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Fatty Acid Methyl Ester Analysis of Microorganisms in Soil Amended with Biochar

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Abstract:

Objectives: To identify the bacteria, fungi and actinomycetes isolated from soil samples augmented with biochar by fatty acid methyl ester method. **Methods and Statistical analysis:** The fatty acid profiles of microbial populations were identified by FAME analysis. Analysis of variance (ANOVA) and principal component analysis of microbial populations were carried out using SYSTAT 9. **Findings:** Three bacteria, two fungi, three actinomycetes isolated from soil samples augmented with biochar, were identified by FAME analysis based on the presence of particular fatty acid biomarkers. The current study showed that the major fatty acid was 15:0 iso followed by 15:0 anteiso for the bacterial isolates *Stenotrophomonas maltophila* and *Bacillus megaterium*. The major fatty acids present in *Bacillus cereus* was 15: iso followed by 17:0 iso. The results showed that the major fatty acids in fungi (*Cladosporium carrionii*, *Phoma* spp) were18:2 CIS 9,12/18:0a followed by summed feature 8 and 16:0. The major fatty acids in actinomycetes was 15:0 anteiso followed by 16:0 iso for *Streptomyces-rochei-rochei* and 17:0 anteiso for *Streptomyces-halstedii-scabies.* PCA (principal component analysis) resulted in clear separation of microbial communities. So, it can be used for the comparison of microbial communities based on fatty acid profile. **Novelty and applications:** Previous studies scarce data of the microbial populations in the rhizosphere soil of rubber in Kerala. The current study helps in the evaluation of major microbial population in the rhizosphere soil of rubber in Kerala by FAME analysis.

Keywords: *Bacillus spp, Cladosporium carrionii*, FAME analysis, rubber, *Streptomyces spp*.

INTRODUCTION

Soil is the key component for furnishing water, nutritional components and mechanical assistance for the plants. The continuous changes in the agricultural fields affect the physiochemical and biological properties of the ecosystem and this affect the soil health and crop productivity [(1)]. Biochar is a charcoal like substance that can be incorporated into soil for the enhancement of carbon, NPK and water holding capacity [(2)]. Its high nutrient efficiency, porous structure and CEC helps to improve the microbial activities [(3,4-6)]. They enhance the growth of microorganisms and nutrient cycling [(5)]. Thus, the biochar application in soil act as a soil

conditioner and enhance the microbial growth and diversity. Microbial diversity indicates the microbial abundance in a particular habitat. The composition of different bacterial community is affected by soil treatment with biochar and the number of harvesting years [7,8].

The vital parts of each and every living cell consists of membrane lipids and their fatty acids. These chemical components present in the microorganisms (microbial biomarkers) can be evaluated in a given sample and can be explained by quantitative and qualitative methods. They have great configurational variation and high biological accuracy. So, they are used as the most powerful biomarkers for the identification of microorganism [9].

Fatty acids of bacteria are the crucial component of the lipopolysaccharides and the lipid bilayer of the cell membranes. As the bacterial fatty acids takes part in the cell structure and functions, they are extremely preserved and can be used for identification of microorganisms. Therefore, the total cellular FAME composition gives an impression of cellular genome which act as a bacterial sketch or outline. The phenotypic character of a microorganism is shown by cellular fatty acids. The use of fatty acid methyl ester analysis by GC is used for the identification of both environmental and medical microbial isolates ([10,11-14).

The particular type of fatty acids in the specimens are compared and are classified based on the presence or quantity of fatty acids. FAME assay technique is an important mechanism in the study of microbial phylogeny due to the precision of these fatty acids. In this technique cellular lipids are saponified into their methyl esters and are identified by GC[11].

The similarity index (SI) is a numerical value that helps in the comparison of the fatty acid composition of an unknown sample with that of the strains present in the library. A sim index of 0.6 is an indication of good match [15]. So, to compare the profile of an unknown microorganism, FAME library should contain a known bacterial profile [16]. The present work aims in the fatty acid methyl ester identification of bacteria, fungi and actinomycetes obtained from biochar augmented soil and their PCA analysis.

2 MATERIALS & METHODS

2.1 Study site

Soil samples were collected from the rubber plantation at Nellimattom, Ernakulam (Dist.), Kerala, India (9^0 53’ 56.79” N and 76^0 22’ 5.05” E) which are amended with biochar [Figure 1].

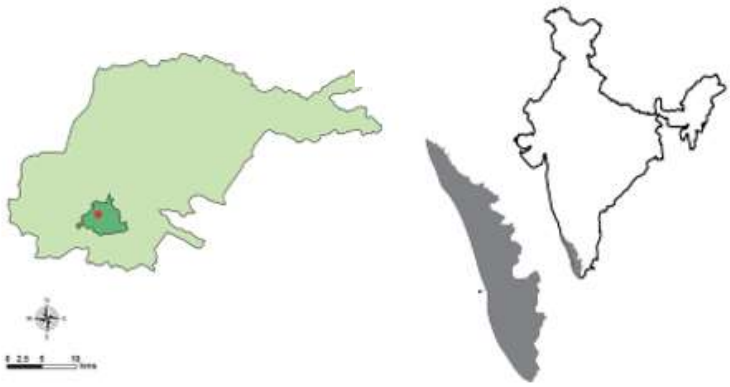

Fig 1. Location map showing the study site

2.2 Sampling

Samples were collected from different biochar augmented soil. The bacteria, fungi and actinomycetes were isolated by spread plate technique using nutrient agar, potato dextrose agar and actinomycete isolation agar. The petri plates were incubated at 37°C overnight for bacteria, at room temperature for 3-5 days for fungi and actinomycetes [17, 18]. The isolates showing maximum frequency were taken for FAME analysis.

2.2.1 Fatty acid methyl ester analysis

Sherlock microbial identification system by gas chromatographic technique was used for the identification of bacteria, fungi and actinomycetes. About 40 mg of bacterial cells were taken and added 1.0 ml of saponification to each cell containing tubes and vortexed. It is heated in a boiling water bath for 5 minutes, during that time the tubes are strongly vortexed for 5-10 seconds and again put back to the water bath for 30 minutes heating.

The tubes were cooled and 2 ml of methylation reagent was added (325 ml 6.0N HCl and 275 ml methyl alcohol). The tubes were vortexed and heated at 80° ± 1°C for 10 ± 1 minutes. 1.25 ml of extraction reagent (200 ml hexane and 200 ml methyl tert-butyl ether) was added to the cooled tubes and gently tumbled for 10 minutes on a rotator. The fatty acid methyl esters at this stage are extracted into the organic phase. About 3ml of clean up reagent (10.8g NaOH dissolved in 900ml distilled water) is added to the organic phase, and tumbled for 5 minutes. The organic phase was used for the GC analysis.

2.3 Statistical Analysis

Results were presented as mean ± SD. The means were separated using Tukey's Honestly Significant Difference (HSD) test with a significant level P <0.05, n=3 using SYSTAT 9.

RESULTS AND DISCUSSION

3.1 Fatty acid composition of the microbial isolates

Fungi have greater fatty acid composition followed by actinomycetes and bacteria. Percentage of fatty acid composition in the microbial isolates were given in Table1.

Table 1. Percentage of fatty acid composition in the microbial isolates

Microorganisms		Percentage of PLFA composition
Bacteria	*Stenotrophomonas maltophila*	1.24±4.67
	B.cereus	1.23±2.8
	B.megaterium	1.23±5.53
Fungi	*Cladosporium carrionii*	5±12.03
	Phoma spp	5.17±13.52
Actinomycetes	*S. rochei. rochei*	2.78±8.76
	Streptomyces. halstedii. scabies	2.78±7.5
	S.rochei. rochei	2.78±5.96

Results are given as mean±SD

3.2 Fatty acid composition of the bacterial isolates

The bacterial spp. obtained after biochar amendment were *Stenotophomonas maltophila*, *Bacillus cereus* and *Bacillus megaterium*. The bacterial FAME analysis showed eighty- one different fatty acids with 8 to 20 carbon atoms. Out of the bacterial isolates analysed, two belong to *Bacillus* family and one belong to *Stenotrophomonas*.

Biomarkers such as 14:00, 14:0 iso, 15:0 iso/anteiso, 16:00, 16:0 iso, 17:0 iso/ anteiso, 18:00, 18:1 w9c were present in all the bacterial isolates. The common and major fatty acids present only in genus *Bacillus* were 13:0 iso, 13:0 anteiso, 15:0 iso/anteiso cellular fatty acids. Among this, fatty acids13:0 iso/anteiso were more in *Bacillus cereus* and 15:0 iso/anteiso were more in *Bacillus megaterium.*

Sharmili and Ramasamy reported that iso and anteiso branched saturated cellular fatty acids were present mainly in *Bacillus* spp [16, 19]. Gram-positive bacteria contain fatty acids like 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 iso/anteiso as their major cellular fatty acids [20]. *Stenotophomonas maltophila* showed cellular fatty acids 15:0 iso/ anteiso (39.94% and 12.46%) as major biomarkers.

Fatty acid methyl ester profile of *Stenotophomonas maltophila* showed a sim index of 0.480. In a study conducted by Shaikh *et al.*, the *Stenotophomonas maltophila* showed a similarity index of 0.973 [15]. The gas chromatography profile is shown Figure 2.

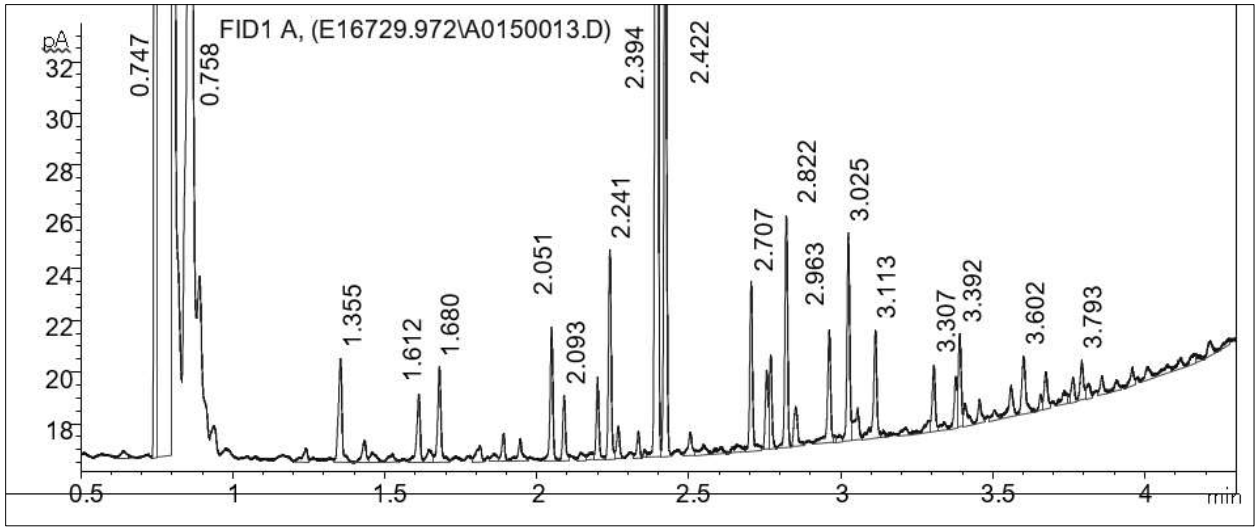

Fig 2. Gas chromatograph profile of cellular fatty acids of *Stenotrophomonas-maltophilia*

Fatty acid methyl ester profile of strain *Bacillus -cereus*-GC subgroup B showed a sim index of 0.114. Cellular fatty acid methyl esters in this strain revealed the presence of 15:0 iso (14.23%), 17:0 iso (13.63%), 16:00 (9.48%), 15:0 anteiso (7.76%), 17:0 anteiso (6.67%), 16:0 iso (6.58%) and 13:0 anteiso (6.32%) as major fatty acids. Fatty acid methyl ester profile of strain *Bacillus megaterium*- GC subgroup A showed a sim index of 0.249. Cellular fatty acid methyl esters in this strain revealed the presence of 15:0 iso (45.4%) and 15:0 anteiso (19.45%) as major fatty acids.

The fungi obtained after biochar amendment were *Phoma* spp. *Cladosporium carionnii*. The results of FAME analysis showed thirty-five biomarkers with 8 to 22 carbon atoms were detected. Cellular fatty acids, 18:2 CIS 9,12/18:0a and 18:00 were present in all the isolates. Eukaryotic cells constitute monounsaturated or saturated fatty acids having C10 to C18 carbon chain [21, 22]. Cellular fatty acids such as 18:2 CIS 9,12/18:0a, 16:00, were present commonly in all the fungal isolates. Of these 18:2 CIS 9, and 12/18:0a and were the major fatty acids present. In our present study, *Phoma* spp. contains only five cellular fatty acids like16:0, 16:1 Cis 9 (w7), 18:0, 18:2 CIS 9,12/18:0a and showed a sim index of 0.928. *Cladosporium carionnii* showed the presence of eleven fatty acids. Of this major percentage fatty acids were 18:2 CIS 9,12/18:0a, 16:0, 18:0. It showed a sim index of 0.839.

3.4 Fatty acid composition of the actinomycete isolates

The results of FAME analysis showed thirty-six biomarkers with 10 to 20 carbon atoms. The actinomycete isolates belongs to *Streptomycetes* family. Hence, we can assume *streptomycetes* as

the major group of cultivable actinomycetes in our soil samples. The common fatty acids for the identified streptomyces isolates were saturated iso/anteiso- fatty acids like 15:0 anteiso, 16:0 iso, 15:0 iso, 14:0 iso, 17:0 anteiso. Vítězová isolated 115 actinomycetes strains from soil sample [23]. According to Vítězová, due to specific characteristic features of actinomycetal fatty acids, FAME method can be used only for differentiating Streptomyces genus from other members of actinomycetes.

In a study done by Korn-Wendisch and Kutzner, the chromatogram patterns of *streptomyces* have C14 to C17 long chain (branched saturated iso-/anteiso- fatty acids). In our study also the common and major fatty acids are iso-/anteiso with C14 to C17 long chain [24]. The main actinomycetes isolated from soil were of genus *Streptomyces* [25, 26].

Fatty acid methyl ester profile of *S. rochei rochei* revealed the presence of 15:0 anteiso (49.58%), 16:0 iso (14.77%), 14:0 iso (9.63%) and 15:00 (9.13%) as major fatty acids. It has a sim index of 0.018. It is identified using the library, ACTIN13.80. Fatty acid methyl ester profile of strain *Streptomyces-halstedii-scabies* revealed the presence of 18:2 CIS 9,12/18:0a (28.94%), 15:0 anteiso (16.12%), Summed Feature 8 (11.19%), 17:0 anteiso (10.26%) and 16:00 (8.54%) as major fatty acids. It has a sim index of 0.017. It is identified using the library, ACTIN1 3.80. Fatty acid methyl ester profile of strain A14, *Streptomyces-rochei-rochei* revealed the presence of 15:0 anteiso (30.85%), 16:0 iso (15.39%), 15:0 iso (9.89%), 17:0 anteiso (9.72%) and 14:0 iso (6.35%) as major fatty acids. It has a sim index of 0.018. It is identified using the library, ACTIN1 3.80.

3.5 Principal component analysis

PCA analysis illustrates the variation in the PLFA distribution among the bacteria, fungi and actinomycetes identified by FAME method. PC1 and PC2 account for 59.59% and 27.11% variation for bacteria, 92.92% and 5.88% variation for fungi and 71.62% and 24.62% variation for actinomycetes. Microorganisms having a sim index of less than 0.3 was not able to identify by FAME analysis.

Stenotrophomonas maltophila, *Bacilus cereus* and *Bacillus megaterium* form clustur and had more positve scores for PC1. Unidentified bacteria B15 and B18 form cluster and had more positve scores for PC2. *Cladosporium-carrionii* and *Phoma* spp. form cluster and had more positve scores for PC1. Unidentifies F1 aso had more positive scores for PC1. Unidentified F2 and F10 form cluster and had more positve scores for PC2. Two *Streptomyces-rochei-rochei* and *Streptomyces-halstedii-scabies* form cluster and had more positve scores for PC1. A1 had more positive scores for PC2 (figure 3).

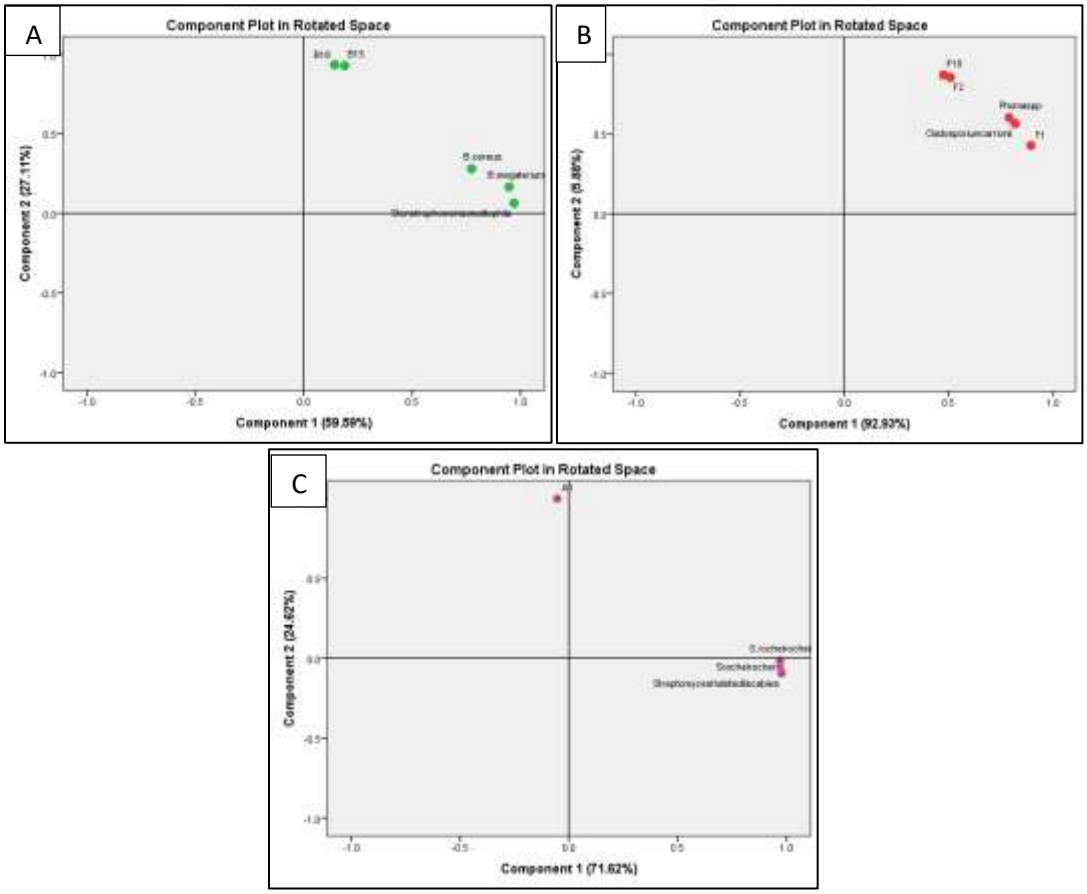

Fig 3. PCA analysis of solvent peaks in A) bacteria, B) fungi and C) actinomycetes identified by FAME

CONCLUSION

The FAME analysis of bacteria revealed, eighty- one different fatty acids with 8 to 20 carbon atoms. 15:0 iso, 15:0 anteiso, 16:0, 16:0 iso, 17:0 ,17:0 anteiso were the major fatty acids in bacteria. After the of biochar augmentation, the major bacteria present in the samples were *Stenotrophomonas maltophila***,** *B. cereus* and *B. megaterium*. Major cellular fatty acids in fungal isolates were 18:2 CIS 9,12/18:0a and18:00. the major fungi identified were *Cladosporium-carrionii* and *Phoma* spp. 15:0 iso, 15:0 anteiso, 16:0, 16: iso, 17:0 anteiso were the major fatty acids in actinomycetes. After biochar amendment the major actinomycetes were of the genus Streptomyces (*Streptomyces-rochei-rochei* and *Streptomyces-halstedii-scabies*). PCA analysis helped to compare the similarities and variations in different taxonomic groups of microorganisms.

CONFLICT OF INTEREST

The authors declare no conflict of interest

Reference:

- Shahane AA, Shivay YS. Soil Health and Its Improvement Through Novel Agronomic and Innovative Approaches. Frontiers in Agronomy. 202; 3: 680456. https://doi.org/10.3389/fagro.2021.680456
- Agegnehu G, Bass AM, Nelson PN, Bird MI. Benefits of biochar, compost and biochar–compost for soil quality, maize yield and greenhouse gas emissions in a tropical agricultural soil. Science of the Total Environment. 2016; 543:295-306. https://doi.org/10.1016/j.scitotenv.2015.11.054
- Fryda L, Visser R. Biochar for soil improvement: Evaluation of biochar from gasification and slow pyrolysis. Agriculture. 2015; 5(4):1076-115. https://doi.org/10.3390/agriculture5041076.
- Manyà JJ. Pyrolysis for biochar purposes: a review to establish current knowledge gaps and research needs. Environmental science & technology. 2012; 46(15):7939-54. https://doi.org/10.1021/es301029g
- Qian K, Kumar A, Zhang H, Bellmer D, Huhnke R. Recent advances in utilization of biochar. Renewable and Sustainable Energy Reviews. 2015; 42:1055-64. https://doi.org/10.1016/j.rser.2014.10.074
- Schimmelpfennig S, Glaser B. One step forward toward characterization: some important material properties to distinguish biochars. Journal of environmental quality. 2012; 41(4):1001-13. https://doi.org/10.2134/jeq2011.0146.
- Khodadad CL, Zimmerman AR, Green SJ, Uthandi S, Foster JS. Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments. Soil Biology and Biochemistry. 2011; 43(2):385-92. https://doi.org/10.1016/j.soilbio.2010.11.005
- Su P, Lou J, Brookes PC, Luo Y, He Y, Xu J. Taxon-specific responses of soil microbial communities to different soil priming effects induced by addition of plant residues and their biochars. Journal of Soils and Sediments. 2017 Mar;17(3):674-84. https://doi.org/10.1007/s11368-015-1238-8
- Salomonová S, Lamacova J, Rulík M, Rolcik J, Cap L, Bednar P, Barták P. Determination of phospholipid fatty acids in sediments. Acta Univ. Palacki. Olomuc. Facultas Rerum Nat. Chem. 2003; 42:39-49.
- Neyts K, Huys G, Uyttendaele M, Swings J, Debevere J. Incidence and identification of mesophilic Aeromonas spp. from retail foods. Letters in Applied Microbiology. 2000; 31(5):359-63. https://doi.org/10.1046/j.1472-765x.2000.00828.x
- Hinton Jr A, Cason JA, Ingram KD. Tracking spoilage bacteria in commercial poultry processing and refrigerated storage of poultry carcasses. International Journal of Food Microbiology. 2004; 91(2):155-65. https://doi.org/10.1016/S0168-1605(03)00377-5.

- Kunitsky C, Osterhout G, Sasser M. Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock Microbial Identification System. Encyclopedia of rapid microbiological methods. 2006; 3:1-8.
- Hoffmann M, Fischer M, Whittaker P. Evaluating the use of fatty acid profiles to identify deep-sea Vibrio isolates. Food chemistry. 2010;122(4):943-50. https://doi.org/10.1016/j.foodchem.2010.04.015.
- Himelbloom BH, Oliveira AC, Shetty TS. Rapid methods for the identification of seafood micro-organisms. Handbook of seafood quality, safety and health applications. 2010:226-36.
- Shaikh SS, Wani SJ, Sayyed RZ, Thakur R, Gulati A. Production, purification and kinetics of chitinase of Stenotrophomonas maltophilia isolated from rhizospheric soil.
- Sharmili AS, Ramasamy P. Fatty Acid Methyl Ester (FAME) Analysis of Moderately Thermophilic Bacteria Isolated from the Coramandal Coast. Chennai, Tamilnadu. European Journal of Experimental Biology. 2016; 6(6:5).
- Mary C, Vaj EA, Subash A, Paul N M. Comparative study on the extracellular amylase activity of amylolytic bacteria isolated from different soil types and earthworms gut. International journal of botany studies. 2021; 6(2): 115-121.
- Prabhakaran H, Paul NM, John E. Screening of economically important microorganisms from Thrissur kol paddy wetland. In AIP Conference Proceedings 2020 (Vol. 2263, No. 1, p. 030009). AIP Publishing LLC.
- Kaneda T. Fatty acids of the genus Bacillus: an example of branched-chain preference. Bacteriological reviews. 1977 Jun;41(2):391-418.
- White DC, Stair JO, Ringelberg DB. Quantitative comparisons ofin situ microbial biodiversity by signature biomarker analysis. Journal of Industrial Microbiology. 1996;17(3):185-96.
- Sasser M. Identification of bacteria by gas chromatography of cellular fatty acids. 1990.
- Giacomini M, Ruggiero C, Calegari L, Bertone S. Artificial neural network based identification of environmental bacteria by gas-chromatographic and electrophoretic data. Journal of Microbiological Methods. 2000; 43(1):45-54. https://doi.org/10.1016/S0167-7012(00)00203-7
- Vítězová M. Characterisation of actinomycetes community from the heavy metals-polluted soil. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis. 2013; 61(5):1471-8. http://dx.doi.org/10.11118/actaun201361051471
- Korn-Wendisch F, Kutzner HJ. The family Streptomycetaceae. The Prokaryotes.1992(Ed. 2):921-95.
- ŞAHİN N, IŞIK K, KARİPTAŞ E, GOODFELLOW M. The evaluation of alkalitolerant-mesophilic streptomyces by gas chromatography and pyrolysis mass spectrometry. Turkish Journal of Biology. 2001;25(4):463-75.

- Huddleston AS, Cresswell N, Neves MC, Beringer JE, Baumberg S, Thomas DI, Wellington EM. Molecular detection of streptomycin-producing streptomycetes in Brazilian soils. Applied and environmental microbiology. 1997 Apr;63(4):1288-97. https://doi.org/10.1128/aem.63.4.1288-1297.1997