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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

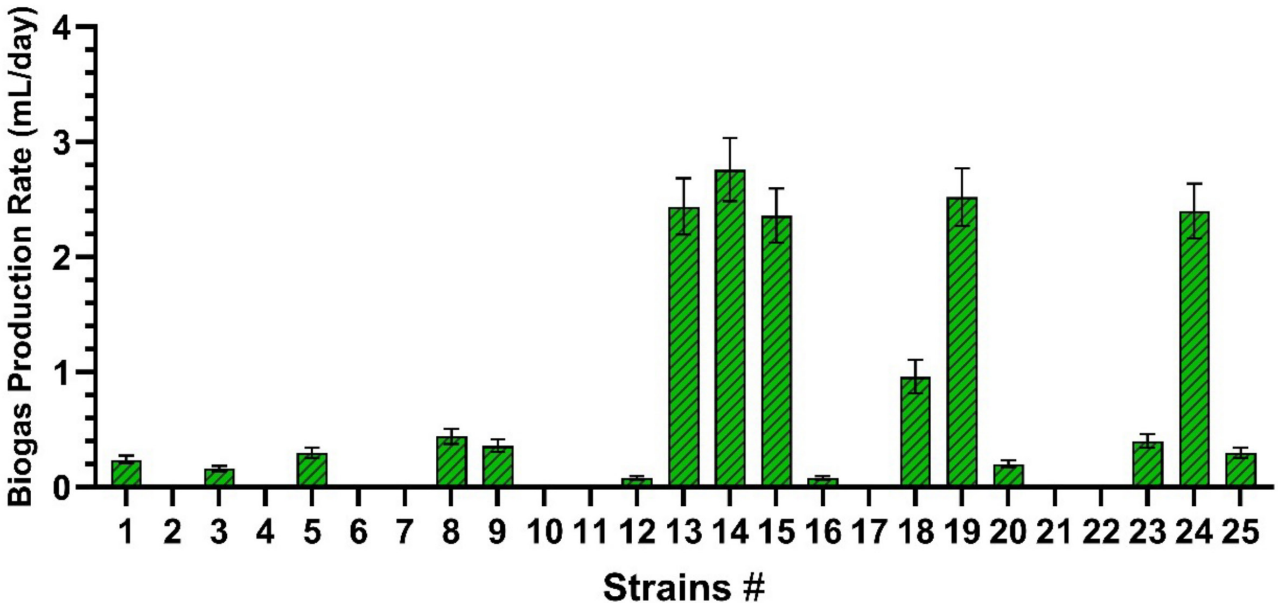

Fig. 1. Biogas production rate of different yeast strains over 5 days. Values represent mean ± SD.

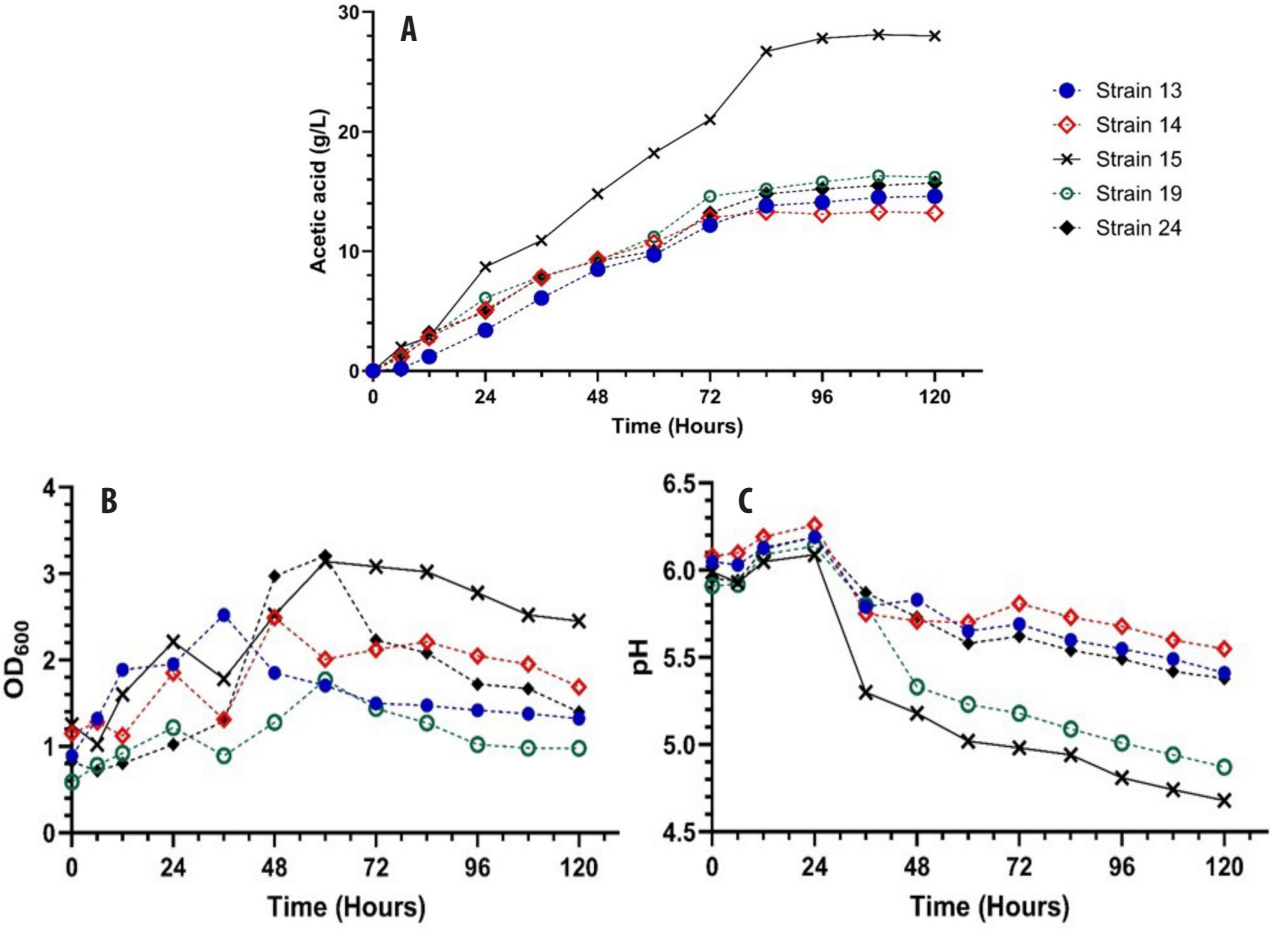

Fig. 2. Acetic acid production (A), OD_{600} (B) and pH (C) over time by selected *Bacillus* strains.

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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