The aim of the study was to determine the cellular lipids fatty acid composition for identification of the Bacillus subtilis ONU551 strain bacteria, which is a phenol destructor. Fatty acids analysis of B. subtilis ONU551 strain was performed using an automatic system for microorganisms’ identification MIDI Sherlock (MIDI, USA) based on gas chromatograph Agilent 7890. Chromatograms analysis showed that the fatty acid spectrum of the strain B. subtilis ONU551 consisted predominately of branched structural isomers of saturated acids: 13-methyltetradecanoic (15:0 iso; 34.72%) and 12-methyltetradecanoic (15:0 anteiso; 33.72%) acids. The total content of the branched saturated fatty acids was 88.16% – 14:0 iso (0.52%), 15:0 iso (34.72%), 15:0 anteiso (33.72%), 16:0 iso (1.85%), 17:0 iso (7.11%), 17:0 anteiso (10.24%). The saturated fatty acids of the normal structure were also detected – 12:0 (0.36%), 14:0 (0.28%), 16:0 (1.30%). No 2- and 3-hydroxy acids and no cyclic fatty acids were detected in the fatty acid profile of B. subtilis ONU551 strain. Unsaturated fatty acid isomers – 15:1 w5c (1.85%), 16:1 w11c (1.21%), 16:1 w7c alcohol (1.08%), 17:1 iso w10c (3.18%), ∑17:1 iso 1/anteiso B (2.57%) were shown to be the distinctive biomarkers of the B. subtilis ONU551 strain. According to the fatty acid profile analysis with MIDI Sherlock system, the studied strain was identified as Bacillus subtilis with high level of similarity index (0.563).

Keywords: Bacillus subtilis ONU551, fatty acids composition, saturated acids isomers, strain identification.

Analysis of the composition of fatty acids in cellular lipids of biochemically active bacteria, in particular, bacteria of the genus Bacillus, which have broad biotechnological potential, is necessary for their chemodifferentiation (fatty acid composition of bacteria as an important chemotaxonomic criterion is largely correlated with the results of the identification of molecular genetic indicators) [1, 2]. Microbial communities of Bacillus genus are distinguished by high autonomy and close cooperative bonds, organic substances are used by them in a multi-stage process of destruction [3]. Some components of the lipid composition of bacteria, for example, branched and unsaturated fatty acids are autoinducers in a quorum-sensitive system that ensures the cooperative interaction of microorganisms in the biofilm between members of the population [4].

The literature contains data on the composition of fatty acids in cellular lipids of bacteria of the genus Bacillus, isolated from the rhizosphere, oil-contaminated soil, etc. [5-9]. It was shown that depending on the growth phase of the bacteria, the content of fatty acids 15:0 anteiso, 15:0 iso, 17:0 anteiso and 17:0 iso varies from 86.5 to 88.9% of the total fatty acids [8]. However, in the literature, there is no information on the composition of fatty acids of bacteria strains of the genus Bacillus – destructor of phenol.

The aim of the work was to determine the fatty acid composition in cellular lipids for identification of the bacteria Bacillus subtilis ONU551 strain – destructor of phenol, isolated from wastewater pharmaceutical plant.

Materials and Methods

The object of the research was the biochemically-active phenol bacterial strain B. subtilis ONU551. The strain was isolated in 2017 (southern region of Ukraine) from wastewater pharmaceutical plant [10].
Determination of the taxonomic position of the strain *B. subtilis* ONU551 was carried out according to phenotypic characters and the composition of fatty acids in cellular lipids. Morphological, physiological, biochemical, cultural characteristics of the strain *B. subtilis* ONU551 was determined using classical bacteriological methods and API 50 CHB Medium test system (bioMerieux, France). Analysis of the composition of fatty acids of the strain *B. subtilis* ONU551 was performed using the Sherlock MIDI system, which allows automating the process of identifying microorganisms by chemotaxonomic trait using fatty acid profile libraries [11]. To determine the fatty acid composition of total lipids of the studied strain and to identify it, we used the MIDI Sherlock 4.5 software and the RSTBA6 version 6.2 library of fatty acid profiles of aerobic microorganisms.

Bacteria were cultured on Tryptic soy agar (Merck, Germany) at 28 ± 1 °C for 24 h. To analyze the composition of cellular lipids, one complete loop of wet biomass was placed in glass vials for further chemical lysis of cells and saponification of lipids of the studied organism. Saponification was performed by adding 50% methanol and 3.7 M NaOH.

The prepared sample was kept for 30 min at a temperature of 95–100 °C. Fatty acid methylation was carried out by heating the reaction mixture at 80 °C for 10 min after adding a solution of acidic methanol. Extracted methyl esters of fatty acids were neutralized with 0.3 M NaOH solution [12].

Chromatographic separation of fatty acid methyl esters was performed on an Agilent 7890 gas chromatograph (Agilent Technologies, USA) with an ULTRA-2 capillary column (25 m×0.2 mm×0.33 μm) and a flame ionization detector. A sample of 2 μl was injected in split mode with a ratio of 40:1, the evaporator temperature was 250 °C. The separation of fatty acids of cellular lipids was carried out in the temperature programming mode from 170 to 270 °C with a gradient of 5 °C/min.

Chromatographic analysis of the fatty acid composition of the *B. subtilis* ONU551 strain, the content of which is expressed as a percentage of the total peak areas in the chromatograms obtained, was carried out in several replications. We cultivated the strain three times (three samples, the number of options – (n) – 3) and analyzed the FAME extracts under the highly standardized conditions described in the Microbial Identification System Protocol. In general, data for the most common fatty acids ranged by less than 5%, which indicates that the procedure had good reproducibility.

Statistical processing of the research results was performed using MS Excel computer program with Student’s *t*-test. The difference was considered statistically significant at *P* < 0.05. The tables show the average values of three experiments. The calculation of the biomarker ratios of 15:0 anteiso/15:0 iso and 17:0 anteiso/17:0 iso for the studied *B. subtilis* ONU551 strain was within 7-8%.

**Results and Discussion**

The morphological, cultural and physiological-biochemical properties of the strain *B. subtilis* ONU551 – destructor of phenol and other cyclic aromatic compounds were determined. The strain of *B. subtilis* ONU551 is represented by movable, large gram-positive rods with the size (1.5–1.7)×(5.5–5.8) μm with rounded ends, forming round, oval endospores, which are placed subterminal. Cells are arranged singly, and also in the form of a chain, V-shaped. The colonies are light brown, dense, smooth with a smooth edge. Bacteria grow in meat-peptone broth with clouding of the medium and the formation of a loose film; with growth in MPB with 7.5% NaCl, loose sediment is formed. The strain *B. subtilis* ONU551 gives a negative reaction with methyl red and a negative reaction Voges-Proskauer, hydrogen sulfide does not form. Catalase-positive, oxidase-negative bacteria hydrolyze starch and urea, peptonize milk. The strain *B. subtilis* ONU551 does not reduce nitrates to nitrites, does not liquefy gelatin, does not ferment lactose, maltose, arabinose, galactose, rhamnose; it is characterized by the fermentation of glucose, sucrose, mannitol with the formation of acid. Metabolism of the strain *B. subtilis* ONU551 is oxidative and fermentation. According to the phenotypic, physiological, biochemical, cultural properties of the investigated strain of *B. subtilis* ONU551 was previously classified as *B. subtilis* [13].

To clarify the species of the observable strain *B. subtilis* ONU551 has been studied the fatty acid composition of the cell wall of bacteria – destructor of phenol and other cyclic aromatic compounds.

As a result of the analysis of the fatty acid profile of the *B. subtilis* ONU551 strain were found fatty acids 17:1 (total ∑17:1 iso I/anteiso B) and 17 fatty acids with 12 to 17 carbon atoms, mostly branched (Table 1). The total fatty acids content (saturated and unsaturated) with branched structure, which are presented in Table 1 (where the average values of
the data, obtained from three independent samples, \( n = 3 \) are 91.34% of the total fatty acid pool.

It is known that the prevalence of branched fatty acids in the spectrum is a characteristic feature of bacteria of the genus *Bacillus* [2]. The fatty acid composition of the studied *B. subtilis* ONU551 strain was dominated by 13-methyltetradecanoic (15:iso; 34.72%) and 12-methyltetradecanoic (15:0 anteiso; 33.72%) acids. This is typical for the majority of the representatives of the genus *Bacillus* – for representatives of rRNA group 1 in particular [14].

Fatty acids 17:0 in the form of -iso (17:0 iso, 15-methylhexadecanoic acid) and –anteiso (17:0 anteiso, 14-methylhexadecanoic acid) were recorded compared to fatty acids 15:0 iso and 15:0 anteiso in 4.9 and 3.3 times less, respectively. The chromatographic data obtained by us in deciphering the tested strain of bacteria –destructor of phenol and other cyclic aromatic compounds are in good agreement with the literature data. It is known that the exaggeration of branched fatty acids in the fatty acid profile is a characteristic feature of bacteria of the genus *Bacillus* [2, 5]. The authors of the work [2], referring to the research of T. Kaneda, indicate that the content of fatty acids of branched structure in bacteria of the genus *Bacillus* varies from 54 to 85% of the total fatty acid pool with a predominant content of acids 15:0 in the form of -iso and -anteiso. Bacilli is also characterized by the content of 17:0 iso and 17:0 anteiso fatty acids [5].

There is a higher content of saturated and unsaturated fatty acids of a branched structure (91.34%) in the fatty acid profile of the studied *B. subtilis* ONU551 strain compared to the content of branched fatty acids in Bacilli (85%) [2]. This is due to the fact that the bacterial strain we studied was isolated from the wastewater of a pharmaceutical plant with a predominant content of organic pollutants. According to [15], various stress factors may serve as inducers of fatty acid isomerization: presence of heavy metal salts or organic xenobiotics, low pH, high temperature.

The presence in the fatty acid profile 15:0 and 17:0 branched structure both in iso form and in anteiso form made it possible to calculate biomarker ratios for the studied strain *B. subtilis* ONU551 [15:0 anteiso/15:0 iso; 0.97 ± 0.05 at \( P < 0.05 \) and [17:0 anteiso/17:0 iso; 1.38 ± 0.08 at \( P < 0.05 \) and compare them with available literature data. Thus, in [6], was shown that for *B. subtilis* 170221, the biomarker ratio [15:0 anteiso/15:0 iso] is very close to our result and is \( \sim 1.1 \).

It should be noted that the chromatograms of the *B. subtilis* ONU551 strain we studied did not record, even in trace amounts, isomers of saturated fatty acids of a branched structure: 12:0 iso, 13:0 iso and 13:0 anteiso, which are mainly characteristic of

![Chromatogram of cellular fatty acids of Bacillus subtilis ONU551](image-url)
Table 1. Cellular fatty acid (%) composition of Bacillus subtilis ONU551

| Fatty acid         | % of the total peak areas |
|--------------------|--------------------------|
| 12:0               | 0.36 ± 0.01              |
| 14:0 iso           | 0.52 ± 0.04              |
| 14:0               | 0.28 ± 0.01              |
| 15:0 iso           | 34.72 ± 1.82             |
| 15:0 anteiso       | 33.72 ± 1.74             |
| 15:1 w5c           | 1.85 ± 0.10              |
| 16:1 w7c alcohol   | 1.08 ± 0.04              |
| 16:0 iso           | 1.85 ± 0.09              |
| 16:1 w11c          | 1.21 ± 0.04              |
| 16:0               | 1.30 ± 0.05              |
| 17:1 iso w10c      | 3.18 ± 0.17              |
| 17:0 iso           | 7.11 ± 0.38              |
| 17:0 anteiso       | 10.24 ± 0.54             |
| 17:1 iso I/anteiso B | 2.57 ± 0.12           |
| Total              | 99.99                    |

Bacillus cereus [9]. Fatty acids 13:0 iso and 13:0 anteiso in a minor amount of 0.50% and 0.25%, respectively, were recorded earlier in the analysis of the fatty acid profile of the strain Bacillus megaterium OZ-5, a toxic organic compounds destructor [7].

The saturated fatty acid content of the strain B. subtilis ONU551 was 88.16% (of the total fatty acid pool): 14:0 iso (0.52%), 15:0 iso (34.72%), 15:0 anteiso (33.72%), 16:0 iso (1.85%), 17:0 anteiso (10.24%). 12-methyltridecanoic (14:0 iso) and 14-methylpentadecanoic (16:0 iso) acids should not be considered markers for the detection of the strain B. subtilis ONU551 at the species level. Fatty acids 14:0 iso, 16:0 iso were recorded by us earlier also on the chromatograms of the strain B. megaterium OZ-5 [7].

The total content of saturated and unsaturated fatty acids of the branched structure in the form –iso: reached 47% (14:0 iso, 0.52%; 15:0 iso, 34.72%; 16:0 iso, 1.85%; 17:1 iso w10c, 3.18%; 17:0 iso, 7.11%) and very slightly prevailed over the total content of branched fatty acids in the form –anteiso (∑w = 44%): 15:0 anteiso (33.72%), 17:0 anteiso (10.24%), that is, they were almost equal. For comparison, it can be noted that in the fatty acid profile of bacteria belonging to another genus, for example, Microbacterium barkeri OZ-3 and Microbacterium barkeri OZ-2-destructors of oil and petroleum products, unlike the strain B. subtilis ONU551 studied by us, saturated fatty acids prevailed branched structure in the form – anteiso: (∑w = 72.3-73.4%) 12-methyltetradecanoic (15:0 anteiso) and 14-methylhexadecanoic (17:0 anteiso) acids [16].

Of the saturated fatty acids of normal structure, the maximum was hexadecanoic acid (16:0; 1.30%). In general, it is necessary to take into account that hexadecanoic acid, like other saturated fatty acids of normal structure, found in small quantities on chromatograms - 12:0 (0.36%), 14:0 (0.28%), is not specific and is most often used only for verification calculations when conducting a material balance of substances and identifying microorganisms not recorded in the data bank.

The fact that this strain is attributed to grampositive bacteria is also indicated by the absence of chemical markers of 3-hydroxy acids in their fatty acid profile. In the spectrum of fatty acids of total cellular lipids of the peak culture studied by us, corresponding to a series of 2- and 3-hydroxy acids from the biomass composition (odd and branched hydroxy acids with 15 and 17 carbon atoms, it is possible to identify bacteria to the type of gliding), was not found even in trace amounts (as we found earlier, for example, when identifying the strain B. megaterium OZ-5 - w (18:1 2OH) = 0.15% [7]. This testifies to the fact that hydroxy acids are completely absent in the lipid fractions, which are marker for most other bacteria-destructors of toxic organic pollutants, for example, bacteria of the genus Pseudomonas, which we studied earlier [17].

In the fatty acid profile of the strain B. subtilis ONU551, cyclic fatty acids were not recorded. They are characteristic, according to the research of T. Kaneda, to the fatty acid profile of the representatives of the Bacillus genus of the seventh group D. In the fatty acid pool of representatives of the seventh group D, cyclohexane fatty acids with a chain length of 17 to 19 carbon atoms, w > 50%, predominate [6, 18].

The results of the analysis of the fatty acid profile of the studied strain B. subtilis ONU551 showed the presence of unsaturated fatty acid isomers (∑FA unsaturated = 9.86%): 15:1 w5c (1.85%); 16:1 w11c (1.21%); 16: 1 w7c alcohol (1.08%); 17:1 iso w10c (3.18%) and the amount of acids ∑17:1 iso I/anteiso B (2.57%). This distinguishes it from members of the genus Bacillus of group 2 (B. sphaericus, B. fusiformis, B. insolitus, B. pasteurii, B. psychrophilus),
which are characterized by a significant content of isomers with unsaturated bonds (\(w = 17-28\%\)) and can be attributed the \(B. subtilis\) ONU551 strain studied by us to the bacteria of group 1. From literature data it is known that the content of unsaturated fatty acid isomers in representatives of the genus \(Bacillus\) group 1 (\(B. amyloliquefaciens, B. atrophaeus, B. azotoformans, B. megaterium, B. licheniformis,\) etc.) is less than 10% [14].

We found the distinctive features of the fatty acid composition of the strain \(B. subtilis\) ONU551 and \(B. megaterium\) OZ-5, which we studied earlier [7]. Both bacterial strains belong to the Bacilli of group 1, and are good destructors of toxic organic compounds (phenol, petroleum products, etc.). Biomarkers and a biomarker value are proposed – saturation coefficient (\(\Sigma FA_{unsaturation,\%} / FA_{unsaturation,\%}\)) for detecting the above strains at the species level (Table 2).

Table 2 shows that the distinctive biomarkers of the \(B. subtilis\) ONU551 strain at the species level may be unsaturated isomers of 15:1 w5c fatty acids (1.85%) and the amount of acids \(\Sigma 17:1\) iso I/anteiso B (2.57%). Monoenic 11-hexadecenoic acid 16:1 w11c (palmitic acid) \(\text{CH}_3-(\text{CH}_2)_3-\text{CH} = \text{CH}-(\text{CH}_2)_9-\text{COOH}\) was detected in 1.21% of the unsaturated fatty acids in the fatty acid profile of the studied strain \(B. subtilis\) ONU551; 4.84 times more compared to the minor content of 16:1 w11c - in the strain \(B. megaterium\) OZ-5 (0.25%). The total content of all unsaturated fatty acids in cellular lipids of the strain \(B. subtilis\) ONU551 was 2.26 times the total content of unsaturated fatty acids in the strain \(B. megaterium\) OZ-5, and was 9.86%: 15:1 w5c (1.85%); 16:1 w11c (1.21%); 16:1 w7c alcohol (1.08%); 17:1 iso w10c (3.18%); \(\Sigma 17:1\) iso I/anteiso B (2.57%). Thus, both of the above bacterial strains at the species level differ among themselves by their biomarker value – the saturation coefficient (Table 2). Thus, the saturation coefficient for the strain \(B. subtilis\) ONU551 was 2.4 times less than (9.13) saturation coefficient for strain \(B. megaterium\) OZ-5 (21.84).

The presence of w(9Z)-9-Hexadecen-1-ol (16:1 w7c alcohol), albeit in small quantities (\(w = 1.08\%\)) in the fatty acid profile of the strain \(B. subtilis\) ONU551 can serve as a biomarker for the recognition of this strain among other microorganisms. It also gives the possibility of a large probability to predict the ability of the strain \(B. subtilis\) ONU551 to dispose of alcohols - the intermediate products of the oxidation of most organic compounds. This assumption is indicated by the fact that it was the waste water of a pharmaceutical plant with a wide range of organic pollutants (phenol, surfactants, etc.) that isolated the strain under study.

Attention should be paid to the fact that when deciphering the fatty acid profile of other strains – destructors of phenolic compounds – \(Aeromonas ichthiosmia\) ONU552, \(Brevibacillus centrosporus\) F-14 (allocated by us from pharmaceutical plant wastewater), 16:1 w7c alcohol was also detected [19, 20]. Its content in cellular lipids varied from 3.45% in \(A. ichthiosmia\) ONU552 to 7.71% in \(Brevibacillus centrosporus\) F-14 [19, 20].

However, unlike them, the amount of unsaturated fatty acids in the strain \(B. subtilis\) ONU551 – destructor of phenol and other cyclic aromatic compounds was 5 times less (\(\Sigma w = 9.89\%\)) compared with the amount of unsaturated fatty acids found in the fatty acid pool. For example, \(\Sigma FA\) unsaturation in strain \(A. ichthiosmia\) ONU552 was 50% [19].

The detected features of the fatty acid profile of the studied strain \(B. subtilis\) ONU551 – (biomarkers 15:1 w5c, \(\Sigma 17:1\) iso I/anteiso B and the biomarker value – saturation coefficient) can be used as an auxiliary key for its differentiation at the species level.
level (by results of studies of fatty acids composition). The identified features of the fatty acid composition of B. subtilis ONU551 strain are necessary to verify the purity of the culture in the manufacture of biological products intended for cleaning the effluent of medical institutions, the pharmaceutical plant, and other industries containing biological and chemical pollutants, in particular phenol.

Thus, by the fatty acid composition, the spectrum of which was obtained on an Agilent 7890 gas chromatograph (Agilent Technologies, USA) (Figure), and decoded using the RTSBA6 6.21 library of the Sherlock MIDI program, the Bacillus sp. ONU551 with a similarity index (Sim Index = 0.563) was identified as B. subtilis (Table 1). The non-pathogenic strain B. subtilis ONU551 isolated from wastewater pharmaceutical plant with a wide range of biotechnological properties has been added to the museum collection of non-pathogenic microorganisms strains from the Department of Microbiology, Virology and Biotechnology of Odessa National I.I. Mechnykov University – B. subtilis ONU551 [21].

According to phenotypic, physiological, biochemical, cultural characteristics, the strain isolated from wastewater pharmaceutical plant was previously classified as Bacillus subtilis. Specification of the species composition of cellular lipid fatty acids, carried out using the MIDI Sherlock microorganism identification system based on the Agilent 7890 gas chromatograph, confirmed its belonging to the B. subtilis species with the fatty acid profile similarity index with library data Sherlock MIDI -0.563. Chromatograms analysis allowed to assert that feature of the fatty acid composition of the non-pathogenic strain of B. subtilis ONU551 – destructor of phenol and other cyclic aromatic xenobiotics is dominance 13-Methyltetradecanoic (15:0 iso; 34.72%) and 12-Methyltetradecanoic (15:0 anteiso; 33,72%) acids. The distinctive biomarkers of B. subtilis ONU551 can be unsaturated fatty acid isomers: 15:1 w5c (1.85%), 16:1 w11c (1.21%), 16:1 w7c alcohol (1.08%), 17:1 iso w10c (3.18%), Δ17:1 iso/anteiso B (2.57%), recorded in small quantities. The found features of the fatty acid composition of the non-pathogenic strain Bacillus ONU551 – destructor of phenol and other cyclic aromatic xenobiotics as biomarkers and biomarker ratios [15:0 anteiso/15:0 iso, 0.97 ± 0.05; 17:0 anteiso/17:0 iso; 1.38 ± 0.08] are systematized and can be used as an auxiliary key for differentiating the B. subtilis ONU551 strain from other microorganisms.

**Conflict of interest.** Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

**ЖИРНОКИСЛОТНИЙ СКЛАД ЛІПІДІВ Bacillus subtilis ONU551**

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У роботі визначали жирнокислотний склад клітинних ліпідів для ідентифікації бактерій штаму Bacillus subtilis ONU551, які є деструкторами фенолу. Аналіз жирних кислот штаму B. subtilis ONU551 проводили з використанням автоматичної системи ідентифікації мікроорганізмів MIDI Sherlock (MIDI, США) на базі газового хроматографа Agilent 7890. Аналіз хроматограм показав, що домінуючими в жирнокислотному спектрі штаму B. subtilis ONU551 є розгалужені структурні ізомери насичених кислот, з яких переважали 13-метилтетрадеканова (15:0 iso; 34,72%) і 12-метилтетрадеканова (15:0 anteiso; 33,72%) кислоти. Сумарний вміст насичених жирних кислот розгалуженої будови дорівнював 88,16% і був таким: 14:0 iso (0,52%), 15:0 iso (34,72%), 15:0 anteiso (33,72%), 16:0 iso (1,85%), 17:0 iso (7,11%), 17:0 anteiso (10,24%). Із насичених жирних кислот нормальної будови виявлено 12:0 (0,36%), 14:0 (0,28%), 16:0 (1,30%). У жирнокислотному профілі штаму B. subtilis ONU551 відсутня низка 2- і 3-гідроксикислот і не зафіксовано жирні кислоти циклічної будови. Встановлено, що біомаркерами штаму B. subtilis ONU551 можуть слугувати ненасичені ізомери жирних кислот – 15:1 w5c (1,85%), 16:1 w11c (1,21%), 16:1 w7c alcohol (1,08%), 17:1 iso w10c (3,18%), Δ17:1 iso/anteiso B (2,57%). Аналіз жирнокислотного профілю досліджуваного штаму з використанням системи MIDI Sherlock дозволив віднести його до виду Bacillus subtilis із високим індексом подібності (0,563).
Ключові слова: Bacillus subtilis ONU551, склад жирних кислот, ізомери насичених кислот, ідентифікація виду.

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