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Nitrogen source and pH interact and modulate lipase secretion in a non-clinical strain of *Candida parapsilosis*

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ABSTRACT. Lipases (E.C. 3.1.1.3) are serine-hydrolases, and act on long chain fatty acid ester bonds. They exhibit specific and enantioselective activities, which are desirable for many industrial applications. This study aimed at screening and optimizing the production of lipases by wild yeast strains from a variety of substrates, as well as characterizing the enzyme. An initial selection was made in oxygenated oil-supplemented minimum medium, and the enzymatic activity of the supernatant was tested over p-nitrophenyl palmitate. One-hundred and twenty-four yeast strains from different substrates were tested, and twenty-three showed significantly higher lipolytic activity ($p < 0.01$). One yeast in particular, QU110, showed best lipase production and therefore was selected for the optimization and characterization processes. This yeast exhibits enzyme secretion in initial pH 6.0, with olive oil and tryptone as carbon and nitrogen sources, respectively. There was a strong interaction between nitrogen source and initial pH, and pH 9.0 seems to inhibit enzyme secretion. The crude enzyme (cell-free supernatant) shows stability in surfactants and n-hexane, but not in ethanol or methanol. A Response Surface Model was created and optimal enzyme activity conditions were observed at 36°C and pH 8.0. The lipase is appropriate for transesterification reactions, as the enzyme is more stable in strong apolar solvents than moderately apolar ones. Also, secretion by pH was not reported elsewhere, which should be further investigated and contribute for other yeast bioprocesses as well.

Keywords: *p*-NPP; Tryptone; Yeast; Palmitate; Response Surface Model; Serine-hydrolase.

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Introduction

Lipases (E.C. 3.1.1.3) catalyze hydrolysis and synthesis reactions over triacylglycerol esters. The typical structure involves a catalytic triad (Ser-His-Asp), and the main residue, Serine, is commonly found in a (Ser-X-His-X-Asp) sequence (Jain & Naik, 2018). Lipases are distinguished from other esterases especially by the interfacial activity, given by a hydrophobic "lid" that covers the catalytic center and prevents it from acting on water soluble substrates (Gupta, Kumari, Syal, & Singh, 2015). Nonetheless, lipases can hydrolyze many substrates of esterases, and the opposite also occurs (Lopes, Fraga, Fleuri, & Macedo, 2011). Besides acting on lipids, lipases possess interesting properties such as chemo-, regio- and stereoselectivity. Thus, a broad range of applications is possible, such as detergents and emulsifiers, biodiesel transesterification, paper pulp and leather cleaning, resolution of racemic solutions, food nutrition and flavor enhancement (Navvabi, Razzaghi, Fernandes, Karami, & Homaei, 2018; Sharma et al., 2012).

This enzyme is vastly distributed in nature, from Archaea to mammals, but microbial lipases are preferred since microorganisms secrete it to the outside of the cell, producing high yields of enzyme readily available to purification or direct utilization (Sharma et al., 2012). There are several reports of naturally occurring yeasts with high lipase production, especially belonging to the genera *Candida* and *Yarrowia* (Navvabi et al., 2018; Souza, Salgueiro, & Albuquerque, 2012; Thakur, 2012), and substrates like food, plants and soil are common sources of those organisms.

Candida parapsilosis is a cosmopolitan yeast, being reported associated with diverse substrates such as mayonnese and salad toppings (Pitt & Hocking, 2009), goat and cow milk and cheese (Landell, Hartfelder, &

Valente, 2006; Spanamberg, Ramos, Leoncini, Alves, & Valente, 2009), Colombian *chicha*, a fermented beverage (López-arboleda & Ramírez-castrillón, 2010), soil and water (Gadanho & Sampaio, 2005; Wang, You, Bemis, Tegeler, & Brown, 2008). Its lipase production is also considered a virulence factor (Toth, Toth, Vagvolgyi, & Gacser, 2017; Trofa et al., 2011) as well as in other common industrial lipid-producing fungi, like *Aspergillus niger* (Costa, Hermann, Garcia-Roman, Valle, & Tavares, 2017) and *Yarrowia lypolitica* (Boyd, Wheless, Brady, & Ellis, 2017). Due to the ability of transesterification under water activities higher than 0.9 (Neang, Subileau, Perrier, & Dubreucq, 2014; Neugnot, Moulin, Dubreucq, & Bigey, 2002), *C. parapsilosis* lipase is considered a good choice for biodiesel transesterification and other biotechnological applications. It was successfully used for biodiesel transesterification, yielding more than 95% (Rodrigues, Perrier, Lecomte, Dubreucq, & Ferreira-Dias, 2016). Nonetheless, even when the transesterification reaction is desired, preliminary screening tests usually apply lipase hydrolysis as the evaluation parameter (Raoufi & Mousavi Gargari, 2018; Yan, Duan, Liu, Jiang, & Yang, 2016).

Given the interest in naturally produced lipases, the objective of this study was to find a lipase-producing wild yeast, optimize its production conditions and the enzymatic activity conditions.

Material and methods

Chemicals

The reagents were all of analytical grade.

Samples

One-hundred and twenty-four yeast strains were tested, previously isolated from cheese (Landell et al., 2006) and other substrates (Annex, supplementary material). All strains are maintained at the Department of Microbiology, Immunology and Parasitology of the *Universidade Federal do Rio Grande do Sul*, Brazil.

Screening and Lipase induction

One-hundred µL from a suspension of 10^6 cells mL^{-1} were inoculated in 100 mL inductive medium (2% soybean oil, 0.5% peptone, 0.01% magnesium sulphate, 0.1% potassium phosphate), and grown in orbital shaker (Excella E24, New Brunswick, NJ, USA) at 200 rpm for 72 hours at 28°C for lipase induction. Culture supernatants (cell harboring and/or cell-free) were subjected to the *p*-nitrophenyl palmitate (pNPP) enzymatic assay in order to detect extracellular and cell-bound lipases. Cell-free supernatant was prepared by centrifugation at 5000 rpm for 5 min., and cell harboring supernatant was directly used.

Optimizing the conditions for lipase induction by univariate analysis

Different pH values (3, 6 and 9), organic nitrogen sources (peptone and tryptone) and carbon sources (soybean and olive oils, tween 20 and tween 80) were tested in order to verify optimal conditions for lipase induction. The concentrations are the same as above; pH was adjusted by HCl or NaOH, as needed, but was not monitored during yeast growth. Lipase reactions were performed with cell harboring and cell free supernatants.

Enzymatic assay

pNPP assays were performed in triplicate, in Tris-HCl 50 mM Buffer pH 8.0, containing 0.11% Arabic Gum, 0.44% Triton x-100 and 10% pNPP at 3 mg mL^{-1} of 2-propanol, according to Gupta et al. (2002). This substrate solution was added to supernatant (9:1) and kept for 1 hour at 37°C. The released product pNP exhibits yellow color, which absorbs light at 405-410 nm. Absorbance was then measured with Elisa microplate reader at 405 nm (ELx800 – BioTek Instruments Inc, VT USA). One unit of lipase (U) was defined as the amount of enzyme that releases 1 µmol *p*-nitrophenol min^{-1}. A standard curve was prepared using *p*-nitrophenol in previously known concentrations.

Enzyme activity optimization

A surface-response design was created using the Statistica 10 software (Statsoft Inc., 2011). The factors pH and Temperature were taken into account in this design. The pH/Temperature combinations were 14.6/8.0; 25.0/6.0; 25.0/10.0; 37.0/3.5; 37.0/14.14; 37.0/8.0; 37.0/8.0; 49.0/6.0; 49.0/10.0; 59.3/8.0. The

reactions were performed as above, except for the buffers, which were either Tris-HCl 50mM for pH 7.5 or above, or Citrate-Phosphate 50mM for pH under 7.5.

Enzyme stability characterization

The cell-free supernatant (crude enzyme) was co-incubated (concentration of 1:1 v:v) for one hour at 37°C with different Salts [50mM] ($MgCl_2$, KCl, $CaCl_2$, NaCl and EDTA), Solvents (n-Hexane, Acetone or Isopropanol 20%, 50% and 80%; ethanol 20%, 50% and 99.5%) and detergents (1% SDS, Triton X-100 or Tween 20). A control was made with distilled water (1:1 v:v). The residual activity was measured by the pNPP test.

Yeast strain identification

Candida parapsilosis QU110 was aerobically grown in GYP broth (2% glucose, 1% peptone, 0.5% yeast extract) at 28ºC. Total genomic DNA was extracted and purified from 5 mL cultures as described by Osorio-Cadavid, Ramírez, López, and Mambuscay (2009). Sequencing of the D1/D2 domain of the large subunit (LSU) ribosomal DNA was performed according to Kurtzman and Robnett (1998), using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). Amplification conditions were: initial denaturation at 94ºC for 5 min., 30 cycles of denaturation at 94ºC for 1 min., annealing at 55ºC for 30 s, extension at 72ºC for 1 min., and final extension at 72ºC for 10 min. The PCR product was purified by the polyethylene glycol precipitation method (Lis, 1980), and sequenced at the Biotechnology Center of *Universidade Federal do Rio Grande do Sul* (Cbiot/UFRGS), Brazil. The sequence was assembled and compared with sequences reported in GenBank using the basic local alignment search tool (BLAST) algorithm. The sequence was deposited in GenBank under accession number MH938079.

Statistical analysis

Results of screening were subjected to Student's t-test, and results of the univariate optimization were tested by Univariate Analysis of Variance (UNIANOVA) and GLM (Generalized Linear Model) applying hybrid estimation and robust estimation of covariance matrix, with Bonferroni correction for multiple comparisons. The SPSS v.18 was used. All graphic data show deviation bars corresponding to p =0.05.

Results and discussion

Lipase screening and strain selection

Twenty-three strains showed significantly higher lipase activity ($p < 0.001$; supplementary Annex). *Candida parapsilosis* QU110 was selected for optimization of lipase production due to its higher lipase activity when compared to the others, and consistency between the replicates. This yeast was isolated from an artisanal Caccio Cavalo cheese sample (Landell et al., 2006), and was identified as *Candida parapsilosis* by sequencing the D1/D2 region of the 26S rDNA.

Optimization of lipase induction by univariate analysis

Carbon source effect

All carbon sources tested showed different induction values ($p < 0.05$) when cell-free supernatant was tested, but regarding cell-harboring supernatant, only olive oil induced significantly higher lipase production ($p < 0.01$), while soybean oil, Tween 20 and Tween 80 showed no difference ($p > 0.4$). The effect of different carbon sources on lipase production can be seen in Figure 1. *Candida parapsilosis* QU110 showed lipase production of 47.97 u L^{-1} in the presence of olive oil, more than ten times the activity in the presence of soybean oil, Tween 20 or Tween 80. However, the cell-free supernatant showed lipase activity of only 9.23 u L^{-1} (Figure 1).

Both data indicated olive oil as the best carbon source among all four tested, and no interaction with other variables over lipase production was observed.

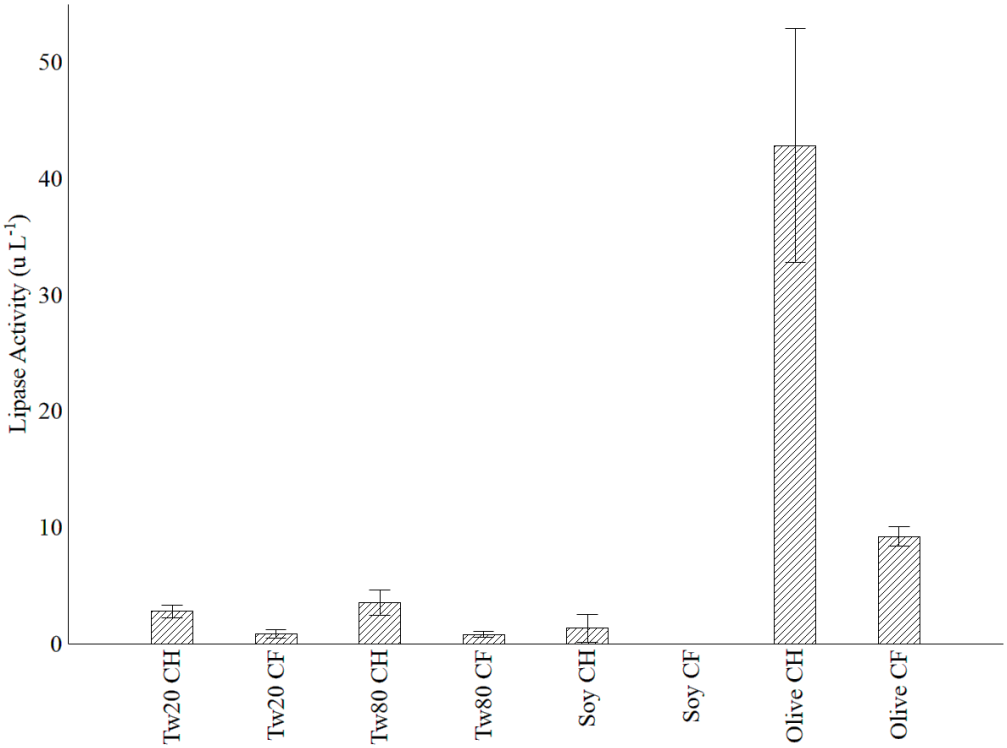

Figure 1. Effect of different carbon sources on lipase production. Tw20: Tween 20; Tw80: Tween 80; Soy: Soybean oil; Olive: Olive oil. CH: cell-harboring supernatant. CF: cell-free supernatant.

Nitrogen source effect

The effect of nitrogen source is strongly subjected to initial pH. Tryptone was the best nitrogen source for lipase production at pH 6.0, inducing an activity of 47.02 u L^{-1} in cell harboring supernatant and 36.98 u L^{-1} in cell-free supernatant (Figure 2). In pH 9.0, there was a slight reduction in lipase activity in the cell harboring supernatant (43.01 u L^{-1}), and there was no lipase secretion (Figure 2).

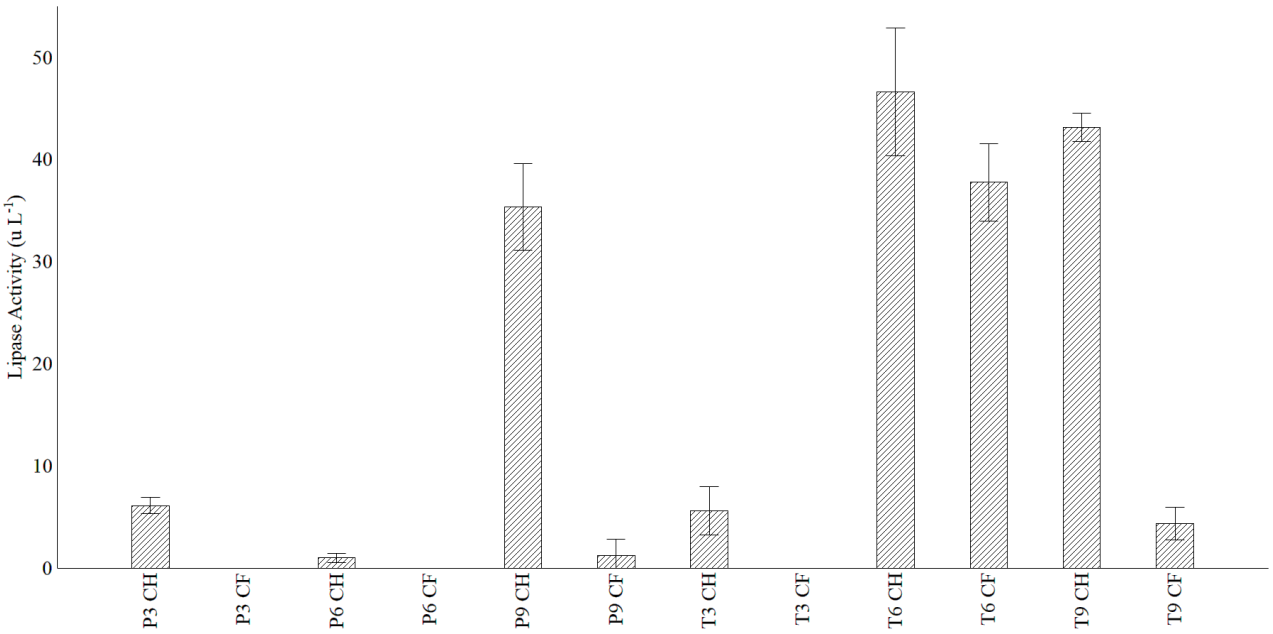

Figure 2. Effect of different pH and nitrogen sources on lipase production. P: Peptone; T: Tryptone; 6 and 9: pH6 and pH 9, respectively; CH: cell-harboring supernatant CF: cell-free supernatant.

The absence of lipase secretion at pH 9.0 was also found with peptone as the nitrogen source, while there was an increase in lipase activity in the cell-harboring supernatant (31.05 u L^{-1}) in comparison to pH 6.0.

These variations are corroborated by Generalized Linear Model statistics, which showed that pH and nitrogen sources have correlated effects, and cannot be estimated separately.

Lipase characterization and stability

According to the Response Surface Model for lipase production, the best hydrolysis activity conditions for the lipase produced by *C. parapsilosis* QU110 were pH 8.0 and temperature of 36°C (Figure 3).

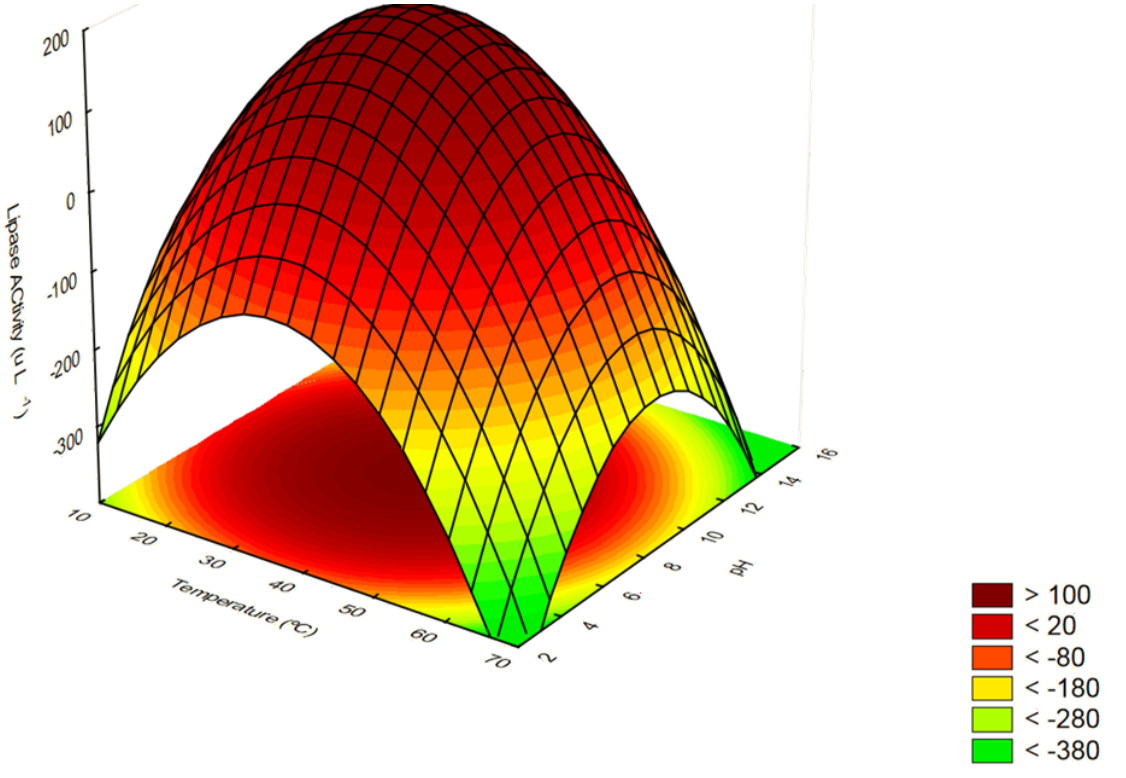

Figure 3. Response surface model for lipase production (u L^{-1}) considering pH and Temperature (°C). Green areas indicate lower lipase activity, and dark red, higher activities.

The crude enzyme exhibited inhibition values greater than 50% for concentrated solvents, and good stability to anionic surfactants, retaining up to 80% activity in the presence of Tween 80 (Figure 4a). In the presence of mono- and divalent ions, the enzyme showed destabilization, with residual activities lower than 40% when treated with $CaCl_2$. Most solvents showed strong inhibition and destabilization of the lipase, especially when concentrated. In contrast, n-Hexane 40% (v:v) increased lipase activity in about 5% (Figure 4c).

The preliminary screening step performed with 124 strains from different sources evidenced that, although lipase production is widespread, most wild yeast strains had low activities. *Candida parapsilosis* QU110 was selected as a good lipase-producing yeast.

It is known that oleic acid induces *C. parapsilosis* lipase gene CpLip2 (Neugnot et al., 2002), and this species has been identified in virgin oils as a spoilage organism (Zullo & Ciafardini, 2008). Recently, a good lipase producing yeast, *Magnusiomyces capitatus* was isolated from an Olive Mill Wastewater, indicating good inducing potential of olive oil (Salgado, Fonseca, Silva, Roseiro, & Eusébio, 2019).

No reports on gene CpLip1 activity were made so far. Since *C. parapsilosis* uses an alternative yeast genetic code, alternative translations are required for heterologous expression, which seems to be a setback in expressing LIP1 gene (Nosek, Holesova, Kosa, Gacser, & Tomaska, 2009).

In most conditions, we observed activity only in the cell harboring supernatant, indicating a cell wall or membrane-bound lipase. Interestingly, alkaline initial conditions seem to inhibit enzyme secretion.

The effect of the nitrogen source on lipase production was highly influenced by the medium pH. Although tryptone induced lipase secretion in pH 6.0 (cell-free supernatant), the same did not occur in pH 9.0. pH 9.0 allowed lipase production with both nitrogen sources, although it seemed to inhibit lipase secretion, probably remaining attached to the plasmatic membrane. To our knowledge, no reports have been made in this regard so far.

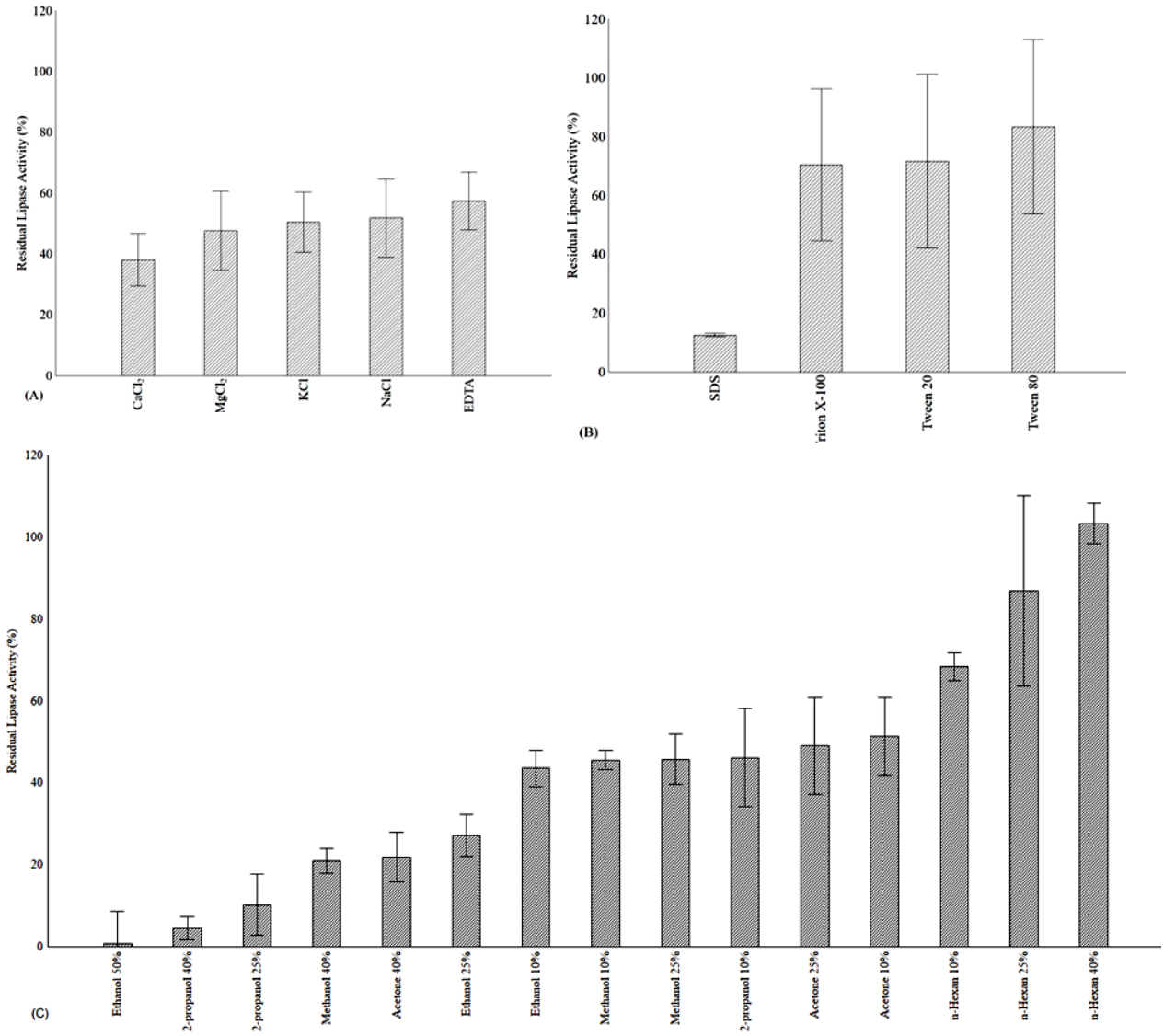

Figure 4. Residual extracellular lipase activity (%) after exposure to chelating salts (A), surfactants (B) and several concentrations of different solvents (C) for 1 hour at 37°C.

An alternative explanation would be that two different lipases (cell-bound and extracellular) are produced at different pH conditions. In accordance to this two-enzyme hypothesis, two lipase genes for *C. parapsilosis* have been described and characterized (Brunel et al., 2004; Subileau et al., 2015). Also, Hlavsová, Zarevúcka, Wimmer, Macková, and Sovová (2009) showed different conditions to induce extracellular and cell-bound lipases in *Geotrichum candidum* 4013, and Alonso, Oliveira, Dellamora-Ortiz, and Pereira-Meirelles (2005) stated that, in *Yarrowia lipolytica* IMUFRJ 50682, lipase secretion is dependent on the growth stage, with extracellular activity found at the late stationary phase, while late logarithmic phase is associated with cell-bound lipase activity.

It is remarkable that *C. parapsilosis* lipase, unlike classical lipases like CAL-A from *Pseudozyma antarctica*, retains its acyltransferase ability even at higher water activities, making it the best candidate for green biotechnology applications like biodiesel transesterification (Subileau et al., 2015). Wang, Chi, Wang, Liu, and Li (2007) showed in pH=7 optimal activity for cell-bound lipase from *C. parapsilosis*, similar to that observed for *C. parapsilosis* QU 110 crude lipase.

Stability characteristics seem to agree with typical lipases, showing stabilization against n-hexane and instability for solvents and ionic surfactants (Hama, Noda, & Kondo, 2018; Hasan, Shah, & Hameed, 2006; Nie, Xie, Wang, & Tan, 2006). Acetone 10% and 25% (v/v) show destabilization of about 50%, lower than other solvents tested, coherent with reports of acetone use for cell-preparation for interesterification and whole-cell biocatalysis (Lin & Tao, 2018)

Conclusion

It is known that lipase production is strongly influenced by culture conditions, which modulate its secretion. Our study showed that *C. parapsilosis* QU110, a wild strain, could be induced to produce lipases that are either secreted or bound to the cell, depending on the medium pH. This lipase shows typical optimal conditions for pNPP hydrolysis and good stability against some solvents. This yeast is a good candidate for further investigations in order to produce and/or degrade hydrophobic compounds and resolution of racemic mixtures, among other uses.

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Annex

List of yeasts screened for p-nitrophenyl palmitate essay indicating lipase activities (units L^{-1}). The “t” student test was performed in order to highlight good lipase producing strains. Values over three standard deviations were considered high producing yeasts when compared to the pool of yeasts tested. All tests were performed in triplicates. Strain code names indicate substrate of origin (QU: artisanal cheese; FA: enrichment growth of soil samples; CB: fatty residues of restaurant exhaustion equipment; LV: raw bovine milk; EI: bromelid endophytic yeast).

Strain	Lipase Activity	"t" value	Higher Value?
QU21	0.063	592.893	Yes
QU10	0.336	6.368.372	Yes
QU110	0.274	5.050.544	Yes
QU84B	0.067	661.864	Yes
QU29	0.103	1.424.678	Yes
QU48	0.116	1.708.502	Yes
QU95	0.047	241.783	No
QU138	0.106	1.487.661	Yes
QU110	0.125	1.897.250	Yes
QU18C	0.063	595.945	Yes
QU95	0.038	0.65542	No
QU68	0.032	-0.67212	No
QU27	0.034	-0.24181	No
QU29	0.038	0.60964	No
QU135	0.128	1.956.623	Yes
QU13	0.115	1.681.022	Yes
QU105	0.015	-429.709	No
QU10	0.094	1.250.171	Yes
QU79	0.061	552.143	Yes
QU 137	0.076	861.040	No
QU125	0.027	-171.125	No
QU33	0.029	-141.370	No
CB1	0.051	339.288	Yes
QU 123	0.071	746.161	No
QU35	0.060	526.602	Yes
QU03	0.069	708.597	Yes
QU22	0.049	295.342	No
QU42	0.023	-253.066	No
QU 132	0.073	804.330	No
QU126	0.047	241.641	No
QU35	0.022	-289.688	No
FA04	0.022	-287.399	No
CB2	0.043	166.251	No
QU04	0.019	-340.500	No
LV102	0.030	-104.138	No
QU103	0.032	-0.70666	No
QU92	0.025	-208.567	No
QU 56	0.040	0.93356	No
QU34	0.027	-175.972	No
QU29	0.043	161.674	No

QU01	-0.019	-1.155.789	No
QU60	0.042	152.983	No
FA05	0.015	-436.632	No
QU54	0.016	-409.166	No
QU55	0.033	-0.39576	No
FA10	0.014	-448.382	No
QU10	0.014	-449.907	No
QU102	0.038	0.63724	No
QU03	-0.016	-1.090.481	No
FA01	0.010	-536.884	No
QU119	0.032	-0.67658	No
QU 91	0.027	-173.237	No
FA06	0.012	-493.853	No
QU115	0.032	-0.74879	No
QU19	0.011	-512.164	No
QU99	0.048	260.546	No
QU30	0.009	-547.413	No
QU39	0.009	-548.786	No
QU114	0.031	-0.94536	No
QU15	-0.011	-981.837	No
QU 77	0.048	280.262	No
QU82	0.042	148.721	No
QU98	0.027	-174.217	No
FA9	0.016	-409.319	No
QU140	0.021	-311.967	No
QU06	-0.010	-965.967	No
QU37	-0.014	-1.039.146	No
FA02	0.009	-546.039	No
QU101	0.011	-504.928	No
QU133	0.033	-0.58030	No
QU128	0.011	-504.126	No
QU108	0.008	-574.129	No
QU15	-0.016	-1.076.187	No
QU96	0.013	-480.356	No
QU28	-0.007	-902.616	No
QU121	0.012	-493.695	No
EI01	0.013	-473.712	No
QU81	0.020	-313.622	No
QU07	0.006	-624.776	No
QU80	0.011	-519.972	No
FA03	0.007	-606.007	No
QU71	0.011	-513.498	No
QU94	0.021	-293.062	No
QU73	0.009	-549.492	No
QU 116	0.011	-520.792	No

FA8	0.003	-674.215	No
QU122	0.011	-514.957	No
QU75	0.001	-725.569	No
QU41	0.024	-236.388	No
QU52	0.015	-434.872	No
QU64	0.010	-536.558	No
QU117	0.005	-629.690	No
QU131	0.006	-624.876	No
QU67	0.005	-644.918	No
QU129	0.004	-666.597	No
QU113	0.010	-529.800	No
QU130	0.004	-665.394	No
QU104	0.001	-718.298	No
QU49	0.003	-685.347	No
QU40	0.002	-704.842	No
QU10	0.002	-707.174	No
QU70	0.002	-695.863	No
QU139	0.006	-621.114	No
QU 63	-0.003	-803.614	No
QU 136	-0.002	-787.567	No
QU 57	0.000	-742.345	No
QU07	-0.001	-761.191	No
QU47	0.008	-583.863	No
FA7	-0.000	-747.916	No
QU19	0.001	-721.022	No
QU110	0.078	903.719	Yes
QU68	0.097	1.296.028	Yes
QU29	0.150	2.423.579	Yes
QU13	0.158	2.599.022	Yes
QU29	0.067	662.016	Yes
QU03	0.048	263.299	No
QU120	0.057	470.211	Yes
QU134	0.063	595.870	Yes