CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

**ISBN** 978-88-95608-56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864058

# Differential Protein Profiles of the Lipolytic Yeast *Candida palmioleophila* under Different Growth Conditions

Leidy Johanna Rincón[a], Bayron Agualimpia[a], German Zafra[a,b]*

[a]Universidad de Santander UDES, Grupo de Investigación en Ciencias Básicas y Aplicadas para la Sostenibilidad – CIBAS. Campus Lagos del Cacique, Calle 70 No. 55-210, Bucaramanga, Santander, 680003, Colombia.
[b]Current address: Universidad Industrial de Santander, Escuela de Microbiología. Bucaramanga, Santander, 680002, Colombia.
gzafra@udes.edu.co

This work aimed to stablish the influence of different carbon and nitrogen sources, including vegetable oils, on the variability of the protein profiles of an oil and grease-degrading strain of *Candida palmioleophila*. Production of biomass and protein by *C. palmioleophila* strain SACL11 was evaluated under eight different culture conditions, which provided palm oil or sunflower oil as sole carbon source and ammonium sulfate as sole nitrogen source, during 48 h at 30 °C. Protein profiles from *C. palmioleophila* crude extracts were obtained by SDS-PAGE every 12 h and analyzed with bioinformatic programs. The results showed that treatments providing the highest concentrations of each of the carbon and nitrogen sources resulted in a higher biomass production, with sunflower oil being the carbon source that produced the highest values and an overall faster growth. Proteins of approximately 63 KDa and 28 KDa were detected only in protein extracts obtained from media containing palm or sunflower oil as carbon source, suggesting a key role of these proteins in the hydrolysis of oils. Furthermore, the molecular weights of these proteins were similar to several reported lipases and esterases from *Candida rugosa* and other related species, reinforcing their possible function. In conclusion, this work identified and reported for the first time differential protein profiles of the lipolytic yeast *C. palmioleophila* in response to different growth conditions, and found evidence of the involvement of lipase-like proteins during the metabolism of vegetable oils. This give insight about the enzymes involved in grease metabolism and reinforces the potential of this promising microorganism to be used as an excellent bioremediation agent in fat, oil and grease-polluted environments.

## 1. Introduction

*Candida palmioleophila,* formerly known as *Torulopsis candida,* is an ascomycetous lipolytic yeast related to *Candida famata* and *Candida saitoana,* with a remarkably ability to assimilate crude palm oil (Nakase et al. 1988). Even though there are only few reports on the biochemical and physiological characterization of *C. palmioleophila*, evidence points to a high potential of this yeast as bioremediation agent of effluents contaminated with fats and oils. Recently, Rodriguez-Mateus et al. (2016) reported the isolation and characterization of native strains of *C. palmioleophila* from solid and liquid wastes of a palm oil refining process, which showed high lipolytic activity and were successfully used to degrade up to 79% of palm oil in liquid medium. Agualimpia et al. (2016) also reported that microbial consortia composed of several strains of *C. palmioleophila* degraded more efficiently palm oil that individual strains, achieving up to 84% palm oil degradation and were successfully used for the bioremediation of Palm Oil Mild Effluents (POME).
Even though the specific enzyme mechanisms and metabolic pathways used by C. *palmioleophila* to degrade oil and grease are not yet described, lipases and esterases are most likely involved in the first steps of this process. Lipases and esterases from *C. palmioleophila* could have a strong potential to be used as biocatalysts for bioremediation purposes. However, little is known about the structure and expression of *C. palmioleophila* lipases or esterases in response to different growth conditions and there is no reports about the characterization or purification of these enzymes for bioremediation purposes. Thus, this work aimed to stablish the influence of different carbon and nitrogen sources, including vegetable oils, on the variability of the protein profiles of an oil and grease-degrading strain of *C. palmioleophila in vitro*.

Please cite this article as: Rincon L.J., Agualimpia B., Zafra G., 2018, Differential protein profiles of the lipolytic yeast candida palmioleophila under different growth conditions, Chemical Engineering Transactions, 64, 343-348 DOI: 10.3303/CET1864058

## 2. Materials and methods

### 2.1 Candida palmioleophila

A native strain of *Candida palmioleophila* (SACL11), previously isolated from a grease trap of a palm oil refining process and showing high oil and grease degradation activity (Rodriguez-Mateus et al. 2016), was used in this study. Growth curve of strain SACL11 was determined in 180 ml of MBS1 medium (g/L: $(NH_4)_2SO_4$, 2; $K_2HPO_4$, 0.9; $KH_2PO_4$, 0.6; $MgSO_4$, 0.2; $CaCO_3$, 0,5; yeast extract 0.1; $FeSO_4$, 0,0002; glucose, 2; lactose, 2; glycerol, 0,5; peptone, 1) during 12 h at 30 °C with constant agitation at 180 rpm. Samples were taken at hours 0, 6, 12, 18, 24, 30, 36 and 42 to determine optical density at 600 nm and dry weight.

### 2.2 Growth of *C. palmioleophila* under different nutrient conditions

To evaluate the biomass and protein production by *C. palmioleophila* SACL11 under different growth conditions, MBS2 medium (g/L: ($K_2HPO_4$, 0.9, $KH_2PO_4$, 0.6, $MgSO_4$, 0.2; $CaCO_3$, 0.5, $FeSO_4$, 0.0002) was supplemented either with refined palm oil (total fat, 90%; saturated fatty acids, 40 %; monounsaturated fatty acids, 35 %; polyunsaturated fatty acids, 15 %) or sunflower oil (total fats, 90%; saturated fatty acids, 10 %; monounsaturated fatty acids, 55 %; polyunsaturated fatty acids, 25%; linolenic acid, 5%; linoleic acid, 20 %; oleic acid, 55 %) as sole carbon source and ammonium sulfate ($(NH_4)_2SO_4$) as sole nitrogen source. Eight different culture media containing different concentrations of these carbon and nitrogen sources were used, as shown in Table 1. As a growth control, *C. palmioleophila* SACL-11 was grown in MBS2 medium containing 0.2 % glucose and 0.1 % yeast extract as sole source of carbon and nitrogen, respectively. The inoculum size consisted of approximately 8.7 $x10^8$ CFU (0.108 absorbance at 600 nm), prepared from 20 ml of a 12-h culture of *C. palmioleophila* SACL11 in MBS1 medium, which was centrifuged, the pellet was recovered and washed with distilled water to remove carbon and nitrogen traces that could alter the results. The washed cells were then inoculated into 180 ml of MBS2 medium containing each of the different carbon and nitrogen sources, and then were incubated at 30 °C for 48 h, with constant agitation at 180 rpm. Samples of 8.5 ml medium were taken every 12 h (hours 0, 12, 24, 36 and 48) to calculate dry weight, absorbance at 600 nm and to obtain crude protein extracts.

*Table 1. Combinations of carbon and nitrogen sources used for the growth of C. palmioleophila SACL11.*

| Treatment | Carbon source | Nitrogen source |
|---|---|---|
| T1 | Palm oil (0.5 %) | Ammonium sulfate (0.5 %) |
| T2 | Palm oil (0.5 %) | Ammonium sulfate (2 %) |
| T3 | Palm oil (1 %) | Ammonium sulfate (0.5 %) |
| T4 | Palm oil (1 %) | Ammonium sulfate (2 %) |
| T5 | Sunflower oil (0.5 %) | Ammonium sulfate (0.5 %) |
| T6 | Sunflower oil (0.5 %) | Ammonium sulfate (2 %) |
| T7 | Sunflower oil (1 %) | Ammonium sulfate (0.5 %) |
| T8 | Sunflower oil (1 %) | Ammonium sulfate (2 %) |
| Control (MBS2 medium) | Glucose (0.2 %) | Yeast extract (0.05 %) |

### 2.3 Preparation of crude protein extracts and SDS-PAGE

Total crude protein extracts of *C. palmioleophila* were prepared from 6 ml culture medium samples, which were centrifuged at 13,500 rpm for 15 min at 4 °C to obtain a cell pellet. Cells were washed with wash buffer (50 mM NaCl, 10 mM EDTA), centrifuged at 4 °C, and then suspended in 200 µl TE buffer (10 mM Tris-Cl pH 7.5; 1 mM EDTA). 200 µl of Laemmli buffer (0.134 M Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue, 50 mM PMSF) were added and vortexed for 1 min. 50 µl of 20 % SDS was then added and samples were homogenized in vortex at maximum speed for 2 minutes. Samples were boiled in a water bath for 5 minutes, vortexed briefly, boiled for 5 additional min and finally centrifuged at 10,000 rpm for 5 min. The resulting supernatant, containing the total protein lysate, was stored at -20 °C until use. Protein separation from lysates was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini Protean4 vertical electrophoresis chamber (Bio-rad, USA) at 100 volts for 1 h, using 15 µl of each protein extract. Separating gels were set up at 12% polyacrylamide, while stacking gels were prepared at 4% polyacrylamide. After electrophoretic separation, gels were stained with a solution of 0.25 % Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid for 90 min with gentle agitation. Destaining was performed in in a solution containing methanol, acetic acid and distilled water (2:3:35) for 3 h with gentle agitation (Zhang et al. 2011). Gels were photographed with a Nikon D3200 camera (Nikon Inc., Japan).

### 2.4 Determination of variability in protein profiles and statistical analysis

The variability of the protein profiles was determined *in silico* and analyzed with bioinformatic programs. First, the molecular weights of the protein bands of each profile were calculated with the PhotocaptMW 10.0 program (Vilber Loumat), by comparison with the Broad Range Protein Molecular Marker molecular weight standard (Promega). Once the bands were identified and their weights calculated, a comparative analysis of the protein patterns based on the Unweighted Pair Group Method With Arithmetic Mean (UPGMA) (Brandus et al., 2012) was performed with the PyElph program version 1.0 (Pavel and Vasile 2012). Data obtained from the growth curves were analyzed using Analysis of Variance (ANOVA) using the IBM SPSS statistics software (IBM). The comparison of means of treatments was performed using the Fisher's Minimum Significant Difference (LSD) method test (Fisher 1939).

## 3. Results and discussion

### 3.1 Influence of carbon and nitrogen sources on the growth of *C. palmioleophila*

Figure 1 shows the growth curve and biomass production of *C. palmioleophila* SACL11 under different culture conditions. Results showed that *C. palmioleophila* lag phase in enriched MBS1 medium was short, as exponential growth was detected already at hour 6. This would indicate that this yeast has a short adaptation time, but could also be explained by the fact that the inoculum was also reactivated in enriched MBS1 medium, containing glucose, lactose, peptone and glycerol as carbon sources. The exponential phase extended until approximately 12 h of growth and from this point the growth decreased, initiating a stationary phase by hour 18 (Figure 1A).

*C. palmioleophila* SACL11 responded differently when cultured in MBS2 medium supplemented with different concentrations of palm oil (as only carbon source) and ammonium sulfate (as only nitrogen source). These conditions (T1-T4) were tested in order to determine the influence of these macronutrients and their concentrations on the growth of *C. palmioleophila*. In general, the best yields of dry biomass were obtained with treatment T4, which provided the highest concentration of both palm oil (1%) and ammonium sulfate (2%) (Figure 1B). Although differences in biomass production were observed from hour 12 of growth, this finding did not become statistically significant until hour 48 in comparison to treatment T1 ($p$= 0.030), treatment T2 ($p$= 0.022) and treatment T3 ($p$= 0.032) (figure 1C). In concordance, the same observations were made when analyzing the results from absorbance measurements (data not shown). The above results indicate that *C. palmioleophila* SACL11 can efficiently use palm oil as sole carbon and energy source and that differences in oil concentrations can influence its growth to a greater extent than the concentration of available nitrogen, although the presence of high concentrations (2%) of ammonium sulfate in the medium favored its growth. The efficient use of palm oil by strain SACL11 agrees with previous studies, in which this strain was the one presenting the highest efficiency in the removal of crude palm oil between different strains of *C. palmioleophila* and degrading bacteria (Agualimpia et al. 2016; Rodriguez-Mateus et al. 2016). We observed also that, although the log phase occurred during hours 6 to 18, strain SACL11 is able to sustain its growth more than 48 h since the stationary phase was not reached at hour 48. On the other hand, and similar to the observed with palm oil, *C. palmioleophila* SACL11 was able to efficiently use sunflower oil as sole carbon source. Figure 1E and F shows the growth behavior of strain SACL11 in culture media supplemented with different concentrations of sunflower oil and ammonium sulfate. The highest biomass production was obtained with treatment T8, which in turn had the highest concentration of both sunflower oil (1%) and of ammonium sulfate (2%) (Figure 1D). These differences were detected since hour 12, but were only statistically significant until hour 36 of growth with respect to treatment T5 ($p$= 0.002), treatment T6 ($p$= 0.002) and treatment T7 ($p$= 0.001).

The results showed that conditions providing the highest concentrations of each of the carbon and nitrogen sources resulted in a higher biomass production by *C. palmioleophila*. Interestingly, when comparing among treatments producing the highest biomass yields for each of the carbon sources (1% palm oil and 1% sunflower oil), it was sunflower oil (treatment T8) which promoted and overall faster growth and significantly highest biomass yields compared to palm oil (treatment T4) by hour 48 ($p$=0.001). These findings could be explained by differences in the fatty acids composition of both palm and sunflower refined oil and their metabolism by *C. palmioleophila* enzymes. During the degradation of vegetable oils by several lipolytic organisms, unsaturated fatty acids (oleic, linoleic, linolenic, etc.) are the first substrates used by microbial lipases while the long-chain saturated fatty acids are hydrolyzed subsequently (Liu and Kokare 2017). Sunflower oil has a higher content of mono and polyunsaturated fatty acids (55 % and 25 % respectively) in comparison to palm oil (35 % and 15 % respectively), as well as a lower content of saturated fatty acids (10% vs 40 %). This indicate that *C. palmioleophila* lipases have different substrate specificities, suggesting a higher selectivity toward mono and polyunsaturated fatty acids. Even though sunflower oil induced a biomass

production significantly higher than palm oil, the latter was also efficiently used by *C. palmioleophila* as growth support. Since the refined palm oil contained a high proportion of saturated fatty acids, this could be indicative of a selectivity of certain *C. palmioleophila* lipases towards saturated fatty acids and a potential use in PUFA enrichment, in agreement with previous studies describing that *Candida* lipases are among the few microbial lipases reported to have an adequate stability and selectivity to concentrate polyunsaturated fatty acids (Fregolente et al. 2009). However, the number, structure and specificity of *C. palmioleophila* lipases is yet to be determined.

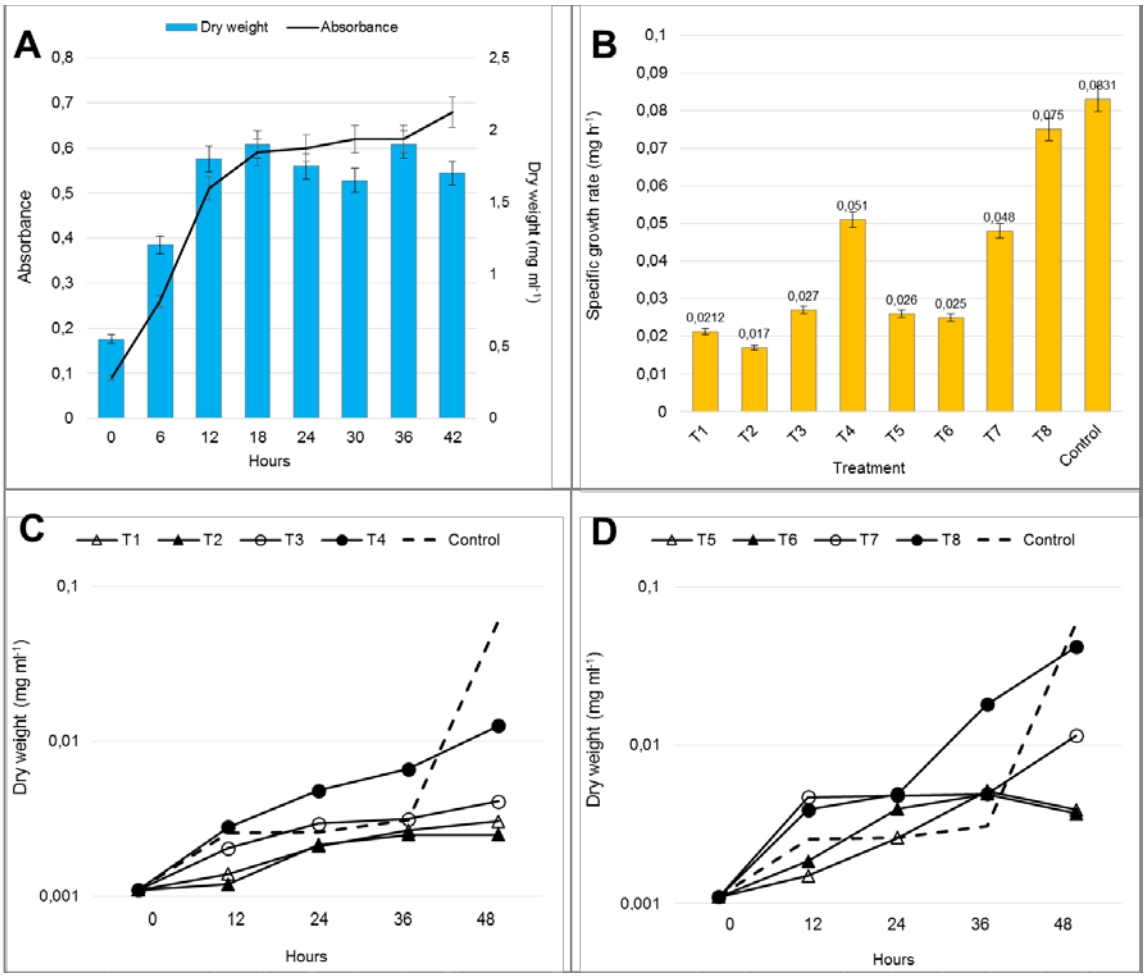


*Figure 1. Growth of C. palmioleophila SACL11 cultured under different nutrient conditions. A) Growth curve in MBS1 medium. B) Specific growth rate in 8 different MBS2 media formulations. C) Biomass dry weight measurement in media containing palm oil as sole carbon source. D) Biomass dry weight measurements in media containing sunflower oil as sole carbon source*

### 3.2 Variability of protein expression by *C. palmioleophila* during oil degradation

Variations in protein expression of *C. palmioleophila* in response to the different growth conditions were detected by SDS-PAGE. As can be observed in figure 2, *C. palmioleophila* protein profiles were complex and contained up to 32 different bands, containing proteins ranging from 25 to 154 KDa while cultured in media containing palm or sunflower oil. We detected slight changes in band patterns during the course of cultures, especially when comparing protein profiles from hour 0 (biomass not exposed to palm or sunflower oil) with respect to those obtained from hours 12, 24, 36 and 48 of oil exposition.

Interestingly, a protein band of approximately 63 KDa was exclusively detected during the degradation of both palm and sunflower oil (Figure 2, red rectangles). Furthermore, an additional 28 KDa band was detected only during the degradation of sunflower oil. These two bands appeared to become more intense over time, indicating a higher expression at hour 48 compared to hour 12. Because at the time of this study there were virtually no information about *C. palmioleophila* genes, proteins or enzymes in public databases such a NCBI,

we compared the calculated molecular weights of band profiles with respect to known lipases of related *Candida* species, including *Candida albicans*, *Candida parapsilosis, Candida rugosa*, *Candida antarctica* and *Candida cylindracea*. Due to similarities in the molecular weight of *C. palmioleophila* protein bands and the reported lipases of 60, 62 and 64 KDa from *C. rugosa* (Benjamin and Pandey 2001), the 62 KDa lipase from *C. cylindracea* (Shaw et al. 1989), the 60 KDa lipase from *C. parapsilosis* (Toth et al. 2017) and the 33 KDa lipase from *C. antarctica* (Høegh et al. 1995; Rotticci-Mulder et al. 2001), as well as the presence of these bands only when *C. palmioleophila* was exposed to oil, we can speculate that the 63 and 28 KDa protein bands could correspond to lipases involved in palm and sunflower hydrolysis.

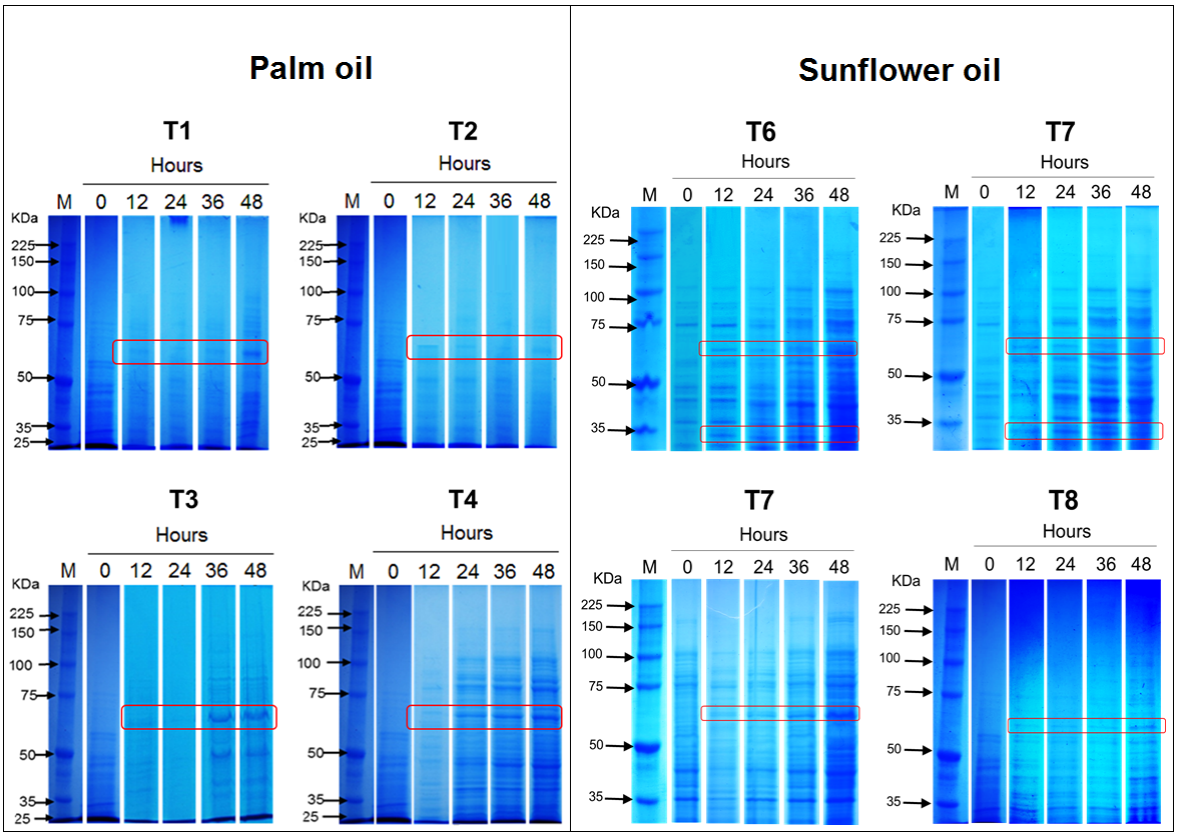


*Figure 2. SDS-PAGE analysis of protein expression by C. palmioleophila cultured in media containing palm and sunflower oil as sole carbon source. T1: 0.5 % palm oil and 0.5 % ammonium sulfate. T2: 0.5 % palm oil and 2 % ammonium sulfate. T3: 1 % palm oil and 0.5 % ammonium sulfate. T4: 1 % palm oil and 2% ammonium sulfate. T5: 0.5 % sunflower oil and 0.5 % ammonium sulfate. T6: 0.5 % sunflower oil and 2 % ammonium sulfate. T7: 1 % sunflower oil and 0.5 % ammonium sulfate. T8: 1 % sunflower oil and 2 % ammonium sulfate. M: Broad Range Protein Molecular Marker molecular weight standard (Promega). Red rectangles highlights protein bands detected only when C. palmioleophila was exposed to either palm or sunflower oil.*

Agualimpia et al. (2016) reported that palm oil removal efficiency by *C. palmioleophila* significantly varied during time, changing from 61 % by hour 48 to 79% by hour 72 of culture. This, summed to the observed differences in protein profiles and band intensities during oil degradation by *C. palmioleophila* SACL11 reinforces a possible role of these proteins in palm and sunflower oil degradation.

## 4. Conclusions

In conclusion, in this work we identified and reported differential protein profiles of the lipolytic yeast *Candida palmioleophila* in response to different growth conditions, and found evidence of the involvement of lipase-like proteins during the metabolism of vegetable oils. Evidence shows that *C. palmioleophila* efficiently uses both palm and sunflower oil as sole carbon sources, and produces 63 KDa and 28 KDa proteins only when exposed to oil, suggesting a key role of these proteins in oil hydrolysis. Furthermore, the molecular weights of

these proteins were similar to several reported lipases and esterases from *C. rugosa* and other related species, indicating their possible hydrolytic function. This reinforces the potential of this promising microorganism to be used as an excellent bioremediation agent in fat, oil and grease-polluted environments, and give insight about the enzymes involved in fat, oil and grease degradation by *C. palmioleophila*. To the best of our knowledge, this is the first report of the influence of different vegetable oils on the growth and protein expression of *C. palmioleophila in vitro*.

**Acknowledgments**

This research was supported by Universidad de Santander project PINL0117207733321EJ.

**References**

Agualimpia B., Otero J., Zafra G., 2016, Evaluation of native microorganisms for biodegradation of oil and grease in palm oil refinery effluents, Biotecnología Aplicada, 33, 1221-1226.

Benjamin S., Pandey A., 2001, Isolation and characterization of three distinct forms of lipases from *Candida rugosa* produced in solid state fermentation, Brazilian Archives of Biology and Technology, 44, 213-221.

Fisher R.A., 1939, The Comparison of Samples with Possibly Unequal Variances, Annals of Eugenics, 9, 174-180. DOI:10.1111/j.1469-1809.1939.tb02205.x

Fregolente P.B.L., Fregolente L.V., Maciel M.R.W., Carvalho P.O., 2009, Screening of microbial lipases and evaluation of their potential to produce glycerides with high gamma linolenic acid concentration, Braz J Microbiol, 40, 747-756. DOI:10.1590/S1517-83822009000400004

Høegh I., Patkar S., Halkier T., Hansen M.T., 1995, Two lipases from *Candida antarctica*: cloning and expression in Aspergillus oryzae, Canadian Journal of Botany, 73, 869-875. DOI:10.1139/b95-333

Liu X., Kokare C. (2017) Chapter 11 - Microbial Enzymes of Use in Industry A2 - Brahmachari, Goutam. In: Biotechnology of Microbial Enzymes. Academic Press, pp 267-298. DOI:https://doi.org/10.1016/B978-0-12-803725-6.00011-X

Nakase T., Itoh M., Suzuki M., Komagata K., Kodama T., 1988, *Candida palmioleophila* sp. nov., a yeast capable of assimilating crude palm oil, formerly identified as *Torulopsis candida*, The Journal of general and applied microbiology, 34, 493-498.

Pavel A.B., Vasile C.I., 2012, PyElph - a software tool for gel images analysis and phylogenetics, BMC bioinformatics, 13, 9-9. DOI:10.1186/1471-2105-13-9

Rodriguez-Mateus Z., Agualimpia B., Zafra G., 2016, Isolation and Molecular Characterization of Microorganisms with Potential for the Degradation of Oil and Grease from Palm Oil Refinery Wastes, Chemical Engineering Transactions, 49, 517-522. DOI:10.3303/CET1649087

Rotticci-Mulder J.C., Gustavsson M., Holmquist M., Hult K., Martinelle M., 2001, Expression in *Pichia pastoris* of *Candida antarctica* Lipase B and Lipase B Fused to a Cellulose-Binding Domain, Protein expression and purification, 21, 386-392. DOI:http://dx.doi.org/10.1006/prep.2000.1387

Shaw J.F., Chang C.H., Wang Y.J., 1989, Characterization of three distinct forms of lipolytic enzyme in a commercial *Candida* lipase preparation, Biotechnology Letters, 11, 779-784. DOI:10.1007/bf01026096

Toth R., Toth A., Vagvolgyi C., Gacser A., 2017, *Candida parapsilosis* secreted lipase as an important virulence factor, Current protein & peptide science, 18, 1-7. DOI:10.2174/1389203717666160813163054

Zhang T., Lei J., Yang H., Xu K., Wang R., Zhang Z., 2011, An improved method for whole protein extraction from yeast *Saccharomyces cerevisiae*, Yeast, 28, 795-798. DOI:10.1002/yea.1905