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Lipase Extracellular Activity from Non-Conventional Yeasts

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Abstract

The purpose of this study is to establish if the non-conventional yeasts *Debaryomyces hansenii*, *Rhodotorula glutinis* and *Candida guilliermondii* present lipase activity under saline stress growth conditions using NaCl and KCl, evaluating growing rate and adapting capacity to saline culture medium and enzymatic activity. A comparative analysis was done for the three yeasts in different growing conditions, with and without salt media in order to determine the best conditions for growing and enzymatic activity. This research pretends to look for a new biotechnological tool for the expanding applications in biotransformation area, such as the use of lipases in several industries, and a new source to obtain these enzymes. In order to do so, it is necessary to make an analysis of viability of extraction, and determine salt concentration in the incubation media as an important factor for growing, metabolism and enzymatic activity. *D. hansenii* ISA 1508 was the non-conventional strain with more enzymatic activity in saline stress condition at 30°C, using 0.6 M NaCl and 1.2 M NaCl, respectively. The p-nitrophenol method was used to measure lipase activity. The non-conventional yeast *D. hansenii* ISA 1508*C* showed tolerance to saline conditions and lipase activity. Yeasts *C. guilliermondii*, *R. glutinis* L-033 were found to have a similar enzymatic activity and did not show any preference for any of the experimental conditions probed herein.

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Introduction

Enzymes are called nature's catalysts, extensively used in industry: proteases and lipases are used in detergents; amylases and glucose isomerase are useful in starch processing, and in several organic synthesis due to their principal characteristics of extreme specificity and high reaction rate (Hasan, *et al.*, 2006).

Lipases (E.C. 3.1.1.3.) are part of the hydrolases family, they catalyze triglycerides hydrolysis in lipid-water phase. Lipases catalyze enantio- and regio-selective hydrolysis or synthesis of a wide variety of natural substrates as soy, fish oil, ricin and citric fruits (Dheeman *et al.*, 2010; Fang *et al.*, 2014), they can also perform esterification, interesterification and trans esterification reactions in non-aqueous media (Hasan *et al.*, 2006).

Most lipases are isolated from bacteria and some few yeasts, plants and animals (Hasan *et al.*, 2006). Since the 90's, lipases have attracted attention toward other applications such as solving quiral compounds racemic mixtures of pharmaceutical interest (Skupin *et al.*, 1997), through inter- and transesterification reactions (Jaeger *et al.*, 1994), and pure enantiomeric product synthesis (Gil

et al., 1997; Lee *et al.,* 2008), also as synthesis of products of interest for the perfume industries (Dandavate *et al.,* 2008), applications in food industry, such as fat production with desirable physical and chemical properties, and also low level of trans fat in final products, unlike the traditional method by chemical hydrogenation and esterification (Beisson *et al.,* 2000), among others.

Isolated Enzymes from yeasts are few and there is little information about lipases obtained under saline stress conditions in the non-conventional yeast *D. hansenii*, *R. glutinis* and *C. guilliermondii* (yeasts that do not belong to *Saccharomyces* gender).

The present study quantified lipase activity in *Debaryomyces hansenii*, *Rhodotorula glutinis* and *Candida guilliermondii* yeasts to determine if it is feasible to obtain lipases for biotechnological applications under saline stress conditions using NaCl and KCl, evaluating growing rate, adapting capacity to saline culture medium, and enzymatic activity. Lipase activity was found under saline conditions in the three yeasts, standing out the activity of *D. hansenii*, opening opportunities of a new source to obtain a lipase capable of working under saline conditions.

Materials and Methods

Microorganisms and preparation of inoculums

Each of one the yeasts *Debaryomyces hansenii*, *Rhodotorula glutinis* and *Candida guilliermondii* were treated separately under the same grown conditions as described. Each yeast was grown in sterile YPD medium (20% dextrose, 20% agar, 10% peptone y 10% yeast extract). The stored yeast was transferred by bacteriological loop to 100 mL of YPD medium in a 250 mL Erlenmeyer flask for the preparation of the inoculum, then incubated at 30 °C for 16 h and shaken at 200 rpm. A sample of 100 µL was taken for counting in Neubauer plate as described in to establish the amount of cultured medium to obtain a 3 x 10^6 cells / mL concentration (González-Hernández *et al.*, 2015).

Plate culture with serial dilution

The inoculum was diluted in an essay tube with sterile water to obtain serial dilutions from 1:10 to 1:100 000, one 3 µL drop of each different dilution was placed by spotting on a Petri dish with solid YPD medium as control and four Petri dishes with solid YPD medium with different salt concentrations (NaCl 0.6 M, NaCl 1.2 M, KCl 0.6 M y KCl 1.2 M), cultures were incubated at 30 °C, 72 hours (n = 4) (González-Hernández *et al.*, 2015).

Cell growing kinetics

Erlenmeyer flasks with 100 ml of sterile YPD medium alone and with several concentrations of salt were inoculated with $3x10^6$ cells / mL, incubated at 30 °C and 200 rpm for 16 hours, cell growth was measured every 2 hours, counting viable cells in a Neubauer plate using Tripan or Metilen Blue as described in González-Hernández *et al.,* (2015) to identify growing phases (n=4), pH was also measured during 60 hours. Results were analyzed using ANOVA and a Tukey-Kramer statistical analysis.

Yeast extracts

Non-conventional yeasts were cultured in YPD medium plus an specific salt concentration (0.6 M NaCl, 1.2 M NaCl, 0.6 M KCl y 1.2 M KCl) and were incubated at 30 °C and 200 rpm, samples of 5 mL were taken at 2, 6, 10, 12, 24, and 48 hours, samples were centrifuged at 3500 rpm for 5 minutes, supernatant was separated (yeast extract) and kept in Eppendorf tubes for further quantification of p-nitrophenol release and protein (n = 4).

pH measurement

A Hanna Instruments potentiometer (pH 20, pH meter) was used to measure pH changes of the samples taken as yeast extracts. 2 mL of the supernatant of each sample were placed into Falcon tubes for pH determination at room temperature (25°C) (n = 4).

Lipase activity quantification

To determine enzymatic activity was necessary to quantify the p-nitrophenol (4-nitrophenol) released by the action of a lipase that can act on the substrate triacilgliceride p-nitrophenol butirate. Lipases perform hydrolysis of the ester bond between the triglyceride and the p-nitrophenol, quantification of p-nitrophenol is used to determine units of enzymatic activity as described by Frank (2002). 100 µl of yeast extract were incubated for 30 min at 37°C in pH 7,0 0,05M phosphate buffer and 0,025M p-nitrophenol butyrate, reaction is stopped with 0.1M NaOH, the mixture obtained is centrifuged for 10 min at 11000 rpm (n=4). Quantification of released p-

nitrophenol is done using a spectrophotometer (Perkin Elmer Lamda35) UV at 400 nm. The amount of p-nitrophenol released is determined based on the Lambert-Beer law, since the specific lipase activity by 1 µmol of p-nitrophenol/h is proportional to 1 mg of protein in the sample. A calibration curve was done with n=3. Results were analyzed using ANOVA and a Tukey-Kramer statistical analysis.

Protein quantification

Protein was quantified with the Bradford (1976) method, using a spectrophotometer (Perkin Elmer Lamda35) UV at 595 nm.

Results and Discussion

Plate growing in saltine conditions with serial dilution

Seven different yeast strains were used: *C. guilliermondii, R. glutinis L-033, C. shehatae, D. hansenii ISA 1510, D. hansenii ISA 1508, D. hansenii ISA 1507* and *D. hansenii Y7426,* in order to rule out those strains which could not grow in saline conditions as described in methodology. Plate culturing by spotting was done using 3 µL drops of dilutions from 1:10 to 1:100 000, drops were placed over YPD solid medium using a micropipette. YPD solid medium alone (control) and with added salt in the following concentrations: 0.6 M NaCl, 1.2 M NaCl, 0.6 M KCl and 1.2 M KCl (Fig. 1). Salt concentrations were chosen because *D. hansenii* is halophilic by nature, this yeast is found in sea water where NaCl is about 0.6 M (González-Hernández, 2004). 1.2 M NaCl was also tested as a higher concentration to check salt tolerance. Results of this experiment allowed us to discard those strains which were inhibited by saline conditions. It can be observed that *D. hansenii* grows in saline conditions, four strains were cultured, but strain 1508 was chosen for its best growing (Fig.1). In the case of *C. shehatae* it is observed that saline conditions inhibit its growing so it was discarded. *C. guilliermondii* showed tolerance to saline stress, such capacity has not been described previously. *R. glutinis L-033* showed moderated tolerance to saline stress.

Cell growing kinetics

Non- conventional yeasts chosen were *C. guilliermondii, R. glutinis L-033 and D. hansenii ISA 1508,* all were grown in Erlenmeyer flasks on a rotatory shaker at 30 °C, 200 rpm for 60 hours, taking samples every two hours. Initial cell concentration was 3.0 x 10^6 cells/mL, a graphic was done with obtained data to identify latency, exponential growing and stationary phases. pH measurement was also determined every two hours, showing that as microorganisms reproduce, pH diminishes; this might be due to the proton ATPases of the yeast membrane pump H^+ to extracellular medium (Eraso and Portillo, 1994). During the stationary phase pH was kept constant, then a light increase is observed, perhaps because of the production of non-identified metabolites or a probable reenter of protons into the cell due to a cellular effort to maintain homeostasis through special proton-cation interchangers (Prior *et al.*, 1996; Ramírez *et al.*, 1998).

Duplication and cell division rate

D. hansenii ISA 1508 shows a diminished duplication time versus control in presence of a saline medium. Otherwise, *C. guilliermondii* shows a higher duplication time under saline conditions. *R. glutinis L-033* shows no difference at 0.6 M of NaOH or KCl, but at 1.2 M of either salt a light decrease in duplication time is shown (Table 1).

Table.1 Duplication rate of non-conventional yeasts in YPD medium at different saline conditions, 30 °C and 200 rpm

Duplication time (h^{-1})					
YEAST	**YPD CONTROL**	**0.6 M NaCl**	**1.2 M NaCl**	**0.6 M KCl**	**1.2 M KCl**
D. hansenii ISA 1508	3.7	3.0	3.4	3.3	3.3
C. guilliermondii	3.5	3.7	4.0	3.9	4.0
R. glutinis L-033	3.9	3.9	3.7	3.8	3.6

Table.2 Lipase activity in extracts of *D. hansenii* ISA 1508 at 2, 10 and 24 h of microbial growing

***Debaryomyces hansenii ISA 1508* [µM / mg protein * min]**			
Culture condition	Hour 2	Hour 10	Hour 24
YPD	0.36^{A}	0.38^{A}	0.46^{B}
0.6 M NaCl	0.40^{AB}	0.50^{B}	0.68^{B}
1.2 M NaCl	0.41^{A}	0.46^{B}	0.62^{A}
0.6 M KCl	0.35^{B}	0.33^{D}	0.51^{B}
1.2 M KCl	0.36^{AB}	0.34^{D}	0.51^{B}

Different letters mean significant differences; equal letters mean there is NOT significant difference according to Tukey-Kramer analysis.

Table.3 Lipase Activity determination in 2, 10 and 24 h extracts of microbial growth of *C. guilliermondii*

***Candida guilliermondii* [µM / mg protein * min]**			
Culture condition	Hour 2	Hour 10	Hour 24
YPD	0.48^{A}	0.66^{A}	0.42^{D}
0.6 M NaCl	0.39^{AB}	0.49^{C}	0.50^{B}
1.2 M NaCl	0.35^{ABC}	0.45^{C}	0.52^{B}
0.6 M KCl	0.36^{ABC}	0.59^{C}	0.46^{C}
1.2 M KCl	0.30^{ABC}	0.67^{C}	0.43^{D}

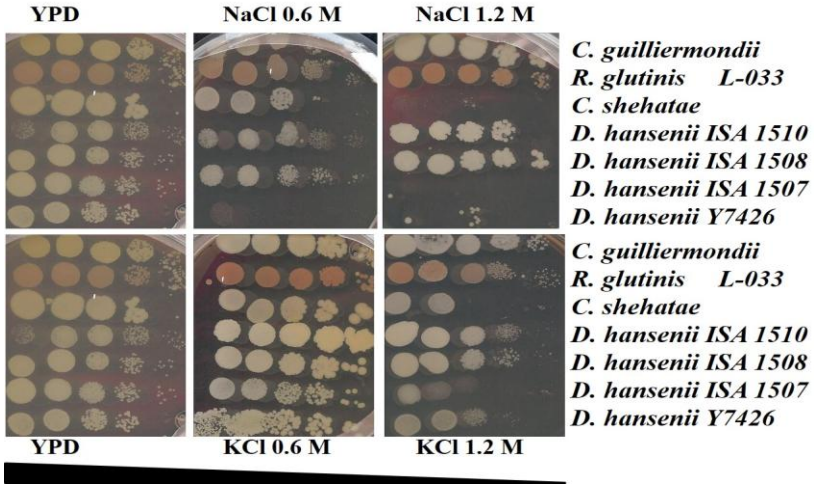
Different letters mean significant differences; equal letters mean there is NOT significant difference according to Tukey-Kramer analysis.

Table.4 Lipase Activity determination in 2, 10 and 24 h extracts of microbial growth of *R. glutinis*

***Rhodotorula glutinis L-033* [µM / mg protein * min]**			
Culture condition	Hour 2	Hour 10	Hour 24
YPD	0.55^{A}	0.49^{A}	0.42^{A}
0.6 M NaCl	0.43^{A}	0.37^{A}	0.39^{A}
1.2 M NaCl	0.34^{A}	0.43^{A}	0.42^{A}
0.6 M KCl	0.44^{A}	0.39^{A}	0.35^{A}
1.2 M KCl	0.33^{A}	0.37^{A}	0.32^{A}

Different letters mean significant differences; equal letters mean there is NOT significant difference according to Tukey-Kramer analysis.

Fig.1 Plate growing by serial dilution of seven strain yeasts. Plates were spot inoculated (3 µL drops) and incubated at 30 °C for 72 h. Shown images are representative data of n = 4



Enzymatic activity quantification

Samples at 2, 10 and 24 hours were chosen as representative samples of the latency, exponential growing or stationary phases to determine if the lipase activity showed any differences depending on the growing phase. Results are shown in tables (2, 3 y 4) where it can be seen that *D.hansenii ISA 1508* has higher enzymatic activity when cell extracts are from cells cultured in presence of 0.6 M NaCl, which supports the saline preferences of this yeast. *C. guilliermondii* showed similar behavior of enzymatic activity in either of the tested conditions. *R. glutinis L-033* also showed similar growing in any of the used salt concentrations, but the lipase activity decreased in the cell extracts in saline conditions.

Lipase production is better when cultures use lipids as carbon source in comparison to the use of glicerol, starch or glucose (Crueger *et al.*, 1993). Positive obtained results for enzymatic activity found in this study using non-conventional yeasts and glucose as primary carbon source open the posibility of changing the carbon source for lipids as oleic acid to stimulate lipase production and corresponding enzymatic activity.

In comparision to previous research in other microorganisms as *Candida antarctica* and *Pichia pastoris* (yeasts), *Hypothenemus hampei*, *Aspergillus niger* and *Aspergillus fumigatus* (fungi), our results are similar.

Therefore, it is possible that some of these non-conventional yeasts can be considered as a feasible source of lipases. Since most industrial lipases are from bacterial origin, this study may expand diversity of these enzyme sources. Furthermore, these lipases could be able to act in saline conditions for industries processes that require it. Obtaining costs can be smaller considering that lipases are secreted into the medium and that biological risks are minor.

Salt concentration in the incubating medium is an important factor for growing, metabolisms and enzymatic activity, especially for. *D. hansenii ISA 1508*, being this non-conventional yeast the one with best enzymatic activity under saline stress conditions at 30 °C. The most favorable conditions for lipase activity for *D. hansenii ISA 1508* are 0.6 M NaCl and 1.2 M NaCl. Non-conventional yeasts *C. guilliermondii* and *R. glutinis L-033* have similar enzymatic activity at different saline conditions, but none of them showed better enzymatic activity than *D. hansenii ISA 1508.*

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Conflict of interest

There is no conflict of interest.

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