academicJournals

Vol. 13(11), pp. 1287-1294, 12 March, 2014
DOI: 10.5897/AJB2013.13586
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Screening of *Candida boidinii* from *Chemlal* spent olive characterized by higher alkaline-cold adapted lipase production

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Received 20 December 2013; Accepted 25 February, 2014

A total of 24 lipolytic yeasts were isolated from the spent olive derived from olive fruits of the Algerian variety *Chemlal*. One strain, G5, had the highest lipolytic activity (20 mm) on the tributyrin agar plate. The morphological, biochemical characterization and 18S rDNA gene analysis of the selected strain, confirms that it is *Candida boidinii* KF156789. The production of lipase and biomass were carried out in liquid and solid (spent olive) media. In submerged fermentation, it seemed that the production of enzyme reached its maximum 7.3 U/ml, whereas; the growth cells reached its maximum at 1.9 × 10^8 cell/ml. That can be explained by the assimilation of free fatty acids by this strain after degradation of olive oil by the enzyme. The production of lipase and biomass, in solid state fermentation, gave the maximum yield for cell growth (1.3 × 10^9 cell/ml), while lipolytic activity reached 4.8 U/g. The highest activity of the studied enzyme was at pH 7.0 and 37°C. The enzyme maintained more than 90% of its activity at pH 8.0-9.0 and 70% at temperature range of 4-40°C; it was concluded that the lipase from *C. boidinii* KF156789 has the potential to be an alkaline cold-adapted enzyme.

Key words: *Candida boidinii* KF156789, higher lipase, alkaline cold-adapted lipase, *chemlal* spent olive, solid state fermentation.

INTRODUCTION

Lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications. They have immense applications in various fields, such as food, pharmaceutical, cosmetic, agrochemical, feedstock, detergent, textile, biodiesel and oil processing industries (Treichel et al., 2009). These enzymes are ubiquitous in nature and are widely distributed in plants, animals and microorganisms. According to Vakhlu and Kour (2006), the main lipolytic yeasts isolated from terrestrial environment are: *Candida rugosa, Candida tropicalis, Candida antarctica, Candida cylindracea, Candida parapsilopsis, Candida deformans, Candida curvata,*

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Candida valida, Yarrowia lipolytica, Rhodotorula glutinis, Rhodotorula pilimornae, Pichia bispora, Pichia mexicana, Pichia sivicola, Pichia xylosa, Pichia burtonii, Saccharomycopsis crataegenesis, Torulaspora globosa, and Trichosporon asteroids. Other strains, such as *Candida boidinii* 1638, have lipase negative as described by Ciafardini et al. (2006). After that, Rodriguez-Gomez et al. (2010, 2011) remarked that all strains of *C. boidinii*, isolated during process of fermentation of olive table, can produce lipase.

In this work, *C. boidinii* was isolated from local oily environment as spent olive; because it may provide a good environment for lipolytic strains to flourish and for isolation of lipase producing microorganisms (Ohimain et al., 2012). In fact, Olive cultivation (*Oleaeuropea L*.) and olive oil production are important in Algeria, where several varieties of olives trees are grown. The most important one is *Oleae uropea L*. variety of *Chemlal*, where it represents the most widespread in Algeria (more than 50% of the olive groves) (Tamendjari et al., 2009). Therefore, the objective of this study was to isolate the highest lipolytic yeast from olive residue (spent olive, *Chemlal* variety), collected from manufacturers of Skikda (East of Algeria). One isolated strain *Candida boidinii* KF156789 was found to be able to produce highly extracellular lipase, which was detected qualitatively and quantitatively in liquid and solid media (as shown in the following sections). To our knowledge, this is the first report about isolation and characterization of highest lipolytic yeast, *Candida boidinii* KF156789, from spent olive of *Chemlal* variety. In addition, it was important to investigate the production of lipase on the spent olive *Chemlal* (SOC).

MATERIALS AND METHODS

Site description and sample collection

Samples of spent olive (*Chemlal variety*) used in this study were collected from manufacturers of olive oil located in Skikda, in the North East of Algeria, known as an important olive oil producers. The samples were collected during pressing of olive oil. Samples were kept in sterile bottle containers.

Isolation of yeasts from spent olive

The isolation process was performed by serial dilution of samples on Sabouraud Chloramphenicol medium (Pasteur institute, Algeria).The purification of strains was established on yeast extract, peptone, dextrose (YPD) agar medium, incubated at 28°C and examined during five days (Harju et al., 2004). Colonies with distinct morphological differences such as colour, shape, and size were picked and purified by streaking at least three times on YPD agar; the isolates were stored at 4°C and sub-cultured for 15 days intervals.

Screening of lipolytic yeasts

The colonies of isolated yeasts were examined on modified tributyrin plate agar and clear zones around the colonies indicate production of lipase (Cardenas et al., 2001). This medium composed of (g/l): 10 peptone; 12 yeast extract; 30 tributyrin and 10 agars (pH 6.2). The Petri dishes were incubated at 28°C for 3 days. The diameter (d) of the colonies and the diameter (D) of total hydrolytic halos including the colonies were determined. The strain that yielded higher halos (D-d) was selected as potential microorganism for lipase production using tributyrin as substrate (Griebeler et al., 2009; Hassan et al., 2009).

Morphological characteristics

The cultural morphology of the selected isolate was examined on YPD liquid and agar plates after incubation at 28°C for 3 days. The diameter colony, morphology and texture on plates were analyzed. The formation of filaments was characterized as described by Guiraud (1998).

Physiological and biochemical approach

Yeasts were identified using the conventional methods described by Wickerham (1951), Van der and Yarrow (1984), Deak and Beuchat (1996) and also by using the API 20C AUX test strips (Bio-Merieux, Belgium). The tests included the fermentation of sugars, liquid assimilation of carbon compounds, liquid assimilation of nitrogen compounds, growth at 30 and 37°C, growth in media containing 16% NaCl, resistance to 0.01% Cycloheximide and detection of urease activity.

Molecular approach

The total genomic DNA of the yeast strain was isolated, purified and amplified using the methods as described by Promega. The used primers for amplification of 18S ribosomal DNA in selected yeast were 5'-CTT-TCG-ATG-GTA-GTG-TAT-TGG-ACT-AC-3' and 5'-TGA-TCC-TTC-TGC-AGG-TTC-ACC- TAC-3'. The sequencing of the PCR products was performed in Progenus (Belgium). The sequences were corrected by the Bio-edit program and deposited in Genbank data base. The DNA sequences were compared to those previously published in Genbank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Production of lipase in submerged medium (SmF)

The yeast strain grew in the liquid medium YPD for 24 h at 30°C, for reactivation of strain. 10 ml of this culture were used as the inoculum for production of enzyme. The lipase enzyme was produced in flasks as described by Destain et al. (1997). It was cultivated in 250 ml Erlen meyer flasks containing 100 ml medium composed of (g/l): 10 glucose, 30 whey powder, 8 $(NH_4)_2 SO_4$, 10 corn steep liquor and 5 olive oil (extra virgin) (pH 6.5). The production broth (100 ml) was incubated at 30°C under shaking (120 rpm, 120 h) conditions. The cell numbers were estimated and lipase activity was measured.

Production of lipase in solid state fermentation (SSF)

The SOC was used as a natural substrate for the solid state fermentation. The used method was followed as described by Moftah et al. (2011). For the fermentation, spent olive samples were taken and dried in a general purpose oven for 1 h at 105 ± 5°C. Next, the sample was grounded and sieved to provide particle sizes >800 µm. SSF of spent olive was carried out in 150 ml Erlen meyer

Table 1. Hydrolytic activity on agar plates after three days.

Strain	Tributyrin use	Strain	Tributyrin use
G4	++	**G5**	**++++**
G9	++	G13	++
Gb1	++	Gb2	++
Gb3	+	Gb4	+
Gb5	++	Gb6	+
Glb1	++	Glb2	+
Glb3	+++	Glb33	++
Glb7	++	GG1	+
GG2	++	GG3	++
GG4	+	GG5	+
Gg1	-	Gg2	++
Gg3	++	Gg4	++
Gg6	+		

Symbols are related to the diameter of halos: + < 5 mm; ++ between 5 and 10 mm; +++ between 10 and 15 mm; ++++ is 20 mm.

flasks to study the effects of the selected strain in its origin media for lipase activity and biomass yield. The media in flasks (10 g of dry substrate) were autoclaved at 121°C for 20 min. One milliliter of autoclaved distilled water was added to this autoclaved preparation before inoculation. Further, SSF was carried out by inoculating 10 g of SOC (moisture content adjusted to 90%) with 1 ml of inoculum (approximately 1.7×10^7 cells) followed by incubation at 30°C. The basic medium was enriched with YPD medium supplemented with olive oil (0.5%) and tween 80 (0.1%). The water added with the inoculum was also considered in moisture correction. The pH of the prepared medium was adjusted to 6.5. The samples are aseptically withdrawn at various time intervals (one, two, three, four and five days). The cell numbers were estimated and lipase activity was measured.

Extraction of crude enzyme from SSF

To extract the enzyme, a known quantity of the fermented media was mixed with distilled water (1:4, w/w) by shaking on a rotary shaker (180 rpm, 30 min, 30°C); then, the whole contents were centrifuged at 8,000 rpm for 10 min (4°C), and the supernatant is used as a crude enzyme extract (Moftah et al., 2011).

Lipase assay

After centrifugation of samples at 8000 rpm for 20 min at 4°C, the supernatant was used as the source of extracellular crude enzyme. Lipase activity was assayed by titration using olive oil as substrate as described by Mafakher et al. (2010). The assay mixture consisted of 5 ml of emulsion (olive oil 15 ml, NaCl 30 ml and completed until 200 ml with polyvinyl alcohol), 4 ml of phosphate buffer (0.1 M; pH 7.0) and 1 ml of the crude enzyme. The preparation was incubated for 15 min at 37°C. The reaction was terminated by adding 20 ml stop solution (1:1 norvanol/acetone), and the amount of liberated fatty acids during incubation was tittered with 0.05 N NaOH in the presence of phenolphthalein as an indicator. The enzyme assay was analyzed in triplicate. One unit of lipase activity was defined as the amount of enzyme that librated 1 µmole of Free Fatty Acids (FFAs) per ml under the assay conditions.

Cell growth

Determination of yeast cell growth was performed by spreading suitably diluted cell suspensions on YPD plates and counting the yeast cell colonies after 48 h of incubation at 30°C.

Partial characterization of lipase: Effects of pH and temperature

Lipases are known to be diversified in their catalytic properties, and therefore, it is important to characterize them. The partial characterization of crude enzyme obtained by submerged fermentation tested at the large range of pH and temperature. pH optimum was determined by carrying out the enzyme assay at 37°C by titrimetric method. The phosphate buffer (0.1 M) was used at pH 5-8, adding acetic acid (0.1 M) at pH 3-4 and NaOH (0.1 M) at pH 9. The temperature optimum of the enzyme was evaluated by measuring the lipase activity at different temperatures (4-70°C) in 0.1 M phosphate buffer pH 7.0. The enzyme assay was analyzed in triplicate.

RESULTS

Screening of highest lipolytic yeast from the SOC

In this preliminary study, 25 colonies with morphology typical of yeast were isolated from the SOC samples that can utilize glucose as the sole carbon source. Most of the isolated strains (24 strains) grew on medium contains tributyrin as carbon source; which prove that the majority of isolates have a lipase activity. Table 1 shows the average diameter of extracellular lipolytic activities halos zone of the isolated yeasts on tributyrin agar plates. The strain G5 showed better lipolytic activity than the others strains. The diameter of its halos zone reached 20 mm.

Morphological characteristics of the selected strain

The colony types and cells morphology of selected strain which were investigated in this study are depicted in Figure 1. This strain has aerobic growth and formed white, butyrous colonies with convex elevation and entire margin; the diameter of colonies is around 2-3 mm (Figure 1a and b). In microscopically appearance, it has oval and cylindrical multilateral budding cells (Figure 1d). The diameter of cells is 3 µm and 8 µm of length, after 48 h of culture in YPD broth medium (Figure 1c). The filamentation test showed that the strain develops a pseudo-hyphae consisting of long branched chains of cells with blastoconidia (Figure 1e and f).

Physiological characterization

The selected strain (G5) was identified by biochemical and physiological tests (Table 2). This identification was carried out by using API 20C AUX (Bio-Merieux, Belgium) and other residual biochemical tests. API test was

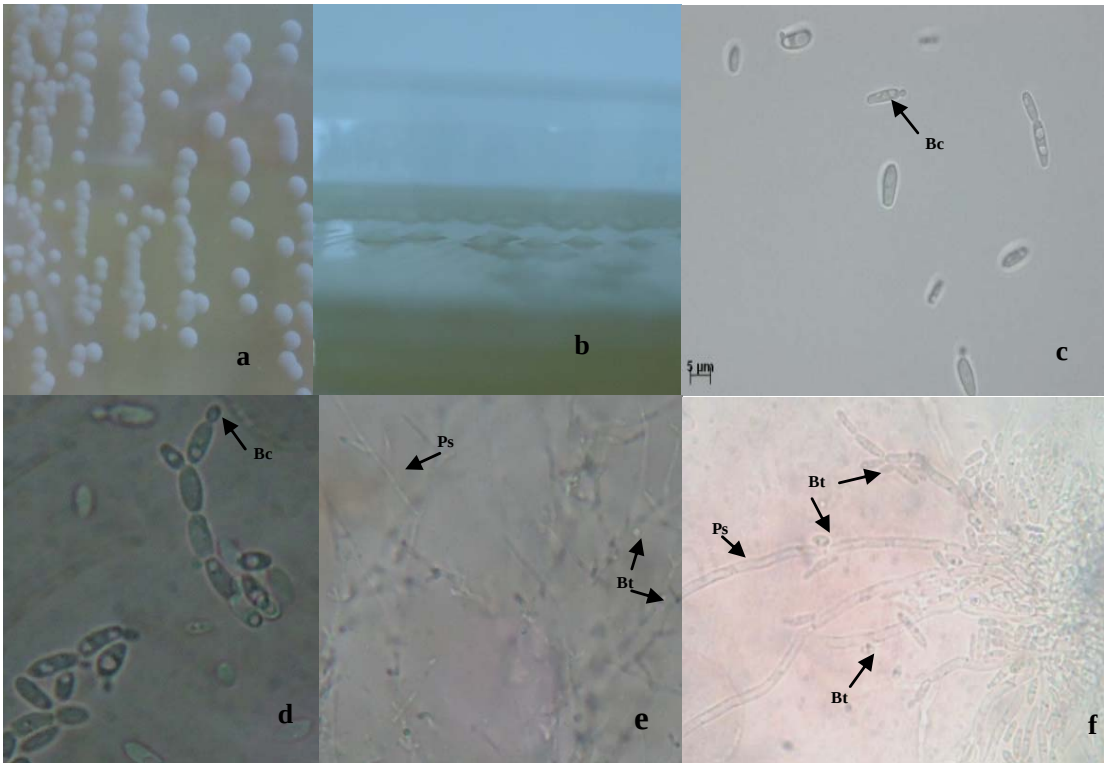

Figure 1. Morphological aspect of G5 strainisolated from the Algerian spent olive *Chemlal* variety: **a,** culture on YPD agar after 48 h at 30°C; **b,** convex elevation of colony on YPD after 48h at 30°C; **c** and **d,** microscopically arrangement of the cell in wet mount (G×100); **e** and **f,** filamentation test on PDA medium after 48h (G×100); **Ps**: pseudo-hyphae, **Bt**: blastoconidia, **Bc**: budding cell; (Photos taken in laboratory of Mycology, Biotechnology and Microbial Activity, Constantine, Algeria and in Wallon Centre of Industrial Biology, Gembloux Agro Biotech, Belgium).

classified G5 as *Candida boidinii*, with probability of 0.99; considered as a good score. In addition, the results of the rest biochemical test shows that *C. boidinii*i was able to ferment just glucose and fructose. However, the strain can assimilate a diversity of carbon compounds such as glucose, fructose, xylose, glycerol, adonitol, xylitol, sorbitol, trehalose and mannitol but not the other used substrates. Otherwise, other tests had revealed that the strain can use the potassium nitrate and had exhibited a good growth at 30°C but not at 37°C.

Molecular identification

In order to deepen more in identification study of the selected strain, the regions of 18 S ribosomal DNA was amplified, and PCR products were analyzed. The sequences were compared with available DNA sequence databases using BLAST program (Figure 2). After comparing the sequences to the GenBank database, it was demonstrated clearly in the phylogenetic tree that the strain (G5) was identified as *C. boidinii* KF156789 with a similarity of about 100% of the 813-nucleotide sequence.

The kinetics of enzyme production and cell growth in SmF

The results concerning the production of extracellular lipase and biomass of *C. boidinii* KF156789, in liquid medium were depicted in Figure 3. The results shows that the *C. boidinii* KF156789 produces the maximum activity of extracellular lipase (7.3 U/ml) after 26 h of fermentation; Whereas, the maximum growth cells (1.9×10^{08} cell/ml) was reached after 72 h of fermentation. At the end of fermentation, the lipase activity diminished gradually, leading to the gradual reduction in the growth of *C. boidinii* KF156789.

The kinetics of enzyme production and cell growth in SSF

Using basal medium formulation, the kinetics of the production of lipase and biomass, were investigated (Figure 4). It appears that the production of lipase and biomass increased steadily with the cultivation time. The lipase activity and biomass yield reached its maximum, 4.8 U/g and 1.3×10^{09} CFU/g, respectively, at the end of cultivation.

Table 2. Biochemical and physiological tests of high lipolytic yeast G5 isolated from the Algerian spent olive *Chemlal* variety.

Substrate	Fermentation of sugars	Assimilation of carbon compounds	Other tests
Glucose	+	+	
Fructose	+	+	
Galactose	-	-	
Xylose	-	+	
L- Arabinose		-	
Saccharose	-		
Lactose	-		
Maltose	-	-	
Starch	-	-	
Glycerol		+	
calcium 2-Keto-Gluconate		-	
Adonitol		+	
Xylitol		+	
Inositol		-	
D-Sorbitol		+	
Methyl-αD-Glucopyranoside		-	
N-Acetyl-Glucosamine		-	
D-Cellobiose		-	
D-Trehalose		-	
D-Melezitose		-	
D-Raffinose		-	
Mannitol		+	
Potassium nitrate			+
16%NaCl			-
Cycloheximide 0,01%			-
Urease			-
Growth at 30°C			+
Growth at 37°C			-

Partial enzyme characterization: Effects of pH and temperature

The influence of pH and temperature in the enzyme activity is presented in Figures 5 and 6, respectively. The highest activity (7 U/ml) was found at pH 7.0 and at 37°C. The enzyme was most active in pH range between 7.0 and 9.0, that it showed more than 90% of its activity at pH 9. In addition, the enzyme kept just 50% of its activity at pH 4. On other hand, the lipase kept about 90% of its activity between 30 and 40°C, noted that this enzyme present 70% of its activity at 4°C.

DISCUSSION

Algeria has about 32 million olive trees spread over 300,000 hectares and produce 4.7 million quintals of olives. The local variety *Olea europea L*. *Chemlal* exists with highest values 80% in the territories reserved to oil olive trees, especially, in the north eastern of Algeria. The spent olive is a by-product of oil extraction; this residue consists of the solid constituents of the skins, pulp, seeds and fragments of core (Moussaoui et al., 2008). In this work, several yeasts were isolated from Algerian spent olive (*Chemlal*) collected from olive oil mills in Skikda (Eastern of Algeria). One strain (G5) of them was selected as the highest extracellular producer of lipase. The combination of morphological, biochemical and molecular properties can lead to identifying G5 as *C. boidinii* KF156789.

Usually, the cited strain was isolated from soil, but, several authors have isolated it during industrial fermentation of table olives (Bautista-Gallego et al., 2011; Rodriguez-Gomez et al., 2011). In addition, *C. boidinii* was known in literature as methylotrophic yeast (Janatova, 1992), however, recent investigations have shown its capacity of lipase production (Rodriguez-Gomez et al., 2010). The production of lipase was carried out on two different systems of fermentation SmF and SSF. As shown in Figure 3, the surge of exponential growth, which could have been the result of the lipase

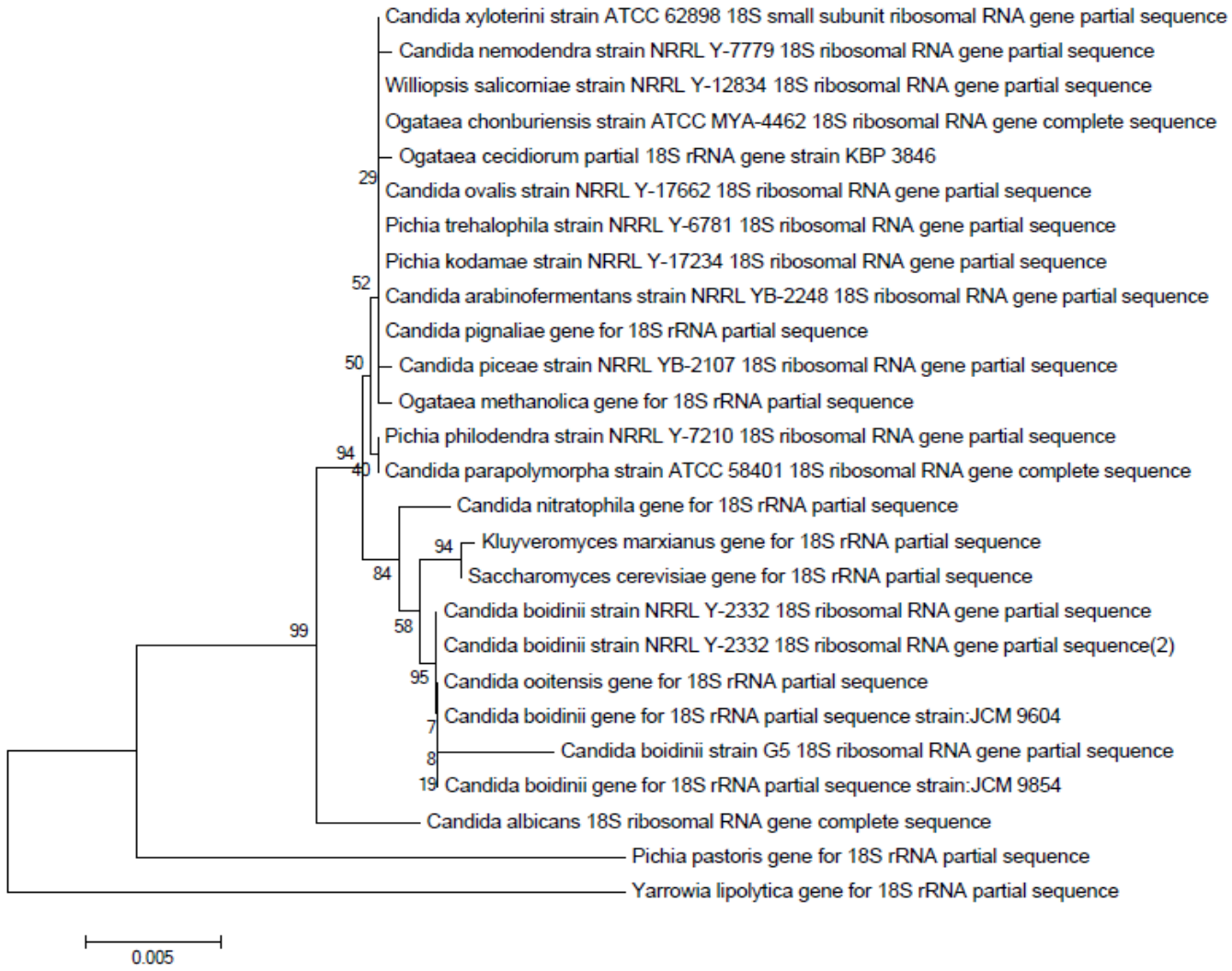

Figure 2. Evolutionary tree of the selected strain (*C. boidinii*) based on 18S rDNA gene sequences obtained in this study.

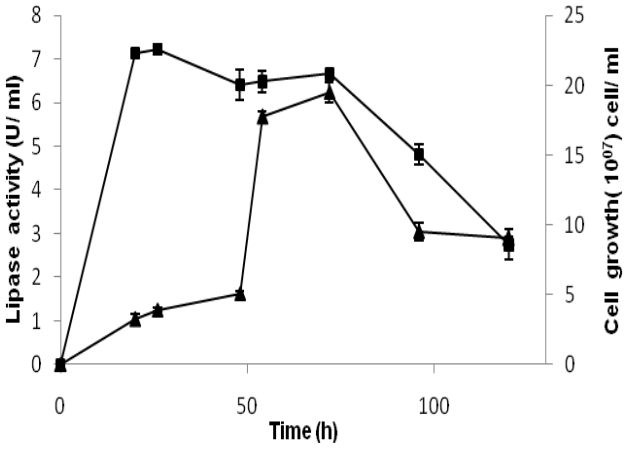

Figure 3. Production of lipase and biomass in SmF for 120 h at 30°C: (▲) cell growth; (■) lipase activity.

activity, breaking down of olive oil, leading to the creation of fatty acids that could be assimilated easily by the used strain after 26 h of incubation. The extra virgin olive oil was found to be the most suitable enhanced source of carbon and considered as inducer for lipase production (Mobarak-Qamsari et al., 2011).

In addition, it was important to investigate the production of lipase and biomass of the cited strain in its origin medium (SOC). Lipase production in SSF process seems to have the most cell growth and low lipolytic production (Figure 4), in comparison with SmF. The decay of extracellular lipase activity during the later period of both cultures may be related to the effect of pH and temperature, or it was probably caused by proteo-lysis. Several recent studies reported that SSF has many preferences to SmF for microbial enzyme pro-duction including: high yield and productivities, enzyme stability

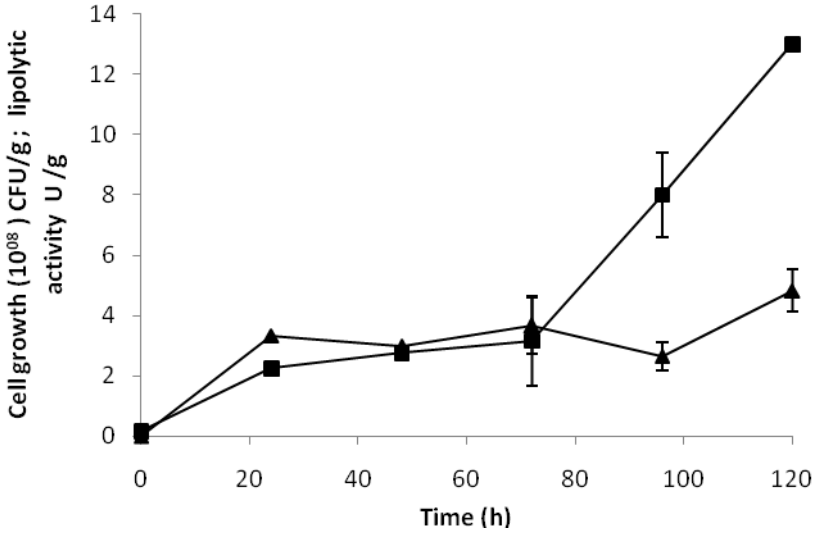

Figure 4. Production of lipase and biomass in SSF for 120h at 30°C; (▲) Lipolytic activity; (■) Cell viability.

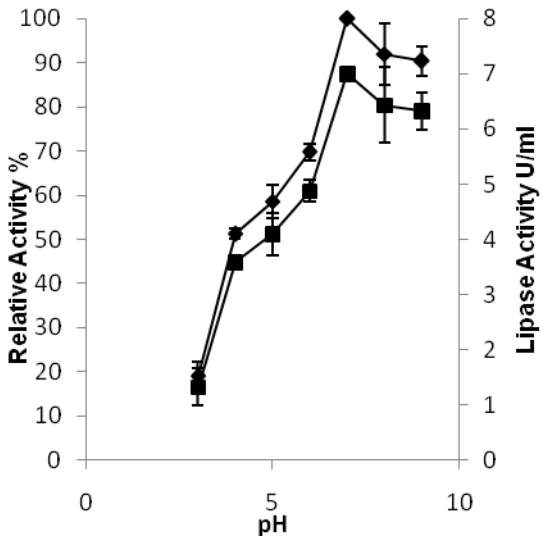

Figure 5. Effect of pH on lipase activity: (■) lipase activity; (♦) relative activity. The experiments were performed in triplicate and bars represent the standard deviation.

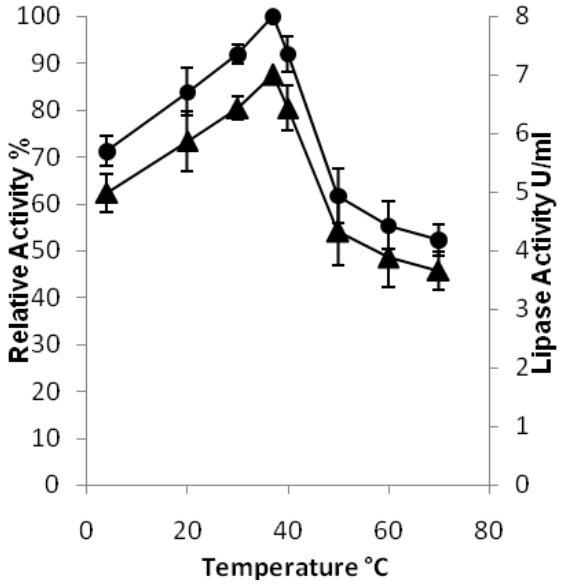

Figure 6. Effect of temperature on lipase activity: (▲) lipase activity; (●) relative activity. The experiments were performed in triplicate and bars represent the standard deviation.

and low cost (Hosseinpoura et al., 2012); whereas in other research, in particular, for the production of laccase obtained by the fungus *Trametes versicolor* ATCC200801, the production in submerged fermentation is seen maximum benefit of production facilities and the minimum cost (Demir et al., 2011). Other investigations about the production of lipase on the SOC are still needed. It was interesting also to know that lipase from *C. boidinii* KF156789 is active in a pH range of 7-9, with a maximum lipase activity at pH 7, and between temperatures of 30-40°C with an optimum temperature for the lipase activity at 37°C. These results are similar to that reported for lipase from *Y. lipolytica, A. johnsonii LP28* and *Aeromonas sp. LPB 4* (Lee et al., 2003; Brigida et al., 2007; Wang et al., 2010). So, lipase from *C. boidinii* KF156789 may be good in industrial applications (as industry of detergent), at different range of temperature and alkaline pH.

Conflict of interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

I would like to thank Mr. BOUTEBBA Samir, MOULA Nassim and BENHASSINE Sara for their help to complete this work.

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