

Research Paper

Occurrence of *Candida orthopsilosis* in Brazilian tomato fruits (*Lycopersicon esculentum* Mill.)

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Abstract

We aimed to isolate and identify yeasts found in the tomato fruit in order to obtain isolates with biotechnological potential, such as in control of fungal diseases that damage postharvest fruits. We identified *Candida orthopsilosis* strains LT18 and LT24. This is the first report of this yeast on *Lycopersicon esculentum* fruits in Brazil.

Key words: *Lycopersicon esculentum* Mill, *Candida orthopsilosis*, tomato fruits.

The tomato fruit (*Lycopersicon esculentum*), which is used as a vegetable, is one of the most consumed food ingredients in the world (Mata *et al.*, 2003), and Brazil is one of the major producers of tomato. In 2009, the national production reached 4.3 million tons and the world production was more than 152 million tons (FAOSTAT, 2011).

Yeasts are a large and diverse group of microorganisms consisting of more than 1000 species and can be found in soil, air, water, and food (Mislivec *et al.*, 1992). Some species also co-exist with the natural microflora of fruits and vegetables and their colonization is influenced by environmental, harvest, or storage conditions (Skinner, 1980). Generally, many fruits and vegetables present nearly ideal conditions for the survival and growth of several types of microorganisms (Barth *et al.*, 2009). Ferreira *et al.* (2010) studied the postharvest quality of the tomato fruit and revealed that the yeast and mold count decreases with the ripening of the fruit, but remains around the order of 10³ CFU/g, indicating that the tomatoes preserved at room temperature need to be cleaned efficiently before consumption.

A diverse community of epiphytic microorganisms also colonizes the tomato fruit surface, effectively provid-

ing a further competitive barrier against the spoilage organisms (Barth *et al.*, 2009). Therefore, the yeast microflora in the tomato fruit may present a biotechnological potential, such as in the fungal diseases control on postharvest fruit. The main mechanisms for the control of postharvest diseases by using microbial antagonists exploit microbial competition for nutrients and space, induced resistance, production of antibiotics, and direct parasitism (Sharma *et al.*, 2009).

Pichia guilliermondii presents potential biocontrol activity against *Botrytis cinerea* in apples (Trofa *et al.*, 2008) and against *Rhizopus nigricans* in tomato fruits (Zhao *et al.*, 2008). *Pichia anomala*, isolated from the surface of coffee berries, is able to inhibit the spore production of *Aspergillus ochraceus* and *Penicillium roqueforti* (Ramos *et al.*, 2010). *Candida lambica* is able to reduce up to 95.87% of *A. ochraeus* biomass in submerged culture (Beux, 2004). Therefore, we aimed to isolate and identify yeasts from the tomato fruit, which display characteristics similar to the *Pichia* genus in order to obtain isolates with biotechnological potential.

Ten tomato fruit samples were collected from different markets in Curitiba, Paraná State, Brazil, on October

2006. Tomato broth was prepared by disintegration and homogenization of tomatoes by using a sterile metal shredder. The broth was separated into sterile Erlenmeyer flasks and cultivated in a biochemical oxygen demand (BOD) incubator at 28°C for 5 days. Subsequently, the microorganisms were isolated on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) by serial dilution in 0.1% peptone water.

The yeast groups were identified by the ability to ferment sugars such as glucose, galactose, sucrose, maltose, fructose, mannose, and raffinose according to the method described by Rocha (2006). The results were compared with those of a prior study (Back, 2006; Barnett and Pankhurst, 1974). Later, the yeasts were identified by growing them on CHROMAgar Candida (BD), by using the API 20C AUX system (bioMérieux) and by sequencing the ITS of their rDNA.

About 1 cm² colonies of 5-day-old cultures were transferred to 2-mL Eppendorf tubes, each containing 300 µL cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB [w/v], 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.2% β-mercaptoethanol [v/v]) and approximately 80 mg of a silica mixture (silica gel H; Merck/Celite 545-Macherey Nagel & Co.; 2:1, w/w). The cells were disrupted manually using a sterile pestle for approximately 5 min. Subsequently, 200 µL CTAB buffer was added again, the mixture was vortexed, and then incubated for 10 min at 65 °C. After adding 500 µL chloroform, the solution was mixed and centrifuged for 5 min at 20,500 g force value and the supernatant was transferred to a new tube containing 2 volumes of ice-cold 96% ethanol. The DNA was allowed to precipitate for 30 min at -20 °C and then centrifuged again for 5 min at 20,500 g force value. Subsequently, the pellet was washed with cold 70% ethanol. After drying it at room temperature, the pellet was resuspended in 97.5 µL TE-buffer with 2.5 µL RNase (20 U/mL) and incubated for 5 min at 37 °C; thereafter, it was stored at -20°C (Gerrits and Hoog, 1999).

The ITS of the ribosomal DNA (rDNA) was amplified using the primers V9G (5'-TTACGTCCCTGCCCT TTGTA-3') and LS266 (5'-GCATTCCCAAACAAC TACTC-3') (9) and sequenced using ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Amplicons were cleaned using the GFX PCR DNA purification kit (GE Healthcare) and sequenced using an ABI 3130 automatic sequencer (Applied Biosystems). Sequences were edited and aligned using the Staden sequence analysis package v. 1.6.0 (Staden, 1996). Sequence analysis was performed using the sequence alignment software BLASTn run against the NCBI database. Phylogenetic analysis was performed using the software Mega 4.0.2 (Tamura *et al.*, 2007) by applying the neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor correct distance model (Jukes and Cantor, 1969). The nucleo-

tide sequence obtained in this study was submitted to GenBank.

Seventeen species of filamentous fungi were isolated from the tomato broth, and the analysis of macromorphology and reproductive structures revealed that they belonged to 4 genera: *Penicillium*, *Aspergillus*, *Acremonium*, and *Trichoderma* (Data not shown). Some species (*e.g.*, *Trichoderma harzianum*) of these genera have previously shown the ability to control postharvest diseases in fruits (Batta, 2007), whereas others such as *Penicillium expansum* were agents of postharvest diseases (Yu *et al.*, 2012). However, we chose to focus this work on yeast, so that the future implementation and bioprocess viability could be easily achieved.

We isolated 2 strains of yeast labeled LT18 and LT24, whose colonies were bright white, flat, and smooth, with regular, rounded borders and no pseudohyphae. Fermentation of the carbon sources was assessed in a previous identification of the yeast isolates, revealing that the strains could potentially belong to *Pichia*.

The morphological and physiological characters of LT18 and LT24 were similar to *Pichia* sp.; hence, a further identification was carried out. The strains LT18 and LT24 presented pink pigmentation and smooth texture on the chromogenic medium CHROMAgar, and they were identified as *Candida parapsilosis* by using the API 20C AUX system.

Sequences of ITS1-5.8S-ITS2 revealed a fragment of 422 bp for LT18 and 423 bp for LT24. Sequences of both strains presented with 98% (LT18) and 99% (LT24) similarity with the *Candida orthopsilosis* sequence EU557371. The sequences of the isolates LT18 and LT24 were deposited in GenBank with the respective accession numbers JN797502 and JN797503.

Twenty sequences previously deposited at GenBank (Table 1), which showed similarity in the range of 95-99% to the sequences of the isolates obtained in this study, were employed for constructing a phylogenetic tree (Figure 1). Three groups that showed high bootstrap values were obtained. Group A, 88% consistent, was composed of isolates of *C. orthopsilosis*, including the ones obtained in our study, LT18 and LT24; group B, with a bootstrap value of 92%, is represented by members of *Candida metapsilosis*; and finally group C, with a bootstrap value of 100%, contained isolates from *C. parapsilosis*.

Identification via conventional biochemical systems corroborated with the data obtained by Tay *et al.* (2009), who also used the API 20C AUX system for preliminary identification of the isolates from the bloodstream of infected patients. All isolates were initially identified as *C. parapsilosis*, but were later differentiated into *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*, and *Lodderomyces elongisporus* through RAPD and ITS sequencing. Others studies (Silva *et al.*, 2009; Toro *et al.*, 2010) also differentiated a large set of *C. parapsilosis* iso-

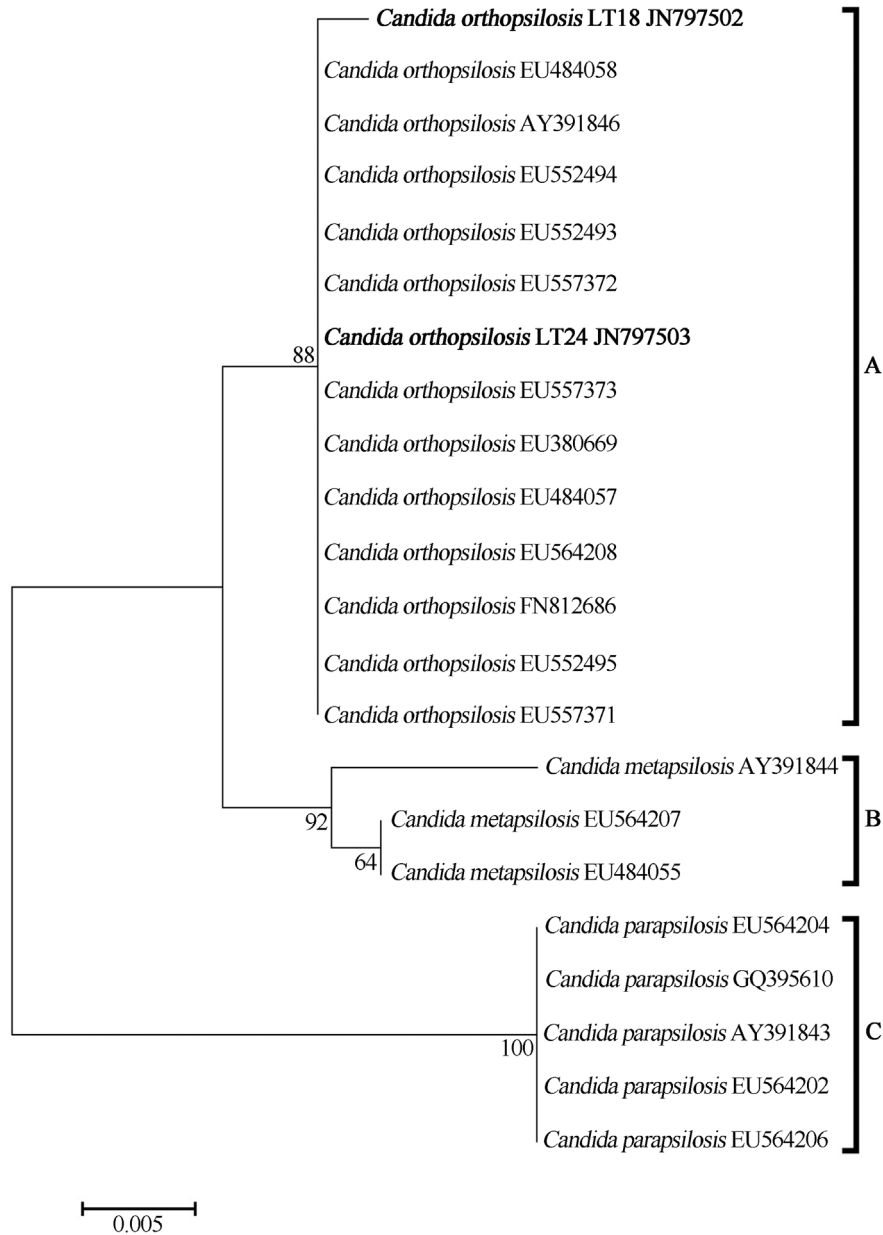


Figure 1 - Phylogenetic analysis of yeasts LT18 and LT24 belonging to the *C. orthopsilosis* specie. Neighbor-joining method. Numbers on the tree branches indicate the *bootstrap* value found from 10000 replicates. Mega Software 4.0.2 release. Strains available in GenBank accession Nos JN797502 and JN797503.

lates, previously identified by biochemical tests, into *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* species by molecular identification (SADH gene restriction profile).

Further, Pitt and Hocking (1997) have isolated *C. parapsilosis* from fruit juices, olives, meat, and seafood. This particular species is well known for causing nosocomial blood infections, especially among neonates and the immuno-compromised, and it is associated with Candidemia due to contaminated intravascular devices and parenteral nutrition (Almirante *et al.*, 2006; Girmenia *et al.*,

1996; Pfaller and Diekema, 2002, 2007; Safdar *et al.*, 2002; Trofa *et al.*, 2008). This yeast was also isolated from robusta coffee samples from the Congo Republic, akin to others species from this genera including *Candida pelliculosa*, *Candida famata*, and *Candida tropicalis* (Pee and Castelein, 1971).

The taxon *C. parapsilosis* was traditionally divided into 3 groups, I, II, and III. Recently, Tavanti *et al.* (2005) investigated the genetic heterogeneity of the taxon and proposed replacing groups II and III with *C. orthopsilosis* and *C. metapsilosis*, respectively.

Table 1 - Sequences from *Candida* spp. deposited in GenBank used in the phylogenetic analysis.

Name	GenBank	Source	Origin	Strain Number
<i>Candida orthopsilosis</i>	JN797502	Tomato fruit	Brazil	LT18
<i>Candida orthopsilosis</i>	JN797503	Tomato fruit	Brazil	LT24
<i>Candida orthopsilosis</i>	EU557371	Bloodstream infection	Brazil	L8201A
<i>Candida orthopsilosis</i>	EU564208	Human blood culture	USA	ATCC 96141
<i>Candida orthopsilosis</i>	EU484057	Human Blood	Brazil	L7941
<i>Candida orthopsilosis</i>	EU557372	Bloodstream infection	Brazil	840
<i>Candida orthopsilosis</i>	FN812686	Unknown	USA	90-125
<i>Candida orthopsilosis</i>	EU557373	Bloodstream infection	Brazil	L7956
<i>Candida orthopsilosis</i>	EU552495	Human blood culture	Brazil	L7786
<i>Candida orthopsilosis</i>	EU380669	Human Blood	Brazil	L6786
<i>Candida orthopsilosis</i>	EU552494	Human blood culture	Brazil	L8106A
<i>Candida orthopsilosis</i>	EU484058	Human Blood	Brazil	L6785
<i>Candida orthopsilosis</i>	AY391846	-	USA	MCO456
<i>Candida orthopsilosis</i>	EU552493	Human blood culture	Brazil	L8068A
<i>Candida parapsilosis</i>	EU564206	Human Blood	USA	ATCC 90018
<i>Candida parapsilosis</i>	CG395610	Feeding production sample	China	A005
<i>Candida parapsilosis</i>	EU564204	Human blood culture	Brazil	L8367
<i>Candida parapsilosis</i>	EU564202	Human blood culture	Brazil	L8096
<i>Candida parapsilosis</i>	AY391843	Feces	Puerto Rico	CBS 604
<i>Candida metapsilosis</i>	EU564207	Unknown	USA	ATCC 96143
<i>Candida metapsilosis</i>	AY391844	Human sputum	Norway	CBS 2916
<i>Candida metapsilosis</i>	EU484055	Human Blood	Brazil	L8521

The phylogenetic tree clusterings of all 3 species for the ITS region are consistent with those reported by Gomez-Lopez *et al.* (2008), Tavanti *et al.* (2005) and Tay *et al.* (2009), in which the differences in the sequence alignment validated the species separation. This result showed that *C. orthopsilosis* and *C. metapsilosis* are more closely related to each other than to *C. parapsilosis*, as shown in Figure 1.

In conclusion, our study is likely the first report of *C. orthopsilosis* found in *L. esculentum* Mill. fruits in Brazil. We recommend further studies on these strains in order to screen for biotechnologically important characteristics and exploit their use in postharvest fungal diseases control.

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