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ORIGINAL ARTICLE

# Influence of carbon and nitrogen sources on lipase production by a newly isolated *Candida viswanathii* strain

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**Abstract** Microorganisms can produce lipases with different biochemical characteristics making necessary the screening of new lipase-producing strains for different industrial applications. In this study, 90 microbial strains were screened as potential lipase producers using a sensitive agar plate method with a suitable medium supplemented with Tween 20 and also a liquid culture supplemented with olive oil. The highest cell growth and lipase production for *Candida viswanathii* were observed in triolein and oleic acid when used as the only pure carbon source. Renewable low-cost triacylglycerols supported the best cell growth, and olive oil was found to be the best inducer for lipase production (19.50 g/L and 58.50 U). The selected conditions for enzyme production were found with yeast extract as nitrogen source and 1.5 % (w/v) olive oil (85.70 U) that resulted in a good cell growth yield ($Y_{X/S}$=1.234 g/g) and lipase productivity (1.204 U/h) after 72 h of shake-flask cultivation. *C. viswanathii* lipase presented high hydrolytic activity on esters bonds of triacylglycerols of long-chain, and this strain can be considered an important candidate for future applications in chemical industries.

**Keywords** *Candida viswanathii* · Triacylglycerol · Lipase · Lipolytic yeast · Olive oil

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## Introduction

Lipases (triacylglycerol acyl hydrolases E.C. 3.1.1.3) are hydrolases that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil–water interface (Sarda and Desnuelle 1958; Seitz 1974; Antonian 1988; Björkling et al. 1991; Sharma et al. 2001; Treichel et al. 2010). The complex lipases mechanism of action includes a process of interfacial activation, which comprises two different conformations: an open and active conformation and a closed and inactive conformation (Palomo et al. 2005). Due to their ability to hydrolyze fats, lipases are commonly used as additives in industrial laundry and household detergents; however, they can also be applied in the food, pulp and paper, pharmaceutical, and chemical industries, and for the resolution of racemic chemicals (Sharma et al. 2001; Houde et al. 2004). More recently, this enzyme has been investigated for biodiesel production (Li and Yan 2010).

Microbial lipases have received special attention because of their biochemical properties and broad substrate specificity. Extracellular yeasts lipases for industrial applications can be produced by submerged and solid state fermentations, and also by immobilized cell cultures (Sharma et al. 2001; Treichel et al. 2010). Many bacteria, yeasts and molds can produce lipases (Wang et al. 2007), and among terrestrial yeasts, several *Candida* spp., *Yarrowia lipolytica*, *Rhodotorula* spp., *Pichia* spp., and *Trichosporum* spp., are known lipases producers (Vakhlu and Kour 2006; Treichel et al. 2010). Molecular biology techniques have also been successfully utilized to increase lipase production by microorganisms (Chang et al. 2006; Darvishi et al. 2011; Sheng et al. 2011). Despite the large number of lipase producers, only a few yeast strains, such as *Candida* spp., *Torulopsis* spp., *Y. lipolytica*, *Trichosporum* spp., *Geotrichium* sp., and *Pichia* sp. can use hydrophobic substrates (Papanikolaou and

Aggelis 2010). The multiplicity and complexity of the genes involved in microorganisms' catabolic pathways significantly valorize hydrophobic substrates like vegetable oils, waste-cooking oil, waste edible oil, and tallow allowing the synthesis of high value metabolites such as biosurfactants, tailor-made single cell-oils, organic acids and biodiesel by these strains (Adamczak et al. 2009; Beopoulos et al. 2009; Papanikolaou and Aggelis 2010). *Y. lipolytica* is a model-microorganism capable of breaking-down hydrophobic substrates such as *n*-alkanes, fatty acids, fat, and oils through specific metabolic pathways, for the production of single cell protein, single cell oil, organic acids, and lipases (Fickers et al. 2005; Darvishi et al. 2009; Papanikolaou and Aggelis 2010).

Currently, research on lipases is focused mainly on the production and biochemical characterization of crude or purified enzymes. An important new approach is the incorporation of carbon (especially hydrophobic substrates) and nitrogen sources, into culture media for a better understanding of the physiology of lipase-producing strains in submerged fermentations and also in order to obtain higher enzyme production (Lakshmi et al. 1999; Dalmau et al. 2000; Fickers et al. 2004; Tan et al. 2004; Messias et al. 2009). Some agro-industrial by-products have been evaluated as substrates for the production of fine chemicals and some enzymes, including lipases (Papanikolaou et al. 2007; Darvishi et al. 2009). In other cases, agro-industrial wastewaters were incorporated in culture media for lipase production, consequently allowing the removal environmental pollutants from contaminated water (Vargas et al. 2008; Mafakher et al. 2010). Organic and inorganic nitrogen sources play an important role in enzymes synthesis as inorganic sources can be used quickly, while organic nitrogen sources can supply cells with growth factors and amino acids required for cell metabolism and enzymes synthesis (Tan et al. 2004). In addition, other parameters such as agitation, pH, and temperature are also be important.

Although some of the above-mentioned lipase producers have been extensively investigated, screening programs have found some emerging lipase-producing yeasts and novel biocatalysts that represents a promise for biotechnological innovation in many areas (Bussamara et al. 2010). The Brazilian ecosystems biodiversity is a promising source of new microorganisms that can produce a variety of biocatalysts with desirable properties for industrial applications.

The aim of this work was to select the best lipase-producing microbial strain isolated from different Brazilian ecosystems or from industrial effluents, and also to determine the effect of various carbon and nitrogen sources on the lipase production by the selected strain.

## Materials and methods

### Microorganisms: origin and maintenance

Filamentous fungi strains isolated from soil of Atlantic rainforest (Ruegger and Tauk-Tornisielo 2004) and Caatinga areas (Simões and Tauk-Tornisielo 2006) were obtained from the Culture Collection of the Environmental Studies Center (CEA), UNESP, Rio Claro, São Paulo, Brazil. Yeast strains isolated from the Cerrado environment were provided by the Laboratory of Environmental and Biotechnological Microbiology, Federal University of Tocantins, Palmas, Tocantins, Brazil. Other filamentous fungi strains were isolated from poultry slaughterhouse effluent (Fricock, Rio Claro, São Paulo, Brazil). *Candida viswanathii* was isolated from wastewater of a Brazilian oil refinery (Replan/Petrobras, Paulínia, São Paulo, Brazil) (Soares et al. 2008). These strains were considered as from industrial environment. All strains were grown on MEA-slants for five days at 28 °C and subsequently kept in a refrigerator.

### Screening of lipolytic microorganisms

Preliminary lipolytic microorganisms screening was carried out in Petri dishes containing the medium proposed by Hankin and Anagnostakis (1975) composed of (g/L): peptone, 10; NaCl, 5; $CaCl_2 \cdot 2H_2O$, 0.1, agar, 20, supplemented with 1 % (w/v) Tween 20. The final pH was adjusted to 7.4 and the medium was autoclaved at 121 °C, for 20 min. Cultures were incubated at 28 °C and examined daily during 7 days. The enzymatic activity was measured by an enzymatic index, i.e. the rate between the halo diameter including the colony and the diameter of the colony expressed in percentage. The best periods of cultivation for measurements was established and colonies showing an opaque area due to calcium salts precipitation were selected for the subsequent screening in liquid medium.

### Inoculum preparation and lipase production conditions

Liquid medium was prepared using Vogel's medium (Vogel 1956). Trace elements solution (solution A) was prepared containing (g/L): citric acid monohydrate·$H_2O$, 50, $ZnSO_4 \cdot 7H_2O$, 50, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 10; $CuSO_4 \cdot 5H_2O$, 2.5; $MnSO_4 \cdot H_2O$, 0.05; $H_3BO_3$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0,05. Salt solution (solution B) was prepared containing (g/L): sodium citrate pentahydrate·$5H_2O$, 150; $KH_2PO_4$, 250; $NH_4NO_3$, 100; $MgSO_4 \cdot 7H_2O$, 10; $CaCl_2 \cdot 2H_2O$, 5 and biotin solution (0.1 mg/mL), 5 mL; solution A, 5 mL; 0.2 mL chloroform. The solutions were maintained at 4 °C. Medium preparation consisted in 50-fold dilution of the solution B, adding glucose or other carbon sources and the final pH was adjusted to 6.0.

Cultures were performed in Erlenmeyer flasks (125 mL) containing 25 mL of culture medium. Soluble pure carbon sources (glucose, galactose, lactose, mannitol, sorbitol, glycerol, citric acid, lactic acid), hydrophobic pure sources (lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, tributyrin and triolein) and natural triacylglycerols (canola, castor, corn, linseed, olive, palm, soybean, sunflower oils, chicken fat, lard and beef tallow) were used initially at 2 % (w/v). Hydrophobic substrates were mechanically emulsified allowing the dispersion of fat particles into the aqueous phase. Nitrogen sources [corn steep liquor, peptone, tryptone, yeast extract, urea, $NH_4Cl$, $(NH_4)_2SO_4$ and $NH_4NO_3$] were added to the culture media at 0.2 % (w/v). Different concentrations of triacylglycerols (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 %, w/v) were also evaluated with yeast extract or $NH_4Cl$.

Time-course of lipase production using the best carbon source concentration (olive oil, 1.5 % w/v) and nitrogen source (yeast extract) was carried out during 120 h. Samples were collect each 12 h.

All media were autoclaved at 121 °C for 20 min. Inocula were prepared using 5-day-old cultures. Media were inoculated with 1 mL yeast cells or fungal suspension ($1\times10^7$ spores or cells per mL) and incubated at 28 °C, and 180 rpm for the same period of those used in solid cultures. Biomass was separated from the fermentation broth by filtration (membrane cellulose acetate 0,45 μm cut-off) and dried at 105 °C until constant weight. Cell-free broth was used for lipase activity assays. The results were assayed for biomass, maximum lipase activity, substrate, biomass yield on substrate, lipase yield on substrate, lipase productivity, and specific rate of lipase production.

### Lipase activity assays on synthetic substrate

Lipase activity was assayed with ρ-nitrophenyl-palmitate (pNPP) as substrate (Yang et al. 2002). pNPP was firstly solubilized in 0.5 mL of dimethyl sulfoxide, then diluted to 50 mM with 50 mM sodium phosphate buffer pH 7.0, containing 0.5 % Triton X-100. Lipase activity was determined by following the hydrolysis of pNPP at 37 °C. The reaction was stopped at different intervals by heat shock (90 °C, 1 min), followed by the addition of 1 mL saturated sodium tetraborate solution. The *p*-nitrofenolate formed was measured spectrophotometrically at 405 nm. The molar extinction coefficient for ρ-nitrophenol (pNP) at 405 nm was determined as $1.8\times10^4$ $M/cm^{-1}$. Controls were prepared without enzyme. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of pNP per min. Results were presented as total units or specific activity in units by milligram of protein.

### Hydrolytic activity of lipase on triacylglycerols

Hydrolytic lipase activity was determined using a modified titration method (Leal 2000). The hydrolysis of tributyrin, triolein or olive oil was determined at 37 °C, following the release of fatty acids by titration. Each 10 % substrates (w/v) were individually emulsified in 0.5 mM sodium phosphate buffer pH 7.0, containing 6 % (w/v) Triton X-100. The reaction was started by adding 1 mL samples to 5 mL of this emulsion, and then maintained in orbital shaker at 300 rpm for 30 min. The reaction was stopped by adding 16 mL of an acetone:ethanol solution (1:1, v/v). The released fatty acids were titrated to pH 11.0 with a 0.1 M NaOH solution. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of fatty acid per min. The results were expressed as percentage of the hydrolysis product of the pNPP.

### Determination of residual olive oil after cultivation

The culture supernatant was transferred to Erlenmeyer flasks (125 mL) and acidified to pH 1.0 with concentrated sulfuric acid. Then, 10 mL of hexane were added to the samples, which were vigorously agitated and maintained in a separating funnel until the separation of organic and aqueous phases. The organic phase was oven-dried at 40 °C and the mass was measured in analytical balance.

### Protein analysis

The protein was determined by the Lowry method (Lowry et al. 1951), using bovine serum albumin as standard.

## Results and discussion

### Selection of lipolytic microorganisms

Initially, a total of ninety microorganisms isolated from natural and industrial environment, including filamentous fungi (33 strains from Atlantic rainforest, 34 strains from the Caatinga area, 9 from industrial environment) and yeasts (7 strains from the Cerrado environment, 7 strains from industrial environment) were employed in this work. All the microorganisms were screened for lipase activity in solid medium plates containing Tween 20. In the first step, 11 filamentous fungi and 8 yeast strains produced an opaque halo around them and were selected as lipase producers, representing 21 % of the microorganisms (Table 1). The *Aspergillus niger, Aspergillus* sp. F2, *Lipomyces starkeyi* I and II, *Metharhizium anisopliae, Penicillium simplicissimum, Rhodotorula graminis*, *Rhodotorula glutinis* I and II, *Yarrowia lipolytica* I and II, *Rhizopus* sp. L04, and *C.*

**Table 1** Evaluation of lipase production from selected filamentous fungi and yeasts

| Strain | Origin | Time of cultivation (h) | Solid cultures Enzimatic index (%) | Liquid cultures Lipase activity (U) |
|---|---|---|---|---|
| *Penicillium simplicissimum* | Atlantic rainforest | 120 | 74.96±4.11 | ND |
| *Penicillium verruculosum* | | 120 | 40.42±4.38 | 0.75±0.01 |
| *Penicillium janthinellum* | | 120 | 38.64±6.86 | 9.50±0.12 |
| *Verticillium lecani* | | 120 | 26.76±1.09 | 0.75±0.02 |
| *Metharryzhium anisopliae* | | 120 | 70.10±0.46 | 23.75±2.25 |
| *Gliocephalotrichum simplex* | | 120 | 55.78±6.14 | ND |
| *Aspergillus* sp. | | 120 | 48.60±1.61 | 6.44±0.83 |
| *Aspergillus niger* | Caatinga | 120 | 80.53±2.08 | 7.50±0.81 |
| *Yarrowia lipolytica* I | Cerrado | 72 | 70.58±4.88 | ND |
| *Yarrowia lipolytica* II | | 72 | 68.24±2.90 | ND |
| *Rodothorula graminis* | | 72 | 79.24±2.97 | ND |
| *Rodothorula glutinis* I | | 72 | 74.50±1.52 | 2.25±0.75 |
| *Rodothorula glutinis* II | | 72 | 72.24±1.83 | 2.76±0.50 |
| *Lipomyces starkeyi* I | | 72 | 77.76±4.69 | ND |
| *Lipomyces starkeyi* II | | 72 | 73.30±5.92 | ND |
| *Rhizopus* sp. L04 | Industrial environment | 120 | 68.48±8.05 | 0.75±0.00 |
| *Rhizopus* sp. L05 | | 120 | 26.40±0.01 | 0.25±0.01 |
| *Aspergillus* sp. F2 | | 120 | 71.33±2.84 | 1.25±0.09 |
| *Candida viswanathii* | | 72 | 66.67±2.15 | 52.50±5.50 |

*viswanathii* strains showed the highest enzymatic index reaching over 65 %. Lower enzymatic index were observed for *Aspergillus* sp., *Gliocephalotrichum simplex*, *Penicillium verruculosum*, *Penicillium janthinellum*, *Rhizopus* sp. L05, and *Verticillium lecani* strains (below 56 %). These strains were cultured in liquid medium with olive oil and 63 % of them presented lipase activity as verified by pNPP hydrolysis (Table 1).

No lipase activity was observed in liquid cultures with *P. simplicissimum*, *G. simplex*, *Y. lipolytica* I, *Y. lipolytica* II, *R. graminis*, *L. starkeyi* I, or *L. starkeyi* II. These results can be attributed to the medium composition used for lipase production since the *Y. lipolytica* and *P. simplicissimum* are known good lipase producers in submerged and solid state cultivations (Gutarra et al. 2007; Papanikolaou et al. 2007; Wang et al. 2007; Vargas et al. 2008; Sheng et al. 2011). *Aspergillus* sp., *Aspergillus* spp. F2, *A. niger*, *P. janthinellum, P. verruculosum, R. glutinis* I, *R. glutinis* II, *V. lecani*, *Rhizopus* sp. L04. and *Rhizopus* sp. L05 strains produced only low levels of lipase activity. The greatest lipase activity was verified with *C. viswanathii* that presented values of 52.50 U, significantly higher than that from *M. anisopliae* (23.75 U). *C. viswanathii* is reported here for the first time as an extracellular lipase producer because no information about the lipase produced and its properties were described before. A biotechnological application of *C. viswanathii* was described for the production of carbonil reductase, which presents high enantioselectivity (Fatima et al. 2007) and high potential for hydrocarbons and biodiesel degradation (Soares et al. 2008). Therefore, due to its biotechnological potential as well as due to the high lipase production, *C. viswanathii* was selected for further optimization studies.

## Effect of pure carbon sources

*Candida viswanathii* can use various carbon compounds for energy production and biomass formation (Kurtzman et al. 2011). When cultivated in medium supplemented with glucose, galactose, mannitol, sorbitol or glycerol *C. viswanathii* growth was verified but no lipase production was observed (Table 2). Cultures with lactose, lactic acid and citric acid also showed low cell growth (1.20, 1.52. and 2.33 g/L, respectively) and no lipase production.

Among the fatty acids and triacylglycerols, the highest *C. viswanathii* growth was observed with oleic acid and triolein. *C. viswanathii* growth on hydrophobic substrates was critically influenced by composition of the fatty acids in the medium. Cultivation with saturated fatty acids (lauric, myristic, palmitic and stearic) provided low growth (values between 2.00 and 4.56 g/L, respectively), and low lipase production (0.25 U). One possible reason is that all these fatty acids are solids, and cannot be dispersed completely in the broth. Similar results were also observed for the lipase production by *Rhizopus chinensis* (Teng et al. 2009), and a possible solution for this problem would be the addition of emulsifiers or ethanol into the culture medium (Shimada et al. 1992). The highest *C. viswanathii* growth and lipase

**Table 2** Lipase production and *Candida viswanathii* growth in different pure carbon source

| Carbon source | Lipase activity (U) | Specific activity (U/mg) | Biomass (g/L) |
|---|---|---|---|
| Carbohydrates | | | |
| Glucose | ND | ND | 11.85±0.07 |
| Galactose | ND | ND | 9.32±0.68 |
| Lactose | ND | ND | 1.20±0.01 |
| Alcohols | | | |
| Mannitol | ND | ND | 13.6±0.99 |
| Sorbitol | ND | ND | 8.83±0.52 |
| Glycerol | ND | ND | 15.47±0.35 |
| Organic acids | | | |
| Citric acid | ND | ND | 2.33±0.35 |
| Lactic acid | ND | ND | 1.52±0.39 |
| Fatty acids | | | |
| Lauric (12:0) | 0.25±0.01 | 0.22±0.02 | 4.56±0.45 |
| Myristic (14:0) | 0.25±0.01 | 0.15±0.01 | 3.25±0.04 |
| Palmitic (16:0) | 0.25±0.01 | 0.12±0.00 | 3.32±0.51 |
| Estearic (18:0) | 0.25±0.01 | 0.04±0.00 | 2.06±0.03 |
| Oleic (18:1) | 6.75±0.75 | 2.88±0.71 | 9.06±0.11 |
| Linoleic (18:2) | ND | ND | ND |
| Triacylglycerols | | | |
| Tributyrin | ND | ND | 2.88±0.05 |
| Triolein | 10.25±0.98 | 3.43±0.90 | 10.82±0.08 |

Culture conditions: cultivations were performed in Vogel's medium with 2 % (w/v) carbon sources, pH 6.0, 72 h, 180 rpm at 28 °C. *ND* not detected

production were observed with triolein (10.82 g/L and 10.25 U, respectively), followed by acid oleic acid (9.06 g/L and 6.75 U, respectively), and the highest specific activities were also observed with this triacylglycerol and fatty acid (3.43 U/mg and 2.88 U/mg, respectively). Usually, saturated fatty acids support microbial growth and lipase production by yeasts as observed in several other studies (Obradors et al. 1993; Dalmau et al. 2000; Saravanan et al. 2007); however, oleic acid has been considered the most important inducer for some microorganisms (Sharma et al. 2001; Treichel et al. 2010). These results suggest this enzyme is inducible and can be subjected to catabolic repression, as observed for the lipase production by *Candida rugosa* (Dalmau et al. 2000). No microbial growth was observed with linoleic acid as the only carbon sources. This fact indicates that this fatty acid, differently from others, did not promote growth or lipase production. Lipases can be produced with this carbon source by filamentous fungi (Shimada et al. 1992; Ohnishi et al. 1994; Pogori et al. 2008; Teng et al. 2009).

In this work, in medium with tributyrin as only carbon source, *C. viswanathii* grew, but did not produce lipase, indicating the production of some isozymes or an esterase responsible for the hydrolysis of this triacylglycerol and cell development. Although tributyrin was good carbon source for induction of lipase in some microorganisms, in others this carbon source and short- and medium-chain fatty acids did not induce the production of the lipases (Kok et al. 1996).

## Effect of natural triacylglycerols

Lipase production and *C. viswanathii* growth in different natural triacylglycerols, as well as the predominant fatty acids presence in these oils and fats, are shown in Table 3. The choice of inducers was based on the presence of high percentage of long-chain fatty acids, their availability and cost. The cultures prepared with natural triacylglycerols were more effective for lipase production and cell growth in comparison with pure fatty acids and triacylglycerols (Table 2). These findings could be related to the intrinsic composition of the vegetable oils, and also to the presence of several tocopherols and other liposoluble vitamins that are important for microbial growth.

The highest lipase activity (58.50 U) and cell growth (19.50 g/L) were achieved in medium with olive oil. The lowest lipase activity was detected, respectively, in castor oil, beef tallow and lard. The results found for lipase production with castor oil could be related to the anti-nutritional effect of the ricinoleic acid (present in 90 % of total composition) an unsaturated omega-9 fatty acid that has in its structure an unusual hydroxyl linked to the 12-carbon. Besides this fatty acid, castor oil is composed by 0.7 % palmitic acid, 0.9 % stearic acid, 2.8 % oleic acid, 4.4 % linoleic acid, and 0.2 % linolenic acid (Salimon et al. 2010). These results were also found for the lipase production by *C. rugosa* with coconut oil that present the hydroxy substituted C-14 fatty acids (Lakshmi et al. 1999). As beef tallow and lard are solid in ambient temperature, mechanical emulsion could not promote adequate dispersion of these substrates in the liquid medium, resulting in low lipase production, and low biomass formation.

The parameters biomass yield on substrate consumed, biomass productivity, lipase yield on substrate consumed, lipase productivity, and specific rate of lipase production were calculated for all carbon sources (Table 3). Olive oil indicates that this carbon source is a suitable hydrophobic substrate for lipase production showing $Y_{X/S}$ 1.476 g/g, $P_X$ 0.270 g/h, $Y_{L/S}$ 4.417 U/g, $P_L$ 0.812 U/h, and $q_L$ 0.041 U/g.h. The highest specific activity was also found in cultures with olive oil (10.63 U/mg of protein) followed by palm oil (10.54 U/mg of protein). These results could be justified by the fact that *C. viswanathii* was lipase induced preferentially by triacylglycerol of monounsaturated (18:1 $\Delta^9$) fatty acids, as also observed in Table 2. Olive oil contains approximately

**Table 3** Lipase production and *Candida viswanathii* growth in different natural mixtures of triacylglycerols

| Natural triacylglycerol | Predominant fatty acids[a] | X | L | Specific activity (U/mg prot.) | $Y_{X/S}$ | $P_X$ | $Y_{L/S}$ | $P_L$ | $q_L$ |
|---|---|---|---|---|---|---|---|---|---|
| Castor oil | 18:1 ω-OH (90 %) | 6.55±0.13 | 0.75±0.01 | 0.43±0.01 | 0.481 | 0.091 | 0.055 | 0.010 | 0.001 |
| Olive oil | 18:1 (76–79 %) | 19.50±3.25 | 58.50±5.25 | 10.63±0.66 | 1.472 | 0.270 | 4.417 | 0.812 | 0.041 |
| Soybean oil | 18:2 (54 %); 18:1 (25 %) | 13.90±2.04 | 14.64±1.50 | 6.77±0.72 | 1.198 | 0.193 | 1.262 | 0.203 | 0.014 |
| Canola oil | 18:1 (61 %); 18:2 (21 %) | 10.46±1.47 | 16.33±1.32 | 8.88±0.50 | 0.970 | 0.145 | 1.514 | 0.226 | 0.021 |
| Maize oil | 18:2 (57 %); 18:1 (28 %) | 16.94±1.42 | 11.28±1.62 | 3.91±0.48 | 1.176 | 0.235 | 0.806 | 0.156 | 0.009 |
| Sunflower oil | 18:2 (66 %); 18:1 (22 %) | 16.13±0.18 | 25.99±1.75 | 7.53±0.89 | 1.353 | 0.224 | 2.180 | 0.360 | 0.022 |
| Palm oil | 16:0 (48 %); 18:1 (36 %) | 12.30±0.27 | 29.00±2.12 | 10.54±0.98 | 0.707 | 0.170 | 1.667 | 0.402 | 0.032 |
| Linseed oil | 18:3 (53 %); 18:1and 18:2 (~20 %) | 18.95±1.02 | 21.50±1.03 | 7.16±0.28 | 0.972 | 0.263 | 1.103 | 0.298 | 0.015 |
| Chicken fat | 18:1 (55 %); 16:0 and 18:2 (18 %) | 14.71±0.31 | 19.25±1.00 | 6.41±0.99 | 1.218 | 0.204 | 1.594 | 0.267 | 0.018 |
| Lard | 18:1 (44 %); 16:0 (29 %) | 4.33±0.07 | 2.75±0.25 | 1.22±0.12 | 0.881 | 0.060 | 0.557 | 0.038 | 0.008 |
| Beef tallow | 18:1 (35 %); 18:0 (26 %); 16:0 (30 %) | 5.46±0.95 | 2.50±0.09 | 0.10±0.02 | 1.092 | 0.075 | 0.125 | 0.034 | 0.006 |
| P+O (1:1) | – | 18.36±3.09 | 42.75±4.00 | 9.00±0.85 | 0.991 | 0.255 | 2.309 | 0.593 | 0.032 |
| P+O (0.75:0.25) | – | 19.28±0.82 | 26.75±1.75 | 5.94±0.42 | 1.027 | 0.267 | 1.425 | 0.371 | 0.019 |
| P+O (0.25:0.75) | – | 16.39±3.27 | 36.75±3.07 | 7.73±0.96 | 1.673 | 0.227 | 3.751 | 0.510 | 0.031 |

Culture conditions: cultivations were performed in Vogel's medium with 2 % (w/v) vegetable oils or animal fats, pH 6.0, 72 h, 180 rpm at 28 °C

*P+O* Palm oil and olive oil mixtures; *X* biomass (g/L); *L* lipase (U); $Y_{X/S}$ biomass yield on consumed substrate (g/g); $Y_{L/S}$ lipase yield on consumed substrate; $P_X$ biomass productivity (g/h); $P_L$ lipase productivity (U/h); $q_L$ specific rate of lipase production (U/g biomass.h)

[a] Salimon et al. (2010); Kalo and Kemppinem (2003)

80 % oleic acid and is considered a good inducer for lipase synthesis by many microorganisms (Sharma et al. 2001; Darvishi et al. 2009; Wang et al. 2007; Mafakher et al. 2010), which could be related to the fact that the lipases preferentially hydrolyze fatty acids residues at positions 1 and 3 of the glycerides, and some extracellular lipase requires oleic acid as stabilizer/activator (Barth and Gaillardin 1997).

Other triacylglycerols sources, i.e. maize, soybean, canola, linseed, sunflower, palm oils, and chicken fat, promoted cell growth but were not efficient to induce the secretion of high levels of lipase into the fermentation medium. The combined effect of olive and palm oil (1:1) was effective for lipase production (42.75 U) and biomass formation (18.36 g/L) by *C. viswanathii*. Yield parameters for this combined carbon source presented good growth ($Y_{X/S}$= 0.992 g/g) and lipase yield ($Y_{L/S}$=2.309 U/g, $P_L$=0.593 U/h, and $q_L$=0.032 U/g.h), but it would be important to became this process cheaper.

The incorporation of vegetable oil with different fatty acids composition influenced the lipase production and cell growth in flasks fermentation due to enzyme specificity for some fatty acids. The results in Fig. 1 indicate that the *C. viswanathii* lipase presents high hydrolytic activity on triolein and olive oil suggesting that this enzyme preferentially hydrolyzes ester bounds of long-chain monounsaturated fatty acids (C18:1 $\Delta^9$). Thus, the quantity of C18:1 $\Delta^9$ of the vegetable oils clearly enhanced the lipase production of *C. viswanathii*. This finding was also reported for the lipase production by many microorganisms, for which olive oil serves not only as inducer for lipase production but also as the carbon source for microorganism growth (Lakshmi et al. 1999; Azeredo et al. 2007; Teng et al. 2009).

## Effect of nitrogen sources

The effect of various mineral and organic nitrogen sources were evaluated on the lipase production and *C. viswanathii*

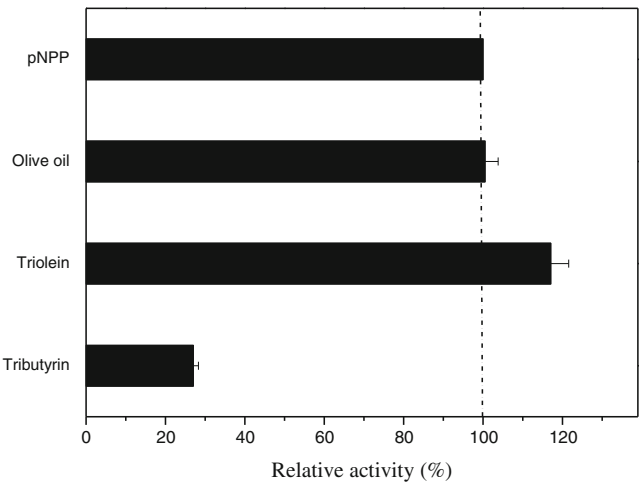


**Fig. 1** Hydrolytic activity of *C. viswanathii* lipase on different triacylglycerols. Culture conditions: cultivations were performed in Vogel's medium with 2 % (w/v) olive oil, pH 6.0, 72 h, 180 rpm at 28 °C

**Table 4** Lipase production and *Candida viswanathii* growth in different nitrogen sources

| Nitrogen source (0.2 %, w/v) | X | L | Specific activity (U/mg prot.) | $Y_{X/S}$ | $P_X$ | $Y_{L/S}$ | $P_L$ | $q_L$ |
|---|---|---|---|---|---|---|---|---|
| Peptone | 20.98±3.05 | 59.25±0.39 | 3.76±0.62 | 1.625 | 0.291 | 4.590 | 0.822 | 0.039 |
| Tryptone | 20.84±4.44 | 62.00±0.35 | 4.73±0.98 | 1.075 | 0.289 | 3.199 | 0.861 | 0.041 |
| Yeast extract | 21.12±1.25 | 68.75±0.25 | 10.57±0.86 | 1.543 | 0.340 | 4.683 | 0.954 | 0.039 |
| Cor steep liquor | 10.79±0.55 | 55.75±0.34 | 8.25±0.18 | 0.969 | 0.149 | 4.245 | 0.774 | 0.072 |
| Urea | 3.80±0.89 | 0.55±0.00 | 0.07±0.00 | 0.310 | 0.052 | 0.044 | 0.006 | 0.001 |
| $NH_4Cl$ | 14.06±0.67 | 2.00±0.00 | 0.24±0.00 | 1.210 | 0.195 | 0.172 | 0.030 | 0.001 |
| $(NH_4)_2SO_4$ | 14.56±1.08 | 4.25±0.01 | 0.54±0.04 | 1.227 | 0.202 | 0.366 | 0.060 | 0.004 |
| $NH_4NO_3$ (control) | 19.50±3.25 | 58.50±0.33 | 10.63±0.66 | 1.472 | 0.270 | 4.417 | 0.812 | 0.041 |

Culture conditions: cultivations were performed in Vogel's medium with 2 % (w/v) olive oil, pH 6.0, 72 h, 180 rpm, at 28 °C

*X* Biomass (g/L); *L* lipase (U); $Y_{X/S}$ biomass yield on consumed substrate (g/g); $Y_{L/S}$ lipase yield on consumed substrate; $P_X$ biomass productivity (g/h); $P_L$ lipase productivity (U/h); $q_L$ specific rate of lipase production (U/g biomass.h)

growth and the results are shown in Table 4. Among mineral nitrogen sources, ammonium chloride and ammonium sulfate supported good cell growth (14.06 and 14.56 g/L, respectively) but a negative effect on lipase production was observed. Moreover, ammonium nitrate supported good cell growth (19.50 g/L) and lipase production (58.50 U) and also resulted in the highest specific activity (10.63 U/mg of protein). Organic nitrogen sources (peptone, tryptone, and yeast extract) increased lipase and cell productivity, except for corn steep liquor. Yeast extract was the best nitrogen source for lipase production (68.75 U) and cell growth (21.12 g/L). Specific activity (10.57 U/mg) similar to the control was also observed with this nitrogen source. For other analyzed parameters, *C. viswanathii* presented high cell growth and lipase production with peptone ($Y_{X/S}$=1.625 g/g; $Y_{L/S}$=4.590U/g), tryptone ($Y_{X/S}$=1.075 g/g; $Y_{L/S}$=3.199 U/g), and yeast extract ($Y_{X/S}$=1.543 g/g; $Y_{L/S}$=4.683 U/g). These organic nitrogen sources are generally used as they provide additional nutritional factors such as amino acids, vitamins, and cofactors to the cells (Barth and Gaillardin 1997; Tan et al. 2004; Darvishi et al. 2009).

The high specific rate of lipase production (0.072 U/g.h) and biomass production ($Y_{X/S}$=0.969) with corn steep liquor indicates that this low-cost nitrogen source could be a strong candidate for lipase production in a large-scale process, since in the literature it is suggested that lower $Y_{X/S}$ values around 0.5–0.7 g/g obtained from high-lipase production are very satisfactory for single-cell protein fermentation with fatty substrates (Kamzolova et al. 2005; Papanikolaou et al. 2007; Darvishi et al. 2009). Urea provided the lowest growth (3.80 g/L) and lipase production (0.55 U). Although this nitrogen source was used for lipase

**Table 5** Effect of olive oil concentrations on lipase production by *C. viswanathii* in medium with ammonium nitrate or yeast extract

| Olive oil concentration (%, w/w) | X | L | Specific activity (U/mg prot.) | $Y_{X/S}$ | $P_X$ | $Y_{L/S}$ | $P_L$ | $q_L$ |
|---|---|---|---|---|---|---|---|---|
| Ammonium nitrate (0.2 %, w/w) | | | | | | | | |
| 0.5 | 8.56±0.23 | 21.25±2.42 | 5.08±0.49 | 1.958 | 0.118 | 4.860 | 0.295 | 0.034 |
| 1.0 | 12.10±0.16 | 46.50±6.20 | 9.01±0.93 | 1.628 | 0.168 | 6.256 | 0.645 | 0.053 |
| 1.5 | 14.66±0.24 | 55.75±5.77 | 10.27±0.57 | 1.141 | 0.203 | 4.340 | 0.774 | 0.052 |
| 2.0 | 15.95±0.31 | 58.50±6.42 | 10.46±0.66 | 1.557 | 0.221 | 4.417 | 0.812 | 0.050 |
| 2.5 | 19.47±0.63 | 59.75±6.67 | 10.54±0.68 | 1.807 | 0.270 | 4.634 | 0.829 | 0.042 |
| 3.0 | 19.82±1.97 | 64.50±6.15 | 10.58±1.02 | 1.537 | 0.275 | 4.642 | 0.895 | 0.045 |
| 3.5 | 19.18±0.97 | 58.00±6.20 | 8.35±0.76 | 1.439 | 0.266 | 4.353 | 0.805 | 0.041 |
| Yeast extract (0.2 %, w/w) | | | | | | | | |
| 0.5 | 7.95±0.52 | 58.25±3.65 | 8.50±0.53 | 1.590 | 0.110 | 5.295 | 0.809 | 0.101 |
| 1.0 | 13.1±0.36 | 78.75±7.72 | 11.00±0.75 | 1.419 | 0.182 | 7.825 | 1.093 | 0.083 |
| 1.5 | 18.44±0.11 | 86.50±9.62 | 12.79±0.19 | 1.234 | 0.256 | 5.791 | 1.201 | 0.065 |
| 2.0 | 20.58±0.79 | 68.75±1.40 | 10.63±0.77 | 1.402 | 0.285 | 4.683 | 0.954 | 0.046 |
| 2.5 | 22.04±0.22 | 66.00±5.67 | 8.91±0.79 | 1.351 | 0.306 | 4.451 | 0.916 | 0.041 |
| 3.0 | 24.05±0.92 | 66.25±5.70 | 8.88±0.73 | 1.751 | 0.334 | 4.824 | 0.920 | 0.038 |
| 3.5 | 26.35±0.93 | 65.00±5.35 | 8.50±0.31 | 1.347 | 0.365 | 3.323 | 0.902 | 0.034 |

Culture conditions: cultivations were performed in Vogel's medium, 0.2 % (w/v) nitrogen source, pH 6.0, 72 h, 180 rpm at 28 °C

*X* Biomass (g/L); *L* maximum lipase activity (U); $Y_{X/S}$ biomass yield on consumed substrate (g/g); $Y_{L/S}$ lipase yield on consumed substrate; $P_X$ biomass productivity (g/h); $P_L$ lipase productivity (U/h); $q_L$ specific rate of lipase production (U/g biomass.h)

production in other studies (Corzo and Revah 1999; Rodriguez et al. 2006), during the test of assimilation, the *C. viswanathii* strain showed growth in urea medium as well as low lipase production.

### Effect of selected inducer concentrations

The effects of substrate concentration (0.5–3.5 %, w/v) were studied while maintaining the ammonium nitrate and yeast extract sources constant. These nitrogen sources were chosen for these experiments because they presented very similar specific activity values (~10 U/g of protein) and they are readily available at low cost. Lipase production and growth were evaluated using several concentrations of olive oil in the fermentation medium. The olive oil concentration has a strong influence on lipase production and growth at both nitrogen sources. The highest lipase production and the highest specific activity were observed with 1.5 % (w/v) olive oil and yeast extract (Table 5), corresponding to 86.50 U and 12.8 U/mg of protein, respectively, while it was necessary to have 3.0 % (w/v) olive oil to produce 64.50 U in the cultures with ammonium nitrate. Fermentation parameters analyzed for ammonium nitrate and yeast extract indicated an excellent biomass and lipase yield for all olive oil concentrations studied; however, 1.5 % (w/v) in medium with yeast extract provided the highest lipase productivity ($P_L$=1.201 U/h). Above these concentrations, lipase activity decreased possibly due to the inhibitory effect of the glycerol produced (Lakshmi et al. 1999). In similar studies, maximal lipase production was achieved in cultures with 0.75–2.5 % (w/v) vegetable oils (Mahadik et al. 2002; Tan et al. 2004; Gulati et al. 2005).

The *C. viswanathii* growth behavior on hydrophobic substrates was commonly found in oleaginous microorganisms that are capable of consuming fats and at the same time accumulating high quantities of intracellular lipids (Papanikolaou and Aggelis 2011). The excellent biomass yield for all olive oil concentrations ($Y_{X/S}$ >1.1 g/g) in both nitrogen sources used in this study suggests that this strain may be used in future investigations for lipid formation or accumulation. *Y. lipolytica* cultures in olive oil supplemented with ammonium sulfate presented 0.710 g/g biomass yield (Darvishi et al. 2009), which is considered a good single-cell growth on hydrophobic substrates.

### Time-course of lipase production

The time-course of lipase production and olive oil consumption were assayed for 120 h in medium with 1.5 % (w/v) olive oil and 0.2 % (w/v) yeast extract. The highest enzyme production and specific activity values were observed in 72 h cultures (86.50 U, 14.31 U/mg of protein, respectively) (Fig. 2a). Typical microbial growth and olive oil consumption curves were observed (Fig. 2b). After 24 h, the olive oil was exhausted from the fermentation medium and after 48 h the growth reached the stationary phase. Lipase production was cell growth associated, and lipase production decreased from middle of stationary phase. These results are similar to those with *Rhizopus arrhizius* and *C. rugosa* in relation to cell growth and lipase production (Elibol and Ozer 2000; Takaç et al. 2010).

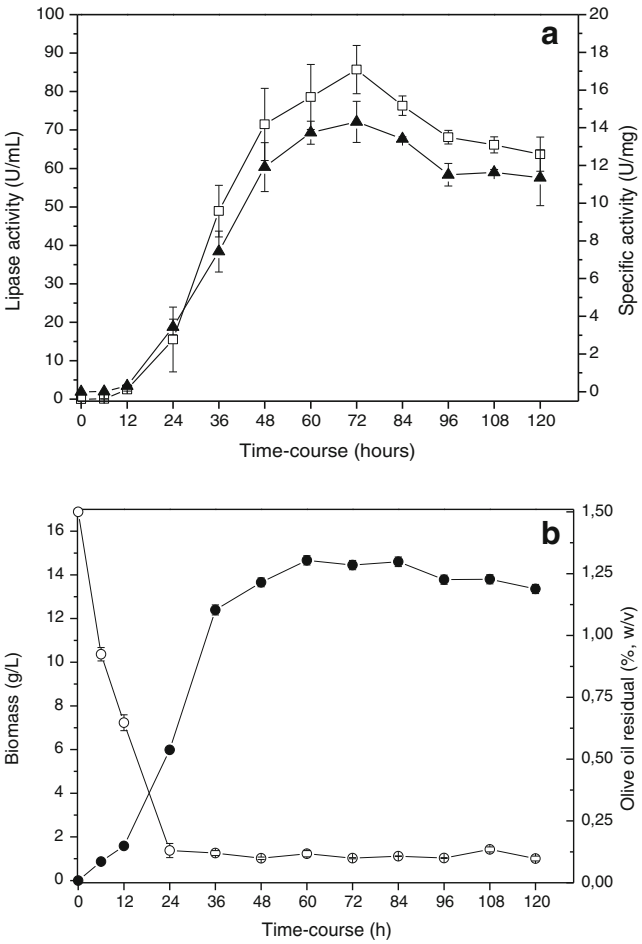


**Fig. 2** Time-course of lipase production (**a**), residual olive oil and growth (**b**) of *C. viswanathii*. Legend: (□) lipase activity, (▲) specific lipase activity, (●) biomass, (○) olive oil residual. Culture conditions: Vogel's medium, 1.5 % (w/v) olive oil and 0.2 % (w/v) yeast extract, pH 6.0, 180 rpm at 28 °C

## Concluding remarks

*C. viswanathii* was selected as the best lipase producer among 90 strains assayed. The lipase production in media with hydrophobic substrates indicated that this enzyme is preferentially induced by monounsaturated long-chain fatty acids and triacylglycerol such as oleic acid and triolein. Renewable low-cost substrates, such as vegetable oils, were more effective for lipase production and could support

expressive cell growth. Due to these characteristics, the *C. viswanathii* strain can be an important candidate for hydrophobic substrates degradation and lipid accumulation. Our results suggest that a more detailed study should be carried out to improve the lipase production and to investigate the potential of this strain for lipid accumulation.

The high hydrolytic activity of *C. viswanathii* lipase on olive oil and triolein is important for a future application of this enzyme in order to obtain unsaturated fatty acids. Due to its biotechnological importance, further studies are underway aiming to optimize the production of this lipase and also to purify and biochemically characterize this enzyme. This way, we expect a better exploitation of the potential of this microorganism and its lipase.

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