International Journal of Food Microbiology 215 (2015) 95–100

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Development of an experimental apparatus and protocol for determining antimicrobial activities of gaseous plant essential oils

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ARTICLE INFO

Article history:
Received 19 May 2015
Received in revised form 20 August 2015
Accepted 27 August 2015
Available online 2 September 2015

Keywords:
Antimicrobial
Gaseous essential oils
Escherichia coli O157:H7
Minimal inhibitory concentration
Minimal lethal concentration

ABSTRACT

There is a growing interest in the use of naturally-occurring antimicrobial agents such as plant essential oils (EOs) to inhibit the growth of hazardous and spoilage microorganisms in foods. Gaseous EOs (EO gases) have many potential applications in the food industry, including use as antimicrobial agents in food packaging materials and sanitizing agents for foods and food-contact surfaces, and in food processing environments. Despite the potentially beneficial applications of EO gases, there is no standard method to evaluate their antimicrobial activities. Thus, the present study was aimed at developing an experimental apparatus and protocol to determine the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of EO gases against microorganisms. A sealed experimental apparatus was constructed for simultaneous evaluation of antimicrobial activities of EO gases at different concentrations without creating concentration gradients. A differential medium was then evaluated in which a color change allowed for the determination of growth of glucose-fermenting microorganisms. Lastly, an experimental protocol for the assessment of MIC and MLC values of EO gases was developed, and these values were determined for 31 EO gases against *Escherichia coli* O157:H7 as a model bacterium. Results showed that cinnamon bark EO gas had the lowest MIC (0.0391 μl/ml), followed by thyme-thymol EO gas (0.0781 μl/ml), oregano EO gas (0.3125 μl/ml), peppermint EO gas (0.6250 μl/ml), and thyme-linalool EO gas (0.6250 μl/ml). The order of the MLC values of the EO gases against the *E. coli* O157:H7 was thyme-thymol (0.0781 μl/ml) < cinnamon bark (0.1563 μl/ml) < oregano (0.3125 μl/ml) < peppermint (0.6250 μl/ml) = thyme-linalool (0.6250 μl/ml). The experimental apparatus and protocol enable rapid and accurate determination of the MIC and MLC values of EO gases and perhaps other types of gaseous antimicrobial agents.

1. Introduction

There is an increasing demand among health-conscious consumers for nutritious foods and naturally occurring food ingredients, including natural antimicrobial compounds (Sofos et al., 1998). As a result, a number of attempts have been made to inhibit the growth of undesirable microorganisms in foods using naturally occurring plant essential oils (EOs) (Jun et al., 2013; Sofos et al., 1998; Tyagi et al., 2012). EOs are volatile and aromatic oily extracts primarily obtained from plant materials, mostly by steam distillation of flowers, buds, seeds, leaves, stems, twigs, barks, fruits, and roots, and many are known to have antimicrobial properties (Burt, 2004; Laird and Phillips, 2011; López et al., 2005).

EOs are available in liquid and gaseous forms. The advantages of using EOs in liquid phase are that their minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) can be easily evaluated and they can be directly used in foods as antimicrobial preservatives. One concern associated with evaluating liquid EOs for antimicrobial activity in laboratory culture media is that activities are comparatively lower in food matrices (Laird and Phillips, 2011). When added to foods, higher concentrations of EOs are required to have the same antimicrobial effect. Another disadvantage of using liquid EO is their influence of sensorial properties of foods. Strong odors and flavors of many EOs are likely to exceed acceptable thresholds in foods to which they are added (Hsieh et al., 2001; Nazer et al., 2005; Tyagi and Malik, 2010).

Compared to EOs in liquid phase, gaseous EOs (EO gases) are not directly added to foods but can be used as primary antimicrobial agents in food packaging materials and also as sanitizing agents for food surfaces, food-contact surfaces, and surfaces of storage rooms and transport containers. Another advantage of using EO gases is that they often cause minimal alteration in aroma and flavor of foods because there is minimal penetration into subsurface areas and they are released into the atmosphere after application (Goñi et al., 2009; Tyagi et al., 2012). Compared with EO in liquid phase, some EO gases have higher antimicrobial activity against foodborne pathogens and spoilage bacteria (Tyagi and Malik, 2010). However, there are some disadvantages in using EO gases to eliminate or control foodborne microorganisms. It is

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.08.021
0168-1605/

difficult to maintain EO gases at specific concentrations during application to food surfaces or food-contact surfaces because they easily diffuse into the atmosphere. This behavior makes it difficult to measure antimicrobial activities of EO gases against microorganisms. To date, there is no standard method available to determine the antimicrobial activities (MIC and MLC values) of gaseous antimicrobial agents (Avila-Sosa et al., 2012; Nedorostova et al., 2009).

Various methods have been used to measure antimicrobial activities of EO gases (Kloucek et al., 2012; Nedorostova et al., 2009). Most of these methods were designed to separately position liquid EO and target microorganisms on solid support materials without direct contact in a sealed container. In these experimental systems, EO gases are vaporized from liquid EOs and the concentration of gas is calculated based on two assumptions: i) the liquid EO is completely vaporized in the sealed container and, ii) the EO gas is evenly distributed in the head space of the container. A Petri dish or a jar (airtight box) has been used as sealed treatment containers, but different antimicrobial activities have been observed, depending on the type of container used and the treatment method employed. When a Petri dish was used, a solid medium containing the test microorganisms was placed above liquid EO and incubated at a specific temperature for 24 to 48 h. Researchers determined the MIC by measuring the concentration of EO gas which produced a zone of inhibition of growth on the solid medium or they simply compared antimicrobial activities of different types of EO gases by measuring the size of zones without determination of MIC. Examples of this experimental system include the disc volatilization method (López et al., 2005; Tyagi and Malik, 2010, 2012), modified disc volatilization method (Nedorostova et al., 2009), vapor contact assay (Tullio et al., 2007), vapor diffusion assay (Shannon et al., 2011), agar vapor assay (Inouye et al., 2006), and micro-atmosphere diffusion method (Mondello et al., 2009). When liquid EO and the medium containing the target microorganism are located close to each other and have different plane areas, a concentration gradient of EO gas will be generated, creating an inhibition zone on the medium. If a concentration gradient of EO gas is created on the surface of the medium on which the target microorganism has been placed, then it is not possible to accurately determine the gas concentration that will cause inhibition. In a recent effort to solve this problem, Kloucek et al. (2012) developed an experimental method in which the same concentration of EO gas comes in contact with the entire surface of a medium on which the test microorganism has been placed. Another disadvantage of using Petri dish as a sealed container is that only one concentration of EO gas can be evaluated in each experiment. To measure MIC values of EOs accurately and rapidly, measurement of antimicrobial activities of several concentrations of a serially diluted EO gas should be done simultaneously. In studies using a jar or airtight box as a sealed container, the source of EO gas and the medium containing target microorganisms are placed in parallel. In this experimental system, researchers considered the concentration of EO gas that inhibited every microorganism on the medium surface as MIC (Inouye et al., 2001; Ward et al., 1998). Compared to the experimental system using Petri dishes, a relatively accurate measurement of the MIC would be possible because the jar and airtight box may reduce the concentration gradient of EO gas. However, as with the Petri dish method, the antimicrobial activity of only one EO concentration can be measured in each jar or airtight box. Ideally, a method for measuring the antimicrobial activities of EO gases should not create a concentration gradient of gas within the sealed container and should allow for simultaneous evaluation of antimicrobial activities of a given gas at different concentrations.

The primary goal of the present study was to develop a method for the determination of MIC and MLC values of EO gases against foodborne pathogenic and spoilage microorganisms. First, we constructed an experimental apparatus for simultaneous measurement of antimicrobial activities of EO gases at various concentrations without creating a concentration gradient of the gas within the test chamber. We then prepared a differential medium for detecting antimicrobial activity, using glucose-fermenting microorganisms as a model. Finally, we developed an experimental protocol and measured the MIC and MLC values of 31 EO gases against enteropathogenic *Escherichia coli* O157:H7.

2. Materials and methods

2.1. Bacterial strains

Five strains of *E. coli* O157:H7 from laboratory stock cultures were used: ATCC 43895 (isolated from ground beef), E0018 (isolated from cattle feces), F4546 (isolated from a patient in an alfalfa sprout-associated outbreak), H1730 (isolated from a patient in a lettuce-associated outbreak), and 932 (isolated from a patient with hemorrhagic colitis). Cryopreserved cells of each strain were activated separately in 10 ml of nutrient broth (NB; BBL/Difco; Sparks, MD, USA). Cultures were incubated at 37 °C for 24 h and transferred into 10 ml of NB using an inoculation loop (ca. 10 μl) at 24-h intervals. After three consecutive transfers, 2 ml of each culture were combined to yield 10 ml of a five-strain *E. coli* O157:H7 cocktail. The cocktail was diluted 10-fold in NB to prepare an inoculum (ca. 7.0 log CFU/ml).

2.2. Essential oils

Thirty-one EOs in gaseous form were evaluated for antimicrobial activities against a five-strain *E. coli* O157:H7 cocktail: EOs of *Allium sativum* (garlic), *Anethum graveolens* (dill), *Melaleuca alternifolia* (tea tree), *Pinus sylvestris* (pine), *Rosmarinus officinalis* (rosemary), and *Syzygium aromaticum* (clove bud) were purchased from EuroAroma Co. Ltd. (Gewerbegebiet, Germany). EOs of *Cinnamomum zeylanicum* (cinnamon bark), *Cistus ladaniferus* (cistus), *Citrus aurantifolia* (lime), *Citrus medica limonum* (lemon), *Citrus paradisi* (grapefruit), *Cupressus sempervirens* (cypress), *Cymbopogon citratus* (lemongrass), *Cymbopogon nardus* (citronella), *Eucalyptus globulus* (eucalyptus globulus), *Foeniculum vulgare* (fennel sweet), *Hyssopus officinalis* (hyssop), *Lavandula angustifolia* (lavender), *Chamomilla recutita* (chamomile blue), *Mentha piperita* (peppermint), *Mentha spicata* (spearmint), *Ocimum basilicum* (basil sweet), *Origanum majorana* (marjoram sweet), *Origanum vulgare* (oregano), *Piper nigrum* (black pepper), *Salvia lavandulifolia* (Spanish sage), *Salvia sclarea* (clary sage), *Thymus mastichina* (thyme Spanish), *Thymus zygis* CT *thymol* (thyme thymol), *Thymus zygis* CT *linalool* (thyme linalool), and *Zingiber officinale* (ginger) were purchased from Neumond-Düfte der Natur GmbH (Raisting, Germany).

2.3. Construction of the experimental apparatus

An airtight experimental apparatus was designed and constructed for simultaneous evaluation of antimicrobial activities of EO gases at various concentrations without creating a concentration gradient. Fig. 1 shows a schematic design of the experimental apparatus: Fig. 1A shows a top view of the apparatus and Fig. 1B shows a side view of one of seven wells. The apparatus consists of an upper chamber (diameter 8.2 mm, height 9.3 mm) with seven wells, each containing a solid medium on which the target microorganism has been uniformly distributed, and a lower chamber (diameter 8.2 mm, height 19.0 mm) with seven wells containing gaseous EO generated from liquid EO. The internal volumes of the empty upper and lower wells are approximately 0.49 and 1.0 ml, respectively. Since wells in the upper chamber contain 0.49 ml of surface-inoculated solid medium, the combined volume of headspace in the two wells where the EO gas is infused is 1.0 ml. To prevent leakage of EO gas, O-rings are positioned at the juncture of the upper and lower well rims and around the entire set of wells. The apparatus is constructed of autoclavable polycarbonate and the four corners and center are tightly sealed with nuts and bolts.