Biotechnology Reports 8 (2015) 116–123

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

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

Mara Cea[a,b], Naiane Sangaletti-Gerhard[c], Pedro Acuña[a], Idi Fuentes[a], Milko Jorquera[a,d], Karina Godoy[a], Francisco Osses[a], Rodrigo Navia[a,b,e,*]

[a] Scientific and Technological Bioresource Nucleus, University of La Frontera, P.O. Box 54-D, Temuco, Chile
[b] Department of Chemical Engineering, University of La Frontera, P.O. Box 54-D, Temuco, Chile
[c] Laboratory of Oils and Fats, Department of Agro-food Industry, Food and Nutrition, College of Agriculture "Luiz de Queiroz" (ESALQ), University of São Paulo, Piracicaba, São Paulo, Brazil
[d] Department of Chemical Sciences and Natural Resources, University of La Frontera, P.O. Box 54-D, Temuco, Chile
[e] Centre for Biotechnology & Bioengineering (CeBiB), University of La Frontera, P.O. Box 54-D, Temuco, Chile

ARTICLE INFO

*Article history:*
Received 16 May 2015
Received in revised form 22 September 2015
Accepted 29 October 2015
Available online 4 November 2015

*Keywords:*
Activated sludge
*Bacillus* sp.
Biodiesel
Lipids
Fatty acid methyl esters

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

* Corresponding author at: University of La Frontera, Department of Chemical Engineering, P.O. Box 54-D, Temuco, Chile. Fax: +56 45 2732402.
*E-mail addresses:* mara.cea@ufrontera.cl (M. Cea), naisangaletti@yahoo.com.br (N. Sangaletti-Gerhard), pacunaquijada01@gmail.com (P. Acuña), i.fuentes01@ufromail.cl (I. Fuentes), milko.jorquera@ufrontera.cl (M. Jorquera), karina.godoy@ufrontera.cl (K. Godoy), f.osses03@ufromail.cl (F. Osses), rodrigo.navia@ufrontera.cl (R. Navia).

http://dx.doi.org/10.1016/j.btre.2015.10.008

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

samples to stand for about 6 h with occasional manual agitation. Phase separation of the biomass-solvent mixtures in the separation funnels was achieved by adding chloroform and water to obtain a final chloroform:methanol:water ratio of 1:1:0.9. Chloroform phase (bottom phase) was evaporated and recovered lipids were determined by gravimetric method

### 2.7. Lipid content in isolated strains by Nile red fluorescence method

Neutral (transesterifiable) and polar lipids content were determined using flow cytometry according to the methodology described by Lopes da Silva et al. [27]. Briefly, 1 mL of cell suspension ($\sim 10^6$ cells/mL) was mixed with 10 μL of a Nile Red solution (0.033 mg/mL) and incubated for 2 min at 37 °C in darkness. Nile Red fluorescence was determined using a FACs Canto II flow cytometer of double laser (Becton Dickinson instrument). Upon excitation by the 488 argon laser, Nile Red exhibits yellow and red fluorescence when dissolved in neutral and polar lipids, detected by the channels FL2 and FL3. Non-staining cells were used as autofluorescence control. A semi-quantitative determination of the transesterifiable lipids was performed using this methodology by Chen et al. [28]. A standard curve based in the fluorescence generated by triolein in a range of concentration (0–30 $\mu g\,mL^{-1}$) was used to establish the transesterifiable lipid content of each strain in percentage of total biomass.

### 2.8. Bacillus *sp. V10 growth kinetics and lipids characterization*

Based on lipid content determinations, *Bacillus* sp. V10 was selected as the most suitable strains for biodiesel production and was therefore cultured for biomass accumulation and lipids profile characterization. *Bacillus* sp. V10 was cultured using urban wastewater (kinetic trial K1 in Table 1) supplemented with glucose as carbon source (kinetic trials K2 and K3 in Table 1) to achieve a medium with higher C/N ratio for an efficient lipids accumulation yield. In addition, *Bacillus* sp. V10 was cultured in milk processing wastewater as a low-cost C source (kinetic trial K4 in Table 1). Kinetic trials were then performed in shaken (150 rpm) Erlenmeyer flasks at 30 °C during 10 days, taking samples every 12 h in a laminar flow chamber and analyzing biomass concentration spectrometrically at 660 nm. Specific growth rate (μ), biomass productivity ($g\,L^{-1}\,day^{-1}$) and lipid productivity ($mg\,L^{-1}\,day^{-1}$) were calculated from the obtained data to assess growth characteristics of *Bacillus* sp. V10. The quantification of lipids produced by *Bacillus* sp. V10 was performed using the Nile red fluorescence method, as described before.

## 3. Results and discussion

### 3.1. Content and characterization of lipids in sewage sludge

Lipids content of sewage sludge (SS) from four different wastewater treatment plants ranged between of 7.7–12.6%, where the high lipid contents not necessarily implies a high content of transesterifiable lipids (Fig. 1). This result is expected as lipids extraction is related to solvents characteristics such as polarity, volatility, immiscibility with water and boiling point. In our case, the extraction method used considered a chloroform:methanol mixture. Using this mixture it was possible to extract glycerides and other compounds, resulting in a higher lipids extracted mass compared to transesterifiable lipids (neutral lipids). Transesterifiable lipids were however present in all samples, demonstrating a high potential of transesterifiable lipids synthesis by SS microorganisms. The Vilcún locality showed the highest content of transesterifiable lipids in SS, representing about 50% of the total extracted lipids. On the opposite, transesterifiable lipids content in SS of the other wastewater treatment facilities represent not more than 20% of total lipids (Fig. 1). Given these results, bacteria from SS were isolated and identified for further evaluation of their ability to produce transesterifiable lipids.

**Table 1**
Chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN) in urban wastewater (K1), in wastewater supplemented with glucose (K2 and K3) and in milk processing wastewater (K4).

| Kinetic trial | COD (mg/L) | TKN (mg/L) | COD/TKN (C/N) |
|---|---|---|---|
| K1 | 272 | 47 | 5.8 |
| K2 | 1686 | 47 | 35.9 |
| K3 | 2528 | 47 | 53.8 |
| K4 | 6743 | 187 | 36.1 |

### 3.2. Screening of bacteria from sewage sludge

Sixteen different bacterial strains were isolated from the four SS through culture in sludge-media and agar. The morphological and some physiological characteristics of the cultures are given in Table 2. Most of the isolates were rod-shape, both gram negative and positive, followed by isolates with coccus and spherical morphology. The facilities of Pucón and Traiguén presented rod-shape and mainly gram negative isolates. The isolates belonging to Lonquimay and Vilcún facilities presented the more diverse morphology including filamentous, rod and spherical shape morphologies.

The results of 16S rRNA gene sequencing indicated that 16 strains we characterized, belonging to eight different genus (see the phylogenetic tree in Fig. 2). Three of the eight genus showed similarity to *Acinetobacter*, *Pseudomonas* and *Bacillus* genera, which have previously been studied on biodiesel production or lipids accumulators [20,29]. In particular, *Acinetobacter* has been already reported as lipids accumulators present in the biomass of activated sludge [20]. *Acinetobacter* has been previously defined as oleaginous bacteria and produces a high oil content of more than 20% of its total biomass [29]. *Acinetobacter sp.* accounts for less than 10% of the sewage sludge bacteria [30]; however, it is a bacterium commonly present in wastewater treatment plants and therefore, it is available as a microbial lipids source for biodiesel production [31].

In the case of *Pseudomonas*, enzymes from *Pseudomonas* have been used as biological catalysts in the transesterification process for biodiesel production. These enzymes include lipases from *Pseudomonas fluorescens* [32], *Pseudomonas cepacia* [33] and *Pseudomonas aeruginosa* [34], acting in lipids biosynthesis and promoting lipids solubilization by hydrolyzing triglycerides. Regarding *Bacillus*, *Bacillus subtilis* HB1310 has been described as an oleaginous microorganism isolated from thin-shelled walnut. *B. subtilis* H1310 is able to reach a lipid content of 39.8% in 48 h, when cultured in cotton stalk hydrolysate as substrate [35]. In addition, *Bacillus* lipases are easily produced and display high tolerance toward organic solvents, proving them useful in the synthesis of esters for food industry, cosmetics and biodiesel production [36].

### 3.3. Lipids characterization of isolated bacterial strains

Fig. 3 shows total lipids content in dry biomass of each isolated strain. Total lipids were found in the range between 3.1 and 10.7%. Clearly, relevant differences exist in neutral or transesterifiable lipids between isolated strains. Higher neutral lipids content were observed in strains isolated from Vilcún (V) and Traiguén (T) wastewater treatment plants, suggesting that microbial oils in terms of neutral lipids from strains *Pseudomonas* sp. T2 (3.6%), *Pseudomonas* sp. T15 (4.8%), *Acinetobacter* sp.V4 (3.0%), and *Bacillus* sp. V10 (7.4%) are suitable for biodiesel production. In particular, *Bacillus sp.* V10 is the strain with the highest neutral lipids content and therefore the most suitable for biodiesel production. In this

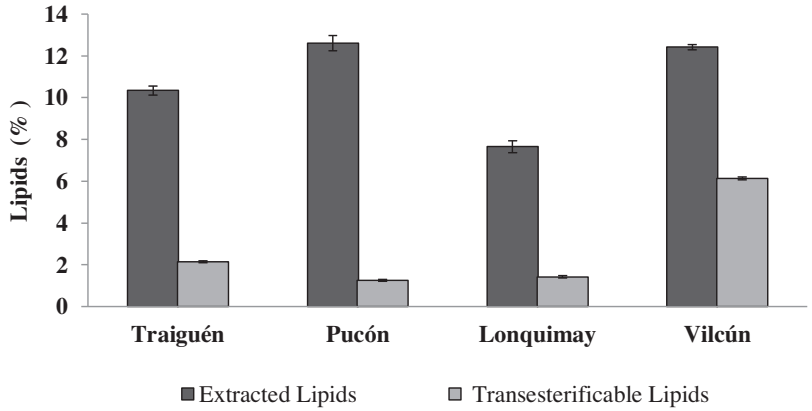


**Fig. 1.** Total and transesterifiable lipids contained in sludge biomass. The error bars represent the standard deviation of three independent replicates.

sense, *Bacillus* sp. V10 was selected for kinetic trials growth for both, biomass accumulation performance and lipids accumulation and characterization. The results of 16S rRNA gene sequencing and BLAST indicate that *Bacillus* sp. V10 matches in a 99% with *Bacillus thuringiensis* and *Bacillus cereus. B. thuringiensis* can be naturally found on leaf surfaces, aquatic environments, animal feces, insect-rich environments, flour mills and grain-storage facilities, contrary to this, *B. cereus* in responsible of alimentary intoxications and is naturally present in soils [37]. In addition, *Bacillus* sp. V10 matches in a 93% with the aforementioned oleaginous endophyte *B. subtilis* HB1310.

### 3.4. Bacillus *sp. V10 growth kinetics and lipids characterization*

*Bacillus* sp. V10 was selected as the most suitable strain for biodiesel production and was therefore cultured in urban

**Table 2**
Bacterial strains isolated from sewage sludge belonging to the Lonquimay, Pucón, Vilcún and Traiguén wastewater treatment facilities.

| Locality | Strain | Colony characteristics on nutritive agar | Morphology | Gram reaction | Closet relatives or cloned sequences (accession no.) | Similarity[a] (%) | Accession no. |
|---|---|---|---|---|---|---|---|
| Pucón | *Acinetobacter* sp. P4 | White, dry | Coccobacilary rod | – | *Acinetobacter* sp. with oil activity isolated from coastal and marine ecosystems (KM370367) | 99 | KP099626 |
| | *Citrobacter* sp. P5 | Small white transparent | Rod | – | *Citrobacter freundii* from membrane bioreactor activated sludge (KF938666) | 98 | KP099627 |
| | *Klebsiella* sp. P9 | White and small | Rod | – | *Klebsiella oxytoca* from treatment of cosmetic wastewater by submerged membrane bioreactor (KC593550) | 99 | KP099628 |
| | *Pseudomonas* sp. P11 | Yellow, dry | Rod | – | *Pseudomonas* sp. from oil production water (JX997893) | 99 | KP099629 |
| | *Microvirgula* sp. P14 | Yelow | Rod | – | *Microvirgula aerodenitrificans* from wastewater treatment plants (NR029204) | 99 | KP099630 |
| Lonquimay | *Citrobacter* sp. L11 | Brown, creamy | Coccus | – | *Citrobacter freundii* from membrane bioreactor activated sludge (KF938666) | 98 | KP099625 |
| | *Lysinibacillus* sp. L4 | Light brown, creamy | Sphere | + | *Lysinibacillus* sp. from stored swine manure (KF856718) | 97 | KP099624 |
| | *Lysinibacillus* sp. L6 | Transparent white, dry | Sphere | + | *Lysinibacillus* sp. from stored swine manure (KF856718) | 99 | KP099639 |
| Traiguén | *Pseudomonas* sp. T1 | Filamentous white | Filamentous | – | *Pseudomonas frederiksbergensis* crude oil degrading bacteria from Qinghai-Tibet (KF704095) | 99 | KP099631 |
| | *Pseudomonas* sp. T2 | Transparent white | Rod | – | *Pseudomonas* sp. from Antarctic lakes (KF301575) | 99 | KP099632 |
| | *Pseudomonas* sp. T15 | Transparent white, dry | Rod | – | *Pseudomonas mandelii* crude oil degrading bacteria from Qinghai-Tibet (KF704105) | 99 | KP099633 |
| Vilcún | *Acinetobacter* sp. V4 | White transparent | Rod | – | *Acinetobacter* sp. with oil activity isolated from coastal and marine ecosystems (KM370367) | 99 | KP099635 |
| | *Bacillus* sp. V7 | White | Rod filamentous | + | *Bacillus subtilis* from wastewater treatment plants (KF453784) | 95 | KP099367 |
| | *Bacillus* sp. V8 | Brown | Coccus | + | *Bacillus subtilis* from wastewater treatment plants (KF453784) | 95 | KP099634 |
| | *Microbacterium* sp. V9 | Yellow | Coccus | + | *Microbacterium luticocti* from sewage sludge compost (AM747814) | 91 | KP099636 |
| | *Bacillus* sp. V10 | White, dry | Coccobacilary | + | *Bacillus subtilis* from wastewater treatment plants (KF453784) | 94 | KP099638 |

[a] Based on partial sequencing of 16S gene and comparison with those present in GenBank by using BLAST. The search was done using the non-redundant nucleotide collection and optimized for highly similar sequences using Megablast.

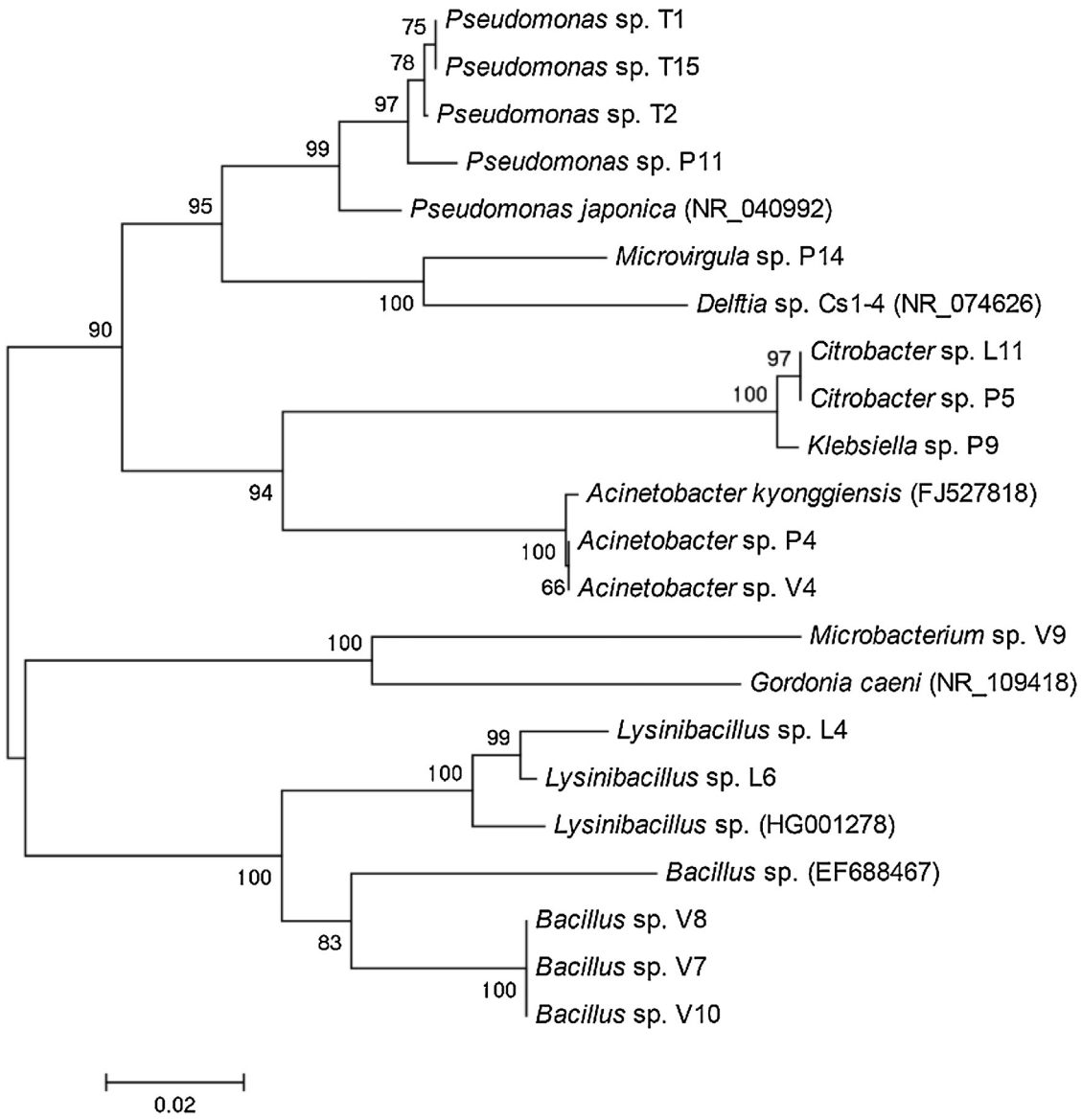


**Fig. 2.** Phylogenetic tree showing the taxonomic affiliation of selected strains in relation to the presentative 16S rRNA gene sequences of bacteria from sewage sludge and deposited in Genbank. The neighbor-joining tree was constructed based on some sequences of control taken from the NCBI database and by using Mega 6 software. Scale of bar indicate 2% of divergence and bootstrap analysis was performed with 1000 trials.

wastewater supplemented with glucose as carbon source (kinetic trials K2 and K3 in Table 1) to achieve a medium with different C/N ratios. In addition, *Bacillus* sp. V10 was cultured in milk processing wastewater as a low-cost C source (trial K4 in Table 1). The results show that no cellular growth occurred during kinetic trial K1 due to the low nutrients availability. However, kinetic trials K2–K4 showed a significant growth of *Bacillus* sp. V10.Lag phase for K2 and K3 ends at about 96 h, while the exponential growth phase ended for both trials at about 190 h. Afterwards, the stationary phase ended at about 230 h, followed by a declining phase for both trials due to nutrients consumption. Contrary to this, a faster acclimation of *Bacillus* sp. V10 was observed in trial K4, where the lag phase was not observed and the exponential phase started before 12 h and ended at 48 h approximately. After that, trial K4 showed a stationary phase of around 48 h, similar to those observed for trials K2 and K3. The calculated specific growth rates ($\mu$) of 0.018, 0.019 and 0.016 for trials K2, K3 and K4, respectively, indicate that when *Bacillus* sp. V10 reached the exponential phase, a slightly growth increase in urban waste water supplemented with glucose was observed.

K2–K4 trials presented a high neutral (transesterifiable) lipids content (Fig. 4). K4 presented the highest neutral lipid content in microbial cells (6.1%) at 48 h, followed by K3 which reached a neutral lipid content of 5.3% at 180 h and, K2 with about 4.3% at 192 h. This behavior can be compared with the results obtained by Singh et al. [38] in their study performed with the microalgae *Chlorella vulgaris*. In their study authors state that fast growth rarely correlates with high total lipid content. Our results show that *Bacillus* sp. V10 is able to accumulate up to 6.1% of neutral lipids in 48 h with a biomass and lipid productivity of 0.046 g $L^{-1}$ $day^{-1}$ and 2.81 mg $L^{-1}$ $day^{-1}$. Singh et al. [38] found a total lipid content ranging between 7.066 and 27.6% and total lipid productivity between 0.748 and 5.381 mg $L^{-1}$ $day^{-1}$ for sixteen isolates of *Chlorella vulgaris*. Moreover, they determined a biomass productivity ranging between 0.006 and 0.019 g $L^{-1}$ $day^{-1}$. Considering all these values we could suggest that *Bacillus* sp. V10 could

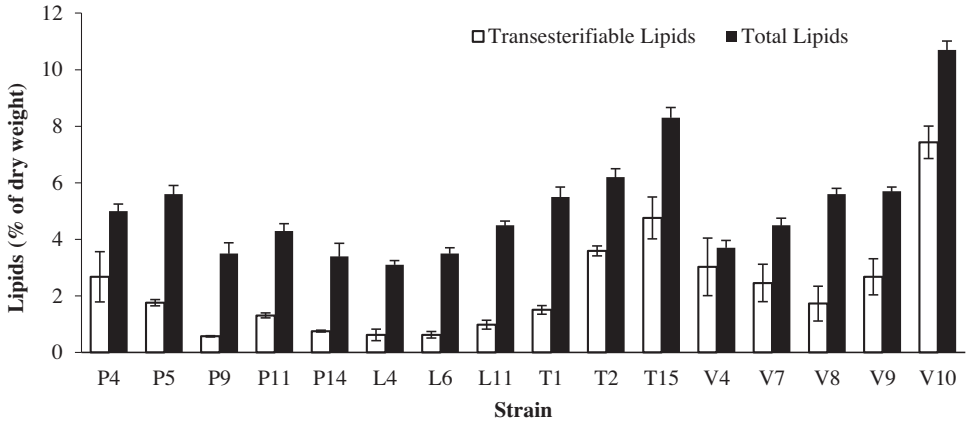


**Fig. 3.** Total and transesterifiable lipids contained in strains isolated from different sewage sludge samples determined by gravimetric and Nile Red methods, respectively. The error bars represent the standard deviation of three independent replicates. P4: *Acinetobacter* sp. P4, P5: *Citrobacter* sp. P5, P9: *Klebsiella* sp. P9, P11: *Pseudomona* sp. P11, P14: *Microvirgula* sp. P14, L11: *Citrobacter* sp. L11, L4: *Lysinibacillus* sp. L4, L6: *Lysinibacillus* sp. L6, T1: *Pseudomona* sp. T1, T2: *Pseudomona* sp. T2, T5: *Pseudomona* sp. T5, V4: *Acinetobacter* sp. V4, V7: *Bacillus* sp. V7, V8: *Bacillus* sp. V8, V9: *Microbacterium* sp. V9, V10: *Bacillus* sp. V10.

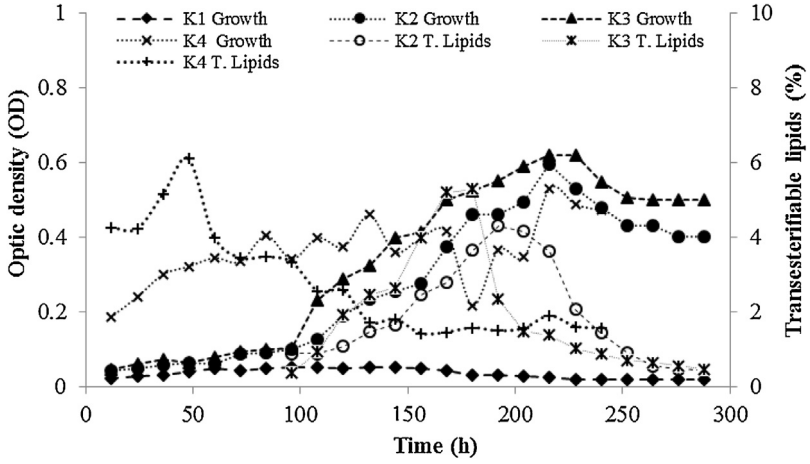


**Fig. 4.** Growth kinetic trials (K1–K4) and transesterifiable lipids (K2–K4) of *Bacillus* sp. V10 using different COD/NTK ratios and substrates.

be a proper candidate as a lipid accumulator for biodiesel production.

Normally, lipids accumulation in microbial cells starts when a specific nutrient concentration such a nitrogen drastically declines in the growth medium and the carbon excess (in the form of glucose in our case) is transformed by microorganisms in lipids mainly in form of TAG, wax esters and polyhydroxyalkanoates (PHA) [6]. Similar to our results, the oleaginous *B. subtilis* HB1310 tends to accumulate lipids when glucose is provided as the carbon source [35]. With limited nitrogen concentration (or a

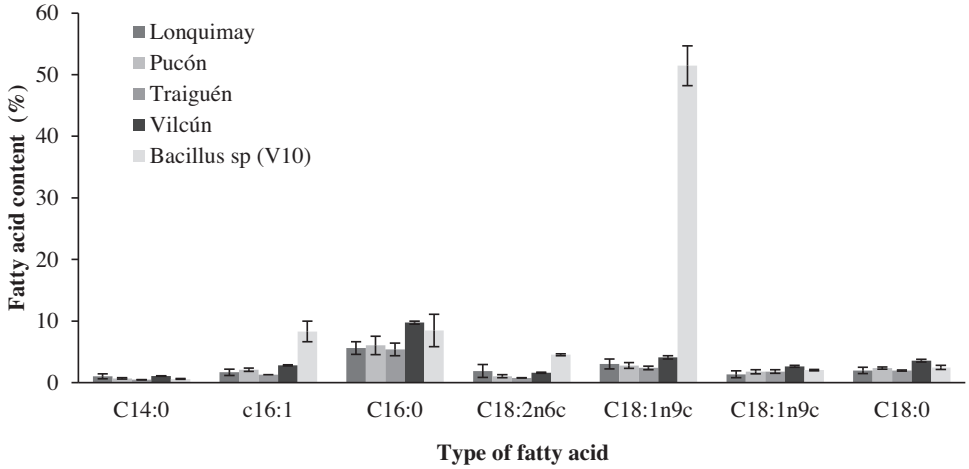


**Fig. 5.** Lipids profile of the different sewage sludge samples compared to *Bacillus* sp. V10. The error bars represent the standard deviation of three independent replicates.

high C/N ratio) in the growth medium lipids accumulate inside the cells which are not able to divide themselves, promoting lipids accumulation [29]. With a C/N ratio of 50/1, high lipid content was obtained by *Bacillus* sp. V10, showing a similar behavior compared to *B. subtilis* HB1310 [35] and *B. subtilis* (RRL-8) from of marine sponges [39]. These microorganisms were able to accumulate a lipid content of 39.8% and 33.4%, respectively. However, *Bacillus* sp. V10 was able to growth and accumulate a higher lipid content in milk processing wastewater with C/N ratio of 36/1. Therefore, growing *Bacillus* sp. V10 in this type of wastewater can be used simultaneously for nutrients removal as well as bacterial biomass growth. This approach can contribute to wastewater treatment technology by providing an environmentally sustainable process, as the harvested biomass can be used as feedstock for biodiesel production thus reducing the total cost associated to the wastewater treatment.

Under limited nitrogen conditions, genera such as *Streptomyces* and *Rhodococcus* accumulate TAG predominantly during the stationary growth phase, while during the exponential growth phase the synthesis of phospholipids is predominant [18]. Also, the aforementioned *B. subtilis* HB1310 and *B. subtilis* (RRL-8) started to accumulate lipids during the stationary phase. In our case, we found that *Bacillus* sp. V10 reached its maximum neutral lipids accumulation at the mid-exponential phase (see Fig. 4). Finally, at the end of the kinetic trials, chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN) were determined in all growth media. In all kinetic trials *Bacillus sp.* V10 consumed the available energy sources being these consumption values of 68.4, 68.7, 47.9 and 63.4% for COD in K1–K4 respectively. In the case of TKN, the observed consumption values were 16.0, 38.9, 41.9, and 58.2%, reaching final COD/TKN ratios of 2.2, 17.1, 48.3 and 31.7 for K1–K4, respectively.

In addition to total and neutral lipids content, the lipids profile of *Bacillus sp.* V10 compared to SS samples from the four facilities was determined (see Fig. 5). The lipids profile from *Bacillus* sp. V10 indicates that low degree unsaturated long chain fatty acids such as C18:1 may account for approximately 50% of the lipids content, showing that this lipids profile is suitable and it could be used as raw material for biodiesel production. *B. subtilis* (RRL-8) screened from marine sponges for single cell oil and polyunsaturated fatty acids (PUFA), presented a similar lipid profile compared to *Bacillus sp.* V10 with C18:1 accounting for up to 43.6% [38]. On the contrary, *B. subtilis* HB1310 isolated from thin-shell walnut and grown in cotton stalk hydrolysate, presented a C18:1 content of only 3.8%, showing a higher C16:0 content of 28.33% [35].

## 4. Conclusions

Sewage sludge from wastewater treatment facilities can be considered as a high available and low cost microbial lipids feedstock for biodiesel production, promoting a more cost-effective production process. Sludge samples presented total lipids content between 7.7 and 12.6%, being Vilcún wastewater treatment sludge that with the highest transesterifiable lipids content of about 50% of the total extracted lipids. *Bacillus* sp. V10, from Vilcún wastewater treatment plant, presented the highest transesterifiable lipids content of 7.4%. *Bacillus sp.* V10 was also cultured using urban wastewater supplemented with glucose to achieve a medium with higher C/N ratio. In addition, *Bacillus* sp. V10 was cultured in milk processing wastewater as a low cost C source. *Bacillus* sp. V10 lipids profile indicates that low degree unsaturated long chain fatty acids such as C18:1 may account for approximately 50% of the lipids content, showing a suitable potential for biodiesel production. Future research will be focused on growing *Bacillus* sp. V10 at larger scale for biodiesel production, refining and characterization.

## Acknowledgements

This work was supported by CONICYT Chile through FONDECYT Projects 1120812 and 1150707.

## References

[1] U.S. Energy Information Administration (EIA). International Energy Outlook (DOE/EIA-0484/2014). 2014. www.eia.gov/forecasts/ieo/pdf/0484(2014).pdf. (accessed January 2015).
[2] V.K. Shahir, C.P. Jawahar, P.R. Suresh, Comparative study of diesel and biodiesel on CI engine with emphasis to emissions—a review, Renew. Sust. Energy Rev. 45 (2015) 686–697.
[3] M.K. Lam, K.T. Lee, A.R. Mohamed, Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: a review, Biotechnol. Adv. 28 (2010) 500–518.
[4] M.P. Dorado, in: A. Nag (Ed.), Raw Materials to Produce Low Cost Biodiesel. Biofuels Refining and Performance, McGraw-Hill, New York, USA, 2008, pp. 107–147.
[5] L. Azócar, G. Giudad, H. Heipieper, R. Navia, Biotechnological processes for biodiesel production using alternative oils, Appl. Microbiol. Biotechnol. 88 (2010) 621–636.
[6] E. Revellame, R. Hernandez, W. French, W. Holmes, T. Benson, J. Pham, A. Forks, R. Callahan, Lipid storage compounds in raw activated sludge microorganisms for biofuels and oleochemicals production, RSC Adv. 2 (2012) 2015–2031.
[7] S. Raimondi, M. Rossi, A. Leonardi, M. Bianchi, T. Rinaldi, A. Amaretti, Getting lipids from glycerol: new perspectives on biotechnological exploitation of *Candida freyschussii*, Microb. Cell Fact. 13 (2014) 83.
[8] P. Hidalgo, C. Toro, G. Ciudad, S. Schober, M. Mittelbach, R. Navia, Evaluation of different operational strategies for biodiesel production by direct transesterification of microalgal biomass, Energy Fuels 28 (2014) 3814–3820.
[9] X. Zhang, S. Yan, R.D. Tyagi, R.Y. Surampalli, J.R. Valéro, Wastewater sludge as raw material for microbial oils production, Appl. Energy 135 (2014) 192–201.
[10] D. Mattanovich, M. Sauer, B. Gasser, Yeast biotechnology: teaching the old dog new tricks, Microb. Cell Fact. 13 (2014) 34.
[11] F. Markis, J.C. Baudez, R. Parthasarathy, P. Slatter, N. Eshtiaghi, Rheological characterisation of primary and secondary sludge: Impact of solids concentration, Chem. Eng. J. 253 (2014) 526–537.
[12] R. Willson, Z. Wiesman, A. Brenner, Analyzing alternative bio-waste feedstocks for potential biodiesel production using time domain (TD)-NMR, Waste Manage. 30 (2010) 1881–1888.
[13] E. Jardé, L. Mansuy, P. Faure, Characterization of the macromolecular organic content of sewage sludges by thermally assisted hydrolysis and methylation-gas chromatography–mass spectrometer (THM-GC/MS), J. Anal. Appl. Pyrol. 68–69 (2003) 331–350.
[14] V. Reveille, L. Mansuy, E. Jardé, E. Garnier-Sillam, Characterization of sewage sludge-derived organic matter: lipids and humic acids, Org. Geochem. 34 (2003) 615–627.
[15] E. Jardé, L. Mansuy, P. Faure, Organic markers in the lipidic fraction of sewage sludges, Water Res. 39 (2005) 1215–1232.
[16] D.M. Kargbo, Biodiesel production from municipal sewage sludges, Energy Fuel 24 (2010) 2791–2794.
[17] M.K. Gouda, S.H. Omar, L.M. Aouad, Single cell oil production by Gordonia sp. DG using agro-industrial wastes, World J. Microb. Biotechnol. 24 (2008) 1703–1711.
[18] H. Alvarez, A. Steinbuchel, Triacyl glycerols in prokaryotic microorganisms, Appl. Microbiol. Biotechnol. 60 (2002) 367–376.
[19] Y.H. Seo, I.G. Lee, J.I. Han, Cultivation and lipid production of yeast Cryptococcus curvatus using pretreated waste active sludge supernatant, Bioresour. Technol. 135 (2013) 304–308.
[20] E. Muller, A.R. Sheik, P. Wilmes, Lipid-based biofuel production from wastewater, Curr. Opin. Biotechnol. 30 (2014) 9–16.
[21] A. Mondala, K. Liang, H. Toghiani, R. Hernandez, T. French, Biodiesel production by in situ transesterification of municipal primary and secondary sludges, Bioresour. Technol. 100 (2009) 1203–1210.
[22] O.K. Choi, J.S. Song, D.K. Cha, J.W. Lee, Biodiesel production from wet municipal sludge: Evaluation of in situ transesterification using xylene as a cosolvent, Bioresour. Technol. 166 (2014) 51–56.
[23] N. Sangaletti-Gerhard, M. Cea, V. Risco, R. Navia, In situ biodiesel production from greasy sewage sludge using acid and enzymatic catalysts, Bioresour. Technol. 179 (2015) 63–70.
[24] E.G. Bligh, W.J. Dyer, A rapid metod of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[25] A. Sathish, R. Sims, Biodiesel from mixed culture algae via a wet lipid extraction procedure, Bioresour. Technol. (2012) 643–647.
[26] T. Hamaki, M. Suzuki, R. Fudou, Y. Jojima, T. Kajiura, A. Tabuchi, K. Sen, H. Shiba, Isolation of novel bacteria and actinomycetes using soil-extract agar medium, J. Biosci. Bioeng. 99 (2005) 485–492.
[27] Lopes, S. da, T. ilva, D. Feijão, A. Reis, Using multi-parameter flow cytometry to monitor the yeast Rhodotorula glutinis CCMI 145 batch growth and oil production towards biodiesel, Appl. Biochem. Biotechnol. 162 (2010) 2166–2176.
[28] W. Chen, C.W. Zhang, L.R. Song, M. Sommerfeld, Q. Hu, A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae, J. Microbiol. Methods 77 (2009) 41–47.

[29] X. Meng, J. Yang, X. Xu, L. Zhang, Q. Nie, M. Xian, Biodiesel production from oleaginous microorganisms, Renew. Energy 34 (2009) 1–5.
[30] M. Wagner, R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi, K: Schleifer Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge, Appl. Environ. Microbiol. 60 (1994) 792–800.
[31] K. Phuong, S. Hanazaki, K. Kakii, T. Nikata, Involvement of Acinetobacter sp. in the floc-formation in activated sludge process, J. Biotechnol. 157 (2012) 505–511.
[32] A. Salis, M.S. Bhattacharyya, M. Monduzzi, V. Solinas, Role of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase used for biodiesel synthesis, J. Mol. Catal. B-Enzym. 57 (2009) 262–269.
[33] Q. Li, Y. Yan, Production of biodiesel catalyzed by immobilized *Pseudomonas cepacia* lipase from *Sapium sebiferum* oil in micro-aqueous phase, Appl. Energy 87 (2010) 3148–3154.
[34] Q. Ji, S. Xiao, B. He, X. Liu, Purification and characterization of an organic solvent-tolerant lipase from *Pseudomonas aeruginosa* LX1 and its application for biodiesel production, J. Mol. Catal. B-Enzym. 6 (2010) 264–269.
[35] G. Zhang, Y. Li, L. Xia, An oleaginous endophyte *Bacillus subtilis* HB1310 isolated from thin-shelled walnut and its utilization of cotton stalk hydrolysate for lipid production, Biotechnol. Biofuels 752 (1) (2014) .
[36] M. Guncheva, D. Zhiryakova, Catalytic properties and potential applications of Bacillus lipases, J. Mol. Catal. B-Enzym. 68 (2011) 1–21.
[37] M.T. Madigan, J.M. Martinko (Eds.), Brock Biology of Microorganisms, 11th ed., Prentice Hall, U.K, 2005.
[38] R. Singh, A. Kant, R. Singh, Screening of micro algae for Growth and lipid accumulation properties, J. Algal Biomass Util. 5 (1) (2014) 38–46.
[39] S. Patnayak, A. Sree, Screening of bacterial associates of marine sponges for single cell oil and PUFA, Lett. Appl. Microbiol. 40 (2005) 358–363.