Ph.D. thesis

Effect of essential oils and their combinations on food-spoilage microorganisms

Tserennadmid Rentsenkhand

Supervisers:
Dr. Judit Krisch, Assistant Professor
Dr. Csaba Vágvölgyi, Professor,
Head of Department of Microbiology

Biology Ph.D. Program

University of Szeged
Department of Microbiology

Szeged
2010

INTRODUCTION

Essential oils (EOs) are volatile liquids obtained from herbs, spices and different plants mainly by steam distillation. They can have more than 50 components in different ratios. Some of the essential oils and their constituents are known to possess antibacterial, antifungal, antiparasitic and insecticidal properties. There is a growing interest in using EOs by the food industry as natural preservatives against food spoilage- and food-borne pathogen microbes, in order to meet consumer demands for avoiding synthetic components in food. It has been often observed that the preservative effect in food can be achieved by higher doses of EOs than *in vitro*. It is supposed that foodstuffs with high protein and fat content can protect bacteria from the antibacterial effect of EOs and other natural antimicrobials. The food matrix can represent a physical hurdle and an essential oil dissolved in the fat of the food will be less available to act on bacteria present in aqueous phase. Regarding that higher amount of essential oils are required for food preservation, their application can negatively influence taste or odor. To avoid this unwanted side effect, several EOs can be combined.

Products that cannot be pasteurized are usually treated with weak acid preservatives: sorbic or benzoic acid or their salts. However, there is a strong consumer demand to avoid or diminish the use of artificial preservatives. It was recently reported that benzene can be formed from benzoic acid in foods. *Saccharomyces cerevisiae* and *Pichia anomala* are able to decarboxylate sorbic acid to 1,3-pentadiene causing a kerosene-like off-odour (Stratford et al., 2007). In these cases, the application of essential oils (EOs) can be a good alternative. Most EOs are regarded as safe (GRAS) and are accepted by consumers (Burt, 2004). They have wide antimicrobial spectra against bacteria (Dorman and Deans, 2000, Burt, 2004), yeasts (Conner et al., 1984; Belletti et al., 2010) and moulds (Pinto et al., 2006; Kamble et al., 2008; Viuda-Martos et al., 2008).

The aim of this study was to evaluate the antimicrobial effects of four essential EOs from clary sage, juniper, lemon and marjoram containing mainly monoterpenes or terpene alcohols (α-pinene, limonene, linalool and terpinen-4-ol) against food spoilage microorganisms. The interaction between the EOs and their main components was also studied. The influence of food ingredients - hydrolyzed proteins of animal and plant origin

(meat extract and soy peptone) and sucrose - on the antibacterial effect of EOs was also investigated.

Mechanism of action of essential oils and their main components

Essential oils are aromatic, concentrated, hydrophobic liquids containing more than 50 different components. It has been suggested that EOs containing phenolic compounds possess the strongest antimicrobial activities. The alcohol terpenoids can act as protein denaturing or dehydrating agents, but they are more active when the cell membrane has increased permeability, suggesting that their main target may be within the cell. Hydrophobic EO compounds can increase the permeability of the membrane, leading to leakage of the cell contents, because the cell membrane is considered to be their main target both in bacterial and in fungal cells. Membrane damage and the loss of cytoplasmic components (absorbing at 260 nm) have been reported for terpinen-4-ol in *S. aureus* and α-terpinene and (+)-limonene in *C. tropicalis*. Other targets of the EOs are enzymes in the energy regulation. Uribe et al. (1985) reported that β-pinene and limonene inhibited the respiratory activity in intact yeast cells and also in isolated mitochondria.

Antibacterial effects of essential oils

Essential oils have substantial antibacterial and antifungal effect. MIC values are in the range of 0,5 – 4 µl/ml; the MIC of the main components are generally higher than the parent EOs. Considering the effects of EOs, Gram-positive bacteria are more susceptible than the Gram-negative ones (Burt, 2004; Hammer et al., 1999). This difference is due to the presence of the outer membrane which functions as a barrier for small hydrophobic compounds (Burt, 2004; Longbottom és mtsai, 2004). Essential oils are effective not only as supplements in the medium but also as volatiles in a closed space.

Essential oils in foodstuffs

Application of EOs as natural food preservatives is an intensively searched area. In foodstuffs, higher concentrations of the EOs are usually required for the antimicrobial action than *in vitro* because some food ingredients, like proteins and fats, decrease their effect (Burt, 2004). The food matrix can represent a physical hurdle and an essential oil dissolved in the fat of the food will be less available to act on microorganisms present in aqueous phase. Regarding that higher amount of essential oils are required for food preservation, their application can

negatively influence taste or odor. To avoid this unwanted side effect, several EOs can be combined with each other or with other antimicrobials. Combinations of EOs with different preservation techniques seem to be good alternatives for future issues in food protection.

There are examples in the literature of the application of EOs or their components to improve the shelf-life of fruit-based acidic foods or beverages. EO compounds combined with mild heat treatment inhibited the growth of *S. cerevisiae* in non-carbonated soft drinks (Belletti et al., 2007).

The effects of EOs were mainly tested in food spoilage- susceptible meat products (fish, minced meat, and sausages). They were frequently associated with other preservative methodologies like vacuum or modified atmosphere packaging, or refrigeration.

AIMS

The main aim of the study was to investigate antimicrobial effects of EOs, and their main components, alone or in combinations against food spoilage microorganisms.

Aims of the study were:

1. Testing of antimicrobial effects of selected essential oils and their combinations against Gram positive and Gram negative bacteria and yeasts with agar hole diffusion assay. Selection of the most effective oils for the further detailed experiments.

2. Investigation of the effects of the selected EOs on the growth parameters of bacteria and yeasts in liquid media. Investigation of the bactericid effects of the EOs.

3. Investigation the antifungal effects of EOs against some filamentous fungi.

4. Determination of the minimal inhibitory concentrations (MICs) of the selected EOs and their main components with macro- és microdilution methods.

5. Investigation of the combined effects of EOs and their main components against bacteria and yeasts.

6. Investigation of the effects of selected EOs in the presence of different food components, and in different foods.

MATERIALS AND METHODS

Microorganisms:

Gram positive bacteria: *Bacillus subtilis, B. cereus*

Gram negative bacteria: *Escherichia coli* és *Serratia marcescens*

Yeasts: *Saccharomyces cerevisiae, Pichia anomala, Geotrichum candidum, Schizosaccharomyces pombe*

Moulds: *Penicillium chrysogenum, Aspergillus niger, Rhizopus stolonifer, Fusarium sporotrichioides*

Essential oils and components

Juniper (*Juniperus communis*) - α-pinene

Lemon (*Citrus lemon*) - limonene

Marjoram (*Origanum majorana*) - terpinen-4-ol

Clary sage (*Salvia sclarea*) - linalool

Culture media and solutions

1. Yeast extract-tryptone-glucose medium (TGE)
2. Supplemented meat medium (MEE)
3. Luria-Bertani (LB) medium
4. Malt extract medium (MEA)
5. Hydrolysed meat extract supplemented medium
6. Hydrolysed soy peptone supplemented medium
7. Sucrose supplemented medium

Culture conditions

MEE, LB and MEA were used for the maintenance of *Bacillus*, *E. coli* and fungal strains, respectively. Growth temperature were 30 °C, 37 °C, 28 °C, and 25 °C for *Bacillus*, *E. coli*, yeasts and moulds, respectively.

Testing of antimicrobial susceptibility

1. Agar hole diffusion assay
2. Determination of growth curves
3. Determination of growth parameters

4. Time-kill method for Gram positive and Gram negative bacteria
5. Determination of the sporocid effect of EOs.
6. Effect of pH on the effectiveness of marjoram EO.
7. Testing of antifungal effect against moulds
8. Determination of the minimal inhibitory concentrations (MICs) of the selected EOs and their main components with macro- és microdilution methods.
9. Investigation of the combined effects of EOs and their main components by *checkerboard* method
10. Determination of the fractional inhibitory index (FICI)

Interaction of essential oils with food constituents

Effectiveness of essential oils in foods

Sensory studies

Statistical analysis - ANOVA

RESULTS AND DISCUSSION

1. In the experiments, it was found that the length of the lag phase is the growth parameter most affected by the essential oils in case of bacteria and yeasts. The lengthening was typically proportional to the concentration, and in some cases no growth was observed in the 48 hours observation period. Growth rate was often not influenced by the presence of the essential oil, or was lowered only by the higher doses. Gram positive bacteria were more sensitive than Gram negative ones.
2. In case of moulds, the EOs influenced more the size of the colony and less the colony forming rate. Also, the growth of the moulds was more influenced by EO vapour space than by EOs mixed to the medium.
3. Minimal inhibitory concentrations (MIC) were between 0.25 - 2 µl/ml for bacteria and yeasts. None of the EOs proved to be especially efficient on the basis of the microbes' individual sensitivity. Generally it can be stated that the EO of marjoram and clary sage exerted stronger inhibitory effect on bacteria, and juniper and lemon EO, on yeasts and moulds.
4. Combination of EOs containing main components from the same group (monoterpens or terpene alcohols) showed additive interaction. Combining of EOs with different type main components frequently showed no interaction.
5. Combination of the main components of EOs resulted in different interactions than the combination of the parent EOs, emphasizing the role of the minor components.
6. In testing the components, monoterpenes (α- pinene and limonene) were more efficient than terpene alcohols (linalool and terpinene-4-ol).
7. High concentrations of animal protein hydrolysates exerted protection against the growth inhibitory effect of EOs but similar effect was not observed in the case of soy peptone. Apparently we are the first to describe the difference between animal and vegetable proteins in the interaction with EOs. The hydrophobic side chain of the animal proteins probably bound the components of the EOs so these could not reach the bacteria in the aqueous phase. The peptone of plant origin, instead, promoted the even distribution of the EO in the medium. Sucrose had no effect on the antimicrobial effect of the EOs.

8. On testing the EOs in real foods, the best effects were achieved in fruit juices of low pH, high sugar, but low protein and fat content. Organoleptic test also indicated that fruit juices can provide the starting point for product development of foodstuffs with added EOs. For packaged bread, EO-containing active packaging may be a significant advance in technology and food safety.

DISCUSSION

The aim of this work was to examine the effect of certain essential oils, selected in preliminary experiments, on some bacteria, yeasts and filamentous fungi causing food spoilage. The use of essential oils is limited by their strong aroma, altering the taste of the food, therefore, combinations of oils were also tested.

The mode of action of these is thus also of interest, so the effect of the main components of the selected essential oils (such as α-pinene of juniper oil, limonene of lemon iol, linalool of marjoram oil and terpinen-4-ol of clary sage), as well as the combinations of these, were also investigated.

It has been suggested that EOs containing phenolic compounds possess the strongest antimicrobial activities. The EOs used in our study were declared by the manufacturer not to contain phenolics. The main components of our EOs were alcohol terpenoids, linalool (clary sage) and terpinen-4-ol (marjoram), and the cyclic monoterpenes α-pinene (juniper) and limonene (lemon). To sum up the outcomes of the work, it can be stated that substantial antimicrobial effect could be verified for each of the EOs and their main components investigated *in vitro* and also in real foods.

Our experiments with lemon EO given to apple juices at a concentration less than MIC showed that the “open” storage time at ambient temperature could be prolonged and also a novel, refreshing taste could be achieved. The beverage was suggested for product innovation.

As a result of our work the shelf life of sliced bread coud be prolonged by the use of EOs in an active packaging of the product.

Publications in connection with the Ph.D. thesis

Full papers:

1. **Tserennadmid, R**., Takó, M., Galgóczy, L., Papp, T., Vágvölgyi, Cs., Gerő, L., Krisch, J.(2010) Antimicrobial effects of essential oils and interaction with food components. Cent. Eur. J. Biol. 5(5): 641-648.(If: 0,918)
2. **Tserennadmid, R**., Takó, M., Galgóczy, L., Papp, T., Miklós, P., Vágvölgyi, Cs., Almássy, K., Krisch' J. (2010) Anti yeast activities of some essential oils in growth medium, fruit juices and milk. International Journal of Food Microbiology (*in press*) (If: 3,011)
3. Krisch, J., Pardi, Zs., **Tserennadmid, R**., Papp, T., Vágvölgyi', Cs. (2010) Antimicrobial effects of commercial herbs, spices and essential oils in minced pork. Acta Biologica Szegediensis. (*in press*)
4. Krisch, J., Pardi, Zs., Kovács, K., Takó, M., Papp, T., Vágvölgyi, Cs., **Tserennadmid, R**. (2010) Effect of essential oils in food systems. Analecta Technica Szegediensis. 2-3: 128-132.

Abstract in referred journal:

1. **Tserennadmid, R**., Takó, M., Galgóczy, L., Papp, T., Vágvölgyi, Cs., Krisch, J. (2009) Essential oils against food spoilage bacteria and yeasts. *Acta Microbiol. Immunol. Hung.* 56, 233.

Abstracts in conference proceedings:

1. Krisch, J., Pardi, Zs., Kovács, K., Takó, M., Papp, T., Vágvölgyi, Cs., **Tserennadmid, R**. (2010) Effect of essential oils in food systems. ICoSTAF2010, Abstracts 39.
2. **Tserennadmid, R.,** Krisch, J., Takó, M., Galgóczy, L., Vágvölgyi, Cs. (2009) Antimicrobial effects of essential oils and their combinations. 11th DKMT Regional Conference on Environment and Health. Abstracts, pp. 102.
3. Krisch, J., Horváth, G., Vágvölgyi, Cs., **Tserennadmid, R**., Dugarsuren, Ts. (2010) Antimicrobial action of essential oils against food-related moulds. ISIRR, Abstracts: pp. 96. (CD- ISBN. 978-963-508-600-9)

Other conference presentations:

1. **Tserennadmid, R**., **Takó, M.**, Galgóczy, L., Papp, T., Vágvölgyi, Cs., Krisch, J. (2009) Essential oils against food spoilage bacteria and yeasts. 2nd Central European Forum for Microbiology (CEFORM), Okt. 7-9. Keszthely, Hungary.
2. **Tserennadmid, R.,** Krisch, J., Takó, M., Galgóczy, L., Vágvölgyi, Cs. (2009) Antimicrobial effects of essential oils and their combinations. 11th DKMT Regional Conference on Environment and Health, DKMT 15/16 May 2009, Szeged, Hungary.
3. Krisch, J., Pardi, Zs., Kovács, K., Takó, M., Papp, T., Vágvölgyi, Cs., **Tserennadmid, R**. (2010) Effect of essential oils in food systems. International Conference On Science And Technique In The Agri-Food Business, ICoSTAF, 3-5 Nov. 2010. Szeged, Hungary
4. Krisch, J., Horváth, G., Vágvölgyi, Cs., **Tserennadmid, R**., Dugarsuren, Ts. (2010) Antimicrobial action of essential oils against food-related moulds. 11th International Symposium Interdisciplinary Regional Research, ISIRR, 13-15. Okt. 2010, Szeged, Hungary.

Other publications

Full papers:

1. Tserendulam, D., **Tserennadmid, R**., Dulamsuren, Ch. The beverage of the whey. (1996) Scientific journal of National University of Mongolia. 10 (163), 124-126.
2. Tserendulam, D., Bayarlhagva, D., **Tserennadmid, R.,** Badrakh, B. Studies of utilization of ethanol by yeasts. (2000) Scientific journal of The development of the food industry of Mongolia. 11, 156-159.
3. Tserendulam, D., Dulamsuren, Ch., **Tserennadmid, R.** The chemical studies of beverages from the whey. (2000) Scientific journal of National University of Mongolia .10 (163), 127-130.
4. Badrakh, D., Puntsag, T., **Tserennadmid, R.,** Tserendulam, D. Experiments for increasing of continuous phaseolotoxin production of *Pseudomonas syringae* pv. Phaseolicola. (2000), Proceedings of the Institute of Biology. Ulaanbaatar. 22, 195-198.

5. Tserendulam, D., Mongonsukh, O., Puntsag, T., **Tserennadmid, R**., Badrakh, D. Some results of identification of relationship for the several yeast species. (2000) Proceedings of the Institute of Biology. Ulaanbaatar. 22, 217-220.
6. Tserendulam, D., Bayarlhagva, D., Mongonsukh, O., **Tserennadmid, R**. The choosing of raw materials for growth of yeast for fodder production, (2000) Proceedings of the Institute of Biology. Ulaanbaatar. 22, 213-216.
7. Tserendulam, D., Narantsetseg, T., **Tserennadmid, R**., Dulamsuren, Ch. Wine from the whey. (2000) Proceedings of the Institute of Biology, Ulaanbaatar. 22, 221-224.
8. Tserendulam, D., **Tserennadmid, R**., Badrakh, D., Selenge, B. Isolation and identification of yeast strains. (2002) Proceedings of the Institute of Biology. Ulaanbaatar. 23, 219-224.
9. Tserendulam, D., **Tserennadmid, R**., Dulamsuren, Ch., Bayarlkhagva, D., Badrakh, B. Some results of the experiments for the estimation of the growth dynamics and the carotinoide synthesis activity of *Rhodototula glutinis* DM-3. (2002) Proceedings of the Institute of Biology. Ulaanbaatar. 23, 213-218.
10. **Tserennadmid, R**., Tserendulam, D. Studies of tocoferol (Vitamin E) synthesizing yeast. (2006) Proceedings of the Institute of Biology, Ulaanbaatar. 26, 213-218.
11. Tserendulam, D., **Tserennadmid, R**., Dulamsuren, Ch. Studies of fungi in soil of the Gobi. (2006) Proceedings of the Institute of Biology. Ulaanbaatar. 26, 213-218.
12. **Tserennadmid, R**., Tserendulam, D. Production of bioingredients from *Kluyveromyces marxianus-33* grown on whey. (2007) Proceedings of the Institute of Biology. Ulaanbaatar. 27, 209-214.
13. **Tserennadmid, R**., Tserendulam, D. Production of bioingredients from *Saccharomyces uvarum (S-1)*. (2008) Proceedings of the Mongolian University of Science and Technology. 12, 103-106.
14. **Tserennadmid, R**., Tserendulam, D. Study of biologically active microorganisms. (2008) Indian studies in Mongolia. 4, 80-82.

Abstracts in referred journals:

15. Tserendulam D., **Tserennadmid, R**., Ariunaa J. (1999) "The whey microflora". Conference review: Biotechnology-1999, 45-48, Ulaanbaatar, Mongolia

16. Lung, Sz., Takó, M., **Tserennadmid, R.**, Krisch, J., Papp, T., Vágvölgyi, Cs. (2009) Cellulolytic enzymes on agricultural waste in solid state fermentation by Zygomycetes. *Acta Microbiol. Immunol. Hung.* 56, 199-200.

17. **Tserennadmid, R.**, Takó, M., Lung, Sz., Krisch, J., Papp, T., Vágvölgyi, Cs. (2009) Purification and partial characterization of an extracellular beta-glucosidase from *Mucor corticolus*. *Acta Microbiol. Immunol. Hung.* 56, 232-233.

Other conference presentations:

18. Tserendulam D., **Tserennadmid, R**., Badamkhand.D., Narantsetseg T. Characterization of the protein producing yeasts. (2006), "The supplemental food in Mongolia" . The Hunstekh corporation 35-th anniversary conference review, 57-66.

19. **Tserennadmid, R.** Spirulina-desaturase gene manipulation for polyunsaturated fatty acid production in yeast, *Saccharomyces cerevisiae* (2007) report, BIOTEC, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand. .

Patents:

1. Puntsag T., Tserendulam D., Dulamsuren Ch., Badrakh B., **Tserennadmid, R**., "Production of azotobacter dry fertilizer" 1997. №1301
2. Tserendulam D., **Tserennadmid, R**., Dulamsuren, Ch., Badrakh, B. "*Saccharomyces cerevisiae* Z-9" 2002. №2126
3. Tserendulam, D., **Tserennadmid, R**., Dulamsuren, Ch., Damdaingaa Ts. "*Saccharomyces carlsbergensis* 34/70". 2002. №2127
4. Tserendulam, D., **Tserennadmid, R**., Dulamsure,n Ch., Narantsetseg, T. "Nutritional media for cultivation yeast strain *Kluyveromyces marxianus*-33" 2002 №2166
5. **Tserennadmid, R**., Tserendulam D., Avdai Ch., Odonmajig P., Delgermaa B. "*Saccharomyces oviformus* Y-20". 2003. №2225
6. **Tserennadmid, R**., Tserendulam D., Avdai Ch., Odonmajig P., Delgermaa B., Enkhjargal., Tsevegmed P. "*Saccharomyces cerevisiae*-CH" 2003 №2226
7. Tserendulam, D., **Tserennadmid, R**., Bayarlkhagva,.D *"Rhodotorula glutinis DM-3"* 2006. №2779
8. Tserendulam, D., Mongon, O., **Tserennadmid, R**., Narantsetseg, T. „*Saccharomyces lactis*-54" 2006. №2780