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Screening local marine habitats suggests a novel *Bacillus subtilis* MZ1 strain as a potential fatty acid producer

El cribado de hábitats marinos locales sugiere una nueva cepa, *Bacillus subtilis* MZ1, como un posible productor de ácidos grasos

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ABSTRACT

Within this academic research article, a bacterium isolated from a marine soil environment near the Mediterranean Sea is observed to possess the potential for producing various fatty acids, particularly *n*-caproic and oleic acids, as evidenced by FAME profiling. Furthermore, the introduction of glucose into the growth medium enhances the production of caprylic acid rather than caproic acid. The 16S rDNA sequencing suggested that the MZ1 strain belongs to *Bacillus subtilis* and is closely related to many halophilic species. FAME profiling reveals that the isolated MZ1 is competent in the total production of fatty acids compared to other marine bacterial candidates. Furthermore, the results indicate that MZ1 is efficient in the production of many other fatty acids. This exploration suggests that the marine bacterium *Bacillus subtilis* MZ1 can be used for fatty acids synthesis, which could be valuable for biodiesel production and other applications.

Keywords: *Bacillus subtilis*, fatty acids, gas chromatography, caproic acid, caprylic acid, reverse β-oxidation.

RESUMEN

Dentro de este artículo de investigación académica, se observa que una bacteria aislada de un entorno de suelo marino cerca del mar Mediterráneo posee el potencial de producir diversos ácidos grasos, especialmente los ácidos n-caproico y oleico, según lo evidencia el perfil FAME. Además, la introducción de glucosa en el medio de crecimiento potencia la producción de ácido caprílico en lugar de ácido caproico. La secuenciación del 16S rDNA sugiere que la cepa MZ1 pertenece a *Bacillus subtilis* y está estrechamente relacionada con muchas especies halófilas. El perfil FAME revela que la cepa MZ1 aislada es competente en la producción total de ácidos grasos en comparación con otros candidatos bacterianos marinos. Además, los resultados indican que MZ1 es eficiente en la producción de muchos otros ácidos grasos. Esta exploración sugiere que la bacteria marina *Bacillus subtilis* MZ1 puede ser utilizada para la síntesis de ácidos grasos, lo cual podría ser valioso para la producción de biodiesel y otras aplicaciones.

Palabras clave: *Bacillus subtilis*, ácidos grasos, cromatografía de gases, ácido caproico, ácido caprílico, oxidación β inversa.

INTRODUCTION

Since the 1800s, heavy dependence on fossil fuels for industrial activities has greatly hastened global climate change. This has led to a hotter and drier climate, higher atmospheric carbon dioxide levels, ozone depletion, rising sea levels, and habitat loss. Ecosystems have transitioned from carbon sinks to carbon sources, emitting gases like carbon dioxide and methane, ultimately jeopardizing the food supply. One solution to minimize the adverse effects of climate change, is to replace fossil fuels with renewable sources such as solar energy or other alternative like biofuels. For example, the synthesis of fatty acids by microorganisms could contribute to the preservation of agricultural land. In addition to their nutraceutical properties, microbial fatty acids have been employed in medicine for their antibacterial activity (Hegazy *et al.*, 2020; Shaaban *et al.*, 2021; 2022; 2023; Zayed *et al.*, 2022).

Harnessing microbes for fatty acids production was well-studied by German scientists during World War II (Ratledge and Lippmeier, 2017). Since then, numerous microorganisms have undergone screening for their capacity to produce fatty acids, revealing several notable candidates such as *Clostridium*, recognized for synthesizing and storing middle-chain n-alkanes, and *Vibrio furnissii*, known for hydrocarbons resembling kerosene and light oil. Actinomycetes such as *Streptomyces*, *Nocardia*, *Rhodococcus*, and *Mycobacterium* have demonstrated enzymatic biosynthesis and accumulation of triacylglycerols (TAG), a crucial precursor for microbial biodiesel production. Noteworthy producers of Eicosapentaenoic acid (EPA) include *Shewanella*, *Photobacterium*, *Colwellia*, *Vibrio*, and *Psychromonas*. Additionally, *E. coli* and green algae are recognized for TAG production. *Yarrowia lipolytica*, an oleaginous yeast, is renowned for TAG production and has been genetically engineered for enhanced yield (Shulse and Allen, 2011; Lennen and Pfleger, 2013; El Razak *et al.*, 2014; Janßen and Steinbüchel, 2014). These microorga-

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nisms accumulate lipids, subsequently converted into fatty acids, which serve as feedstocks for biodiesel production via trans-esterifying fatty acids with alcohol to yield biodiesel (fatty acid methyl or ethyl esters) (Hoekman *et al*., 2012; Diomandé *et al*., 2015; Rana *et al*., 2019). Herby, microbes can be harnessed as a renewable alternative to conventional diesel fuel and aligns with sustainability goals by offering an environmentally friendly alternative to fossil fuels (Kumar *et al*., 2020).

Although microbial production of fatty acids saves agricultural land, it remains comparatively costly when compared to counterparts derived from plants (Osorio-González *et al*., 2019). Therefore, the search for microbial species that produce massive amounts of fatty acids on inexpensive nutrients is still ongoing to minimize production costs. This research aimed to identify a competent bacterial species for efficient lipid production. Salinity has been found to trigger lipid accumulation in plants, algae, and bacteria (Monteoliva-Sanchez *et al*., 1993; Aziz *et al*., 2015; Hounslow *et al*., 2016; Atikij *et al*., 2019; El-Halmouch, 2019). However, salinity may induce the biosynthesis of saturated fatty acids rather than unsaturated ones (Atikij *et al*., 2019; El-Halmouch, 2019). As a result, the main target of this research was to explore bacteria that live in saline environments to identify a microbe with a natural ability to augment fatty acids as an adaptive response to salinity.

Until now, gas chromatography has served as an accurate tool for estimating fatty acid content; however, new cost-effective methods have been developed to identify lipid-producing candidates. For example, microbial lipids, especially polyunsaturated fatty acids (PUFAs), enable PUFA-producing microbes to withstand the damaging effects of H_2O_2 in a NaN_3-containing growth medium. These lipids act as shields against the H_2O_2 oxidative damage (Tilay and Annapure, 2012). Additionally, the ability of the microbe to reduce the colorless 2,3,5-triphenyl tetrazolium chloride (TTC) into the red-colored triphenyl formazan (TPF) expresses its capacity on lipid biosynthesis. Only cells with active dehydrogenases can convert TTC to red TPF, thus active dehydrogenases indicate lipid production and promote TTC reduction (Ke *et al*., 2000; Xue *et al*., 2017). Herby, these methods were employed to identify the most efficient lipid-producing candidate.

MATERIALS AND METHODS

Sampling procedure

Soil samples were amassed from three different geographic marine locations adjacent to the Mediterranean Sea. These locations are from Alexandria, Baltim, and Damietta cities (Fig. 1). After removing a 2 cm from the surface of the soil with a sterilized spatula, each sample was taken with a sterilized Peterson grab sampler, preserved in a sterilized polythene bag, and labeled appropriately. The collected samples were preserved in the dark at 4 °C until further analyses.

Bacterial isolation, purification, and culture conditions

The collected soil samples were serially diluted with sterilized seawater to get 10^{-1} to 10^{-6} CFU (Colony Forming Units) per one-gram soil on plates containing nutrient agar growth media that contains 5 g NaCl, 2 g beef extract, 1 g yeast extract, 5 g peptone, and 20 g agar dissolved in distilled water for a total volume of 1 L (Downes and Ito, 2001). After a two days incubation period at 30 °C, pure cultures of different isolates were acquired according to the colony morphological characters. Later, pure strains were saved. For storage purposes, the isolates were preserved in 50 % glycerol and 50 % nutrient broth at - 20°C (Downes and Ito, 2001; Oliveira *et al*., 2021). In either nutrient agar or nutrient broth, the seawater was used in place of the distilled water to maintain the proper growth of marine isolates.

Figure 1. A Google map of Egyptian Nile Delta reaching the Mediterranean Sea where soil sample were collected. Blue stars indicate the geographic sample collection.

Figura 1. Un mapa de Google del Delta del Nilo egipcio que se extiende hasta el Mar Mediterráneo, donde se recopilaron muestras de suelo. Las estrellas azules indican la ubicación geográfica de la recopilación de muestras.

Sorting out the potential fatty acids-producing isolates

Potential isolates with the competence of producing fatty acids were selected according to two assays. Initially, the potency of each strain to survive over different H_2O_2 concentrations (0.01, 0.5, 1.0 % prepared from 30 % H_2O_2 stock solution) was evaluated by H_2O_2-plate assay (Tilay and Annapure, 2012). Moreover, the ability of each H_2O_2-resistant isolate to reduce TTC into TPF was tested by spectrophotometer at 485 nm, according to Ryan *et al*. (2010). Only candidates that showed resistance against H_2O_2 and reduced TTC into TPF were selected for GC-MS analysis. An example of the result of these two tests is shown in Fig. 2 for the bacterial isolate #2.

FAME profiling

Bacterial isolates that can produce fatty acids were grown on 50 mL seawater-based nutrient broth (SNB) cultures for 24 h, and the pellets were collected by centrifugation at 4,000 *g* for 15 m. Afterward, the pellets were rinsed and prepared according to Rogers and Burns (1994). Total lipids were extracted from the collected pellets according to Folch *et al*. (1957). Finally, samples were subjected to saponification and methylation to generate fatty acid methyl esters (FAME). He-

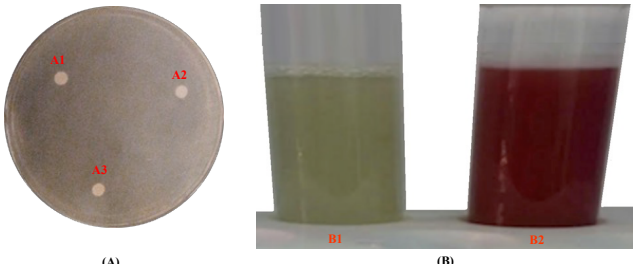

Figure 2. (A) The H_2O_2 plate assay for the bacterial isolate #2, where PUFAs producers generate no inhibition zone at different H_2O_2 concentrations (A1, A2 and A3). (B) The 2,3,5-triphenyl tetrazolium chloride (TTC) assay for the bacterial isolate #2 as shown in B1. After incubation at 25 ºC for 1h.; the formation of red color is considered as a positive result due to the reduction of TTC to the red-colored triphenyl formation as seen in B2.

Figura 2. (A) La prueba de placa de H_2O_2 para el aislado bacteriano #2, donde los productores de PUFAs no generan una zona de inhibición a diferentes concentraciones de H_2O_2 (A1, A2 , A3). (B) La prueba de cloruro de 2,3,5-trifenil tetrazolio (TTC) para el aislado bacteriano #2, como se muestra en B1. Después de incubarse a 25 ºC durante 1 h, la formación de color rojo se considera un resultado positivo debido a la reducción de TTC a la formación trifenil roja, como se ve en B2.

reafter, the FAME were extracted and then 1 mL was injected into the gas chromatography device H.P. (Hewlett Packard) 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50 – 550); source at 230 °C and quadruple at 150 °C) in the EI mode with an HP-5ms capillary column (30 m 0.25 mm i.e., 0.25 mm film thickness; J and W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C/min., and maintained for 20 min at 300 °C (Watanabe *et al*., 1996). Based on the interpretation of mass spectrometric fragmentation, confirmed by comparing our compounds' retention times and fragmentation patterns with the Supelco 37-component FMAE standard mixture. The microbial candidate with the peak of total fatty acid production was selected for molecular identification and further experiments.

Microbial growth optimization

The study selected the most promising candidate based on its proficiency in augmenting fatty acid production, as revealed by FAME analysis. The chosen candidate's growth was then assessed using various growth media that were inoculated by 0.5 McFarland of the candidate isolate. The seawater-based nutrient broth medium (SNB) emerged as the most favorable, and its components were fine-tuned to enhance and optimize the growth conditions.

First, the optimal pH was determined from 6 - 6.5 - 7 - 7.5 - 8. Second, optimal temperature was evaluated from 15 - 40 C. Finally, various carbon and nitrogen sources were evaluated to optimize the candidate's ability to reduce TTC into TPF. In the case of the carbon source, the yeast extract of the SNB was replaced by 2 % of different carbon sources such as lactose, mannitol, glucose, fructose, sucrose, starch, and dextrose. For the nitrogen source, peptone and beef extract were replaced by four different nitrogen sources, at 2 % of either potassium nitrate, ammonium sulfate, ammonium chloride, or ammonium nitrate. Carbon or nitrogen surrogates were sterilized via a sterile Millipore filter (0.45 µm) before using them within the growth media (Wang *et al*., 2007). Natural wastes like sugarcane and banana peels also replaced both the carbon and nitrogen sources. All treatments were incubated for 48 h at the optimum temperature and the optimum pH that was noted from the previous results. Later, all optimal conditions were employed to collect the culture biomass of the selected candidate, and FAME profiling was estimated under the best growth conditions as previously explained.

DNA barcoding

The 16S rDNA sequencing was performed at the Macrogen Inc., Seoul, South Korea. In brief, DNA extraction and purification were performed via the DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), as described by the supplier's protocol. Purified DNA was considered pure when the OD_{260}/OD_{280} ratio was around 1.8, and the OD_{260}/OD_{230} ratio was about 2.0. Accordingly, the polymerase chain reaction (PCR) was performed with 27F/1492R primers according to Zayed *et al.* (2022). The PCR thermal gradient program was a single cycle of 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 120 s, and 68 °C for 90 s, followed by a final extension at 68 °C for 10 min, and the reaction was held at 4 °C. Afterwards, the product was purified and sequenced using Sanger (dideoxy) technology via the sequencing kit BigDye (R) Terminator v3.1 Cycle Sequencing Kit with four different primers, which were 785F, 907R, 337F, and 1100R. Sequencing was performed using ABI PRISM 3730XL Analyzer (96 capillary type) at the Macrogen Inc., Seoul, South Korea. The sequences from ABI 3730 were assembled and the obtained contig was deposited in the GenBank of the National Center for Biotechnology Information (NCBI Resource Coordinators 2016) with accession number OM946589.

The sequence was used as a query in a blastn algorithm with a cutoff E-value 1e-11 to retrieve the authentic 16S rDNA homologs (Zhang *et al*., 2000; Zayed and Badawi, 2020). The retrieved sequences were aligned using MAFFT version 7 (Katoh *et al*., 2019). Maximum likelihood phylogenetic tree was inferred assuming the lowest BIC (Bayesian Information Criterion) score model which is F81+F+I (Nguyen *et al*., 2015; Kalyaanamoorthy *et al*., 2017; Hoang *et al*., 2018). The phylogenetic tree was visualized by using iTOL (Interactive Tree of Life) webserver (Letunic and Bork, 2016). The same phylogenetic analysis was repeated using the blast algorithm against bacterial type strains only and with the Maximum likelihood phylogenetic tree was inferred assuming the lowest BIC model which is HKY+F+I to reveal the closest type strain to MZ1.

RESULTS AND DISCUSSION

Lipids are high-energy storage molecules, and microorganisms foster their lipid biosynthesis as a response to stress conditions such as salinity, light, and temperature (Venkata Mohan and Devi, 2014). Although salinity may promote membrane fluidity by enhancing the desaturation levels of

the membrane lipids, it has been reported that the saturated fatty acids are accumulated dramatically in response to salinity (Venkata Mohan and Devi, 2014). For example, the decline in the PUFAs content and the accumulation of saturated fatty acids (SFAs) and Mono saturated fatty acids (MUFAs) in the Antarctic *Chlamydomonas* sp. L4 can intensify the membrane's rigidity as affected by long-term salinity stress (Kan *et al.*, 2012). The increase in the inelasticity of the membrane in response to high salt concentrations enhances the cell's ability to control the passive permeability. Thus, the cell can uphold its internal osmotic pressure, which may protect it from shrinkage (Hosono, 1992). Therefore, salinity stress triggers lipid-accumulation, or fatty acids biosynthesis is a biochemical adaptive evolutionary trait of organisms dwelling in marine environments.

Thirty-five distinct bacterial species were purified according to their morphological characteristics on nutrient agar plates, as a result of screening bacterial species dwelling in the saline soil of three different geographic locations North of Egypt near the Mediterranean Sea (Fig. 1). To Select competent lipid-producing candidates, two characteristics were chosen from literature, the microbe's ability to tolerate H_2O_2 toxic effects, and its ability to reduce TTC (Ryan *et al.*, 2010; Tilay and Annapure, 2012). Previous studies indicated that the total fatty acid accumulation has a significant positive correlation with the ability of the microorganism to reduce TTC into TPF. As such, TTC can be absorbed by living cells (Zhu *et al.*, 2004; Ryan *et al.*, 2010; El Razak *et al.*, 2014). Active dehydrogenases are thought to be involved in reducing TTC. For example, the dehydrogenase Δ5-desaturase is hypothesized as a TTC oxidizing agent (Zhu *et al.*, 2004; Gad *et al.*, 2016; Romano *et al*., 2020) as summarized in Fig. 3. Moreover, the TTC staining of *Mortierella alpina* M-23 mycelia is positively correlated with the total lipid content (Zhu *et al.*, 2004).

Fatty acids composition of the six selected candidates

Our target was then to identify which of the six candidates exhibits the highest efficiency in lipid production. The H_2O_2-assay plate, being a qualitative method, is insufficient to address this question. Additionally, TPF formation from TTC may lack the precision required to differentiate the lipid production capacities of efficient candidates. Therefore, gas chromatography was utilized to quantitatively assess the biosynthesized lipid quantities by each species under consistent pre-set conditions. FAME profiling was performed to reveal the bulk of intracellular and extracellular fatty acids compositions for all the six selected candidates (Figs. 4 and 5).

FAME profiles of the six bacterial candidates reveal that all of them are gram-positive due to the absence of liposaccharides and hydroxy fatty acids in their FAME profiles (Bisen *et al.*, 2012). It has been noted that candidate II recorded the highest lipid accumulation (78.5 %). The production of caproic acid attained 46.1 %, followed by oleic acid 3.1 %, which indicates the efficiency of this candidate to manufacture caproic acid when grown on nutrient agar. Other

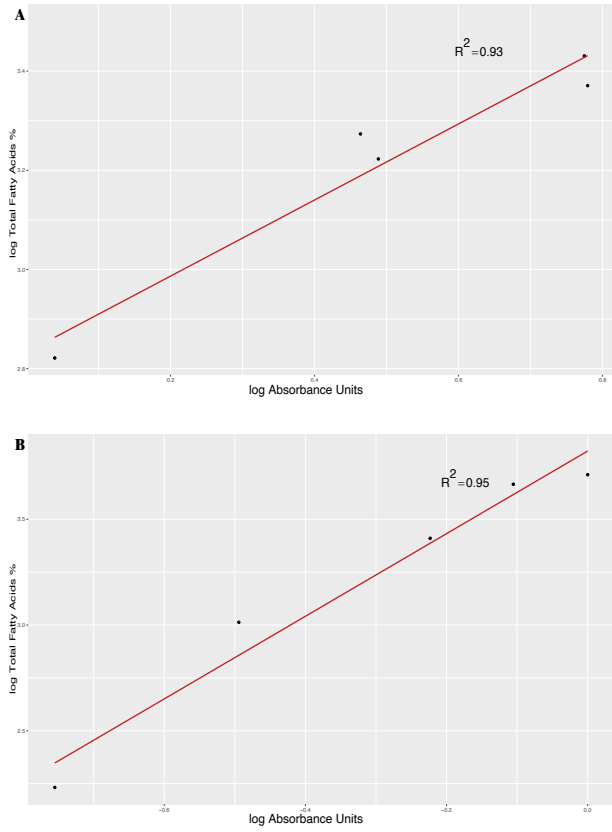

Figure 3. Predictions of total fatty acid contents from the TPF formation as measured by absorbance units as revealed from previous studies (Zhu *et al.*, 2004; Gad *et al.*, 2016). Data were extrapolated by using WebPlotDigitizer (Rohatgi, 2019). **(A)** The correlation of total fatty acids and the TPF absorbance units at 450 nm on the log scale as conducted by five different marine bacterial strains, which are AG9, AG10, AG18, AG26, and AG28 (Gad *et al.*, 2016). The adjusted R^2 of 0.93 at a P-value less than 5 % indicates a significant positive correlation. **(B)** The relationship between the total fatty acids and the absorbance units of TPF at 450 nm on the log scale after the TTC staining of the *Mortierella alpina* M-23 mycelia (Zhu *et al.*, 2004). The adjusted R^2 of 0.95 at a P-value less than 5 % indicates a significant positive correlation.

Figura 3. Las predicciones de los contenidos totales de ácidos grasos a partir de la formación de TPF medida en unidades de absorbancia, según se revela en estudios previos (Zhu et al., 2004; Gad et al., 2016). Los datos se extrapolaron utilizando WebPlotDigitizer (Rohatgi, 2019). **(A)** La correlación de los ácidos grasos totales y las unidades de absorbancia de TPF a 450 nm en escala logarítmica, realizada por cinco cepas bacterianas marinas diferentes, que son AG9, AG10, AG18, AG26 y AG28 (Gad et al., 2016). El R2 ajustado de 0.93 con un valor de P inferior al 5 % indica una correlación positiva significativa. **(B)** La relación entre los ácidos grasos totales y las unidades de absorbancia de TPF a 450 nm en escala logarítmica después de la tinción con TTC de las micelias de *Mortierella alpina* M-23 (Zhu et al., 2004). El R2 ajustado de 0.95 con un valor de P inferior al 5 % indica una correlación positiva significativa.

candidates were also seen to accumulate lipids but with fewer amounts. For example, candidates XIII and XVI have a total lipid production of 25.5 % and 36.3 %, respectively, with a pronounced production of monounsaturated fatty acids (MUFAs) such as erucic acid that reached 5.6 %, and oleic acid that reached 2.9 %. Also, candidate V has a total lipid production of 25 % with efficiency in producing oleic acid that attained 2.9 %. Although candidates XI and XIV recorded the lowest total lipid production (18.6 % and 15.2 %, respectively), they efficiently produced oleic acid by 2.9 %.

The creation of oleic acid was also maintained by candidate II (3.1 %) with efficiency higher than any other candidates. The results emphasize candidate II as the most potent for most fatty acids production, specifically caproic and oleic acids (Fig. 4).

Although this study used methods that look for PUFAs-producing bacterial species, it highlights a bacterial species that is most efficient in producing SFAs, mainly *n*-caproic acids. Substantially, the results of screening the fatty acids contents of six marine microbes showed salinity as an adaptive evolutionary regulator, which stabilizes the evolutionary trait of the MUFAs and SFAs accumulation in comparison with PUFAs, at least for these screened microbes (Figs. 4 and 5). Both MUFAs and SFAs are industrially valuable in enhancing fuel properties. Thus, finding a microbial species with an evolved trait of SFAs production will minimize the cost of biodiesel production, and will help improve its features.

Bacterial identification and phylogenetics

The partial 16S rDNA revealed that candidate II is *Bacillus subtilis* and its 16S rDNA sequence was deposited on NCBI (accession number OM946589.1). The Blastn algorithm showed that its closest subspecies is *Bacillus subtilis* spizizenii TU-B-10, with no gaps and a 100 % identity, which covered the query sequence that includes all 16S rDNA hypervariable regions from V1 to V9 (Fig. 6). However, the phylogenetic relationship of MZ1 with bacterial type strains revealed its uniqueness and its close affinity with numerous halophilic *Bacillus* species such as *B. swezeyi*, *B. haynessi* and *B. mojavensis*, where they all clustered in a distinct clade (Fig. 7). The *B. subtilis* MZ1 is phylogenetically closely related to many other halotolerant/marine *Bacilli* species (Figs. 6 and 7). For examples, *B. cabrialesii* and *B. tequilensis* can tolerate up to 2 % and 5 % NaCl, respectively. Additionally, the closest subspecies to MZ1 strain, which is *B. spizizenii* can tolerate 7 % NaCl (de los Santos Villalobos *et al.*, 2019). Also, *B. swezeyi*, *B. haynessi* and *B. mojavensis* that are clustered in a distinct clade with MZ1 (Fig. 7) can tolerate 12 %, 12 % and 10 % NaCl (w/v), respectively (Reimer *et al.*, 2022).

Lipid-production optimization

Bacillus subtilis MZ1 had the highest levels of total lipid production (78.5 %) and the highest levels of production for each individual fatty acid when compared to the other candidates studied. *Bacillus subtilis* MZ1 was screened for its ability to grow on a variety of different media, and it was found to prefer nutrient broth, and seawater-based nutrient broth over the other growth media tested, particularly those based on natural wastes like sugarcane and banana peels (Fig. 8). The seawater-based nutrient broth was then chosen to adjust its components to maximise lipid production by *B. subtilis* MZ1 on it. First, the ability of lipid biosynthesis to reduce TTC into TPF was adjusted utilising diverse carbon and nitrogen sources, as well as variable pH and temperature degrees. The optimal conditions for *B. subtilis* MZ1 to reduce TTC into TPF were a pH of 7 and a temperature of 37 °C (Fig. 9). However, giving

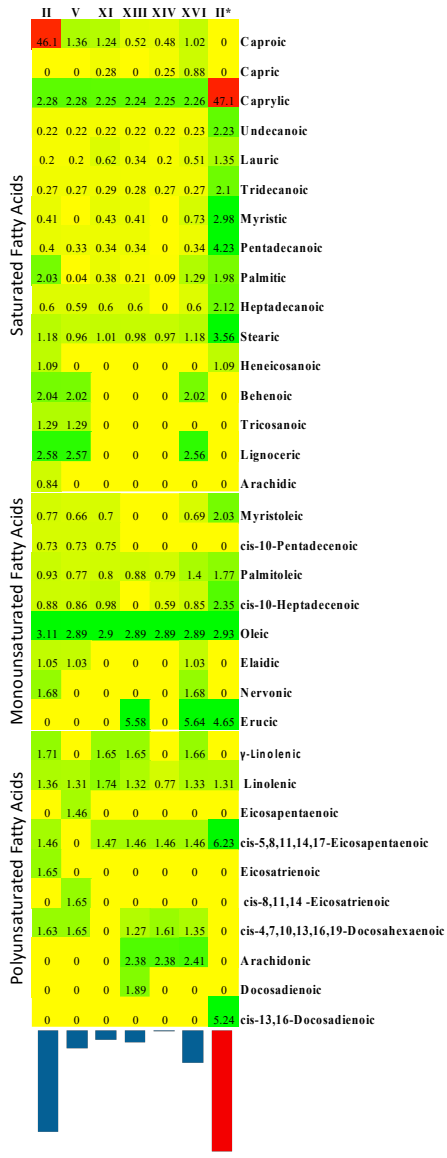

Figure 4. Heatmap displaying the FAME compositions of the selected bacterial candidates, after one day of growth on seawater-based nutrient broth cultures. Each column represents the fatty acid methyl ester composition for a bacterial pellet besides the supernatant. Each bacterial candidate has a Latin number, and the showed candidates were selected out of thirty-five unknown bacterial species. The last column (II*) represents candidate II that has been cultured on modified nutrients were glucose and ammonium sulfate were used as alternative carbon and nitrogen sources, respectively. The inverted bars represent the strength of the total fatty acid production for each candidate. The number shown in each color represents the concentration of each specified fatty acid in percentage.

Figura 4. Un mapa de calor que muestra las composiciones de FAME de los candidatos bacterianos seleccionados, después de un día de crecimiento en cultivos de caldo de nutrientes a base de agua de mar. Cada columna representa la composición de éster metílico de ácido graso para un pellet bacteriano además del sobrenadante. Cada candidato bacteriano tiene un número latino, y los candidatos mostrados fueron seleccionados de entre treinta y cinco especies bacterianas desconocidas. La última columna (II*) representa el candidato II que ha sido cultivado en nutrientes modificados, donde se utilizaron glucosa y sulfato de amonio como fuentes de carbono y nitrógeno alternativas, respectivamente. Las barras invertidas representan la intensidad de la producción total de ácidos grasos para cada candidato. El número mostrado en cada color representa la concentración de cada ácido graso especificado en porcentaje.

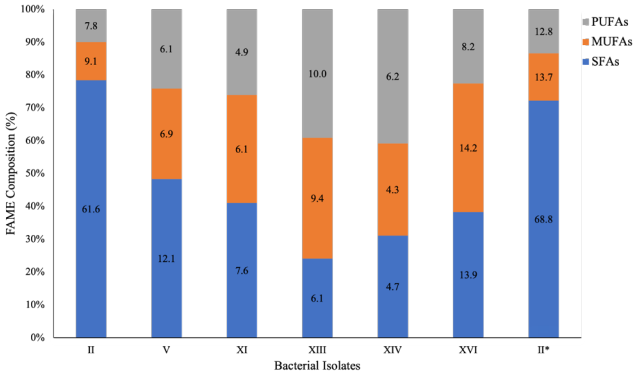

Figure 5. The total concentrations of saturated, monounsaturated, and polyunsaturated fatty acids as indicated by the FAME profiles.
Figura 5. Las concentraciones totales de ácidos grasos saturados, monoinsaturados y poliinsaturados según se indica en los perfiles de FAME.

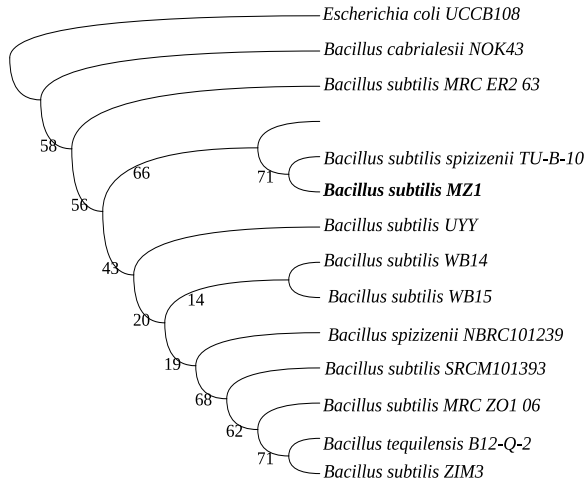

Figure 6. The Maximum likelihood phylogenetic tree constructed based on 16S rDNA sequences of different *Bacilli* species. The 16S rDNA sequence of the *E. coli* UCCB108 is the outgroup. The accession numbers for the utilized sequences are mentioned beneath the respective genus names.
Figura 6. El árbol filogenético de máxima verosimilitud construido en base a secuencias de ADNr 16S de diferentes especies de Bacilli. La secuencia de ADNr 16S de *E. coli* UCCB108 es el grupo externo. Los números de acceso de las secuencias utilizadas se mencionan debajo de los nombres de los géneros respectivos.

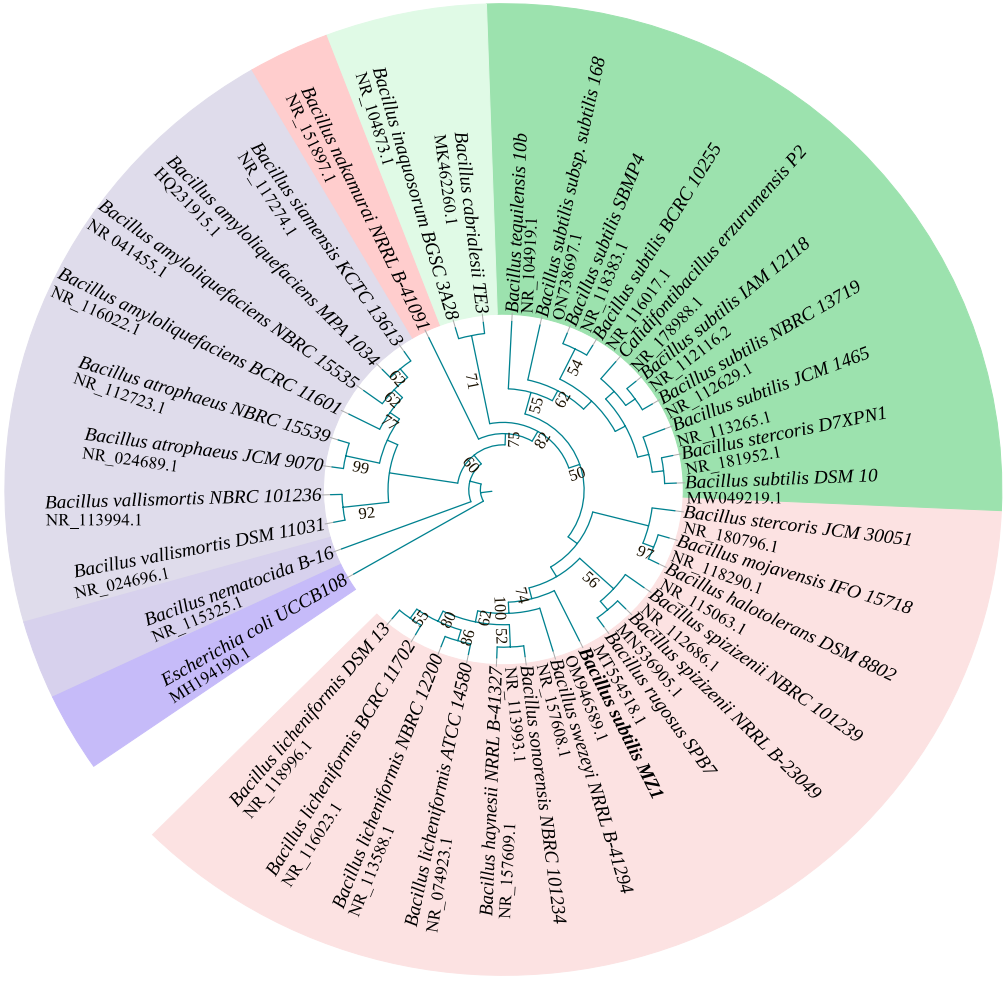

Figure 7. The Maximum likelihood phylogenetic tree based on 16S rDNA sequences of different bacterial strains. The 16S rDNA sequence of the *E. coli* UCCB108 is used as an outgroup. The accession numbers for the utilized sequences are mentioned beneath the respective genus names.
Figura 7. El árbol filogenético de máxima verosimilitud construido en base a secuencias de ADNr 16S de diferentes cepas bacterianas tipo. La secuencia de ADNr 16S de *E. coli* UCCB108 se utiliza como grupo externo. Los números de acceso de las secuencias utilizadas se mencionan debajo de los nombres de los géneros respectivos.

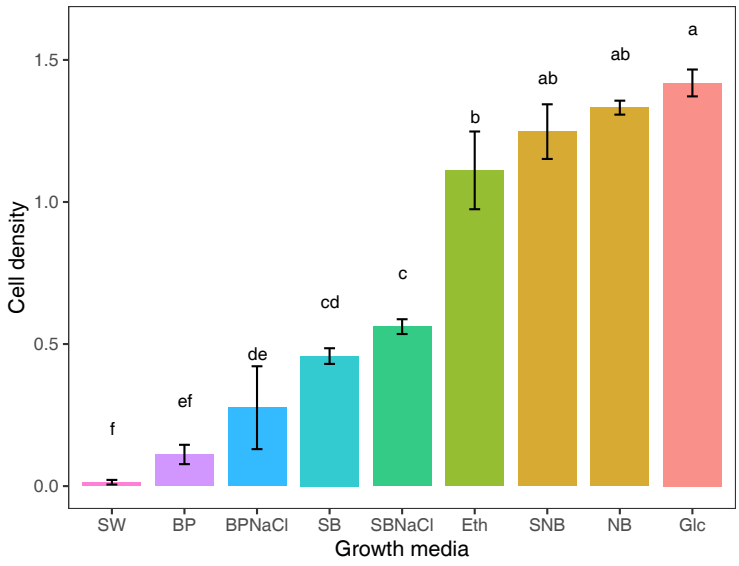

Figure 8. Growth density of *B. subtilis* MZ1 on different growth media. Glc stands for nutrient broth medium where glucose was used instead of the yeast extract; NB nutrient broth; SNB is nutrient broth where seawater was used to dissolve its components; Eth stands for nutrient broth medium where ethanol was used instead of the yeast extract; SBNaCl implicates growth medium that contains only sugarcane bagasse extract dissolved in seawater; SB implicates growth medium that contains only sugarcane bagasse extract; BPNaCl refers to growth medium that encloses only banana peel extract dissolved in seawater; BP denotes to growth medium that has banana peel extract only; and SW implicates seawater only.

Figura 8. La densidad de crecimiento de *B. subtilis* MZ1 en diferentes medios de crecimiento, donde Glc representa el medio de caldo de nutrientes donde se utilizó glucosa en lugar del extracto de levadura; NB caldo de nutrientes; SNB es caldo de nutrientes donde se utilizó agua de mar para disolver sus componentes; Eth representa el medio de caldo de nutrientes donde se utilizó etanol en lugar del extracto de levadura; SBNaCl implica medio de crecimiento que contiene solo extracto de bagazo de caña de azúcar disuelto en agua de mar; SB implica medio de crecimiento que contiene solo extracto de bagazo de caña de azúcar; BPNaCl se refiere a medio de crecimiento que contiene solo extracto de cáscara de plátano disuelto en agua de mar; BP denota medio de crecimiento que tiene solo extracto de cáscara de plátano; y SW implica solo agua de mar.

diverse nitrogen sources had no obvious influence on TTC reduction, as demonstrated in Fig. 9. Surprisingly, glucose, rather than the other sources of carbon, shown a significant impact on enhancing TTC to TPF conversion (Fig. 9).

To test the effect of modifying the growth medium composition on lipid profile, the nutritional growth medium was employed with glucose as an extra carbon source and ammonium sulphate as another nitrogen source at pH 7 and 37 °C. Lipid production of *B. subtilis* MZ1 is mostly rich with caproic acid, when grown on SNB or caprylic acid, when glucose was used as an additional carbon source in the SNB growth medium. From the applied perspectives, *n*-caproic acids are vital in the biodiesel industry, as flavor compounds, and as antimicrobial agents (Zhu *et al.*, 2017). Additionally, the MZ1

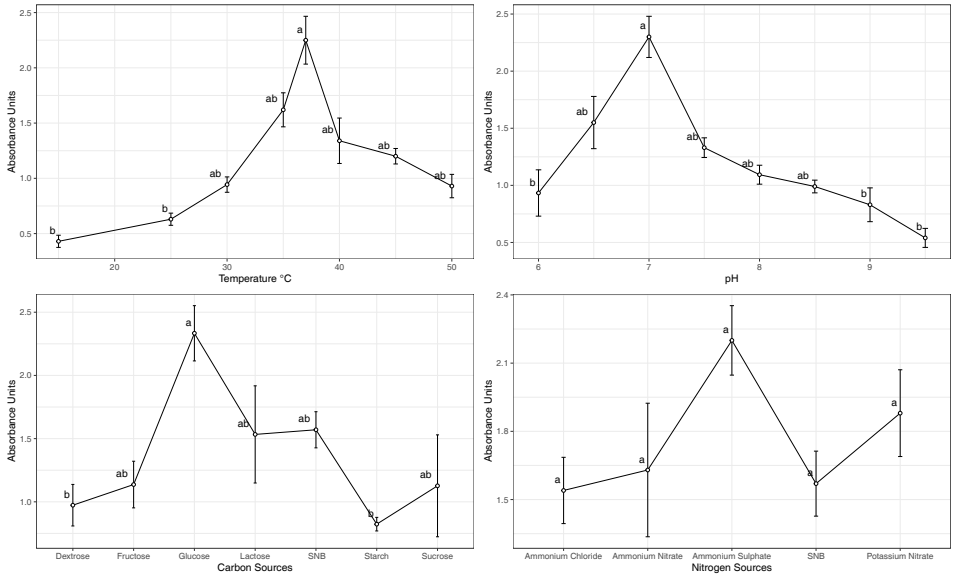

Figure 9. Effect of different temperature, pH points, carbon and nitrogen sources, on *B. subtilis* MZ1 ability to reduce TTC into TPF as expressed by the absorbance of TEF at 450 nm on SNB growth media. Each represented value is the average of three replicas, and each bar shows the standard error among these replicas. The significance among different conditions was measured by Tukey's Honest Significant Difference (Tukey's HSD, $P < 0.05$) using Agricolae-package in R language (R Development Core Team, 2013; De Mendiburu and Simon, 2015). Identical letters indicate that the difference is not statistically significant.

Figure 9. El efecto de diferentes temperaturas, puntos de pH, fuentes de carbono y nitrógeno en la capacidad de *B. subtilis* MZ1 para reducir TTC a TPF, expresado por la absorbancia de TEF a 450 nm en medios de crecimiento SNB. Cada valor representado es el promedio de tres réplicas, y cada barra muestra el error estándar entre estas réplicas. La significancia entre las diferentes condiciones se midió mediante la Prueba de Diferencia Significativa Honestidad de Tukey (Tukey's HSD, $P < 0.05$) utilizando el paquete Agricolae en el lenguaje R (R Development Core Team, 2013; De Mendiburu y Simon, 2015). Las letras idénticas indican que la diferencia no es estadísticamente significativa.

strain produces SFAs such as myristic, pentadecanoic, stearic, besides other MUFAs such as oleic, erucic; and other PUFAs such as cis-5,8,11,14,17-eicosapentaenoic, and cis-13,16-docosadienoic in considerable amounts. The results demonstrated that the modified growth medium efficiently changed the *B. subtilis* MZ1 metabolic pathways to create octanoic acid (C8:0, caprylic) instead of hexanoic acid (C6:0, caproic). This metabolic shift suggests that by managing the given feedstock, it is possible to improve carbon chain elongation and produce more valuable longer-chain carboxylic acids (Figs. 4 and 8), and as is shown by the gas chromatograms. We suppose that elongation is the most likely to happen in our case throughout the process of the reverse β-oxidation (Magdalena *et al*., 2020).

CONCLUSIONS

In sum, this paper reports that the marine *B. subtilis* MZ1 is a good source for *n*-caproic acids production, identified by FAME analysis and 16s rDNA sequencing. Additionally, the results show that the creation of the fatty acids can be maintained by using the seawater-based nutrient broth. Furthermore, manipulating growth media composition controls the profile of the produced fatty acids. For example, using glucose as an additional carbon source enhances the production of caprylic acid rather than caproic acid, because glucose oxidation may add one molecule of Acetyl-CoA to the C6:0, caproic acid. Also, many other PUFAs and MUFAs have been noted to accumulate. This research suggests the *B. subtilis* MZ1 as a competent strain to produce fatty acids, and this finding may help improve biodiesel production or using this microbial strain, as mentioned above, as an energy source for various kinds of applications.

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