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The effect of thyme and tea tree oils on morphology and metabolism of *Candida albicans**

Katarzyna Rajkowska[1]✉, Alina Kunicka-Styczyńska[1], Marta Maroszyńska[1,2] and Mariola Dąbrowska[3]

[1]Institute of Fermentation Technology and Microbiology, Lodz University of Technology, Łódź, Poland; [2]Biotechnology Laboratory, Regional Park of Science and Technology Ltd., Łódź, Poland; [3]Institute of General Food Chemistry, Lodz University of Technology, Łódź, Poland

Members of *Candida* species cause significant problems in medicine and in many industrial branches also. In order to prevent from *Candida* sp. development, essential oils are more and more frequently applied as natural, non-toxic, non-pollutive and biodegradable agents with a broad spectrum of antimicrobial activity. The aim of the research was to determine changes in morphology and metabolic properties of *Candida albicans* in the presence of thyme and tea tree oils. Changes of enzymatic activity of isolates were observed in the presence of both tested essential oils, and they were primarily associated with loss or decrease of activity of all enzymes detected for control. Furthermore, only for 3 out of 11 isolates additional activity of N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and trypsin was detected. Vivid changes in biochemical profiles were found after treatment with tea tree oil and they were related to loss of ability to assimilate D-xylose, D-sorbitol and D-trehalose. The main differences in morphology of isolates compared to the control strain concerned formation of pseudohyphae structures. Both examined essential oils caused changes in cell and colony morphology, as well as in the metabolism of *Candida albicans*. However, the extent of differences depends on the type and concentration of an essential oil. The most important finding is the broad spectrum of changes in yeast enzymatic profiles induced by thyme and tea tree oils. It can be supposed that these changes, together with loss of ability to assimilate saccharides could significantly impact *Candida albicans* pathogenicity.

Key words: *Candida albicans*, essential oils, morphology, metabolism, enzymatic activity, biochemical properties

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INTRODUCTION

Candida species are currently the most common cause of fungal infections worldwide (Manolakaki *et al*., 2010), and the first Polish multicentre candidaemia study revealed that the most frequent fungal pathogen is *Candida albicans* (Nawrot *et al*., 2013). Many fungal species are harmless commensals or endosymbionts of hosts including humans. However, when mucosal barriers disrupted or the immune system is compromised they can invade and cause disease (Kourkoumpetis *et al*., 2010). In last 30 years there has been a significant increase in the incidence of fungal infections in humans (Lass-Flörl, 2009). A number of factors have been implicated in this increased occurrence of fungal disease. Specific conditions of the organism and in particular during predisposing situations like: diabetes, pregnancy, genetic factors and the increased and widespread use of certain medical practices, such as immunosuppressive therapies, invasive surgical procedures and the use of cortisones, contraceptives, estrogen and in particular broad-spectrum antibiotics are significant (Samaranaykae *et al*., 2002; Hagerty *et al*., 2003; Kojic & Darouiche, 2004; Selvia *et al*., 2012). In order to prevent from *Candida* sp. development, essential oils are more and more frequently applied in food, cosmetic, pharmaceutical industry as well as in medicine and in the processes of washing and disinfection (Dorman & Deans, 2000; Batish *et al*., 2008; Kotzekidou *et al*., 2008). Essential oils are aromatic oily liquids plant origin, forming multicomponent mixtures of terpenes and terpenoids (Burt, 2004). Due to the broad spectrum of antimicrobial activity, low risk of side effects after their use and low risk of resistance development by microorganisms, the essential oils can provide a valuable alternative to synthetically produced substance (Budzyńska *et al*., 2011; Kalemba & Kunicka, 2003). Moreover, the essential oils are natural, non-toxic, non-pollutive and biodegradable compounds (Donaldson *et al*., 2005; Adorian & Buchbauer, 2010).

The literature data focused rather on antimicrobial activity of essential oils and their active compounds than their mechanisms of action. Therefore, the aim of this study was the evaluation of effect of thyme and tea tree oils on morphology and metabolic properties of yeast *Candida albicans*.

MATERIALS AND METHODS

Yeast. The study was carried out for collection strain *Candida albicans* ATCC 10231, which is typically used as a reference strain in the analysis of disinfectants and antifungal agents. The strain was maintained on Sabouraud dextrose agar (peptone 10 g/l, dextrose 20 g/l, agar 20 g/l) and activated through double passaging in Sabouraud liquid medium at 37°C for 24 h.

Essential oils. The effect of essential oils was estimated for thyme (*Thymus vulgaris* L.) and tea tree oil (*Melaleuca alternifolia* L.), obtained from Pollena Aroma S.A. in Warsaw (Poland).

✉e-mail: katarzyna.rajkowska@p.lodz.pl

*The preliminary report on the same subject was presented at MIKROBIOT 2013 Workshop, Łódź, Poland

Abbreviations: *C. albicans, Candida albicans*

Table 1. Percentage composition of examined essential oils estimated by GC-MS analysis

Compound	RI	Tea tree oil	Thyme oil
		Content (%)	
α-Thujene	922	0.8	0.9
α-Pinene	931	2.4	0.9
Campehen	940	–	0.4
Sabinene	968	0.1	–
β-Pinene	970	0.8	0.2
β-Myrcene	985	0.6	1.8
α-Phellandrene	996	0.5	0.3
Car-2-ene	1003	–	0.1
Car-3-ene	1008	–	2.0
α-Terpinene	1010	8.0	–
p-Cymene	1012	4.6	18.4
β-Phellandrene	1019	–	0.4
1,8-Cineole	1020	4.4	–
Limonene	1022	1.8	0.9
γ-Terpinene	1054	17.8	8.8
α-Terpinolen	1080	3.0	–
Linalool	1086	–	3.2
(E)-p-Menth-2-en-1-ol	1112	0.3	–
(Z)-p-Ment-2-en-1-ol	1130	0.2	–
Borneol	1150	–	0.7
Terpinen-4-ol	1171	41.9	0.3
α-Terpineol	1177	3.8	0.3
(Z)-Piperitol	1202	0.1	–
Ascaridol	1207	0.3	–
Carvacrol methyl ether	1230	–	0.3
Cumin alcohol	1271	–	0.1
Thymol	1281	–	48.6
Carvacrol	1285	–	5.5
α-Copaene	1374	0.2	–
α-Gurjunene	1406	0.3	–
(E)-β-Caryophyllene	1415	0.3	2.3
Aromadendrene	1436	0.7	–
α-Humulene	1449	0.1	0.1
allo-Aromadendrene	1456	0.4	0.1
γ-Muurolene	1484	0.1	0.1
Ledene	1489	1.2	–
Viridiflorene	1490	–	0.1
α-Muurolene	1492	0.2	–
γ-Cadinene	1505	–	0.1
δ-Cadinene	1513	0.8	0.2
Spathulenol	1564	–	0.1
(E)-β-Caryophyllene oxide	1568	–	0.4
Globulol	1574	0.2	–

RI — retention index, — not detected

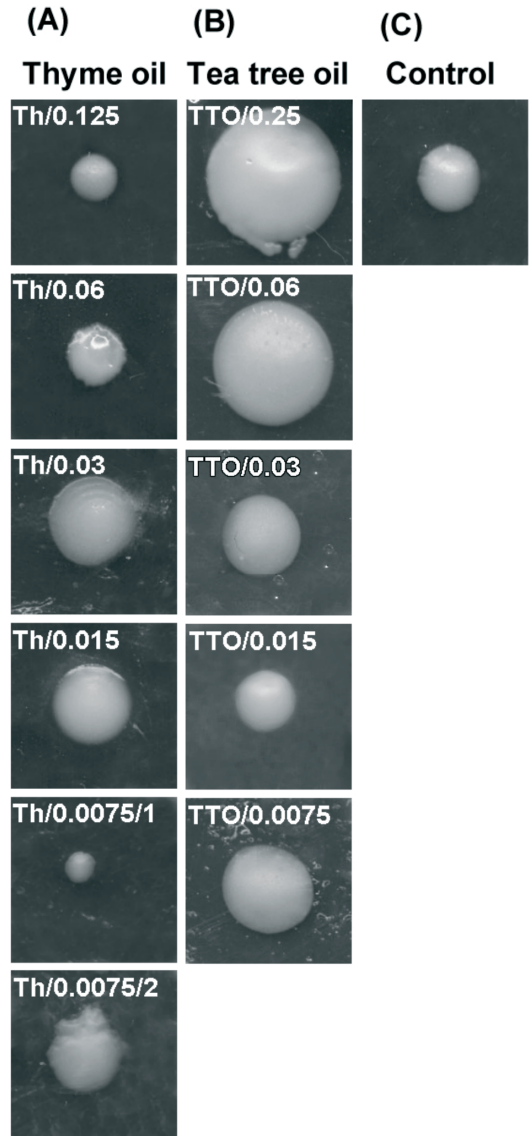

Figure 1. *Candida albicans* ATCC 10231 colony morphology in the presence of (A) thyme oil (Th) and (B) tea tree oil (TTO) in concentration from 0.0075 to 0.25% v/v; (C) control.
Isolate symbol is composed as follows: essential oil type/ the oil concentration.

Essential oils were analyzed using Trace GC Ultra (Thermo Scientific) equipment combined with DSQ II mass spectrometer and with flame ionization detector (FID) throughout MS-FID splitter. Analysis was provided using nonpolar chromatography column Rtx-1 ms (60 m×0.25 mm, film thickness 0.25 μm, Restek). The oven temperature was programmed as followed: 50–300°C at 4°C/min; injector temp. 280°C; carrier gas helium with regular pressure 200 kPa, ionization energy 70 eV, ion source temperature 200°C. Identification of components was based on the comparison of their MS spectra with those in a laboratory made MS library, commercial libraries (NIST 98.1 and Mass Finder 4) along with the retention indices associated with a series of alkanes with linear interpolation (C8-C26). A quantitative analysis (expressed as percent ages of each component) was carried out by peak area normalization measurements without correction factors. The components of essential oils are presented in Table 1.

Colony morphology assay. *Candida albicans* cell suspension (approximately 30 cells on plate) was streaked on Sabouraud dextrose agar with addition of thyme or tea tree oil in a concentration from 0.0075 to 0.5% v/v. Plates were incubated at 37°C for up to 14 days and colonies of different morphology, in comparison with control grown only on Sabouraud dextrose agar, were used for further studies.

Morphology index. Yeast colonies of different morphology was transferred to 5 ml of sterile saline (sodium chloride 8.5 g/l), harvested by centrifugation (5000×*g*, 5 min) and resuspended in saline to prepare for micro-

scopic examination. Cell dimensions in all samples were measured with Olympus BX-41 computerized image analysis system. The length (l), maximum diameter (d) and the diameter of septal junctions (s) were determined for at least 100 randomly chosen cells in each sample. For each cell a morphology index (Mi) was calculated from the formula: $Mi = 2 + 1.78 \times \log_{10}(l \times s/d^2)$, according to Merson-Davies & Odds (1989).

Biochemical and enzymatic profiles. The biochemical ability of the *C. albicans* strain and isolates to assimilate 19 substrates (D-glucose, glycerol, 2-keto-D-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-α D-glucopyranoside, N-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose, D-raffinose) as sole carbon sources was checked with API 20C AUX tests (bioMerieux) according to the producer's guidelines.

In order to determine enzymatic profiles of yeasts API-ZYM test (bioMerieux) was used and activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase was analyzed.

RESULTS AND DISCUSSION

The growth of *Candida albicans* strain was observed on solid medium supplemented with thyme oil in concentration below 0.25% v/v and tea tree oil (TTO) below 0.5% v/v, and these values correspond to minimal inhibitory concentrations (MICs) of examined essential oils. In analyzed oil concentration ranges colonial morphology differed from control, especially in size (Fig. 1). However, there was no correlation between the size of colonies and essential oils concentrations, and the smallest colonies were detected in the presence of thyme oil in concentration 0.125, 0.06, 0.0075 and tea tree oil — 0.015% v/v. In turn, colonies of larger diameter than the control were formed after treatment with 0.03, 0.015% thyme oil and 0.25, 0.06 and 0.0075% TTO. The number of morphologically different colonies varied from 15% after treatment with 0.03% v/v TTO to 100% with 0.015% v/v thyme oil.

Colony morphology of yeast is strongly influenced by growth condition, including agar and nutrient concen-

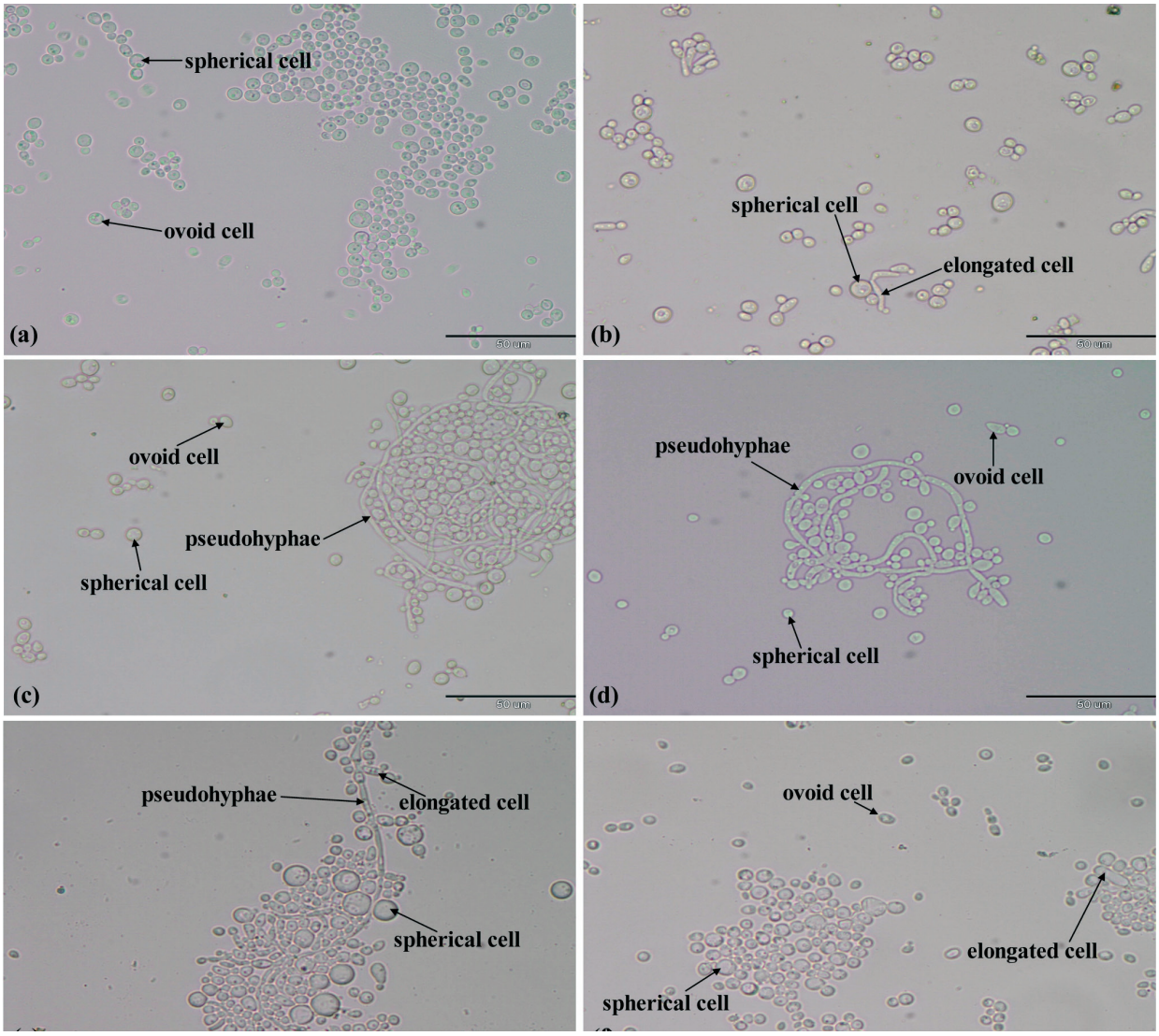

Figure 2. Examples of different morphologies of *C. albicans* after treatment with essential oils; (a) control, (b, c) thyme oil 0.015% v/v, (d) thyme oil 0.03% v/v, (e) thyme oil 0.06% v/v, (f) tea tree oil 0.03% v/v; bar 50 µm.

Table 2. Morhology index (Mi) values for *C. albicans* after treatment with thyme (Th) and tea tree oil (TTO). Isolate symbol is composed as follows: essential oil type/ the oil concentration.

Yeast	Cell shape	Morphology index			Frequency distributions of Mi (%)
		Range	Average	S.D.	
Control	spherical	0.58–1.32	0.95	0.52	39.0
	ovoid	1.29–1.72	1.51	0.30	50.5
	elongated	1.61–3.47	2.54	1.32	10.5
Th/0.125	spherical	0.48–1.33	0.91	0.60	51.0
	ovoid	1.29–1.61	1.45	0.23	49.0
Th/0.06	spherical	1.12–1.38	1.25	0.18	37.0
	ovoid	1.29–1.72	1.51	0.30	25.0
	elongated	1.92–2.41	2.17	0.35	4.0
	pseudohyphae	3.58–4.09	3.84	0.36	34.0
Th/0.03	spherical	0.79–1.35	1.07	0.40	13.5
	ovoid	1.07–1.83	1.45	0.54	32.0
	elongated	1.92–2.99	2.46	0.76	13.5
	pseudohyphae	3.67–4.07	3.87	0.28	41.0
Th/0.015	spherical	0.48–1.35	0.92	0.62	25.0
	ovoid	1.29–1.46	1.38	0.12	16.5
	elongated	2.70–3.13	2.92	0.30	6.5
	pseudohyphae	3.73–4.12	3.93	0.28	52.0
Th/0.0075/1	spherical	0.58–1.12	0.85	0.38	58.5
	ovoid	1.29–1.61	1.45	0.23	41.5
Th/0.0075/2	spherical	0.48–1.48	0.98	0.71	44.5
	ovoid	1.29–1.46	1.38	0.12	55.5
TTO/0.25	spherical	0.86–1.35	1.11	0.35	55.5
	ovoid	1.29–1.83	1.56	0.38	44.5
TTO/0.06	spherical	1.25–1.39	1.32	0.10	60.5
	ovoid	1.46–1.72	1.59	0.18	39.5
TTO/0.03	spherical	0.79–1.49	1.14	0.49	58.5
	ovoid	1.46–1.61	1.54	0.11	34.0
	elongated	2.31–2.94	2.63	0.45	7.5
TTO/0.015	spherical	1.09–1.42	1.26	0.23	36.0
	ovoid	1.46–1.83	1.65	0.26	64.0
TTO/0.0075	spherical	1.12–1.35	1.23	0.16	59.0
	ovoid	1.29–1.61	1.45	0.23	41.0

tration, pH, osmotic pressure (Vopálenská *et al*., 2005; Voordeckers e*t al*., 2012). Yeasts respond to environmental conditions with a common gene-expression response, termed the environmental stress response (ESR). However, in addition to ESR response, each species responds to environmental changes with a great deal of precision in terms of the genes affected by each condition, the magnitude of their expression changes, and the kinetics of the response (Gasch, 2007). It seems that *C. albicans* may have evolved to respond to environmental conditions rather by triggering a change in cellular states than by ESR (Ernst, 2000).

Yeast colony morphology is controlled by a very large number of genes that are involved in different signalling cascades, including the MAPK, TORC, SNF1, RIM101 pathways (Granek & Magwene, 2010; Voordeckers e*t al*., 2012). Other genes that affect colony morphology control protein sorting and epigenetic regulation (Voordeckers e*t al*., 2012). Furthermore, many of the genes that are implicated in colony formation have been previously reported to control adhesion, mat formation and invasive growth (Madhani, 2000; Reynolds *et al*., 2006). Changes in *C. albicans* colony morphology are associated not only with differences in gene expression but also with the rearrangements of chromosomal DNA, and the most frequent changes involves the long chromosome VIII, which carries ribosomal DNA cistrons (Rustchenko-Bulgac, 1991). It cannot be excluded, that colonial morphology changes observed by us after treatment with essential oils may results from modifications of chromosomal DNA or gene expression, which will be checked in the further research.

Hyphae formation in *C. albicans* is linked to the colony morphology and associated with the processes of endocytosis and vacuolar sorting (Sudbery, 2011). This observation on dependence between colony and cell morphology is consistent with our data.

In our study differences in colony morphology were associated with different sizes of isolates' cells, expressed as morphology index, though changes did not have directional character. After treatment with 0.06, 0.03 and 0.015% v/v thyme oil in microscopic preparations, besides spherical, ovoid and elongated cells as in control, the pseudohyphae structures of morphology index ranged 3.84±0.36–3.93±0.28 (Table 2, Fig. 2) occurred. Isolates Th/0.125, Th/0.0075/1, Th/0.0075/2, TTO/0.25, TTO/0.06, TTO/0.015 and TTO/0.0075 were characterized only by cells of spherical and ovoid shapes. For isolates of elongated cells, frequency distributions of these cells were usually lower compared with control and equaled from 4.0 to 13.5% (Table 2).

Cells of *C. albicans* are capable to develop in many diverse morphological forms, two of which are the most common i.e. ovoid, budding yeast form and the filamentous hyphal form (Odds, 1988). However, intermediate morphologies between these two forms, of morphology index in the range 2.5–4.0 occur frequently (Merson-Davies *et al*., 1991), which is in accordance with our results.

Table 3. *Candida albicans* ATCC 10231 biochemical and enzymatic profiles after treatment with thyme oil (Th) and tea tree oil (TTO) at the concentration of 0.0075–0.25% v/v. Isolate symbol is composed as follows: essential oil type/ the oil concentration.

Control	*assimilation of GLU, 2KG, XYL, ADO, XLT, GAL, SOR, MDG, NAG, MAL, SAC, TRE	**2, 3, 4, 6, 7, 8, 11, 12, 16
Isolates	Changes in biochemical profiles	Changes in enzymatic profiles
Th/0.125	no changes	3(**L**), 4(↓), 6(**L**), 7(**L**), 8(**L**), 11(↓), 12(↓), 16(**L**)
Th/0.06	no changes	3(↓), 4(↓), 6(↓), 7(**L**), 8(**L**), 12(↓),16(**L**)
Th/0.03	no changes	2(**L**), 4(↓), 6(↓), 7(**L**), 8(**L**), 12(↓), 16(↓)
Th/0.015	no changes	2(**L**), 3(↓), 7(↓), 8(**L**), 11(↓), 12(↓),16(↓)
Th/0.0075/1	no changes	2(**L**), 3(↓), 4(↓), 6(↓), 11(↓), 12(↓), 16(**L**)
Th/0.0075/2	no changes	2(**L**), 8(**L**), 11(↓), 12(**L**), 16(↓), **18**, **19**, **20**
TTO/0.25	no changes	2(↑), 8(↑), 11(↑), 12(↑), **18**
TTO/0.06	no changes	2(**L**), 3(**L**), 4(**L**), 6(↓), 7(**L**), 8(**L**), 11(↓), 16(**L**)
TTO/0.03	XLT(**L**), TRE(**L**)	3(**L**), 4(↓), 7(**L**), 8(**L**), 11(↓), 12(↓), 16(↓)
TTO/0.015	XLT(**L**), SOR(**L**), TRE(**L**)	2(**L**), 3(**L**), 4(↓), 6(↓), 7(**L**), 8(**L**), 11(↓), 16(**L**)
TTO/0.0075	no changes	2(**L**), 3(**L**), 4(↓), 6(↓), 7(↓), 8(**L**), **9**, 12(↓),16(↓)

*Biochemical substrates (API 20C AUX, bioMerieux): GLU D-glucose, 2KG calcium 2-keto-D-gluconate, XYL D-xylose, ADO adonitol, XLT xylitol, GAL D-galactose, SOR D-sorbitol, MDG methyl-αD-glucopyranoside, NAG N-acetyl-glucosamine, MAL D-maltose, SAC D-saccharose, TRE D-trehalose; (**L**) loss of ability to assimilate substrate. **Enzymes (API-ZYM, bioMerieux): 2 alkaline phosphatase, 3 esterase (C4), 4 esterase lipase (C8), 6 leucine arylamidase, 7 valine arylamidase, 8 cystine arylamidase, 9 trypsin, 11 acid phosphatase, 12 naphthol-AS-BI-phosphohydrolase, 16 α-glucosidase, 18 N-acetyl-β-glucosaminidase, 19 α-mannosidase, 20 α-fucosidase; (↓) decrease of enzymatic activity, (↑) increase of enzymatic activity, (**L**) loss of enzymatic activity, **bold** enzyme activity not detected in control

In the presence of examined essential oils *C. albicans* strain showed changes in biochemical and enzymatic properties, but extensive differences were found for enzymatic profiles. Yeast ability to assimilate different sources of carbon changed for D-xylose and D-trehalose assimilation after treatment with 0.03% TTO and additionally for D-sorbitol in the presence of 0.015% TTO (Table 3). Changes in enzymatic profiles were primarily associated with loss or decrease of activity of all enzymes detected for control, i.e. alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Furthermore, for isolate Th/0.0075/2 additional activity of N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase was detected. Interestingly, for isolate TTO/0.25 as the only one an increase of 4 enzymes activities and additional activity of N-acetyl-β-glucosaminidase was found. And isolate TTO/0.0075 showed proteolytic activity of trypsin, which was not observed for control strain (Table 3).

Decrease of enzymatic activity of *C. albicans* strains could significantly reduce their pathogenicity. Batura-Gabryel and Młynarczyk (2000) have found correlation between presence of oral candidosis and secretion of lipase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and β-glucosidase by *C. albicans* strains. Similarly, *C. albicans* isolates from patients with bullous and connective tissue diseases revealed high activity of acid phosphatase, and those isolated from patients with neoplasms — acid phosphatase and naphthol-AS-BI-phosphohydrolase (Kwaśniewska *et al*., 2001). Yeast enzymes produced and released during reproduction and cell death initiate the inflammatory process by cytopathic effect, leading to epithelial cell damage (Louie *et al*., 1994). It has been shown that enzymes which play a key role in the pathogenesis of *Candida* species are proteases and phospholipases (Abaci, 2011, Schaller *et al*., 2005). Phospholipases damage mucosal cells and hydrolases are responsible for degradation of macromolecules. Both of them facilitate fungal colonization process (Zhu & Filler, 2010). Proteases cause degradation of epithelial cells, cytokines and immunoglobulins, and modification of the fungal surface antigens, thus contribute to the process of adhesion and colonization of host tissues (Abaci, 2011, Schaller *et al*., 2005). Furthermore, alkaline phosphatase, N-acetyl-β-glucosaminidase and α-mannosidase inhibit the migration of neutrophils to sites of infection and lipase is considered as particularly important in the early stages of infection, when lipids may be used by fungi as a carbon source necessary to their further growth and development of infection (Nowicki & Korting, 1995). On the other hand, it has been shown that lipolytic activity of *Candida* spp. depends on carbon sources available in medium (Wróblewska *et al*., 2011).

In our study, in the presence of thyme and tea tree oils changes in *C. albicans* colony and cell morphology as well as, in most cases, reduction of enzymatic activity and loss of ability to assimilate saccharides were observed. These modifications may be caused by principal active constituents of examined essential oils, i.e. pinene, terpinen-4-ol, γ-terpinene, thymol, *p*-cymene. The antimicrobial action of essential oils and their monoterpenoid components is generally explained by toxic effects on membrane structure and function (Cox *et al*., 2000; Uribe *et al*., 1985). In yeast cells α-pinene and β-pinene, being compounds inter alia tea tree oil, destroy cellular integrity, inhibit respiration and ion transport processes and increase membrane permeability (Uribe *et al*., 1985). Apart of the cytotoxic effects, the interactions with the cell membrane, the antimicrobial action of monoterpenes such as thymol, carvacrol, *p*-cymene, and γ-terpinene may also result from a loss of ATP synthesis capacity required to support the regulation of several cell functions (Custódio *et al*., 2011). Moreover, essential oils show a concentration dependent post-antifungal effect (PAFE) and significantly reduce tolerance to oxidative stress (Budzyńska *et al*., 2013).

In recent years, there has been an increase in the number of scientific publications concerning the thera-

peutic abilities of essential oils. It has been shown that thyme oil significantly enhances intracellular killing activity of human polymorphonuclear granulocytes against *C. albicans* and this antifungal activity is comparable to fluconazole efficacy (Tullio *et al*., 2012). The therapeutic effectiveness of essential oils for mucosal candidiasis has been evaluated in a murine oral candidiasis model. The treatment with a tea tree oil and its main component terpinen-4-ol showed a decrease in the symptom score of tongues and in the viable *Candida* cell number (Ninomiya *et al*., 2012). It has been demonstrated, that the reduction of antifungal agents concentrations required to achieve lethal effect is possible by the application of essential oils into liposome-encapsulated combined preparations with silver ions (Low *et al*., 2013). These data may encourage the development of essential oils products for a practical and safe approach to topical treatment. The explanation of the mechanism of *Candida albicans* response to the essential oils requires multiplatform research, including rarely reported in the literature data phenotypic changes in yeast morphology and particularly changes within the metabolic profile.

Changes that we observed for *C. albicans* in the presence of essential oils, especially in yeast enzymatic activity and carbon sources assimilation, may be associated with yeast response to environmental stress conditions, and may contribute to the reduction of *C. albicans* pathogenicity.

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