissner, 2020). This transformation not only reduces the environmental burden of organic waste but also contributes to circular bioeconomy strategies through the generation of high-value bioproducts under mild, low-energy conditions (Ashokkumar et al., 2022).

I.6.1. Enzymatic hydrolysis of complex substrates

Enzymatic hydrolysis is a pivotal step in the bioconversion of agro-industrial by-products, facilitating the breakdown of complex macromolecules into fermentable monomers (Fagundes et al., 2024). This process employs specific enzymes to cleave the bonds within polysaccharides, rendering them into simpler sugars that microorganisms can readily assimilate for subsequent fermentation or biosynthesis (Houfani et al., 2020).

I.6.1.2. Cellulose and hemicellulose degradation

Cellulose, a linear polymer of β-1,4-linked glucose units, and hemicellulose, a heterogeneous polysaccharide composed of various sugar monomers, constitute significant portions of lignocellulosic biomass found in materials like olive pomace and agricultural residues (Achyuthan et al., 2010). The enzymatic hydrolysis of these polymers involves a synergistic action of cellulases and hemicellulases (Khamassi & Dumon, 2023):

- **Cellulases**, including endoglucanases, exoglucanases, and β-glucosidases, collaboratively degrade cellulose into glucose units (Lambertz et al., 2014).

- **Hemicellulases**, such as xylanases and mannanases, target hemicellulose, releasing sugars like xylose and mannose (Shrivastava et al., 2020).

Effective hydrolysis of lignocellulosic materials often necessitates pretreatment methods (e.g., steam explosion, acid hydrolysis) to disrupt the complex matrix and enhance enzyme accessibility (X. Li et al., 2022; Yu et al., 2022).

I.6.1.3. Starch hydrolysis

Starch-rich by-products, such as molasses and certain cereal residues, are hydrolyzed by amylolytic enzymes:

- **α-Amylase** initiates the process by randomly cleaving internal α-1,4-glycosidic bonds, producing dextrins (de Souza & de Oliveira Magalhães, 2010).
- **Glucoamylase** further hydrolyzes these dextrins into glucose by cleaving both α-1,4 and α-1,6 linkages (Marín-Navarro & Polaina, 2011).

This sequential enzymatic action efficiently converts starch into glucose, serving as a substrate for various fermentation processes (Alias et al., 2021).

I.6.1.4. Lactose hydrolysis

Whey, a by-product of the dairy industry, contains lactose, a disaccharide composed of glucose and galactose (Tsermoula et al., 2021). The enzyme **β-galactosidase** (lactase) catalyzes the hydrolysis of lactose into its constituent monosaccharides, facilitating their fermentation into value-added products such as ethanol and lactic acid (Saqib et al., 2017a).

I.6.1.5. Enhancing hydrolysis efficiency

To improve the efficiency of enzymatic hydrolysis, several strategies are employed:

- **Enzyme immobilization**: Immobilizing enzymes on various supports can enhance their stability and reusability, reducing operational costs (Mirsalami et al., 2024).
- **Membrane bioreactors (MBRs)**: Integrating enzymatic hydrolysis with membrane separation allows continuous removal of hydrolysis products, alleviating product inhibition and enhancing overall conversion rates (Al-Mardeai et al., 2022).

- **Synergistic enzyme cocktails**: Utilizing a combination of enzymes tailored to the specific substrate composition can lead to more efficient hydrolysis (Agrawal et al., 2018).

I.6.2. Production of microbial biomass

During bioconversion, the microbial growth that occurs as microorganisms consume agro-industrial residues not only drives product formation but also results in the accumulation of microbial biomass, which can itself be harvested and utilized as a commercially valuable output. This biomass, depending on the microbial species and cultivation conditions, can serve nutritional, industrial, or environmental functions (Stikane et al., 2022).

I.6.2.2. Single cell proteins

One of the most promising applications of microbial biomass is in the production of Single-Cell Protein (SCP)—a protein-rich biomass derived from yeasts, fungi, algae, or bacteria. SCP offers a sustainable and high-quality source of protein that can be used in animal feed, aquaculture, and potentially even human nutrition, particularly in regions with limited access to conventional protein sources (del Carmen Carranza-Méndez et al., 2022; Y. P. Li et al., 2024). Agro-industrial residues such as molasses, whey, and starch-rich effluents have been successfully used as substrates for SCP production by strains like *Candida utilis*, *Kluyveromyces marxianus*, and *Aspergillus oryzae* (Kim et al., 2020; Li et al., 2024)

In addition, Lactic Acid Bacteria (LAB) grown on whey or lactose-rich media produce not only lactic acid but also probiotic biomass. These bacteria, including *Lactobacillus plantarum* and *Streptococcus thermophilus*, are increasingly utilized in functional foods, nutraceuticals, and gut microbiome therapeutics, thanks to their proven benefits in digestive health, immune support, and metabolic regulation (Berisvil et al., 2021; Choi et al., 2021; Fenster et al., 2019; Martinović et al., 2023).

Filamentous fungi, such as *Rhizopus*, *Trichoderma*, and *Aspergillus* spp., grown in solid-state fermentation (SSF) systems using residues like olive pomace or fruit peels, produce mycelial biomass that can be utilized as a source of enzymes, dietary fiber, or functional bioactive compounds. Mycelial extracts may also contain immunomodulatory polysaccharides, phenolic antioxidants, and other secondary metabolites valuable in pharmaceutical and food industries (Soccol et al., 2017; Yafetto, 2022).

Beyond nutritional and biochemical value, microbial biomass also plays a key role in circular bioresource utilization. Incorporating microbial biomass recovery into agro-industrial biorefineries helps close the nutrient loop, reduces the need for external inputs, and supports zero-waste strategies (Stikane et al., 2022).

I.6.3. Synthesis of high value-added products

In parallel with microbial biomass production, many microorganisms are capable of converting agro-industrial by-products into a wide range of structurally diverse and functional biomolecules, which are of great interest in sectors such as bioenergy, bioplastics, green chemistry, cosmetics, and food processing. These bioconversions offer sustainable and competitive alternatives to petroleum-derived products, especially when optimized for cost and scalability.

I.6.3.2. Organic Acids

Organic acids are among the most common and economically important fermentation products:

- **Lactic acid** is primarily produced by *Lactobacillus* spp. through the fermentation of lactose-rich whey. Global demand for lactic acid continues to rise due to its role in the biodegradable plastics industry (Mejia-Gomez & Balcázar, 2020).
- **Citric acid,** produced from molasses or hydrolyzed starchy wastes by *Aspergillus niger*, is widely used in the food and beverage industry (as a flavor enhancer and preservative), as well as in pharmaceuticals and detergents. Citric acid production accounts for over 1.7 million tons annually, making it one of the most produced organic acids via microbial fermentation (Deme & Asfaw, 2020; Książek, 2023).
- **Acetic acid** is obtained by aerobic oxidation of ethanol by *Acetobacter* spp. in a two-stage process where sugars are first fermented to ethanol and then oxidized to acetic acid. This acid is a key ingredient in vinegar, synthetic fibers, plastics, and solvents, and has also shown promise in antimicrobial surface treatments (Gomes et al., 2018).

I.6.3.3. Alcohols

Fermentative production of alcohols from agro-residues is a well-established and scalable strategy in biofuel technology:

- **Bioethanol** is typically produced from molasses, but co-fermentation with whey and olive mill wastewater (OMW) has shown improved productivity by balancing nutrient levels and diluting inhibitory compounds (Nikolaou & Kourkoutas, 2018; Tesfaw et al., 2021). Bioethanol serves as a transportation fuel, fuel additive, and precursor for green chemical synthesis (Falowo & Betiku, 2023).
- **Butanol**, produced via acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum*, offers higher energy content than ethanol and is compatible with current fuel infrastructure. Molasses and starch-based wastes serve as ideal feedstocks when pretreated appropriately, making butanol an attractive next-generation biofuel (Antolinez et al., 2016; Kumar et al., 2024).

I.6.3.4. Industriel enzymes

Microbial production of industrial enzymes is a growing application of bioconversion, especially when using agro-wastes as low-cost substrates:

- Amylases, cellulases, and proteases produced by *Aspergillus*, *Bacillus*, and *Trichoderma* spp (Simair et al., 2017). are used extensively in detergent formulations, textile desizing, paper bleaching, and food processing (e.g., baking, juice clarification). Substrates like wheat bran, whey protein, and olive pomace have been successfully used to grow these enzyme-producing microbes in both SmF and SSF systems (de Souza & de Oliveira Magalhães, 2010).
- For example, *Bacillus subtilis* grown on dairy wastewater has shown high yields of proteases, and *Trichoderma reesei* on lignocellulosic biomass can yield commercial cellulase preparations (Keshapaga et al., 2023; Xue et al., 2025).

I.6.3.5. Bio surfactants and natural pigments

Emerging bioproducts such as biosurfactants and natural pigments are gaining interest due to their biodegradability, low toxicity, and broad-spectrum bioactivity:

- Biosurfactants, such as rhamnolipids and sophorolipids, can be produced by *Pseudomonas*, *Bacillus*, and *Candida* spp. grown on olive mill wastewater, molasses, or glycerol-rich effluents (Russo-Martínez et al., 2025; Tan & Li, 2018). These compounds reduce surface and interfacial tension and are increasingly used in

bioremediation, cosmetics, oil recovery, and pharmaceuticals (Gudiña et al., 2016) (Bjerk et al., 2021).

- Natural pigments, including carotenoids, melanins, and anthocyanins, can be extracted from microbial fermentation on agro-residues (Grewal et al., 2022; Usmani et al., 2020). *Monascus purpureus*, for instance, can produce red pigments from rice bran, molasses, or fruit waste providing natural alternatives to synthetic food dyes and antioxidants (Srianta et al., 2021).

These bioconversions typically require only a few enzymatic reactions, often facilitated by highly specific microbial pathways, allowing for targeted production under mild process conditions. The efficiency of product yield depends on multiple factors:

- Microbial strain and genetic stability,
- Nutrient and inhibitor content of the substrate,
- Fermentation system (batch, fed-batch, continuous),
- Immobilization or co-culture techniques.

In recent years, integrated biorefineries have sought to combine multiple processes e.g., producing enzymes, acids, and biomass in parallel to maximize value extraction from a single feedstock. This approach not only enhances economic feasibility but also aligns with sustainable development goals (SDGs) by reducing waste generation, replacing petrochemical products, and promoting bio-based industrialization.

I.7. Cell immobilization

I.7.1. Principles and advantages

Cell immobilization is a widely used biotechnological strategy that involves restricting the free movement of microbial cells by attaching them to solid supports or entrapment within matrices, while retaining their biological activity and metabolic functionality. The goal is to create a reusable, stable, and efficient biocatalyst system suitable for a wide range of industrial and environmental applications (Willaert, 2011).

This technique has evolved as a solution to limitations associated with free-cell systems, such as poor operational stability, biomass washout, and high inoculum costs in repeated-batch

or continuous fermentation. The immobilized cells retain their capacity to grow, reproduce, and function, although in a more spatially constrained environment (Willaert, 2011).

In addition to the well-known benefits of operational stability and reusability, recent research highlights several physiological and process-level advantages associated with immobilized cell systems:

I.7.1.2. Prolonged cell activity and microenvironment optimization

Immobilization creates a favorable microenvironment for microbial cells, including nutrient gradients, pH stabilization, and cell–cell contact, all of which help maintain high metabolic activity over longer periods. This buffering effect is particularly beneficial for fermenting complex or inhibitory substrates like olive mill wastewater (OMW), which can vary in pH and contain toxic compounds (Ayadi et al., 2022a; Ge et al., 2017; Nikolaou & Kourkoutas, 2018)

I.7.1.3. Reuse of biomass and reduced lag phase

Immobilized cells can be reused over multiple fermentation cycles without loss of viability, significantly reducing costs related to inoculum preparation. Additionally, the presence of metabolically active biomass at the start of each batch often leads to a shorter lag phase, accelerating fermentation onset and improving throughput (Willaert, 2011).

I.7.1.4. Higher cell density and volumetric productivity

By retaining cells within the reactor space, immobilization allows for high cell loading, enabling greater product output per unit volume. This enhances volumetric productivity and makes continuous or fed-batch fermentation more efficient, especially in space-constrained bioreactors (Najim et al., 2024).

I.7.1.5. Improved substrate utilization and yield

Immobilized cells often exhibit more efficient substrate conversion, especially when operating under high dilution rates in continuous systems. This leads to better resource use and higher product yields compared to free-cell fermentations (Bai et al., 2011).

I.7.1.6. Resistance to shear stress and toxicity

The physical barrier provided by the matrix or support protects the cells from mechanical shear stress—an important feature for shear-sensitive organisms (e.g., certain yeast strains or

mammalian cells)—as well as from toxic metabolites or substrate overload. This is particularly useful for fermenting molasses or whey, which may have osmotic or inhibitory challenges (Guo et al., 2020).

I.7.1.7. Simplified separation and reduced contamination risk

Because immobilized biomass remains fixed, it simplifies cell recovery, product separation, and reduces the need for costly centrifugation (Yoshimoto et al., 2017). Moreover, by maintaining a stable microbial population within the reactor, immobilization helps reduce microbial contamination risks, especially in open or semi-continuous processes (Najim et al., 2024).

I.7.1.8. Enhanced genetic and phenotypic stability

Some studies also suggest that long-term immobilization can promote genetic stability and reduce the emergence of undesired mutations, particularly in engineered strains, making immobilization useful in high-precision bioprocessing environments (Willaert, 2011).

I.7.2. Types of solid supports used

The effectiveness of an immobilization system is largely influenced by the nature of the solid support material used (Hassan et al., 2019). Solid supports serve as physical anchors for microbial cells, and their chemical, structural, and mechanical properties determine not only the immobilization efficiency but also the stability, productivity, and reusability of the system (Ge et al., 2017). Supports can be broadly classified into natural and synthetic materials, each offering distinct advantages and limitations depending on the intended application.Natural: alginate, agar, volcanic rocks (pozzolana), sponges, biochar (Willaert, 2011).

I.7.2.2. Natural supports

Natural supports are often biodegradable, biocompatible, and low-cost, making them attractive options for environmentally sustainable and economically feasible processes especially when using agro-industrial by-products as substrates. Examples include:

- **Alginate**: One of the most widely used materials for cell entrapment, sodium alginate is a polysaccharide extracted from brown algae (Giese, 2020). It forms gel beads upon contact with calcium ions (Ca^{2+}), offering a gentle environment for microbial immobilization. It is ideal for lactic acid bacteria, yeasts, and fungi, but suffers from poor mechanical strength and gradual bead degradation over time (Ge et al., 2017).

- **Agar and Carrageenan**: These polysaccharide gels are also used for microbial entrapment, particularly in enzyme production and food fermentation. While relatively inexpensive and biocompatible, they are less stable at elevated temperatures (Najim et al., 2024).
- **Volcanic rocks (e.g., Pozzolan)**: Inert, porous, and chemically stable, volcanic materials like pozzolan offer excellent surface area and mechanical durability. Their rough texture enhances cell adhesion, making them particularly suited for adsorption-based immobilization in aerobic or anaerobic fermenters. They are ideal for ethanol or acetic acid production, as demonstrated in olive oil wastewater valorization (Najim et al., 2024).
- **Sponges and biochar**: These porous natural supports have high surface-to-volume ratios and can absorb and retain significant quantities of microbial cultures. Biochar, derived from pyrolyzed biomass, has recently gained attention for its adsorptive and buffering capacity, particularly in wastewater treatment and bioremediation systems (R. Li et al., 2022).

I.7.2.3. Synthetic supports

Synthetic supports are often engineered to achieve greater mechanical strength, controlled porosity, and chemical resistance, making them suitable for repeated industrial-scale use (Zdarta et al., 2018). Common synthetic supports include:

- **Polyacrylamide beads:** These polymeric supports are chemically stable and can be tailored for specific porosity and rigidity. However, they are non-biodegradable and may involve higher production costs (Mahajan et al., 2010).
- **Polyurethane foam:** Highly porous and inert, polyurethane is widely used for adsorption immobilization. It supports aerobic biofilm development and is commonly used in packed-bed and trickling filter reactors (de Ory et al., 2020).
- **Silica and glass beads:** These materials offer high thermal and chemical resistance, making them ideal for harsh processing environments or solvent-rich fermentation. Their surface can also be chemically modified to improve microbial adhesion (Giese, 2020; Zhang et al., 2016).

I.7.2.4. Criteria for support selection

The choice of immobilization support depends on multiple criteria as shown in the following table:

Table 4. Main criteria for the selection of immobilization supports in biotechnological applications (Abdelmajeed et al., 2012; Ge et al., 2017; Hassan et al., 2019; Zdarta et al., 2018).

Parameter	Relevance
Compatibility	Should be non-toxic and suitable for the microorganism used
Porosity	Affects nutrient diffusion and surface area for adhesion
Mechanical Stability	Important for long-term use and reactor agitation
Reusability	Influences operational cost and long-term sustainability
Surface Chemistry	Determines cell adhesion and potential for functionalization
Cost and Availability	Key for large-scale or rural/agro-industrial settings

I.8. Immobilization techniques

Microbial immobilization involves a range of physical and chemical methods that allow for the stable retention of viable or non-viable cells on or within a support material, while maintaining their biological activity (Najim et al., 2024). These techniques are not limited to microorganisms—they are also applicable to plant cells, insect cells, and mammalian cells, making them highly versatile for biotechnological and biomedical applications (Ge et al., 2017).

Consequently, the choice and optimization of immobilization methods for cells must consider the physiological properties and the purpose of application.

A comprehensive overview of cell immobilization strategies and their material-based classifications is presented in Figure 2. This framework illustrates how techniques such as adsorption, entrapment, and containment vary depending on the use of natural vs. synthetic materials and physical vs. chemically induced retention mechanism.

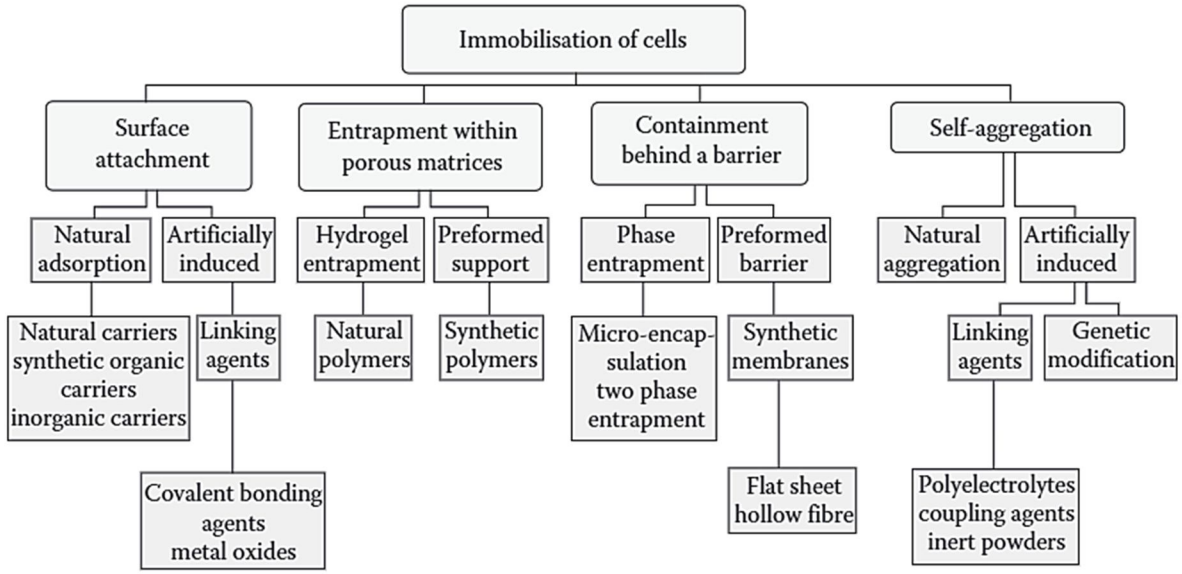


Figure 2. Classification of cell immobilization techniques based on mechanism and material type (Willaert, 2011).

I.8.1. Surface attachement

Surface attachment (Figure 3a) involves the adsorption or binding of microbial cells onto a solid carrier through electrostatic interactions, hydrophobic forces, or covalent bonds. The efficiency of attachment is influenced by several factors, including the physicochemical properties of the support (e.g., surface charge, roughness), the cell surface characteristics (such as hydrophobicity, presence of fimbriae or flagella), and the composition of the growth medium. Some microorganisms also secrete extracellular polymeric substances (EPS) that facilitate adhesion. While adsorption is simple and economical, the binding may be unstable under agitation unless reinforced by chemical linkers or pretreated supports (Abdelmajeed et al., 2012; Ge et al., 2017).

I.8.1.2. Adsorption

Adsorption represents one of the earliest and simplest techniques for cell immobilization. This method relies on non-covalent interactions (primarily electrostatic attractions and van der Waals forces) between the microbial cell surface and a charged or textured support material (Figure 3a). These interactions are influenced by the surface properties of both the microorganism and the carrier, including factors such as hydrophobicity, surface roughness, and ionic charge (Jesionowski et al., 2014).

In general, hydrophobic surfaces tend to promote stronger cell adhesion than hydrophilic ones, a factor often exploited in reactor design. For example, in animal cell culture, microcarriers provide a large surface area that supports the adhesion of anchorage-dependent cells, enabling high-density growth (Ferrari et al., 2019).

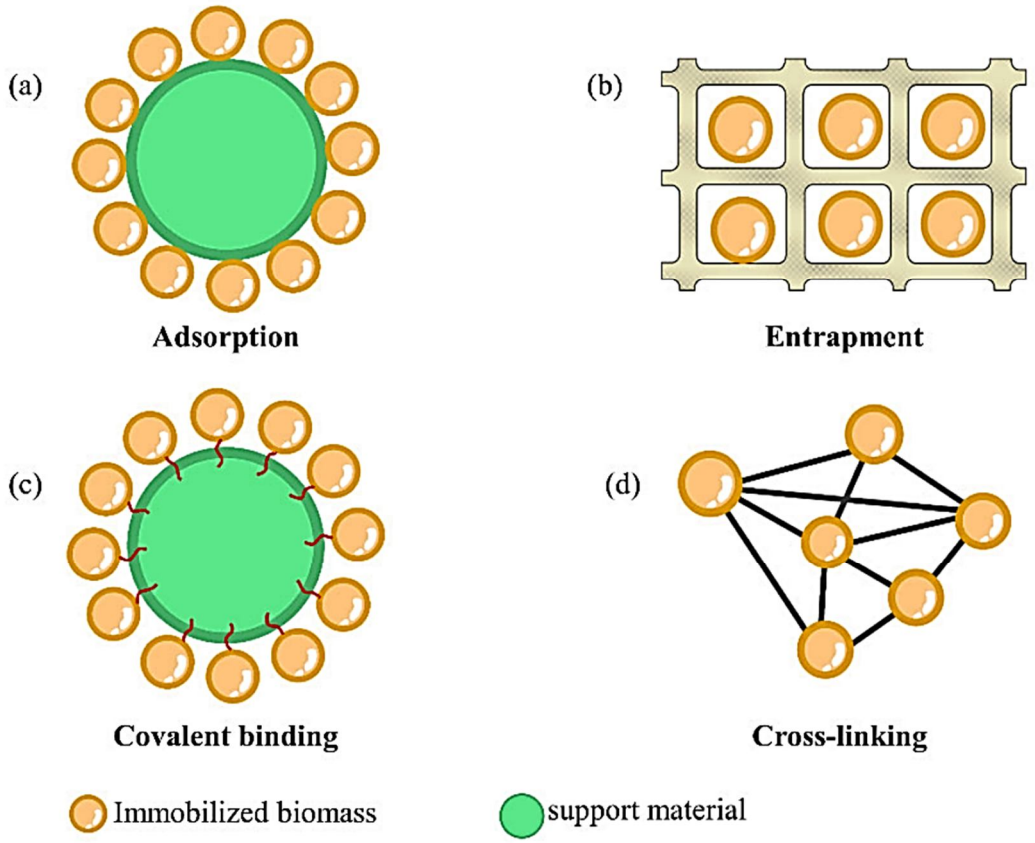


Figure 3. Schematic representation of key microbial immobilization methods: (*a) adsorption, (b) entrapment, (c) covalent binding, (d) cross-linking* (D. Liu et al., 2022).

Adsorption is favored for its operational simplicity, low cost, and minimal impact on cell viability, as it does not require chemical modification or high temperatures. Common supports used include natural rocks (e.g., pozzolan), biochar, glass beads, and synthetic foams, all of which offer high surface area and porosity conducive to microbial retention (Willaert, 2011).

However, one key limitation of this technique is its reversible nature. A dynamic equilibrium may develop between cell attachment and detachment, especially in systems with agitation or hydraulic flow, leading to the coexistence of free-floating and immobilized cells. As a result, adsorption may be less suitable for fast-growing microorganisms such as *Saccharomyces cerevisiae* or *E. coli*, which can dominate the broth as free cells (Ge et al., 2017).

I.8.1.3. Covalent binding

Covalent binding involves the chemical attachment of cells to a solid support via strong covalent bonds (Figure 3c), typically formed using crosslinking agents like glutaraldehyde or carbodiimide (Prabhakar et al., 2025). This technique generally uses functionalized inorganic carriers (e.g., silica or modified polymers) that can react with groups on the microbial cell surface (Zucca & Sanjust, 2014).

While it can offer greater immobilization stability than adsorption, its use in whole-cell immobilization is limited. The low availability of suitable reactive groups on both the cell membrane and carrier often reduces efficiency, and toxic crosslinkers may impair cell viability during or after immobilization (D. Liu et al., 2022).

As a result, covalent binding is more appropriate when working with non-viable cells, especially for biocatalytic applications where intracellular enzymes are utilized in place of purified ones. In such cases, this method helps avoid the cost of enzyme extraction and provides added protection to intracellular enzymes against environmental degradation (Hoarau et al., 2017).

I.8.1.4. Entrapment

Entrapment is one of the most widely used and studied cell immobilization methods, involving the physical confinement of cells within a gel matrix or porous particle as shown in Figure 3b, without direct chemical bonding to the support (Bassani et al., 2019). This technique provides a protective environment that minimizes the impact of external stressors and is particularly suited to sensitive microbial systems (D. Liu et al., 2022).

a. Gel-Based entrapment

Cells are suspended in a gel-forming solution (e.g., alginate, κ-carrageenan, gelatin, polyvinyl alcohol) and solidified into beads or sheets. These gels are semi-permeable, allowing substrate and product diffusion while retaining the cells inside. Calcium alginate is the most commonly used material due to its biocompatibility, mild gelling conditions, and ease of preparation (Ge et al., 2017; Willaert, 2011). However, mass transfer limitations and cell leakage particularly from cell growth and bead degradation can reduce efficiency over time (D. Liu et al., 2022).

b. Entrapment in porous particles

Alternatively, cells can be immobilized within the pores of solid support materials such as porous glass, ceramics, or zeolites. These carriers offer higher mechanical strength and abrasion resistance, making them ideal for use in stirred-tank and packed-bed reactors (Bassani et al., 2019; Willaert, 2011). This method supports large-scale operations and maintains high cell viability during immobilization, though it may suffer from internal diffusion resistance and lower volumetric cell density (Bouabidi et al., 2019).

Overall, entrapment methods are cost-effective, scalable, and highly compatible with repeated-batch and continuous fermentation systems. However, they are best suited for reactions involving small-molecule substrates, as diffusion limitations can affect performance in more complex systems.

I.8.1.5. Cross-linking

Cross-linking is a chemical immobilization technique where microbial cells or enzymes are linked together using multifunctional cross-linking agents (Figure 3d), forming stable, insoluble aggregates. Unlike covalent binding, cross-linking does not require a support matrix, as the cells are immobilized by forming intercellular bonds. Common agents include glutaraldehyde, diacetamide, maleic anhydride, and dialdehyde cellulose (DAC) (D. Liu et al., 2022).

This method is widely applied in non-viable cell systems or for enzyme immobilization, as the cytotoxic nature of the cross-linking agents can significantly impair live cells (Dzionek et al., 2021). However, it provides high mechanical and thermal stability and simplifies the downstream recovery process (C. H. Lee et al., 2021).

Cross-linking is an irreversible process, making it suitable for processes where biomass reuse and high resistance to stress are more critical than cell viability. It is also commonly used in combination with other methods such as entrapment or adsorption to improve overall immobilization strength and durability (Bouabidi et al., 2019)

Section II:
Experimental Methodology

Chapter II:

Isolation and characterization of polyphenol-tolerant yeast strains

II.1. Introduction

The increasing urgency to address climate change, environmental degradation, and fossil fuel dependency has led to growing global interest in alternative, renewable energy sources. Among these, bioethanol has emerged as a key candidate for replacing conventional petroleum-based fuels due to its clean-burning properties and renewable nature (Devi et al., 2022). Bioethanol is primarily generated through the microbial fermentation of sugars and starches, with *Saccharomyces cerevisiae* being the most widely used organism, owing to its well-documented ethanol tolerance and robust fermentation efficiency (Perruca Foncillas et al., 2023).

Historically, bioethanol production has depended heavily on first-generation feedstocks such as sugarcane, maize, and other starchy crops. While effective, this strategy raises ethical and economic concerns, as it may intensify competition with food supplies and exert pressure on agricultural land use, undermining the very sustainability objectives that biofuels are intended to support (Appelt et al., 2022; H. Huang et al., 2023; Wardani et al., 2023b).

In this context, agro-industrial by-products present an attractive alternative. One such substrate, olive oil wastewater (OOW) a major effluent generated during olive oil extraction holds considerable promise due to its high content of fermentable organic matter, particularly sugars (Alkhalidi et al., 2023; Massadeh et al., 2022). Algeria, being a significant olive-producing country, generates large volumes of this effluent annually. However, OOW also presents significant challenges: it is rich in polyphenols and fatty acids, which possess antimicrobial properties that can inhibit microbial metabolism and compromise fermentation performance (Canal et al., 2019; Sar & Akbas, 2023). These compounds disrupt cellular membranes and interfere with key metabolic processes, posing a major obstacle to the direct bioconversion of OOW into bioethanol (Abu-Lafi et al., 2017; Cuffaro et al., 2023).

Effectively valorizing OOW not only addresses the environmental concerns associated with its disposal but also aligns with the principles of the circular economy transforming waste

streams into value-added products. This requires the development or identification of resilient microbial strains capable of fermenting OOW under its native, harsh conditions. In particular, yeast strains with high tolerance to both polyphenols and elevated ethanol concentrations are needed. These strains must exhibit specialized adaptations, such as fortified membrane integrity, efficient efflux systems, and polyphenol-detoxifying pathways (Parapouli et al., 2020; Villarreal et al., 2022).

To date, most bioethanol studies involving OOW have relied on strategies such as detoxification, dilution, or the use of commercial, lab-adapted yeast strains. While these methods have shown promise, they involve added processing steps and costs, which reduce economic feasibility at larger scales (Calabrò et al., 2018; H. Zhang et al., 2023). In contrast, the present study explores a cost-effective and eco-friendly alternative by isolating native yeast strains already adapted to the polyphenol-rich environment of OOW that can perform fermentation without pretreatment or chemical supplementation.

Specifically, this chapter presents the isolation, morphological identification, and molecular characterization via rRNA-based sequencing of yeast strains obtained directly from olive oil wastewater samples. The focus is placed on assessing their tolerance to inhibitory compounds, particularly polyphenols, and evaluating their bioethanol production performance across a gradient of untreated OOW concentrations.

The approach taken here differs from conventional methods by emphasizing the potential of native microbial strains, already adapted to harsh environmental conditions, thereby eliminating the need for detoxification or external additives. Through this work, the chapter contributes to the broader thesis objective of developing sustainable, low-cost bioprocesses for the valorization of agro-industrial residues. It specifically highlights a strategy for converting a highly polluting effluent olive oil wastewater into a renewable source of bioethanol, offering a practical application within the context of Algerian agro-industrial waste management and the global pursuit of greener energy alternatives.

II.2. Materials and methods

II.2.1. Sample collection and preparation

II.2.1.1. Location of the sampling site:

In this chapter, the olive oil wastewater (OOW) used for microbial isolation and fermentation studies was sourced from the Ennakhla olive oil production facility, located in the commune of Medjadja, within the Wilaya of Chlef, Algeria. This site is representative of traditional olive oil processing in the region and was selected due to the high volume of wastewater generated during the olive oil extraction season from December to February.

Figure 4. Geographic location of the Ennakhla olive oil mill in Medjadja (Chlef, Algeria), showing the sampling site for olive oil wastewater.

II.2.1.2. Sampling

A total of three lots of 5 liters' containers each were collected immediately after the decantation stage a phase where granular solids naturally settle ensuring the samples retained both their chemical complexity and native microbial load with minimal external contamination. Samples were transported in clean containers and preserved at 4°C to maintain their original properties until laboratory processing.

Figure 5. Photograph of a 5-liter container filled with freshly collected olive oil wastewater (OOW).

II.2.1.3. Samples preparation

Before being used in the experiments, the raw olive oil wastewater (OOWW) underwent a two-step pretreatment process aimed at removing large particulates and reducing inhibitory components. First, it was passed through a fine mesh sieve to eliminate visible solid debris. Then, the filtrate was subjected to centrifugation at 5,000 rpm for 10 minutes to allow sedimentation of suspended solids. During this step, the upper oil layer was carefully removed to reduce the hydrophobic load and potential fermentation inhibitors. The resulting clarified supernatant was collected and designated as the working substrate for yeast isolation, physiological tests, and fermentation assays.

For experiments involving tolerance assessment, the clarified OOW was diluted with distilled, autoclaved water to prepare a concentration series of 10%, 25%, 50%, 75%, and 100% (v/v). This dilution series enabled a stepwise evaluation of yeast performance across increasing levels of potential inhibitors, particularly polyphenols and fatty acids, which are known to be abundant in OOW.

To establish a baseline understanding of the substrate's composition, comprehensive physicochemical analyses were conducted. The pH was measured using a calibrated digital pH meter (Hanna Instruments). Organic load parameters, namely Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand over 5 days (BOD□), were determined according to standard protocols published by the American Public Health Association (APHA, 2017a). The total acidity was assessed through acid-base titration, using 0.1 M NaOH and phenolphthalein as a visual endpoint indicator.

Additional analytical methods were employed to quantify key chemical constituents. Nitrite (NO_2^-) concentrations were measured via a colorimetric reaction with Griess reagent, adhering to APHA guidelines. The total polyphenol content, which plays a critical role in microbial inhibition, was quantified using the Folin–Ciocalteu assay, following the procedure detailed by (Russo et al., 2022) Carbohydrate content was evaluated by determining both glucose and total reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method, as described by (Jain et al., 2020). All measurements were performed in triplicate to ensure both reproducibility and data reliability.

II.2.2. Yeast isolation and culturing

In this chapter, yeast strains were isolated directly from the collected untreated olive oil wastewater (OOWW) samples to assess their tolerance and fermentation capacity in this complex substrate. The wastewater was first subjected to serial dilution using sterile saline solution (0.9% NaCl), with dilutions ranging from 10^{-1} to 10^{-6}. From each dilution, 100 µL was aseptically spread onto Sabouraud Dextrose Agar (SDA) plates (Merk KGaA, Germany). The medium was prepared with 40 g/L dextrose, 10 g/L peptone, 20 g/L agar, and 1 L of sterile distilled water, and adjusted to a pH of 5.4 to support yeast growth.

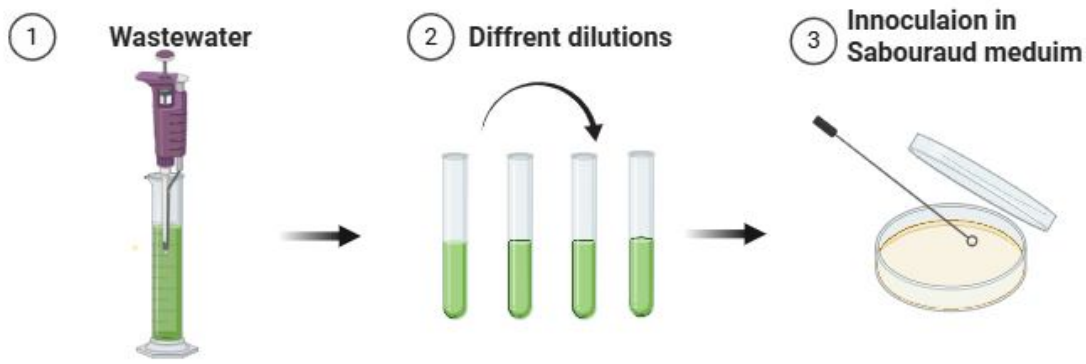


Figure 6: Process of dilution and inoculation of the yeast strains.

To prevent bacterial contamination, the medium was supplemented with chloramphenicol (0.05 g/L). Inoculated plates were incubated at 30°C for 48 hours, allowing for the development of yeast colonies.

After incubation, individual colonies were selected based on distinct morphological traits such as colony size, elevation, surface appearance, pigmentation, and edge structure. These candidate colonies were sub-cultured on fresh SDA plates to ensure purity and were maintained on Sabouraud agar slants at 4°C for long-term storage. Regular sub-culturing was performed to maintain cell viability and activity throughout the study.

In parallel, a commercial *Saccharomyces cerevisiae* strain (Saf-instant®, Lesaffre, France), commonly used in industrial baking, was employed as a reference strain. This strain was rehydrated in sterile distilled water according to the manufacturer's instructions and used under identical fermentation conditions to the selected isolate (Y17), enabling direct performance comparisons during fermentations conducted in undiluted (100%) OOWW for 72 hours.

II.2.3. Morphological identification

The initial characterization of the isolated yeast strains and the commercial strain was conducted using morphological criteria, which provided essential preliminary data for differentiating and selecting promising candidates for further testing.

A. Colony morphology

Colony-level traits were assessed after cultivating the isolates on Sabouraud Dextrose Agar (SDA) plates, incubated at 30°C for 48 hours. Key phenotypic descriptors such as colony diameter, pigmentation, margin definition, surface texture (smooth, wrinkled, or mucoid), elevation, and overall shape were carefully recorded. These macroscopic features helped distinguish between different strains and facilitated the identification of potentially robust fermentative yeasts.

B. Cellular morphology

To observe microscopic characteristics, a small portion of each yeast colony was transferred using a sterile loop and suspended in a drop of sterile distilled water on a clean microscope slide. The cells were stained using methylene blue, which allows for the assessment of cell viability as well as structure. Under 400× magnification using a light microscope, cell size, shape (spherical, ovoid, or elongated), budding patterns (single, multilateral), and internal clarity were examined.

This morphological screening provided valuable preliminary insight into yeast identity and vitality before progressing to molecular-level identification. Particular attention was paid to the presence of budding cells and typical features of ascomycetous yeasts, which are indicative of the *Saccharomyces* genus.

II.2.4. Fermentability testing

The fermentative potential of the yeast strains isolated from olive oil wastewater was evaluated using the Durham's tube method, as outlined by (Reiner, 2012). In this approach, each yeast isolate was inoculated into test tubes containing 10 mL of Sabouraud Dextrose Broth, supplemented with 1% (w/v) glucose to provide a carbon source for fermentation. A Durham tube, placed in an inverted position within each test tube, was used to trap the carbon dioxide (CO_2) gas released during the fermentation process.

Figure 7. Prepared fermentability test tubes containing Sabouraud Dextrose Broth supplemented with 1% (w/v) glucose. Inverted Durham tubes were placed inside to capture carbon dioxide (CO_2) produced during fermentation.

The test tubes were then incubated at 30°C for 48 hours under anaerobic conditions to promote fermentation. The accumulation of gas in the Durham tube was monitored periodically, with the presence of CO_2 serving as an indicator of successful fermentation. Yeast isolates that demonstrated clear evidence of CO_2 production were considered positive for fermentation activity and were subsequently selected for further studies on ethanol production.

II.2.5. Assessment of yeast tolerance to olive oil wastewater polyphenols

To assess the ability of selected yeast strains to tolerate the polyphenol-rich environment of olive oil wastewater (OOW), an adaptation assay was performed using Sabouraud Dextrose medium supplemented with increasing concentrations of OOW. Only isolates that showed positive results in the fermentability test were included in this experiment.

The test media consisted of Sabouraud broth amended with OOW supernatant at concentrations of 10%, 25%, 50%, 75%, and 100% (v/v). Each selected yeast strain was inoculated into 10 mL of the respective medium at an initial cell density of $1 \times 10^{\square}$ CFU/mL. This concentration was determined through serial dilution and colony counting on solid media. Cultures were incubated at 30°C for 48 hours under anaerobic conditions.

Yeast growth was monitored both visually by checking turbidity and quantitatively, through optical density measurements at 600 nm (OD□□□) using a spectrophotometer. The ability of a strain to grow at higher OOW concentrations was taken as an indicator of polyphenol tolerance. A strain was considered tolerant if it showed significant growth in 100% OOW, defined as an increase in OD□□□ of ≥0.15 from the initial value of 0.05, indicating biomass accumulation.

II.2.6. DNA extraction and 5.8S-ITS rRNA sequencing

To enable molecular identification, the most promising yeast isolate was subjected to sequencing of the 5.8S-Internal Transcribed Spacer (ITS) region of ribosomal RNA (rRNA), a common marker for fungal taxonomy.

Genomic DNA was extracted from freshly cultivated yeast using the method outlined by (Fazio et al., 2024), with slight adjustments to improve yield and purity. The extracted DNA served as the template for polymerase chain reaction (PCR) amplification of the ITS region.

PCR was carried out in 50 µL reaction mixtures using a Thermal Cycler 2720 (Applied Biosystems, Waltham, MA, USA). The PCR mix consisted of :

- 25 µL of DreamTaq™ Green PCR Master Mix 2X (Thermo Fisher Scientific),
- 2 µL each of the universal fungal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′),
- 16 µL of nuclease-free ultrapure water,
- 5 µL of the DNA template.

The thermal cycling protocol included:

- An initial denaturation step at 95 °C for 15 minutes to activate the Taq polymerase,
- 35 amplification cycles consisting of denaturation at 95 °C for 1 minute, primer annealing at 54 °C for 2 minutes, and extension at 72 °C for 2 minutes,
- Followed by a final extension step at 72 °C for 10 minutes.

PCR products were separated on a 1.5% agarose gel prepared with 1× TBE buffer (Lonza, Switzerland) and stained with 3 µL of GelRed nucleic acid stain (Biotium, USA).

Electrophoresis was conducted, and the bands were visualized under ultraviolet illumination using a gel documentation system (Axygen).

The PCR-amplified ITS products were further analyzed through Restriction Fragment Length Polymorphism (RFLP) to differentiate among yeast isolates at the molecular level. For this purpose, two restriction endonucleases HaeIII and HinfI (Thermo Fisher Scientific, Waltham, MA, USA) were selected due to their frequent use in yeast genotyping.

Each restriction reaction was carried out in a total volume of 20 µL and incubated at 37 °C for 2 hours to ensure complete digestion. The resulting DNA fragments were resolved on a 2.0% agarose gel (1× TBE buffer), stained with GelRed, and subjected to electrophoresis at 100 V for 3 hours. Distinct RFLP patterns were visualized under UV light and analyzed to identify genetic polymorphisms among the isolates.

Based on the RFLP profiles, representative isolates from each observed cluster were selected for further identification via Sanger sequencing. The selected PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Sequencing was carried out using the same ITS primers (ITS1 and ITS4) by an external sequencing provider (Eurofins Genomics, Vimodrone, Italy).

The sequence of the most promising strain, Y17, was deposited in the GenBank database (NCBI Resource Coordinators, 2018) and assigned an accession number. To determine its phylogenetic position, the sequence was compared with reference sequences using the BLAST tool available at the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

For phylogenetic analysis, 12 type strains were selected based on their similarity to Y17, and one strain, *Torulaspora globosa* CBS 764, was included as an outgroup. The sequences were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) as implemented by (Edgar, 2004), providing a robust multiple alignment for evolutionary inference.

To elucidate the evolutionary relationships of the selected yeast isolate Y17, a phylogenetic tree was constructed using the Maximum Likelihood (ML) method, employing the Kimura 2-parameter (K2P) model for nucleotide substitution (Tamura & Nei, 1993). This

model was chosen for its ability to account for different rates of transitions and transversions, thereby providing a more accurate estimate of evolutionary distances.

The tree with the highest log-likelihood score (-1566.20) was retained as the most representative of the dataset. The bootstrap support values, indicating the percentage of trees in which the associated taxa clustered together, are displayed at each branch node, reflecting the reliability of the inferred phylogenetic relationships.

To initiate the heuristic search, initial tree topologies were generated automatically by applying both Neighbor-Joining (NJ) and BioNJ algorithms to a matrix of pairwise distances. These distances were computed using the Maximum Composite Likelihood (MCL) approach. Among the resulting trees, the one with the highest log-likelihood value was selected for further analysis.

To better capture the variation in substitution rates among different sites, a discrete Gamma distribution was employed, divided into five rate categories (+G), with a shape parameter (α) of 0.5515. Additionally, the model incorporated a proportion of sites considered evolutionarily invariable (+I), which represented 49.09% of the alignment positions.

The dataset used for this analysis included 13 nucleotide sequences, consisting of 12 closely related type strains identified through BLAST analysis and one outgroup species (Torulaspora globosa CBS 764). Prior to tree construction, all positions containing gaps or missing data were excluded, resulting in a final alignment of 632 positions.

All phylogenetic computations and tree visualizations were performed using MEGA7 software (S. Kumar et al., 2016), a widely adopted tool for molecular evolutionary genetics analysis.

II.2.7. Ethanol productivity measurement

The ethanol production capacity of the selected yeast strains was evaluated in free cell batch fermentation experiments. Yeast cells were inoculated into 1,000 mL fermentation flasks containing 700 mL of olive oil wastewater (OOW) as a fermentation medium. The flasks were equipped with an exhaust system allowing gas to escape through a one-way valve fitted with a 22-micron filter to prevent contamination. Additionally, a second outlet was incorporated to facilitate sampling while minimizing the risk of contamination. Fermentation was carried out

at 30°C for 72 hours under continuous shaking at 150 rpm to ensure proper mixing and uncontrolled pH, as shown below in Figure 7.

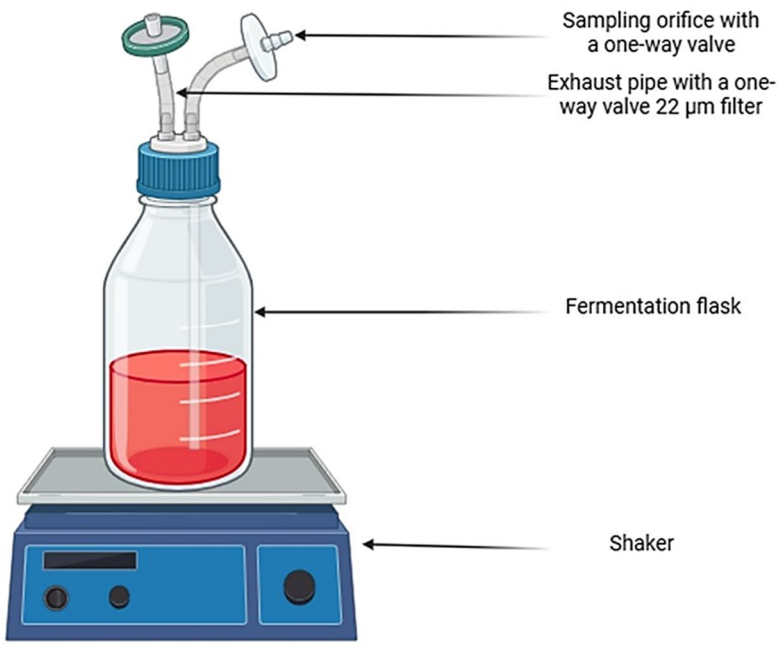


Figure 8. Fermentation Setup: Actual Photograph and Schematic Representation of the Experimental Configuration.

Samples were collected at predetermined time intervals: t=0, t=3h, t=12h, t=24h, t=48h, and t=72h. At each time point, pH, optical density at 600 nm (OD_{600}), and glucose concentration were measured to monitor fermentation progress.

Glucose concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method, where reducing sugars react with DNS to produce a reddish-brown color. The absorbance at 540 nm was measured and compared against a glucose calibration curve to quantify glucose levels (Jain et al., 2020).

The ethanol in the fermentation broth was first distilled using a rotary evaporator (rotavapor) to separate it from other components. Ethanol concentration was then determined using the permanganate method, which involves oxidation of ethanol by potassium permanganate ($KMnO_4$) in an acidic medium. The reduction of $KMnO_4$ from purple to colorless was monitored, and ethanol concentration was quantified by comparing against a calibration curve prepared using standard ethanol solutions (Geies & Abdelazim, 2021; P. Zhang et al., 2019).

II.3. Results

II.3.1. Physicochemical characteristics of Olive Mill Wastewater (OMW)

The olive mill wastewater (OMW) used in this study was analyzed for key physicochemical parameters and is summarized in Table 5. The effluent exhibited an acidic pH, high organic load, and a significant concentration of polyphenols, which are known inhibitors of microbial fermentation.

Table 5. Physicochemical Characteristics of Olive Mill Wastewater (OMW)

Parameter	Mean ± SD	Range
pH	4.6 ± 0.2	4.3 – 4.7
Chemical Oxygen Demand (COD)	178 ± 5 gO□/L	175 – 185
Biochemical Oxygen Demand (BOD□)	7 ± 0.3 gO□/L	6.7 – 7.3
Average Acidity (%)	1.65 ± 0.05	1.6 – 1.7
Nitrite	30 ± 2 mg/L	28 – 32
Total Polyphenols	5.77 ± 0.1 g/L	5.6 – 5.8
Glucose concentration	26.0 ± 0.4 g/L	25.6 – 26.4
Total reducing sugars	~29.5 ± 0.7 g/L (as glucose equivalents)	28.8 – 30.2

II.3.2. Colony and cellular morphology

The morphological characteristics of yeast isolates obtained from olive oil wastewater were assessed to provide a preliminary identification and to support strain selection for further fermentation trials. Both macroscopic (colony) and microscopic (cellular) features were examined.

Colony morphology was evaluated after 48 hours of incubation on Sabouraud Dextrose Agar at 30°C. The isolates displayed variations in color, surface texture, elevation, and margin characteristics, which are important for distinguishing between yeast species.

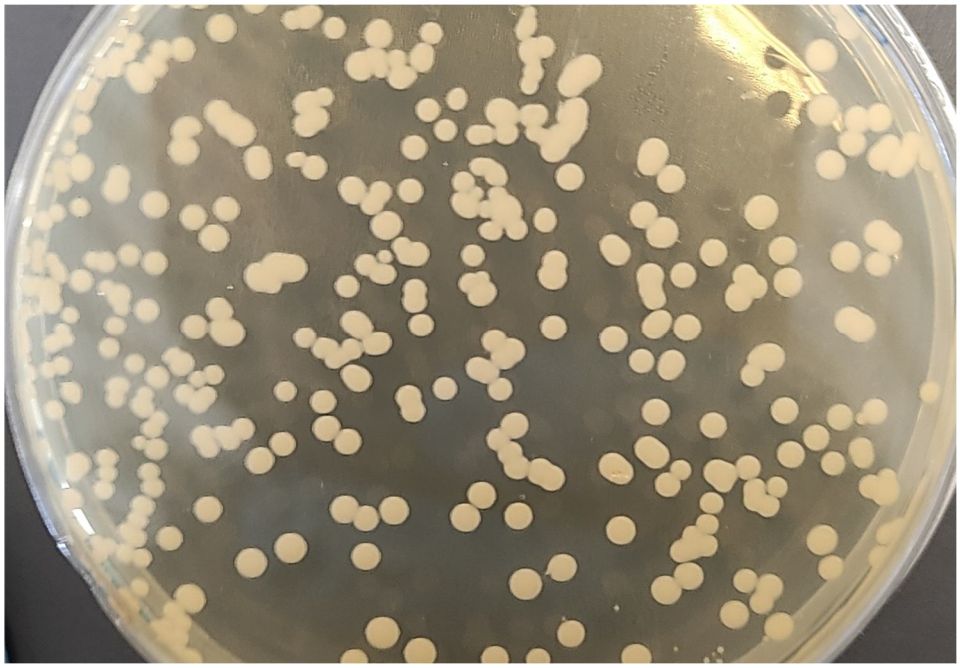
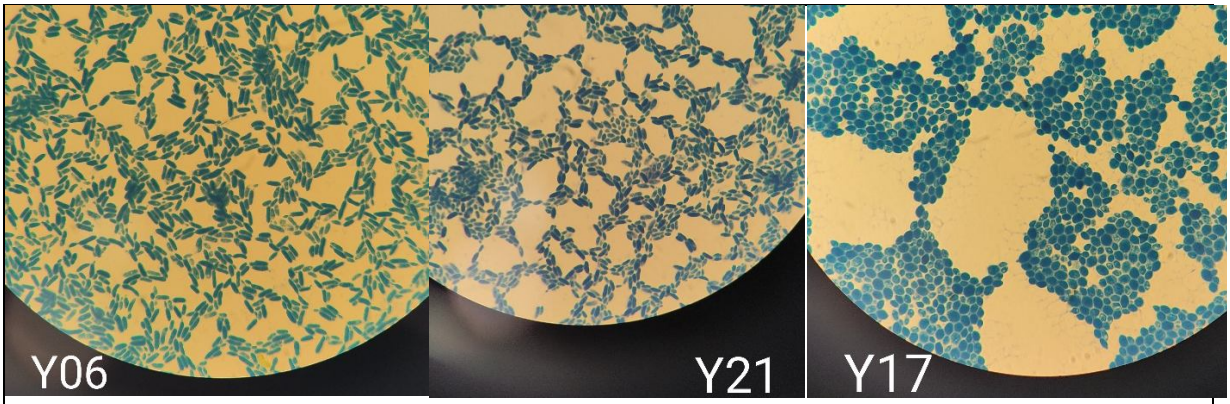


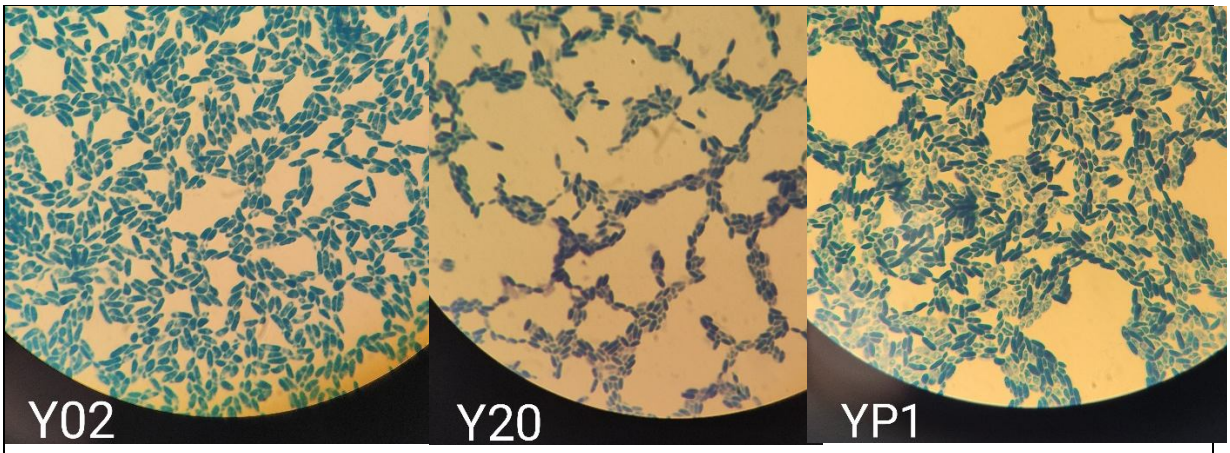
Figure 9. Representative images of yeast isolates Y17: Colony morphology on Sabouraud Dextrose Agar (top view).

Microscopically, cells were stained with methylene blue to assess shape, budding patterns, and viability. Observations were made under 400× magnification using a light microscope. Most isolates showed ovoid to ellipsoidal cells, with either unipolar or multipolar budding. No hyphal or pseudohyphal structures were detected, supporting the classification of isolates as true yeasts rather than dimorphic fungi.

Representative examples of both colony and microscopic morphology are shown in Figure 9, and a summary of morphological traits is provided in Table 6.



Y06 strain	Y21 strain	Y17 strain



Y02 strain	Y20 strain	YP1 strain

Figure 10. Microscopic images of some yeast isolates stained with methylene blue, illustrating cell morphology ×400

Table 6. Morphological Characteristics and arrangement mode on Yeast Strains Isolated from OOW

Strain	Cell Shape	Arrangement	Morphological Features
Y02	Spherical to ovoidal	Evenly distributed	Slightly wrinkled surface
Y03	Ovoidal	Scattered and paired	Smooth surfaces, budding
Y06	Ovoidal	Scattered cells	Smooth surface, moderate budding
Y07	Cylindrical to oval	Chains	Clear budding, uniform arrangement
Y15	Ovoidal	Dense clusters	Smooth surface, distinct budding
Y16	Round to oval	Small dense clusters	Thick cell walls, budding
Y17	Ovoidal	Small clusters	Uniform size, smooth surface
Y18	Elongated	Dense chains and clusters	Irregular budding scars
Y19	Elongated	Dense clusters	Compact pseudohyphal-like structures
Y20	Elongated with chains	Moderate clustering	Polar budding visible
Y21	Ovoidal	Moderate clustering	Smooth surface, budding
YP1	Ovoid to elongated	Dense clusters	Thin cell walls, budding
YP2	Elongated	Chains	Pseudohyphal structures

II.3.3. gas production assessment

In this chapter, the fermentability of the isolated yeast strains was evaluated through biogas production testing, which serves as an indirect measure of yeast metabolic activity and fermentative capacity under anaerobic conditions. The test was performed using Durham tube assays, where the presence of gas in the inverted vial indicated active fermentation.

Among the thirteen yeast isolates tested, only strain Y17 showed a positive gas production, forming visible gas bubbles in the Durham tubes, thereby confirming its

fermentative potential in the olive oil wastewater medium. This result highlights Y17 as a promising candidate for subsequent bioethanol production trials.

All other strains—Y02, Y03, Y06, Y07, Y15, Y16, Y18, Y19, Y20, Y21, YP1, and YP2—failed to exhibit any detectable biogas production, suggesting limited or no fermentative activity under the test conditions.

These findings are summarized in Table 7.

Table 7: Results of biogas production by yeast strains using Dhuram's tubes.

Strain	Biogas Production
Y02	Negative (-)
Y03	Negative (-)
Y06	Negative (-)
Y07	Negative (-)
Y15	Negative (-)
Y16	Negative (-)
Y17	Positive (+)
Y18	Negative (-)
Y19	Negative (-)
Y20	Negative (-)
Y21	Negative (-)
YP1	Negative (-)
YP2	Negative (-)

II.3.4. Assessment of yeast tolerance to olive oil wastewater polyphenols

The tolerance of the selected yeast strain Y17 to the potentially inhibitory compound particularly polyphenols present in olive oil wastewater (OOW) was assessed. This was achieved by culturing the strain in media containing increasing concentrations of OOW (10%, 25%, 50%, 75%, and 100% v/v), diluted with sterile Sabouraud medium.

The growth kinetics of Y17 in each condition were monitored spectrophotometrically by measuring optical density at 600 nm over a 48-hour incubation period. These measurements

served as an indicator of cell proliferation and general metabolic activity under each level of polyphenol stress.

As shown in Figure 10, the strain was able to grow across all tested concentrations, though a decline in growth rate was observed at higher OOW concentrations. This result reflects the inhibitory nature of polyphenols on yeast metabolism, yet underscores Y17's robust tolerance, even in undiluted OOW (100%).

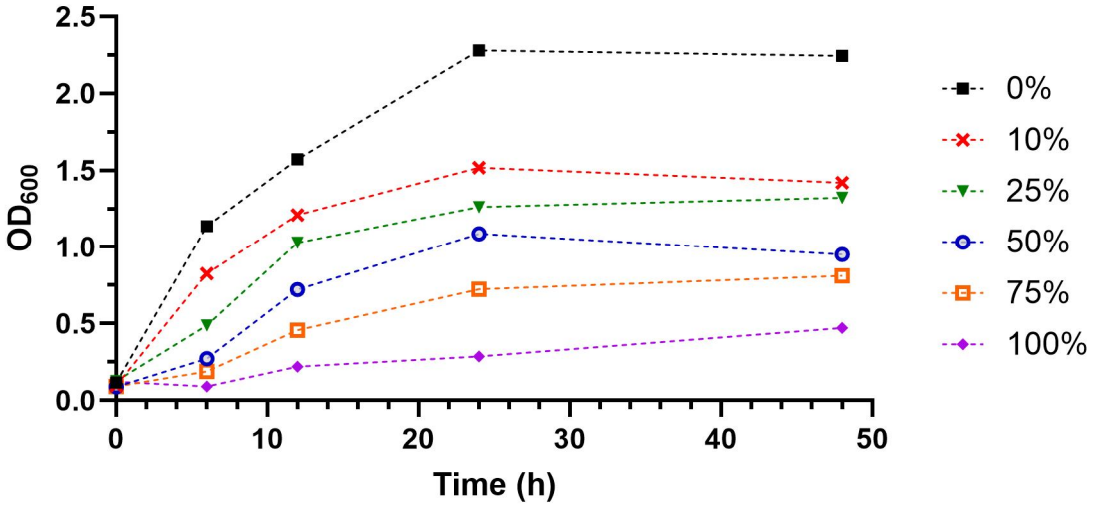


Figure 11. Growth of Y17 strain in olive oil wastewater at different concentrations over time

II.3.5. DNA sequencing results

The retained yeast strain Y17 was preliminarily identified based on its distinct morphological characteristics and its capacity for biogas and ethanol production. To confirm its taxonomic affiliation, DNA sequencing was conducted using the 5.8S-ITS rRNA region as a molecular marker. The sequence analysis confirmed that Y17 belongs to the *Saccharomyces* genus, and it was thus classified as *Saccharomyces sp. Y17*.

Members of the *Saccharomycetaceae* family are unicellular fungi typically exhibiting oval to spherical cells, ranging in size from 2.5–10 µm × 4.5–21 µm. These yeasts are facultative anaerobes, meaning they can thrive in both aerobic and anaerobic environments. Under aerobic conditions, they efficiently convert sugars into biomass, CO□, and energy, while under anaerobic conditions, they shift metabolism toward alcoholic fermentation, primarily producing ethanol (Stanzer et al., 2023).

Saccharomyces species possess the metabolic versatility to assimilate various sugars, including glucose, fructose, maltose, sucrose, galactose, and raffinose, but are typically unable to utilize lactose and cellobiose (Bušić et al., 2018). These characteristics make them ideal candidates for fermentation processes involving complex agro-industrial wastewaters such as olive oil wastewater.

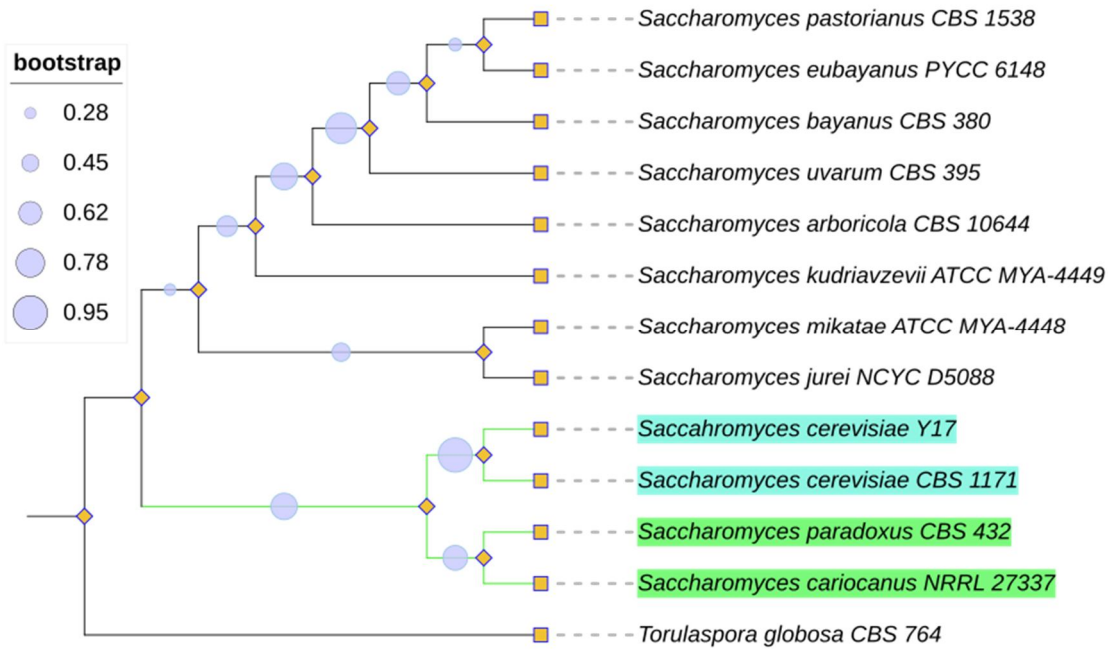


Figure 12. Maximum likelihood (ML) phylogenetic tree generated from the 5.8S-ITS sequence of the selected strain Y17 (shaded in blue) and its closest type strains. Numbers on branches represent bootstrap values. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Torulaspora globosa CBS 764* served as an outgroup.

The retained yeast strain Y17 was initially identified based on its distinct morphological characteristics and promising fermentation performance. For molecular confirmation, the 5.8S-Internal Transcribed Spacer (ITS) region of rRNA was sequenced, as this region is widely used for fungal and yeast identification due to its high variability among species.

The resulting sequence was deposited in the GenBank database under the accession number PQ566690. Phylogenetic analysis was performed to determine the evolutionary relationship between Y17 and closely related yeast species. As illustrated in Figure 12, the strain Y17 clustered closely with *Saccharomyces cerevisiae* CBS 1171, *Saccharomyces paradoxus* CBS 432, and *Saccharomyces cariocanus* NRRL 27337, forming a distinct green clade.

Sequence alignment revealed a 98.68% similarity between Y17 and *S. cerevisiae* CBS 1171, supporting its identification as a member of the *Saccharomyces* genus. This high degree of similarity, combined with morphological and physiological traits, confirms that strain Y17 belongs to the *Saccharomyces cerevisiae* species complex.

II.3.6. Batch fermentation results with 100% Olive Oil Wastewater (OOW)

Batch fermentation trials with free cells were conducted using undiluted olive oil wastewater (OOW) as the sole substrate, employing both the *Saccharomyces cerevisiae* strain Y17 (Figure 13a) and a commercial yeast strain (Figure 13b) under identical conditions. The performance of each strain was assessed over a 72-hour period by monitoring glucose consumption, ethanol production, and pH changes (Figure 13).

At the onset of fermentation (t = 0), both systems exhibited a glucose concentration of 26.0 ± 0.4 g/L and a pH of 4.8 ± 0.1, with no detectable ethanol. After 12 hours, strain Y17 produced 3.2 ± 0.2 g/L of ethanol, while the commercial strain yielded only 1.2 ± 0.1 g/L.

By 48 hours, ethanol concentration in the Y17 fermentation reached 10.8 ± 0.5 g/L, with residual glucose reduced to 7.0 ± 0.3 g/L. In comparison, the commercial strain produced 6.0 ± 0.4 g/L of ethanol and retained a significantly higher glucose level of 20.0 ± 0.5 g/L.

At the conclusion of the fermentation (72 h), Y17 attained a maximum ethanol concentration of 11.3 ± 0.5 g/L, with glucose nearly depleted (1.0 ± 0.1 g/L). Conversely, the commercial yeast reached 8.6 ± 0.4 g/L of ethanol and left 5.2 ± 0.2 g/L of residual glucose.

Throughout fermentation, a progressive decline in pH was observed for both strains. However, Y17 exhibited a more pronounced acidification, reaching a final pH of 3.8 ± 0.1, compared to 3.9 ± 0.1 for the commercial strain.

The ethanol yield for Y17 was calculated at 0.45 g/g of glucose consumed, slightly surpassing that of the commercial strain (0.41 g/g). These findings highlight the enhanced fermentative potential and stress tolerance of the native Y17 strain in untreated OOW, demonstrating superior sugar assimilation and ethanol productivity under harsh environmental conditions.

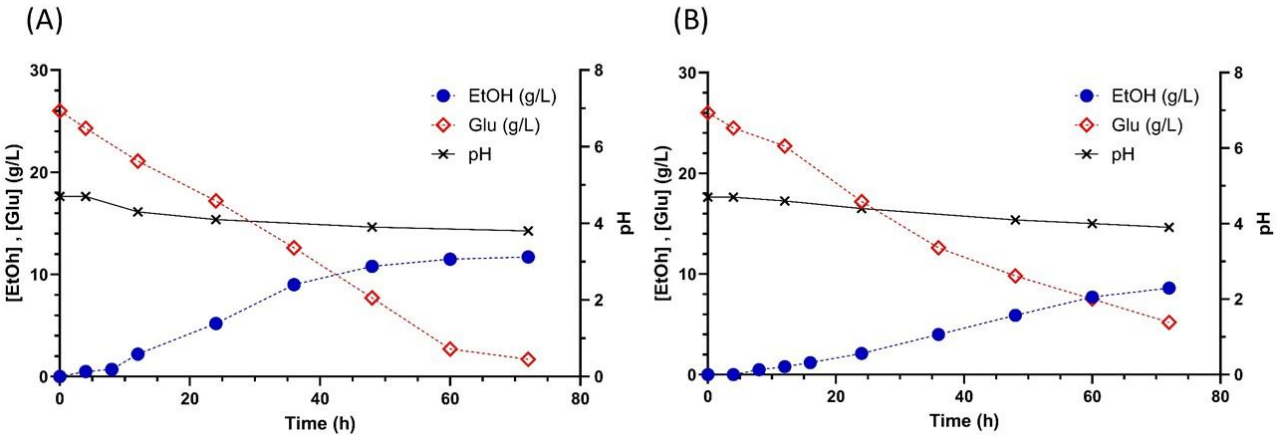


Figure 13. Comparative fermentation profiles of strain Y17 (A) and commercial *S. cerevisiae* (B) in 100% olive oil wastewater (OOW). The graph illustrates glucose consumption, ethanol production, and pH variation over 72 hours.

II.4. Discussion

II.4.1. Physicochemical characteristics of Olive Mill Wastewater (OMW)

The physicochemical parameters of the olive mill wastewater (OMW) analyzed in this study are consistent with those commonly reported for similar agro-industrial effluents. The measured pH of 4.6 ± 0.2 falls within the typical acidic range of 4.5–5.5 cited in previous studies (Bougherara et al., 2021b; Bouharat et al., 2018; Bouknana et al., 2014). The chemical oxygen demand (COD) was determined to be 178 ± 5 gO□/L, while the biochemical oxygen demand over five days (BOD□) was 7 ± 0.3 gO□/L, yielding a COD/BOD□ ratio greater than 25. This elevated ratio indicates the predominance of recalcitrant organic matter, which is poorly biodegradable—an observation that aligns with findings reported by (Gueboudji et al., 2022b) and with our prior investigations on OMW from the same geographical region (Rouam & Meziane, 2025).

The polyphenol content measured in the OMW was 5.77 ± 0.1 g/L, placing it within the inhibitory range (0.5–8 g/L) commonly associated with microbial growth suppression (Vavouraki et al., 2020). The antimicrobial nature of these phenolic compounds is well established, and their presence can significantly hinder the development of non-tolerant microbial populations during fermentation (Russo et al., 2022; Sar & Akbas, 2023).

These findings emphasize the complex and inhibitory composition of OMW, underscoring its challenges as a fermentation substrate. Such environmental stress likely played

a critical role in selecting for resilient yeast strains, particularly the polyphenol-tolerant *Saccharomyces cerevisiae* strain Y17 isolated in this study.

II.4.2. Colony and cellular morphology

The morphological characterization of yeast strains isolated from olive oil wastewater (OOW) provides valuable insights into their potential performance in fermentation processes. As summarized in Table 6, the isolates exhibited significant diversity in cellular shape, arrangement, and structural features traits that may influence their metabolic efficiency and adaptability to harsh environmental conditions.

A variety of cell morphologies were observed, including spherical, ovoidal, cylindrical, and elongated forms. For example, strains Y02 and Y21 exhibited spherical cells, arranged in loose clusters and evenly dispersed populations, respectively. Spherical morphology is generally associated with a stable growth phase, whereas ovoidal forms, as seen in strains Y03 and Y06, often reflect active metabolic states, characterized by smooth surfaces and evident budding activity (J. Kim & Rose, 2015). Similar correlations between morphology and metabolic activity have been reported in previous studies, highlighting the potential impact of cell structure on fermentation outcomes (Sinigaglia et al., 2010).

The occurrence of elongated cells, particularly in strains Y18, Y19, Y20, and YP2, suggests a possible stress-induced morphological shift. Such pseudohyphal or filamentous structures are typically associated with environmental stress responses, such as nutrient limitation or toxic compound exposure (conditions commonly found in OOW). These forms indicate adaptive strategies that enable the strains to persist under adverse conditions (Ben Sassi et al., 2008; Bleve et al., 2011; J. Kim & Rose, 2015).

Furthermore, certain strains exhibited structural adaptations that may confer additional resilience. For instance, strain Y16, characterized by thickened cell walls, is likely to possess enhanced tolerance to environmental stressors, which could contribute to increased survival and performance during fermentation (Ben Sassi et al., 2008; Boutafda et al., 2019).

Overall, the morphological heterogeneity observed among the isolated strains underscores their diverse physiological states and adaptive capacities which are factors that are critical for selecting robust candidates for industrial bioconversion processes.

II.4.3. Gas production assessment

To evaluate the fermentative potential of the isolated yeast strains, a gas production assay was performed using Durham tubes, which serve as indicators of biogas generation during anaerobic fermentation. among the thirteen yeast strains isolated from olive oil wastewater (OOW), only strain Y17 demonstrated measurable gas production, suggesting its active engagement in fermentative metabolism under the tested conditions. This finding is particularly notable, as it positions Y17 as a promising candidate for bioethanol production utilizing OOW as a substrate.

This observation is visually supported by the positive result seen in Figure 14, where gas accumulation is clearly visible in the inverted Durham tube inoculated with strain Y17.

Figure 14. Gas production observed in Durham tube inoculated with strain Y17, showing positive biogas formation as an indicator of fermentative activity.

The absence of gas formation in the remaining strains may indicate a limited capacity for anaerobic sugar metabolism, despite the presence of viable and morphologically active cells. This phenomenon can be attributed to several factors. First, although Sabouraud medium supports general yeast growth, it may be suboptimal for inducing fermentation due to possible deficiencies in micronutrients or cofactors essential for the activation of fermentative pathways (Villarreal et al., 2022). Additionally, the high glucose concentration typically found in this medium might have triggered catabolite repression, a regulatory mechanism where yeast cells

suppress alternative metabolic routes in favor of glucose respiration, thereby inhibiting fermentation (H. Zhang et al., 2023).

It is also important to recognize that visible growth indicators, such as budding or colony expansion, do not necessarily correlate with fermentative activity. Yeasts can proliferate under aerobic or semi-aerobic conditions without producing biogas if energy metabolism is routed through oxidative pathways ((G. dos S. Costa et al., 2024; Cuffaro et al., 2023). Moreover, the genetic heterogeneity among environmental isolates likely played a role in the observed variability. Some strains may lack key enzymes, such as pyruvate decarboxylase or alcohol dehydrogenase, required for efficient ethanol and gas production (Ndubuisi et al., 2023; Perruca Foncillas et al., 2023).

This aligns with previous findings where not all yeast strains exhibit equal fermentative capabilities, especially when challenged with non-standard substrates or environmental stressors, such as those present in agro-industrial effluents like OOW (Ben Sassi et al., 2008; Kieliszek et al., 2017). The positive result observed with strain Y17 thus represents a critical step in selecting efficient yeast candidates for valorizing OOW through bioethanol production.

II.4.4. Assessment of yeast tolerance to olive oil wastewater polyphenols

The ability of yeast strains to withstand the toxic effects of polyphenols is a crucial factor when utilizing olive oil wastewater (OOW) as a substrate for bioethanol production. Due to the inherently high polyphenolic load in OOW, only robust microbial strains can effectively ferment such substrates. In the current study, the isolate Y17 demonstrated strong tolerance across a range of OOW concentrations, indicating its suitability for fermentation in polyphenol-rich environments.

Polyphenols, while valuable for their antioxidant properties in food and health contexts, exhibit potent antimicrobial activity. They can compromise microbial viability by altering membrane integrity, impairing transport systems, and interfering with enzymatic activity, ultimately hindering cellular metabolism (Cuffaro et al., 2023; De Rossi et al., 2025; Sar & Akbas, 2023).

Strain Y17's ability to grow and ferment under elevated polyphenol concentrations suggests that it harbors inherent or adaptive mechanisms for resistance. These may include

reinforced cell wall and membrane structures, active efflux pumps, or enzymatic systems capable of neutralizing phenolic compounds (De Rossi et al., 2025; Villarreal et al., 2022). The strain's resilience underscores the importance of screening for polyphenol-tolerant yeasts when designing bioconversion processes using agro-industrial residues like OOW.

II.4.5. DNA Sequencing results

The phylogenetic analysis based on the internal transcribed spacer (ITS) rRNA region (Figure 12) confirmed the taxonomic identity of yeast strain Y17. This strain clustered within a well-supported monophyletic group alongside *Saccharomyces cerevisiae* CBS 1171, *S. paradoxus* CBS 432, and *S. cariocanus* NRRL 27337. Notably, Y17 was positioned in the same clade as the reference strain *S. cerevisiae* CBS 1171, indicating a close evolutionary relationship.

These molecular findings confirm that strain Y17 belongs to the genus *Saccharomyces*, and more specifically, to the species *Saccharomyces cerevisiae*. Its affiliation with this taxon is further supported by phenotypic traits observed during the initial morphological identification. On Sabouraud Dextrose Agar, Y17 formed colonies and cellular structures typical of *S. cerevisiae*, including ellipsoidal cells with multilateral budding patterns.

The confirmed identity of Y17 as *S. cerevisiae* Y17 reinforces its relevance as a promising bioethanol-producing strain. This species is well-documented for its robustness and adaptability to stressful fermentation environments, such as high ethanol levels and the presence of antimicrobial polyphenols both characteristic of olive oil wastewater (OOW) (Devi et al., 2022; Wardani et al., 2023b). In addition, *S. cerevisiae* possesses a highly versatile metabolism, allowing it to efficiently ferment a range of sugars even in the presence of inhibitory substances (H. Huang et al., 2023).

Overall, these results emphasize the potential of Y17 as a high-performing, stress-tolerant yeast strain suitable for the conversion of complex agro-industrial by-products like OOW into bioethanol, contributing both to waste valorization and sustainable energy production.

II.4.6. Batch fermentation with 100% Olive Oil Wastewater (OOW)

In fermentation experiments utilizing undiluted olive oil wastewater (OOW), *Saccharomyces cerevisiae* Y17 demonstrated high fermentative performance. After 72 hours,

the strain produced a peak ethanol concentration of 11.3 g/L, corresponding with a sharp glucose depletion from 26.0 g/L to just 1.0 g/L and a pH drop from 4.8 to 3.8. This yielded an ethanol conversion efficiency of 0.43 g ethanol per g glucose consumed—placing it within the upper range of yields previously reported for untreated or minimally processed OOW (Ayadi et al., 2022a; Nikolaou & Kourkoutas, 2018; Sarris et al., 2013). The production of over 10 g/L ethanol by 48 hours closely parallels the kinetics described in batch fermentations conducted by Sarris et al., where final ethanol levels ranged from 12 to 15 g/L.

Under the same fermentation conditions, the commercial *Saccharomyces cerevisiae* Saf-instant® strain produced a lower ethanol concentration (8.6 g/L), with a residual glucose level of 5.2 g/L and a slightly lower yield (0.41 g/g). These results underscore Y17's superior fermentative capacity in complex substrates, a trait likely derived from its environmental origin and natural resistance to phenolic toxicity. This aligns with the observations of Parapouli et al. (2020), who reported that wild *S. cerevisiae* isolates often outperform industrial strains in challenging or inhibitor-rich media.

Significantly, fermentation was achieved without prior detoxification or physicochemical treatment of the OOW, and the process remained effective despite a steady acidification of the medium. The final pH of 3.8 falls within the typical range observed in OOW fermentations, where acid accumulation is a natural consequence of yeast metabolic activity (Ayadi et al., 2022a). Unlike many protocols that require immobilization to stabilize fermentation in such media, strain Y17 performed efficiently in free-cell form, suggesting a high level of metabolic resilience.

Comparative data from studies using non-*Saccharomyces* yeasts (e.g., *Pichia*, *Candida*) further highlights the strength of Y17. While certain non-conventional strains exhibit phenolic detoxification capabilities, they often display poor tolerance to ethanol accumulation and reduced productivity under stress (Abdelhadi et al., 2010; Dragičević et al., 2010; Foti et al., 2021; Jamai & Ettayebi, 2015; Ndubuisi et al., 2023). In contrast, Y17 maintained high ethanol output and growth throughout the fermentation period.

These findings confirm the promising potential of *S. cerevisiae* Y17 as a robust biocatalyst for direct ethanol production from OOW. Its ability to withstand inhibitory conditions and achieve substantial ethanol yields without additional treatment makes it an attractive candidate for sustainable bioethanol applications. Future studies may focus on scaling

up the process, investigating the genetic and biochemical basis of Y17's phenol tolerance, and exploring co-culture strategies with organisms capable of enhancing phenolic degradation prior to fermentation.

II.5. Conclusion

This study successfully isolated and characterized a native yeast strain, *Saccharomyces cerevisiae* Y17, from olive oil wastewater (OOW)—a substrate recognized for its complex composition and inhibitory load due to high polyphenol content. Among the isolates tested, Y17 exhibited outstanding tolerance to 100% untreated OOW and demonstrated efficient ethanol production, reaching 11.3 g/L after 72 hours of fermentation.

The performance of Y17 underscores its strong adaptability to harsh environmental conditions, effective glucose metabolism, and sustained fermentative activity even under acidifying conditions. Notably, this wild-type strain outperformed the commercial *S. cerevisiae* Saf-instant® under identical fermentation settings, reinforcing the potential of indigenous strains in processing non-conventional, inhibitor-rich feedstocks.

These findings support the valorization of OOW as a promising substrate for sustainable bioethanol production, offering the dual advantage of renewable energy generation and eco-friendly waste management. The identification of *S. cerevisiae* Y17 as a robust, phenol-tolerant strain opens avenues for future research, including process scale-up, fermentation optimization, and potential integration with detoxification or immobilization technologies. Ultimately, this work contributes to advancing circular bioeconomy strategies through the biotechnological valorization of agro-industrial byproducts.

Chapter III

Bioethanol production from agro-industrial by-products using immobilized yeasts

III.1. Introduction

In Algeria, the agro-food sector generates millions of tons of by-products and residues each year. Although often treated as waste, these materials are rich in organic compounds and fermentable sugars, presenting significant potential for biotechnological valorization. Among the most prominent examples are cheese whey from dairy industries, olive mill wastewater (OMW) from olive oil production, and sugarcane or sugar beet molasses from the sugar industry. Instead of contributing to environmental burden, these by-products can serve as low-cost substrates for the production of bioethanol, a renewable energy source (Abu Tayeh et al., 2014; Álvarez-Cao et al., 2020; Pasotti et al., 2017). In recent years, considerable attention has been given to the bioconversion of agro-industrial residues, with particular interest in strategies that combine multiple waste streams to enhance fermentation efficiency.

The production of bioethanol from such waste not only addresses pressing environmental issues associated with waste disposal but also contributes to the development of sustainable, low-carbon energy alternatives. Bioethanol, as a renewable fuel, holds significant promise for reducing greenhouse gas emissions and decreasing reliance on fossil fuels (Falowo & Betiku, 2023). Although various methods exist for bioethanol production, fermentation remains the most widely studied approach due to its ability to utilize a broad range of carbon-rich substrates (Duque et al., 2021). Nonetheless, most existing studies have focused on single-waste fermentation, overlooking the potential advantages of co-fermentation systems.

Enzymatic hydrolysis has emerged as a crucial pre-treatment step for enhancing the fermentability of complex waste materials. Through enzymatic breakdown, polysaccharides are converted into simpler sugars, thereby improving the efficiency of subsequent fermentation processes (Vasić et al., 2021). Despite its success in single-substrate systems, the application of enzymatic hydrolysis in multi-waste fermentations remains relatively unexplored (Cheng et al., 2020). Furthermore, the immobilization of yeast cells a technique that offers enhanced

stability, reusability, and tolerance to fermentation stresses has not been widely investigated in the context of mixed-substrate fermentations (de Araujo et al., 2024).

This Chapter focuses on evaluating the synergistic effects of co-fermenting olive oil mill wastewater, milk whey, and sugarcane molasses for bioethanol production. Special emphasis is placed on optimizing enzymatic hydrolysis using a commercial multi-enzyme complex, Natuzyme, by testing different enzyme concentrations to maximize the release of fermentable sugars. Additionally, the impact of yeast immobilization on pozzolan, a highly porous volcanic rock, is assessed to determine improvements in fermentation performance compared to free-cell systems (Indira et al., 2015).

The objectives of this research are to optimize the enzymatic hydrolysis process for enhanced sugar availability, evaluate the influence of immobilized yeast on fermentation kinetics and ethanol yield, and identify the most effective combination of OMW, MW, and molasses in terms of ethanol production. Through an integrated approach combining waste valorization, enzymatic treatment, and cell immobilization, this study aims to develop a more efficient and sustainable strategy for bioethanol production from agro-industrial residues.

III.2. Materials and methods

III.2.1. Sample collection

Agro-industrial by-product samples were collected from various local industries across Algeria. Each sample was coded upon collection and stored at 4°C in a dark environment within the Laboratory of Natural Bio-Resources, University of Hassiba Benbouali, Chlef, until further use. The substrates employed in this study included:

- **Olive Oil Waste-Water (OOWW):** Samples were collected from the El Nakhla olive mill, located in northwestern Algeria (36°26′03″ N, 1°41′32″ E), during the olive harvesting season (October–December) to ensure maximum sugar concentration.
- **Milk Whey (MW):** Sourced from the El Saada dairy production facility, specialized in yogurt and cheese manufacturing, situated in northern Algeria (35°68′63″ N, 0°34′50″ W).
- **Sugarcane molasses (SCM):** Obtained from the Berrahal sugar refinery, located in western Algeria (35°91′53″ N, 0°07′78″ E).

- **Pozzolan Rocks:** Used as a natural immobilization support, collected from the ENG Pozzolan quarry in western Algeria (35°28′58″ N, 1°40′95″ W).
- **Natuzyme:** A commercial multi-enzyme complex, acquired from Safana, an animal nutrition supplier based in eastern Algeria.

III.2.2. Samples preparation

III.2.2.1. Olive Oil Wastewater (OOWW):

The raw OOWW underwent a multi-step pretreatment process to reduce its content of solids, oils, and inhibitory compounds. Initially, it was passed through a fine mesh sieve to remove visible solid debris. The filtrate was then centrifuged at 5,000 rpm for 10 minutes to sediment suspended solids. After centrifugation, the upper oil layer was carefully removed to minimize the hydrophobic load. The clarified aqueous phase was subsequently diluted at a 1:10 ratio with distilled water to further lower the concentration of naturally occurring inhibitory substances.

III.2.2.2. Sugarcane Molasses (SCM):

SCM was diluted at a 1:10 ratio with distilled water to reduce its high viscosity and sugar concentration, thereby standardizing it for fermentation. The diluted solution was mixed thoroughly until homogeneity was achieved before use.

III.2.2.3. Milk Whey (MW):

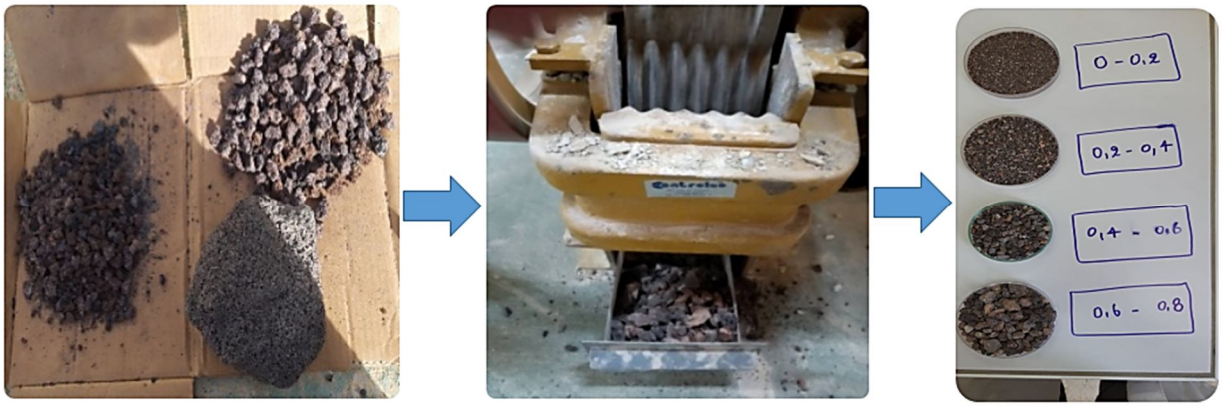
Due to its high water content, milk whey was diluted at a 1:5 ratio with distilled water. This level of dilution was selected to retain sufficient concentrations of fermentable sugars while improving consistency across experiments.

III.2.2.4. Pozzolan Rocks (Immobilization Support):

To prepare the pozzolan rocks for use as immobilization support, a detailed multi-step treatment protocol was applied to ensure the appropriate size, porosity, and sterility of the material. These steps aimed to eliminate physical impurities, organic residues, and microbial contaminants while enhancing the surface characteristics of the support.

- Crushed into small aggregates (4–6 mm in diameter) using a motorized metal hammer.

- Sieved to obtain uniform particle size (see process in Figure 14).
- Heated in a muffle furnace at 500 °C to increase porosity and destroy biological residues.
- Allowed to cool, then rinsed several times with tap water.
- Immersed in alcohol for 24 hours to remove residual organic matter.
- Treated with hydrogen peroxide solution for disinfection and oxidation of remaining contaminants.
- Washed thoroughly with distilled water to eliminate chemical residues.
- Dried in an oven at 105 °C for 10 hours.
- Sterilized by autoclaving at 120 °C for 20 minutes.



- **Figure 14.** The crushing and sieving process of the Pozzolan rocks.

Before enzymatic hydrolysis and fermentation, all liquid substrates and prepared pozzolan supports were sterilized to avoid microbial contamination.

III.2.3. Yeast strain and preparation of inoculum

The yeast strain used in this study was *Saccharomyces cerevisiae* Y17, previously isolated from olive oil wastewater (OOWW) for its polyphenol tolerance.
To prepare the inoculum, the strain was first cultured on Sabouraud agar medium (composed of 40 g/L dextrose, 10 g/L peptone, and 20 g/L agar) and incubated at 30°C for 48 hours. Subsequently, a pre-culture was initiated by inoculating selected yeast colonies into 100 mL of a sterilized mixture of the diluted substrates. The culture was incubated at 30°C under orbital shaking at 150 rpm for 24 hours, allowing the yeast cells to reach the exponential growth phase, which is ideal for fermentation initiation.

III.2.4. Static fermentation tests

Preliminary fermentation tests were performed to evaluate the feasibility of ethanol production under the designed experimental conditions and to troubleshoot potential operational issues. Static fermentation assays were carried out over a 48-hour period using the *S. cerevisiae* Y17 strain. The production of carbon dioxide (CO_2), a by-product of ethanolic fermentation, was monitored as an indirect indicator of ethanol production. This evaluation was based on the stoichiometric relationship where one mole of glucose produces two moles of ethanol and two moles of CO_2, as described by Kumara Behera and Varma (2017).

The CO_2 produced during fermentation was quantified using a gas collection system based on the displacement of a syringe piston connected to a sealed fermentation tube. Each fermentation test was conducted in triplicate to ensure the reliability and reproducibility of the results.

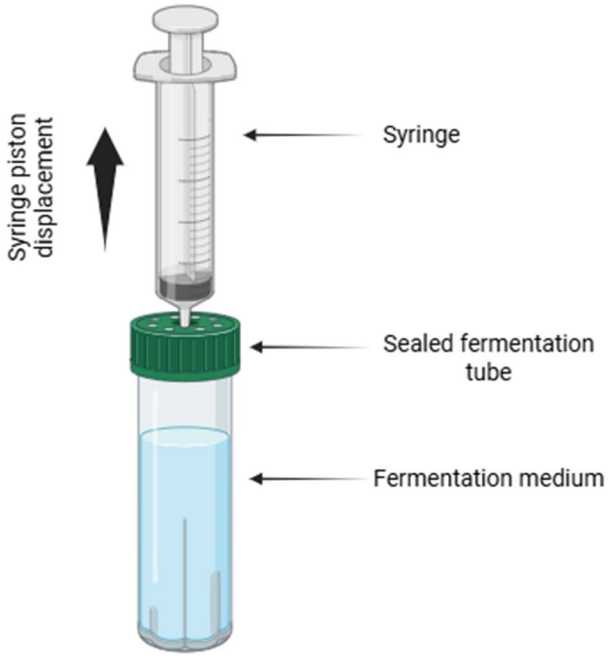


Figure 15. The gas collection system based on the displacement of a syringe piston connected to a sealed fermentation tube.

III.2.5. Formulation of fermentation media using agro-industrial waste mixtures

To optimize fermentation performance and enhance ethanol production, different formulations were prepared by mixing the three pretreated agro-industrial wastes—olive oil wastewater (OOWW), milk whey (MW), and sugarcane molasses (SCM)—in varying proportions. Four mixtures were tested to evaluate the effect of substrate composition on yeast fermentation efficiency (Table 9).

Table 9: Composition of fermentation media using different mixtures of agro-industrial wastes

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

III.2.6. Enzymatic hydrolysis

To enhance sugar availability, enzymatic hydrolysis was carried out using Natuzyme, a commercial enzyme complex from Bioproton, recognized for its broad-spectrum activity on polysaccharides. The enzyme mixture contains phytase, α-amylase, xylanase, β-mannanase, β-glucanase, cellulase, protease, lipase, and pectinase, which together facilitate the breakdown of complex carbohydrates.

Figure 16. The Label for the composition and posology of Natuzyme.

Three different enzyme concentrations were tested: 0.25%, 0.5%, and 0.75% (w/v), based on results from preliminary trials. Enzymatic hydrolysis was performed at 30°C with the pH adjusted to 5.0 using 0.1M HCl or NaOH. The incubation period lasted for 48 hours, during which continuous stirring was maintained at 150 rpm.

Figure 17. The enzymatic hydrolysis setup (0.25%,0.5% and 0.75%).

To quantify glucose concentrations before and after the hydrolysis process, the 3,5-dinitrosalicylic acid (DNS) method was employed, as outlined by (Jain et al., 2020).

III.2.7. Static FreeCell (non-immobilized) fermentations assays

III.2.7.1. Fermentation of individual substrates at varying enzyme doses

To assess the effect of enzymatic hydrolysis on fermentative gas production from each agro-industrial waste, static batch fermentations were conducted using *non-immobilized (free) cells* of *Saccharomyces cerevisiae* Y17. The three substrates olive oil wastewater (OOWW), milk whey (MW), and sugarcane molasses (SCM) were fermented individually.

Each substrate underwent enzymatic pretreatment at four different Natuzyme concentrations: 0% (untreated), 0.25%, 0.5%, and 0.75% (w/v). Hydrolysis was carried out for 48 hours at 30 °C and pH 5.0 with constant agitation at 150 rpm, following the procedure outlined in Section III.2.6. After hydrolysis, 200 mL of each treated substrate was transferred into a 250 mL sealed flask and inoculated with yeast for static fermentation.

CO_2 evolution was monitored over a 72-hour period using a gas collection system based on syringe piston displacement (see Section III.2.4). The cumulative CO_2 volume was used as an indirect measure of ethanol production, based on the stoichiometric fermentation equation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

This equation implies that 1 mole of glucose yields 2 moles of ethanol and 2 moles of CO_2, allowing ethanol yield to be inferred from CO_2 volume. Each fermentation was performed in triplicate to ensure statistical accuracy and reproducibility.

III.2.7.2. Fermentation of substrate mixtures (mix 1–4) with and without enzymatic treatment

To explore the effect of combining agro-industrial wastes on ethanol fermentation efficiency, four different substrate mixtures (Mix 1 to Mix 4; see Table 9) were formulated and tested using free cells of *S. cerevisiae* Y17 and Saf-instant commercial. Each mix was evaluated under two conditions: without enzymatic hydrolysis (0%) and with 0.5% (w/v) Natuzyme treatment.

Following hydrolysis (for enzyme-treated samples) or dilution (for untreated ones), 200 mL of each mix was transferred into 250 mL Erlenmeyer flasks and subjected to static fermentation for 72 hours. CO_2 evolution was monitored using the same displacement system described previously.

Cumulative CO_2 production was measured for each condition and compared across the four mixtures to identify the most efficient substrate combination and the effect of enzymatic treatment. As before, results were used to infer ethanol yield using the glucose fermentation equation, and all assays were performed in triplicate for statistical reliability.

III.2.8. Simultaneous Saccharification and Batch Fermentation (SSF) with Free Cells

Fermentation assays were conducted using batch culture in 1 L glass flasks, each containing 700 mL of the selected substrate mixtures. The fermentations were carried out under agitation at 150 rpm and a constant temperature of 30 °C for a total duration of 72 hours. These

trials were performed using free (non-immobilized) yeast cells, allowing direct contact between the microbial biomass and the fermentation medium.

To maintain sterile and semi-anaerobic conditions, the flasks were equipped with one-way gas release valves and 22-micron filters, permitting the escape of CO□ while preventing external contamination as shown in Figure 19. Sampling was performed aseptically through a dedicated sampling port at regular intervals.

Based on previous optimization studies, a single enzyme concentration of 0.5% (w/v) Natuzyme® was used for all substrate formulations. The process was conducted as a simultaneous saccharification and fermentation (SSF), where enzymatic hydrolysis of complex carbohydrates and ethanol fermentation occurred concurrently.

The four substrate mixtures tested (Mix 1, Mix 2, Mix 3, and Mix 4) were prepared from combinations of olive oil wastewater (OOWW), milk whey (MW), and sugarcane molasses (SCM), as described in Section III.2.5.

Throughout the fermentation, the following parameters were measured:

- pH
- Residual glucose concentration (via the 3,5-dinitrosalicylic acid [DNS] method)
- Ethanol concentration

III.2.9. Simultaneous saccharification and Batch 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 72 hours. To maintain sterility and anaerobic conditions, the flasks were equipped with one-way gas release valves and 22-micron filters to prevent contamination as shown in Figure 19. Sampling was carried out in a sterile zone using a dedicated sampling orifice. Based on preliminary optimization results, the best-performing enzyme dose of **0.5%** was selected and applied uniformly across all four fermentation mixtures (Mix 1, Mix 2, Mix 3 and Mix 4) to enhance the hydrolysis of complex substrates and improve fermentability.

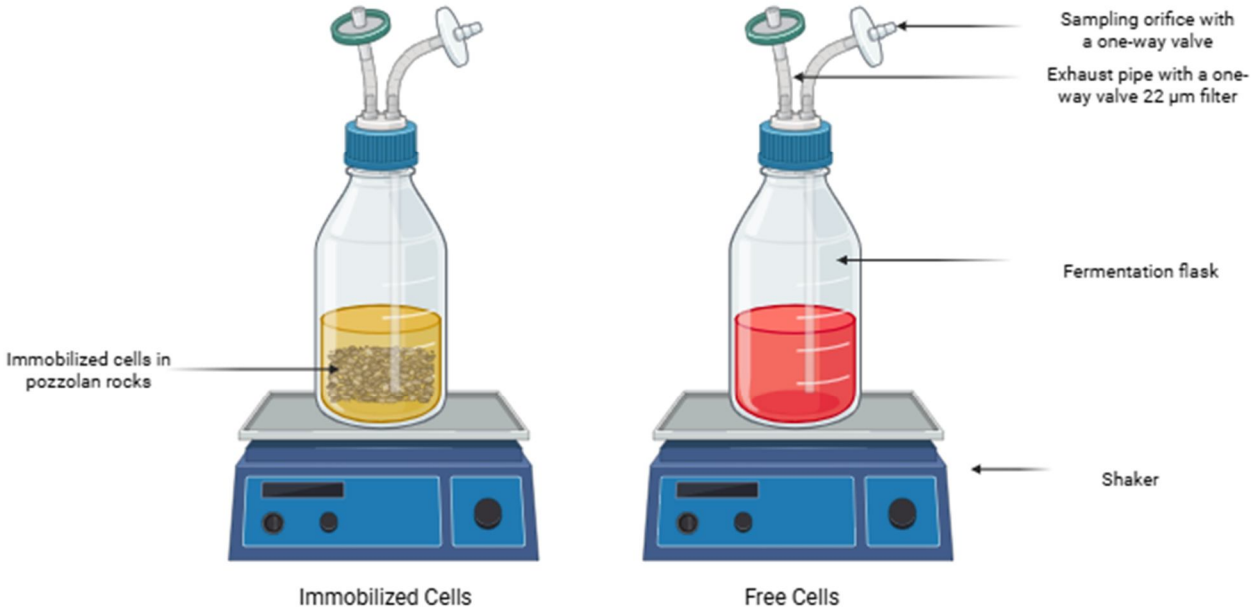


Figure 19. Fermentation setup schematic representation of the experimental configuration for both free and immobilized cells.

III.2.10. Cell immobilization

In a previous study (Ayadi et al., 2022), we developed a method for yeast cell immobilization using pozzolan, a naturally occurring, porous volcanic rock known for its high surface area, which enhances cell attachment and retention. The pozzolan was first washed thoroughly to remove any debris, followed by drying, and then autoclaved at 121°C for 15 minutes to ensure sterility and eliminate any potential contaminants. Sterile pozzolan was subsequently introduced into YPD medium, which had been inoculated with pre-cultured *Saccharomyces cerevisiae* Y17. The mixture was incubated at 30°C for 24 hours to facilitate biofilm formation and effective cell immobilization (Figure 20). To confirm the success of the immobilization process, microscopic observations were conducted to examine the formation of the yeast biofilm on the pozzolan particles. Additionally, viable cell counting was performed to ensure that the immobilized cells were still metabolically active and capable of contributing to the fermentation process.

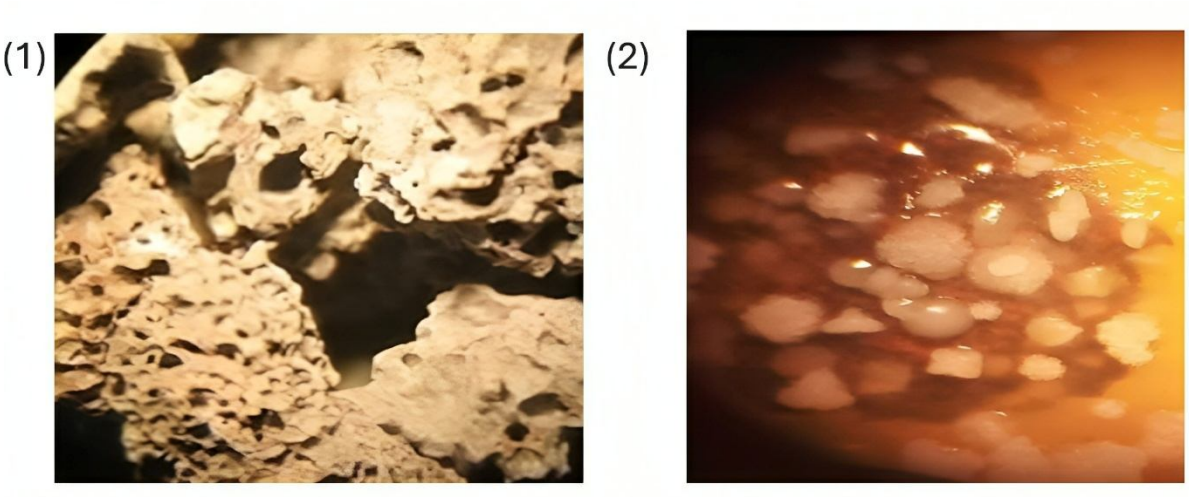


Figure 20. Pozzolane rocks under binocular observation x40 : (1) before yeast immobilization, showing a porous structure, and (2) after immobilization, highlighting yeast clusters formation on the surface.

III.2.11. Analytical methods

To monitor the progress of fermentation, several key parameters were measured:

- **pH**: The pH of the fermentation broth was measured using a BANTE-210 benchtop pH meter.
- **Optical density (OD_{600})**: The optical density at 600 nm (OD600) was measured to estimate cell growth using a Shimadzu UV-1800 spectrophotometer, which was connected to a computer for data analysis.
- **Glucose concentration**: Glucose concentration in the fermentation broth was determined using the 3,5-Dinitrosalicylic Acid (DNS) method, as described by Jain et al. (2020). The reagent used was 3.5-DNS 97+ from Alfa Aesar, Germany.
- **Ethanol concentration**: Ethanol was separated from the fermentation broth using a rotary evaporator (Rotavapor Büchi R-100). The ethanol concentration was then determined via potassium permanganate titration, as outlined by P. Zhang et al. (2019).

These analytical methods enabled a comprehensive assessment of the fermentation process, including cell growth, substrate consumption, and ethanol production.

III.2.12. Statistical analysis

A detailed statistical analysis was performed using **GraphPad Prism 10** to investigate the relationships between enzyme dosage, glucose release, and biogas production. The aim was

to assess both the direct influence of enzyme dosage on glucose concentration and biogas yield, as well as to evaluate the correlation between glucose levels and biogas production.

Linear regression

To evaluate the effect of enzyme dosage on glucose release and biogas production, a simple linear regression model was applied for each substrate (MW, OOWW, and SCM) at two distinct time points (T1: 24 hours and T2: 48 hours). In these models, enzyme dosage was considered the independent variable, while glucose concentration and biogas yield were treated as dependent variables in separate analyses.

The linear regression model used is represented by the following equation:

$$Y = \beta_0 + \beta_1 X + \epsilon \quad (4)$$

Where:

- Y represents the dependent variable (either glucose or biogas),
- X denotes the enzyme dose,
- β_0 is the intercept,
- β_1 is the slope,
- ϵ is the error term.

Significance of the model was assessed using the coefficient of determination (R2R^2R2) and the p-value ($p<0.05p < 0.05p<0.05$).

Pearson correlation analysis

Additionally, Pearson correlation analysis was conducted to explore the relationship between glucose concentration and biogas yield. Prior to this analysis, normality, homoscedasticity, and linearity assumptions were tested to ensure the validity of the data. The statistical analysis highlighted the direct effects of enzyme dosage on glucose availability and biogas production, as well as the correlation between glucose concentration and biogas yield, thus providing insights into the efficiency of the fermentation process.

III.3. Results and discussion

III.3.1. Physicochemical parameters of co-products

The physicochemical characteristics of olive oil wastewater (OOWW), milk whey (MW), and sugarcane molasses (SCM) were analyzed to evaluate their potential as substrates for fermentation (Table 10). These properties are critical because they directly affect yeast growth, enzymatic hydrolysis performance, and ultimately, ethanol yield.

Table 10: 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 (%)	2.19	0.0	0.21	(Clément, 1956)
DBO5 O2/l (g.L-1)	11	52.4	7.3	ISO 5815-1:2019
DCO O2/l (g.L-1)	123	102.2	14	ISO 15705:2002
pH	4.73	4.99	4.89	pH meter (BANTE-210)

a. Olive oil wastewater (OOWW)

The OOWW analyzed in this study presented compositions comparable to those reported in previous literature, though some variations were observed.

The fat content measured was 2.19%, slightly higher than the ranges reported by Esmail et al. (2013) (1–2.5%) and Djeziri et al. (2023) (1.25%). Nevertheless, it remains within the broader interval described by Bouknana et al. (2014) (0.8–27.4 g/L). Variations in fat content are often attributed to differences in olive processing methods, harvest seasons, and geographical origins of the olive cultivars.

The reducing sugar content was 3.42 g/L, falling within the range observed by Bouknana et al. (2014) (3.52–10.48 g/L), suggesting a moderate level of fermentable sugars available for yeast metabolism.

In terms of chemical oxygen demand (COD), OOWW exhibited a value of 123 g/L, higher than that reported by Esmail et al. (2013) (104 g/L) and Djeziri et al. (2023) (90.5 g/L), yet similar to Bouknana et al. (2014) (120 g/L) and lower than the value recorded by Ayadi et al. (2022) (183 g/L).

Regarding biochemical oxygen demand (BOD□), the value obtained (11 g/L) was lower than those reported by Esmail et al. (2013) (35 g/L), Djeziri et al. (2023) (29 g/L), and Bouknana

et al. (2014) (17–25 g/L), but was relatively close to the value found by Ayadi et al. (2022) (7 g/L).

The measured pH of 4.73 was slightly higher than the value reported by El Kafz et al. (2023) (4.09), yet slightly lower compared to Ayadi et al. (2022) (4.88).

Overall, these findings confirm that OOWW remains a challenging but promising substrate, requiring pretreatment or supplementation to optimize its use in fermentation processes.

b. Sugarcane molasses (SCM)

The reducing sugar content of SCM in this study was 37.02%, noticeably lower than the 51.36% reported by S.H.A. Hassan et al. (2019). This discrepancy may be attributed to potential dilution effects or variations in sugar extraction and processing methods.

The COD value measured at 102.2 g/L was lower than the 132.25 g/L reported by Hakika et al. (2019), while the BOD□ of 52.4 g/L was higher than their reported 31.25 g/L. The relatively lower sugar content observed could explain the reduced COD values and suggests that the SCM used might have been of lower concentration or partially diluted.

Regarding pH, the value of 4.99 falls between those reported by previous studies: higher than Hakika et al. (2019) (3.8) and slightly lower than the value reported by S.H.A. Hassan et al. (2019) (5.1). This intermediate pH may favor yeast activity without requiring extensive adjustment.

c. Milk whey (MW)

In the case of MW, the protein content found in this study (1.03%) was higher than that reported by Lievore et al. (2015) (0.84%), but lower compared to Lachebi and Yelles (2018) (6.2%), possibly reflecting differences in whey processing or origin.

The fat content measured at 0.21% was close to the 0.08% reported by Lievore et al. (2015), but much lower than the 1.6% found by Lachebi and Yelles (2018), indicating that partial skimming might have been performed on the MW used in this study.

For reducing sugars, the MW sample contained 4.1%, which was slightly lower than the 6.2% reported by Lachebi and Yelles (2018). This relatively modest sugar concentration

suggests that MW alone may not be sufficient for robust fermentation unless supplemented, for instance, with SCM.

The COD and BOD□ values recorded were 14 g/L and 7.3 g/L, respectively. These values were somewhat higher than those reported by Lachebi and Yelles (2018), who found COD and BOD□ levels of 11 g/L and 6.4 g/L, respectively.

Lastly, the pH of MW (4.89) was slightly higher than those reported by Lievore et al. (2015) (4.37) and Lachebi and Yelles (2018) (4.5), yet remained within a favorable range for yeast fermentation processes.

The physicochemical characterization of OOWW, SCM, and MW revealed significant variability in their composition, reflecting their diverse origins and processing conditions. OOWW presented moderate sugar content and a high COD, indicating potential for fermentation after appropriate pretreatment. SCM exhibited the highest reducing sugar concentration, although slightly lower than literature values, positioning it as a key supplement to enhance fermentability. MW, while richer in protein, contained lower sugar levels, suggesting it would benefit from combination with other substrates. Overall, the complementary profiles of these co-products offer promising potential for their valorization in biotechnological applications such as ethanol production.

III.3.2. Effect of enzymatic hydrolysis on sugar release and biogas production

III.3.2.1. Glucose concentration before and after enzymatic treatment

To evaluate the efficacy of enzymatic hydrolysis, glucose concentrations were measured at T□ (before treatment) and T□ (after 48 hours of treatment) for the different wastewaters (OOWW, SCM, and MW) at varying enzyme doses (0.25%, 0.5%, and 0.75%). The results are summarized in Table 11 and illustrated in Figure 21.

Table 11: 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 enzymatic treatment resulted in a significant increase in glucose concentration ($p < 0.05$) for all substrates and enzyme doses. Linear regression analysis revealed strong correlations ($R^2 > 0.85$) between enzyme dose and glucose release.

Among the substrates, OOWW exhibited the highest relative increase in glucose concentration (up to 225.1%), which can be attributed to the hydrolysis of complex carbohydrates such as cellulose into fermentable sugars. SCM and MW displayed comparable percentage increases (219.5% and 219.6%, respectively), indicating effective enzymatic activity, even though MW primarily contains lactose.

Interestingly, the largest jumps in glucose concentration occurred between enzyme doses of 0.25% and 0.5%, with increases exceeding 190% across all substrates. This suggests that a 0.5% enzyme dose represents the most efficient and economically viable concentration for large-scale hydrolysis applications.

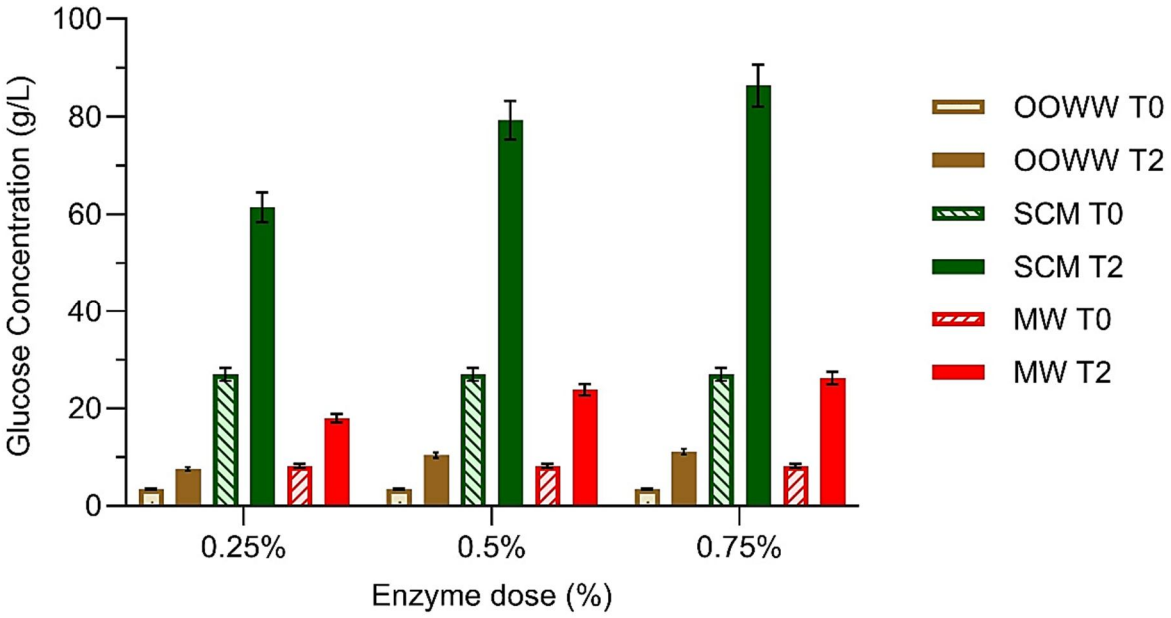


Figure 21. Glucose release after enzymatic hydrolysis at different Natuzyme concentrations.

The ability to sustain a consistent glucose concentration increase of around 200% across all substrates at higher enzyme doses clearly demonstrates the efficiency of the enzymatic hydrolysis process. This performance can be attributed to the diverse enzymatic composition of Natuzyme, where each enzyme specifically targets key substrate components. For OOWW,

enzymes such as cellulase, xylanase, β-glucanase, and pectinase played crucial roles in breaking down complex polysaccharides and structural carbohydrates, enhancing glucose release even in the presence of inhibitory phenolic compounds (Bhardwaj et al., 2021; Nguyen et al., 2018).

In the case of SCM, the high percentage increase in glucose concentration can be explained by the activity of α-amylase, responsible for degrading residual starch, and potentially invertase, which hydrolyzes sucrose into glucose and fructose, thereby promoting rapid sugar availability for fermentation (Manoochehri et al., 2020). Regarding MW, the presence of β-galactosidase likely contributed to the hydrolysis of lactose into glucose and galactose, enhancing the fermentable sugar pool (Saqib et al., 2017b).

These enzymes act synergistically, optimizing the breakdown of complex carbohydrates, increasing substrate accessibility, and maximizing glucose yield, all of which are critical for efficient bioethanol production from agro-industrial residues.

Moreover, the plateau effect observed at the 0.75% enzyme dose suggests a point of substrate saturation, where further enzyme addition results in diminishing returns. This highlights the importance of enzyme dose optimization for industrial-scale applications to balance process efficiency and economic viability (Bisswanger, 2017).

III.3.3. Enzymatic hydrolysis effect on biogas production for each individual substrates

The influence of enzymatic hydrolysis on biogas generation was assessed by measuring biogas volumes at T□ (24 hours) and T□ (48 hours) following the addition of varying enzyme doses (0%, 0.25%, 0.5%, and 0.75%). The evolution of biogas production under different conditions is presented in Figure 22.

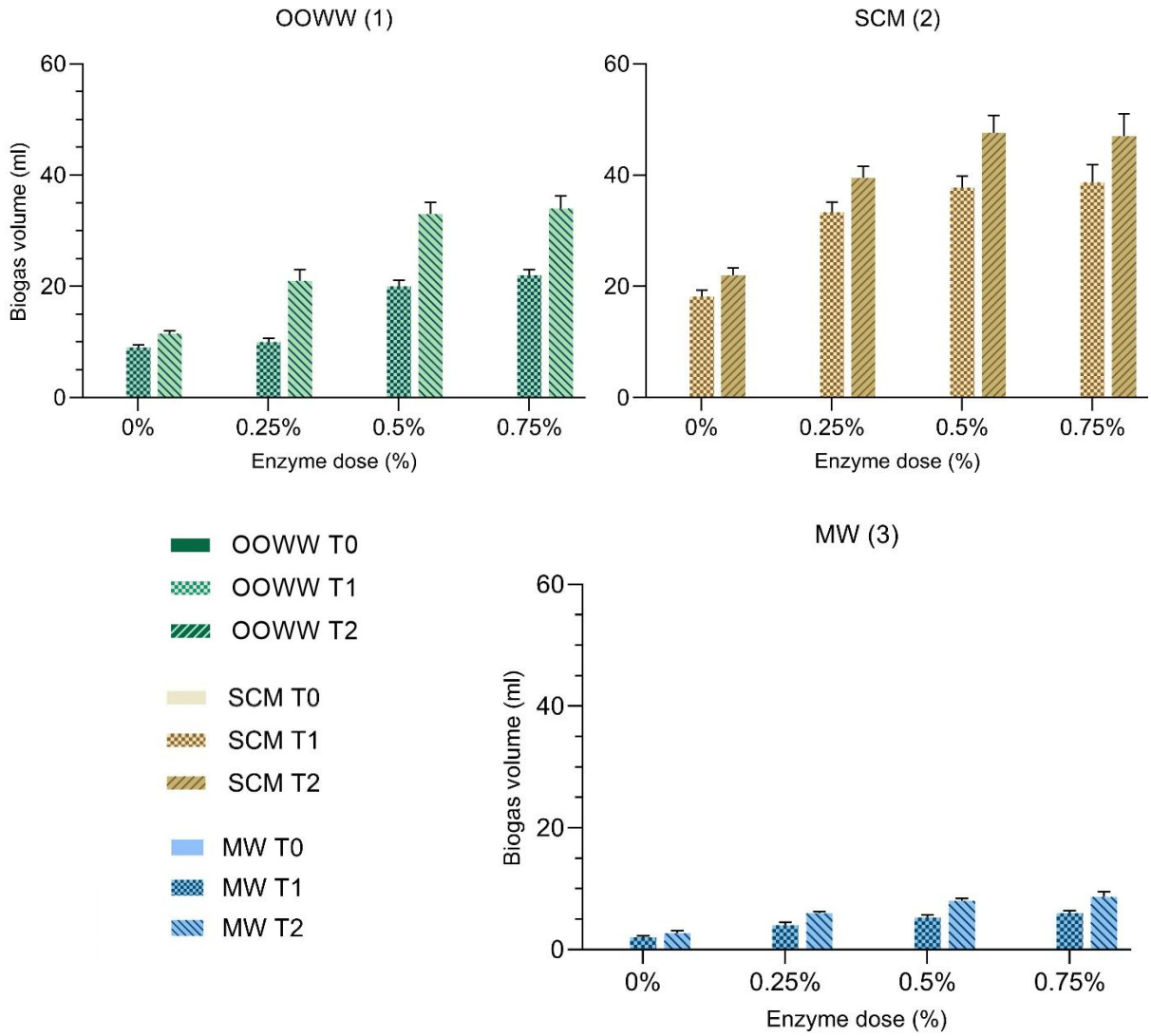


Figure 22. Biogas production (mL) at T1 and T2 Across different enzyme doses for OOWW, SCM, and MW

After 48 hours (T□), SCM exhibited the highest biogas production, reaching 47 ± 2 mL at a 0.75% enzyme dose, followed by OOWW with 34 ± 1.5 mL, and MW with a substantially lower yield of 8.7 ± 1 mL.

The superior performance of SCM can be attributed to its elevated sugar content, which enhances microbial metabolic activity during anaerobic digestion. In contrast, OOWW's moderate biogas yield is likely influenced by the presence of polyphenolic compounds, known microbial inhibitors (Calabrò et al., 2018), which may partially suppress microbial fermentation despite improved sugar availability.

Conversely, MW recorded the lowest biogas output, a result that may be explained by its compositional profile rich in lactose and proteins which are less efficiently converted into biogas compared to simple sugars (Kovács et al., 2013).

Importantly, control samples (0% enzyme dose) exhibited markedly lower biogas production at both T□ and T□, emphasizing the critical role of enzymatic pre-treatment in enhancing fermentative performance.

The most substantial improvement in biogas production was observed between the 0.25% and 0.5% enzyme doses, particularly for SCM, where biogas output increased by over 35%. However, a saturation trend emerged beyond the 0.5% dose, indicating diminishing returns with further enzyme supplementation.

Statistical analysis revealed a significant enhancement in biogas production with increasing enzyme doses ($p < 0.05$). The strong linear relationship between enzyme dose and biogas yield ($R^2 > 0.80$) supports the efficiency of enzymatic hydrolysis in augmenting anaerobic digestion. Additionally, the high correlation observed between glucose release and biogas production ($r > 0.85$) further confirms that substrate availability directly drives microbial activity and subsequent biogas generation.

III.3.4. Fermentation of substrate mixtures (mix 1–4) with and without enzymatic treatment

To evaluate the efficiency of ethanol production using complex agro-industrial by-product mixtures, a comparative study was conducted between the commercial *Saccharomyces cerevisiae* strain (Saf-Instant) and the isolated Y17 strain. Both strains were tested across four fermentation mixtures (Mix 1 to Mix 4), under static conditions at 30 °C for 72 hours. The impact of enzymatic hydrolysis (Natuzyme at 0.5%) was also assessed by comparing treated (ENZ 0.5%) and untreated (ENZ 0%) conditions.

III.3.4.1. CO□ Release and estimated Ethanol production

The ethanol yield was estimated indirectly via the volume of CO□ released, applying the stoichiometric relationship of 1:1 (mol:mol) between ethanol and CO□ production in alcoholic fermentation. The results are presented in Table 12, and they reflect both the improvement due to enzymatic pretreatment and the relative performance of the two yeast strains.

Table 12: Results of the CO_2 release and the estimation Ethanol for the 4 mixes, using both Y17 and Saf-instant strains in the FreeCell fermentation.

Mixture	*Yeast*	*ENZ Dose*	*CO□ (mL ± SD)*	*Estimated Ethanol (mL)*	*% Increase (ENZ 0.5% vs 0%)*
Mix 1	Saf-Instant	0%	11.5 ± 2.5	9.07	—
		0.5%	17.0 ± 1.8	13.41	+46.8%
	Y17	0%	12.5 ± 2.0	9.86	—
		0.5%	17.2 ± 2.3	13.57	+37.6%
Mix 2	Saf-Instant	0%	19.2 ± 2.3	15.15	—
		0.5%	24.7 ± 2.8	19.48	+28.6%
	Y17	0%	18.4 ± 1.5	14.52	—
		0.5%	25.4 ± 3.5	20.04	+38.0%
Mix 3	Saf-Instant	0%	9.8 ± 2.0	7.74	—
		0.5%	14.8 ± 3.0	11.67	+51.0%
	Y17	0%	11.2 ± 1.3	8.84	—
		0.5%	16.2 ± 3.2	12.78	+44.5%
Mix 4	Saf-Instant	0%	13.2 ± 2.5	10.42	—
		0.5%	18.0 ± 2.8	14.20	+36.3%
	Y17	0%	12.0 ± 3.0	9.47	—
		0.5%	17.8 ± 2.8	14.05	+48.4%

***Ethanol estimation**: Based on the theoretical stoichiometry where 1 mole of glucose → 2 mol ethanol + 2 mol CO□, and knowing that CO□ and ethanol are produced in a 1:1 molar ratio, the ethanol volume (in mL) is estimated by:*

$$\text{Ethanol (mL)} = \text{CO}_2\text{ (mL)} \times \frac{\text{Density of ethanol}}{\text{Density of CO2}} \approx CO_2\,(mL) \times 0.789$$

The fermentation performance of the commercial *Saccharomyces cerevisiae* strain (Saf-Instant) and the isolated Y17 strain was evaluated across four substrate mixtures (Mix 1 to Mix 4). The impact of enzymatic hydrolysis using 0.5% Natuzyme was assessed by comparing treated (ENZ 0.5%) and untreated (ENZ 0%) conditions. Ethanol yield was estimated indirectly through the volume of CO□ released, applying the stoichiometric relationship of 1:1 (mol:mol) between ethanol and CO□ production in alcoholic fermentation.

The addition of 0.5% Natuzyme significantly enhanced CO□ production and, consequently, ethanol yield across all mixtures and both yeast strains. The percent increase in estimated ethanol production due to enzymatic pretreatment ranged between +28.6% and +51.0%, confirming the positive effect of enzymatic saccharification in releasing fermentable sugars from complex polysaccharides present in olive oil wastewater, molasses, and milk whey.

III.3.4.2. Effect of enzymatic hydrolysis

The observed enhancement in ethanol yield aligns with findings from previous studies. For instance, (Yamada et al., 2009) reported that the enzymatic hydrolysis of potato processing by-products using a combination of amylase and pectinase increased ethanol concentration from approximately 20 mg/mL to 50 mg/mL, demonstrating the efficacy of enzymatic pretreatment in improving fermentable sugar availability .

Similarly, a study on cassava pulp hydrolysate indicated that optimal enzymatic hydrolysis conditions led to a high glucose concentration (160 g/L), which, upon fermentation, resulted in an ethanol concentration of 68 g/L with a yield of 0.48 g ethanol/g glucose (Valeriano et al., 2018).

In our study, Mix 3, which initially exhibited the lowest ethanol yield, benefited the most from enzymatic treatment, with a 51% increase observed in the Saf-Instant trials. This suggests that Mix 3 was more recalcitrant in its untreated form and required hydrolysis to improve sugar accessibility.

III.3.4.3. Yeast strain comparison

While both strains responded positively to enzymatic treatment, the Y17 strain generally performed better than Saf-Instant in most mixtures after enzymatic hydrolysis. Notably, in Mix 2, Y17 produced 25.4 mL CO□ (estimated 20.04 mL ethanol) compared to 24.7 mL CO□ (19.48 mL ethanol) for Saf-Instant.

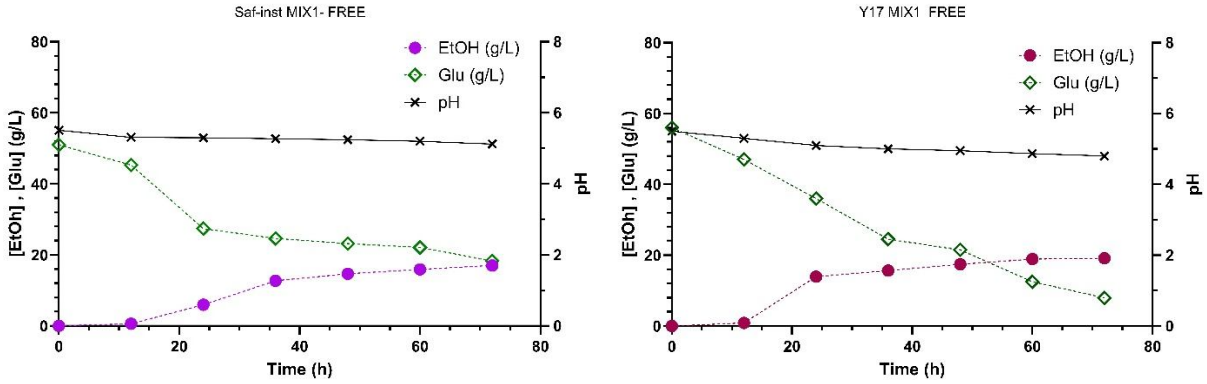
The better performance of Y17 may be attributed to its adaptation to the specific substrates used. Native yeast strains have been reported to exhibit enhanced fermentation capabilities in certain conditions. For example, (Shaghaghi-Moghaddam et al., 2018) found that traditional and industrial *S. cerevisiae* strains had higher bioethanol productivity compared to wild strains, highlighting the importance of strain selection based on substrate compatibility.

III.3.5. Simultaneous Saccharification and Batch Fermentation (SSF) with Free Cells

Fermentation assays were performed batch conditions using free (non-immobilized) yeast cells. All trials were carried out in 1 L glass flasks containing 700 mL of substrate mixture, maintained at 30 °C under agitation (150 rpm) for 72 hours. Anaerobic, sterile conditions were assured throughout the process. The simultaneous saccharification and fermentation (SSF) system used a 0.5% (w/v)

dose of the enzymatic complex Natuzyme®, enabling in-situ hydrolysis of polysaccharides and immediate fermentation of released sugars. Ethanol and glucose concentrations, along with pH (initially adjusted to 5.5), were measured at multiple time points, with ethanol quantified through chemical oxidation and distillation methods, the results are illustrated in Figure 23.

(1)



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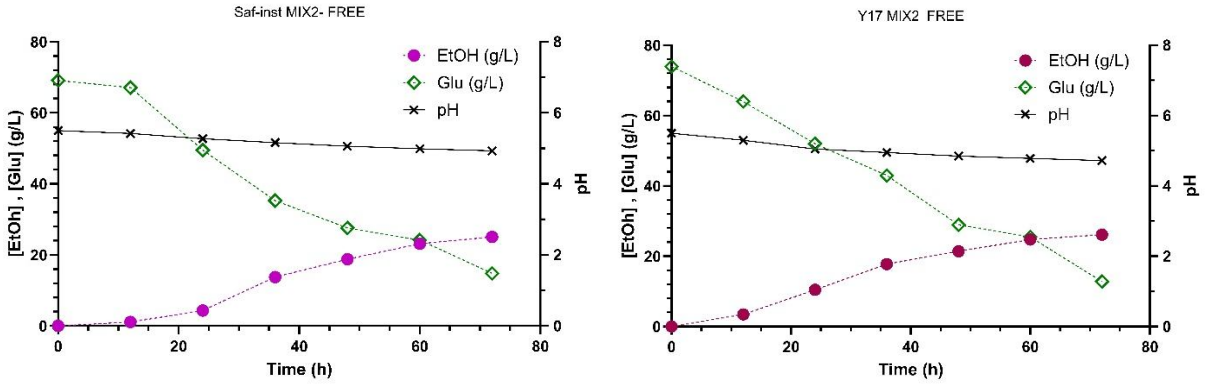

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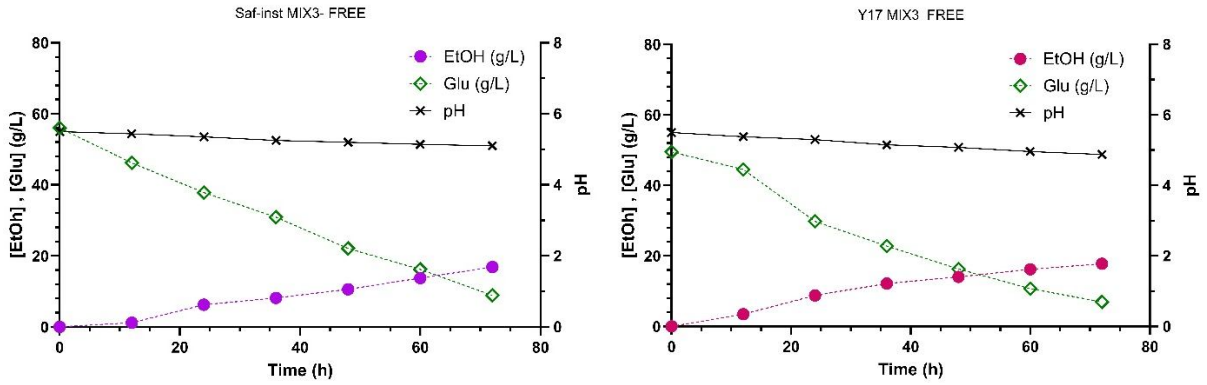

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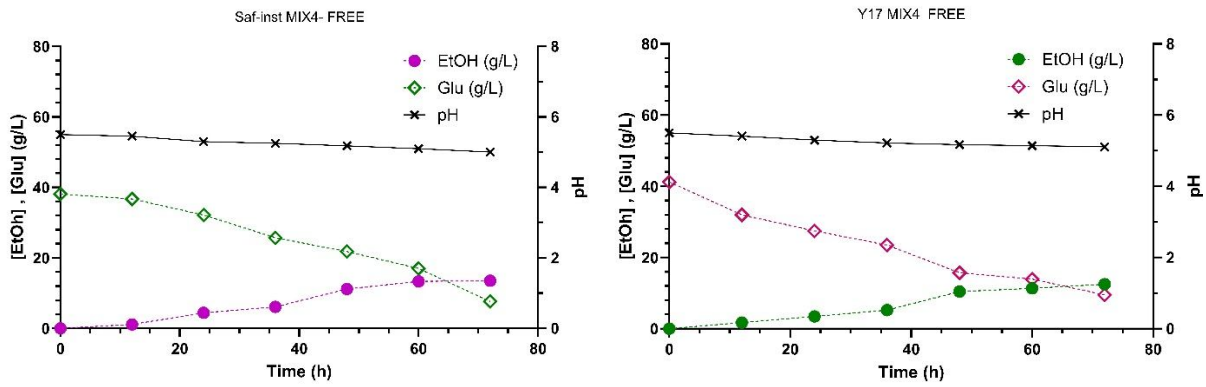


Figure 23. Kinetics of Batch FreeCell fermentation for the 04 mixes, (1) mix1, (2) mix2, (3) mix3 and (4) mix4, for Y17 and the Saf-instant commercial *S. cerevisiae.*

The four substrate mixtures—MIX 1 to MIX 4—were formulated with different proportions of olive oil wastewater (OOWW), milk whey (MW), and sugarcane molasses (SCM). The performance of the strain Y17 was compared to that of the commercial *Saccharomyces cerevisiae* Saf-instant yeast, across these mixtures in free-cell configurations.

As presented in Figure 23, MIX 2 supported the highest ethanol production levels for both strains, with final titers of 26.75 g/L for Y17 and 25.59 g/L for the commercial yeast after 72 hours of fermentation. This can be attributed to the optimized combination of SCM and whey, providing a readily fermentable sugar profile and balanced nutrient composition. These findings are in agreement with results reported by (Abu Tayeh et al., 2014) who obtained up to 30 g/L ethanol from SSF fermentation of OOWW, and (Halema, 2014) who reported yields of approximately 22 g/L from sugarcane molasses. The comparable outcomes indicate that the Y17 strain, isolated from OOWW, is capable of efficiently converting sugars in enriched waste-based media, performing at levels similar to an industrial standard.

In MIX 1, Y17 produced 18.49 g/L of ethanol, slightly higher than the commercial yeast (16.88 g/L). Although this mixture contains potentially inhibitory compounds from OOWW, ethanol production remained significantly above levels typically reported for untreated or singly used OOWW. For instance, (Ayadi et al., 2022a)observed ethanol yields ranging from 8.5 to 14 g/L using OOWW without enzymatic treatment. These results underscore the beneficial effect of enzymatic hydrolysis and substrate mixing, and the robustness of Y17 in complex matrices.

MIX 3, consisting primarily of SCM and MW, resulted in ethanol concentrations of 17.04 g/L (Y17) and 15.20 g/L (commercial yeast). Although this mixture was slightly less rich in fermentable sugars compared to MIX 2, it still sustained high ethanol yields, comparable to those reported by

(Chauhan et al., 2024) for agro-industrial residues (~16 g/L). The consistently better performance of Y17 in MIX 3 suggests enhanced sugar utilization efficiency and potential adaptation to nutrient-limited environments.

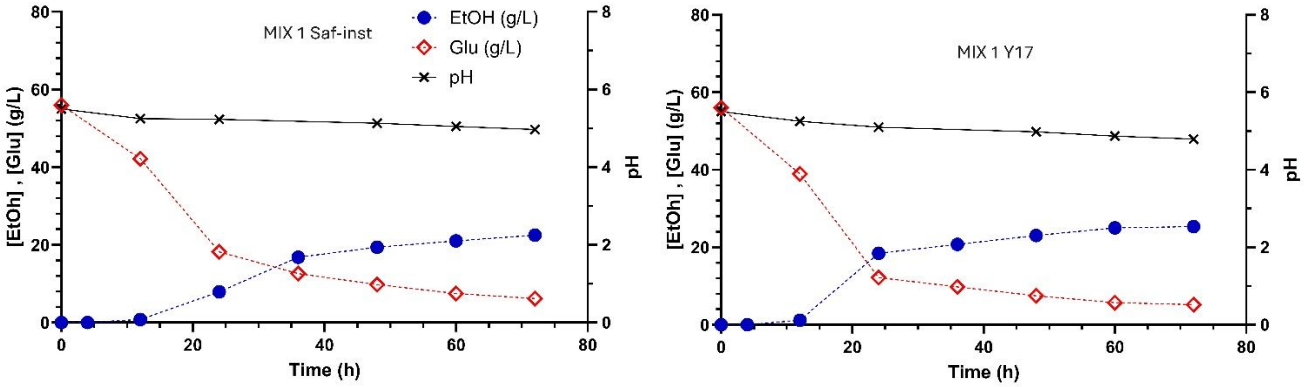
Finally, MIX 4, yielded the lowest ethanol concentrations: 12.10 g/L (Y17) and 12.83 g/L (commercial yeast).

These results highlight the critical role of substrate formulation and yeast strain selection in bioethanol production from agro-industrial residues. The Y17 strain demonstrated equal or higher fermentative capacity compared to a commercial yeast across all mixtures, especially in complex or inhibitory environments (Shaghaghi-Moghaddam et al., 2018). Moreover, the use of SSF with Natuzyme® significantly enhanced sugar availability and conversion, enabling ethanol titers that compare favorably with the literature. These findings affirm the potential of locally isolated, stress-tolerant yeasts for cost-effective bioethanol production from underutilized agro-waste streams.

III.3.6. Simultaneous Saccharification and Batch Fermentation (SSF) with Immobilized Cells

The pH of the fermentation environment plays a pivotal role, influencing both enzymatic activity and microbial growth—key factors essential for maximizing ethanol yields (Yang et al., 2016). In this study, the initial pH across all fermentations was uniformly adjusted to 5.5 to create optimal starting conditions.

(1)



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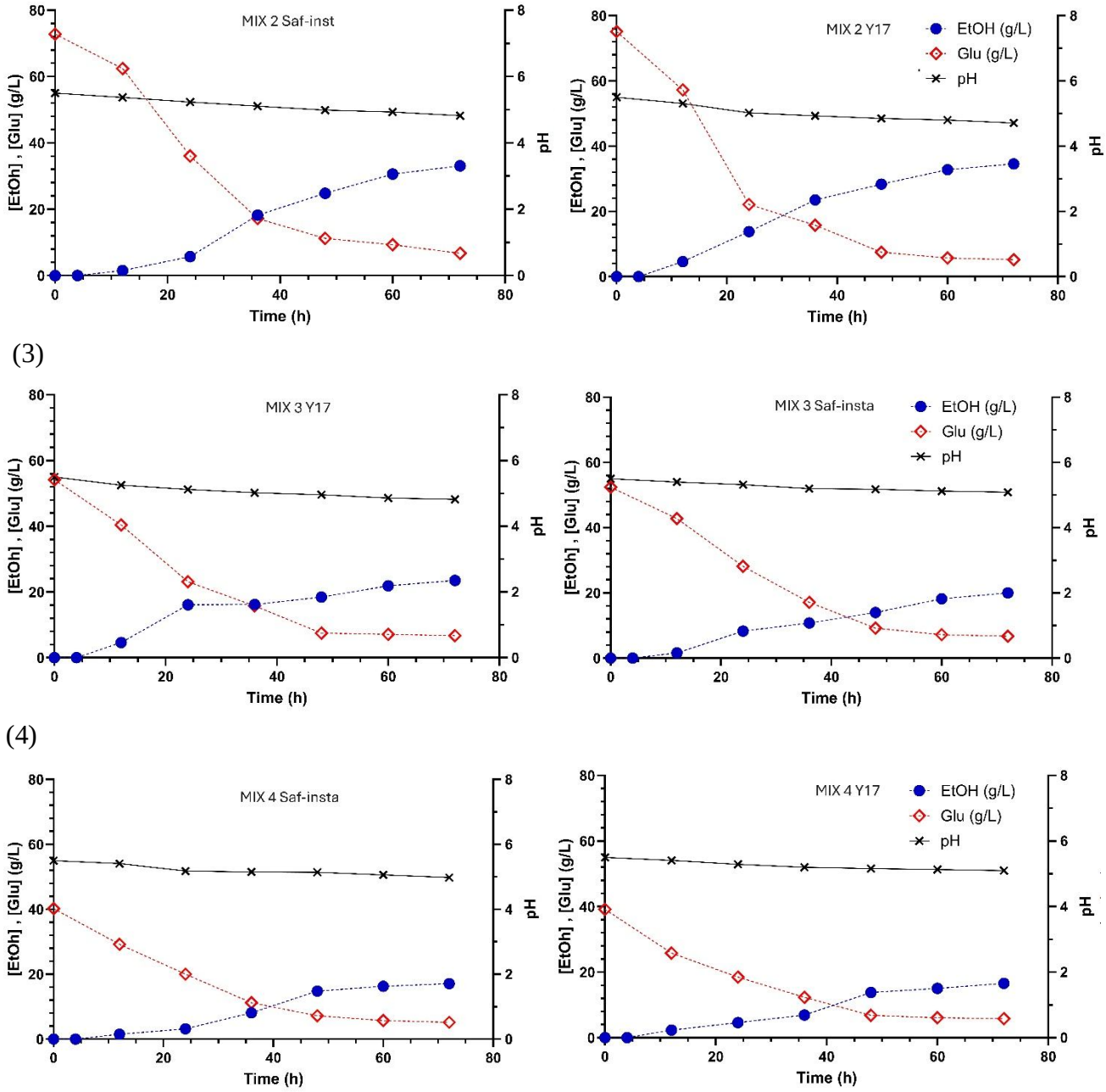

Figure 24. Kinetics of Batch Immobilized fermentation for the 04 mixes, (1) mix1, (2) mix2, (3) mix3 and (4) mix4, for Y17 and the Saf-instant commercial *S. cerevisiae.*

III.3.6.1. pH evolution during fermentation

Throughout the fermentation process, a progressive acidification was observed in all mixtures, consistent with the expected metabolic dynamics of ethanol-producing systems. Organic acid accumulation, particularly pyruvic acid—a central intermediate in ethanol biosynthesis pathways—is a characteristic byproduct of active fermentation (Darwin et al., 2019).

As illustrated in Figure 22, all mixtures exhibited similar downward pH trends. Mix 1 declined from pH 5.5 to 5.02 after 72 hours of fermentation. Mix 2 decreased to 5.05, while Mixes 3 and 4 reached final pH values of 4.98. Notably, the differences between strains (Y17 vs. Saf) were marginal in terms of pH evolution, indicating that both immobilized systems maintained comparable acid-base dynamics during fermentation.

These final pH values remain within a range that supports both yeast viability and enzymatic activity (Mohd-Zaki et al., 2016). The observed acidification patterns confirm active metabolic processing without excessive acid accumulation, which might otherwise inhibit cell function or enzymatic catalysis (Yusuf et al., 2023).

III.3.6.2. Ethanol production

Ethanol concentrations varied significantly across the different substrate mixtures, reflecting the combined influence of substrate composition, enzymatic pre-treatment, and yeast strain performance under immobilized conditions. Among the mixtures, Mix 2 yielded the highest ethanol concentration (34.56 g/L) when fermented with the Y17 strain, clearly outperforming Mix 1 (25.34 g/L), Mix 3 (23.50 g/L), and Mix 4 (16.58 g/L). These results underscore the critical importance of sugar availability and composition, as Mix 2 had the richest glucose profile post-hydrolysis, enhancing substrate assimilation and ethanol synthesis.

Importantly, when comparing the performance of the isolated Y17 yeast strain with that of the commercial *Saccharomyces cerevisiae* Saf strain, Y17 demonstrated better productivity in nearly all cases. For example, in Mix 1, Y17 produced 25.34 g/L compared to 22.51 g/L by Saf, while in Mix 3, it reached 23.5 g/L versus 20.0 g/L. The most pronounced difference was observed in Mix 3, with a +3.5 g/L advantage for Y17. In Mix 2 Y17 still outperformed Saf (34.56 g/L vs. 33.10 g/L). The only exception was in Mix 4, where Saf marginally surpassed Y17 (17.10 g/L vs. 16.58 g/L).

This trend highlights the robust adaptability of Y17 to diverse substrates**,** particularly those with lower initial sugar concentrations or higher inhibitory compounds like polyphenols, which are abundant in OOWW. Its prior isolation and adaptation from OOWW likely conferred greater resistance to phenolic toxicity, osmotic stress, and acidification factors known to impair commercial strains under complex fermentation environments (Ayadi et al., 2022; Darwin et al., 2019). Moreover, these findings suggest that local yeast isolates such as Y17 may

outperform industrial strains under non-conventional fermentation conditions, particularly when optimized enzymatic hydrolysis is used to liberate fermentable sugars from complex matrices.

The results of this study also compare favorably with prior reports. Ayadi et al. (2022) achieved only ~14 g/L of ethanol using immobilized cells on OOWW without enzymatic hydrolysis, emphasizing the value of enzyme supplementation.

In summary, ethanol yield followed the order:

Y17 – Mix 2 (34.56 g/L) > Saf – Mix 2 (33.10 g/L) > Y17 – Mix 1 (25.34 g/L) > Saf – Mix 1 (22.51 g/L) > Y17 – Mix 3 (23.5 g/L) > Saf – Mix 3 (20.0 g/L) > Saf – Mix 4 (17.1 g/L) > Y17 – Mix 4 (16.58 g/L)**.**

III.4. Conclusion

The present study underscores the promising potential of enzymatic hydrolysis and immobilized yeast cells on pozzolane for bioethanol production from heterogeneous agro-industrial waste substrates, namely Olive Oil Wastewater (OOWW), Sugarcane Molasses (SCM), and Milk Whey (MW)**.** Optimization of the fermentation process particularly the application of enzymatic hydrolysis significantly improved sugar release and, consequently, ethanol yields, with a maximum concentration of 34.56 g/L achieved in Mix 2**.** This performance not only surpassed traditional fermentation approaches but also exceeded several reported values in the literature for waste-based fermentation systems.

A key outcome of the study was the comparison between the isolated Y17 yeast strain, isolated from OOWW, and the commercial *Saccharomyces cerevisiae* Saf strain, both under immobilized and free cell conditions. The Y17 strain performed better in most cases then the Saf-instant strain across most mixtures, particularly under complex or low-glucose conditions. These findings confirm the more adaptability and fermentative capacity of the Y17 strain, likely due to its pre-adaptation to the inhibitory compounds present in OOWW, including polyphenols and organic acids.

The immobilization technique enhanced both strains' fermentation stability, reusability, and resistance to contamination, all of which are advantageous for industrial applications. Moreover, the correlation between glucose consumption and ethanol production reinforces the

importance of optimizing both hydrolysis efficiency and initial sugar concentrations. Mixes enriched with SCM, such as Mix 2, demonstrated enhanced ethanol biosynthesis, emphasizing the benefits of co-substrate strategies.

Beyond ethanol production, the residual biomass from fermentation retained sufficient organic load to support biogas generation, supporting the development of integrated biorefinery systems**.** This circular approach not only maximizes resource utilization but also aligns with the goals of waste valorization and renewable energy production.

In conclusion, this research demonstrates the technical and economic viability of using agro-industrial by-products for sustainable and scalable bioethanol production via SSF. It highlights the efficiency of enzymatic pre-treatment, the robustness of immobilized systems, and the critical role of yeast strain selection**.** Collectively, these innovations pave the way for the advancement of eco-friendly, high-yield biofuel technologies rooted in circular economy principles.

Chapter IV

Acetic acid production using *Bacillus* from bovine rumen

IV.1. Introduction

The production of olive oil generates considerable waste, particularly olive oil mill wastewater (OMW), which poses environmental challenges due to its high organic load and potential for pollution (Sar & Akbas, 2023). OMW is characterized by high levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD), along with an acidic pH, making it a candidate for valorization through microbial fermentation (Bouharat et al., 2018).

Microbial fermentation is a crucial biochemical process that converts organic substrates into valuable products, including organic acids, which play essential roles in various industrial applications (Senanayake et al., 2023). Among these, acetic acid is particularly noteworthy due to its wide use as a preservative, flavoring agent, and chemical feedstock (Qiu et al., 2021). The rumen of ruminant animals harbors a diverse microbial community capable of fermenting fibrous plant materials into volatile fatty acids (VFAs) (Mizrahi et al., 2021). This unique microbial ecosystem provides an opportunity to exploit specific strains for efficient fermentation processes (Graham et Knelman, 2023).

Previous research has primarily focused on *Saccharomyces cerevisiae* and acetic acid bacteria for acetic acid production, leaving room for exploration of other microbial candidates like *Bacillus* (Angeloni et al., 2024; Ayadi et al., 2022b; Carmona et al., 2023; Fronteras et al., 2021; Ntougias et al., 2013). Studies on the use of olive oil mill wastewater (OMW) as a substrate have highlighted its potential for producing value-added products such as bioethanol and acetic acid, often relying on traditional microbial strains.

Therefore, this study aims to evaluate the capacity of *Bacillus* strains isolated from bovine rumen to produce acetic acid through fermentation of olive oil mill wastewater. Specifically, it focuses on identifying efficient acid-producing strains, characterizing their species, and assessing their fermentation performance.

The hypothesis is that Bacillus strains isolated from bovine rumen can efficiently ferment OMW to produce acetic acid, offering a sustainable solution for waste management and

bioenergy generation. The use of OMW as a substrate presents a dual opportunity: providing an environmentally sustainable solution for waste valorization while simultaneously producing valuable bioproducts.

IV.2. Material and methods

IV.2.1. Sample collection and analysis

OMW samples were collected from the Nakhla oil mill located in Chlef, Algeria, during the peak olive processing season. Samples were filtered through a fine mesh to eliminate any large contaminants and autoclaved before fermentations, Samples were stored at 4 °C when not in use. Physicochemical analyses were conducted to determine pH using a digital pH meter (Hanna Instruments), while chemical oxygen demand (COD) and biochemical oxygen demand (BOD5) were measured following standard methods (APHA, 2017b). Organic matter content was assessed through gravimetric methods by drying the samples at 105 °C for 24 hours and measuring the weight loss. Acidity was determined by titration with 0.1 M NaOH using phenolphthalein as an indicator. Nitrites (NO_2^-) were quantified using the colorimetric method with Griess reagent, following the protocol described in (APHA, 2017a). Total polyphenols were measured using the Folin-Ciocalteu method, as described by (Russo et al., 2022). All analyses were performed in triplicate to ensure reproducibility.

IV.2.2. Microbial isolation and screening

Bovine rumen juice was obtained from a freshly slaughtered Holstein cow at the Taiba slaughterhouse in Chlef, Algeria. The rumen contents were filtered through sterile gauze to collect the liquid fraction. Serial dilutions were prepared and spread onto MRS (de Man, Rogosa and Sharpe) and M17 agar plates supplemented with 2% CaCO3 to facilitate the identification of acid-producing colonies through the formation of clear halos. The MRS medium composition included peptone (10 g/L), beef extract (10 g/L), yeast extract (5 g/L), glucose (20 g/L), Tween 80 (1 mL/L), ammonium citrate (2 g/L), sodium acetate (5 g/L), magnesium sulfate (0.1 g/L), manganese sulfate (0.05 g/L), and dipotassium phosphate (2 g/L). Anaerobic conditions were maintained using a 2.5 L anaerobic culture jar from MERK. Plates were incubated anaerobically at 37 °C for 48 hours. A total of 25 distinct colonies were isolated and subcultured to obtain pure strains.

IV.2.3. Primary identification and evaluation of isolated strains

Initial identification of the isolated strains was performed through a combination of morphological and biochemical tests. Microscopic observations were conducted to assess cell morphology and Gram staining characteristics. Oxidase and catalase tests were performed to evaluate enzymatic activity, aiding in preliminary strain classification.

A preliminary fermentation assay was conducted to assess the metabolic activity of the isolated strains through gas (H_2 + CO_2) production. The assay used a fermentation medium consisting of 25% (v/v) olive oil mill wastewater (OMW) and 75% MRS broth, supplemented with calcium carbonate ($CaCO_3$) as a pH buffer. Fermentations were carried out in 100 mL airtight bottles equipped with graduated syringes to accurately measure cumulative gas production (Figure 23). Each bottle was inoculated with 5% (v/v) of a 24-hour fresh culture and incubated at 37 °C for five days under static conditions.

The volume of gas produced, primarily composed of hydrogen (H_2) and carbon dioxide (CO_2), was monitored as an indicator of microbial fermentative activity, particularly acidogenesis and acetogenesis processes. During these metabolic stages, fermentable substrates are converted into volatile fatty acids (e.g., acetic acid), hydrogen, and carbon dioxide according to the general reaction (Angelidaki et al., 2018):

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (5)$$

where glucose is converted into acetic acid, carbon dioxide, and hydrogen.

Strains exhibiting the highest gas production were selected for further characterization and analysis.

Figure 25: Primary fermentation setup.

Following these initial tests, the API 50 CHB/E system (bioMérieux), a biochemical assay designed to characterize carbohydrate metabolism, was employed. The system evaluates the fermentation of 49 different carbohydrates, providing a metabolic fingerprint that aids in species-level identification.

IV.2.4. Fermentation trials for acetic acid production

To evaluate long-term acetic acid production, a separate fermentation trial was conducted over a period of 120 hours. Fresh OMW medium (500 mL) with pH adjusted to the value of 6 to favor the Acidogenesis because methanogenesis (methane production) requires a higher pH (6.5–8.5) and longer incubation times as discussed by (Liew et al., 2016). The OMW was inoculated with 5% (v/v) of *Bacillus* strains selected based on their biogas production efficiency. The flasks were incubated at 37 °C with continuous shaking at 150 rpm to ensure optimal oxygenation and nutrient distribution.

Samples were collected at regular intervals to monitor critical parameters, including pH, optical density at 600 nm (OD600), and acetic acid concentration. pH measurements provided insights into acidification dynamics, while OD600 reflected biomass growth over time.

Acetic acid was quantified following the method outlined by (Sode, 2014), This method involves a classic acid–base titration using NaOH.

IV.2.5. Data analysis

All experiments were conducted in triplicate. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10 software. Differences between strains and fermentation parameters were evaluated using analysis of variance (ANOVA), with significance considered at $p < 0.05$.

IV.3. Results

IV.3.1. Physicochemical characteristics of OMW

The physicochemical analysis of olive oil mill wastewater (OMW) (Table 12) revealed several important characteristics that underscore its potential for microbial fermentation. The recorded pH of 4.5 ± 0.2, chemical oxygen demand (COD) of 183 ± 5 gO□/L, and biochemical oxygen demand (BOD□) of 7 ± 0.3 gO□/L indicate a highly organic-rich environment suitable for fermentation processes. The average acidity measured at 1.65 ± 0.05% further emphasizes the high organic load present in the OMW.

Table 12: Results of Physicochemical Characteristics of OMW.

Parameter	Mean ± SD	Range
pH	4.5 ± 0.2	4.3 – 4.7
Chemical Oxygen Demand (COD)	183 ± 5 gO□/L	178 – 188 gO□/L
Biochemical Oxygen Demand (BOD5)	7 ± 0.3 gO□/L	6.7 – 7.3 gO□/L
Average Acidity (%)	1.65 ± 0.05	1.6 – 1.7
Nitrite (mg/L)	31 ± 2	29 – 33
Total polyphenols (g/L)	5.81 ± 0.1	5.7 – 5.9

The high COD/BOD□ ratio observed in this study suggests the presence of non-biodegradable organic compounds. Additionally, the detection of 31 ± 2 mg/L nitrites and 5.81 ± 0.1 g/L total polyphenols in the OMW highlights its complex composition.

IV.3.2. Microbial isolation and characterization

IV.3.2.1. Biochemical and morphological characterization of the isolated strains

The results of the biochemical tests conducted on the isolated bacterial strains are summarized in Table 13. A total of 25 strains were evaluated for Gram staining, catalase activity, oxidase activity, and morphological form, revealing a diverse array of bacterial types

predominantly classified as cocci and bacilli. The majority of strains exhibited Gram-negative characteristics, with notable exceptions among Gram-positive strains.

The identification of specific strains with positive catalase and oxidase activities, such as JR/GN/-4/1/3, JR/GN/-6/1/1, and JR/GN/-6/3/1, suggests their metabolic versatility. The morphological diversity observed among the strains—ranging from cocci to bacilli and coccobacilli—demonstrates their ecological adaptability.

Table 13: Results of Gram staining and biochemical tests for isolated strains.

Strain ID		**Gram Stain**	**Catalase**	**Oxydase**	**Form**
1.	JR /GN/-5 /1 /1/1	-	-	-	Cocci
2.	SR2/M17/SM/1/2	-	-	-	Cocci
3.	JR/GN/-4/1/3	+	+	-	Cocci
4.	JR/GN/-6/1/1	+	+	-	Cocci
5.	JR/GN/-4/3/2	+	-	-	Cocci
6.	JR/GN/-4/4/1	-	+	-	Coccobacilli
7.	JR/GN/-6/3/1	+	+	-	Bacilli
8.	JR/GN/-8/2/2	-	+	-	Cocci
9.	A/GN/SM/1	+	-	-	Coccobacilli
10.	JR/GN/-4/4/4	+	-	-	Cocci
11.	A/GN/-1/1	-	+	-	Cocci
12.	JR/GN/-6/2/1	-	+	-	Coccobacilli
13.	JR/GN/-6/2/5	+	+	-	Bacilli
14.	JR/GN/-8/2/5	+	+	-	Bacilli
15.	A/GN/-7/1	+	+	-	Coccobacilli
16.	JR/GN/-6/2/1	-	+	-	Coccobacilli
17.	JR/GN/-6/2/2	+	+	-	Bacilli
18.	JR/GN/-6/3/3	-	+	+	Coccobacilli
19.	JR/GN/-4/4/1	+	+	-	Bacilli
20.	SR2/SM/M17/1/3	-	-	-	Cocci
21.	JR/GN/-4/2/1	+	+	-	Bacill
22.	SR2/M17/-7/1	+	-	-	Coccobacilli
23.	SR2/M17/-5/4/1	-	-	-	Cocci
24.	SR2/Clm /-9/4	+	-	-	Bacilli
25.	JR/GN/-4/1/1	-	-	-	Coccobacilli

IV.3.2.2. Evaluation of biogas production of the isolated strains

The results expressed in Figure 24 of the batch fermentation trials indicated significant variability in gas production among the isolated bacterial strains. Notably, strains 2, 4, 6, 7, 10, 11, 17, 21, and 22 exhibited no gas production, while strains 1, 3, 5, 8, 9, 12, 16, 18, 20, 23, and 25 showed low levels of gas production. In contrast, strains 13, 14, 15, and 19 demonstrated substantial gas production starting on the third day of incubation. Strain 14 emerged as the highest gas producer with approximately 14 mL, followed closely by strains 19 (12.6 mL) and 13 (12.2 mL), while strain 15 produced around 11.8 mL.

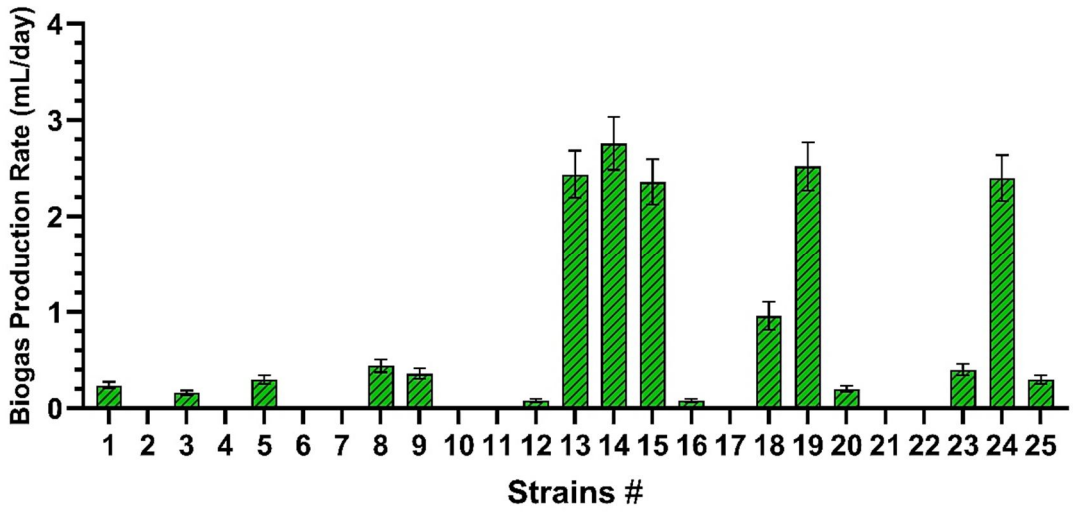

Figure 26*:* Gas production rate (mL/day) of different strains over 5 days. Values represent mean ± SD.

IV.3.2.3. Biochemical identification using API 50CHB/E

The fermentation profiles of strains 13, 14, 15, 19, and 24 were further characterized using the API 50 CHB/E galleries. This analysis identified two *Bacillus* species: *Bacillus licheniformis* (strains 13, 19, and 24) and *Bacillus circulans* (strains 14 and 15). The results obtained from the API galleries confirm their classification at the species level (Table 14).

The acetic acid production of five yeast strains (13, 14, 15, 19, and 24) was monitored over a period of 120 hours (Graph 2). Among the five yeast strains tested, Strain 15 exhibited the highest acetic acid production, achieving 28 g/L at 108 hours.

Table 14: Results of API 50 CHB/B strains identification

Strain N°	Result of API
13	*Bacillus licheniformis*
14	*Bacillus circulants*
15	*Bacillus circulans*
19	*Bacillus licheniformis*
24	*Bacillus licheniformis*

IV.3.3. Acetic acid production and fermentation kinetics

The acetic acid production of five yeast strains (13, 14, 15, 19, and 24) was monitored over a period of 120 hours. The data revealed distinct trends in growth (OD□□□), acid production, and pH variation among the tested strains.

Strain 15 exhibited the highest acetic acid production, reaching 28.1 g/L at 108 hours before slightly stabilizing at 28.0 g/L at 120 hours. This strain demonstrated a continuous increase in acetic acid concentration, with significant production acceleration after 24 hours (8.7 g/L), peaking between 84 and 108 hours. The pH of the fermentation medium gradually decreased, reaching 4.87 at 120 hours, indicating active acidification.

Strains 13 and 14 showed moderate acetic acid production levels, with maximum concentrations of 15.7 g/L and 16.2 g/L, respectively, at 120 hours. Their pH values decreased to 5.38 and 4.87, respectively, by the end of fermentation. Growth patterns differed among the strains, with OD□□□ values peaking earlier (between 48 and 72 hours) and subsequently declining, suggesting possible cellular stress or nutrient depletion.

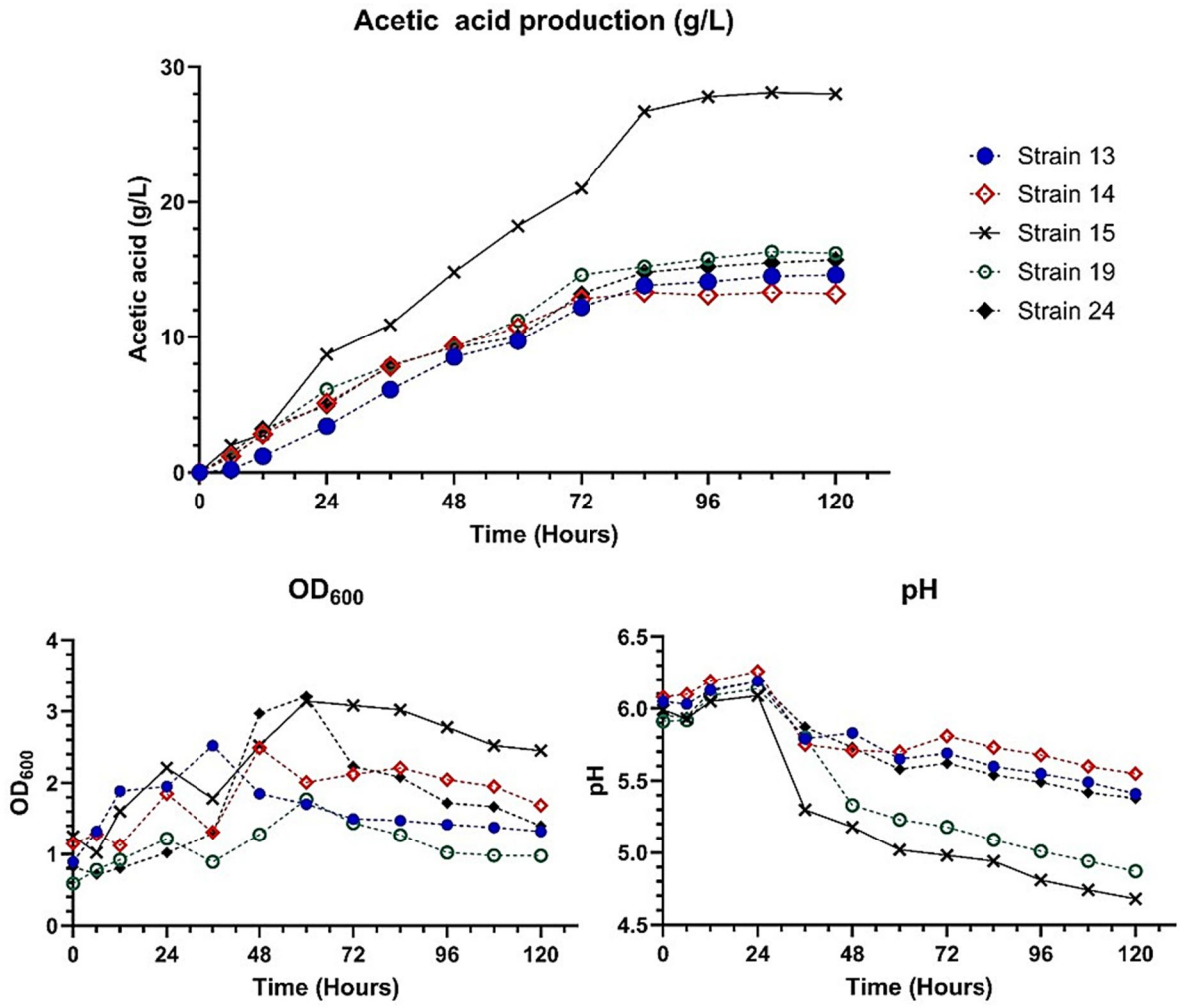

Figure 27: Acetic acid production over time by different *Bacillus* strains (13, 14, 15, 19, 24).

IV.4. Discussion

The physicochemical characteristics of OMW observed in this study align with previous research, reporting similar values for pH (4.5–5.5), COD (40–100 g/L), and BOD□ (20–50 g/L) (Bougherara et al., 2021b; Bouharat et al., 2018; Bouknana et al., 2014; Russo et al., 2022). The high COD/BOD□ ratio (>2.5) indicates the presence of slowly biodegradable or recalcitrant organic compounds, which can hinder biological treatment methods, as previously reported by (Gueboudji et al., 2022b). Additionally, polyphenol concentrations ranging from 0.5 to 8 g/L in OMW (Vavouraki et al., 2020) are known to exert antimicrobial effects, which may have contributed to growth inhibition in certain strains during fermentation (Russo et al., 2022; Sar & Akbas, 2023).

The observed cumulative gas production by strains 13, 14, 15, and 19 (13.8 ± 1.2 mL/100 mL OMW) reflects an active microbial metabolism capable of fermenting olive oil mill wastewater (OMW) components. Although *Bacillus* species are not classical methanogens, they can play an important indirect role in biogas production. Specifically, Bacillus strains are known for their robust hydrolytic and acidogenic activities, facilitating the breakdown of complex organic matter into simpler molecules such as volatile fatty acids (VFAs), hydrogen (H□), and carbon dioxide (CO□) (Al Rabadi et al., 2021; Harirchi et al., 2022).

The gas measured during fermentation is therefore primarily attributed to H□ and CO□ production during acidogenesis and acetogenesis stages, rather than direct methane production. The absence of methanogenic archaea in the system likely limited methane (CH□) formation, which explains why the gas volume remains slightly lower compared to typical full anaerobic digestion systems that include both bacterial and archaeal consortia (Angelidaki et al., 2018; Harirchi et al., 2022).

Nevertheless, the detected gas production confirms that the *Bacillus* strains were metabolically active and could efficiently degrade the OMW substrates into intermediate products essential for biogas generation. In contrast, the absence of gas production in certain strains suggests deficiencies either in substrate utilization pathways (e.g., inability to hydrolyze complex polyphenolic compounds present in OMW) or in key enzymatic activities related to fermentative metabolism. In particular, lack of fermentative pathways leading to H□ and CO□ generation (e.g., via pyruvate fermentation routes) could explain the non-producing profiles (Liew et al., 2016).

This variability between strains highlights the importance of carefully selecting strains not only for substrate tolerance but also for their capacity to drive the early steps of anaerobic digestion. Optimizing the metabolic performance of such strains, possibly through co-cultivation with methanogens or through genetic or process engineering, could further enhance gas production and process stability.

The API 50CHB/E biochemical identification confirmed that *Bacillus licheniformis* and *Bacillus circulans* are key contributors to OMW fermentation. These species are well-documented for their ability to degrade complex carbohydrates and efficiently produce organic acids (lactic acids, α-ketoglutaric acid, and γ-aminobutyric acid) (Park et al., 2021; Serin et al.,

2012). *Bacillus licheniformis*, in particular, is known for its tolerance to extreme environmental conditions, making it a promising candidate for large-scale bioprocess applications (Shleeva et al., 2023; Tamang et al., 2016). Previous studies have reported its use in anaerobic digestion systems, where it enhances hydrolysis and acidogenesis, leading to improved biogas yields (Shleeva et al., 2023).

The acetic acid production observed in this study highlights the potential of microbial fermentation for olive mill wastewater (OMW) valorization. Among the tested strains, Strain 15 exhibited the highest acetic acid production (28.1 g/L at 108 hours), surpassing values typically reported for *Saccharomyces cerevisiae*, which range from 20 to 40 g/L (De Leonardis et al., 2019; Fronteras et al., 2021). This suggests that *Bacillus* strains, particularly *Bacillus licheniformis* and *Bacillus circulans*, could be viable candidates for direct OMW fermentation, without requiring co-culturing with acetic acid bacteria such as *Acetobacter aceti*, which is commonly used in two-step fermentation systems (Fronteras et al., 2021).

The acetic acid production observed in this study highlights the potential of microbial fermentation for OMW valorization. Among the tested strains, Strain 15 exhibited the highest acetic acid production (28.1 g/L at 108 hours), exceeding values typically reported for *Saccharomyces cerevisiae* (which range between 20–40 g/L, depending on substrate composition) (De Leonardis et al., 2019; Fronteras et al., 2021). This suggests that *Bacillus* strains could be viable candidates for direct OMW fermentation, eliminating the need for co-culturing with acetic acid bacteria such as *Acetobacter aceti*, which is typically used in two-step fermentation systems (De Leonardis et al., 2019; Qiu et al., 2021).

The findings of this study indicate that *Bacillus* strains can efficiently ferment OMW to produce acetic acid and biogas, making them strong candidates for industrial-scale waste valorization. Compared to other microbial strains, *Bacillus* species offer advantages such as high environmental adaptability, resilience to acidic pH, and efficient enzyme production, which are essential for large-scale fermentation.

IV.5. Conclusion

This study demonstrated the capacity of five *Bacillus* strains (13, 14, 15, 19, and 24) to produce acetic acid from olive oil mill wastewater (OMW), highlighting their potential for bioconversion of agricultural waste. Among the strains tested, *Bacillus* strain 15 exhibited the

highest acetic acid yield, reaching 28 g/L at 108 hours, significantly outperforming the other strains. Strains 19 and 24 followed with moderate production, while strains 13 and 14 showed lower yet consistent acidification and biomass growth.

The use of *Bacillus* strains for acetic acid production from OMW represents a novel approach, as previous studies have primarily focused on *Saccharomyces cerevisiae* and acetic acid bacteria. This work not only contributes to the valorization of olive oil production by-

products but also introduces *Bacillus* as a promising candidate for sustainable acetic acid fermentation.

Future research should focus on optimizing fermentation conditions to enhance yields, scaling up the process for industrial applications, and further investigating the metabolic pathways involved in *Bacillus*-mediated acid production. This study opens new avenues for biotechnological innovation in the valorization of olive oil wastewater, promoting circular economy principles and reducing environmental impact

General conclusion

General conclusion

This thesis explored the biotechnological valorization of agro-industrial by-products, primarily olive oil wastewater (OOWW), sugarcane molasses (SCM), and milk whey (MW), through innovative microbial strategies to produce molecules of industrial interest such as bioethanol, biogas, and acetic acid.

In the first phase, a native yeast strain, *Saccharomyces cerevisiae* Y17, was successfully isolated from olive oil wastewater. The strain demonstrated remarkable tolerance to the toxic environment of untreated OOWW and outperformed commercial yeast strains in ethanol production. This highlighted the potential of indigenous microbial resources for sustainable bioprocessing of inhibitor-rich substrates, offering an eco-friendly solution for waste management while contributing to renewable energy generation.

Building upon these findings, the second phase of the work integrated simultaneous saccharification and fermentation (SSF) using immobilized *S. cerevisiae* Y17 on pozzolane supports. This strategy, combined with enzymatic hydrolysis, significantly enhanced glucose availability and fermentation efficiency, resulting in a maximum ethanol concentration of 34.56 g/L under optimized conditions. The immobilization technique provided improved process stability, reduced contamination risks, and enabled potential biomass reuse, demonstrating its industrial relevance for scalable and economically viable bioethanol production.

Expanding the valorization approach, the third phase assessed the capacity of selected *Bacillus* strains to convert OOWW into acetic acid. *Bacillus* strain 15 achieved the highest production yield (28 g/L), establishing the feasibility of using alternative bacterial systems for acidogenesis from agro-industrial residues — a relatively novel avenue compared to traditional acetic acid fermentation practices.

Overall, this thesis demonstrates that integrating microbial strain selection, enzymatic enhancement, and immobilization technologies can create efficient and sustainable bioprocesses for transforming agro-industrial wastes into value-added bioproducts. The results underline the potential of developing scalable, circular bioeconomy models that address both environmental challenges and renewable energy needs. Future research directions include process scale-up, detailed metabolic profiling, and the design of integrated biorefineries capable of sequentially producing multiple bio-based products from a single waste stream.

Through these contributions, this work advances the field of microbial biotechnology and opens new perspectives for the sustainable and innovative exploitation of agro-industrial by-products. Future research directions could include:

- Exploring mixed cultures (e.g., yeast and *Bacillus* or acetic acid bacteria) to improve substrate utilization and product yield.
- Evaluating the long-term stability and reusability of immobilized cells to enhance industrial applicability.
- Investigating additional agro-industrial wastes (e.g., date palm residues or fruit peels) to diversify feedstocks.
- Testing alternative immobilization materials or genetically enhanced strains to further improve process efficiency.

References

Abdelhadi, B., benlemlih, M., Saad, I., Ahnsal, L., Aayah, H., & Boussaid, A. (2010). Suitable of yeasts for the Treatment of Olive Mill Wastewater. *Terrestrial and Aquatic Environmental Toxicology, 4*, 113–117.

Abdelmajeed, N. A., Khelil, O. A., & Danial, E. N. (2012). Immobilization technology for enhancing bio-products industry. *African Journal of Biotechnology*, *11*(71), 13528–13539. https://doi.org/10.5897/AJB12.547

Abu Tayeh, H., Najami, N., Dosoretz, C., Tafesh, A., & Azaizeh, H. (2014). Potential of bioethanol production from olive mill solid wastes. *Bioresource Technology*, *152*, 24–30. https://doi.org/10.1016/j.biortech.2013.10.102

Abubakar, I. R., Maniruzzaman, K. M., Dano, U. L., AlShihri, F. S., AlShammari, M. S., Ahmed, S. M. S., Al-Gehlani, W. A. G., & Alrawaf, T. I. (2022). Environmental Sustainability Impacts of Solid Waste Management Practices in the Global South. *International Journal of Environmental Research and Public Health*, *19*(19), 12717. https://doi.org/10.3390/ijerph191912717

Abu-Lafi, S., Al-Natsheh, M. S., Yaghmoor, R., & Al-Rimawi, F. (2017). Enrichment of Phenolic Compounds from Olive Mill Wastewater and In Vitro Evaluation of Their Antimicrobial Activities. *Evidence-Based Complementary and Alternative Medicine*, *2017*(1), 3706915. https://doi.org/10.1155/2017/3706915

Achyuthan, K. E., Achyuthan, A. M., Adams, P. D., Dirk, S. M., Harper, J. C., Simmons, B. A., & Singh, A. K. (2010). Supramolecular Self-Assembled Chaos: Polyphenolic Lignin's Barrier to Cost-Effective Lignocellulosic Biofuels. *Molecules*, *15*(12), Article 12. https://doi.org/10.3390/molecules15118641

Addai, F. P., Lin, F., Wang, T., Kosiba, A. A., Sheng, P., Yu, F., Gu, J., Zhou, Y., & Shi, H. (2020). Technical integrative approaches to cheese whey valorization towards sustainable environment. *Food & Function*, *11*(10), 8407–8423. https://doi.org/10.1039/D0FO01484B

Agrawal, R., Semwal, S., Kumar, R., Mathur, A., Gupta, R. P., Tuli, D. K., & Satlewal, A. (2018). Synergistic Enzyme Cocktail to Enhance Hydrolysis of Steam Exploded Wheat Straw at Pilot Scale. *Frontiers in Energy Research*, *6*. https://doi.org/10.3389/fenrg.2018.00122

Ahmed, P. M., Fernández, P. M., De Figueroa, L. I. C., & Pajot, H. F. (2019). Exploitation alternatives of olive mill wastewater: Production of value-added compounds useful for industry and agriculture. *Biofuel Research Journal*, *6*(2), 980–994. https://doi.org/10.18331/BRJ2019.6.2.4

Akay, A., & Sert, D. (2020). The effects of whey application on the soil biological properties and plant growth. *Eurasian Journal of Soil Science*, *9*(4), Article 4. https://doi.org/10.18393/ejss.785380

Al Rabadi, S., Alzboon, K., Shawaqfah, M., Damseh, R., & Alzoubi, O. (2021). Biogas Production through Co-Digestion of Olive Mill with Municipal Sewage Sludge and Cow Manure. *Environment and Natural Resources Journal*, *20*, 1–11. https://doi.org/10.32526/ennrj/20/202100162

Albuquerque, M. M., Sartor, G. de B., Martinez-Burgos, W. J., Scapini, T., Edwiges, T., Soccol, C. R., & Medeiros, A. B. P. (2024). Biohydrogen Produced via Dark Fermentation: A Review. *Methane*, *3*(3), Article 3. https://doi.org/10.3390/methane3030029

Alias, N. H., Abd-Aziz, S., Yee Phang, L., & Ibrahim, M. F. (2021). Enzymatic Saccharification with Sequential-Substrate Feeding and Sequential-Enzymes Loading to Enhance Fermentable Sugar Production from Sago Hampas. *Processes*, *9*(3), Article 3. https://doi.org/10.3390/pr9030535

Alkhalidi, A., Halaweh, G., & Khawaja, M. K. (2023). Recommendations for olive mills waste treatment in hot and dry climate. *Journal of the Saudi Society of Agricultural Sciences*, *22*(6), 361–373. https://doi.org/10.1016/j.jssas.2023.03.002

Al-Mardeai, S., Elnajjar, E., Hashaikeh, R., Kruczek, B., Van der Bruggen, B., & Al-Zuhair, S. (2022). Membrane Bioreactors: A Promising Approach to Enhanced Enzymatic Hydrolysis of Cellulose. *Catalysts*, *12*(10), Article 10. https://doi.org/10.3390/catal12101121

Álvarez-Cao, M.-E., Becerra, M., & González-Siso, M.-I. (2020). Chapter 8—Biovalorization of cheese whey and molasses wastes to galactosidases by recombinant yeasts. In N. Krishnaraj Rathinam

& R. K. Sani (Eds.), *Biovalorisation of Wastes to Renewable Chemicals and Biofuels* (pp. 149–161). Elsevier. https://doi.org/10.1016/B978-0-12-817951-2.00008-0

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., & Kougias, P. G. (2018). Biogas upgrading and utilization: Current status and perspectives. *Biotechnology Advances*, *36*(2), 452–466. https://doi.org/10.1016/j.biotechadv.2018.01.011

Angeloni, G., Spadi, A., Corti, F., Calcaprina, M., Carpi, G., Maioli, F., Parenti, A., & Masella, P. (2024). Advancing Circular Economy in Olive Oil Production: Comparing Maturation Systems for Vermicompost Creation from Olive Pomace. *Biomass*, *4*(4), Article 4. https://doi.org/10.3390/biomass4040065

APHA. (2017a). 5210 biochemical oxygen demand (bod). In *Standard Methods For the Examination of Water and Wastewater*. American Public Health Association. https://doi.org/10.2105/SMWW.2882.102

APHA. (2017b). 5220 chemical oxygen demand (cod). In *Standard Methods For the Examination of Water and Wastewater*. American Public Health Association. https://doi.org/10.2105/SMWW.2882.103

Appelt, J. L., Garcia Rojas, D. C., Verburg, P. H., & van Vliet, J. (2022). Socioeconomic outcomes of agricultural land use change in Southeast Asia. *Ambio*, *51*(5), 1094–1109. https://doi.org/10.1007/s13280-022-01712-4

APS. (2023). *Zitouni: Le sucre largement disponible à travers tous les marchés*. https://www.aps.dz/economie/159395-zitouni-le-sucre-largement-disponible-a-travers-tous-les-marches

Argenta, A. B., & and Scheer, A. D. P. (2020). Membrane Separation Processes Applied to Whey: A Review. *Food Reviews International*, *36*(5), 499–528. https://doi.org/10.1080/87559129.2019.1649694

Ashokkumar, V., Flora, G., Venkatkarthick, R., SenthilKannan, K., Kuppam, C., Mary Stephy, G., Kamyab, H., Chen, W.-H., Thomas, J., & Ngamcharussrivichai, C. (2022). Advanced technologies on the sustainable approaches for conversion of organic waste to valuable bioproducts: Emerging circular bioeconomy perspective. *Fuel*, *324*, 124313. https://doi.org/10.1016/j.fuel.2022.124313

Attallaoui, L. (2022). *La filière oléicole en Algérie, état des lieux, opportunités et stratégies d'acteurs ; cas de la wilaya Djelfa* [Doctoral, University of Mohamed Khider Biskra]. http://thesis.univ-biskra.dz/6033/

Ayadi, K., Meziane, M., Rouam, D., Bouziane, M. N., & El-Miloudi, K. (2022a). Olive Mill Wastewater for Bioethanol Production using Immobilised Cells. *Kemija u Industriji : Časopis Kemičara i Kemijskih Inženjera Hrvatske*, *71*(1–2), 21–28. https://doi.org/10.15255/KUI.2021.015

Ayadi, K., Meziane, M., Rouam, D., Bouziane, M. N., & El-Miloudi, K. (2022b). Olive Mill Wastewater for Bioethanol Production using Immobilised Cells. *Kemija u Industriji*, *1–2*. https://doi.org/10.15255/KUI.2021.015

Bader, A. C., Hussein, H. J., & Jabar, M. T. (2022). *BOD: COD Ratio as Indicator for Wastewater and Industrial Water Pollution*.

Bai, F. W., Zhao, X.-Q., & Xu, J. (2011). 2.35 - Immobilization Technology: Cells. In M. Moo-Young (Ed.), *Comprehensive Biotechnology (Second Edition)* (pp. 477–489). Academic Press. https://doi.org/10.1016/B978-0-08-088504-9.00115-X

Bamidele, M. O., Bamikale, M. B., Cárdenas-Hernández, E., Bamidele, M. A., Castillo-Olvera, G., Sandoval-Cortes, J., & Aguilar, C. N. (2025). Bioengineering in Solid-State Fermentation for next sustainable food bioprocessing. *Next Sustainability*, *6*, 100105. https://doi.org/10.1016/j.nxsust.2025.100105

Barba, F. J. (2021). An Integrated Approach for the Valorization of Cheese Whey. *Foods*, *10*(3), Article 3. https://doi.org/10.3390/foods10030564

Bassani, J. C., Queiroz Santos, V. A., Barbosa-Dekker, A. M., Dekker, R. F. H., da Cunha, M. A. A., & Pereira, E. A. (2019). Microbial cell encapsulation as a strategy for the maintenance of stock cultures. *LWT*, *102*, 411–417. https://doi.org/10.1016/j.lwt.2018.12.058

Ben Sassi, A., Ouazzani, N., Walker, G. M., Ibnsouda, S., El Mzibri, M., & Boussaid, A. (2008). Detoxification of olive mill wastewaters by Moroccan yeast isolates. *Biodegradation*, *19*(3), 337–346. https://doi.org/10.1007/s10532-007-9140-8

Benaissa, M. (2018). *Valorisation du lactosérum pour les bactéries lactiques* [Université d'Oran1 - Ahmed Ben Bella]. https://www.pnst.cerist.dz/detail.php?id=33383/

Berisvil, A. P., Astesana, D. M., Zimmermann, J. A., Frizzo, L. S., Rossler, E., Romero-Scharpen, A., Olivero, C. R., Zbrun, M. V., Signorini, M. L., Sequeira, G. J., Drago, S. R., & Soto, L. P. (2021). Low-cost culture medium for biomass production of lactic acid bacteria with probiotic potential destined to broilers. *FAVE Sección Ciencias Veterinarias*, *20*(1), 1–9.

Bettazzi, E., Caretti, C., Caffaz, S., Azzari, E., & Lubello, C. (2007). Oxidative processes for olive mill wastewater treatment. *Water Science and Technology*, *55*(10), 79–87. https://doi.org/10.2166/wst.2007.309

Bhardwaj, N., Kumar, B., Agrawal, K., & Verma, P. (2021). Current perspective on production and applications of microbial cellulases: A review. *Bioresources and Bioprocessing*, *8*(1), 95. https://doi.org/10.1186/s40643-021-00447-6

Bisswanger, H. (2017). *Enzyme Kinetics: Principles and Methods*. John Wiley & Sons.

Bjerk, T. R., Severino, P., Jain, S., Marques, C., Silva, A. M., Pashirova, T., & Souto, E. B. (2021). Biosurfactants: Properties and Applications in Drug Delivery, Biotechnology and Ecotoxicology. *Bioengineering*, *8*(8), 115. https://doi.org/10.3390/bioengineering8080115

Bleve, G., Lezzi, C., Chiriatti, M. A., D'Ostuni, I., Tristezza, M., Di Venere, D., Sergio, L., Mita, G., & Grieco, F. (2011). Selection of non-conventional yeasts and their use in immobilized form for the bioremediation of olive oil mill wastewaters. *Bioresource Technology*, *102*(2), 982–989. https://doi.org/10.1016/j.biortech.2010.09.059

Bolat, E., Sarıtaş, S., Duman, H., Eker, F., Akdaşçi, E., Karav, S., & Witkowska, A. M. (2024). Polyphenols: Secondary Metabolites with a Biological Impression. *Nutrients*, *16*(15), Article 15. https://doi.org/10.3390/nu16152550

Boondaeng, A., Keabpimai, J., Trakunjae, C., Vaithanomsat, P., Srichola, P., & Niyomvong, N. (2024). Cellulase production under solid-state fermentation by *Aspergillus* sp. IN5: Parameter optimization and application. *Heliyon*, *10*(5), e26601. https://doi.org/10.1016/j.heliyon.2024.e26601

Borja, R., Banks ,C. J., Maestro-Durán ,R., & and Alba, J. (1996). The Effects of the Most Important Phenolic Constituents of Olive Mill Wastewater on Batch Anaerobic Methanogenesis. *Environmental Technology*, *17*(2), 167–174. https://doi.org/10.1080/09593331708616373

Bouabidi, Z. B., El-Naas, M. H., & Zhang, Z. (2019). Immobilization of microbial cells for the biotreatment of wastewater: A review. *Environmental Chemistry Letters*, *17*(1), 241–257. https://doi.org/10.1007/s10311-018-0795-7

Bougherara, S., Boucetta, N., Lecheb, F., Medjdoub, F., Delleci, K., & Belkhir, M. (2021a). *Characterization and valorisation of olive by-products from a traditional oil mill in Béjaia.*

Bougherara, S., Boucetta, N., Lecheb, F., Medjdoub, F., Delleci, K., & Belkhir, M. (2021b). Characterization and valorisation of olive by-products from a traditional oil mill in Béjaia. *Algerian Journal of Environmental Science and Technology*, *7*(1). https://www.aljest.net/index.php/aljest/article/view/313

Bouharat, D., Nabila, S. A., Souhail, B., Laghdach, A., & El Yousfi, F. (2018). Physico-chemical characterization of olive-oil mill wastewaters of Ben Karrich area (Tetouan province, North of Morocco) and optimization study of their treatment using activated carbon. *Mediterranean Journal of Chemistry*, *7*, 253–258. https://doi.org/10.13171/mjc74181101-bouharat

Bouizar, R., Mouzai, A., & Boughellout, H. (2021). Impact of milk substitution by sweet whey on chocolate mousse physicochemical, microstructural and sensory properties. *Algerian Journal of Nutrition and Food Sciences*, *1*(4), 17–24.

Bouknana, D., Hammouti, B., Salghi, R., Jodeh, S., Zarrouk, A., Warad, I., Aouniti, A., & Sbaa, M. (2014). *Physicochemical Characterization of Olive Oil Mill Wastewaters in the eastern region of Morocco*.

Bouselsal, B., Satouh, A., Egbueri, J. C., Hashim, M. A., Arafat, A. A., Paramasivam, P., Alzaed, A., & Hussein, E. E. (2025). Groundwater for drinking and sustainable agriculture and public health hazards of nitrate: Developmental and sustainability implications for an arid aquifer system. *Results in Engineering*, *25*, 104160. https://doi.org/10.1016/j.rineng.2025.104160

Boutafda, A., Zegzouti, Y., El Fels, L., Ouhdouch, Y., Lebrihi, A., Bekkaoui, F., & Hafidi, M. (2019). Olive mill wastewater biological assessment: Optimization and identification of compounds

degrading yeasts. *Desalination and Water Treatment*, *149*, 129–137. https://doi.org/10.5004/dwt.2019.23869

Bovina, S., Frascari, D., Ragini, A., Avolio, F., Scarcella, G., & Pinelli, D. (2021). Development of a continuous-flow anaerobic co-digestion process of olive mill wastewater and municipal sewage sludge. *Journal of Chemical Technology & Biotechnology*, *96*(2), 532–543. https://doi.org/10.1002/jctb.6570

Bušić, A., Marđetko, N., Kundas, S., Morzak, G., Belskaya, H., Ivančić Šantek, M., Komes, D., Novak, S., & Šantek, B. (2018). Bioethanol Production from Renewable Raw Materials and Its Separation and Purification: A Review. *Food Technology and Biotechnology*, *56*(3), 289–311. https://doi.org/10.17113/ftb.56.03.18.5546

Calabrò, P. S., Fòlino, A., Tamburino, V., Zappia, G., & Zema, D. A. (2018). Increasing the tolerance to polyphenols of the anaerobic digestion of olive wastewater through microbial adaptation. *Biosystems Engineering*, *172*, 19–28. https://doi.org/10.1016/j.biosystemseng.2018.05.010

Canal, C., Ozen, B., & Baysal, A. H. (2019). Characterization of antimicrobial activities of olive phenolics on yeasts using conventional methods and mid-infrared spectroscopy. *Journal of Food Science and Technology*, *56*(1), 149–158. https://doi.org/10.1007/s13197-018-3468-4

Carmona, I., Aguirre, I., Griffith, D. M., & García-Borrego, A. (2023). Towards a circular economy in virgin olive oil production: Valorization of the olive mill waste (OMW) "alpeorujo" through polyphenol recovery with natural deep eutectic solvents (NADESs) and vermicomposting. *Science of The Total Environment*, *872*, 162198. https://doi.org/10.1016/j.scitotenv.2023.162198

Caroca, E., Serrano, A., Borja, R., Jiménez, A., Carvajal, A., Braga, A. F. M., Rodriguez-Gutierrez, G., & Fermoso, F. G. (2021). Influence of phenols and furans released during thermal pretreatment of olive mill solid waste on its anaerobic digestion. *Waste Management*, *120*, 202–208. https://doi.org/10.1016/j.wasman.2020.11.027

Chang, Y.-H., Chang, K.-S., Chen, C.-Y., Hsu, C.-L., Chang, T.-C., & Jang, H.-D. (2018). Enhancement of the Efficiency of Bioethanol Production by Saccharomyces cerevisiae via Gradually Batch-Wise and Fed-Batch Increasing the Glucose Concentration. *Fermentation*, *4*(2), Article 2. https://doi.org/10.3390/fermentation4020045

Chauhan, S. J., Patel, B., Devliya, B., Solanki, H., & Patel, H. D. (2024). Recent advancement in production of bioethanol from waste biomass: A review. *Clean Technologies and Environmental Policy*, *26*(11), 3739–3759. https://doi.org/10.1007/s10098-023-02710-0

Chen, X., Mao, H., Cui, Y., Deng, H., Zhao, T., Liu, J., Huang, L., & Shen, P. (2024). An algal regulation-based molasses vinasse anaerobic digestion strategy for enhancing organic matter removal and methane production. *Renewable Energy*, *234*, 121257. https://doi.org/10.1016/j.renene.2024.121257

Cheng, D., Liu, Y., Ngo, H. H., Guo, W., Chang, S. W., Nguyen, D. D., Zhang, S., Luo, G., & Liu, Y. (2020). A review on application of enzymatic bioprocesses in animal wastewater and manure treatment. *Bioresource Technology*, *313*, 123683. https://doi.org/10.1016/j.biortech.2020.123683

Chikhoune, A., Bedjou, F., Oubouzid, S., Boukefoussa, R., Bechri, B., Tarmoul, H., Abdeladim, T., Tounsi, A., Hamitri, M., Chikh, S., & Kouadri, L. (2014). Development of Sugar Cane Molasses in Formulations of Madeleines, Mini Croissants, and Buns Incorporated with Interesterified Oil. *Journal of Chemistry*, *2014*(1), 936780. https://doi.org/10.1155/2014/936780

Choi, G.-H., Lee, N.-K., & Paik, H.-D. (2021). *Optimization of Medium Composition for Biomass Production of Lactobacillus plantarum 200655 Using Response Surface Methodology*. *31*(5), 717–725. https://doi.org/10.4014/jmb.2103.03018

Clément, G. (1956). DOSAGE DES LIPIDES DANS LES PRODUITS ALIMENTAIRES ; CONSIDÉRATIONS SUR LEUR VALEUR NUTRITIVE (1). *Annales de Zootechnie*, *5*(3), 237–253.

Costa, C. E., Romaní, A., Teixeira, J. A., & Domingues, L. (2022). Resveratrol production for the valorisation of lactose-rich wastes by engineered industrial *Saccharomyces cerevisiae*. *Bioresource Technology*, *359*, 127463. https://doi.org/10.1016/j.biortech.2022.127463

Costa, G. dos S., Martinez-Burgos, W. J., dos Reis, G. A., Puche, Y. P., Vega, F. R., Rodrigues, C., Serra, J. L., Campos, S. de M., & Soccol, C. R. (2024). Advances in Biomass and Microbial

Lipids Production: Trends and Prospects. *Processes*, *12*(12), Article 12. https://doi.org/10.3390/pr12122903

Cuffaro, D., Bertolini, A., Bertini, S., Ricci, C., Cascone, M. G., Danti, S., Saba, A., Macchia, M., & Digiacomo, M. (2023). Olive Mill Wastewater as Source of Polyphenols with Nutraceutical Properties. *Nutrients*, *15*(17), 3746. https://doi.org/10.3390/nu15173746

Czarnota, J., Masłoń, A., & Pajura, R. (2023). Wastewater Treatment Plants as a Source of Malodorous Substances Hazardous to Health, Including a Case Study from Poland. *International Journal of Environmental Research and Public Health*, *20*(7), 5379. https://doi.org/10.3390/ijerph20075379

Darmawan, R., Dewi, V. G. P., Rizaldi, M. A., Juliastuti, S. R., Gunawan, S., Aparamarta, H. W., & Wiguno, A. (2020). Production of Liquid Bio-Fertilizer from Old Coconut Water and Molasses using Consortium Microbes. *IOP Conference Series: Materials Science and Engineering*, *845*(1), 012007. https://doi.org/10.1088/1757-899X/845/1/012007

Das, B., Das, M., Bhattacharjee, S., & Bhattacharjee, C. (2017). Ethanol production from deproteinized cheese whey powder in a batch fermentation process: Optimization of process and kinetic modelling. *Desalination and Water Treatment*, *64*, 198–206. https://doi.org/10.5004/dwt.2017.20238

De Leonardis, A., Masino, F., Macciola, V., Montevecchi, G., Antonelli, A., & Marconi, E. (2019). A study on acetification process to produce olive vinegar from oil mill wastewaters. *European Food Research and Technology*, *245*. https://doi.org/10.1007/s00217-019-03323-y

de Ory, I., Cabrera, G., Ramirez, M., & Blandino, A. (2020). Immobilization of Cells on Polyurethane Foam. *Methods in Molecular Biology (Clifton, N.J.)*, *2100*, 407–415. https://doi.org/10.1007/978-1-0716-0215-7_27

De Rossi, L., Rocchetti, G., Lucini, L., & Rebecchi, A. (2025). Antimicrobial Potential of Polyphenols: Mechanisms of Action and Microbial Responses—A Narrative Review. *Antioxidants*, *14*(2), Article 2. https://doi.org/10.3390/antiox14020200

de Souza, P. M., & de Oliveira Magalhães, P. (2010). Application of microbial α-amylase in industry – A review. *Brazilian Journal of Microbiology*, *41*(4), 850–861. https://doi.org/10.1590/S1517-83822010000400004

de Araujo, T. M., da Cunha, M. M. L., Barga, M. C., Della-Bianca, B. E., & Basso, T. O. (2024). Production of flavor-active compounds and physiological impacts in immobilized *Saccharomyces* spp. Cells during beer fermentation. *Letters in Applied Microbiology*, *77*(9), ovae083. https://doi.org/10.1093/lambio/ovae083

del Carmen Carranza-Méndez, R., Sepúlveda-Torre, L., Ramos-González, R., Verma, D. K., Baranwal, D., Aguilar, C. N., & Chávez-González, M. L. (2022). Chapter 17—Agro-industrial wastes for production of single-cell protein (SCP). In H. Thatoi, S. Mohapatra, & S. K. Das (Eds.), *Innovations in Fermentation and Phytopharmaceutical Technologies* (pp. 381–396). Elsevier. https://doi.org/10.1016/B978-0-12-821877-8.00012-9

Deme, G., & Asfaw, B. (2020). *Fabrication of Citric Acid Using Molasses by Aspergillus niger*.

Devi, A., Bajar, S., Kour, H., Kothari, R., Pant, D., & Singh, A. (2022). Lignocellulosic Biomass Valorization for Bioethanol Production: A Circular Bioeconomy Approach. *Bioenergy Research*, *15*(4), 1820–1841. https://doi.org/10.1007/s12155-022-10401-9

Diez Antolinez, R., Hijosa-Valsero, M., Paniagua-García, A. I., & Gómez, X. (2016). Effect of Nutrient Supplementation on Biobutanol Production from Cheese Whey by ABE (Acetone–Butanol–Ethanol) Fermentation. *Chemical Engineering Transactions*, *49*, 217–222. https://doi.org/10.3303/CET1649037

Djeziri, S., Taleb, Z., Djellouli, M., & Taleb, S. (2023). Physicochemical and microbiological characterisation of Olive Oil Mill Wastewater (OMW) from the region of Sidi Bel Abbes (Western Algeria). *Moroccan Journal of Chemistry*, *11*(2), Article 2. https://doi.org/10.48317/IMIST.PRSM/morjchem-v11i2.31935

Domingues, L., Guimarães, P. M., & Oliveira, C. (2010). Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation. *Bioengineered Bugs*, *1*(3), 164–171. https://doi.org/10.4161/bbug.1.3.10619

Dong, L., Wu, Y., Li, M., Zhang, C., Cao, J., Ledesma-Amaro, R., Zhao, W., & Kang, D. (2025). Exploring the Fermentation Potential of Kluyveromyces marxianus NS127 for Single-Cell

Protein Production. *Fermentation*, *11*(2), Article 2. https://doi.org/10.3390/fermentation11020070

Doriya, K., Jose, N., Gowda, M., & Kumar, D. S. (2016). Solid-State Fermentation vs Submerged Fermentation for the Production of l-Asparaginase. In S.-K. Kim & F. Toldrá (Eds.), *Advances in Food and Nutrition Research* (Vol. 78, pp. 115–135). Academic Press. https://doi.org/10.1016/bs.afnr.2016.05.003

Dragičević, T., Hren, M., Gmajnić, M., Pelko, S., Kungulovski, D., Kungulovski, I., Čvek, D., Frece, J., Markov, K., & Delaš, F. (2010). Biodegradation of Olive Mill Wastewater by Trichosporon Cutaneum and Geotrichum Candidum. *Archives of Industrial Hygiene and Toxicology*, *61*(4), 399–405. https://doi.org/10.2478/10004-1254-61-2010-2079

Duque, A., Álvarez, C., Doménech, P., Manzanares, P., & Moreno, A. D. (2021). Advanced Bioethanol Production: From Novel Raw Materials to Integrated Biorefineries. *Processes*, *9*(2), Article 2. https://doi.org/10.3390/pr9020206

Dzionek, A., Wojcieszyńska, D., Adamczyk-Habrajska, M., Karczewski, J., Potocka, I., & Guzik, U. (2021). Xanthan gum as a carrier for bacterial cell entrapment: Developing a novel immobilised biocatalyst. *Materials Science and Engineering: C*, *118*, 111474. https://doi.org/10.1016/j.msec.2020.111474

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

El-Aidie, S. A. M., & Khalifa, G. S. A. (2024). Innovative applications of whey protein for sustainable dairy industry: Environmental and technological perspectives—A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *23*(2), e13319. https://doi.org/10.1111/1541-4337.13319

Ellis, J. T., Sims, R. C., & Miller, C. D. (2014). Microbial bioproducts from cheese whey through fermentation with wastewater sludge Clostridium isolates. *Canadian Journal of Microbiology*, *60*(7), 431–435. https://doi.org/10.1139/cjm-2013-0803

Fagundes, V. D., Freitag, J. F., Simon, V., & Colla, L. M. (2024). Enzymatic hydrolysis of food waste for bioethanol production. *Revista Brasileira de Ciências Ambientais*, *59*, e1978–e1978. https://doi.org/10.5327/Z2176-94781978

Falowo, O. A., & Betiku, E. (2023). Introduction: Benefits, Prospects, and Challenges of Bioethanol Production. In E. Betiku & M. M. Ishola (Eds.), *Bioethanol: A Green Energy Substitute for Fossil Fuels* (pp. 1–19). Springer International Publishing. https://doi.org/10.1007/978-3-031-36542-3_1

FAO. (2020). *Ecosystems impairment caused by soil pollution*. https://doi.org/10.4060/cb4894en

FAO, U. (2023). *FAOSTAT*. Food and Agriculture Organization of the United Nation). https://www.fao.org/faostat/fr/#data/QCL/visualize

Fazio, N. A., Pino, A., Foti, P., Esteve-Zarzoso, B., Randazzo, C. L., Torija, M.-J., & Caggia, C. (2024). Screening and characterization of indigenous *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts isolated from Sicilian vineyards. *Food Bioscience*, *62*, 105282. https://doi.org/10.1016/j.fbio.2024.105282

Fenster, K., Freeburg, B., Hollard, C., Wong, C., Rønhave Laursen, R., & Ouwehand, A. C. (2019). The Production and Delivery of Probiotics: A Review of a Practical Approach. *Microorganisms*, *7*(3), Article 3. https://doi.org/10.3390/microorganisms7030083

Ferrari, M., Cirisano, F., & Morán, M. C. (2019). Mammalian Cell Behavior on Hydrophobic Substrates: Influence of Surface Properties. *Colloids and Interfaces*, *3*(2), Article 2. https://doi.org/10.3390/colloids3020048

Fleyfel, L. M., Leitner, N. K. V., Deborde, M., Matta, J., & El Najjar, N. H. (2022). Olive oil liquid wastes–Characteristics and treatments: A literature review. *Process Safety and Environmental Protection*, *168*, 1031–1048. https://doi.org/10.1016/j.psep.2022.10.035

Foti, P., Romeo, F., Russo, N., Pino, A., Vaccalluzzo, A., Caggia, C., & Randazzo, C. (2021). Olive Mill Wastewater as Renewable Raw Materials to Generate High Added-Value Ingredients for Agro-Food Industries. *Applied Sciences*, *11*, 7511. https://doi.org/10.3390/app11167511

Fronteras, J. P., Dacera, D. D. M., Bello, D. D., & Delos Santos, K. J. L. (2021). Utilization of pesticide-free calamansi (*Citrus microcarpa*) and mango (*Mangifera indica*) peels for the production of

acetic acid with potential industrial application. *Bioresource Technology Reports*, *15*, 100806. https://doi.org/10.1016/j.biteb.2021.100806

Ge, X., Yang, L., & Xu, J. (2017). Cell Immobilization: Fundamentals, Technologies, and Applications. In *Industrial Biotechnology* (pp. 205–235). John Wiley & Sons, Ltd. https://doi.org/10.1002/9783527807833.ch7

Geies, A., & Abdelazim, M. (2021). Self-Colorimetric Determination of Bio-Ethanol Using Permanganate in Fermentation Samples. *Open Access Library Journal*, *8*(8), Article 8. https://doi.org/10.4236/oalib.1107762

Gharaibeh, M., Albalasmeh, A., & H.J., A. (2021). Characterization of olive mill wastewater in three climatic zones in the North of Jordan. *International Journal of Recycling of Organic Waste in Agriculture*, *11*. https://doi.org/10.30486/IJROWA.2021.1910881.1143

Giese, E. C. (2020). MINING APPLICATIONS OF IMMOBILIZED MICROBIAL CELLS IN AN ALGINATE MATRIX: AN OVERVIEW. *Revista Internacional de Contaminación Ambiental*, *36*(3), 775–787. https://doi.org/10.20937/rica.53357

Giulianetti de Almeida, M. P., Mockaitis, G., & Weissbrodt, D. G. (2023a). Got Whey? Sustainability Endpoints for the Dairy Industry through Resource Biorecovery. *Fermentation*, *9*(10), Article 10. https://doi.org/10.3390/fermentation9100897

Giulianetti de Almeida, M. P., Mockaitis, G., & Weissbrodt, D. G. (2023b). Got Whey? Sustainability Endpoints for the Dairy Industry through Resource Biorecovery. *Fermentation*, *9*(10), Article 10. https://doi.org/10.3390/fermentation9100897

Gomes, R. J., Borges, M. de F., Rosa, M. de F., Castro-Gómez, R. J. H., & Spinosa, W. A. (2018). Acetic Acid Bacteria in the Food Industry: Systematics, Characteristics and Applications. *Food Technology and Biotechnology*, *56*(2), 139–151. https://doi.org/10.17113/ftb.56.02.18.5593

Goudopoulou, A., Krimitzas, A., & Typas, M. A. (2010). Differential gene expression of ligninolytic enzymes in Pleurotus ostreatus grown on olive oil mill wastewater. *Applied Microbiology and Biotechnology*, *88*(2), 541–551. https://doi.org/10.1007/s00253-010-2750-9

Graham, E. B., & Knelman, J. E. (2023). Implications of Soil Microbial Community Assembly for Ecosystem Restoration: Patterns, Process, and Potential. *Microbial Ecology*, *85*(3), 809–819. https://doi.org/10.1007/s00248-022-02155-w

Grewal, J., Wołącewicz, M., Pyter, W., Joshi, N., Drewniak, L., & Pranaw, K. (2022). Colorful Treasure From Agro-Industrial Wastes: A Sustainable Chassis for Microbial Pigment Production. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.832918

Gueboudji, Z., Addad, D., Kadi, K., Nagaz, K., Secrafi, M., Yahya, L. B., Lachehib, B., & Abdelmalek, A. (2022a). Biological activities and phenolic compounds of olive oil mill wastewater from Abani, endemic Algerian variety. *Scientific Reports*, *12*(1), 6042. https://doi.org/10.1038/s41598-022-10052-y

Gueboudji, Z., Addad, D., Kadi, K., Nagaz, K., Secrafi, M., Yahya, L. B., Lachehib, B., & Abdelmalek, A. (2022b). Biological activities and phenolic compounds of olive oil mill wastewater from Abani, endemic Algerian variety. *Scientific Reports*, *12*(1), 6042. https://doi.org/10.1038/s41598-022-10052-y

Gueboudji, Z., Kadi, K., & Nagaz, K. (2021). Extraction and Quantification of Polyphenols of Olive Oil Mill Wastewater from the Cold Extraction of Olive Oil in the Region of Khenchela-Algeria. *Genetics & Biodiversity Journal*, *5*(2), Article 2. https://doi.org/10.46325/gabj.v5i2.79

Guo, X., Wang, Y., Qin, Y., Shen, P., & Peng, Q. (2020). Structures, properties and application of alginic acid: A review. *International Journal of Biological Macromolecules*, *162*, 618–628. https://doi.org/10.1016/j.ijbiomac.2020.06.180

Gutiérrez-Rivera, B., Ortiz-Muñiz, B., Gómez-Rodríguez, J., Cárdenas-Cágal, A., Domínguez González, J. M., & Aguilar-Uscanga, M. G. (2015). Bioethanol production from hydrolyzed sugarcane bagasse supplemented with molasses "B" in a mixed yeast culture. *Renewable Energy*, *74*, 399–405. https://doi.org/10.1016/j.renene.2014.08.030

Halema, A. (2014). Production of Ethanol from Molasses and Whey Permeate Using Yeasts and Bacterial Strains. *Int.J.Curr.Microbiol.App.Sci*, *3 Number 3 (2014)*, 804–818.

Hamli, S., Addad, D., Mekersi, N., Kadi, K., Kadi, Z., Dib, D., Dali, H., Labbani, F.-Z. K., Boukeria, S., Agoune, E., & Lahouel, A. (2024). Short-Term Effects of Olive Mill Wastewater (OMW)

Spreading on Faba Bean and Lentil Growth and Soil Quality. *Polish Journal of Ecology*, *72*(1–2), 1–13. https://doi.org/10.3161/15052249PJE2024.72.1.001

Harirchi, S., Wainaina ,Steven, Sar ,Taner, Nojoumi ,Seyed Ali, Parchami ,Milad, Parchami ,Mohsen, Varjani ,Sunita, Khanal ,Samir Kumar, Wong ,Jonathan, Awasthi ,Mukesh Kumar, & and Taherzadeh, M. J. (2022). Microbiological insights into anaerobic digestion for biogas, hydrogen or volatile fatty acids (VFAs): A review. *Bioengineered*, *13*(3), 6521–6557. https://doi.org/10.1080/21655979.2022.2035986

Hassan, M. E., Yang, Q., Xiao, Z., Liu, L., Wang, N., Cui, X., & Yang, L. (2019). Impact of immobilization technology in industrial and pharmaceutical applications. *3 Biotech*, *9*(12), 440. https://doi.org/10.1007/s13205-019-1969-0

Hernandez, J. E., & Edyvean, R. G. J. (2008). Inhibition of biogas production and biodegradability by substituted phenolic compounds in anaerobic sludge. *Journal of Hazardous Materials*, *160*(1), 20–28. https://doi.org/10.1016/j.jhazmat.2008.02.075

Hoarau, M., Badieyan, S., & Marsh, E. N. G. (2017). Immobilized enzymes: Understanding enzyme – surface interactions at the molecular level. *Organic & Biomolecular Chemistry*, *15*(45), 9539–9551. https://doi.org/10.1039/C7OB01880K

Houfani, A. A., Anders, N., Spiess, A. C., Baldrian, P., & Benallaoua, S. (2020). Insights from enzymatic degradation of cellulose and hemicellulose to fermentable sugars– a review. *Biomass and Bioenergy*, *134*, 105481. https://doi.org/10.1016/j.biombioe.2020.105481

Huang, C.-H., Chiang, L.-C., Liao, C.-J., & Chen, F.-W. (2024). Evaluation of the Impact of Recycled Distillery Effluent on the Cultivation of Wheat and Sorghum. *Water*, *16*(22), Article 22. https://doi.org/10.3390/w16223220

Huang, H., Ali, S., & Solangi, Y. A. (2023). Analysis of the Impact of Economic Policy Uncertainty on Environmental Sustainability in Developed and Developing Economies. *Sustainability*, *15*(7), Article 7. https://doi.org/10.3390/su15075860

Illanes, A. (2011). Whey upgrading by enzyme biocatalysis. *Electronic Journal of Biotechnology*, *14*(6), Article 6. https://www.ejbiotechnology.info/index.php/ejbiotechnology/article/view/v14n6-11

Ilyas, M., Ahmad, W., Khan, H., Yousaf, S., Yasir, M., & Khan, A. (2019). Environmental and health impacts of industrial wastewater effluents in Pakistan: A review. *Reviews on Environmental Health*, *34*(2), 171–186. https://doi.org/10.1515/reveh-2018-0078

Indira, D., Jijnasa, B., Arati, N., Moumita, S., Ajay, D., Eldin, J., Sachin, K., & Rasu, J. (2015). *Comparative Studies Of Ethanol Production And Cell Viability: Free Cells Versus Immobilized Cells*. https://www.semanticscholar.org/paper/Comparative-Studies-Of-Ethanol-Production-And-Cell-Indira-Jijnasa/c5e6dfa8bff0f8acaa8a50ff519983c50a8cc56d

International Olive Council. (2023). *World Olive Oil and Table olive Figures*. International Olive Council. https://www.internationaloliveoil.org/what-we-do/economic-affairs-promotion-unit/

Jain, A., Jain, R., & Jain, S. (2020). Quantitative Analysis of Reducing Sugars by 3, 5-Dinitrosalicylic Acid (DNSA Method). In A. Jain, R. Jain, & S. Jain (Eds.), *Basic Techniques in Biochemistry, Microbiology and Molecular Biology: Principles and Techniques* (pp. 181–183). Springer US. https://doi.org/10.1007/978-1-4939-9861-6_43

Jamai, L., & Ettayebi, M. (2015). Production of bioethanol during the bioremediation of olive mill wastewater at high temperatures. *2015 3rd International Renewable and Sustainable Energy Conference (IRSEC)*, 1–6. https://doi.org/10.1109/IRSEC.2015.7455034

Jesionowski, T., Zdarta, J., & Krajewska, B. (2014). Enzyme immobilization by adsorption: A review. *Adsorption*, *20*(5), 801–821. https://doi.org/10.1007/s10450-014-9623-y

Jiranuntipon, S. (2008). *Decolorization of molasses wastewater from distilleries using bacterial consortium* [Doctor of Philosophy, Chulalongkorn University]. https://doi.org/10.58837/CHULA.THE.2008.1017

Kanimozhi, J., Sivasubramanian, V., Achary, A., Vasanthi, M., Vinson, S. P., & Sivashankar, R. (2018). Bioprocessing of Agrofood Industrial Wastes for the Production of Bacterial Exopolysaccharide. In *Bioprocess Engineering for a Green Environment*. CRC Press.

Karki, R., Chuenchart, W., Surendra, K. C., Shrestha, S., Raskin, L., Sung, S., Hashimoto, A., & Kumar Khanal, S. (2021). Anaerobic co-digestion: Current status and perspectives. *Bioresource Technology*, *330*, 125001. https://doi.org/10.1016/j.biortech.2021.125001

Kechkar, M., Aziza, M., Bessah, R., Abada, S., Djelal, H., Zaid, T. A., & Amrane, A. (2024). Optimization and kinetic study of the bioethanol production by a locally isolated strain using response surface methodology. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-024-05807-8

Keshapaga, U. R., Jathoth, K., Singh, S. S., Gogada, R., & Burgula, S. (2023). Characterization of high-yield Bacillus subtilis cysteine protease for diverse industrial applications. *Brazilian Journal of Microbiology*, *54*(2), 739–752. https://doi.org/10.1007/s42770-023-00992-6

Khamassi, A., & Dumon, C. (2023). Enzyme synergy for plant cell wall polysaccharide degradation. *Essays in Biochemistry*, *67*(3), 521–531. https://doi.org/10.1042/EBC20220166

Kharayat, Y. (2012). Distillery wastewater: Bioremediation approaches. *Journal of Integrative Environmental Sciences*, *9*(2), 69–91. https://doi.org/10.1080/1943815X.2012.688056

Kharitonov, S., Semenov, M., Sabrekov, A., Kotsyurbenko, O., Zhelezova, A., & Schegolkova, N. (2021). Microbial Communities in Methane Cycle: Modern Molecular Methods Gain Insights into Their Global Ecology. *Environments*, *8*(2), Article 2. https://doi.org/10.3390/environments8020016

Khdair, A., & Abu-Rumman, G. (2017). Evaluation of Environmental Pollution from Olive Mill Wastewater. *Fresenius Environmental Bulletin*, *26*, 2537–2540.

Khonngam, T., & Salakkam, A. (2019). Bioconversion of sugarcane bagasse and dry spent yeast to ethanol through a sequential process consisting of solid-state fermentation, hydrolysis, and submerged fermentation. *Biochemical Engineering Journal*, *150*, 107284. https://doi.org/10.1016/j.bej.2019.107284

Khouli, M. R., Haouchine, A., & Banton, O. (2021). Deterioration of the quality of ground water in agricultural region. Case Mitidja (Algeria). *Journal of Fundamental and Applied Sciences*, *13*(1), Article 1. https://doi.org/10.4314/jfas.v13i1.10

Kieliszek, M., Kot, A. M., Bzducha-Wróbel, A., BŁażejak, S., Gientka, I., & Kurcz, A. (2017). Biotechnological use of *Candida* yeasts in the food industry: A review. *Fungal Biology Reviews*, *31*(4), 185–198. https://doi.org/10.1016/j.fbr.2017.06.001

Kim, J., & Rose, M. D. (2015). Stable Pseudohyphal Growth in Budding Yeast Induced by Synergism between Septin Defects and Altered MAP-kinase Signaling. *PLOS Genetics*, *11*(12), e1005684. https://doi.org/10.1371/journal.pgen.1005684

Kim, K., Choe, D., Lee, D.-H., & Cho, B.-K. (2020). Engineering Biology to Construct Microbial Chassis for the Production of Difficult-to-Express Proteins. *International Journal of Molecular Sciences*, *21*(3), Article 3. https://doi.org/10.3390/ijms21030990

Kirimura, K., Honda, Y., & Hattori, T. (2011). 3.13—Citric Acid. In M. Moo-Young (Ed.), *Comprehensive Biotechnology (Second Edition)* (pp. 135–142). Academic Press. https://doi.org/10.1016/B978-0-08-088504-9.00169-0

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B., & Blanch, H. (2012). The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and Bioengineering*, *109*, 1083–1087. https://doi.org/10.1002/bit.24370

König, H., & Fröhlich, J. (2017). Lactic Acid Bacteria. In H. König, G. Unden, & J. Fröhlich (Eds.), *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 3–41). Springer International Publishing. https://doi.org/10.1007/978-3-319-60021-5_1

Kounbesiou, M., Savadogo, A., Barro, N., Thonart, P., & Sabadenedy, A. (2011). Effect of Minerals Salts in Fermentation Process using Mango Residues as Carbon Source for Bioethanol Production. *Asian Journal of Industrial Engineering*, *3*, 29–38. https://doi.org/10.3923/ajie.2011.29.38

Kovács, E., Wirth, R., Maróti, G., Bagi, Z., Rákhely, G., & Kovács, K. L. (2013). Biogas Production from Protein-Rich Biomass: Fed-Batch Anaerobic Fermentation of Casein and of Pig Blood and Associated Changes in Microbial Community Composition. *PLoS ONE*, *8*(10), e77265. https://doi.org/10.1371/journal.pone.0077265

Książek, E. (2023). Citric Acid: Properties, Microbial Production, and Applications in Industries. *Molecules*, *29*(1), 22. https://doi.org/10.3390/molecules29010022

Kumar, K., Jadhav, S. M., & Moholkar, V. S. (2024). Acetone-Butanol-Ethanol (ABE) fermentation with clostridial co-cultures for enhanced biobutanol production. *Process Safety and Environmental Protection*, *185*, 277–285. https://doi.org/10.1016/j.psep.2024.03.027

Kumar, R., Bohra, V., Mk, M., & Wong, J. W. C. (2022). Food Waste as a Valuable Carbon Source for Bioconversion – How Microbes do Miracles. In *Good Microbes in Medicine, Food Production, Biotechnology, Bioremediation, and Agriculture* (pp. 312–322). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119762621.ch25

Kumar, S., Sahu, S. P., Kumari, S., Sinha, R. R. K., Kumar, P., Kumar, S., & Kumar, K. (2024). Effects of liquid whey supplementation with water on egg production and egg quality in layer chickens. *Indian Journal of Animal Research*, *58*(6), 1053–1057.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Kumar, V., Ahluwalia, V., Saran, S., Kumar, J., Patel, A. K., & Singhania, R. R. (2021). Recent developments on solid-state fermentation for production of microbial secondary metabolites: Challenges and solutions. *Bioresource Technology*, *323*, 124566. https://doi.org/10.1016/j.biortech.2020.124566

Laabidi, F., Besbès, R., & Jamoussi, B. (2023). Production of Biogas from Olive Mill Waste Waters Treated by Cow Manure. *Green and Sustainable Chemistry*, *13*(1), Article 1. https://doi.org/10.4236/gsc.2023.131005

Lambertz, C., Garvey, M., Klinger, J., Heesel, D., Klose, H., Fischer, R., & Commandeur, U. (2014). Challenges and advances in the heterologous expression of cellulolytic enzymes: A review. *Biotechnology for Biofuels*, *7*(1), 135. https://doi.org/10.1186/s13068-014-0135-5

Lee, C. H., Lee, H. S., Lee, J. W., Kim, J., Lee, J. H., Jin, E. S., & Hwang, E. T. (2021). Evaluating enzyme stabilizations in calcium carbonate: Comparing *in situ* and crosslinking mediated immobilization. *International Journal of Biological Macromolecules*, *175*, 341–350. https://doi.org/10.1016/j.ijbiomac.2021.02.028

Lee, H., Sohn, Y., Jeon, S., Yang, H., Son, J., Kim, Y., & Park, S. J. (2023). Sugarcane wastes as microbial feedstocks: A review of the biorefinery framework from resource recovery to production of value-added products. *Bioresource Technology*, *376*, 128879. https://doi.org/10.1016/j.biortech.2023.128879

Leite, P., Belo, I., & Salgado, J. M. (2021). Co-management of agro-industrial wastes by solid-state fermentation for the production of bioactive compounds. *Industrial Crops and Products*, *172*, 113990. https://doi.org/10.1016/j.indcrop.2021.113990

Li, J., Chen, S., Fu, J., Xie, J., Ju, J., Yu, B., & Wang, L. (2022). Efficient molasses utilization for low-molecular-weight poly-γ-glutamic acid production using a novel Bacillus subtilis stain. *Microbial Cell Factories*, *21*, 140. https://doi.org/10.1186/s12934-022-01867-5

Li, R., Wang, B., Niu, A., Cheng, N., Chen, M., Zhang, X., Yu, Z., & Wang, S. (2022). Application of biochar immobilized microorganisms for pollutants removal from wastewater: A review. *Science of The Total Environment*, *837*, 155563. https://doi.org/10.1016/j.scitotenv.2022.155563

Li, S., Zhao, X., Ye, X., Zhang, L., Shi, L., Xu, F., & Ding, G. (2020). The Effects of Condensed Molasses Soluble on the Growth and Development of Rapeseed through Seed Germination, Hydroponics and Field Trials. *Agriculture*, *10*(7), Article 7. https://doi.org/10.3390/agriculture10070260

Li, X., Shi, Y., Kong, W., Wei, J., Song, W., & Wang, S. (2022). Improving enzymatic hydrolysis of lignocellulosic biomass by bio-coordinated physicochemical pretreatment—A review. *Energy Reports*, *8*, 696–709. https://doi.org/10.1016/j.egyr.2021.12.015

Li, Y., Luo, L., Ding, X., Zhang, X., Gan, S., & Shang, C. (2023). Production of Tetramethylpyrazine from Cane Molasses by Bacillus sp. TTMP20. *Molecules*, *28*(6), Article 6. https://doi.org/10.3390/molecules28062640

Li, Y. P., Ahmadi, F., Kariman, K., & Lackner, M. (2024). Recent advances and challenges in single cell protein (SCP) technologies for food and feed production. *Npj Science of Food*, *8*(1), 66. https://doi.org/10.1038/s41538-024-00299-2

Lievore, P., Simões, D. R. S., Silva, K. M., Drunkler, N. L., Barana, A. C., Nogueira, A., & Demiate, I. M. (2015). Chemical characterisation and application of acid whey in fermented milk. *Journal of Food Science and Technology*, *52*(4), 2083–2092. https://doi.org/10.1007/s13197-013-1244-z

Liew, F., Martin, M. E., Tappel, R. C., Heijstra, B. D., Mihalcea, C., & Köpke, M. (2016). Gas Fermentation—A Flexible Platform for Commercial Scale Production of Low-Carbon-Fuels and Chemicals from Waste and Renewable Feedstocks. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00694

Liu, C., & and Cheng, K. (2022). Molasses fermentation to produce low-cost carbon source for denitrification. *Biotechnology & Biotechnological Equipment*, *36*(1), 878–890. https://doi.org/10.1080/13102818.2022.2138781

Liu, D., Yang, X., Zhang, L., Tang, Y., He, H., Liang, M., Tu, Z., & Zhu, H. (2022). Immobilization of Biomass Materials for Removal of Refractory Organic Pollutants from Wastewater. *International Journal of Environmental Research and Public Health*, *19*(21), Article 21. https://doi.org/10.3390/ijerph192113830

López, J. C., Quijano, G., Souza, T. S. O., Estrada, J. M., Lebrero, R., & Muñoz, R. (2013). Biotechnologies for greenhouse gases (CH□, N□O, and CO□) abatement: State of the art and challenges. *Applied Microbiology and Biotechnology*, *97*(6), 2277–2303. https://doi.org/10.1007/s00253-013-4734-z

Lyu, Z., Shao, N., Akinyemi, T., & Whitman, W. B. (2018). Methanogenesis. *Current Biology*, *28*(13), R727–R732. https://doi.org/10.1016/j.cub.2018.05.021

Maestre, K. L., Passos, F. R. dos, Triques, C. C., Fiorentin-Ferrari, L. D., Slusarski-Santana, V., Garcia, H. A., Silva, E. A. da, & Fiorese, M. L. (2021). Cheese whey permeate valorization using sequential fermentations: Case study performed in the Western Region of Paraná. *Research, Society and Development*, *10*(13), Article 13. https://doi.org/10.33448/rsd-v10i13.21082

Mahajan, R., Gupta2, V. K., & Sharma1, J. (2010). *Indian Journal of Pharmaceutical Sciences*. https://doi.org/10.4103/0250-474X.65010

Maicas, S. (2020). The Role of Yeasts in Fermentation Processes. *Microorganisms*, *8*(8), 1142. https://doi.org/10.3390/microorganisms8081142

Maidali, D., Magar, S. T., Ghimire, S., Pun, M., Pandey, S., Chataut, G., Maharjan, S., Subedi, S., & Aryal, P. (2024). Valorization of Cheese Whey through Bioethanol Production and Probiotic Drink Development: A Sustainable Approach for Organic Waste Management. *Nepal Journal of Biotechnology*, *12*(1), Article 1. https://doi.org/10.54796/njb.v12i1.273

Makri, S., Raftopoulou, S., Kafantaris, I., Kotsampasi, B., Christodoulou, V., Nepka, C., Veskoukis, A. S., & Kouretas, D. (2020). Biofunctional Feed Supplemented With By-products of Olive Oil Production Improves Tissue Antioxidant Profile of Lambs. *In Vivo*, *34*(4), 1811–1821. https://doi.org/10.21873/invivo.11976

Manikandan, K., & Thangavelu, V. (2010). Optimization of C/N ratio of the medium and fermentation conditions of ethanol production from tapioca starch using co—Culture of aspergillus niger and sachormyces cerevisiae. *International Journal of ChemTech Research*, *2*.

Manoochehri, H., Hosseini, N. F., Saidijam, M., Taheri, M., Rezaee, H., & Nouri, F. (2020). A review on invertase: Its potentials and applications. *Biocatalysis and Agricultural Biotechnology*, *25*, 101599. https://doi.org/10.1016/j.bcab.2020.101599

Marín-Navarro, J., & Polaina, J. (2011). Glucoamylases: Structural and biotechnological aspects. *Applied Microbiology and Biotechnology*, *89*(5), 1267–1273. https://doi.org/10.1007/s00253-010-3034-0

Martău, G.-A., Unger, P., Schneider, R., Venus, J., Vodnar, D. C., & López-Gómez, J. P. (2021). Integration of Solid State and Submerged Fermentations for the Valorization of Organic Municipal Solid Waste. *Journal of Fungi*, *7*(9), Article 9. https://doi.org/10.3390/jof7090766

Martínez Burgos, W., Sydney, E., Medeiros, A., Magalhaes, A., De Carvalho, J., Karp, S., Vandenberghe, L., Letti, L., Thomaz-Soccol, V., Pereira, G., Rodrigues, C., Woiciechowski, A., & Soccol, C. (2021). Agro-Industrial Wastewater in a Circular Economy: Characteristics, Impacts and Applications for Bioenergy and Biochemicals. *Bioresource Technology*, *341*, 125795. https://doi.org/10.1016/j.biortech.2021.125795

Martínez, O., Sánchez, A., Font, X., & Barrena, R. (2017). Valorization of sugarcane bagasse and sugar beet molasses using *Kluyveromyces marxianus* for producing value-added aroma compounds via solid-state fermentation. *Journal of Cleaner Production*, *158*, 8–17. https://doi.org/10.1016/j.jclepro.2017.04.155

Martinović, A., Chittaro, M., Mora, D., & Arioli, S. (2023). The Ability of Streptococcus thermophilus BT01 to Modulate Urease Activity in Healthy Subjects' Fecal Samples Depends on the Biomass Production Process. *Molecular Nutrition & Food Research*, *67*(6), 2200529. https://doi.org/10.1002/mnfr.202200529

Marwaha, S. S., & Kennedy, J. F. (2007). *Whey—Pollution problem and potential utilization*. https://dx.doi.org/10.1111/j.1365-2621.1988.tb00586.x

Mascarin, G. M., Golo, P. S., de Souza Ribeiro-Silva, C., Muniz, E. R., de Oliveira Franco, A., Kobori, N. N., & Fernandes, É. K. K. (2024). Advances in submerged liquid fermentation and formulation of entomopathogenic fungi. *Applied Microbiology and Biotechnology*, *108*(1), 451. https://doi.org/10.1007/s00253-024-13287-z

Massadeh, M. I., Fandi, K., Al-Abeid, H., Alsharafat, O., & Abu-Elteen, K. (2022). Production of Citric Acid by Aspergillus niger Cultivated in Olive Mill Wastewater Using a Two-Stage Packed Column Bioreactor. *Fermentation*, *8*(4), Article 4. https://doi.org/10.3390/fermentation8040153

Mejia-Gomez, C. E., & Balcázar, N. (2020). Isolation, characterisation and continuous culture of *Lactobacillus spp.* and its potential use for lactic acid production from whey. *Food Science and Technology*, *40*, 1021–1028. https://doi.org/10.1590/fst.29619

Mekki, A., Dhouib, A., & Sayadi, S. (2013). Review: Effects of olive mill wastewater application on soil properties and plants growth. *International Journal Of Recycling of Organic Waste in Agriculture*, *2*(1), 15. https://doi.org/10.1186/2251-7715-2-15

Meziani, M., Arhab, R., Lamraoui, I., & Kaddour, I. (2023). Physicochemical and microbiological characterization of olive oil mill wastewater (OMWW) from Algerian Sahara, region of Ghardaia. *Journal of Biological Studies*, *5*(6), Article 6. https://doi.org/10.62400/jbs.v5i6.7585

Mihajlovski, K. R., Radovanović, N. R., Veljović, Đ. N., Šiler-Marinković, S. S., & Dimitrijević-Branković, S. I. (2016). Improved β-amylase production on molasses and sugar beet pulp by a novel strain *Paenibacillus chitinolyticus* CKS1. *Industrial Crops and Products*, *80*, 115–122. https://doi.org/10.1016/j.indcrop.2015.11.025

Mikucka, W., & Zielińska, M. (2020). Distillery Stillage: Characteristics, Treatment, and Valorization. *Applied Biochemistry and Biotechnology*, *192*(3), 770–793. https://doi.org/10.1007/s12010-020-03343-5

Mirsalami, S. M., Mirsalami, M., & Ghodousian, A. (2024). Techniques for immobilizing enzymes to create durable and effective biocatalysts. *Results in Chemistry*, *7*, 101486. https://doi.org/10.1016/j.rechem.2024.101486

Mizrahi, I., Wallace, R. J., & Moraïs, S. (2021). The rumen microbiome: Balancing food security and environmental impacts. *Nature Reviews Microbiology*, *19*(9), 553–566. https://doi.org/10.1038/s41579-021-00543-6

Mohawesh, O., Al-Hamaiedeh, H., Alqaraleh, S., Haddadin, M., Almajali, D., & Bawalize, A. (2017). *Effect of olive mill wastewater (OMW) application on soil properties and plant growth performance under rain-fed conditions*.

Mollea, C., Marmo, L., Bosco, F., Mollea, C., Marmo, L., & Bosco, F. (2013). Valorisation of Cheese Whey, a By-Product from the Dairy Industry. In *Food Industry*. IntechOpen. https://doi.org/10.5772/53159

Mordenti, A. L., Giaretta, E., Campidonico, L., Parazza, P., & Formigoni, A. (2021). A Review Regarding the Use of Molasses in Animal Nutrition. *Animals : An Open Access Journal from MDPI*, *11*(1), 115. https://doi.org/10.3390/ani11010115

Muñoz-Palazon, B., Gorrasi, S., Rosa-Masegosa, A., Pasqualetti, M., Braconcini, M., & Fenice, M. (2022). Treatment of High-Polyphenol-Content Waters Using Biotechnological Approaches: The Latest Update. *Molecules*, *28*(1), 314. https://doi.org/10.3390/molecules28010314

Najim, A. A., Radeef, A. Y., al-Doori, I., & Jabbar, Z. H. (2024). Immobilization: The promising technique to protect and increase the efficiency of microorganisms to remove contaminants. *Journal of Chemical Technology & Biotechnology*, *99*(8), 1707–1733. https://doi.org/10.1002/jctb.7638

NCBI Resource Coordinators. (2018). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*, *46*(D1), D8–D13. https://doi.org/10.1093/nar/gkx1095

Ndiaye, A., Fliss, I., & Filteau, M. (2024). High-throughput characterization of the effect of sodium chloride and potassium chloride on 31 lactic acid bacteria and their co-cultures. *Frontiers in Microbiology*, *15*, 1328416. https://doi.org/10.3389/fmicb.2024.1328416

Ndubuisi, I. A., Amadi, C. O., Nwagu, T. N., Murata, Y., & Ogbonna, J. C. (2023). Non-conventional yeast strains: Unexploited resources for effective commercialization of second generation bioethanol. *Biotechnology Advances*, *63*, 108100. https://doi.org/10.1016/j.biotechadv.2023.108100

Nguyen, S. T. C., Freund, H. L., Kasanjian, J., & Berlemont, R. (2018). Function, distribution, and annotation of characterized cellulases, xylanases, and chitinases from CAZy. *Applied Microbiology and Biotechnology*, *102*(4), 1629–1637. https://doi.org/10.1007/s00253-018-8778-y

Nikolaou, A., & Kourkoutas, Y. (2018). Exploitation of olive oil mill wastewaters and molasses for ethanol production using immobilized cells of Saccharomyces cerevisiae. *Environmental Science and Pollution Research International*, *25*(8), 7401–7408. https://doi.org/10.1007/s11356-017-1051-6

Ntougias, S., Bourtzis, K., & Tsiamis, G. (2013). The Microbiology of Olive Mill Wastes. *BioMed Research International*, *2013*(1), 784591. https://doi.org/10.1155/2013/784591

Oda, Y., & Nakamura, K. (2009). Production of ethanol from the mixture of beet molasses and cheese whey by a 2-deoxyglucose-resistant mutant of Kluyveromyces marxianus. *FEMS Yeast Research*, *9*(5), 742–748. https://doi.org/10.1111/j.1567-1364.2009.00519.x

Ohstrom, A. M., Buck, A. E., Du, X., & Wee, J. (2023). Evaluation of Kluyveromyces spp. For conversion of lactose in different types of whey from dairy processing waste into ethanol. *Frontiers in Microbiology*, *14*, 1208284. https://doi.org/10.3389/fmicb.2023.1208284

Ozi, F. Z., Boutaleb, N., Hadidi, M., Bahlaouan, B., Bennani, M., Silkina, A., & El Antri, S. (2023). Production of bio-fertilizer by biotransformation of poultry waste enriched with molasses and algae. *Environmental Quality Management*, *32*(3), 123–134. https://doi.org/10.1002/tqem.21868

Pais-Chanfrau, J. M., Acosta, L. D. C., Cóndor, P. M. A., Pérez, J. N., Guerrero, M. J. C., Pais-Chanfrau, J. M., Acosta, L. D. C., Cóndor, P. M. A., Pérez, J. N., & Guerrero, M. J. C. (2018). Small-Scale Process for the Production of Kefiran through Culture Optimization by Use of Central Composite Design from Whey and Kefir Granules. In *Current Topics in Biochemical Engineering*. IntechOpen. https://doi.org/10.5772/intechopen.82257

Parapouli, M., Vasileiadis, A., Afendra, A.-S., & Hatziloukas, E. (2020). Saccharomyces cerevisiae and its industrial applications. *AIMS Microbiology*, *6*(1), 1–31. https://doi.org/10.3934/microbiol.2020001

Park, S. A., Bhatia ,Shashi Kant, Park ,Hyun A., Kim ,Seo Yeong, Sudheer ,Pamidimarri D. V. N., Yang ,Yung-Hun, & and Choi, K.-Y. (2021). Bacillus subtilis as a robust host for biochemical production utilizing biomass. *Critical Reviews in Biotechnology*, *41*(6), 827–848. https://doi.org/10.1080/07388551.2021.1888069

Pasotti, L., Zucca, S., Casanova, M., Micoli, G., Cusella De Angelis, M. G., & Magni, P. (2017). Fermentation of lactose to ethanol in cheese whey permeate and concentrated permeate by engineered Escherichia coli. *BMC Biotechnology*, *17*(1), 48. https://doi.org/10.1186/s12896-017-0369-y

Peinemann, J. C., & Pleissner, D. (2020). Continuous pretreatment, hydrolysis, and fermentation of organic residues for the production of biochemicals. *Bioresource Technology*, *295*, 122256. https://doi.org/10.1016/j.biortech.2019.122256

Perruca Foncillas, R., Sanchis Sebastiá, M., Wallberg, O., Carlquist, M., & Gorwa-Grauslund, M. F. (2023). Assessment of the TRX2p-yEGFP Biosensor to Monitor the Redox Response of an Industrial Xylose-Fermenting Saccharomyces cerevisiae Strain during Propagation and Fermentation. *Journal of Fungi*, *9*(6), Article 6. https://doi.org/10.3390/jof9060630

Perwez, M., & Al Asheh, S. (2025). Valorization of agro-industrial waste through solid-state fermentation: Mini review. *Biotechnology Reports*, *45*, e00873. https://doi.org/10.1016/j.btre.2024.e00873

Prabhakar, T., Giaretta, J., Zulli, R., Rath, R. J., Farajikhah, S., Talebian, S., & Dehghani, F. (2025). Covalent immobilization: A review from an enzyme perspective. *Chemical Engineering Journal*, *503*, 158054. https://doi.org/10.1016/j.cej.2024.158054

Qiu, X., Zhang, Y., & Hong, H. (2021). Classification of acetic acid bacteria and their acid resistant mechanism. *AMB Express*, *11*(1), 29. https://doi.org/10.1186/s13568-021-01189-6

Rajlakshmi, Jadhav, D. A., Dutta, S., Sherpa, K. C., Jayaswal, K., Saravanabhupathy, S., Mohanty, K. T., Banerjee, R., Kumar, J., & Rajak, R. C. (2023). Chapter 7 - Co-digestion processes of waste: Status and perspective. In C. M. Hussain, A. Kushwaha, R. N. Bharagava, & L. Goswami (Eds.), *Bio-Based Materials and Waste for Energy Generation and Resource Management* (Vol. 5, pp. 207–241). Elsevier. https://doi.org/10.1016/B978-0-323-91149-8.00010-7

Ranaei, V., Pilevar, Z., Khaneghah, A. M., & Hosseini, H. (2020). Propionic Acid: Method of Production, Current State and Perspectives. *Food Technology and Biotechnology*, *58*(2), 115–127. https://doi.org/10.17113/ftb.58.02.20.6356

Reiner, K. (2012, November 1). *Carbohydrate Fermentation Protocol*. https://www.semanticscholar.org/paper/Carbohydrate-Fermentation-Protocol-Reiner/414d14a25b14272cce9540a1de0ca295466a701e

Rouam, D., & Meziane, M. (2025). Valorization of olive mill wastewater for acetic acid production by Bacillus strains isolated from bovine rumen. *Environmental and Experimental Biology*, *23*, 19–24. https://doi.org/10.22364/eeb.23.03

Rubio, J. A., Romero, L. I., Wilkie, A. C., & García-Morales, J. L. (2019). Mesophilic Anaerobic Co-digestion of Olive-Mill Waste With Cattle Manure: Effects of Mixture Ratio. *Frontiers in Sustainable Food Systems*, *3*. https://doi.org/10.3389/fsufs.2019.00009

Russo, E., Spallarossa, A., Comite, A., Pagliero, M., Guida, P., Belotti, V., Caviglia, D., & Schito, A. M. (2022). Valorization and Potential Antimicrobial Use of Olive Mill Wastewater (OMW) from Italian Olive Oil Production. *Antioxidants*, *11*(5), Article 5. https://doi.org/10.3390/antiox11050903

Russo-Martínez, N., Vecino, X., Moldes, A. B., & Cruz, J. M. (2025). Enhancing spontaneous biosurfactant production through water steeping of olive mill waste. *Scientific Reports*, *15*(1), 7676. https://doi.org/10.1038/s41598-025-92040-6

Ryan, M. P., & Walsh, G. (2016). The biotechnological potential of whey. *Reviews in Environmental Science and Bio/Technology*, *15*(3), 479–498. https://doi.org/10.1007/s11157-016-9402-1

Saavedra, S., Alejandro-Paredes, L., Flores-Santos, J. C., Flores-Fernández, C. N., Arellano-García, H., Zavaleta, A. I., Saavedra, S., Alejandro-Paredes, L., Flores-Santos, J. C., Flores-Fernández, C. N., Arellano-García, H., & Zavaleta, A. I. (2021). Optimization of lactic acid production by Lactobacillus plantarum strain Hui1 in a medium containing sugar cane molasses. *Agronomía Colombiana*, *39*(1), 98–107. https://doi.org/10.15446/agron.colomb.v39n1.89674

Sadh, P. K., Kumar, S., Chawla, P., & Duhan, J. S. (2018). Fermentation: A Boon for Production of Bioactive Compounds by Processing of Food Industries Wastes (By-Products). *Molecules*, *23*(10), Article 10. https://doi.org/10.3390/molecules23102560

Sahoo, P. K., Datta, R., Rahman, M. M., & Sarkar, D. (2024). Sustainable Environmental Technologies: Recent Development, Opportunities, and Key Challenges. *Applied Sciences*, *14*(23), Article 23. https://doi.org/10.3390/app142310956

Salangsang, M. C., Sekine, M., Akizuki, S.-I., Sakai, H., Kurosawa, N., & Toda, T. (2022). Effect of carbon to nitrogen ratio of food waste and short resting period on microbial accumulation during anaerobic digestion. *Biomass and Bioenergy*, *162*, 106481. https://doi.org/10.1016/j.biombioe.2022.106481

Sandoval-Salas, F., Méndez-Carreto, C., Barrales-Fernández, C., & Ortega-Avila, G. (2021). Bioethanol production of cheese whey in continuous culture. *Revista de La Invención Técnica*, 22–25. https://doi.org/10.35429/JOTI.2021.15.5.22.25

Saqib, S., Akram, A., Halim, S. A., & Tassaduq, R. (2017a). Sources of β-galactosidase and its applications in food industry. *3 Biotech*, *7*(1), 79. https://doi.org/10.1007/s13205-017-0645-5

Saqib, S., Akram, A., Halim, S. A., & Tassaduq, R. (2017b). Sources of β-galactosidase and its applications in food industry. *3 Biotech*, *7*(1), 79. https://doi.org/10.1007/s13205-017-0645-5

Sar, T., & Akbas, M. (2023). Antimicrobial Activities of Olive Oil Mill Wastewater Extracts against Selected Microorganisms. *Sustainability*, *15*, 8179. https://doi.org/10.3390/su15108179

Sarangi, P. K., & Bhatia, L. (Eds.). (2022). *Biotechnology for Waste Biomass Utilization*. Apple Academic Press. https://doi.org/10.1201/9781003277187

Saravanan, A., Senthil Kumar, P., Rangasamy, G., Hariharan, R., Hemavathy, R. V., Deepika, P. D., Anand, K., & Karthika, S. (2023). Strategies for enhancing the efficacy of anaerobic digestion of food industry wastewater: An insight into bioreactor types, challenges, and future scope. *Chemosphere*, *310*, 136856. https://doi.org/10.1016/j.chemosphere.2022.136856

Sarris, D., Giannakis, M., Philippoussis, A., Komaitis, M., Koutinas, A. A., & Papanikolaou, S. (2013). Conversions of olive mill wastewater-based media by Saccharomyces cerevisiae through sterile and non-sterile bioprocesses. *Journal of Chemical Technology & Biotechnology*, *88*(5), 958–969. https://doi.org/10.1002/jctb.3931

Seferlis, P. (2008). 13—Measurement and process control for water and energy use in the food industry. In J. Klemeš, R. Smith, & J.-K. Kim (Eds.), *Handbook of Water and Energy Management in Food Processing* (pp. 387–418). Woodhead Publishing. https://doi.org/10.1533/9781845694678.3.367

Senanayake, D., Torley, P. J., Chandrapala, J., & Terefe, N. S. (2023). Microbial Fermentation for Improving the Sensory, Nutritional and Functional Attributes of Legumes. *Fermentation*, *9*(7), Article 7. https://doi.org/10.3390/fermentation9070635

Serin, B., Akcan, N., & Uyar, F. (2012). Production and Optimization of α-Amylase from Bacillus circulans ATCC 4516 with Solid State Fermentation . *Hacettepe Journal of Biology and Chemistry*, *40*(4), Article 4.

Shaghaghi-Moghaddam, R., Jafarizadeh-Malmiri, H., Mehdikhani, P., Jalalian, S., & Alijanianzadeh, R. (2018). Screening of the five different wild, traditional and industrial Saccharomyces cerevisiae strains to overproduce bioethanol in the batch submerged fermentation. *Zeitschrift Für Naturforschung C*, *73*(9–10), 361–366. https://doi.org/10.1515/znc-2017-0180

Shah, F., Ranawat, B., & Mishra, S. (2021). Chapter 3 - Marine fungal biocatalysts: The bionomical standpoint. In V. K. Sharma, M. P. Shah, S. Parmar, & A. Kumar (Eds.), *Fungi Bio-Prospects in Sustainable Agriculture, Environment and Nano-technology* (pp. 45–67). Academic Press. https://doi.org/10.1016/B978-0-12-821925-6.00003-4

Shleeva, M. O., Kondratieva, D. A., & Kaprelyants, A. S. (2023). Bacillus licheniformis: A Producer of Antimicrobial Substances, including Antimycobacterials, Which Are Feasible for Medical Applications. *Pharmaceutics*, *15*(7), 1893. https://doi.org/10.3390/pharmaceutics15071893

Shrivastava, M., Maibam, P. D., Aishwarya, A., & Goyal, A. (2020). Hemicellulases and Auxiliary Activities for Biomass Hydrolysis. In V. Bisaria (Ed.), *Handbook of Biorefinery Research and Technology* (pp. 1–23). Springer Netherlands. https://doi.org/10.1007/978-94-007-6724-9_36-1

Siciliano, A., Stillitano, M. A., & De Rosa, S. (2015). Increase of the anaerobic biodegradability of olive mill wastewaters through a pre-treatment with hydrogen peroxide in alkaline conditions. *Desalination and Water Treatment*, *55*(7), 1735–1746. https://doi.org/10.1080/19443994.2014.928797

Simair, A. A., Qureshi, A. S., Khushk, I., Ali, C. H., Lashari, S., Bhutto, M. A., Mangrio, G. S., & Lu, C. (2017). Production and Partial Characterization of α-Amylase Enzyme from Bacillus sp. BCC 01-50 and Potential Applications. *BioMed Research International*, *2017*, 9173040. https://doi.org/10.1155/2017/9173040

Singh, A., Dikshit, R., & Singh, N. (2023). Valorization of Agro-Industrial Wastes for Biorefinery Products. In A. S. Mathuriya, S. Pandit, & N. K. Singh (Eds.), *Green Technologies for Industrial Waste Remediation* (pp. 101–123). Springer Nature Switzerland. https://doi.org/10.1007/978-3-031-46858-2_6

Sinigaglia, M., Di Benedetto, N., Bevilacqua, A., Corbo, M. R., Capece, A., & Romano, P. (2010). Yeasts isolated from olive mill wastewaters from southern Italy: Technological characterization and potential use for phenol removal. *Applied Microbiology and Biotechnology*, *87*(6), 2345–2354. https://doi.org/10.1007/s00253-010-2684-2

Sivasubramanian, V. (Ed.). (2018). *Bioprocess Engineering for a Green Environment*. CRC Press. https://doi.org/10.1201/b22021

Smeti, E., Kalogianni, E., Karaouzas, I., Laschou, S., Tornés, E., De Castro-Català, N., Anastasopoulou, E., Koutsodimou, M., Andriopoulou, A., Vardakas, L., Muñoz, I., Sabater, S., & Skoulikidis,

N. Th. (2019). Effects of olive mill wastewater discharge on benthic biota in Mediterranean streams. *Environmental Pollution*, *254*, 113057. https://doi.org/10.1016/j.envpol.2019.113057

Soccol, C. R., Costa, E. S. F. da, Letti, L. A. J., Karp, S. G., Woiciechowski, A. L., & Vandenberghe, L. P. de S. (2017). Recent developments and innovations in solid state fermentation. *Biotechnology Research and Innovation*, *1*(1), 52–71. https://doi.org/10.1016/j.biori.2017.01.002

Sode, F. (2014). Simultaneous determination of peracetic acid and acetic acid by titration with NaOH. *Analytical Methods*, *6*(7), 2406–2409. https://doi.org/10.1039/C3AY41836G

Srianta, I., Kusdiyantini, E., Zubaidah, E., Ristiarini, S., Nugerahani, I., Alvin, A., Iswanto, N., & Zhang, B.-B. (2021). Utilization of agro-industrial by-products in Monascus fermentation: A review. *Bioresources and Bioprocessing*, *8*(1), 129. https://doi.org/10.1186/s40643-021-00473-4

Stanzer, D., Hanousek Čiča, K., Blesić, M., Smajić Murtić, M., Mrvčić, J., & Spaho, N. (2023). Alcoholic Fermentation as a Source of Congeners in Fruit Spirits. *Foods*, *12*(10), Article 10. https://doi.org/10.3390/foods12101951

Stikane, A., Dace, E., & Stalidzans, E. (2022). Closing the loop in bioproduction: Spent microbial biomass as a resource within circular bioeconomy. *New Biotechnology*, *70*, 109–115. https://doi.org/10.1016/j.nbt.2022.06.001

Takacs, F., Brunner, D., & Frankenberger, K. (2022). Barriers to a circular economy in small- and medium-sized enterprises and their integration in a sustainable strategic management framework. *Journal of Cleaner Production*, *362*, 132227. https://doi.org/10.1016/j.jclepro.2022.132227

Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Review: Diversity of Microorganisms in Global Fermented Foods and Beverages. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00377

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*(3), 512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Tan, Y. N., & Li, Q. (2018). Microbial production of rhamnolipids using sugars as carbon sources. *Microbial Cell Factories*, *17*(1), 89. https://doi.org/10.1186/s12934-018-0938-3

Tebbouche, L., Aziza ,Majda, Abada ,Sabah, Chergui ,Abdelmalek, Amrane ,Abdeltif, & and Hellal, A. (2024). Bioethanol production from deproteinized cheese whey powder by local strain isolated from soured milk: Influence of operating parameters. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, *46*(1), 397–408. https://doi.org/10.1080/15567036.2023.2284851

Tesfaw, A. (2023). The current trends of bioethanol production from cheese whey using yeasts: Biological and economical perspectives. *Frontiers in Energy Research*, *11*. https://doi.org/10.3389/fenrg.2023.1183035

Tesfaw, A., Oner, E. T., & Assefa, F. (2021). Evaluating crude whey for bioethanol production using non-Saccharomyces yeast, Kluyveromyces marxianus. *SN Applied Sciences*, *3*(1), 42. https://doi.org/10.1007/s42452-020-03996-1

Tsermoula, P., Khakimov, B., Nielsen, J. H., & Engelsen, S. B. (2021). WHEY - The waste-stream that became more valuable than the food product. *Trends in Food Science & Technology*, *118*, 230–241. https://doi.org/10.1016/j.tifs.2021.08.025

Ugwu, S. N., Biscoff, R. K., & Enweremadu, C. C. (2020). A meta-analysis of iron-based additives on enhancements of biogas yields during anaerobic digestion of organic wastes. *Journal of Cleaner Production*, *269*, 122449. https://doi.org/10.1016/j.jclepro.2020.122449

Usmani, Z., Sharma, M., Sudheer, S., Gupta, V. K., & Bhat, R. (2020). Engineered Microbes for Pigment Production Using Waste Biomass. *Current Genomics*, *21*(2), 80–95. https://doi.org/10.2174/1389202921999200330152007

Utama, G. L., Kurnani, T. B. A., Sunardi, & Balia, R. L. (2017). Reducing Cheese-Making by-Product Disposal Through Ethanol Fermentation and the Utilization of Distillery Waste for Fertilizer. *GEOMATE Journal*, *13*(37), Article 37.

Valeriano, I. H., Marques, G. L. L., Freitas, S. P., Couri, S., Penha, E. das M., & Gonçalves, M. M. M. (2018). Cassava Pulp Enzymatic Hydrolysate as a Promising Feedstock for Ethanol Production.

Brazilian Archives of Biology and Technology, *61*, e18161214. https://doi.org/10.1590/1678-4324-2018161214

Vasić, K., Knez, Ž., & Leitgeb, M. (2021). Bioethanol Production by Enzymatic Hydrolysis from Different Lignocellulosic Sources. *Molecules*, *26*(3), Article 3. https://doi.org/10.3390/molecules26030753

Vavouraki, A., Zakoura, M., Dareioti, M., & Kornaros, M. (2020). Biodegradation of Polyphenolic Compounds from Olive Mill Wastewaters (OMW) During Two-Stage Anaerobic Co-digestion of Agro-industrial Mixtures. *Waste and Biomass Valorization*, *11*. https://doi.org/10.1007/s12649-019-00887-4

Veana, F., Martínez-Hernández, J. L., Aguilar, C. N., Rodríguez-Herrera, R., & Michelena, G. (2014). Utilization of molasses and sugar cane bagasse for production of fungal invertase in solid state fermentation using Aspergillus niger GH1. *Brazilian Journal of Microbiology*, *45*(2), 373–377.

Verma, A. K., Raghukumar, C., & Naik, C. G. (2011). A novel hybrid technology for remediation of molasses-based raw effluents. *Bioresource Technology*, *102*(3), 2411–2418. https://doi.org/10.1016/j.biortech.2010.10.112

Vicentini-Polette, C., Gallo, A., SILVA, J., Bernardi, A., Borges, M. T., & Verruma-Bernardi, M. (2024). Minerals levels in sugarcane syrup. *Food Science and Technology*, *44*. https://doi.org/10.5327/fst.00263

Villarreal, P., Quintrel, P. A., Olivares-Muñoz, S., Ruiz, J. J., Nespolo, R. F., & Cubillos, F. A. (2022). Identification of new ethanol-tolerant yeast strains with fermentation potential from central Patagonia. *Yeast*, *39*(1–2), 128–140. https://doi.org/10.1002/yea.3662

Wagh, M. S., S, S., Nath, P. C., Chakraborty, A., Amrit, R., Mishra, B., Mishra, A. K., & Mohanta, Y. K. (2024). Valorisation of agro-industrial wastes: Circular bioeconomy and biorefinery process – A sustainable symphony. *Process Safety and Environmental Protection*, *183*, 708–725. https://doi.org/10.1016/j.psep.2024.01.055

Walker, G. (2004). Metals in Yeast Fermentation Processes. *Advances in Applied Microbiology*, *54*, 197–229. https://doi.org/10.1016/S0065-2164(04)54008-X

Wang, J., Hu, X., Zhao, Y., Li, X., Zhao, P., & Guo, Y. (2024). Effects of molasses-based microbial dust suppressant on soil dust and microbial community. *Powder Technology*, *441*, 119831. https://doi.org/10.1016/j.powtec.2024.119831

Wardani, A. K., Utami, C. P., Hermanto, M. B., Sutrisno, A., & Nurtyastuti, F. (2023a). Bioethanol Production from Sugarcane Molasses by Fed-Batch Fermentation Systems Using Instant Dry Yeast. *Microbiology and Biotechnology Letters*, *51*(2), 184–190. https://doi.org/10.48022/mbl.2301.01012

Wardani, A. K., Utami, C. P., Hermanto, M. B., Sutrisno, A., & Nurtyastuti, F. (2023b). *Bioethanol Production from Sugarcane Molasses by Fed-Batch Fermentation Systems Using Instant Dry Yeast*. *51*(2), 184–190. https://doi.org/10.48022/mbl.2301.01012

Waterborg, J. H., & Matthews, H. R. (1984). The Lowry Method for Protein Quantitation. In J. M. Walker (Ed.), *Proteins* (pp. 1–3). Humana Press. https://doi.org/10.1385/0-89603-062-8:1

Wechgama, K., Laopaiboon, L., & Laopaiboon, P. (2017). Enhancement of batch butanol production from sugarcane molasses using nitrogen supplementation integrated with gas stripping for product recovery. *Industrial Crops and Products*, *95*, 216–226. https://doi.org/10.1016/j.indcrop.2016.10.012

Willaert, R. (2011). *Cell Immobilization and Its Applications in Biotechnology* (pp. 313–367). https://doi.org/10.1201/b11490-13

Xue, W., Sheng, Y., Tai, Y., Ding, J., He, R., Jiang, Y., Zhong, B., He, J., Ren, A., & He, Q. (2025). Mechanism of improved cellulase production by *Trichoderma reesei* in water-supply solid-state fermentation. *Bioresource Technology*, *419*, 132017. https://doi.org/10.1016/j.biortech.2024.132017

Yafetto, L. (2022). Application of solid-state fermentation by microbial biotechnology for bioprocessing of agro-industrial wastes from 1970 to 2020: A review and bibliometric analysis. *Heliyon*, *8*(3), e09173. https://doi.org/10.1016/j.heliyon.2022.e09173

Yamada, S., Shinomiya, N., Ohba, K., Sekikawa, M., & Oda, Y. (2009). Enzymatic Hydrolysis and Ethanol Fermentation of By-Products from Potato Processing Plants. *Food Science and Technology Research*, *15*(6), 653–658. https://doi.org/10.3136/fstr.15.653

Yang, X., Wang, K., Wang, H., Zhang, J., Tang, L., & Mao, Z. (2016). Control of pH by acetic acid and its effect on ethanol fermentation in an integrated ethanol–methane fermentation process. *RSC Advances*, *6*(63), 57902–57909. https://doi.org/10.1039/C6RA04129A

Yoshimoto, S., Ohara, Y., Nakatani, H., & Hori, K. (2017). Reversible bacterial immobilization based on the salt-dependent adhesion of the bacterionanofiber protein AtaA. *Microbial Cell Factories*, *16*, 123. https://doi.org/10.1186/s12934-017-0740-7

Yu, Y., Wu, J., Ren, X., Lau, A., Rezaei, H., Takada, M., Bi, X., & Sokhansanj, S. (2022). Steam explosion of lignocellulosic biomass for multiple advanced bioenergy processes: A review. *Renewable and Sustainable Energy Reviews*, *154*, 111871. https://doi.org/10.1016/j.rser.2021.111871

Zahra El Hassani, F., El Karkouri, A., Errachidi, F., Merzouki, M., & Benlemlih, M. (2023). The impact of Olive Mill Wastewater spreading on soil and plant in arid and semi-arid areas. *Environmental Nanotechnology, Monitoring & Management*, *20*, 100798. https://doi.org/10.1016/j.enmm.2023.100798

Zandona, E., Blažić, M., & Režek Jambrak, A. (2021). Whey Utilization: Sustainable Uses and Environmental Approach. *Food Technology and Biotechnology*, *59*(2), 147–161. https://doi.org/10.17113/ftb.59.02.21.6968

Zdarta, J., Meyer, A. S., Jesionowski, T., & Pinelo, M. (2018). A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility. *Catalysts*, *8*(2), Article 2. https://doi.org/10.3390/catal8020092

Zhang, D., Hegab, H. E., Lvov, Y., Dale Snow, L., & Palmer, J. (2016). Immobilization of cellulase on a silica gel substrate modified using a 3-APTES self-assembled monolayer. *SpringerPlus*, *5*(1), 48. https://doi.org/10.1186/s40064-016-1682-y

Zhang, H., Zhang, P., Wu, T., & Ruan, H. (2023). Bioethanol Production Based on Saccharomyces cerevisiae: Opportunities and Challenges. *Fermentation*, *9*, 709. https://doi.org/10.3390/fermentation9080709

Zhang, P., Hai, H., Sun, D., Yuan, W., Liu, W., Ding, R., Teng, M., Ma, L., Tian, J., & Chen, C. (2019). A high throughput method for total alcohol determination in fermentation broths. *BMC Biotechnology*, *19*(1), 30. https://doi.org/10.1186/s12896-019-0525-7

Zhang, S., Wang, J., & Jiang, H. (2021). Microbial production of value-added bioproducts and enzymes from molasses, a by-product of sugar industry. *Food Chemistry*, *346*, 128860. https://doi.org/10.1016/j.foodchem.2020.128860

Zhang, W., Kong, T., Xing, W., Li, R., Yang, T., Yao, N., & Lv, D. (2022). Links between carbon/nitrogen ratio, synergy and microbial characteristics of long-term semi-continuous anaerobic co-digestion of food waste, cattle manure and corn straw. *Bioresource Technology*, *343*, 126094. https://doi.org/10.1016/j.biortech.2021.126094

Zucca, P., & Sanjust, E. (2014). Inorganic Materials as Supports for Covalent Enzyme Immobilization: Methods and Mechanisms. *Molecules*, *19*(9), 14139–14194. https://doi.org/10.3390/molecules190914139

Appendices

Appendix 1: API 50CHB Biochemical Test Results

The API 50 CHB/E test (bioMérieux, France) was employed to assess the carbohydrate assimilation profile of the isolated *Bacillus* strains. The test strip contains 50 different carbohydrate substrates and was interpreted according to the manufacturer's instructions. The yeast suspension was prepared in API 50 CHB/E medium and incubated at 30 °C for 48–72 hours. Positive reactions were determined based on turbidity and color change**.**

Composition of the Strip

The composition of the API® 50 CH strip is given below in the list of tests:

Strip 0-9

TUBE	TESTS	ACTIVE INGREDIENTS	STRAIN 13	STRAIN 14	STRAIN 15	STRAIN 19	STRAIN 24
0		Control	-	-	-	-	-
1	GLY	Glycerol	+	+	+	+	+
2	ERY	Erythritol	-	+	-	-	+
3	DARA	D-Arabinose	+	+	+	+	+
4	LARA	L-Arabinose	+	+	+	+	+
5	RIB	D-Ribose	+	+	+	+	+
6	DXYL	D-Xylose	+	+	+	+	+
7	LXYL	L-Xylose	-	+	+	-	+
8	ADO	D-Adonitol	+	+	+	+	+
9	MDX	Methyl-ß-D-xylopyranoside	+	+	-	-	+

Strip 10-19

TUBE	TESTS	ACTIVE INGREDIENTS	STRAIN 13	STRAIN 14	STRAIN 15	STRAIN 19	STRAIN 24
10	GAL	D-Galactose	+	+	+	+	+
11	GLU	D-Glucose	+	+	+	+	+
12	FRU	D-Fructose	+	+	+	+	+
13	MNE	D-Mannose	+	+	+	+	+
14	SBE	L-Sorbose	+	+	+	+	+
15	RHA	L-Rhamnose	+	+	+	+	+
16	DUL	Dulcitol	+	+	+	+	+
17	INO	Inositol	+	+	+	+	+
18	MAN	D-Mannitol	+	+	+	+	+
19	SOR	D-Sorbitol	+	+	+	+	+

Strip 20-29

TUBE	TESTS	ACTIVE INGREDIENTS	STRAIN 13	STRAIN 14	STRAIN 15	STRAIN 19	STRAIN 24
20	MDM	Methyl-α-D-mannopyranoside	-	-	-	-	-
21	MDG	Methyl-α-D-glucopyranoside	+	+	+	+	+
22	NAG	N-Acetylglucosamine	-	+	+	-	+
23	AMY	Amygdalin	-	+	-	-	+
24	ARB	Arbutin	-	+	-	-	+
25	ESC	Esculin Ferric citrate	+	+	+	+	+
26	SAL	Salicin	+	+	+	+	+
27	CEL	D-Cellobiose	+	+	+	+	+
28	MAL	D-Maltose	+	+	+	+	+
29	LAC	D-Lactose (bovine origin)	+	+	+	+	+

Strip 30-39

TUBE	TESTS	ACTIVE INGREDIENTS	STRAIN 13	STRAIN 14	STRAIN 15	STRAIN 19	STRAIN 24
30	MEL	D-Melibiose	+	+	+	+	+
31	SAC	D-Saccharose (sucrose)	+	+	+	+	+
32	TRE	D-Trehalose	+	+	+	+	+
33	INU	Inulin	-	-	-	-	-
34	MLZ	D-Melezitose	-	+	+	-	+
35	RAF	D-Raffinose	+	+	+	+	+
36	AMD	Starch (amidon)	+	+	+	+	+
37	GLYG	Glycogen	-	-	-	-	-
38	XLT	Xylitol	-	-	-	-	-
39	GEN	Gentiobiose	-	-	-	-	-

Strip 40-49

TUBE	TESTS	ACTIVE INGREDIENTS	STRAIN 13	STRAIN 14	STRAIN 15	STRAIN 19	STRAIN 24
40	TUR	D-Turanose	-	-	-	-	-
41	LYX	D-Lyxose	-	-	-	-	-
42	TAG	D-Tagatose	-	+	+	+	+
43	DFUC	D-Fucose	-	+	-	-	+
44	LFUC	L-Fucose	+	+	+	+	+
45	DARL	D-Arabitol	+	+	+	+	+

46	LARL	L-Arabitol	+	+	+	+	-
47	GNT	Potassium gluconate	-	-	-	-	-
48	2KG	Potassium 2-ketogluconate	-	-	-	-	-
49	5KG	Potassium 5-ketogluconate	-	-	-	-	-

The reading of these reactions is done using a reading table, and the identification is obtained through the identification table. The identification is carried out using the API WEB software

Appendix 2: Identification of the 16S rRNA sequences of strain *Saccharomyces cerevisiae* Y17

Saccharomyces cerevisiae isolate Y17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: PQ566690.1

FASTA Graphics

Go to:

```
LOCUS       PQ566690                814 bp    DNA     linear   PLN 01-FEB-2025
DEFINITION  Saccharomyces cerevisiae isolate Y17 internal transcribed spacer 1,
            partial sequence; 5.8S ribosomal RNA gene and internal transcribed
            spacer 2, complete sequence; and large subunit ribosomal RNA gene,
            partial sequence.
ACCESSION   PQ566690
VERSION     PQ566690.1
KEYWORDS    .
SOURCE      Saccharomyces cerevisiae (brewer's yeast)
  ORGANISM  Saccharomyces cerevisiae
            Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina;
            Saccharomycetes; Saccharomycetales; Saccharomycetaceae;
            Saccharomyces.
REFERENCE   1  (bases 1 to 814)
  AUTHORS   Rouam,D. and Mezine,M.A.
  TITLE     Screening of indigenous yeasts isolated in Algeria from olive
            wastewater
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 814)
  AUTHORS   Rouam,D. and Mezine,M.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-NOV-2024) Agriculture, Food and Environment,
            University of Catania, Santa Sofia, Catania, Catania 95123, Italy
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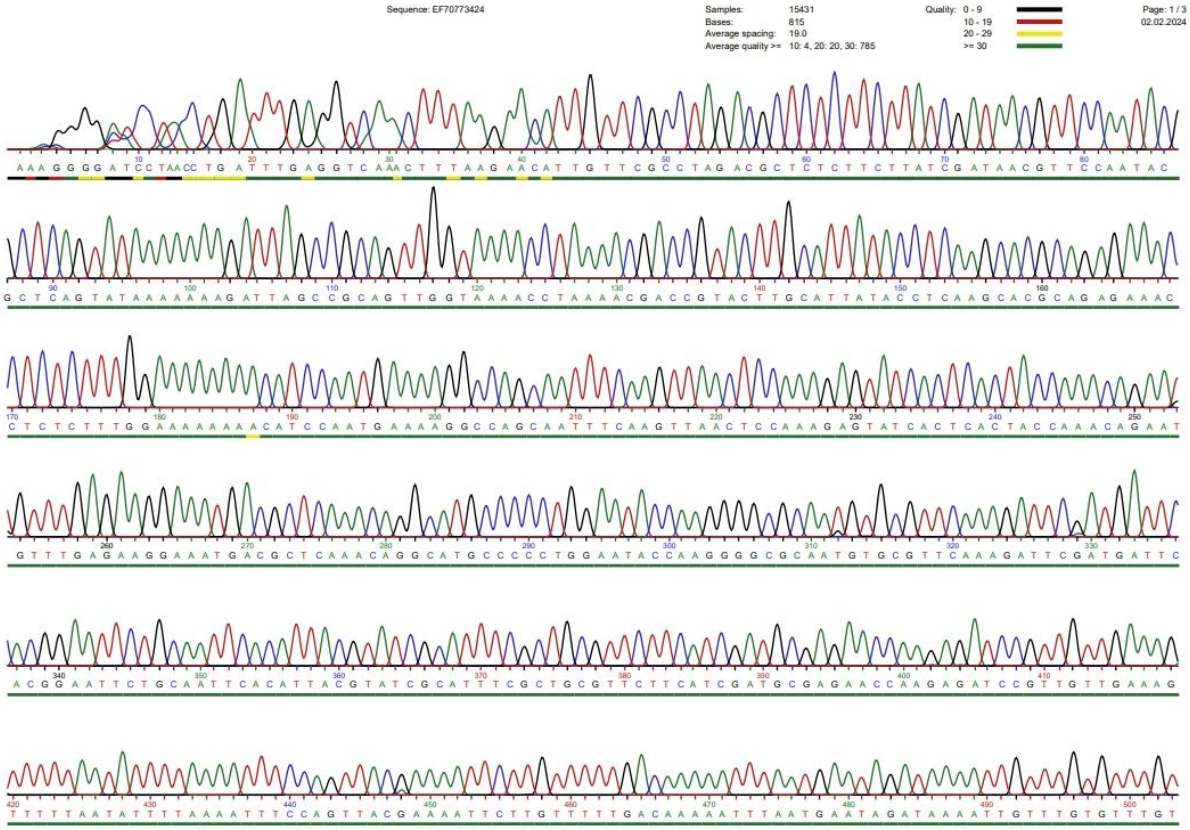
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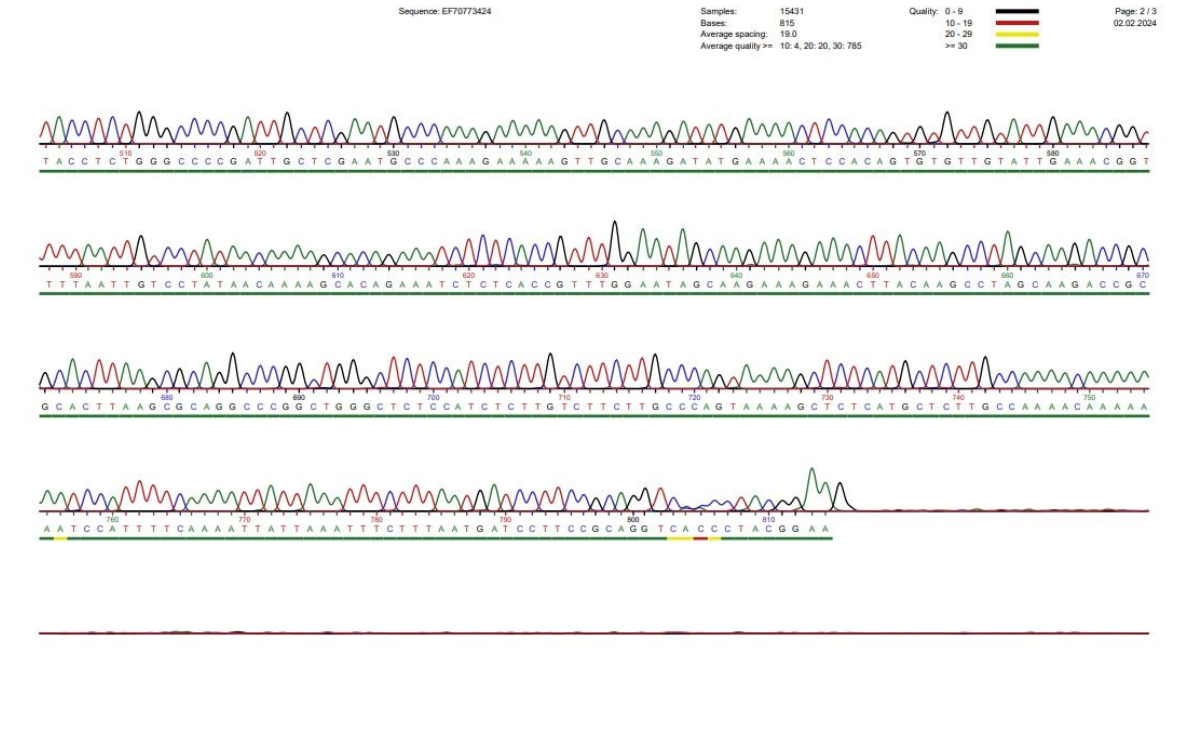
Average quality >= 10: 4, 20: 20, 30: 785
Quality: 0 - 9
10 - 19
20 - 29
>= 30
Page: 1 / 3
02.02.2024


Sequence: EF70773424
Samples: 15431
Bases: 815
Average spacing: 19.0
Average quality >= 10: 4, 20: 20, 30: 785
Quality: 0 - 9
10 - 19
20 - 29
>= 30
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Valorization of olive mill wastewater for acetic acid production by *Bacillus* strains isolated from bovine rumen

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Original Paper

Valorization of olive mill wastewater for acetic acid production by *Bacillus* strains isolated from bovine rumen

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Abstract

The valorization of olive mill wastewater (OMW) through microbial fermentation presents an innovative approach to addressing environmental challenges associated with olive oil production. This study aimed to investigate the potential of *Bacillus* strains isolated from bovine rumen for acetic acid production using olive oil mill wastewater as the primary substrate. Physicochemical analyses revealed high organic load (chemical oxygen demand of 183 g O_2 L^{-1}, biological oxygen demand of 7 g O_2 L^{-1}) and acidic pH (4.5) in olive oil mill wastewater, making it suitable for microbial growth. A total of 25 bacterial strains were isolated, and preliminary screening based on biogas production identified five efficient acid-producing *Bacillus* strains. Species-level identification using the bacterial identification system confirmed the presence of *Bacillus licheniformis* and *Bacillus circulans*. Batch fermentations conducted over 120 h produced up to 14 mL of biogas per 100 mL of culture and acetic acid concentrations of 28 g L^{-1}, highlighting the strains' strong acidification capacity. This study demonstrates the feasibility of bioconverting agricultural waste into valuable bioproducts, contributing to sustainable waste management, bioenergy generation, and promoting circular economy practices.

Key words: acetic acid, *Bacillus*, bovine rumen. olive mill wastewater, wastewater valorization.
Abbreviations: BOD, biological oxygen demand; COD, chemical oxygen demand; OMW, olive mill wastewater.

Introduction

The production of olive oil generates considerable waste, particularly olive mill wastewater (OMW), which poses environmental challenges due to its high organic load and potential for pollution (Sar, Akbas 2023). OMW is characterized by high levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD), along with an acidic pH, making it a candidate for valorization through microbial fermentation (Bouharat et al. 2018).

Microbial fermentation is a crucial biochemical process that converts organic substrates into valuable products, including organic acids, which play essential roles in various industrial applications (Senanayake et al. 2023). Among these, acetic acid is particularly noteworthy due to its wide use as a preservative, flavoring agent, and chemical feedstock (Qiu et al. 2021). The rumen of ruminant animals harbors a diverse microbial community capable of fermenting fibrous plant materials into volatile fatty acids (Mizrahi et al. 2021). This unique microbial ecosystem provides an opportunity to exploit specific strains for efficient fermentation processes (Graham, Knelman 2023).

Previous research has primarily focused on *Saccharomyces cerevisiae* and acetic acid bacteria for acetic acid production, leaving room for exploration of other microbial candidates like *Bacillus* (Ntougias et al. 2013; Fronteras et al. 2021; Ayadi et al. 2022; Carmona et al. 2023; Angeloni et al. 2024). Studies on the use of OMW as a substrate have highlighted its potential for producing value-added products such as bioethanol and acetic acid, often relying on traditional microbial strains.

Therefore, this study aims to evaluate the capacity of *Bacillus* strains isolated from bovine rumen to produce acetic acid through fermentation of olive oil mill wastewater. Specifically, it focuses on identifying efficient acid-producing strains, characterizing their species, and assessing their fermentation performance. The hypothesis is that *Bacillus* strains isolated from bovine rumen can efficiently ferment OMW to produce acetic acid, offering a sustainable solution for waste management and bioenergy generation. The use of OMW as a substrate presents a dual opportunity: providing an environmentally sustainable solution for waste valorization while simultaneously producing valuable bioproducts.

Materials and methods

Sample collection and analysis

OMW samples were collected from the Nakhla oil mill located in Chlef, Algeria, during the peak olive processing season. Samples were filtered through a fine mesh to eliminate any large contaminants and autoclaved before fermentation. Samples were stored at 4 °C when not in use. Physicochemical analyses were conducted to determine pH using a digital pH meter (Hanna Instruments), while chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5) were measured following standard methods (APHA 2017b). Organic matter content was assessed through gravimetric methods by drying the samples at 105 °C for 24 h and measuring the weight loss. Acidity was determined by titration with 0.1 M NaOH using phenolphthalein as an indicator. Nitrites (NO_2^-) were quantified using the colorimetric method with Griess reagent, following the standard protocol (APHA 2017a). Total polyphenols were measured using the Folin-Ciocalteu method, as described by Russo et al. (2022). All analyses were performed in triplicate to ensure reproducibility.

Microbial isolation and screening

Bovine rumen juice was obtained from a freshly slaughtered Holstein cow at the Taiba slaughterhouse in Chlef, Algeria. The rumen contents were filtered through sterile gauze to collect the liquid fraction. Serial dilutions were prepared and spread onto de Man, Rogosa and Sharpe medium and M17 agar plates supplemented with 2% $CaCO_3$ to facilitate the identification of acid-producing colonies through the formation of clear halos. The medium composition included peptone (10 g L^{-1}), beef extract (10 g L^{-1}), yeast extract (5 g L^{-1}), glucose (20 g L^{-1}), Tween 80 (1 mL L^{-1}), ammonium citrate (2 g L^{-1}), sodium acetate (5 g L^{-1}), magnesium sulfate (0.1 g L^{-1}), manganese sulfate (0.05 g L^{-1}), and dipotassium phosphate (2 g L^{-1}). Anaerobic conditions were maintained using a 2.5 L anaerobic culture jar from Merk. Plates were incubated anaerobically at 37 °C for 48 h. A total of 25 distinct colonies were isolated and subcultured to obtain pure strains.

Primary identification and evaluation of isolated strains

Initial identification of the isolated strains was performed through a combination of morphological and biochemical tests. Microscopic observations were conducted to assess cell morphology and Gram staining characteristics. Oxidase and catalase tests were performed to evaluate enzymatic activity, aiding in preliminary strain classification.

A preliminary fermentation to evaluate the capacity of the isolated strains to ferment OMW was conducted using a mix of 25% OMW and 75% de Man, Rogosa and Sharpe medium supplemented with $CaCO_3$ as a pH buffer in 100 mL sealed bottles equipped with graduated syringes to measure the volume of biogas produced. The medium was inoculated with 5% (v/v) of a 24 h fresh culture and incubated in 37 °C for a period of five days. The strains demonstrating the highest biogas production were selected for further analysis.

Following these initial tests, the API 50 CHB/E system (bioMérieux), a biochemical assay designed to characterize carbohydrate metabolism, was employed. The system evaluates the fermentation of 49 different carbohydrates, providing a metabolic fingerprint that aids in species-level identification.

Fermentation trials for acetic acid production

To evaluate long-term acetic acid production, a separate fermentation trial was conducted over a period of 120 h. Fresh OMW medium (500 mL) was pH adjusted to the value of 6 to favour acidogenesis because methanogenesis (methane production) requires a higher pH (6.5 to 8.5) and longer incubation times as discussed previously (Liew et al. 2016). The OMW was inoculated with 5% (v/v) of *Bacillus* strains selected based on their biogas production efficiency. The flasks were incubated at 37 °C with continuous shaking at 150 rpm to ensure optimal oxygenation and nutrient distribution.

Samples were collected at regular intervals to monitor critical parameters, including pH, optical density at 600 nm (OD_{600}), and acetic acid concentration. pH measurements provided insights into acidification dynamics, while OD_{600} reflected biomass growth over time.

Acetic acid was quantified following the method outlined by Sode (2014). This method involves a classic acid-base titration using NaOH.

Data analysis

All experiments were conducted in triplicate. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10 software. Differences between strains and fermentation parameters were evaluated using analysis of variance (ANOVA), with significance considered at $p < 0.05$.

Results

Physicochemical characteristics of OMW

The physicochemical analysis of olive mill wastewater (OMW) (Table 1) revealed several important characteristics that underscore its potential for microbial fermentation. The recorded pH 4.5, COD of 183 g O_2 L^{-1}, and BOD_5 of 7 g O_2 L^{-1} indicate a highly organic-rich environment suitable for fermentation processes. The average acidity measured at 1.65% further emphasizes the high organic load present in the OMW.

The high COD/BOD_5 ratio observed in this study suggests the presence of non-biodegradable organic compounds. Additionally, the detection of 31 mg L^{-1} nitrites and 5.81 g L^{-1} total polyphenols in the OMW highlights its complex composition.

Table 1. Physicochemical characteristics of olive mill wastewater samples

Parameter	Mean ± SD	Range
pH	4.5 ± 0.2	4.3 – 4.7
COD (g O_2 L^{-1})	183 ± 5	178 – 188
BOD_5 (g O_2 L^{-1})	7.0 ± 0.3	6.7 – 7.3
Average acidity (%)	1.65 ± 0.05	1.60 – 1.70
Nitrite (mg L^{-1})	31 ± 2	29 – 33
Total polyphenols (g L^{-1})	5.81 ± 0.1	5.70 – 5.90

Microbial isolation and characterization

The results of the biochemical tests conducted on the isolated bacterial strains are summarized in Table 2. A total of 25 strains were evaluated for Gram staining, catalase activity, oxidase activity, and morphological form, revealing a diverse array of bacterial types predominantly classified as cocci and bacilli. The majority of strains exhibited Gram-negative characteristics, with notable exceptions among Gram-positive strains.

The identification of specific strains with positive catalase and oxidase activities, such as JR/GN/-4/1/3, JR/GN/-6/1/1, and JR/GN/-6/3/1, suggests their metabolic versatility. The morphological diversity observed among the strains – ranging from cocci to bacilli and coccobacilli – demonstrates their ecological adaptability.

The results of the batch fermentation trials shown in Fig. 1 indicate significant variability in gas production among the isolated bacterial strains. Notably, strains 2, 4, 6, 7, 10, 11, 17, 21, and 22 exhibited no gas production, while strains 1, 3, 5, 8, 9, 12, 16, 18, 20, 23, and 25 showed low levels of gas production. In contrast, strains 13, 14, 15, 19 and 24 demonstrated substantial gas production starting from the third day of incubation. Strain 14 emerged as the highest gas producer with approximately 14 mL, followed closely by strains 19 (12.6 mL), 24 (12.0 mL) and 13 (12.2 mL), while strain 15 produced around 11.8 mL within 120 h.

The fermentation profiles of strains 13, 14, 15, 19, and 24 were further characterized using the API 50 CHB/E galleries. This analysis identified two *Bacillus* species: *Bacillus licheniformis* (strains 13, 19, and 24) and *Bacillus circulans* (strains 14 and 15). The results obtained from the API galleries confirm their classification at the species level (Table 3).

The acetic acid production of five yeast strains (13, 14, 15, 19, and 24) was monitored over a period of 120 h. The data revealed distinct trends in growth (shown as OD_{600}), acid production, and pH variation among the tested strains (Fig. 2).

Strain 15 exhibited the highest acetic acid production, reaching 28.1 g L^{-1} at 108 h before slightly stabilizing at

Table 2. Results of Gram staining and biochemical tests for isolated strains

No.	Strain ID	Gram stain	Catalase	Oxidase	Form
1	JR/GN/-5/1/1/1	–	–	–	Cocci
2	SR2/M17/SM/1/2	–	–	–	Cocci
3	JR/GN/-4/1/3	+	+	–	Cocci
4	JR/GN/-6/1/1	+	+	–	Cocci
5	JR/GN/-4/3/2	+	–	–	Cocci
6	JR/GN/-4/4/1	–	+	–	Coccobacilli
7	JR/GN/-6/3/1	+	+	–	Bacilli
8	JR/GN/-8/2/2	–	+	–	Cocci
9	A/GN/SM/1	+	–	–	Coccobacilli
10	JR/GN/-4/4/4	+	–	–	Cocci
11	A/GN/-1/1	–	–	–	Cocci
12	JR/GN/-6/2/1	–	+	–	Coccobacilli
13	JR/GN/-6/2/5	+	+	–	Bacilli
14	JR/GN/-8/2/5	+	+	–	Bacilli
15	A/GN/-7/1	+	+	–	Coccobacilli
16	JR/GN/-6/2/1	–	+	–	Coccobacilli
17	JR/GN/-6/2/2	+	+	–	Bacilli
18	JR/GN/-6/3/3	–	+	+	Coccobacilli
19	JR/GN/-4/4/1	+	+	–	Bacilli
20	SR2/SM/M17/1/3	–	–	–	Cocci
21	JR/GN/-4/2/1	+	+	–	Bacille
22	SR2/M17/-7/1	+	–	–	Coccobacilli
23	SR2/M17/-5/4/1	–	–	–	Cocci
24	SR2/Clm /-9/4	+	–	–	Bacilli
25	JR/GN/-4/1/1	–	–	–	Coccobacilli

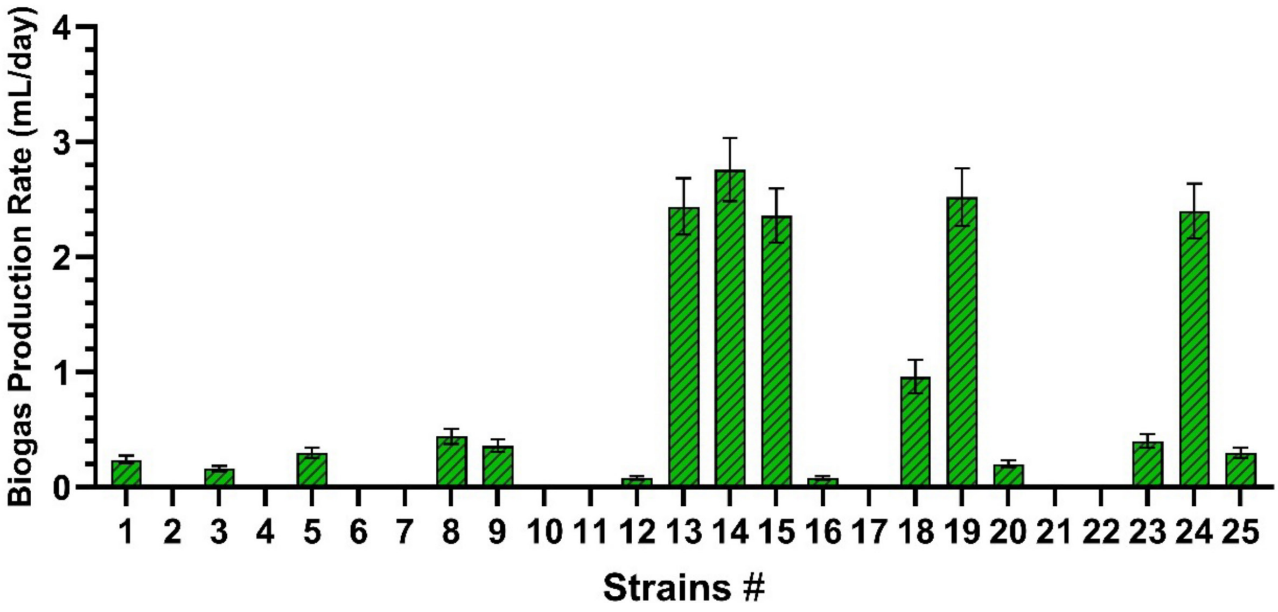

Fig. 1. Biogas production rate of different yeast strains over 5 days. Values represent mean ± SD.

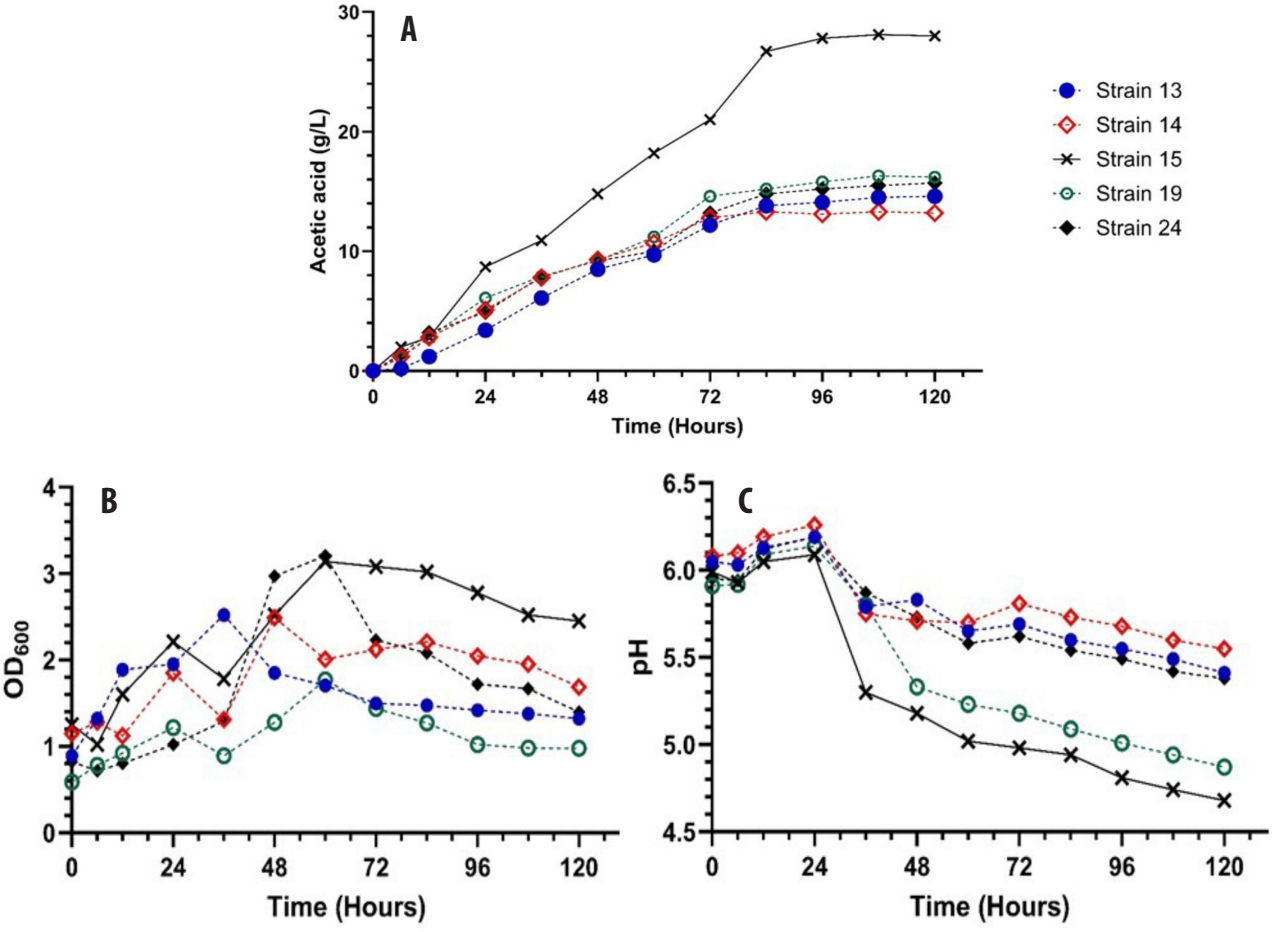

Fig. 2. Acetic acid production (A), OD_{600} (B) and pH (C) over time by selected *Bacillus* strains.

28.0 g L^{-1} at 120 h (Fig. 2A). This strain demonstrated a continuous increase in acetic acid concentration, with significant production acceleration after 24 h (8.7 g L^{-1}), peaking between 84 and 108 h. The pH of the fermentation medium gradually decreased, reaching 4.87 at 120 h, indicating active acidification (Fig. 2C).

Table 3. Results of API 50 CHB/B strains identification

Strain No.	Result of API
13	*Bacillus licheniformis*
14	*Bacillus circulans*
15	*Bacillus circulans*
19	*Bacillus licheniformis*
24	*Bacillus licheniformis*

Strains 13 and 14 showed moderate acetic acid production levels, with maximum concentrations of 15.7 and 16.2 g L^{-1}, respectively, at 120 h (Fig. 2A). Their pH values decreased to 5.38 and 4.87, respectively, by the end of fermentation (Fig. 2C). Growth patterns differed among the strains, with OD_{600} values peaking earlier (between 48 and 72 h) and subsequently declining, suggesting possible cellular stress or nutrient depletion (Fig. 2B).

Discussion

The physicochemical characteristics of OMW observed in this study align with previous research, reporting similar values for pH (4.5 to 5.5), COD (40 to 100 g L^{-1}), and BOD_5 (20 to 50 g L^{-1}) (Bouknana et al. 2014; Bouharat et al. 2018; Russo et al. 2022; Bougherara et al. 2021). The high COD/BOD_5 ratio (> 2.5) indicates the presence of slowly biodegradable or recalcitrant organic compounds, which can hinder biological treatment methods, as previously reported by Gueboudji et al. (2022). Additionally, polyphenol concentrations ranging from 0.5 to 8.0 g L^{-1} in OMW (Vavouraki et al. 2020) are known to exert antimicrobial effects, which may have contributed to growth inhibition in certain strains during fermentation (Russo et al. 2022; Sar, Akbas 2023).

In terms of biogas production, strains 13, 14, 15, 19, and 24 exhibited the most efficient metabolic pathways for OMW fermentation, with cumulative gas production reaching 13.8 mL 100 mL^{-1} of OMW, comparable to values reported in anaerobic digestion studies, where biogas yields typically range between 20 to 45 mL CH_4 100 mL^{-1} (Al Rabadi et al. 2021; Laabidi et al. 2023). The absence of gas production in certain strains suggests either an inability to metabolize the available substrates or a lack of key enzymes involved in methanogenesis (Liew et al. 2016). This variability underscores the importance of strain selection and metabolic optimization to enhance biogas yields and improve process stability.

The API 50CHB/E biochemical identification confirmed that *Bacillus licheniformis* and *Bacillus circulans* were key contributors to OMW fermentation. These species are well-documented for their ability to degrade complex carbohydrates and efficiently produce organic acids (lactic acids, α-ketoglutaric acid, and γ-aminobutyric acid) (Serin et al. 2012; Park et al. 2021). *Bacillus licheniformis*, in particular, is known for its tolerance to extreme environmental conditions, making it a promising candidate for large-scale bioprocess applications (Tamang et al. 2016; Shleeva et al. 2023). Previous studies have reported its use in anaerobic digestion systems, where it enhances hdrolysis and acidogenesis, leading to improved biogas yields (Shleeva et al. 2023).

The acetic acid production observed in this study highlights the potential of microbial fermentation for OMW valorization. Among the tested strains, strain 15 exhibited the highest acetic acid production (28.1 g L^{-1} at 108 h), surpassing values typically reported for *Saccharomyces cerevisiae*, which range from 20 to 40 g L^{-1} (De Leonardis et al. 2019; Fronteras et al. 2021). This suggests that *Bacillus* strains, particularly *Bacillus licheniformis* and *Bacillus circulans*, could be viable candidates for direct OMW fermentation, eliminating the need for co-culturing with acetic acid bacteria such as *Acetobacter aceti*, which is typically used in two-step fermentation systems (De Leonardis et al. 2019; Qiu et al. 2021).

Conclusions

The findings of this study indicate that *Bacillus* strains can efficiently ferment OMW to produce acetic acid and biogas, making them strong candidates for industrial-scale waste valorization. Compared to other microbial strains, *Bacillus* species offer advantages such as high environmental adaptability, resilience to acidic pH, and efficient enzyme production, which are essential for large-scale fermentation.

The use of *Bacillus* strains for acetic acid production from OMW represents a novel approach, as previous studies have primarily focused on *Saccharomyces cerevisiae* and acetic acid bacteria. This work not only contributes to the valorization of olive oil production by-products, but also introduces *Bacillus* as a promising candidate for sustainable acetic acid fermentation. Future research should focus on optimizing fermentation conditions to enhance yields, scaling up the process for industrial applications, and further investigating the metabolic pathways involved in *Bacillus*-mediated acid production. This study opens new avenues for biotechnological innovation in the valorization of olive oil wastewater, promoting circular economy principles and reducing environmental impact.

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References

Al Rabadi S., Alzboon K., Shawaqfah M., Damseh R., Alzoubi O. 2021. Biogas production through co-digestion of olive mill with municipal sewage sludge and cow manure. *Environ. Nat.*

Resour. J. 20: 137–147.

Angeloni G., Spadi A., Corti F., Calcaprina M., Carpi G., Maioli F., Parenti A., Masella P. 2024. Advancing circular economy in olive oil production: Comparing maturation systems for vermicompost creation from olive pomace. *Biomass* 4: 65.

APHA. 2017a. Biochemical oxygen demand (BOD). In: *Standard methods for the examination of water and wastewater.* American Public Health Association.

APHA. 2017b. Chemical oxygen demand (COD). In: *Standard methods for the examination of water and wastewater.* American Public Health Association.

Ayadi K., Meziane M., Rouam D., Bouziane M.N., El-Miloudi K. 2022. Olive mill wastewater for bioethanol production using immobilised cells. *Kem. Ind.* 71: 21–28.

Bougherara S., Boucetta N., Lecheb F., Medjdoub F., Delleci K., Belkhir M. 2021. Characterization and valorisation of olive by-products from a traditional oil mill in Béjaia. *Alger. J. Environ. Sci. Technol.* 7: 1819–1824.

Bouharat D., Nabila S.A., Souhail B., Laghdach A., El Yousfi F. 2018. Physico-chemical characterization of olive-oil mill wastewaters of Ben Karrich area (Tetouan province, North of Morocco) and optimization study of their treatment using activated carbon. *Mediterr. J. Chem.* 7: 253–258.

Bouknana D., Hammouti B., Salghi R., Jodeh S., Zarrouk A., Warad I., Aouniti A., Sbaa M. 2014. Physicochemical characterization of olive oil mill wastewaters in the eastern region of Morocco. *J. Mater. Environ. Sci.* 5: 1039–1058.

Carmona I., Aguirre I., Griffith D.M., García-Borrego A. 2023. Towards a circular economy in virgin olive oil production: Valorization of the olive mill waste (OMW) "alpeorujo" through polyphenol recovery with natural deep eutectic solvents (NADESs) and vermicomposting. *Sci. Total Environ.* 872: 162198.

De Leonardis A., Masino F., Macciola V., Montevecchi G., Antonelli A., Marconi E. 2019. A study on acetification process to produce olive vinegar from oil mill wastewaters. *Eur. Food Res. Technol.* 245: 2123–2131.

Fronteras J.P., Dacera D.D.M., Bello D.D., Delos Santos K.J.L. 2021. Utilization of pesticide-free calamansi (*Citrus microcarpa*) and mango (*Mangifera indica*) peels for the production of acetic acid with potential industrial application. *Bioresour. Technol. Rep.* 15: 100806.

Graham E.B., Knelman J.E. 2023. Implications of soil microbial community assembly for ecosystem restoration: Patterns, process, and potential. *Microbiol. Ecol.* 85: 809–819.

Gueboudji Z., Addad D., Kadi K., Nagaz K., Secrafi M., Yahya L.B., Lachehib B., Abdelmalek A. 2022. Biological activities and phenolic compounds of olive oil mill wastewater from Abani, endemic Algerian variety. *Sci. Rep.* 12: 6042.

Laabidi F., Besbès R., Jamoussi B. 2023. Production of biogas from olive mill waste waters treated by cow manure. *Green Sustain. Chem.* 13: 62–71.

Liew F., Martin M.E., Tappel R.C., Heijstra B.D., Mihalcea C., Köpke M. 2016. Gas fermentation – a flexible platform for commercial scale production of low-carbon-fuels and chemicals from waste and renewable feedstocks. *Front. Microbiol.* 7: 694.

Mizrahi I., Wallace R.J., Moraïs S. 2021. The rumen microbiome: Balancing food security and environmental impacts. *Nat. Rev. Microbiol.* 19: 553–566.

Ntougias S., Bourtzis K., Tsiamis G. 2013. The microbiology of olive mill wastes. *BioMed Res. Int.* 2013: 784591.

Park S.A., Bhatia S.K., Park H.A., Kim S.Y., Sudheer P.D.V.N., Yang Y.H., Choi K.Y. 2021. *Bacillus subtilis* as a robust host for biochemical production utilizing biomass. *Crit. Rev. Biotechnol.* 41: 827–848.

Qiu X, Zhang Y, Hong H. 2021. Classification of acetic acid bacteria and their acid resistant mechanism. *AMB Express* 11: 29.

Russo E., Spallarossa A., Comite A., Pagliero M., Guida P., Belotti V., Caviglia D., Schito A.M. 2022. Valorization and potential antimicrobial use of olive mill wastewater (OMW) from Italian olive oil production. *Antioxidants* 11: 5.

Sar T., Akbas M. 2023. Antimicrobial activities of olive oil mill wastewater extracts against selected microorganisms. *Sustainability* 15: 8179.

Senanayake D., Torley P.J., Chandrapala J., Terefe N.S. 2023. Microbial fermentation for improving the sensory, nutritional and functional attributes of legumes. *Fermentation* 9: 7.

Serin B., Akcan N., Uyar F. 2012. Production and optimization of α-amylase from *Bacillus circulans* ATCC 4516 with solid state fermentation. *Hacettepe J. Biol. Chem.* 40: 393–400.

Shleeva M.O., Kondratieva D.A., Kaprelyants A.S. 2023. *Bacillus licheniformis*: A producer of antimicrobial substances, including antimycobacterials, which are feasible for medical applications. *Pharmaceutics* 15: 1893.

Sode F. 2014. Simultaneous determination of peracetic acid and acetic acid by titration with NaOH. *Anal. Methods* 6: 2406-2409.

Tamang J.P., Watanabe K., Holzapfel W.H. 2016. Review: diversity of microorganisms in global fermented foods and beverages. *Front. Microbiol.* 7: 377.

Vavouraki A., Zakoura M., Dareioti M., Kornaros M. 2020. Biodegradation of polyphenolic compounds from olive mill wastewaters (OMW) during two-stage anaerobic co-digestion of agro-industrial mixtures. *Waste Biomass Valor.* 11: 5783–5791.

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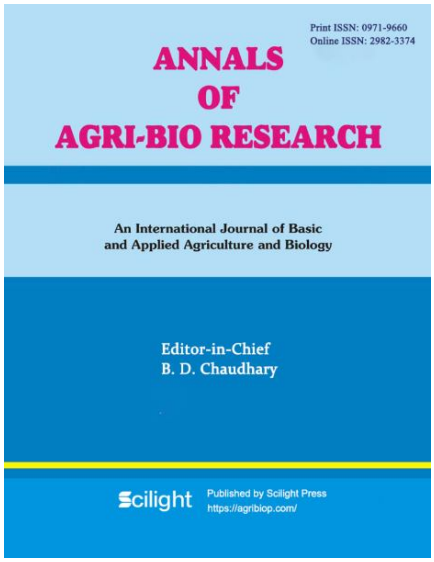

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Innovative approaches to bioethanol production: utilizing olive oil wastewater, milk whey, and sugarcane molasses through enzymatic hydrolysis and yeast immobilization

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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%

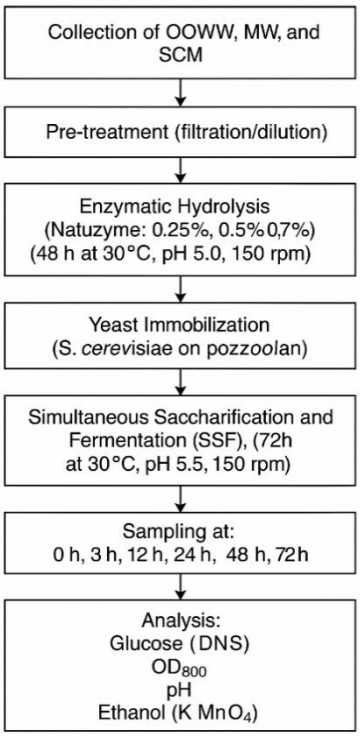


Fig. 1. Schematic representation of the experimental procedure. Three agro-industrial by-products (OOWW: olive oil wastewater, MW: milk whey, and SCM: sugarcane molasses) were pretreated and hydrolyzed enzymatically. Fermentation was carried out using immobilized *S. cerevisiae* on pozzolan. Samples 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.

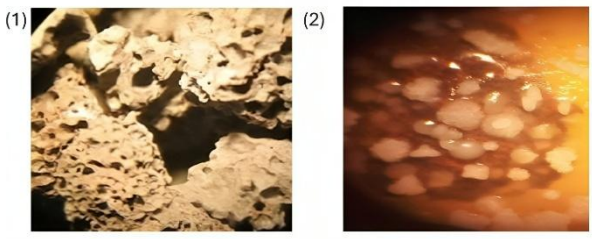


Fig. 2. Pozzolane rocks under binocular observation ×40: (**1**) before yeast immobilization, showing a porous structure, and (**2**) after immobilization, highlighting yeast clusters formation on the surface.

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.

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

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·L^{-1})	11	52.4	7.3	ISO 5815-1:2019
DCO O_2/l (g·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.

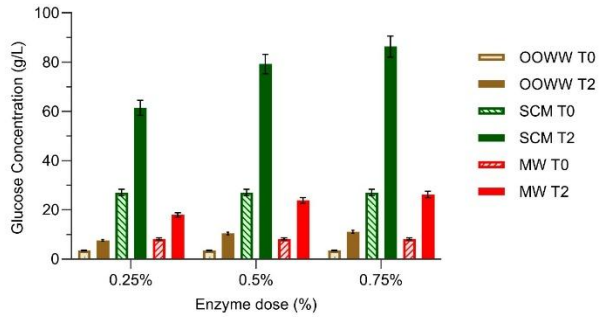


Fig. 3. Glucose release after enzymatic hydrolysis at different Natuzyme concentrations.

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).

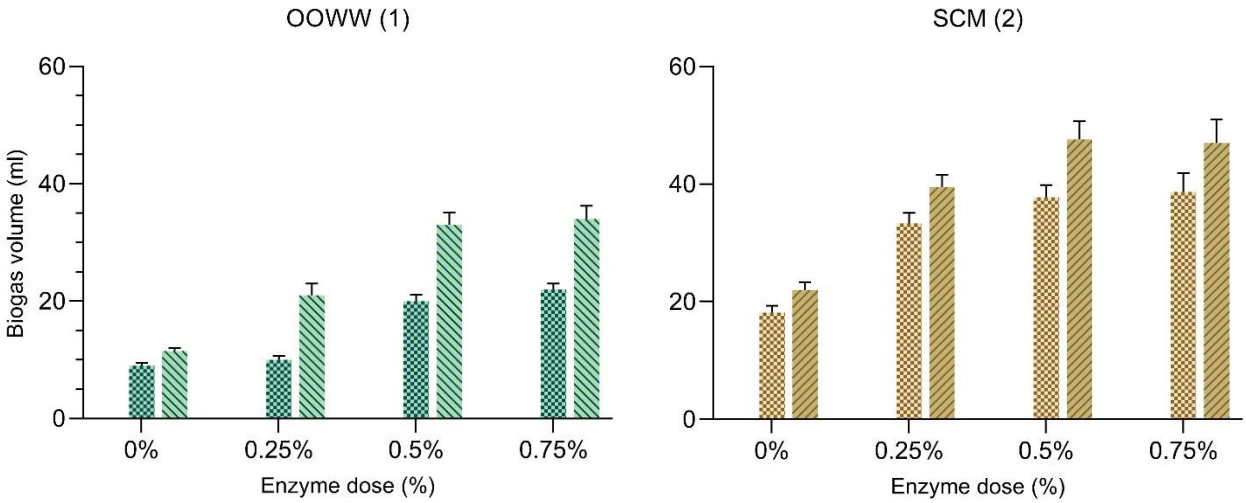
For the SCM, the high percentage increase in glucose concentration is due to the action of α-amylase (breaking down residual starch) and potentially invertase (hydrolyzing sucrose into glucose and fructose), facilitating rapid sugar availability for fermentation (Manoochehri et al., 2020). Lactose in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



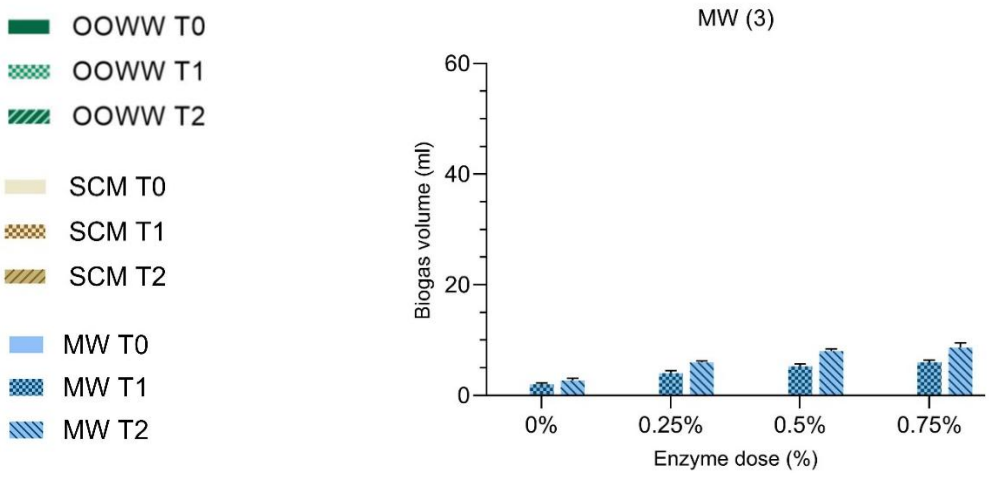


Fig. 4. Biogas production (mL) at T1 and T2 Across different enzyme doses for OOWW, SCM, and MW.

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).

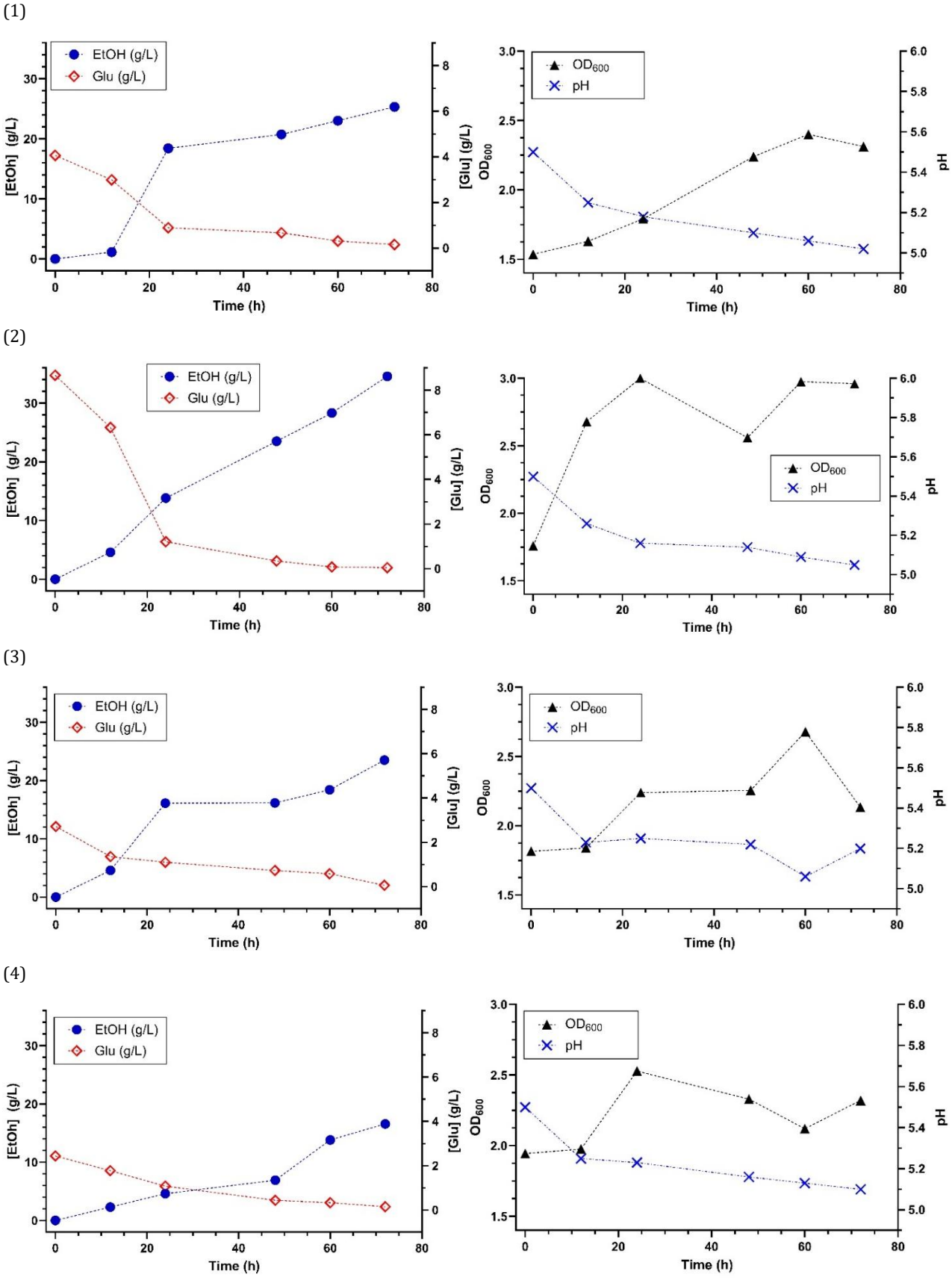


Fig. 5. Variation of ethanol concentration (g/L), glucose concentration (g/L), and optical density (OD_{600}) during fermentation of different waste mixtures. Measurements were taken over 72 h. Mix1 (**1**), Mix2 (**2**), Mix3 (**3**) and Mix4 (**4**).

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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REFERENCES

Abu Tayeh, H., Najami, N., Dosoretz, C., Tafesh, A. and Azaizeh, H. (2014). Potential of bioethanol production from olive mill solid wastes. *Bioresour. Technol.* **152**; 24–30. https://doi.org/10.1016/j.biortech.2013.10.102.

Álvarez-Cao, M.-E., Becerra, M. and González-Siso, M.-I. (2020). Chapter 8—Biovalorization of cheese whey and molasses wastes to galactosidases by recombinant yeasts. In N. Krishnaraj Rathinam & R. K. Sani (Eds.), *Biovalorisation of Wastes to Renewable Chemicals and Biofuels*. Amsterdam, Elsevier; pp. 149–161. https://doi.org/10.1016/B978-0-12-817951-2.00008-0.

Ayadi, K., M., M., Rouam, D., Mohammed, B. and El-Miloudi, K. (2022). Olive Mill Wastewater for

Bioethanol Production using Immobilised Cells. *Kem. Ind.* **71***:* 21–28. https://doi.org/10.15255/KUI.2021.015.

Bhardwaj, N., Kumar, B., Agrawal, K. and Verma, P. (2021). Current perspective on production and applications of microbial cellulases: A review. *Bioresour. Bioprocess.* **8**: 95. https://doi.org/10.1186/s40643-021-00447-6.

Bisswanger, H. (2017). *Enzyme Kinetics: Principles and Methods*. Hoboken: John Wiley & Sons.

Bouizar, R., Mouzai, A. and Boughellout, H. (2021). Impact of milk substitution by sweet whey on chocolate mousse physicochemical, microstructural and sensory properties. *Algerian J. Nutr. Food Sci.* **1**(**4**): 17–24.

Bouknana, D., Hammouti, B., Salghi, R., Jodeh, S., Zarrouk, A., Warad, I., Aouniti, A. and Sbaa, M. (2014). Physicochemical Characterization of Olive Oil Mill Wastewaters in the eastern region of Morocco. *J. Mater. Environ. Sci.* **5**(**4**): 1039–1058.

Calabrò, P. S., Fòlino, A., Tamburino, V., Zappia, G. and Zema, D. A. (2018). Increasing the tolerance to polyphenols of the anaerobic digestion of olive wastewater through microbial adaptation. *Biosyst. Eng.* **172**: 19–28. https://doi.org/10.1016/j.biosystemseng.2018.05.010.

Carteni, F., Occhicone, A., Giannino, F., Vincenot, C. E., de Alteriis, E., Palomba, E. and Mazzoleni, S. (2020). A General Process-Based Model for Describing the Metabolic Shift in Microbial Cell Cultures. *Front. Microbiol.* **11**: 521368. https://doi.org/10.3389/fmicb.2020.521368.

Chang, Y.-H., Chang, K.-S., Chen, C.-Y., Hsu, C.-L., Chang, T.-C. and Jang, H.-D. (2018). Enhancement of the Efficiency of Bioethanol Production by Saccharomyces cerevisiae via Gradually Batch-Wise and Fed-Batch Increasing the Glucose Concentration. *Fermentation* **4**(**2**): 2. https://doi.org/10.3390/fermentation4020045.

Cheng, D., Liu, Y., Ngo, H. H., Guo, W., Chang, S. W., Nguyen, D. D., Zhang, S., Luo, G. and Liu, Y. (2020). A review on application of enzymatic bioprocesses in animal wastewater and manure treatment. *Bioresour. Technol.* **313**: 123683. https://doi.org/10.1016/j.biortech.2020.123683.

Clément, G. (1956). Dosage des lipides dans les produits alimentaires; considérations sur leur valeur nutritive (1). *Ann. De Zootech.* **5**(**3**): 237–253.

Darwin, Charles, W. and Cord-Ruwisch, R. (2019). Anaerobic acidification of sugar-containing wastewater for biotechnological production of organic acids and ethanol. *Environ. Technol.* **40**(**25**); 3276–3286. https://doi.org/10.1080/09593330.2018.1468489.

de Araujo, T. M., da Cunha, M. M. L., Barga, M. C., Della-Bianca, B. E. and Basso, T. O. (2024). Production of flavor-active compounds and physiological impacts in immobilized Saccharomyces spp. Cells during beer fermentation. *Lett. Appl. Microbiol.* **77**(**9**): ovae083. https://doi.org/10.1093/lambio/ovae083.

Djeziri, S., Taleb, Z., Djellouli, M. and Taleb, S. (2023). Physicochemical and microbiological characterisation of Olive Oil Mill Wastewater (OMW) from the region of Sidi Bel Abbes (Western Algeria). *Moroc. J. Chem.* **11**(**2**): 2. https://doi.org/10.48317/IMIST.PRSM/morjchem-v11i2.31935.

Duque, A., Álvarez, C., Doménech, P., Manzanares, P. and Moreno, A. D. (2021). Advanced Bioethanol Production: From Novel Raw Materials to Integrated Biorefineries. *Processes* **9**(**2**): 206. https://doi.org/10.3390/pr9020206.

el Kafz, G., Cherkaoui, E., Benradi, F., Khamar, M. and Nounah, A. (2023). Characterization of Two Olive Mill Wastewater and Its Effect on Fenugreek (*Trigonella foenum-graecum*) Germination and Seedling Growth. *J. Ecol. Eng.* **24**: 207–217. https://doi.org/10.12911/22998993/171545.

Esmail, A., Abed, H., Firdaous, M., Chahboun, N., زكرياء منان, Z. M., Berny, E. and Mohammed, O. (2013). Étude physico-chimique et microbiologique des margines de trois régions du Maroc (Ouazzane, Fès Boulman et Béni Mellal) [Physico-chemical and microbiological study of oil mill wastewater (OMW) from three different regions of Morocco (Ouazzane, Fes Boulman and Béni Mellal)]. *J. Mater. Environ. Sci.* **5**: 121–126.

Galdieri, L., Mehrotra, S., Yu, S. and Vancura, A. (2010). Transcriptional Regulation in Yeast during Diauxic Shift and Stationary Phase. *OMICS A J. Integr. Biol.* **14**(**6**): 629–638. https://doi.org/10.1089/omi.2010.0069.

Gonzalez, J. M. and Aranda, B. (2023). Microbial Growth under Limiting Conditions-Future Perspectives. *Microorganisms* **11**(**7**): 1641. https://doi.org/10.3390/microorganisms11071641.

Hakika, D. C., Sarto, S., Mindaryani, A. and Hidayat, M. (2019). Decreasing COD in Sugarcane Vinasse Using the Fenton Reaction: The Effect of Processing Parameters. *Catalysts* **9**(**11**): 881. https://doi.org/10.3390/catal9110881.

Hassan, S. H. A., el Nasser A. Zohri, A. and Kassim, R. M. F. (2019). Electricity generation from sugarcane molasses using microbial fuel cell technologies. *Energy* **178**: 538–543. https://doi.org/10.1016/j.energy.2019.04.087.

Jain, A., Jain, R. and Jain, S. (2020). Quantitative Analysis of Reducing Sugars by 3,5-Dinitrosalicylic Acid (DNSA Method). In A. Jain, R. Jain, & S. Jain (Eds.), *Basic Techniques in Biochemistry, Microbiology and Molecular Biology: Principles and Techniques*. New York: Springer US; pp. 181–183. https://doi.org/10.1007/978-1-4939-9861-6_43.

Jessen, J. E. and Orlygsson, J. (2012). Production of Ethanol from Sugars and Lignocellulosic

Biomass by Thermoanaerobacter J1 Isolated from a Hot Spring in Iceland. *J. Biomed. Biotechnol.* **2012**: 186982. https://doi.org/10.1155/2012/186982.

Kovács, E., Wirth, R., Maróti, G., Bagi, Z., Rákhely, G. and Kovács, K. L. (2013). Biogas Production from Protein-Rich Biomass: Fed-Batch Anaerobic Fermentation of Casein and of Pig Blood and Associated Changes in Microbial Community Composition. *PLoS ONE* **8**(**10**): e77265. https://doi.org/10.1371/journal.pone.0077265.

Kumara Behera, B. and Varma, A. (2017). Material-Balance Calculation of Fermentation Processes. In B. Kumara Behera & A. Varma (Eds.), *Microbial Biomass Process Technologies and Management*. New York: Springer International Publishing; pp. 257–298. https://doi.org/10.1007/978-3-319-53913-3_5.

Lachebi, S. and Yelles, F. (2018). Valorisation du lactosérum par technique membranaire. *Alger. J. Environ. Sci. Technol.* **4**(**3**). Available online: https://www.aljest.net/index.php/aljest/article/view/55 (accessed on 07 February 2025).

Lievore, P., Simões, D. R. S., Silva, K. M., Drunkler, N. L., Barana, A. C., Nogueira, A. and Demiate, I. M. (2015). Chemical characterisation and application of acid whey in fermented milk. *J. Food Sci. Technol.* **52**(**4**): 2083–2092. https://doi.org/10.1007/s13197-013-1244-z.

Maier, R. M. and Pepper, I. L. (2015). Chapter 3—Bacterial Growth. In I. L. Pepper, C. P. Gerba, & T. J. Gentry (Eds.), *Environmental Microbiology (Third Edition)*. Cambridge, Academic Press; pp. 37–56. https://doi.org/10.1016/B978-0-12-394626-3.00003-X.

Manoochehri, H., Hosseini, N. F., Saidijam, M., Taheri, M., Rezaee, H. and Nouri, F. (2020). A review on invertase: Its potentials and applications. *Biocatal. Agric. Biotechnol. 25*, 101599. https://doi.org/10.1016/j.bcab.2020.101599.

Mohd-Zaki, Z., Bastidas-Oyanedel, J. R., Lu, Y., Hoelzle, R., Pratt, S., Slater, F. R. and Batstone, D. J. (2016). Influence of pH Regulation Mode in Glucose Fermentation on Product Selection and Process Stability. *Microorganisms* **4**(**1**): 2. https://doi.org/10.3390/microorganisms4010002.

Nguyen, S. T. C., Freund, H. L., Kasanjian, J. and Berlemont, R. (2018). Function, distribution, and annotation of characterized cellulases, xylanases, and chitinases from CAZy. *Appl. Microbiol. Biotechnol.* **102**(**4**): 1629–1637. https://doi.org/10.1007/s00253-018-8778-y.

Pasotti, L., Zucca, S., Casanova, M., Micoli, G., Cusella De Angelis, M. G. and Magni, P. (2017). Fermentation of lactose to ethanol in cheese whey permeate and concentrated permeate by engineered Escherichia coli. *BMC Biotechnol.* **17**(**1**): 48. https://doi.org/10.1186/s12896-017-0369-y.

Rouam, D., & Meziane, M. (2025). Valorization of olive mill wastewater for acetic acid production by Bacillus strains isolated from bovine rumen. Environmental and Experimental Biology, 23(1), Article 1. https://doi.org/10.22364/eeb.23.03

Saqib, S., Akram, A., Halim, S. A. and Tassaduq, R. (2017). Sources of β-galactosidase and its applications in food industry. *3 Biotech* **7**(**1**): 79. https://doi.org/10.1007/s13205-017-0645-5.

Tebbouche, L., Aziza, M., Abada, S., Chergui, A., Amrane, A, and Hellal, A. (2024). Bioethanol production from deproteinized cheese whey powder by local strain isolated from soured milk: Influence of operating parameters. *Energy Sources Part A: Recovery Util. Environ. Eff.* **46**(**1**): 397–408. https://doi.org/10.1080/15567036.2023.2284851.

Vasić, K., Knez, Ž. and Leitgeb, M. (2021). Bioethanol Production by Enzymatic Hydrolysis from Different Lignocellulosic Sources. *Molecules* **26**(**3**): 753. https://doi.org/10.3390/molecules26030753.

Waterborg, J. H. and Matthews, H. R. (1984). The Lowry Method for Protein Quantitation. In J. M. Walker (Ed.), *Basic protein and peptide protocols*. Totowa: Humana Press; pp. 1–3. https://doi.org/10.1385/0-89603-062-8:1.

Yang, X., Wang, K., Wang, H., Zhang, J., Tang, L. and Mao, Z. (2016). Control of pH by acetic acid and its effect on ethanol fermentation in an integrated ethanol–methane fermentation process. *RSC Adv.* **6**(**63**): 57902–57909. https://doi.org/10.1039/C6RA04129A.

Yusuf, U., Usman, U. G., Abubakar, A. Y. and Mansir, G. (2023). Effect of pH and Temperature on Bioethanol Production: Evidences from the Fermentation of Sugarcane Molasses using Saccharomyces cerevisiae. *Dutse J. Pure Appl. Sci.* **8**: 9–16. https://doi.org/10.4314/dujopas.v8i4b.2.

Zhang, P., Hai, H., Sun, D., Yuan, W., Liu, W., Ding, R., Teng, M., Ma, L., Tian, J. and Chen, C. (2019). A high throughput method for total alcohol determination in fermentation broths. *BMC Biotechnol.* **19**(**1**): 30. https://doi.org/10.1186/s12896-019-0525-7.