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Research on antifungal and inhibitory effects of DL-limonene on some yeasts

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Abstract: This study was undertaken to investigate the antifungal effect of DL-limonene on yeasts using disk diffusion and the minimum inhibitory concentration (MIC). The effect of DL-limonene on ethyl alcohol fermentation by *Saccharomyces cerevisiae* was also investigated. According to disk diffusion results, the development of an inhibition zone was observed in all microorganisms tested. The inhibition zones were larger than those occurring with the Fungizone antibiotic used as the control. Inhibition zone diameters increased with increasing amounts of DL-limonene. MIC results varied between 500 and 4000 µg mL^{-1} depending on the yeast strain. DL-limonene at a concentration of 0.20% (w v^{-1}) inhibited cell growth, ethanol formation, and sugar utilization by *S. cerevisiae*.

Key words: Antifungal effect, DL-limonene, fermentation, yeasts

Introduction

Since the Middle Ages, essential oils have been widely used in bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, medicinal, and cosmetic applications. Because of the mode of extraction, generally distillation from aromatic plants, these oils contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components, and aliphatic components (Bakkali et al. 2008). The essential oils of plants show antimicrobial activity against a wide range of bacteria, including antibiotic-resistant species and fungal species. They can affect both gram-positive and gram-negative bacteria in addition to yeasts and filamentous fungi (Prashar et al. 2003). Because of their relatively safe status, there is increasing interest in the use of essential oils and their components as antimicrobial agents (Feng and Zheng 2007).

The antimicrobial effects of various plant extracts and essential oils have been investigated against many microorganisms (Schwob et al. 2002; Uzel et al. 2004; Rasooli et al. 2006). However, little is known about the effects of citrus essential oils on the yeasts that commonly cause food spoilage or are used in food and beverage fermentations. Chaibi et al. (1997) studied the antimicrobial effects of 9 plant essential oils, including some from orange and grapefruit, on *Bacillus cereus* T and *Clostridium botulinum* 62A (strains). Caccioni et al. (1998) investigated the relationship between the volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. Belletti et al. (2004) evaluated the antimicrobial activity of citrus essences on *S. cerevisiae*.

Citrus oils are found primarily in oval-shaped sacs in the flavedo or colored portion of the peel and

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act as a natural toxic barrier to many microorganisms and insects. Citrus oils are composed primarily (more than 90%) of D-limonene, a sesquiterpene, with other monoterpenes and sesquiterpenes being found in trace amounts (Kimball 1999). D-limonene is one of the most common terpenes in nature. The typical concentration of D-limonene in orange juice is 100 ppm (Sun 2007). It was reported that the presence of orange peel oil in fermentation medium inhibited ethanol fermentation when using *S. cerevisiae* (Grohmann et al. 1994).

Wine is an alcoholic beverage produced from grapes as well as from other fruits, including oranges. Orange is the third largest fruit crop grown in Turkey after grape and apple, with an annual production of 970,000 t in 1998 (Selli et al. 2002). Orange wine is a novel product that is not yet marketed in China (Fan et al. 2009). Different yeast species participate in spontaneous alcoholic fermentation even when sulfur dioxide is present. *Kloeckera*, *Hanseniaspora*, and *Candida* usually predominate in the early stages of alcoholic fermentation; *Pichia* and *Metschnikowia* prevail in the middle stages. Finally, during the latter stages of fermentation, *S. cerevisiae* becomes the predominant yeast because of its greater resistance to high ethanol concentrations. Some other yeasts, such as *Torulaspora*, *Kluyveromyces*, *Schizosacchaomyces*, *Zygosaccharomyces*, and *Brettanomyces*, may also be present during alcoholic fermentation (Zamora 2009). The study reported herein was designed to determine the antifungal and inhibitory effects of DL-limonene on yeasts and alcohol fermentation by *S. cerevisiae*.

Materials and methods

Materials

DL-limonene (density: 0.842 g mL^{-1}) and ethanol were purchased from Sigma-Aldrich (Germany). Tween 80, malt extract agar, and malt extract broth were purchased from Merck (Germany). Whatman filter paper disks (10 mm in diameter) were used. Fungizone (50 mg) was used as a control (Bristol-Myers Squibb, Taiwan).

The following yeasts were used as test organisms: *S. cerevisiae* (Zymoflore VL1; Laffort Inc., France), *S. cerevisiae* (Fermiblanc SM; DSM Inc., INRA, France), *S. cerevisiae* (Fermirouge 303; DSM Inc., INRA), *S. cerevisiae* (Maurivin SW; Mauri Inc., Australia), *S. cerevisiae* (Lalvin 2226; Lallemand Inc., Canada), and *S. cerevisiae* (Actiflore PM; Laffort Inc.). The following yeast strains were kindly supplied by Dr H. Erten (Department of Food Engineering, Çukurova University, Adana, Turkey): *Kluvyeromyces thermotolerans*, *K. apiculata*, *Candida datilla*, *C. pulcherima*, *C. bolmii*, *Rhodotorula glutinis*, *Hanseniaspora uvarum* NCYC 25, *S. bayanus* N387, and *Pichia subpelliculuse* NCYC 436. They were maintained on malt extract agar at 4 °C.

Disk diffusion method

The filter paper disk agar method was used in this study. A single colony of pure culture on agar was transferred to malt extract broth (5 mL), which was then incubated at 25 °C for 48 h. Sterile malt extract agar (Merck) was prepared and distributed into petri plates of 10 cm in diameter. After allowing the plates to solidify, 300 µL of the test strains that were grown in broth were streaked. Sterilized Whatman filter paper No. 1 disks (10 mm in diameter) were thoroughly moistened with DL-limonene, and 3 disks were placed in each plate. The volumes of DL-limonene tested were 10, 25, and 50 µL. The zone of inhibition was measured in millimeters after incubation at 25 °C for 72 h, and the average of 3 replicates was reported. The results are expressed as the net zone of inhibition, which represents the diameter (10 mm) of the paper disk subtracted from the measured zone. The commercially available Fungizone 50, as a control, was also evaluated under similar conditions against all test microorganisms (Janseen et al. 1987).

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values were determined by the broth dilution method in flasks in triplicate. Each flask contained malt extract broth and 0.5% Tween 80 (v v^{-1}) plus a fixed volume of serially diluted DL-limonene (ranging from 62.5 to 4000 µg mL^{-1}) dissolved in ethanol. Tween 80 was incorporated into the liquid medium to enhance the solubility of DL-limonene. The maximum amounts of ethanol and Tween 80 that were carried into the culture medium did not affect cell multiplication. Each flask was inoculated with a 3-mL aliquot of the yeast inoculum containing 10^6 cells mL^{-1}. The controls contained malt extract broth, 0.5% Tween 80

($v\ v^{-1}$), and ethyl alcohol. The flasks were subjected to continuous agitation at 25 °C for 48-72 h. The lowest concentration of DL-limonene that prevented visible growth was defined as the MIC (Johnston et al. 2001; Yu et al. 2004).

Inhibitory effects of DL-limonene on ethanol fermentation

The effect of DL-limonene on ethanol fermentation by *S. cerevisiae* (Actiflore PM) was studied using a fermentation medium containing ($g\ L^{-1}$): glucose, 150; yeast extract, 10; KH_2PO_4, 1; and $MgSO_4.7H_2O$, 0.5. Fermentation trials were performed in triplicate and the results are expressed as means. The initial pH of the medium was adjusted to 4.0 by using 0.1 N HCl. Fermentations were conducted in 250-mL flasks containing 200 mL of fermentation medium; 0%, 0.05%, and 0.20% ($w\ v^{-1}$) DL-limonene (1 mL of DL-limonene dissolved with 4 mL of ethanol); and 0.5% ($v\ v^{-1}$) Tween 80 at 25 °C for 7 days in a rotary shaker (agitation speed: 140 rpm). Samples (10 mL) were withdrawn aseptically to measure viable cells, fermentation products, and degrees Brix. The samples were centrifuged at 5000 × *g* for 10 min. The supernatant was frozen at −25 °C after degrees Brix was determined. The viability of cells was measured indirectly by staining the cells with methylene blue on a microscope slide, and viable cells were counted under a microscope (Euromex, Holland) according to the methods of Bakker (1991).

Determination of ethanol

Fermentation broth was centrifuged at 5000 × *g* for 10 min. Supernatant (100 mL) was distilled after the addition of 30 mL of distilled water. When 100 mL of distillate was obtained, ethanol was determined by measuring the density of the distilled sample with electronic densimetry (Mettle Toledo RE 50, Switzerland) (European Council 2004).

Analysis of esters and higher alcohols

Fermentation products were determined by direct injection of 1-µL samples into a gas chromatograph (GC) (Agilent 7890A, USA) equipped with a split injector and flame ionization detector (Erten and Campbell 2001; Yilmaztekin et al. 2009). Esters and higher alcohols were separated using a Chrompack CP-WAX-57CB capillary column (0.25 mm i.d. × 60 m × 0.4 µm film thickness) (Chrompack, the Netherlands).

The GC settings were as follows: injection temperature, 160 °C; oven temperature, 4 min at 40 °C then increased by 1.8 °C min^{-1} up to 94 °C, 40 °C min^{-1} up to 180 °C, and, finally, 4 min at 180 °C; detector temperature, 180 °C; carrier gas, He (1.3 mL min^{-1}); and split rate, 1:50. The quantification was performed by the internal standard (3-pentanol) method. Standard solutions containing all compounds were prepared and analyzed in duplicate. Relative response factors (RRFs) were calculated from peak areas for each compound using the following equation:

$$RRF: [(A_{is}) / (A_c)] \times [(C_{is}) / (C_c)],$$

where A_{is} is the area of the internal standard, A_c is the area of the compound, C_{is} is the concentration of the internal standard, and C_c is the concentration of the compound. A linear plot was obtained with a correlation coefficient of at least 0.999 for all compounds. The results given represent the means for 2 determinations with standard deviations.

Results

Antifungal activity

The antifungal activity of DL-limonene was assayed against 14 yeasts. The inhibitory zones and minimum inhibition concentration values are presented in Table 1. The results were compared with standard antibiotics. Inhibitory zones were observed at all DL-limonene concentrations in all organisms tested. The inhibitory zone diameters changed in a concentration-dependent manner. *K. thermotolerans*, with the smallest inhibition zones, was the least sensitive test organism to DL-limonene; *P. subpelliculuse* NCYC 436, with the largest inhibition zones, was the most sensitive organism. The antifungal activity of the lowest DL-limonene concentration applied (10 µL) was better than that of standard antibiotics except on *K. thermotolerans* and *C. pulcherima*. Data on MIC values revealed that DL-limonene was effective against all the organisms tested in this study.

Inhibitory effects on ethanol fermentation

Inhibitory effects of DL-limonene on ethanol fermentation by *S. cerevisiae* (Actiflore) was studied using a fermentation medium containing 0.05% and 0.20% ($w\ v^{-1}$; 500 and 2000 µg mL^{-1}) DL-limonene.

Table 1. Antifungal activity of DL-limonene.

Yeast strains	Inhibitory zone (mm)				MIC (μg mL^{-1})
	10 µL	25 µL	50 µL	Fungizone (50 µL)	
K. thermotolerans	13.0	15.0	18.6	16.0	2000
K. apiculata	26.0	31.3	41.0	21.0	500
C. datilla	32.0	37.6	43.3	15.0	2000
C. pulcherima	17.6	21.0	25.5	20.0	2000
C. bolmii	27.5	30.0	39.3	25.0	500
R. glutinis	25.0	32.0	40.0	22.0	4000
H. uvarum NCYC 25	23.0	33.3	39.0	19.0	2000
S. bayanus N387	25.0	31.0	40.0	20.0	1000
P. subpelliculuse NCYC 436	30.3	36.3	48.0	19.0	500
S. cerevisiae Zymoflore	25.0	33.0	43.3	23.0	1000
S. cerevisiae Maurivin	30.0	35.6	46.3	17.0	500
S. cerevisiae Lalvin	29.3	34.6	42.3	20.0	500
S. cerevisiae Fermiblanc	23.0	31.0	35.6	18.0	1000
S. cerevisiae Fermirouge	27.6	32.6	44.3	14.0	500

The effect of DL-limonene on cell viability is shown in Figure 1. As seen in Figure 1, yeast growth started after a lag period of 12 h in the samples containing 0.05% (500 µg mL^{-1}) DL-limonene, which was similar to the control. However, there was no yeast growth in the samples containing 0.20% (2000 µg mL^{-1}) DL-limonene, indicating that yeast growth at this concentration was completely inhibited.

The effect of DL-limonene on sugar utilization is given in Figure 2. Utilization of sugar followed the trend of cell growth. There was no sugar utilization in the samples containing 0.20% (2000 µg mL^{-1}) DL-limonene, confirming the inhibitory effect of DL-limonene on yeast growth at this concentration. In the control and samples containing 500 µg mL^{-1} DL-limonene, sugar utilization started after 12 h and all the sugar was used up within 48 h.

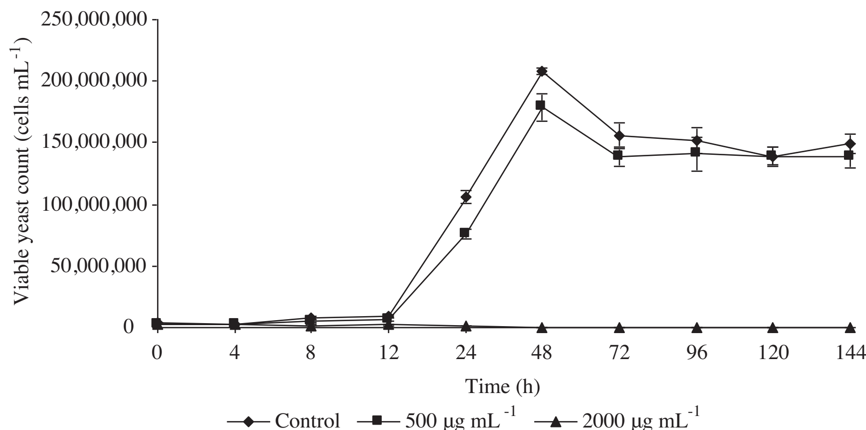

Figure 1. Effect of DL-limonene on *Saccharomyces cerevisiae* Actiflore growth.

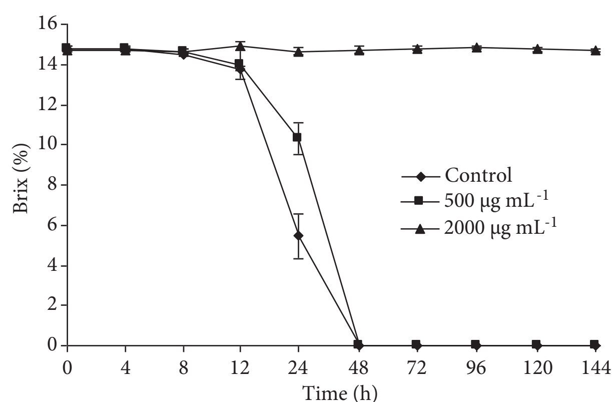

Figure 2. Effect of DL-limonene on sugar utilization by *Saccharomyces cerevisiae* Actiflore.

The products formed during fermentations, given in Table 2, were similar in the control and samples containing 0.05% (500 µg mL^{-1}) DL-limonene. Since yeast growth and sugar utilization were inhibited in the fermentation containing 0.20% (2000 µg mL^{-1}) DL-limonene, the fermentation products examined were either undetected or at insignificant levels. The concentrations of ethanol in the control and the fermentation with 0.05% DL-limonene were similar. However, in the fermentation experiments with 0.20% DL-limonene, the concentration of ethanol detected was very low compared to the others. The amounts of acetaldehyde detected in the control and fermentation experiments with 0.20% DL-limonene were similar, whereas a greater amount of acetaldehyde was produced in the fermentation with 0.05% DL-limonene. There were also differences in the amounts of methyl acetate and ethyl acetate produced between the control and fermentations with DL-limonene (Table 2).

Discussion

No clear correlation between MIC values and inhibition diameters was found, suggesting that the results gained with these 2 methods are not necessarily comparable; this was also reported by others (Manou et al. 1998). Caccioni et al. (1998) studied the antimicrobial action of citrus fruit essential oils on *Penicillium digitatum* and *P. italicum*, and they reported that there was a positive correlation among the oils' content of monoterpenes, other than limonene and sesquiterpene, and pathogen fungi inhibition. In a study carried out by Cvetnic and Vlademir-Knezevic (2004) on antimicrobial activity of grapefruit seed and pulp ethanolic extract against bacteria and yeasts, it was found that grapefruit seed ethanolic extract exhibited antimicrobial activity against all of the organisms tested.

DL-limonene at 0.20% completely inhibited yeast growth. Inhibitory effects of D-limonene on *S. cerevisiae* were attributed to a disruption of the cellular membrane, which causes the cellular contents to leak out of the cell and the disruption of H^+ and K^+ transport (Wilkins et al. 2007b). In a study carried out by Wilkins et al. (2007a) with *S. cerevisiae* and *Kluyveromyces marxianus*, the effect of citrus peel oil

Table 2. Effect of DL-limonene on fermentation products.

	Control	0.05% DL-limonene (w v^{-1})	0.20% DL-limonene (w v^{-1})
Ethanol (% v v^{-1})	7.43 ± 0.06	6.97 ± 0.15	0.47 ± 0.12
Acetaldehyde (mg 100 mL^{-1})	1.57 ± 0.31	2.68 ± 0.42	1.51 ± 0.08
Methyl acetate (mg 100 mL^{-1})	1.58 ± 0.05	1.78 ± 0.07	1.50 ± 0.08
Ethyl acetate (mg 100 mL^{-1})	2.05 ± 0.12	3.99 ± 0.04	0 ± 0
1-Propanol (mg 100 mL^{-1})	8.68 ± 0.67	7.59 ± 0.25	0 ± 0
2-Methyl-1-propanol (mg 100 mL^{-1})	14.5 ± 1.04	14.4 ± 0.42	0 ± 0
2-Methyl-1-butanol (mg 100 mL^{-1})	8.0 ± 0.94	9.30 ± 0.15	0 ± 0
3-Methyl-1-butanol (mg 100 mL^{-1})	20.5 ± 1.03	22.0 ± 0.90	0 ± 0

was investigated at a range of 0.05%-0.20% (v v^{-1}). They reported that peel oil had an effect on cell mass yields, and no inhibition of cell mass production was observed after 72 h.

D-limonene also inhibited alcohol fermentation by *S. cerevisiae* Actiflore at 0.20%. In a study with *Zymomonas mobilis*, minimum inhibitory orange peel oil concentrations for ethanol production at 37 °C were 0.05% after 24 h, 0.10% after 48 h, and 0.20% after 72 h. Orange peel oil did not inhibit ethanol production after 96 h at a temperature of 37 °C (Wilkins 2009). Wilkins et al. (2007a) studied the inhibitory effects of orange peel oil that contained large amounts of limonene (<95%) on ethanol fermentation by *S. cerevisiae* and *K. marxianus*. The minimum peel oil concentration that inhibited ethanol production was determined after 24, 48, and 72 h, and the 2 yeasts were compared in terms of ethanol yield. Minimum inhibitory peel oil concentrations for ethanol production were 0.05% at 24 h, 0.10% at 48 h, and 0.15% at 72 h for both yeasts. *S. cerevisiae* produced more ethanol than *K. marxianus* at each time point. Conner et al. (1984) investigated the effects of essential oils and plant oleoresins on ethanol production by yeasts and reported that essential oils of allspice, cinnamon, and clove had little or no effect on ethanol production. They also reported that oils of onion, oregano, savory, and thyme delayed and/or reduced the production of ethanol.

A greater amount of acetaldehyde was produced in fermentation with 0.05% DL-limonene. Acetaldehyde is an intermediary of alcoholic fermentation obtained by the decarboxylation of pyruvate. Later, acetaldehyde is mainly reduced to ethanol, but small quantities of it may be released into the wine. Acetaldehyde can also be produced from ethanol by chemical or biological oxidation (Zamora 2009). The amount of methyl acetate and ethyl acetate produced between the control and fermentations with DL-limonene differed. Esters are synthesized from acyl-coA and alcohols by a group of enzymes, the alcohol-acylcoA transferases. Basically, there are 2 types of esters in wine: the acetates of higher alcohols and the esters of fatty acids and ethanol. The first group is synthesized from acetyl-coA and the different higher alcohols. These esters give off different odors such as glue (ethyl acetate), banana (isoamyl acetate), or rose (phenylethanol acetate). The other group of esters is synthesized from different acyl-coAs and ethanol. The different esters of fatty acids and ethanol give off a fruity aroma. All esters, with the exception of ethyl acetate, give off an agreeable smell and contribute positively to the wine aroma. Other esters, such as ethyl lactate and diethyl succinate, do not have any sensory impact at normal concentrations (Zamora 2009).

Inhibitory zones were observed at all DL-limonene concentrations with all organisms tested. The inhibitory zone diameters changed in a concentration-dependent manner. The inhibition zones were larger than those created by the Fungizone antibiotic that was used as a control. MIC results ranged from 500 to 4000 µg mL^{-1} depending on the yeast strain. DL-limonene at a concentration of 0.20% (w v^{-1}) inhibited cell growth, ethanol formation, and sugar utilization by *S. cerevisiae*.

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