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Production of Surface-Active Compounds by a Hydrocarbon-Degrading Actinobacterium: Presumptive Relationship with Lipase Activity

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Abstract The replacement of synthetic surface-active compounds (SACs) by their microbial counterparts is carving out a niche for themselves in the field of bioremediation. However, the high cost of microbial products has limited their application at a realistic scale. In the current study, several hydrocarbon-degrading microorganisms were assayed as potential SAC producers in low-cost liquid media. Only the strain CC10, placed within the class *Actinobacteria*, was able to produce emulsifying molecules by using a combination of sugarcane vinasse or crude glycerol (as cheap carbon substrates) with urea or peptone (as nitrogen sources). The emulsifying activity of the supernatants and the stability of emulsions formed with motor oil depended on the carbon and nitrogen sources. However, the biodegradability of these metabolites was only associated with the carbon substrate, and it was always higher than the two tested synthetic surfactants, sodium dodecyl sulfate and Triton X-100. Also, a positive linear association between emulsifying and lipase activities of the CC10 supernatants was detected ($r = 0.781$; $p = 0.219$), with the maximum activities detected in the glycerol-peptone supernatant. Interestingly, this supernatant was able to emulsify different oily substrates, a property that could be used to increase the efficiency of the treatment of effluents with high fat content.

Keywords Actinobacterium · Bioemulsifier · Lipase · Oily substrates · Vinasse · Raw glycerol

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1 Introduction

Surface-active compounds (SACs) are amphipathic molecules that exhibit activity at interfaces, by lowering the tension between them. According to their mode of action, there exist two main types of SACs: those that reduce the surface tension at air-water level (surfactants) and those that are more effective in a reduction in the interfacial tension between two immiscible liquids (emulsifiers) and that may display or not surfactant activity (Uzoigwe et al. 2015).

Singular properties of SACs enable their application in diverse biotechnological sectors. However, the tight regulations of the use of environmentally compatible products have encouraged the application of SACs derived from microbial sources to replace chemically synthesized compounds, since they are more acceptable because of their natural occurrence (Motevasel 2014). In the field of bioremediation, the critical role of microbial SACs in emulsification and solubilization of oily compounds by increasing their availability to microbial degradation has been well-documented (Franzetti et al. 2011; Meliani and Bensoltane 2014; Motevasel 2014;

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Ayed et al. 2015). At the same time, lipolytic enzymes can facilitate the SAC action by reducing the surface tension of liquids and/or improving the solubility of water-immiscible substrates (Sekhon et al. 2012). Among these enzymes are lipases (triacylglycerol-acyl-hydrolases) (EC 3.1.1.3), which are able to catalyze the hydrolysis of triacylglycerols and the esterification, transesterification, and interesterification of lipids. Some studies have reported on the microbial release of SACs and lipolytic enzymes under the form of complexes (Hanna et al. 2009; Sekhon et al. 2011). In fact, the combined use of both products for the treatment of effluents with a high fat content has been documented (Cammarota and Freire 2006; Damasceno et al. 2012; Silva et al. 2013).

Despite their singular properties, microbial SACs are not widely commercialized because of the high costs of production based upon the use of synthetic media (Colin et al. 2013a, 2014, 2016). For example, the lipopeptide surfactin produced by *Bacillus subtilis* has an estimated cost of over US$50 per milligram of product. In turn, it is estimated that the fermentation medium can represent almost 30% of the overall cost of a microbial fermentation (George and Jayachandran 2013). Therefore, the manufacture of these biomolecules from cheap feedstock could be the first step to ensure the sustainability of the biotechnological process. Biodegradability is also a property that should be considered for environmental risk assessment associated with the routine use of any product (European Parliament Regulation (EC) 2004). The real challenge is then to find new producer microorganisms as well as optimal production conditions to obtain competent and safe SACs.

Strains of various genera belonging to the class *Actinobacteria* were reported as SAC producers. For example, marine actinobacteria such as *Nocardiopsis* sp. B4 and *Brachybacterium paraconglomeratum* MSA21 produced biosurfactants when they were grown on different carbon and/or nitrogen sources (Khopade et al. 2012; Kiran et al. 2014). Species of the genus *Streptomyces* (*S.* sp. MC1 and *S.* sp. SS20) demonstrated also their ability to produce molecules with emulsifying activity using different carbon substrates (Colin et al. 2013b, 2016; Hayder et al. 2014). An indigenous actinobacterium of the genus *Amycolatopsis* was also reported as an effective emulsifier producer when it is grown on different carbon/nitrogen sources (Colin et al. 2013a).

It is almost an assumed property that microorganisms isolated from hydrocarbon-contaminated environments are able to produce SACs (Ruggeri et al. 2009). Based upon this presumption, a first study on the potential of hydrocarbon-degrading actinobacteria to produce SACs from cheap feedstock is presented. The presumptive relationship between the production of SACs and a lipolytic enzyme such as lipase was also evaluated.

2 Materials and Methods

2.1 Surfactant Activity Screening

The following hydrocarbon-degrading actinobacteria were assayed for production of SACs: *Amycolatopsis tucumanensis* DSM 45259 (ABO^T), isolated by Albarracín et al. (2005); *Streptomyces* sp. A2 and A12, isolated by Fuentes et al. (2010); and six new isolates recovered from the chronically hydrocarbon-contaminated soil in Patagonia, Argentina (Bourguignon 2016). The strains were screened on blood agar plates containing 5% (*v*/*v*) goat blood and incubated at room temperature for 7 days. Hemolytic activity was detected as the occurrence of a defined clear zone around a colony (Carrillo et al. 1996).

2.2 Formulation of Low-Cost Liquid Media and Culture Conditions

Spores of each microorganism selected after hemolytic activity were harvested from starch-casein agar slants and inoculated in 100 mL of liquid media at a final concentration of 1×10^5 CFU/mL. The basal liquid media (in g/L) contained K_2HPO_4, 0.5; $MgCl_2 \cdot 7H_2O$, 0.20; and $FeSO_4 \cdot 7H_2O$, 0.01. The carbon and nitrogen sources, named as C and N variables, were assayed at two levels (Table 1): 1% (*v*/*v*) sugarcane vinasse or raw glycerol was used as low-cost carbon source while 1.0 g/L urea or peptone was used as nitrogen source. The pH of the culture media was adjusted to 7.0 with NaOH prior to sterilization. After inoculation of spores, cul-

Table 1 Culture medium factors and their levels

Factors	Level 1	Level 2
Carbon source (C)	Vinasse	Glycerol
Nitrogen source (N)	Urea	Peptone

tures were incubated at 30 °C on an orbital shaker (150 rpm) for 96 h.

2.3 Microbial Biomass

After 96 h of incubation, cultures were centrifuged at 10,000×*g* for 15 min at 4 °C. Microbial biomass was washed with distilled water, and dry weight was determined using aluminum foil cups dried at 80 °C to constant weight. Supernatants were stored at − 20 °C for subsequent analysis.

2.4 Surfactant and Emulsifying Activitities

Stored supernatants were assayed for surfactant activity by qualitative hemolytic test according to Carrillo et al. (1996) with minimal modifications: 5 μL of each culture supernatant was sown on blood agar plates and incubated for 7 days in order to detect the presence/absence of hemolysis halos.

The emulsification index (E_{24}) was measured using the method described by Cooper and Goldenberg (1987), also with minimal modifications: a mixture of equal volumes of culture supernatant and a hydrophobic substrate such as motor oil was vortexed for 2 min. After being left to settle for 24 h, the E_{24} value was calculated as the percentage of the height of the emulsified layer (mm), divided by the total height of the liquid column (mm). Aging time of the emulsion was estimated by measuring the emulsification index again after being left to settle until 28 days.

Finally, the microbial SACs and two commercial synthetic surfactants, sodium dodecyl sulfate (SDS) and Triton X-100 (TX-100), were comparatively tested to emulsify oily substrates such as sunflower, soy, canola, olive, and grape oils.

2.5 Lipase Activity

Lipase activity in the supernatants was determined by spectrophotometric measurement using *p*-nitrophenyl palmitate (*p*-NPP) (Winkler and Stuckman 1979). One international unit of enzyme activity (IU) was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol/min.

2.6 Biodegradability Assay

Culture supernatants of strains identified as SAC producers were filtered through a dialysis tubing cellulose membrane (typical molecular weight cutoff = 14,000 Da) at 4 °C for 24 h. The concentrated aqueous extracts were used as partially purified SAC sources and assayed for biodegradability using the BOD/COD ratio, with BOD and COD as the biological and chemical oxygen demands, respectively. BOD and COD parameters were determined according to the Standard Methods for the Examination of Water and Wastewater (A.P.H.A. et al. 2012) and compared with the BOD/COD ratio of the SDS and TX-100.

2.7 Hydrolytic Treatment

Partially purified SACs were subjected to hydrolytic treatments with proteinase K (30 U/mg at 37 °C for 4 h), commercial lipase from *Candida rugosa* (100 U/mg at 37 °C for 1 h), and acid hydrolysis (10% HCl (*v*/*v*) at 100 °C for 10 min) in order to estimate the role of peptides, lipids, and sugars on the SAC nature. After each treatment, residual *E* values were measured again, with the extracts without treatments used as controls representing 100% emulsifying activity.

2.8 Molecular Identification

Total DNA extraction of the SAC-producing bacterium strain was performed using the CTAB method as described previously Ellis et al. (1999) with slight modifications. Universal primers 8F and 1493R (corresponding to positions 8–27 and 1492–1509, respectively, in the 16S rDNA sequence of *Escherichia coli*) were used to amplify the 16S rDNA by PCR, as described previously Quillaguamán et al. (2004). Amplification reactions were carried out in an automated thermal cycler (Perkine Elmer, model 9700, Applied Biosystems). PCR products were run on a 1.0% agarose gel stained with SYBR® Safe DNA gel stain and then visualized by image analysis using a Gel Doc system (Bio-Rad). The amplicons obtained were purified and sequenced by Macrogen Inc. (Seoul, Korea). The 16S rRNA gene sequence of the isolates was analyzed according to Tamura et al. (2011) and compared with databank 16S rRNA sequences of the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. (2012)) and the Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/; Cole et al. (2014)). Alignment was performed

by using MEGA 5 software. A phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei 1987), and the Jukes-Cantor correction method (1969) was used for distance correction. Only unambiguously aligned positions from all sequences were used, and gaps were not included in the match/mismatch count. The nucleotide sequences identified in this study were deposited in the NCBI nucleotide sequence database (GenBank/NCBI) under accession number KU987933.

2.9 Statistical Analysis

Statistical analysis was performed using Infostat (version 2004) and Minitab (version 16) softwares for Windows. Results are presented as means ± standard deviation, conducting all assays in triplicate. Statistically significant values for the means were assayed using one-way analysis of variance. Subsequent comparisons were performed using Tukey's post hoc test. Differences were accepted as significant when $p < 0.05$. The main effects and interactions of C and N variables on growth and metabolite production were evaluated using a 2^2 full-factorial design (Table 1). Associations between variables were assessed using Pearson's correlation coefficient (r).

3 Results and Discussion

3.1 SAC-Producing Microorganisms in Low-Cost Liquid Media

The ability of hydrocarbon-degrading strains to produce SACs was first assayed on blood agar plates. Clear hemolysis halos were only detected for the strains CC8, CC10, and N25 (Fig. 1). Consequently, these strains were selected for subsequent cultures in low-cost liquid media.

The three strains were able to grow in the liquid media formulated (data not shown), but the presence of SACs was only detected in the CC10 supernatants. Therefore, this strain was used for subsequent experiments.

The CC10 cell growth and metabolite production, measured as E_{24} and lipase activity, are shown in Table 2. Maximum growth was observed in the glycerol-peptone medium; biomass increased threefold compared to the vinasse-urea culture medium. Surfactant activity of the CC10 supernatants was not detected by the qualitative method applied in this study. However, the four supernatants showed emulsifying

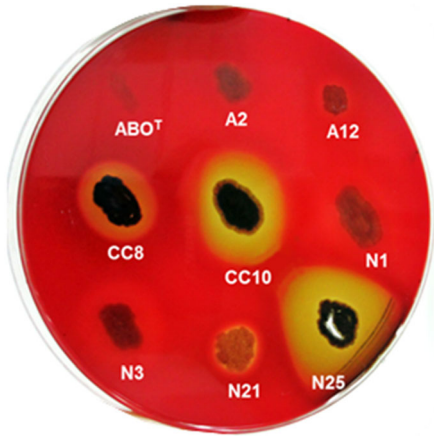

Fig. 1 Hemolysis halos produced by the isolates on a blood agar plate

properties on the motor oil, with a maximum E_{24} value detected for the glycerol-peptone supernatant (Table 2). Based on these findings, it was inferred that SACs produced in these conditions would be molecules with only emulsifying action. Interestingly, the presence of extracellular lipase activity was also detected, which greatly depended on the culture conditions (Table 2).

To estimate the standardized effects of C and N variables on growth and production of metabolites, a Pareto chart was performed (Fig. 2). From this analysis, it was inferred that biomass was mainly affected by the C variable and to a lesser extent by the N variable and CN interaction (Fig. 2a). However, both variables, as well as the interaction between them, had significant effects on the emulsifying ability of the supernatants (Fig. 2b). Interestingly, lipase activity mostly depended on the N substrate and it was less affected by the C source and the CN interaction (Fig. 2c).

As a result of the increased awareness of the use of natural products, microbial SACs are carving out a niche for themselves on the market. These biomolecules are especially used for environmental applications since their presence enhances the solubility and removal of the major contaminants from soil and water (Motevasel 2014; Montero-Rodríguez et al. 2015; Usman et al. 2016). Despite their singular properties, large-scale use of microbial SACs is often restricted because of economic obstacles.

The concept of circular economy considers the reuse of any type of waste as cheap feedstock, contributing to the development of a bio-based economy (Pleissner et al. 2016). For example, the northwest of Argentina has an important concentration of sugar industries coupled with

Table 2 Cell growth and metabolite production by strain CC10 in low-cost liquid media

C-N	Biomass (g/L)	E_{24} (%)	Lipase activity (IU/mL)
Vinasse-urea	0.30 ± 0.03^{a}	16 ± 0^{a}	0.59 ± 0.04^{b}
Vinasse-peptone	0.40 ± 0.01^{b}	18 ± 1^{a}	2.71 ± 0.13^{c}
Glycerol-urea	0.65 ± 0.01^{c}	19 ± 1^{a}	0.10 ± 0.01^{a}
Glycerol-peptone	0.91 ± 0.01^{d}	41 ± 3^{b}	4.00 ± 0.14^{d}
p values	< 0.0001	0.0038	< 0.0001

Values with different superscript letters (a–d) are significantly different ($p < 0.05$)

autonomous distilleries that generate a large vinasse volume during the ethylic alcohol production. This liquid effluent has a high organic load, representing a pollution hazard for areas where it is discharged. The use of vinasse as an alternative feedstock could be profitable for the competent manufacture of microbial SACs while the volume of effluent is significantly reduced (Colin et al. 2016). Regarding glycerol, the major by-product of the biodiesel production process, several companies are experiencing severe problems in how to get rid themselves of the excess glycerol as its disposal is quite expensive. As an alternative, a variety of projects are being conducted for the use in the crude form, as it has a very low value (de Souza Monteiro et al. 2012; Samul et al. 2014; Valerio et al. 2015).

Some studies have reported on the biosynthesis of microbial SACs from industrial wastes. For example, species of the genus *Bacillus*, such as *B. pumilus* and *B. subtilis* PC, demonstrated their ability to produce biosurfactants using vinasse as sole carbon source (Guerra de Oliveira and Garcia-Cruz 2013; de Lima and Rodríguez de Souza 2014). Colin et al. (2016) reported on the biosynthesis of emulsifying molecules by *Streptomyces* sp. MC1 when the strain was grown in a vinasse-based culture medium. On the other hand, the actinobacterium ABO^{T} was also reported as a bioemulsifier-producing strain when it is cultivated with glycerol as carbon substrate (Colin et al. 2013a). Similarly, taxa such as *Trichosporon mycotoxinivorans* CLA2 and *Pseudomana aeruginosa* demonstrated their potential to produce SACs when they were grown on crude glycerol (de Souza Monteiro et al. 2012; Eraqi et al. 2016). In the current work, the feasibility of strain CC10 to produce molecules with emulsifying activity using vinasse or raw glycerol as low-cost carbon substrates was effectively demonstrated. However, a preliminary study of the characteristics and functional properties of these biomolecules could help elucidate their potential for environmental applications.

3.2 Emulsification of Oily Substrates

The performance of three emulsifying agents to form and stabilize oily substrates in water was tested (Fig. 3). The glycerol-peptone supernatant was used as SAC microbial source as it showed the maximum emulsifying activity (see Table 2). As synthetic emulsifying agents, 0.01% SDS and 0.01% TX-100 were tested, since at this concentration, their emulsifying activities on the motor oil were similar to that detected for microbial supernatant (data not shown).

Under such assay conditions, synthetic surfactants were not able to emulsify fatty substrates (Fig. 3a, b). However, they showed a differential affinity for the aqueous and oil phases: While anionic surfactant SDS migrated towards the aqueous phase which turned white in color (Fig. 3a), non-ionic surfactant TX-100 showed a similar affinity for both phases (Fig. 3b). In contrast to synthetic surfactants, CC10 supernatant was able to emulsify all tested oils (Fig. 3c); with E_{24} values of 60, 58, 48, 37, and 22% for sunflower, soy, canola, olive, and grape oils, respectively.

In the present study, a positive linear association between emulsifying and lipase activities of the CC10 supernatants can be established ($r = 0.781$; $p = 0.219$). The joint action of these metabolites could contribute to the formation and stabilization of oily substrates in water. In fact, Gutnick et al. (2003) observed that mixtures containing an apoemulsan and a recombinant lipolytic enzyme (esterase) isolated from cell extracts formed very stable emulsions with highly hydrophobic substrates under conditions in which the emulsan itself was ineffective. The combination of lipolytic enzymes and SACs is often used to increase the treatment efficiency of hydrophobic wastewater (e.g.,

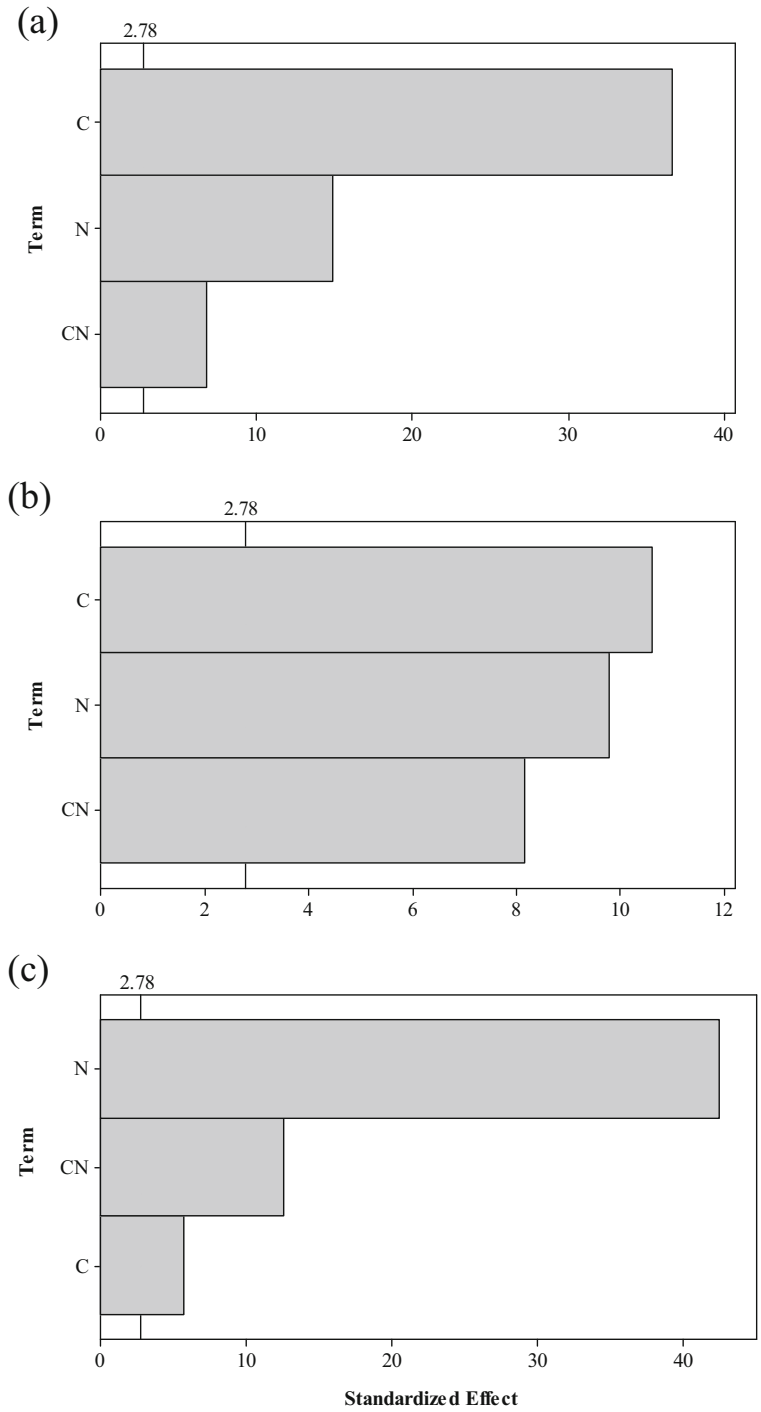

Fig. 2 Pareto chart of the standardized effects of C and N variables on the biomass (**a**), emulsification index (**b**), and lipase activity (**c**) of strain CC10

slaughterhouses wastewater) where lipids can represent until 70% of the COD. With respect to this, Silva et al. (2013) reported on the effective treatment of a fatty effluent using a homemade conformed by an extracellular lipase produced by *Penicillium simplicissimum* and a rhamnolipid surfactant obtained from *Pseudomona aeruginosa* PA1. Fortunately, synthesis and synchronized release of SACs and lipolytic enzymes was reported as beneficial to certain microorganisms (Colla et al. 2010; Doshi et al. 2010; Sekhon et al. 2011).

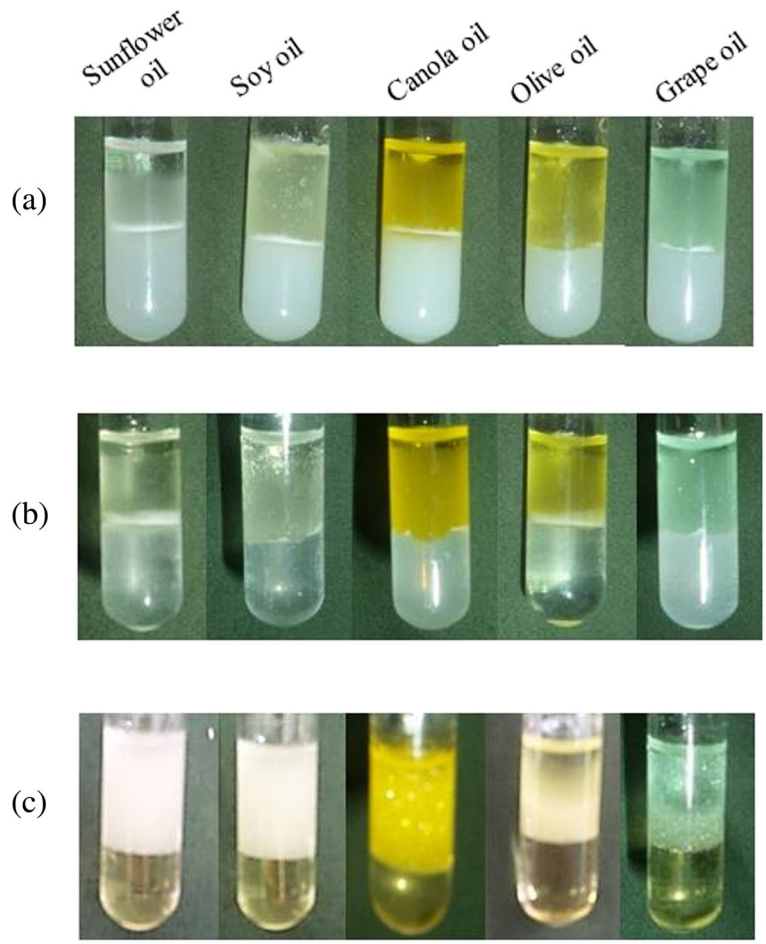

Fig. 3 Emulsification of fatty substrates using three emulsifying agents. 0.01% SDS (**a**). 0.01% TX-100 (**b**). CC10 supernatant harvested from a glycerol-peptone medium (**c**)

Simultaneous release of these metabolites is easily understood if one considers that SACs can emulsify hydrophobic compounds, facilitating the action of enzymes in the biodegradation process. Based upon this background, simultaneous production of emulsifying compounds and lipase enzyme by strain CC10 could be projected as a valuable biotechnological tool for the treatment of fatty effluents, simplifying both operational steps and treatment costs.

3.3 Properties and Presumptive Nature of SACs

Besides the attainment of low production costs, stability and ecotoxicological behavior of microbial SACs are also highly desirable characteristics. The destabilization of emulsions over time, known as the aging effect, often reflects a coalescence process resulting in an increase in drop size which can lead to their disappearance (Tcholakova et al. 2004). However, the tight linkage of SACs to dispersed oily pollutants in an environment is desirable, because this prevents them from merging together to facilitate the subsequent removal. In the current study, the stability of the emulsions formed between CC10 supernatants and motor oil was estimated by measurement of *E* value after being left to settle until 28 days. Glycerol-peptone supernatant showed the highest emulsifying activity on motor oil (see Table 2). However, a sudden drop in the emulsion height (about 27%) was detected after 7 days of setting (Fig. 4). Longer settling periods (14, 21, and even 28 days) did not significantly change the stability of this emulsion. Glycerol-urea, vinasse-urea, or vinasse-peptone supernatants had a different behavior since they showed less emulsifying properties (see Table 2). However, *E* values remained virtually unchanged throughout the assay time (Fig. 4). Despite this differential behavior, bioemulsifiers produced in the four conditions can be considered as acceptable in terms of stability, because their emulsions

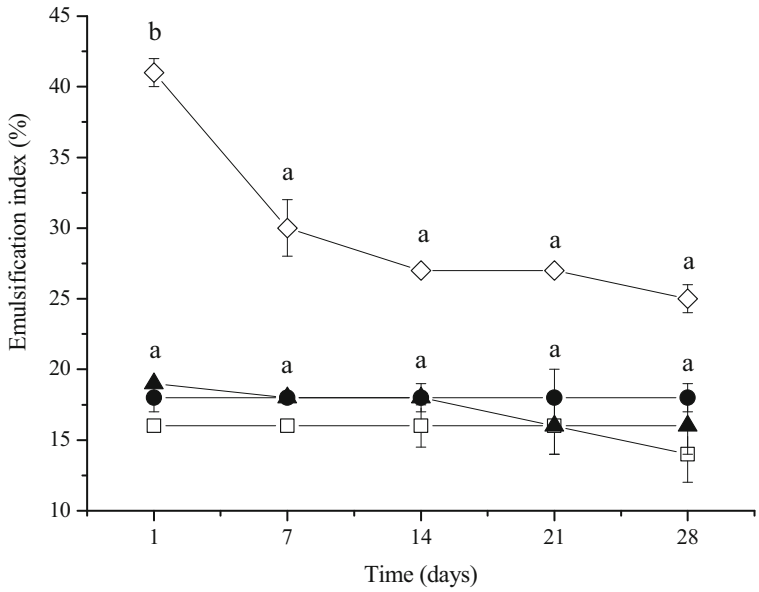

Fig. 4 Aging effect of the emulsions formed between motor oil and CC10 culture supernatants obtained from different carbon and nitrogen sources. Glycerol-peptone (diamond), glycerol-urea (triangle), vinasse-peptone (circle), and vinasse-urea (square). Values with different letters (a, b) are significantly different ($p < 0.05$)

retained more than 50% of the initial height even after long settling periods (Bosch et al. 1988).

Some years ago, the most representative European associations manifested their initiative to assess the environmental risk of SACs before their intensive application (Gheorghe et al. 2013). It is almost an assumed property that microbial SACs are biodegradable as a direct result of their natural origin. However, it is necessary to perform additional studies in order to confirm this feature (Gudiña et al. 2015). In the present work, a comparative study of the biodegradable character of the SDS, TX-100, and SACs produced by CC10 revealed important differences among them (Fig. 5). A virtually negligible BOD/COD ratio was detected for synthetic SACs, confirming their extremely low biodegradability. However, this ratio increased for SACs produced by CC10, particularly for cultures with glycerol as carbon substrate. The nitrogen source (urea or peptone) did not seem to have a significant effect on the biodegradability of the microbial products. These findings suggest the

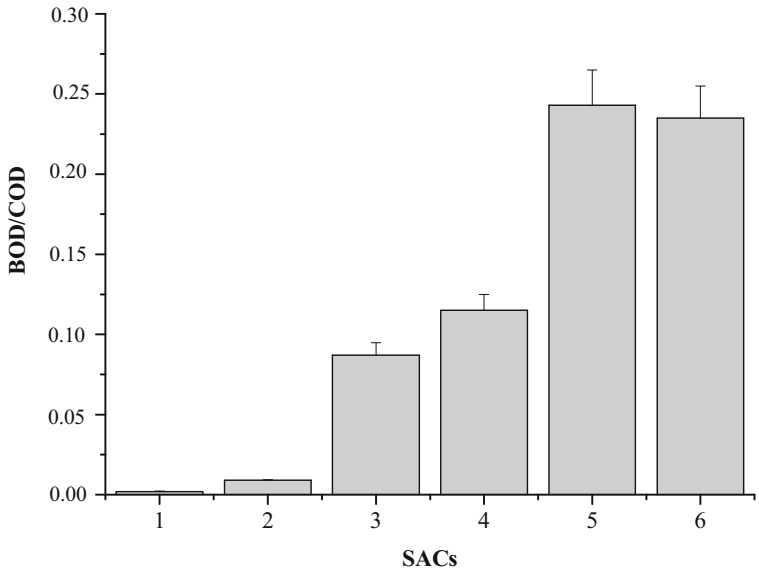

Fig. 5 Estimation of the biodegradability of SACs: 0.01% SDS (1), 0.01% TX-100 (2), bioemulsifiers from strain CC10 produced with vinasse-urea (3), vinasse-peptone (4), glycerol-urea (5), and glycerol-peptone (6)

Table 3 Effect of hydrolytic treatments on the E values of aqueous CC10 extracts

C-N	Hydrolytic treatment		
	Proteinase K	Commercial lipase	Acid hydrolysis
Vinasse-urea	–	–	++
Vinasse-peptone	–	–	++
Glycerol-urea	–	+	+
Glycerol-peptone	–	+	+

The notation +/− represents the presence/absence of hydrolytic treatment effects on the emulsifying ability of extracts

safety of these microbial products, encouraging their routine application for the development of bioremediation strategies, without detriment to the environment.

Finally, the role of peptides, lipids, and sugars on the emulsifying activity of SACs produced by CC10 was assayed with different hydrolytic treatments. As shown in Table 3, proteinase K and commercial lipase had no significant effect on the emulsifying properties of the SACs produced from vinasse. However, acid hydrolysis significantly reduced their emulsifying power. SACs synthesized from glycerol were not affected by treatment with proteinase K, but both lipolytic activity and acid hydrolysis decreased the emulsifying ability of the respective extracts (Table 3). Based upon hydrolysis assays, it was inferred that the chemical nature of the bioemulsifiers would be subjected to modifications according to the carbon substrate: While the use of vinasse seemed to encourage the biosynthesis of emulsifiers of polysaccharide nature, molecules obtained from glycerol seemed to have both lipid and sugar fractions. Similar findings were provided by Colin et al. (2013a) who demonstrated that the emulsifiers synthesized by actinobacterium ABO^T are chemically different depending upon the carbon substrate.

3.4 Phylogenetic Analysis of Strain CC10

To elucidate the taxonomic position of strain CC10, a potential producer of bioemulsifiers, finally, phylogenetic analysis was carried out. Macroscopic and microscopic observations, chemotaxonomic analysis, and 16S rDNA sequencing confirmed the placement of this strain within the class *Actinobacteria*. Sequence analysis of

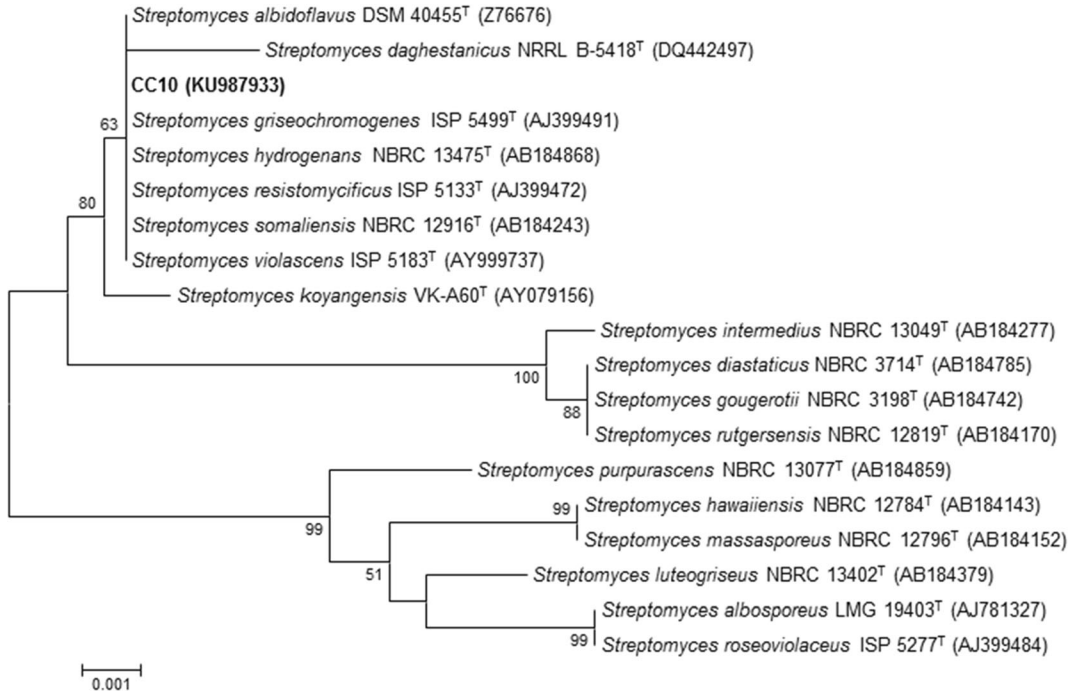

Fig. 6 Phylogenetic tree of a new actinobacterium isolate inferred by the neighbor-joining method (Saitou and Nei 1987) based on a comparison of nearly complete 16S rDNA sequences. Accession numbers of 16S rDNA sequences are given in parentheses. Numbers at the nodes indicate the level of bootstrap support; only values more than 50% are given. Bar corresponds to 0.001 substitutions per nucleotide position

the 16S rRNA gene of CC10 allowed to determine that the strain belonged to the genus *Streptomyces* (Fig. 6); it was closely related to *Streptomyces albidoflavus* DSM 40455T (100%), *Streptomyces griseochromogenes* ISP 5499 T (100%), *Streptomyces hydrogenans* NBRC 13475T (100%), *Streptomyces resistomycificus* ISP 5133 T (100%), *Streptomyces somaliensis* NBRC 12916 T, and *Streptomyces violascens* ISP 5183 T (100%).

4 Conclusions

In the present study, the feasibility of a novel actinobacterium to produce bioemulsifiers using sugarcane vinasse or raw glycerol as cheap carbon substrates was demonstrated. Chemical nature and properties of the bioemulsifiers depended on the carbon and nitrogen sources, with the biodegradability always higher than the two tested synthetic surfactants.

A correlation about 80% between the emulsifying and lipase activities of the CC10 supernatants was also detected, which could be used as a valuable biotechnological tool for the treatments of oily pollutants. In fact, an effective emulsification of oily substrates was only achieved by using CC10 supernatant as the emulsifying agent.

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