Anti-biofilm and Antiradical Activity of Different Essential Oils

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Abstract
The formation of biofilms is determined by the type of microrganisms, availability of nutrients and the characteristics of substrate. In the present study, we aimed to use matrix-assisted laser desorption ionization time-of-flight mass spectrometry profiling as a methodology to monitor *Stenotrophomonas maltophilia* biofilm development and evaluate the antibacterial, anti-biofilm and antioxidant activity of *Mentha piperita* and *Pimpinella anisum* essential oils against *S. maltophilia*. Biofilms were grown within polypropylene tubes containing a glass slide and wooden toothpick, and were cultivated 3, 5, 7, 9, 12 or 14 days of inoculation and with essential oils experiment also. Planktonic cells were obtained separately by centrifugation as control. MALDI-TOF experiment were performed, one by collecting biofilms from both the glass slide and the wooden toothpick external surface, and the other by acquiring biofilms from these surfaces after essential oils influence. The molecular results showed that MALDI profiling is able to distinguish between different biofilm stages and also to indicate when the biofilm cells are released at the dispersion stage, which occurred first on glass slide and wooden toothpick. The present study found out that MALDI profiling may emerge as a promising tool for the biofilms formation and control.

Keywords: antioxidant activity, anti-biofilm activity, mass spectrometry, *S maltophilia*, essential oils

1. Introduction

The essential oils are mixtures of complex volatile substances that can be found in many vegetal species. They are considered very important natural products from an economic point of view, mainly due to their medicinal properties, fragrance, and flavor; they have important role in pharmaceutical, cosmetic and food industries. This special raw material is usually extracted by hydrodistillation or steam distillation methods, but essential oil also can be obtained directly by expression and centrifugation of citric fruits. Chemically, essential oils are mainly constituted of monoterpenes and sesquiterpenes, but they also can be constituted by aromatic substances, such as phenylpropanoids, and aliphatic substances [1].
The genus *Mentha* is represented by six species in India of which four species, *Mentha arvensis* L., *Mentha longifolia* (L.) Huds., *Mentha piperita* L., and *Mentha spicata* L. are common in Uttarakhand [2]. Among these *M. longifolia* (Horse mint) is exclusively temperate wild specie with considerable variation in morphology and three varieties of it *M. longifolia* (L.) Huds. var *longifolia*, *M. longifolia* var. *incana* (Willd.) Dinsm., *M. longifolia* var. *royleana* (Benth.) Hook.f. are known between 1200 and 3200 m altitude range in Uttarakhand. *Mentha arvensis* (corn mint), *M. piperita* (Peppermint), and *M. spicata* (Spearmint) are commonly cultivated up to 2500 m altitude as pot herbs, or sometimes

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occur in a semiwild state. All *Mentha* species have been known to the natives for a long time and are called *Podina* in Uttarakhand. These species are commonly used to provide flavoring and coolant, in sauces, and are medicinally used in indigestion, vomiting, malarial fever, or as culinary herbs [3].

One of the most important species that produces essential oil is *Pimpinella anisum*, commonly known as anise. This is one of the oldest species used by people; it has been cultivated since ancient times, initially in Egypt and later in Greece, Rome, and the Middle East. Anise essential oil is usually extracted from dried ripe fruits by hydrodistillation, but it also can be obtained by supercritical fluid extraction. It is mainly constituted by anethole, an aromatic substance that appears as the major compound of the oil, usually corresponding to more than 80% (w/w) of the oil [4-8]. Other substances, such as methyl chavicol, himachalene, linalool, and α-terpineol, are also found as important constituents of this oil [9].

Plant essential oils have been used for hundreds of years a natural medicine to combat a variety of bacterial infection and other ailments. Several essential oils have been documented for their antibacterial, antifungal, antimutagenic and anti-quorum sensing activities [10-15]. However little efforts have been made to assess their anti-biofilm activity and therapeutic potential against bacterial infection [11-16]. Antimicrobial action of essential oils has been attributed due to the damage to cell wall and cell membrane leading to cell lysis leakage of cell contents and inhibition of proton motive force. Many essential oils have relatively low mammalian toxicity and degrade quickly making them safe and eco-friendly.

The biofilms have important role in protection of microorganism against external aggression and predator attacks [17]. Gupta et al. [18] mention that some populations of biofilm-associated bacteria are resistant to antibiotics that is problematic for the use of antimicrobial drugs [19]. New research is needed for alternative solutions in treatment of infections cause by resistant microorganisms [20]. The medicinal plants could be the solution to identify new alternative drugs [21].

Thus, the present study aims to evaluate the anti-biofilm and antioxidant activity of *Mentha piperita* L. and *Pimpinella anisum* L. essential oils against biofilm producing strain *Stenotrophomonas maltophilia*.

2. Materials and methods

2.1. Microorganisms

Stenotrophomonas maltophilia with biofilm formation from milk company was acquired.

2.2. Essential oils

Mentha piperita and *Pimpinella anisum* essential oils from Slovak company Hanus were evaluated. *Mentha piperita* L. essential oil contained limonene (1-3.5%), cineol (3.5-8%), menthon (14-32%), menthofuran (1-8%), isomenthon (1.5-10%), menthylacetate (2.8-10%), isopulegol (max. 0.2%), menthol (30-55%), pulegol (max. 3%), carvone (max. 1%), cineol (min. 2%) and *Pimpinella anisum* L. obtained anethol (80%).

2.3. Free radical scavenging activity

Free radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The sample (0.4 mL) was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL methanol). Absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-100 mg/L; R^2=0.989) was used as the standard, and the results were expressed in μg/mL Trolox equivalents [22].

2.4 Minimum biofilm inhibitory concentration (MBIC) assay

Minimum biofilm inhibitory concentration (MBIC) is described as the lowest concentration at which the antimicrobial agents prevents the biofilm formation [23]. In order to test the anti-biofilm activity of EOs against *S. maltophilia*, the microtiter plate assay was used. The MBIC test was carried out using the method of Adukwu et al. [24]. An aliquot (100 μL) from an overnight culture (diluted with Muller Hinton broth MHB to 10^8 CFU/mL) was dispensed into each test well of a 96-well plate. Then 100 μL of different concentrations of EOs (0.3125-10 μL) were dispensed into different wells. The negative control contained only MHB whereas the positive control contained cell cultures alone without EOs.

The supernatant of the wells was decanted and each well gently rinsed three times with 300 µL of sterile distilled water and discarded after incubation at 37 °C for 24 h. The plates were dried in air for 30 min and stained with 0.1 % (w/v) crystal violet at room temperature for 30 min, washed 3 times with distilled water and dried. Thereafter the crystal violet was solubilized in 96 % ethanol and absorbance was read in a microplate reader Biotek EL808 with shaker (Biotek Instruments, USA) at 570 nm. MBIC was determined as the EOs concentration at which the absorbance is equal to or less than that of the negative control. This test was performed in triplicate and the mean (n=3) was taken.

2.5. Biofilm development stages by MALDI-TOF MS

For the analyses by MALDI-TOF MS Biotyper two experiments were performed. The first analyze was evaluated to assess whether or not this method would be able to discriminate the stages of biofilm development as a function of the growth time. The second analyze aimed to use MALDI-TOF MS Biotyper to evaluate if biofilms grown in different substrates and essential oils would exhibit any detectable phenotypical distinction. Growing planktonic cells and biofilms: Five polypropylene plastic tubes of 50 ml capacity received 20 ml each of Mueller Hinton (MH) culture medium. A rectangular microscope glass slide and wooden toothpick were placed vertically inside the tube and experimental group with 0.1% EOs. A pre-inoculum of *S. maltophilia* was incubated in MH culture medium at 37 °C during 24 h. Ten ml of the pre-inoculum and EOs were added to the 50 ml polypropylene tubes, which were placed in a shaker with an inclination of 45 °C with a shaking speed of 170 rpm at 37 °C. The biofilms formed in each of the tubes were collected 3, 5, 7, 9, and 12 days after the inoculation. The culture medium of the remaining tubes was replaced in the same intervals. For the analysis of the biofilm, the slide and toothpick were washed two times with ultrapure water and bacteria from the glass slide and wooden toothpick were collected with a sterile toothpick. Planktonic cells were collected by centrifuging 300 ml of the medium of the 5-day tube at 3000 g for 3 min. The supernatant was removed and the pellet was resuspended in ultrapure water and centrifuged again. This procedure was repeated and the resulting pellet was resuspended in 25 ml of ultrapure water (one ml of this suspension was used for each well of the MALDI target plate). MALDI-TOF MS Biotyper: The intact materials (biofilm and planktonic cells) were then spread in 24 wells for each sample in a polished 96-well MALDI target plate (Bruker Daltonics, Germany). One µL of a-cyano-4-hydroxycinnamic acid saturated matrix solution (10 mg/ml) was used to cover every sample and dried at room temperature prior to MALDI-TOF MS Biotyper analysis. After crystallization, the samples were analyzed onto a commercial MALDI-TOF mass spectrometer MicroFlex (Bruker Daltonics, Germany) in the linear and positive mode for a range of m/z 2000-20000. The spectra were acquired automatically using a standard procedure. MALDI Biotyper approach: The similarities among the acquired spectra of the same sample were used for generating a standardized global spectrum (MSP), so all stages of biofilm development were represented by 40 spectra, using the software MALDI Biotyper 3.0 (Bruker Daltonics). From the MSPs of samples, it was generated a dendrogram by the MALDI Biotyper method following standard procedures [25].

2.6 Use of MALDI-TOF MS to evaluate bacterial biofilm growth on glass and on toothpick surfaces

Eighteen propylene tubes of 50 ml were prepared in the same way as described in the previous section. Bacteria materials of three tubes were gathered in each interval of 3, 5, 7, 9, 12, and 14 days. The culture media of the remaining tubes was replaced in the same intervals. However, in this experiment, the biological materials grown on glass and toothpick were collected separately with a sterile toothpick, and spread over 12 wells of the MALDI-TOF target plate. Furthermore, from the tubes of the 14th day, only planktonic cells were collected in the same way as described in the previous section, and one ml of the material was spread over each of the 36 wells. All MALDI target plate wells were always covered with one ml of a-cyano-4-hydroxycinnamic acid and analyzed within 24 h. In summary, 36 spectra were acquired in every group, and one was chosen from these as the most representative based on its common features and displayed for comparison among the experimental groups using

FlexAnalysis 3.0 software (Bruker Daltonics). With the MALDI Biotyper 3.0 software, 11 MSPs were created, and subsequently clustered by a dendrogram using Euclidean distances [25].

3. Results and discussion

3.1 Antioxidant activity of EOs

The reduction ability of DPPH radicals' formation was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The highest antioxidant activity was observed at *Mentha piperita* (59.56±2.75µg TEAC/ml) and lower at *Pimpinella anisum* (28.45±3.44 µg TEAC/ml). The same results with same EOs was described in study Kačániová et al. [26].

3.2 Anti-biofilm activity with MBIC

The MIC values of EO *Mentha piperita* was 2.5 µl/mL and of EO *Pimpinella anisum* was 2.5 µl/mL. The same results of *M. piperita* was found on study of Tutar et al. [27] against *A. baumani*.
In the study at Bazargani and Rohloff [28] was found anti-biofilm activity against *E. coli* and *S. aureus* at 17.4%.

3.3 Anti-biofilm activity with MALDI-TOF MS

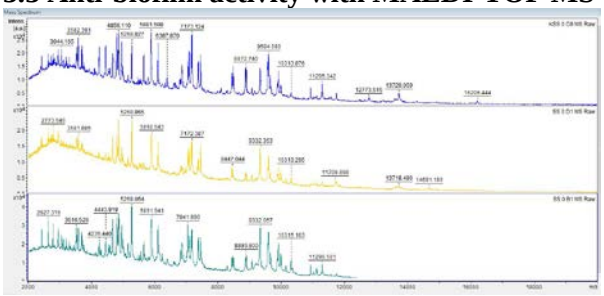

Figure 1 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 3 days

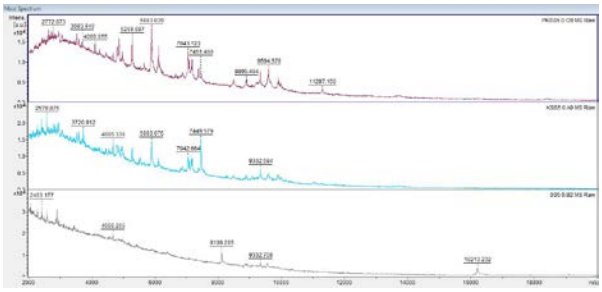

Figure 2 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 5 days

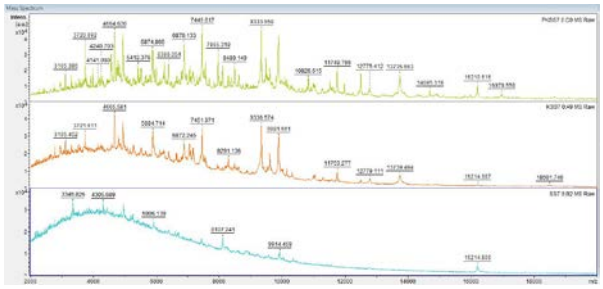

Figure 3 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 7 days

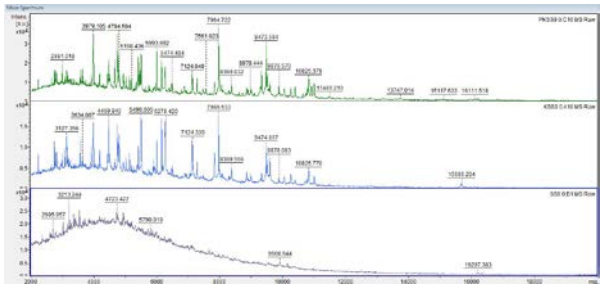

Figure 4 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 9 days

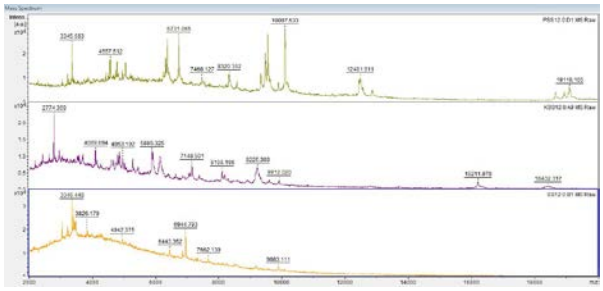

Figure 5 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 12 days

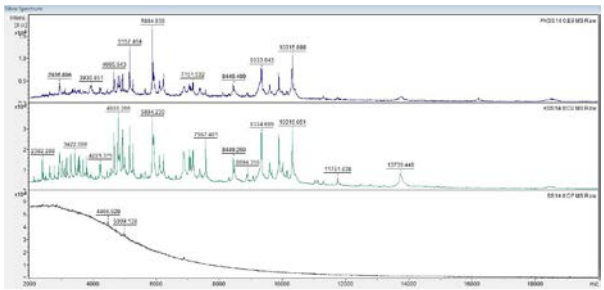

Figure 6 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 14 days

The spectra chosen as representative for each biofilm development stage in this experiment are shown in Fig. 1-6. With the exception of the spectrum representing planktonic cells, which

were obtained from the culture medium, the remaining spectra were grouped in pairs according to their time of growth, so that biofilms obtained from different surfaces could be viewed together.

3.4 Biofilm development stages by MALDI-TOF MS

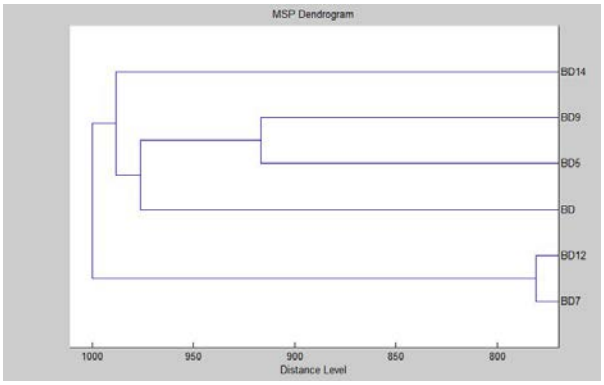

Figure 8. Dendrograms generated using the MSPs of *S. maltophilia* biofilms at different developmental stages collected from the glass surface

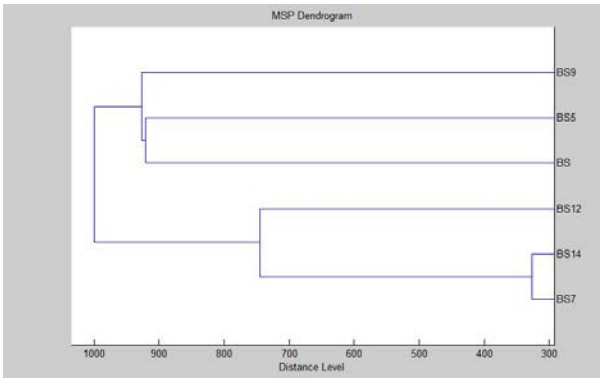

Figure 8. Dendrograms generated using the MSPs of *S. maltophila* biofilms at different developmental stages collected from the wooden toothpick

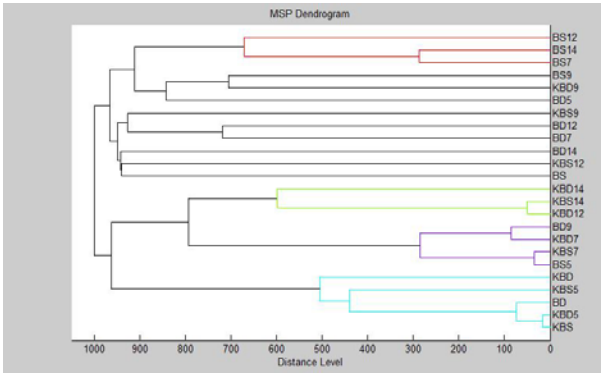

Figure 9. Dendrograms generated using the MSPs of *S. maltophila* biofilms at different developmental stages collected from both the glass surface and wooden toothpick

Fig. 7-9 presents the dendrogram generated by the first MALDI-TOF MS Biotyper experiment. These results indicated that the MSPs of the groups were distinguishable by the use of MALDI profiling approach, since they have been separated into different clusters. It can be observed that the planktonic stage of *S. maltophilia* showed the lowest difference, in terms of MSP distance level, from the 7- and 12-day biofilms on wooden toothpick and 7- and 14-day biofilms on glass surface.

Pereira et al. [25] studied bacterial biofilm of clinical relevance is formed by the gram-negative bacteria *Pseudomonas aeruginosa*. They observed that during the formation of a biofilm, the initial adhesion of the cells is of crucial importance, and the characteristics of the contact surface have great influence on this step. Pereira et al. used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling as a new methodology to monitor *P. aeruginosa* biofilm development. Biofilms were grown within polypropylene tubes containing a glass slide, and were harvested after 3, 5, 7, 9, or 12 days of inoculation. Two independent MALDI-TOF experiments were performed, one by collecting biofilms from both the glass slide and the polypropylene tube internal surface, and the other by acquiring biofilms from these surfaces separately. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to evaluate the morphological progression of the biofilm. The molecular results showed that MALDI profiling is able not only to distinguish between different biofilm stages, but it is also appropriate to indicate when the biofilm cells are released at the dispersion stage, which occurred first on polypropylene surface. Finally, the present study pointed out that MALDI profiling may emerge as a promising tool for the clinical diagnostic and prognostic workup of biofilms formation and control.

4. Conclusions

MALDI-TOF MS profiling is a very useful tool for microorganisms identification. In the present study we decided to apply a MALDI-TOF profiling method to evaluate different stages of *S. maltophilia* biofilm development. The results showed that the method can detects changes in the

biofilm progression, and also it can detect some distinct characteristics related to the surface where the bacteria were grown.

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