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# Screening transesterifiable lipid accumulating bacteria from sewage sludge for biodiesel production

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ABSTRACT

Sewage sludge was evaluated as high available and low cost microbial oils feedstock for biodiesel production. Samples from four different wastewater treatment plants from La Araucanía Region in Southern Chile presented total lipids content ranging between 7.7 and 12.6%, being Vilcún sewage sludge that with the highest transesterifiable lipids content of about 50% of the total extracted lipids. The most relevant identified bacteria present in sludge samples were *Acinetobacter*,*Pseudomonas* and *Bacillus*, being *Bacillus* sp. V10 the strain with the highest transesterfiable lipids content of 7.4%. *Bacillus* sp. V10 was cultured using urban wastewater supplemented with glucose to achieve nitrogen depleted medium and using milk processing wastewater as a low-cost carbon source. *Bacillus* sp. V10 lipid profile indicates that low degree unsaturated long chain fatty acids such as C18:1 may account for approximately 50% of the lipids content, indicating its suitability to be used as raw material for biodiesel production.


## 1. Introduction

In 2010, liquid fuel consumption in the world reached 87 million barrels per day and it is projected to increase up to 122 million barrels of liquid fuel per day in 2040, reducing fossil fuels reserve and acting as a driving force behind the search for alternative fuels [1]. Nowadays, biodiesel is one of the most important alternative biofuel due to lower emissions generation (particularly hydrocarbons, CO and particulate matter) compared to diesel performance [2] and the absence of sulfur content. Biodiesel is mainly produced by transesterification, reaction that occurs between an acylglycerol (from vegetal oils or animal fats), and a short chain alcohol (methanol or ethanol) in the presence of a catalyst.

So-called "first generation biodiesel" is produced from virgin edible vegetable oils as soybean, rapeseed, sunflower, palm and coconut oil, where feedstock costs may account for about 80% of the total biofuel production cost [3]. Non-edible vegetable oils used in second generation biodiesel production such as jatropha, castor, karanja, pongamia, babassu, neem, tobacco and rubber seed oil, may have lower prices than edible oils and could be available to produce biodiesel without competing with food oils [4]. Third generation biodiesel production is nowadays focused on the use of microbial oils such as microalgae, bacteria, yeast and fungi [5–10].

Municipal sewage sludge is a by-product generated in wastewater treatment facilities after primary and secondary treatment processes and could be considered as one of the most interesting potential feedstock for biodiesel production in the future. Wastewater treatment processes produce two main types of sludge: a primary sludge, normally a combination of organic and inorganic matter with gas bubbles trapped within the suspension and a secondary sludge, also called activated sludge, mainly composed of microbial cells and suspended solids produced during

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aerobic biological wastewater treatment [11]. In addition, a third kind of sludge can be considered, namely digested sludge, which is a mixture of primary and activated sludge that has been stabilized through the anaerobic digestion process [11]. Raw primary sludge lipids content on a dry basis could be in the range of 20–26% [12], while activated sludge lipids content may range between 2 and 54% [13–15]. Nevertheless, as pointed out by [16] there are still challenges to be faced in the production of biodiesel from sewage sludge, such as determining the best way to collect and treat the different fractions for increasing the lipids extraction yield.

In particular, activated sludge contains a microbial population responsible for wastewater treatment and is mainly composed by heterotrophic bacteria. These bacteria use the organic compounds contained in wastewater to grow or as energy and carbon storing compounds, mainly as lipid droplets such as triacylglycerol (TAG) [6,17]. The biosynthesis of TAG is common between some filamentous bacterial species belonging to the Actinomycetales order (*Mycobacterium*, *Streptomyces, Nocardia* and *Rhodococcus*), which have been defined as oleaginous bacteria since they can accumulate more than 20% of their biomass as lipids [17,18]. It is well known that microbial culture conditions (carbon and nitrogen sources, aeration, temperature, etc.) can affect microbial intracellular lipids concentration and composition [9]. In this regard, the use of wastewater sludge has demonstrated to be a suitable inoculum for TAG biosynthesis by oleaginous microorganisms using a wide range of inexpensive carbon and nutrients sources [19,20]. In addition, sewage sludge has been recently considered as a source of lipids for biodiesel production by using direct transesterification [21–23].

The purpose of this research was to screen transesterifiable lipid accumulating bacteria from sewage sludge obtained from four wastewater treatment plants belonging to the Araucanía Region of southern Chile and to explore the potential of selected bacteria from these sewage sludge samples to accumulate lipids for biodiesel production under specific culture conditions.

## 2. Materials and methods

### 2.1. Sewage sludge

Sewage sludge (SS) was collected from the sedimentation tanks of wastewater treatment plants belonging to four localities of the Araucanía Region in southern Chile. The samples were transported to the laboratory and stored at −20 °C.

### 2.2. Lipids extraction from sewage sludge

Bligh and Dyer [24] modified method was used to extract the lipids contained in sewage sludge samples. Briefly, 3 g (wet weight) with a solids content ranging between 13.2 and 16.7%, were mixed with a 15 mL of chloroform ($CHCl_3$):methanol (MeOH) mixture (1:2 v/v ratio). The mixture was shaken in vortex for 1 min. Then 15 mL chloroform was added and shaken for 1 min. Finally, 10 mL distilled water were added to the mixture, shaken in vortex for 1 min followed by centrifugation at 13,000 × *g* for 10 min. The chloroform phase containing lipids (bottom phase) was separated and the solvent was removed by evaporation.

### 2.3. Transesterifiable lipids fraction from sewage sludge

Identification and quantification of transesterifiable lipids of sewage sludge was achieved by esterification of the extracted lipids to methyl esters according to the methodology described by Sathish and Sims [25] with some modifications and subsequent analysis by gas chromatography. Briefly, lipids (50 μL) were firstly hydrolyzed by the addition of 1 mL of a 0.5 M potassium hydroxide solution in methanol at 100 °C during 5 min, followed for the addition of 400 μL of a 4:1 (v/v) HCl/methanol solution and heated to 100 °C for 5 min. The resulting FAMEs were extracted from the acidified methanol phase after the reaction with 3 mL of petroleum ether (boiling point between 35 °C and 60 °C). Chromatographic analysis was carried out using a Clarus 600 chromatograph coupled to a flame ionization detector from PerkinElmer (GC-FID) according to the method described by the Comité Européen de Normalization (EN14103). An Elite-5MS capillary column with a length of 30 m, thickness of 0.1 μm and internal diameter of 0.25 mm was used. The vials were prepared by adding 10 μL of sample to 233 μL methyl heptadecanoate as an internal standard (initial concentration of 2060 mg $L^{-1}$). FAME yield (% based on lipid content) was calculated as the ratio between methyl ester mass (g) and lipid mass (g) multiplied by 100.

### 2.4. Bacteria isolation and culture

Bacteria isolation was performed from collected SS using the method described by Hamaki et al. [26] with some modifications. A solid culture media based on SS extract was prepared using 150 g of dry SS and incubated in 300 mL NaOH 50 mM overnight at room temperature. After incubation, the mixture was centrifuged at 13,000 × *g* for 40 min and the supernatant was filtered (1.2 μm) obtaining a sludge-media. The pH of the sludge-media was adjusted to 6.8. Finally, 1 g agar–agar was added for each 100 mL of sludge-media and autoclaved at 121 °C for 15 min at 1 atm.

Activated sludge samples (5 g) were suspended in distilled water (50 mL) and serial dilutions ($10^{-1}$ and $10^{-5}$) were performed. Portions of 50 μL of each dilution were spread onto agar plates containing the sludge-media and incubated at 30 °C for 96 h. Single colonies were randomly picked up from the culture on SS agar, which represented the most abundant phenotypes (color, brightness, form, elevation and margin). Then, the selected colonies were purified by streaking on new agar plates for 24 h at 30 °C. After that, pure single colonies were grown on plates with nutritive media and stored in glycerol at −80 °C.

### 2.5. Bacteria characterization

Firstly, a preliminary characterization of isolated strains was carried out by microscopic observations and gram staining. Genetic characterization of each isolated strains was also carried out by partial sequencing of 16S rRNA genes (Macrogen Inc., Korea). The sequences obtained were compared with those present in GenBank database from the National Center for Biotechnology Information (NCBI) by using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The search was done using the non-redundant nucleotide collection and optimized for highly similar sequences using Megablast. The nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers from KP099624 to KP099639.

### 2.6. Lipid content in isolated strains by gravimetric method

Microbial lipids extraction was performed following the Bligh and Dyer [24] methodology with some modifications. Briefly, 50 L of a bacterial culture were centrifuged at 13,000 × *g* for 15 min and the cell pellet was washed with deionized water and suspended in 100 mL of sodium chloride solution (1.0% NaCl). After that, the cell suspension was centrifuged and the final pellet was stored at −20 °C overnight. Frozen biomass was freeze-dried and subsequently stored at −20 °C.

In covered flasks, 100 mg of freeze-dried cells were blended with 114 mL solvent in the following sequence: chloroform, methanol and water, to achieve a final ratio of 1:2:0.8. Samples were shaken for 15 s after adding each solvent, allowing then