EUROPEAN Journal of Molecular Biotechnology

Has been issued since 2013.
ISSN 2310-6255. E-ISSN 2409-1332
2015. Vol.(7). Is. 1. Issued 4 times a year

The journal is registered by Federal Service for Supervision of Mass Media, Communications and Protection of Cultural Heritage (Russia). Registration Certificate **ПИ № ФС77-55114** 26.08.2013.

Journal is indexed by: **CiteFactor** – Directory of International Reseach Journals (Canada), **Cross Ref** (UK), **EBSCOhost Electronic Journals Service** (USA), **Global Impact Factor** (Australia), **International Society of Universal Research in Sciences** (Pakistan), **Journal Index** (USA), **Electronic scientific library** (Russian Federation), **Open Academic Journals Index** (Russian Federation), **Sherpa Romeo** (Spain), **ULRICH's WEB** (USA), **Universal Impact Factor** (Australia).

All manuscripts are peer reviewed by experts in the respective field. Authors of the manuscripts bear responsibility for their content, credibility and reliability.

Editorial board doesn't expect the manuscripts' authors to always agree with its opinion.

Postal Address: 26/2 Konstitutcii, Office 6
354000 Sochi, Russian Federation

Website: http://ejournal8.com/
E-mail: ejm2013@mail.ru

Founder and Editor: Academic Publishing House *Researcher*

Passed for printing 16.03.15.
Format 21 × 29,7/4.
Enamel-paper. Print screen.
Headset Georgia.
Ych. Izd. l. 4,5. Ysl. pech. l. 4,2.

Circulation 500 copies. Order № 7.

European Journal of Molecular Biotechnology

2015

Is. 1

Европейский журнал молекулярных биотехнологий

2015

№ 1

Европейский журнал молекулярных биотехнологий

Издается с 2013 г. ISSN 2310-6255. E-ISSN 2409-1332
2015. № 1 (7). Выходит 4 раза в год.

Журнал зарегистрирован Федеральной службой по надзору в сфере массовых коммуникаций, связи и охраны культурного наследия (Российская Федерация). Свидетельство о регистрации средства массовой информации ПИ № ФС77-55114 от 26.08.2013 г.

Журнал индексируется в: **CiteFactor** – Directory of International Reseach Journals (Канада), **Cross Ref** (Великобритания), **EBSCOhost Electronic Journals Service** (США), **Global Impact Factor** (Австралия), **International Society of Universal Research in Sciences** (Пакистан), **Journal Index** (США), **Научная электронная библиотека** (Россия), **Open Academic Journals Index** (Россия), **Sherpa Romeo** (Испания), **ULRICH's WEB** (США), **Universal Impact Factor** (Австралия).

Адрес редакции: 354000, Российская Федерация, г. Сочи, ул. Конституции, д. 26/2, оф. 6
Сайт журнала: http://ejournal8.com/
E-mail: ejm2013@mail.ru

Учредитель и издатель: ООО «Научный издательский дом "Исследователь"» - Academic Publishing House *Researcher*

Подписано в печать 16.03.15.
Формат 21 × 29,7/4.
Бумага офсетная.

Печать трафаретная.
Гарнитура Georgia.
Уч.-изд. л. 4,5. Усл. печ. л. 4,2.
Тираж 500 экз. Заказ № 7.

CONTENTS

Published in the Russian Federation
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN 2409-1332
Vol. 7, Is. 1, pp. 4-11, 2015

DOI: 10.13187/ejmb.2015.7.4
www.ejournal8.com

UDC 631

Bactericidal Effect of Aqueous Extracts of the Bark of the Pomegranate (*Punica granatum* L.) on Bacteria

[1] Rabah Chadli
[1] Aman Bouzid
[2] Khadidja Bouzid
[1] Hamida Nader

[1] University Abdelhamid Ibn Badis, Algeria
Faculty of Natural Sciences and Life, Mostaganem, 27000
[2] Djillali Liabes University, Algeria
Laboratory eco-development areas
Faculty of Natural Sciences and life, Sidi Bel Abbes, 22000
Corresponding author: Chadli Rabah
E-mail**:** chadli_rabah@yahoo.fr

Abstract

This research concerns the study of antibacterial properties of different aqueous extracts of the bark of the pomegranate (*Punica granatum* L.). Three bacterial strains were used in this test: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella*. Very interesting bactericidal properties of aqueous extracts of the bark of the pomegranate were found on bacteria. The inhibition zones have a very large diameter up to 20 mm and the MIC and MBC are low, of the order of 0.78 mg/ml. This work has shown antibacterial activity against three bacteria may contribute to the fight against infectious diseases, and possibly offer the possibility of using pomegranate peel pharmaceutical and food industries.

Keywords: *Punica granatum* L.; bark; antibacterial activity; aqueous extracts; inhibition.

Introduction

The use of medicinal plants for therapeutic purposes is an ancient practice. The pomegranate (*Punica granatum* L.) has fascinated all civilizations by its beauty and juiciness of the fruit. Used empirically in traditional medicines, to treat gastrointestinal diseases and parasitic diseases, pomegranate has anti-oxidant properties.

The bark of the pomegranate fruit is the hard part of the fruit. It is generally used dried in the form of brownish pieces. These fragments are leathery consistency. They are formed of parenchyma cells with thin walls, in the middle of which there are groups of stone cells and vascular bundles. The flavor of pomegranate rind is bitter and astringent. The crust is an anthelmintic and astringent and useful in the treatment of diarrhea, dysentery and stomach pains [1]. For centuries, bark, leaves, flowers and fruits of *Punica granatum* L. (Punicaceae), known as the grenade name, were used to treat many diseases [2]. Bacterial and fungal infections due to multidrug-resistant microorganisms are a major concern for both health problem in developing countries where they are the main cause of high mortality rates in industrialized countries where

resistance to existing antibiotics grow alarmingly [3]. This situation creates a growing need to find new compounds; identifying new sources of antibiotic natural products and the expansion of the chemical diversity of antibiotics provide leads for new chemical drugs. Medicinal plants, particularly those used traditionally, are a potential source of such compounds. One strategy for this research is to explore the plants used in traditional medicine to treat many infectious origin pathologies. Capable of colonizing a wide variety of tissue causing, among others, respiratory, intestinal, skin or urinary [4]. They have enormous therapeutic potential to cure many infectious diseases [5]. Thus, for two decades studies have been conducted on the development of new applications and the exploitation of natural properties of essential oils in different areas. The use of essential oils is relevant today. As part of our effort to achieve this goal, we evaluated the antibacterial activity of the plant used traditional medicine Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae. It is a shrub native to Asia and has been cultivated since ancient times throughout the Mediterranean region of Africa and parts of Europe.

Materials and methods

1. Biological material

1. 1. Harvesting of grenade and preparation of the powder

Fresh fruit of the grenade were collected during the month of October 2013 from a grenadier field. The barks were cleaned, peeled and dried in the shade for two weeks and then crushed in a traditional mortar then they were ground to a fine powder using an electric coffee grinder. The powder was stored in clean vials before proceeding with the extraction.

1. 2. Method of study

Extraction processes. Three different extracts (chloroform, acetone, ethanol) were prepared from the powdered bark of the grenade.

Chloroform extraction

A mixture of 100 g of bark powder *Punica granatum* L. 1Litre with chloroform and placed in a Soxhlet apparatus and then the mixture was heated at 70 °C for one hour. After cooling, the homogenate was filtered under vacuum on Whatman paper (n°3), then the filtrate was concentrated under reduced pressure with a rotary evaporator [6].

Acetone extraction

100 g of the powder was mixed with 1L of distilled water in an Erlenmeyer flask, then the mixture was stirred at laboratory temperature (25 °C) for 24 hours [7]. After 24 hours, filtered the mixture under vacuum Whatman paper (n°3) and then the filtrate concentrated under reduced pressure with a rotary evaporator [6].

Ethanol extraction

For the ethanol extract, 100 g of the powder was mixed with 1L of ethanol and the mixture was placed under stirring at room temperature (25 °C) for 24 hours [7]. The mixture was filtered on Whatman paper, then the filtrate was concentrated under reduced pressure with a rotary evaporator [6].

2. Microbiological study

2.1. Study of the antimicrobial activity

The antimicrobial study consists in determining the antibacterial parameters (MIC) of our extracts of pomegranate, for it was used the agar diffusion method [8].

2.2. Preparation of inoculum

The bacterial inoculum. Using a handle previously outbreak then cooled, was taken a colony of S. *aureus*, P. *aeruginosa*, then were seeded on a nutrient agar slope and incubated the tube at 37 ° C for 24 hours.

2.3. Preparing extracts dilutions

After their concentration by rotary evaporation the extracts taken a pasty form prior to use they were solubilized in DMSO [9, 10] to give a stock solution of 100 mg / ml. From this solution, dilutions up to 6.25 % were achieved.

2. 4. Agar diffusion

Microbial suspensions were prepared in physiological saline and then the optical densities were adjusted from 0.08 to from 0.1 to a wavelength of 600 nm for the bacterial strains. Seeding was done by flooding on Petri dishes containing 10 ml of Mueller-Hinton agar in a thickness of

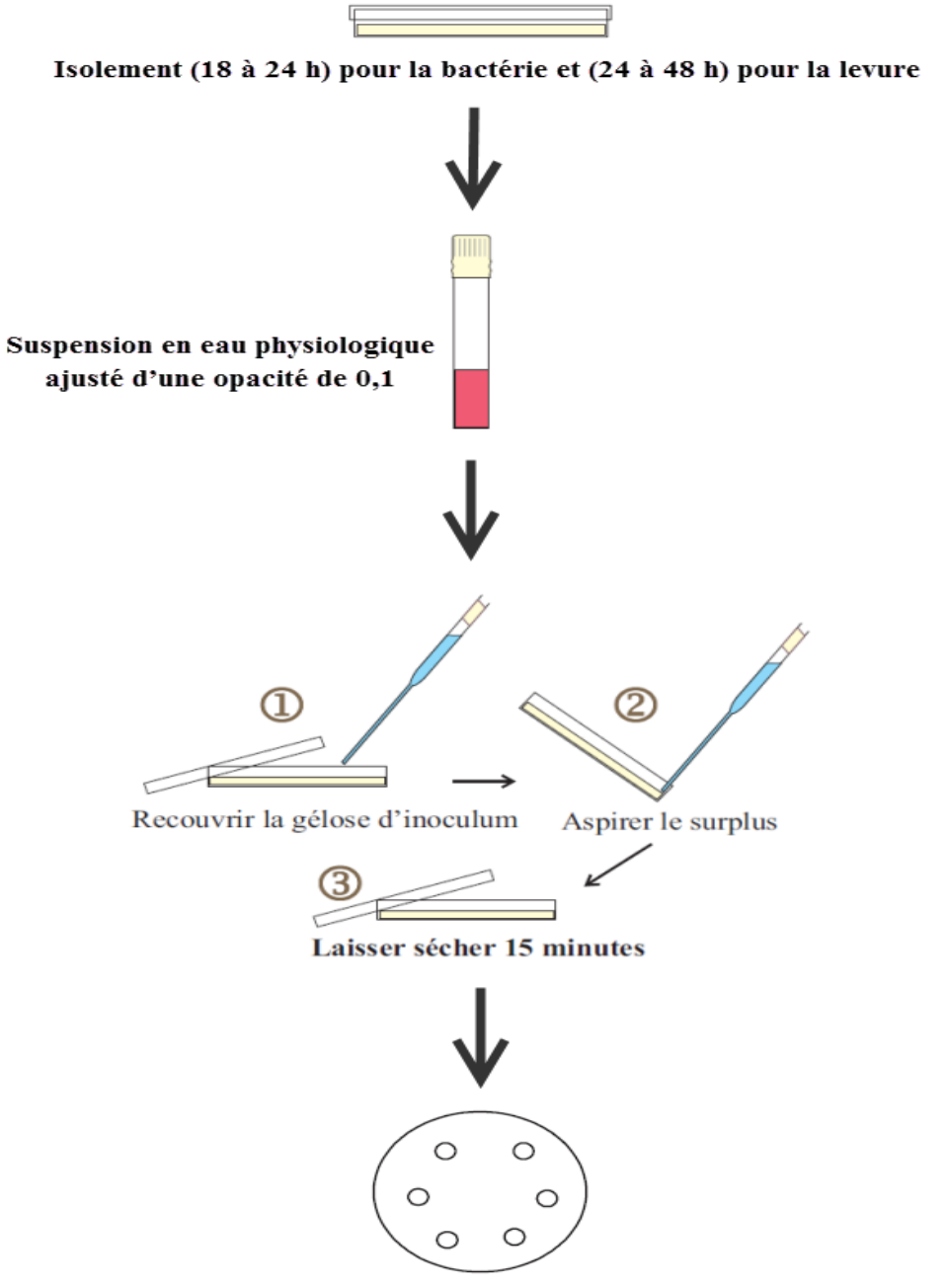
4 mm [11, 12] then the boxes were left open to dry aseptically [9]. Sterile disks of 06 mm diameter were placed on the agar using sterile forceps [13], then pressed to ensure their application then they were impregnated with 10 µl of each extract at different concentrations using a micropipette.

2.5. Antibiotics (control)

They are discs of 06 mm diameter were placed on the agar with sterile forceps, and then pressed to make their application. It has 04 deferent antibiotics (Penicillin P, PG, Streptomycin, Netroxolin).

2.6. Minimum inhibitory concentration (MIC)

The evaluation of the minimum inhibitory concentration is to determine the lowest concentration of an antimicrobial agent required to inhibit any visible culture. It is determined by using a dilution series of the antimicrobial agent, added to a series of tubes of a liquid culture medium [14, 15].



Extraction and preparation of plants

A local variety of grenadier, Punica granatum L., was chosen as plant material. Bark fruits were dried in ambient air and then reduced in the form of powder. Grenadier extracts were extracted in 80 % hot ethanol. After centrifugation, the supernatant was filtered and dried and stored in eppendorf at 4°C. The tested extracts were resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg / ml. After dissolving in the water bath, the mixture was centrifuged at 3000 RPM for 15 minutes at room temperature. The supernatant was then separated and stored at -20°C. And the frozen extracts were thawed at 37°C and diluted to the required concentration in the culture medium before each experiment in order to obtain a final concentration of 0.1 to 0.2 % DMSO.

Results

1. 1. Extraction

The yields of different extracts of *Punica granatum* bark obtained are:

- 34,27 g dark brown for the ethanol extract.
- 29,68 g dark brown for the acetone extract.
- 26,47 g light brown color to the chloroform extract.

1. 2 Microbiological study

Diameters of inhibition zones. The acetone extract exhibited a considerable antimicrobial activity against three bacterial strains tested against two other extracts (ethanol and chloroform), with diameters of the inhibition zones s 20 mm for *Salmonella* by a dose of 75 mg, 17 and 18 mm (*S. aureus, P. aeruginosa*) 50 mg, respectively.

Table 1: The diameters of the inhibition zones for different antibiotics

Antibiotics / Bacterial strains	Penicillin P diameter in mm	Penicillin G diameter in mm	Streptomycin diameter in mm	Nitroxolin diameter in mm
Salmonella	0	11	13	21
Staphylococcus aureus	10	14	15	11
Pseudomonas aeruginosa	12	14	18	12

Table 2: The diameters of the inhibition zones for different extracts by a dose of 100 mg

Bacterial strains / Extracts		Wells method, mm	Disk method, mm
Staphylococcus *Aureus*	Acetone	16	13
	Ethanol	12	10
	Chloroform	0	0
Salmonella	Acetone	17	14
	Ethanol	16	15
	Chloroforme	5	6
Pseudomonas Aeruginosa	Acetone	15	10
	Ethanol	15	14
	Chloroform	0	0

Table 3: The diameters of the inhibition zones for different extracts

Extracts Bacterial strains		Wells method, mm	
		Dose of 75 mg	Dose of 50 mg
Staphylococcus *Aureus*	Acetone	16	17
	Ethanol	15	16
Salmonella	Acetone	20	18
	Ethanol	18	15
Pseudomonas Aeruginosa	Acetone	17	18
	Ethanol	16	18

MIC

In the procedure of the determination of the minimum inhibitory concentration of the ethanolic extract of the biological assay was used to graded concentrations of 50,75 and 100 mg/ml.

Discussions

1. Extraction

Extraction processes have resulted in different yields depending on the nature of the solvent [7], the method and the conditions under which the extraction was carried out [16].

This result could be explained by the difference in solubility of the components of the crude extracts which vary depending on the solvent used and the way in which they are prepared.

Susceptibility testing. In this study, the antibacterial activity was evaluated the ethanol extract, acetone and chloroform *Punica granatum* pericarp of the growth of three strains of bacteria (*Salmonella, S aureus* and *P aeruginosa*) by the agar diffusion method in measuring the diameters of inhibition of bacterial growth areas (Figure 1 - 6).

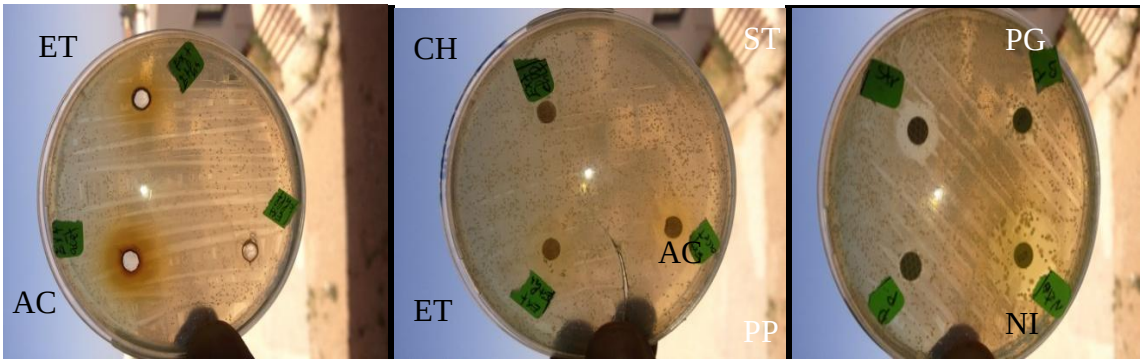


Figure 1. Sensitivity of *S. aureus* to the different extracts (100 mg) and antibiotics.

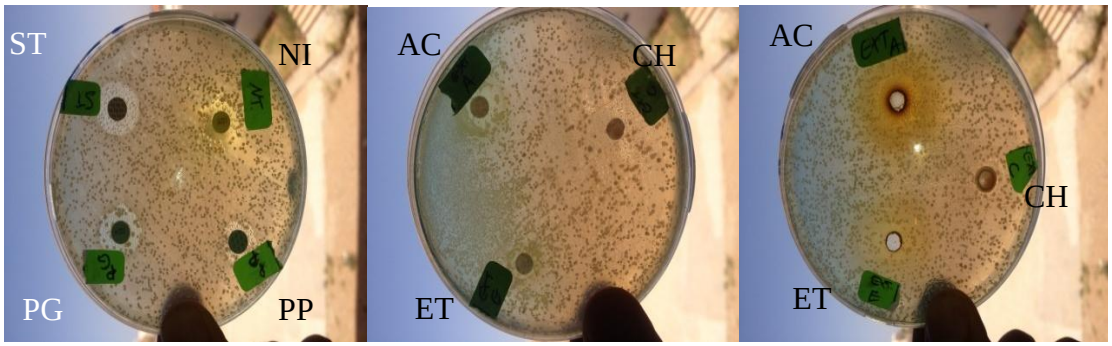


Figure 2. Sensitivity of *Salmonella* to the different extracts (100 mg) and antibiotics.

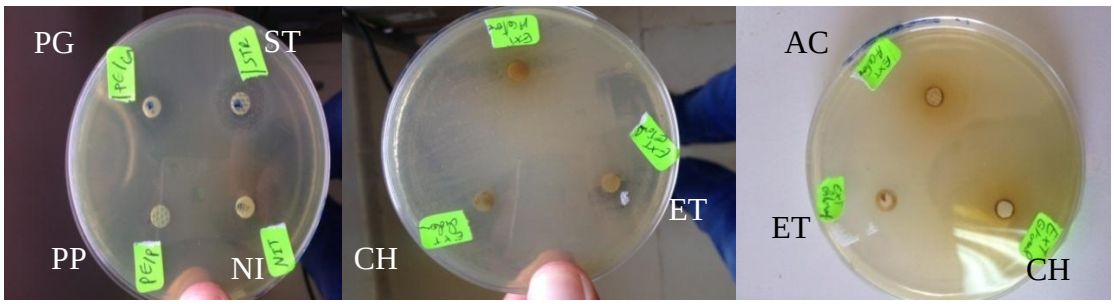


Figure 3. Sensitivity of *P. aeroginosa* to the different extracts (100 mg) and antibiotics

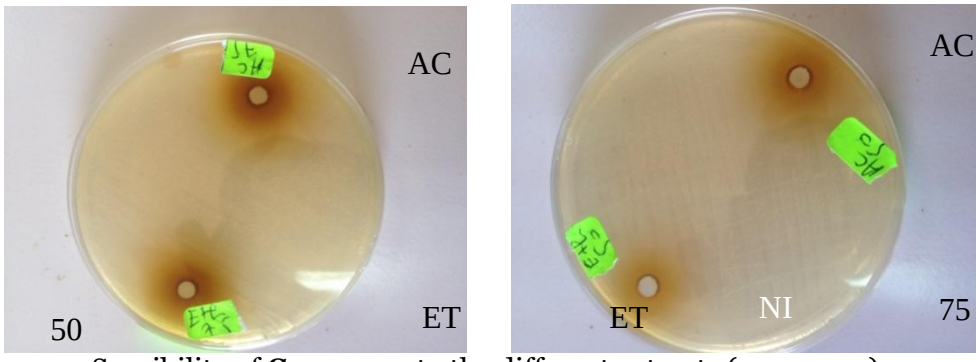


Figure 4. Sensibility of ***S. aureus*** to the different extracts (50–75 mg)

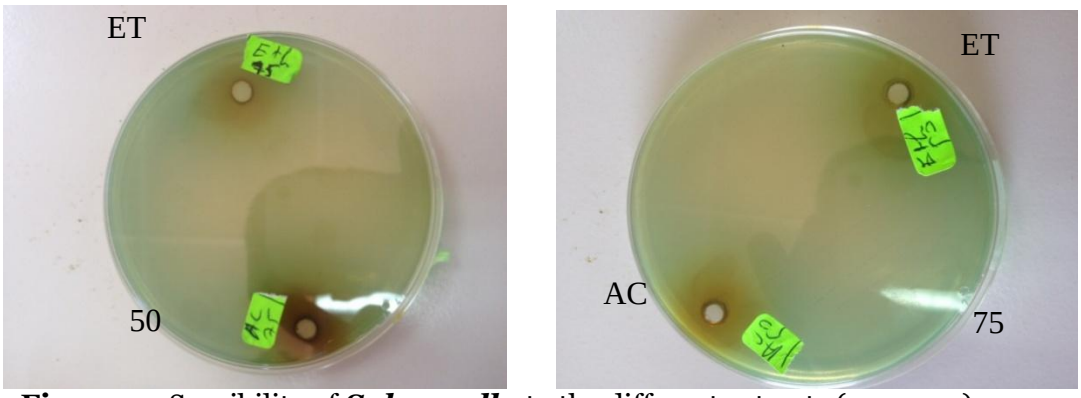


Figure 5. Sensibility of ***Salmonella*** to the different extracts (50-75mg)

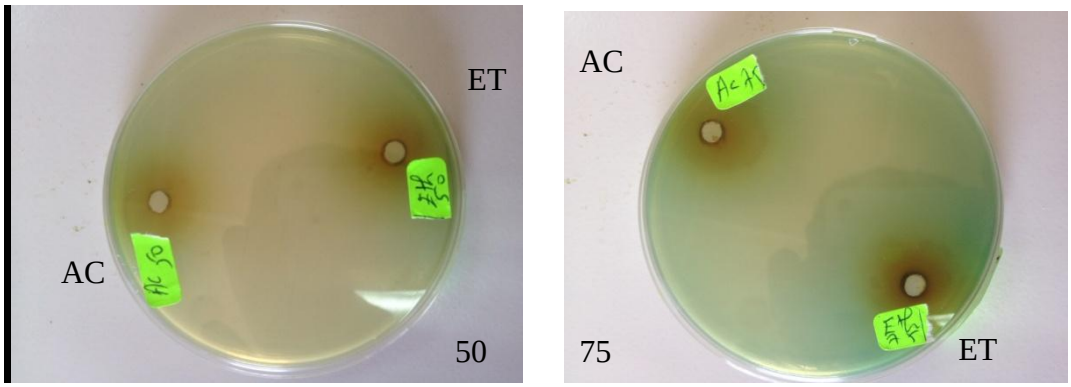


Figure 6. Sensibility of *P. aeroginosa* to the to the different extracts (50–75 mg)

Subsequently, our choice fell on the ethanol extract, which showed better antimicrobial activity. Then we proceeded to the determination of the MIC values of the three strains tested.

Salmonella strain has shown the most sensitive to the extracts studied with diameters of 20 mm for the acetone extract, 18 mm for the ethanol extract and 05 mm for the chloformique extract.

For bacterial strain *S. aureus* showed a sensitivity screw overlooked the acetone extract with a diameter of 17 mm and a diameter of 16 mm for the ethanol extract and for *P. aeruginosa,* has the same sensitivity of 18 mm diameter towards the two extracts (acetone, ethanolic).

Punica granatum (pomegranate) contains tannins. Tannins are known to precipitate the protein and might be involved in the extracts inhibitory mechanism, however, the exact mechanism of inhibition is not known [18].

The data obtained on the three bacteria are similar to those carried out by Vasconcelos et al. [19] and Beckman et al. [17], where the pomegranate extract showed significant inhibition. Melendez and Capriles [20] and Senhaji et al. [13] also reported that pomegranate fruit extracts have antibacterial activity in vitro against many bacteria tested, including our bacterial strains, confirming our results.

In general, the acetone extract has a high antimicrobial activity, it can be linked to the power of acetone to dissolve one or more compounds contained in the homogenate of the pericarp [13]. The efficiency of an extract depends on its concentration, the sole of which it arose and strain tested [21].

Results

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated the search for new antimicrobial substances from other sources, including plants and microbes [22]. This antibacterial activity may be indicative of the presence of metabolic toxins or compounds with broad spectrum antibiotics. This is consistent with previous reports of several researchers [1, 23, 24]. Methanol extracts showed a higher degree of antimicrobial activity against extracted with acetone. Voravuthikunchai et al. [24] reported that *P. granatum* contains large quantities of tannin (25 %) and the antibacterial activity can be indicative of the presence of certain secondary metabolites. The ethanol extract of *P. granatum* showed antibacterial activity against *E. coli* [24] and *S. aureus* [23].

Due to the increasing development of drug resistance and adverse effects caused by existing antifungal agents, search for new antimicrobial compounds has been the subject of a number of studies [25]. Interest in natural products has increased over the years, and medicinal plants have been identified as sources of bioactive compounds. These data support the observations of some research such as those of [12, 18, 26–31], demonstrated that pomegranate extract inhibits affects tannins, the ability to inhibit the growth of the Candida yeast species due to their action in the cell, particularly in the cell membrane, protein precipitation.

References

1. Prashanth D, Asha MK, Amit A (2001) Antibacterial activity of Punica granatum. Fitoterapia, 72: 171-173.

2. Gracieuse RR, Selvasubramanian S, Jayasunder S (2001) actvity immunomodulateur de Punica granatum chez les lapins -A étude préliminaire. J Ethnopharmacol. 78: 85-87.

3. OMS (1999) Rapport d'Organisation mondiale de la Santé.

4. De Billerbeck VG (2007) Huiles essentielles et bactéries résistantes aux antibiotiques. Phytothérapie, 5: 249–253.

5. Janovska D, Kubikova K, Kokoska L (2003) Screening for antimicrobial activity of some medicinal plants species of traditional Chinese medicine. Czech J. of Food Sciences, 21:107-110.

6. Tianchai N, Petlada D, Sukon T (2012) Antibacterial activity of pomegranate fruit peels and arils. *ScienceAsia,* 38: 319–322.

7. Sanogo R, Germano MP, D'Angelo V, Biasini T, De Pasquale R, Catania S (2006) Evaluation of the antioxidant properties and bioavailability of free and bound phenolic acids from Trichilia emetica Vahl. *Journal of Ethnopharmacol* 105: 368-373.

8. Bauer AW, Kirby WM, Sherris JC, Jurck M (1996) Antibiotic susceptibility testing by a standard single disc method. Am J. Clin. Pathol. 451: 493-496.

9. Berahou A, Auhmani A, Fdil N, Benharref A, Jana M, Ghadi CA (2007). Antibacterial activity of Quercus ilex bark's extracts. Journal of Ethnopharmacology. pp.427-428.

10. Amoo SO, Finnie JF, van Staden J (2009) In vitro pharmacological evaluation of three *Barleria* species. J. Ethanopharmacol., 121: 274-277.

11. Hammer KA, Carson CF, Riley TV (1996) Susceptibility of transient and commensal skin flora to the essential oil of Melaleuca alternifolia (tea tree oil). American Journal of Infection control. 24: 186-9.

12. Al-Zoreky NS (2009) Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. International Journal of Food Microbiology, 134:244-248.

13. Senhaji O, Faid M, Elyachioui M, Dehhaoui M (2005) Antifungal activity of different cinnamon extracts. Mycologie Medicale 15 (4): 220-229

14. Andrews JM (2001) Determination of minimum inhibitory concentration. Journal of Antimicrobial chemotherapy (Suppl-1), 48:5-16.

15. Sarker SD, Nahar L, Kumarasamy (2007). Microtiter plate-based antibacterial assay incorporating resazurin as an indicator of cell growth and its application in the in vitro antibacterialscreening of phytochemicals. Methods. 42 (4): 321-324.

16. Lee SE, Hwang HJ, Ha JS, Jeong HS, Kim JH (2003) Screening of medicinal plant extracts for antioxidant activity. *Life Sci*., 73: 167-179.

17. Beckman J (2007) Antimicrobial properties of pomegranate fruit. A thesis. Willamette University.

18. Vasconcelos LCS, Sampaio FC, Sampaio MCC, Pereira MSV, Higino JS, Peixoto MHP (2006) concentration minimale inhibitrice de l'adhésion de *Punica granatum* Linn (grenade) gel contre *S. mutans, S.* et *C. mitis albicans*. Braz Dent J, 17: 223-227.

19. Vasconcelos LC, Sampaio MC, Sampaio FC, Higino JS (2003) Utilisation de *Punica granatum Linn* comme un agent antifongique contre les candidoses associé à stomatite prothétique. Mycoses 46: 192-196.

20. Melendez PA, Capriles VA (2006) Antibacterial properties of tropical plants from Puerto Rico. Phytomedicine. 13: 272-276.

21. Klervi LL (2005) Connaissance chimiotaxonomique du genre Turbinaria et etude des composes de défense de différents espèces de Sargassacées des Iles Salmon (Pacific sud). pp.210.

22. Erdogrul OT (2002) Antibacterial activities of some plant extracts used in Folk medicine. Pharm. Biol. 40, 269-273.

23. Machado TB, Leal ICR, Amaral ACF (2002) Antimicrobial ellagitannin of Punica granatum fruits. J Braz Chem Soc, 13(5): 606–610.

24. Voravuthikunchai S, Lortheeranuwat A, Jeeju W, Sririrak T, Phongpaichit S, Supawita T (2004) plantes médicinales efficaces contre enterohaemorrhagica *Escherichia coli* O157: H7. Ethnopharmacol J, 94: 49-54.

25. Pfaller MA, Messer SA, Boyken L, Hollis RJ, riz C, Tendolkar S, Hollis RJ, Diekema DJ (2004) *dans* les activités *in vitro* de voriconazole, le posaconazole et le fluconazole contre 4169 isolats cliniques de *Candida* spp. et *C.neoformans* recueillies en 2001-2002 dans le programme de surveillance mondiale antifongique ARTEMIS. Diagn Microbiol Infect Dis, 48: 201-205.

26. Anibal PC (2010) Estudo da Composição química e ação inibitória dos extratos obtidos de *Punica granatum* L. (Roma) sobre *Candida* spp. Piracicaba, Brésil. (D.Sc. thèse. Faculdade de Odontologia de Piracicaba. UNICAMP. pp. 112.

27. Duman AD, Ozgen M, Dayisoylu KS, Erbil N, Durgac C (2009) L'activité antimicrobienne de six grenade *(Punica granatum* L.) variétés et leur relation avec certaines de leurs caractéristiques pomologiques et phytonutriments. Molécules, 14: 1808-1817.

28. Duraipandiyan V, Ayyanar M, Ignacimuthu S (2006) L'activité antimicrobienne de certaines plantes utilisées par ethnomédicales Paliyar tribu du Tamil Nadu, en Inde. BMC Complet Altern Med, 6: 1-7.

29. Navarro V, Villarreal ML, Rojas G, Lozoya X (1996) l'évaluation antimicrobienne de certaines plantes utilisées en médecine traditionnelle mexicaine pour le traitement des maladies infectieuses. Ethnopharmacol J, 53: 143-147.

30. Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D (2007) Antioxydant, activités antipaludiques et antimicrobiennes de fractions riches en tanins, acides phénoliques et ellagitannins de *Punica granatum* L. Planta Med, 73: 461-467.

31. Catão RMR, Antunes RMO, Arruda TA, Pereira MSV, Higino JS, juge Alves, Passos MGVM, Santos VL (2006) Atividade antimicrobiana *in vitro* faire extrato etanólico de *Punica granatum Linn.* (Roma) sobre isolados ambulatoriais de *Staphylococcus aureus*. Rev Bras Anal Clin, 38: 111-114.

Published in the Russian Federation
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN 2409-1332
Vol. 7, Is. 1, pp. 12-26, 2015

DOI: 10.13187/ejmb.2015.7.12
www.ejournal8.com

UDC 631.531.027: 635.63

Electrochemically Activited Water: Biophysical and Biological Effects of Anolyte and Catholyte Types of Water

[1*] Georgi Gluhchev
[2] Ignat Ignatov
[3] Stoil Karadzhov
[4] Georgi Miloshev
[5] Nikolay Ivanov
[6] Oleg Mosin

[1] Institute of Information and Communication Technologies
Bulgarian Academy of Sciences, Bulgaria
Assoc. Professor, Ph. D.
1113, Sofia, Acad. G. Bonchev Street, 2
*E-mail: gluhchev@iinf.bas.bg
[2] The Scientific Research Center of Medical Biophysics (SRC MB), Bulgaria
Professor, D. Sc., director of SRC MB
1111, Sofia, N. Kopernik street, 32
E-mail: mbioph@dir.bg
[3] Bulgarian Association of Activated Water, Bulgaria
Professor, D. Sc.
1619, Sofia, Kutuzov blvd., 39
[4] Institute of Molecular Biology, Bulgarian Academy of Science, Bulgaria
Assoc. Professor, Ph. D.
1113, Sofia, Acad. G. Bonchev Street, 21
[5] Institute of Information and Communication Technologies
Bulgarian Academy of Sciences, Bulgaria
Assis. Professor, Dipl. Eng.
1113, Sofia, Acad. G. Bonchev Street, 2
[6] Moscow State University of Applied Biotechnology, Russian Federation
Senior research Fellow of Biotechnology Department, Ph. D.
103316, Moscow, Talalihina ulitza, 33
E-mail: mosin-oleg@yandex.ru

Abstract

This article outlines the results on the antimicrobial action of electrochemically activated water solutions (anolyte/catholyte), produced in the anode and cathode chamber of the electrolitic cell. Under laboratory conditions the cell culture and suspensions of classical swine fever (CSF) virus were treated with the anolyte. After inoculating them with cell cultures, the viral presence

(the presence of viral antigen) was measured using the immunoperoxidase technique. It was found that anolyte did not affect the growth of the cell culture PK-15; viral growth during the infection of a cell monolayer with a cell culture virus was affected in the greatest degree by the anolyte in 1:1 dilution and less in other dilutions; whereas the viral growth at the infection of a cell suspension with the CSF virus was affected by the anolyte in dilution 1:1 in the greatest degree, and less by other dilutions; viral growth at the infection with a virus in suspension of the cell monolayer was affected by the anolyte in all dilutions. Unexpectedly, the stronger biocidal effect of the catholyte was observed when a strain of *E. coli DH5* was treated by the anolyte and catholyte, respectively. In order to provide additional data about the antiviral activity of the electrochemically activated water and the distribution of H_2O molecules according to the energies of hydrogen bonds, the non-equilibrium energy spectrum (NES) and differential non-equilibrium energy spectrum (DNES) of the anolyte and catholyte were measured.

Keywords: anolyte; catholyte; *E. coli DH5*; CSF virus; NES; DNES.

Introduction

The phenomenon of electrochemical activation of water (EAW) is a set of electrochemical and electrical processes occur in water in the electric double layer (EDL) type of electrodes (anode and cathode) with non-equilibrium electric charge transfer through EDL by electrons under the intensive dispersion in water the gaseous products of electrochemical reactions [1]. In 1985 EAW was officially recognized as a new class of physical and chemical phenomena.

As a result of the treatment of water by a constant electric current at electric potentials equal to or greater than the decomposition potential of water (1,25 V), water goes into a metastable state, accompanied by electrochemical processes and characterized by the abnormal activity levels of electrons, the redox potential, and other physical-chemical parameters (pH, E_h, ORP) [2].

The main stage of electrochemical treatment of water is the electrolysis of water or aqueous solutions with low mineralization as aqueous solutions of 0,5–1,0 % sodium chloride (NaCl) [3], which occurs in the electrolysis cell, consisting of the cathode and the anode separated by a special semipermeable membrane (diaphragm) which separates water to alkaline fraction – the catholyte and acidic fraction – the anolyte (Figure 1). When the passing of electric current through water, the flow of electrons from cathode as well as the removal of electrons from water at the anode, is accompanied by series of redox reactions on the surface of the cathode and anode [4]. As the result, new elements are being formed, the system of intermolecular interactions, as well as the composition of water and the water structure are changed [5, 6].

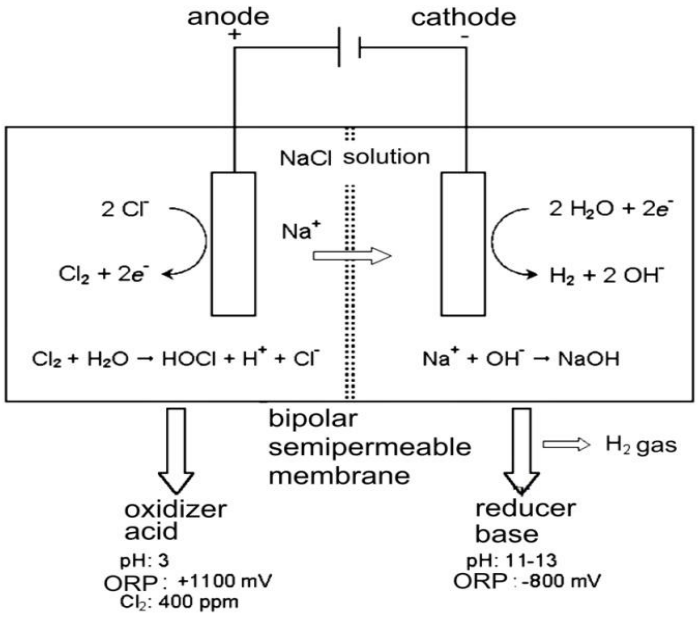


Figure 1. The diaphragm electrolysis method for the preparation of acid (anolyte) and alkali (catholyte) solutions via the electrochemical activation of sodium chloride

The products of electrode reactions are the neutralized aqueous admixtures, gaseous hydrogen and oxygen generated during the electrolytic destruction of H_2O molecules, metal cations (Al^{3+}, Fe^{2+}, Fe^{3+}) in the case of metal anodes made of aluminum and steel, the molecular chlorine. Wherein at the cathode is generated the gaseous hydrogen, and at the anode – oxygen. Water also containes a certain amount of hydronium ions (H_3O^+) depolarizing at the cathode with formation of the atomic hydrogen:

$$H_3O^+ + e^- \rightarrow H + H_2O, \quad (1)$$

In an alkaline environment there occurs the disruption of H_2O molecules, accompanied by formation of the atomic hydrogen and hydroxide ion (OH^-):

$$H_2O + e^- \rightarrow H + OH^-, \quad (2)$$

The reactive hydrogen atoms are adsorbed on the surfaces of the cathode, and after recombination are formed the molecular hydrogen H_2, released in the gaseous form:

$$H + H \rightarrow H_2, \quad (3)$$

At the same time at the anode is released the atomic oxygen. In an acidic environment, this process is accompanied by the destruction of H_2O molecules:

$$2H_2O - 4e^- \rightarrow O_2 + 4H^+, \quad (4)$$

In an alkaline environment, the source of oxygen source is OH^- ions, moving under the electrophoresis from the cathode to the anode:

$$4OH^- \rightarrow O_2 + 2H_2O + 4e^-, \quad (5)$$

The normal redox potentials of these reactions compiles +1,23 V and +0,403 V, respectively, but the process takes place in certain conditions of electric overload.

The cathodes are made of metals that require high electrical voltage (lead, cadmium), allow to generate the reactive free radicals as Cl*, O*, OH*, HO_2*, which react chemically with other radicals and ions.

In bulk oxidative processes a special role plays products of electrolysis of water – oxygen (O_2), hydrogen peroxide (H_2O) and hydrochlorine acid (HClO). During the electrolysis, an extremely reactive compound formed – H_2O_2, the formation of which occurs due to the hydroxyl radicals (OH*), which are the products of the discharge of hydroxyl ions (OH^-) at the anode:

$$2OH^- \rightarrow 2OH^* \rightarrow H_2O_2 + 2e^-, \quad (6)$$

where OH^* – the hydroxyl radical.

The chlorine-anion is transformed to Cl_2:

$$2Cl^- \rightarrow Cl_2 + 2e^-, \quad (7)$$

Gaseous Cl_2 forms highly active oxidants: Cl_2O; ClO_2; ClO^-; HClO; Cl^*; HO_2^*. The parameters of pH, the redox potential, ORP and the electrical conductivity of the anolyte/catholyte depend on different factors including the ratio of water volumes in the two electric chambers, the material of electrodes, NaCl concentration, the temperature, electric voltage and processing time [7,8].

The electrolysis cell can be regarded as a generator of the above mentioned products, some of them, entering into the chemical interaction with each other and water impurities in the interelectrode space, providing additional chemical treatment of water (electrophoresis, electroflotation, electrocoagulation) [9]. These secondary processes do not occur on the electrode surface, but in the bulk water. Therefore, in contrast to the electrode processes they are indicated as the volume processes. They generally are initiated with increasing the temperature of water during the electrolysis process and with increasing the pH value.

As a result of the cathode (catholyte) treatment water becomes alkaline: its ORP decreases, the surface tension is reduced, decreasing the amount of dissolved oxygen in water, increases the concentration of hydrogen, hydroxyl ions (OH^-), decreases the conductivity of water, changes the structure of hydration shells of ions [10]. By external characteristics the catholyte – is a soft, light, with an alkaline taste liquid, sometimes with white sediment; its pH = 10–11, ORP = -200...-800 mV.

On physical and chemical parameters the catholyte has the significantly enhanced electron-donating properties, and getting into the physiological fluids of an organism can enhance the electron-background for a few tens of millivolts [11]. The catholyte reportedly has antioxidant, immunostimulating, detoxifying properties, normalizing ORP, metabolic processes (increases the ATP synthesis, modification of enzyme activity), stimulates the regeneration of tissues, increases the DNA synthesis and stimulates the growth and division of cells by increasing the mass transfer of ions and molecules across the cell membrane, improves trophic processes in tissues and blood

circulation [12]. It was also reported that catholyte with the redox potential at -700…-100 mV favorizes the development of anaerobs, whereas the anolyte with the redox potential at +200…+750 mV supports the growth of aerobs [13]. The antibacterial effect of the catholite is differentiated: the bactericidal effect is appeared relative to Enterobacteriaceae, resistant to it are enterococci and the group of streptococci B, and against Gram-negative microorganisms – only the bacteriostatic effect [14].

The electrochemically activated solutions of the catholite, depending on the strength of the transmitted electric current may be of several types:

C – alkaline catholyte (pH > 9,0; ORP = -700…-820 mV), the active components – NaOH, O_2, HO_2^-, HO_2*, OH^-, OH*, HO_2^-, O_2;

CN – neutral catholyte (pH = 9,0; ORP = -300…-500 mV), the active components – O_2, HO_2^-, HO_2*, H_2O_2, H^+, OH^-.

As a result of the anode (anolyte) treatment water becomes acid reaction, the ORP increases slightly, the surface tension is slightly reduced, the conductivity increases, the amount of the dissolved oxygen and chlorine in water also increases, whereas the amount of hydrogen decreases [15]. The anolyte is a brownish, acid, with a characteristic odor and taste the liquid with a pH = 4–5 and ORP = +500…+1100 mV. The specific anolyte toxicity when being administered in the stomach and applying to the skin refers to the class 4 of harmful substances according to the Russian Standard GOST 12.1.007-76, with the minimal toxicity within this class. When being inhaled the anolyte with oxidants content of 0,02 % and total mineralization 0,25–0,35 % does not irritate the respiratory system and mucous membranes of the eyes. When introduced into the organism, the anolyte has no immunotoxic action and increased chromosomal aberrations in the bone marrow cells and other tissues, and it has no cytogenetic activity. When being heated to 50 °C the bactericidal activity of the anolyte is increased by 30–100 % [16].

The electrochemically activated solutions of the anolyte are divided into four main types:

A – acidic anolyte (pH < 5,0; ORP = +800…+1200 mV), the active components – HClO, Cl_2, HCl, HO_2*;

AN – neutral anolyte (pH = 6,0; ORP = +600…+900 mV), the active components – HClO, O_3, HO^-, HO_2*;

ANK – neutral anolyte (pH = 7,7; ORP = +250…+800 mV), the active components – HClO, ClO^-, HO_2^-, H_2O_2, O_2, Cl^-, HO*;

ANKD – neutral anolyte (pH = 7,3; ORP = +700…+1100 mV), the active components – HClO, $HClO_2$, ClO^-, ClO_2*, HO_2*, H_2O_2, O_2, O_3, Cl^-, HO^-, O*.

The anolyte has antibacterial, antiviral, antifungal, anti-allergic, anti-inflammatory, antiedematous and antipruritic effect, may be cytotoxic and antimetabolite action without harming the human tissue cells [17]. The biocide elements in the anolyte are not toxic to somatic cells, as represented by oxidants, such as those ones produced by the cells of higher organisms.

Studies on the virucidal effect of the anolyte are rare and insufficient, basically on the possibilities of applying the anolyte in the implementation of effective control of viral diseases in humans and animals and especially on particularly dangerous viral infections, as staphylococcal Enterotoxin-A [18]. One of them is the classical swine fever (CSF), prevalent in different regions of the world and inflicting heavy economic losses. It is caused by enveloped viruses belonging to the genus *Pestivirus* of the family *Flaviviridae* [19, 20]. The resistance and inactivation of the virus of CSF virus is a subject of extensive research. Although it is less resistant to external stresses other than non-enveloped viruses, it retains its virulence for a long period of time: in frozen meat and organs – from a few months up to one year; in salted meat – up to three years; in dried body fluids and excreta – from 7 to 20 days. In rotting organs it dies for a few days and in urine and faeces – for approx. 1–2 days. In liquid fertilizer it can withstand 2 weeks at 20 °C, and over 6 weeks at 4 °C. Its thermal resistance may vary depending on the strain type, but the inactivation is dependent mostly on the medium containing the virus. Although the CSF virus loses its infectivity in cell cultures at 60 °C for 10 min, it is able to withstand at least 30 min at t = 68 °C in defibrinated blood. It is relatively stable at pH = 5–10, and the dynamic of the inactivating process below pH = 5 depends on the temperature.

According to J.A. Sands [21] and U.S. Springthorpe [22], the effective disinfection of viruses whose infectivity is associated with the elements of the casing is achieved by disinfectants dissolving fats, surfactants, disinfectants or fatty acids, organic solvents (ether and chloroform),

detergents, proteases, and common disinfectants. It is believed that 2 % solution of sodium hydroxide is most suitable for the disinfection of spaces contaminated with them. It is thought that to achieve the effective electrochemical disinfection it is necessary to irreversibly damage the RNA [23].

Investigations conducted by other authors [24] were carried out with *E. coli*, using as a desinfectant the anolyte with ORP equal or greater than +1100 mV and pH = 5,5, obtained via electrolysis of diluted NaCl solution on planktonic cells of a strain of *E. coli JM109*. It was demonstrated that within 5 min of influence all cells were inflated and burst. Also, it was occurred a full destruction of proteins, DNA and RNA. Supposedly the anolyte enters the cells provoking structural and functional damages on the cell's membrane and cell's wall.

Similar research was performed by S.V. Kumar et al. [25]. They evaluated the inactivation efficacy of anolyte of pH = 2,7 and ORP = + 1100 mV on *Escherihia coli O157:H7*, *Salmonela enteritidis* and *Lusteria monocytogenes*. As it was demonstrated on five strains of *E. coli E06* (milk), E08 (meat), E10 (meat), E16 (meat) and E22 (calf feces), all patogens were significantly reduced (7,0 logCFU/ml) or fully destroied (8,0 logCFU/ml) after 2 to 10 min inactivation by the anolyte in the temperature range from 4 ^{o}C to 23 ^{o}C. Supposedly, the low pH value of the anolyte makes sensitive the outer cell's membrane, thus facilitating HClO to enter the cell and further destroy it.

However, it should be noted that the pharmacological studies of electrochemically activated solutions of water and their virucidal effects and toxicity have not yet been completely evaluated. Therefore, the purpose of this research was to study the antiviral virucidal effect: 1) of the anolyte in different dilutions on classical swine fever virus in cell culture and organ suspensions; 2) of the anolyte/catholyte on a strain of *E. coli DH5a*, and 3) to determine how the virocidal effect relates to local maximums in NES-spectra of the anolyte and catholyte*.

Material and Methods

The studies of the antiviral activity of the anolyte were performed at the National Reference Laboratory of Classical and African Swine Fever, section "Exotic and Especially Dangerous Infections" of the National Diagnostic and Research Veterinary Medical Institute (Sofia, Bulgaria). Experiments were conducted with the anolyte obtained by the electrolysis apparatus "Wasserionisierer Hybrid PWI 2100" equipped with four titanium electrodes coated with platinum. 0,3 % solution of chemically pure sodium chloride (NaCl) in distilled water was used for the electrolysis. The obtained anolyte had pH = 3,2 and ORP = +1070 mV. The interaction of the anolyte with the virus suspension was carried out at a temperature of 22 ^{o}C.

A cell culture of porcine origin sensitive to the CSF virus was used: a continuous cell line was PK-15. Contamination of cell cultures was carried out with the standard cell culture test virus 2,3 (Bulgaria) with a cell titre 107,25 $TCID_{50}$/ml and organ suspension of internal organs (spleen, kidney, lymph node) of wild boar originating from the last outbreak of CSF in Bulgaria in 2009. The titer of the established virus in the suspension was $10^{4,75}$ $TCID_{50}$ ml.

To establish the virucidal activity of the anolyte, the inocula prepared for contamination of cell culture (cell culture virus) were treated with the following dilutions of the anolyte in sterile distilled water: 1:1 (50 %), 1:2 (33,33 %), 1:3 (25 %), 1:4 (20 %). These dilutions were mixed with inocula in proportion 1:1 (100 μl of the CSF virus suspension and 100 μl of the appropriate anolyte concentration). The time of action was conformed to the period, at which it was methodologically necessary to "capture" any viral presence in the cell culture. Upon the infection of a cell monolayer, the mixture was removed after the end of the exposure period of 1 h. Upon the infection of a cell suspension, the mixture, otherwise, was not removed.

To establish the virucidal activity of the anolyte on the CSF virus in the suspension, a different dilution was used: the inoculum was mixed directly with the concentrated anolyte in anolyte-inoculum ratios 1:1; 3:1; 7:1 and 15:1 respectively. Since it is known that the growth of the virus does not cause a cytopathic effect, therefore, for demonstration of its presence, immunoperoxidase plates dyeing were used. The cells were fixed and the viral antigen was detected after binding to a specific antibody labeled with peroxidase. The organs exude 1 cm^3 of tissue,

* Such a dependence was established between the local maximum (-0,1387 eV; 8,95 μm) in the NES-spectrum of the catholyte that suppresses the development of tumor cells (Ignatov & Mosin, 2014).

which was homogenized in a mortar with 9 ml of the cell culture medium containing antibiotics, in order to obtain 10 % of organ suspension. Sterile sand was added to improve the homogenization. The samples were kept at room temperature for 1 h, after that they were centrifuged for 15 min at 2500 g. The supernatant was used to infect the cells. In case of cytotoxic effect, the parallel dilutions of the homogenates were prepared in proportions 1:10 and 1:100. From the suspensions into multi well (24-well) plates were added 200 μl of the inoculums with coverage of 50–80 %. Cell cultures were incubated at t = 37 °C for 1 h in order to "capture" an eventual virus if presented, then they were rinsed once with PBS and fresh media were added. Alternatively, the plate was filled directly (cell suspension), since the preliminary studies had found that the anolyte did not induce a cytotoxic effect.

The cell cultures were incubated for 72–96 h at t = 37 °C in a CO_2 incubator. The procedure with preparation of the positive and negative control samples was similar. The positive control sample was a reference strain of the CSF virus. The immunoperoxidase technique with using a horseradish peroxidase was used for the enzymatic detection of antigen-antibody complexes in cell cultures. The fixation of the plates was carried out thermally for ~3 h at t = 80 °C in a desiccator. In the processing was used a primary monoclonal antibody C 16, diluted in proportion 1:50, and secondary antibody RAMPO, diluted in proportion 1:50. For the immunoperoxidase staining was used 3 % H_2O_2 and AEC (dimethylformamide and 3-amino-9-ethylcarbazole) in acetate buffer. The antibody-antigen complex was visualized by the peroxidase reaction with the substrate.

A polymerase chain reaction (PCR) to amplify the segments of the RNA was carried out in real time scale. The cell culture and organ suspensions were examined for the presence of the CSF viral genome by the PCR in real time (real-time RT-PCR, one step, TagMan), one-step according to Protocol of the Reference Laboratory for CSF of EU. For RNA extraction was used the test QIAamp Vital RNA Mini Kit, Qiagen Hilden (Germany). The initial volume of the biological material was 140 μl, and the elution volume – 60 μl.

For amplification of PCR was used the test Qiagen OneStep RT-PCR Kit in a total volume of 25 μl, and template volume of 5 μl. In the PCR were used primers A 11 and A14, and probe TaqMan Probe–FAM–Tamra.

PCR studies were carried out with a thermo cycler machine "Applied Biosystems 7300 Real Time PCR System" with the temperature control for reverse transcription at t = 50 °C – 30:00 min, inactivation of reverse transcriptase and activation of Taq at t = 95 °C – 15:00 min, denaturation at t = 95 °C – 00:10 min, extension at t = 60 °C – 00:30 min for 40 cycles.

The second study on the antimicrobial activity of the anolyte/catholyte was performed at the Institute of Molecular Biology of the Bulgarian Academy of Sciences (BAS). The two electrochemical solutions were prepared with using the Activator-I, developed at the Institute of Information and Communication Technologies at BAS. For this, drinking water without aditional quantity of NaCl was used. This led to pH = 3,0 and ORP = +480 mV for the anolyte, and pH = 9,8 and ORP = -180 mV for the catholyte.

Bacterial strain used in these experiments was *E. coli* DH5α with genotype: *fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*.

The Colony Forming Units (CFU) technique was used in this study to assess cellular viability. The conditions for the bacterial cultures growth were as described in our previous paper [26]. The bacterial cells were cultivated on the LB-medium (pH = 7,5) with 1 % bactotryptone; 0,5 % yeast extract; 1,0 % NaCl at t = 37 °C. After overnight cultivation of bacteria 100 μl samples of culture liquids were taken, centrifuged for 1 min at 10000 g and the pellet of bacterial cells was resuspended in 100 μl of the anolyte or the catholyte. As control samples were used the bacterial samples, re-suspended in non-electroactivated water. Different dilutions of cells were spread on LB-agar Petri plates. After the overnight incubation at t = 7 °C the appeared bacterial colonies were counted. The viable cells were calculated as a percentage from the CFU. The CFU obtained from culture liguids treated with non-electrochemically activated water were accepted as 100 %.

The NES method was used for the estimation of energy of hydrogen bonds of the anolyte, catholyte and deionized water in order to make a supposition about the spectrum characteristics. The device measures the angle of evaporation of water drops from 72 ° to 0 °. As the main estimation criterion was used the average energy ($\Delta E_{H...O}$) of hydrogen O...H-bonds between individual H_2O molecules in water's samples. The NES-spectrum of water was measured in the

range of energy of hydrogen bonds 0,08–0,387 eV or λ = 8,9–13,8 μm with using a specially designed computer program.

Results and Discussion

Research into the effects of electro-activated aqueous NaCl (anolyte) on the CSF virus

As shown in Figure 2 the cytoplasm of cells infected by the CSF virus was stained in the dark reddish brown color (positive reaction), whereas in the uninfected cells it was colorless. That indicates on the presence of viral antigen in the samples.

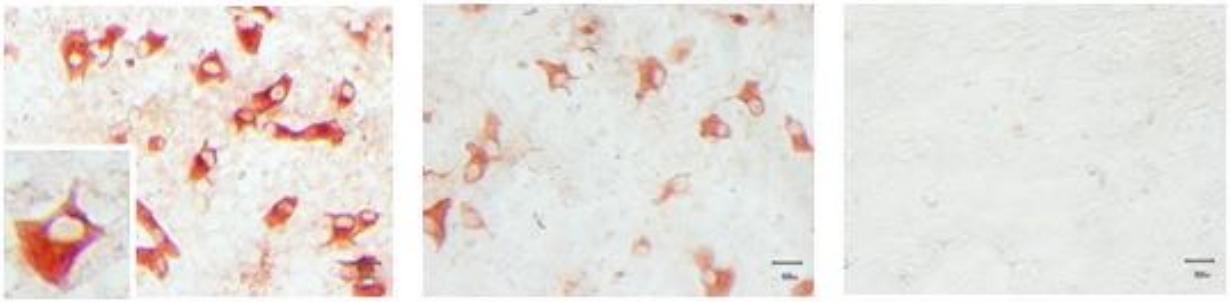


Figure. 2. The established presence of viral antigen in cell cultures (left) and a negative control (right)

Table 1 summarizes the results of different experiments of the virucidal action of the anolyte on the cell culture suspension of the CSF virus upon infecting cell monolayer PK-15. As is shown in Table 1, upon treatment of the viral inoculum with the anolyte in a 1:1 dilution, there was no viral growth in the four infected wells of the plate, upon 1:2 dilution there was no growth in two of the wells, the other two were reported as positive. Upon treatment with the anolyte at dilutions 1:3 and 1:4, the result was identical: no growth in one of the contaminated wells of the plate, and poor growth – in the other three. The results obtained by infection of CSF virus a cell monolayer and cell suspension were identical.

Table 2 summarizes the results of studies aimed at the evaluation of the virucidal effect of the anolyte on organ suspension containing CSF virus upon infecting a cell monolayer PK-15 with the virus. According to the data, upon treatment of the CSF viral inoculum (organ suspension) with the anolyte in all dilutions, there was no viral growth in the four infected wells of the plate.

Table 1: The virucidal action of the anolyte on cell culture suspensions of the CSF virus upon infecting cell monolayer PK-15

Contamination of CC with:	Dilutions of anolyte (100 μl)	Total volume of the inoculum (μl)	Concentration of anolyte in %	Number of wells:	Result: positive/ negative:
Virus 200 μl	–	200	–	4	4/0
Virus 100 μl	1:1	200	25	4	0/4
Virus 100 μl	1:2	200	16,51	4	2/2
Virus 100 μl	1:3	200	12.5	4	3/1
Virus 100 μl	1:4	200	10	4	3/1

Table 2: The virucidal action of the anolyte on organ suspensions containing CSF virus upon infecting cell monolayer PK-15

Contamination of CC with:	Dilutions of anolyte (100 µl)	Total volume of the inoculum (µl)	Concentration of anolyte in %	Number of wells:	Result: positive/ negative:
Virus 200 µl	–	200	–	4	4/0
Virus 100 µl	1:1	200	50	4	0/4
Virus 50 µl	3:1	200	75	4	0/4
Virus 25 µl	7:1	200	87	4	0/4
Virus 12,5 µl	15:1	200	94	4	0/4

Evidently, the anolyte has a destructive influence on the envelope of the CSF virus, wherein the main antigens (proteins) are localized. Studies of the viral inocula used in the tests by means of polymerase chain reaction (PCR) in real time demonstrated the presence of a genome (RNA) in them, also after the treatment with the anolyte. Some shortening of the time was proved (the decreased number of amplification cycles), required for the formation of a fluorescent signal, respectively, a positive reaction for a genome, closely correlated with the exposure under the treatment of the viral inocula. The longer the exposure of processing with the anolyte, the sooner the presence of the viral RNA in the PCR was detected. According to one of our co-authors (Stoil Karadzhov), this may serve as an indirect indication that anolyte destroys the CSF virus envelope, which, in its turn, facilitates the extraction of viral RNA and its more rapid reading by the fluorescent signal. However, there is still no sufficient convincing evidence on the impact of different concentrations of the anolyte on CSF viral particles. The analogous experiments carried out by Russian and German researchers were carried out mainly with the concentrated anolyte. The maximum virucidal effect detected in those experiments confirmed a strong virucidal action of the electrochemically activated aqueous solution of NaCl on the CSF virus. The difference in the results evidently is due to the use of lower concentrations of NaCl in our experiments. We attributed essential significance to the fact that we determined the concentration limit (25 %) of the well demonstrated by the virucidal activity. In this aspect the further studies on reducing the time of the virucidal action, and the conducting of experiments in the presence of biofilms which protect viruses would be promising.

Research into the antibacterial effects of the anolyte and catholyte on a strain of E. coli

In order to assess the effect, if any, of the electrochemically activated water solutions (catholyte/anolyte) on bacterial cells we treated the cultures of a strain of *E. coli DH5a* by the catholyte. After the treatment of bacterial cells the colonies appearing on the plates with 2 % agar were obtained, produced by survived cells, which were further counted by the CFU method. Therefore, the number of colonies was presented on Figure 3 as a percentage of viable cells. It can be seen from Figure 3 that bacterial cells of *E. coli DH5a* treated with the catholyte hardly survived the treatment with only approximately 15 % of the cells being survived. This clearly shows that the electrochemically activated water produced from the cathode possesses a strong bacteriocidal activity on the strain of *E. coli DH5a*.

Notably, the anolyte also showed slight antibacterial effect. Thus, approximately, 73 % of the bacterial cells of *E. coli DH5a* survived the electrochemical treatment with the anolyte. In summary, it is assumed that both types of the electrochemically activated water solutions (catholite/anolyte) possess antibacterial effect on the strain of *E. coli DH5a*, however it is obvious that the catholyte has a stronger bacteriocide effect than the anolyte.

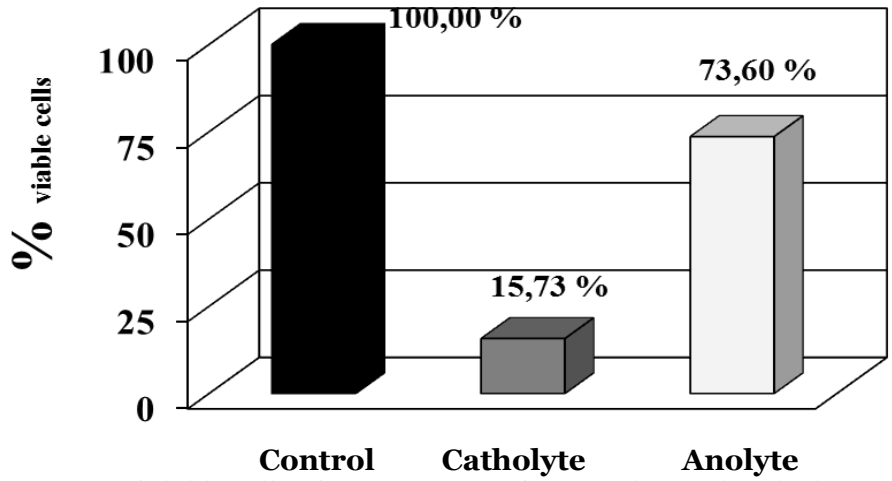


Figure 3. Percentage of viable cells of *E. coli DH5a* after the electrochemical treatment with the catholyte and anolyte relative to the non-electrochemically activated water.

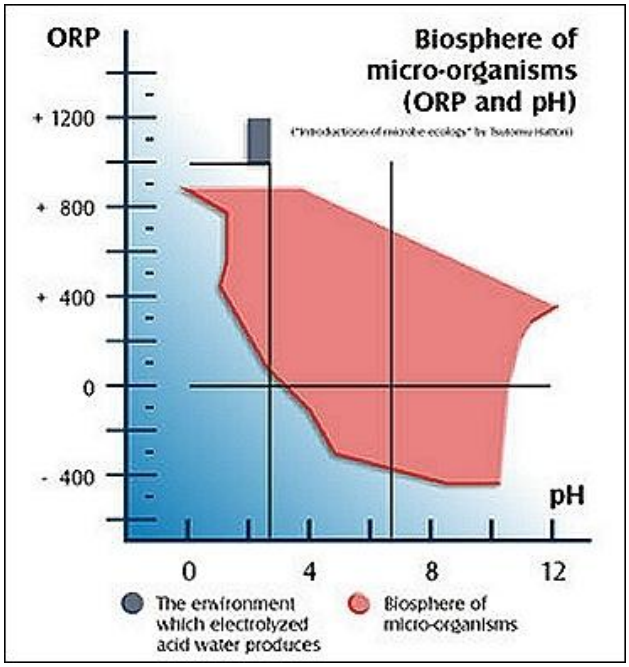


Figure 4. The dependence between the acidity and basicity (pH) of electrochemically activated solution of NaCl and the oxidation-reduction potential (ORP) on the biosphere of microorganisms.

Figure 4 shows the dependence between the acidity and basicity (pH) of electrochemically activated solution of NaCl and the oxidation-reduction potential (ORP). The pH value within the interval from 3 to 10 units and the ORP within the interval from -400 mV to +900 mV characterize the area of the biosphere of microorganisms. Outside these ranges of pH and ORP the microorganisms will hardly survive. The disinfecting effect in this case is strengthened by the residual chlorine in electrochemically activated solution of NaCl, destructing unsaturated fatty acids, phospholipids and protein in the cell membrane.

NES and DNES methods in spectral analysis of the anolyte and catholyte

Other method for obtaining useful information about the structural changes in water and the average energy of hydrogen bonds is the measuring of the energy spectrum of the water state. It was established experimentally that at evaporation of water droplet the contact angle θ decreases

discretely to zero, whereas the diameter of the droplet changes insignificantly [27]. By measuring this angle within a regular time intervals a functional dependence f(θ) can be determined, which is designated as "the spectrum of the water state" (SWS) [28]. For practical purposes by registering the SWS it is possible to obtain information about the averaged energy of hydrogen bonds in an aqueous sample. For this purpose the model of W. Luck was used, which consider water as an associated liquid, consisted of O–H…O–H groups [29]. The major part of these groups is designated by the energy of hydrogen bonds (-E), while the others are free (E = 0). The energy distribution function f(E) is measured in electron-volts (eV^{-1}) and may be varied under the influence of various external factors on water as temperature and pressure.

For calculation of the function f(E) experimental dependence between the water surface tension measured by the wetting angle (θ) and the energy of hydrogen bonds (E) is established:

$$f(E) = bf(\theta) / [1 - (1 + bE)^2]^{1/2}, \qquad (8)$$

where $b = 14{,}33\ eV^{-1}$; $\theta = \arccos(1 - bE)$

The energy of hydrogen bonds (E) measured in electron-volts (eV) is designated by the spectrum of energy distribution. This spectrum is characterized by non-equilibrium process of water droplets evaporation, thus the term "non-equilibrium energy spectrum of water" (NES) is applied.

The difference *Δf(E) = f (samples of water) – f (control sample of water)* – is designated as the "differential non-equilibrium energy spectrum of water" (DNES) [30].

The DNES-spectrum measured in milielectron volts (0,001 eV) is a measure of changes in the structure of water as a result of external factors. Figure 5 shows the characteristic NES-spectrum of deionized water made from 25 independence measurements performed in a period of one year.

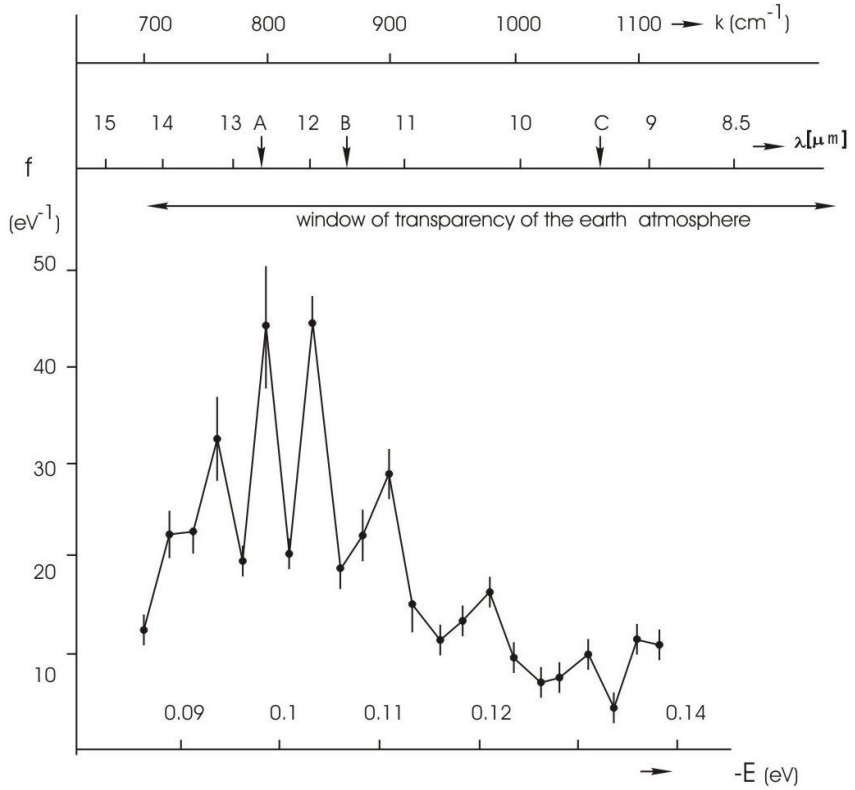


Figure 5. NES-spectrum of deionized water (chemical purity – 99,99 %; pH – 6,5–7,5; total mineralization – 200 mg/l; electric conductivity – 10 μS/cm). The horizontal axis shows the energy of the H…O hydrogen bonds in the associates – E (eV). The vertical axis – energy distribution function – f (eV^{-1}). k – the vibration frequency of the H–O–H atoms (cm^{-1}); λ – wavelength (μm)

The average energy ($\Delta E_{H...O}$) of hydrogen H...O-bonds among individual molecules H_2O was calculated for the catholyte and analyte by NES- and DNES-methods. We studied the distribution of local maximums in catholyte and anolyte solutions. The local maximum for catholyte in the NES-spectrum was detected at -0,1285 eV, for anolyte – at -0,1227 eV, and for the control sample of deionized water – at -0,1245 eV. The calculations of $\Delta E_{H...O}$ for the catholyte with using the DNES method compiles (-0,004±0,0011 eV) and for anolyte (+1,8±0,0011 eV). These results suggest the restructuring of $\Delta E_{H...O}$ values among individual H_2O molecules with a statistically reliable increase of local maximums in DNES-spectra of the catholyte and anolyte (Table 3).

For the catholyte the biggest local maximum was detected at -0,1387 eV, or at 8,95 μm. In 1992 A. Antonov performed experiments with the impact of different types of water on tumor mice cells. It was detected a decrease in the NES-spectrum compared with the control sample of cells from healthy mice. There was also a decrease of the local maximum at -0,1387 eV, or 8,95 μm in DNES-spectra. Notably, the local maximum at 8,95 μm was detected with the negative value. It should be noted that for the catholyte the local maximum in the DNES-spectrum was detected with the positive value at +133,3 eV^{-1}.

For the catholyte the biggest local maximum in the DNES-spectrum was detected at -0,1312 eV, or 9,45 μm. It should be noted that for the treatment of influenza in medical drugs is included $Al(OH)_3$ [31]. The local maximum in this case was measured at -0,1326 eV, or at 9,35 μm.

The evaluation of the possible number of hydrogen bonds as percent of H_2O molecules with different values of distribution of energies is presented in Table 4. These distributions are basically connected with the restructuring of H_2O molecules with the same energies. This serves as the base for evaluating the mathematical model explaining the behavior of the anolyte and catholyte regarding the distribution of H_2O molecules to the energies of hydrogen bonds [32].

Table 3: Local maximums of catholite and anolyte solutions in NES- and DNES-spectra

-E(eV) x-axis	Catholyte	Anolyte y-axis (eV^{-1})	Control sample y-axis (eV^{-1})	DNES Catholyte	DNES Anolyte	-E(eV) x-axis	Catholyte y-axis (eV^{-1})	Anolyte y-axis (eV^{-1})	Control Sample y-axis (eV^{-1})	DNES Catholyte y-axis (eV^{-1})
0,0937	0	0	0	0	0	0,1187	0	66,7	66,7	-66,7
0,0962	0	0	0	0	0	0,1212	66,7	0	0	66,7
0,0987	0	0	0	0	0	0,1237	0	0	0	0
0,1012	66,7	66,7	33,3	33,4	33,4	0,1262	0	0	66,7	-66,7
0,1037	0	0	33,3	-33,3	-33,3	0,1287	0	0	66,7	-66,7
0,1062	0	0	0	0	0	0,1312	33,3	100	33,3	0
0,1087	0	0	0	0	0	0,1337	33,3	33,3	33,3	0
0,1112	0	0	0	0	0	0,1362	0	0	0	0
0,1137	0	66,7	66,7	-66,7	0	0,1387	200	66,7	66,7	133,3
0,1162	0	0	0	0	0	–	–	–	–	–

Table 4: Energy distribution of catholyte and anolyte solutions in electrochemical activation of sodium chloride

-E(eV) x-axis	Catholyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)	Anolyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)	-E(eV) x-axis, % ($-E_{value}$)/($-E_{total\ value}$)	Catholyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)	Anolyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)
0,0937	0	0	0,1187	0	16,7
0,0962	0	0	0,1212	16,7	0
0,0987	0	0	0,1237	0	0

0,1012	16,7	16,7	0,1262	0	0
0,1037	0	0	0,1287	0	0
0,1062	0	0	0,1312	8,4	24,8
0,1087	0	0	0,1337	8,4	8,4
0,1112	0	0	0,1362	0	0
0,1137	0	16,7	0,1387	49,8	16,7
0,1162	0	0	–	–	–

Conclusions

The experimental results prove the strong influence of different types of electrochemically activated water solutions (catholyte/anolyte) on various microbes and viruses. They are in accordance with the results obtained by other researchers, and demonstrate the strong biocidal effect of the anolyte toward the CSF virus. Also, the interesting results on the antibacterial effect were obtained when a strain of *E. coli DH5a* was treated with the catholyte and anolyte, respectively. Unexpectidely, the catholyte with ORP ≈ -180 mV and pH = 9,8 demonstrated the better biocidal effect than the anolyte with ORP ≈ +500 and pH = 3,9. We tried to relate the antimicrobial and antiviral action of electrochemically activated water with the characteristics of the NES-spectrum. There is an indication about such a connection but more thorough research is needed to prove it. For example, the inverse biocidal effect between the catholyte and anolyte in case of a strain of *E. coli DH5a* requires a clear explanation.

The results of the research are formulated as follows.

1. The anolyte did not affect the growth of the cell culture PK-15;
2. The anolyte administered at a concentration of 25 %, exerts a strong virucidal effect on a cell culture virus, and a weaker antiviral activity at concentrations of 16,51 %, 12,5 % and 10 %;
3. The anolyte exerted a strong virucidal effect at concentrations of 50 %, 75 %, 87 % and 94 % over the CSF virus in cell culture suspensions;
4. The catholyte supresses the growth of *E. coli* up to 85 % while anolyte is at least three times less effective;
5. The local maximum in the DNES-spectrum of the catholyte was detected at 9,85 μm; there was a decrease of this local maximum in water with mice tumor cells;
6. The local maximum in the DNES-spectrum of the anolyte was detected at 9,45 μm; at 9,35 μm occurred the effect of inflammation from virus of influenza;
7. The mathematical model of the catholyte and anolyte regarding the distribution of H_2O molecules to the energies of hydrogen bonds was evaluated.

References:

1. Bahir V.M., Liakumovich A.G., Kirpichnikov P.A., Spector L.E., Mamajanov U.D. The physical nature of the phenomena of activation substances // Izv. UzSSR. Ser. Tehn. Sciences. 1983. Vol. 1. P. 60–64.

2. Kirpichnikov P.A., Bahir V.M., Hamer P.U. On the nature of electrochemical activation of media // Dokl. USSR Academy of Sciences. 1986. Vol. 286. № 3. P. 663–666.

3. Morita C., Sano K., Morimatsu S., Kiura H., Goto T., Kohno T. Disinfection potential of electrolyzed solutions containing sodium chloride at low concentrations // Journal of Virological Methods. 2000. Vol. 85(2). P. 163–174.

4. Petrushanko I.Ju., Lobyshev V.I. Non-equlibrium state of electrochemically activated water and its biological activity // Biofizika. 2001. Vol. 46. № 3. P. 389–401.

5. Mosin O.V. Electrochemical treatment of water // Santechnics Heating Air Conditioning. C.O.K. Moscow: Media Technology. 2012. № 12. P. 20–26.

6. Dykstra C.E. External electric field effects on the water trimer // Chemical Physics Letters. 1999. Vol. (2)299. P. 132–136.

7. Bahir V.M. Electrochemical activation. Moscow: All Russian Scientific Research and Experimental Institute of Medical Engineering (VNIIIMT). 1992. Vol. 2. P. 632–657.

8. Hsu S.Y. Effects of flow rate, temperature and salt concentration on chemical and physical properties of electrolyzed oxidizing water // Journal of Food Engineering. 2005. Vol. 66. P. 171–176.

9. Bahir V.M., Zadorozhnii Y.G., Leonov B.I., Panicheva S.A., Prilutsky V.I. Electrochemical activation: water purification and production of useful solutions. Moscow: VNIIIMT. 2001. Vol. 1. P. 100–176.

10. Toropkov V.V., Altschul E.B., Toropkova E.V. Toxicological characterization of catholyte / in 3d International Symposium "Electrochemical Activation". – Moscow-Leningrad. 2001. Vol. 1. P. 57–62.

11. Leonov B.I., Prilutsky V.I., Bahir V.M. Physico-chemical aspects of the biological effect of electrochemically activated water. – Moscow: VNIIIMT. 1999. Vol. 1. P. 224–240.

12. Petrushanko I.Ju., Lobyshev V.I. Physico-chemical properties of aqueous solutions, prepared in a membrane electrolyzer // Biofizika. 2004. T. 49(1). P. 22–31.

13. Kirkpatrick R.D. The Mechanism of antimicrobial action of electro-chemically activated (ECA) water and its healthcare applications. – Doctoral Thesis, University of Pretoria. 2009.

14. Leonov B.I., Bahir V.M., Vtorenko V.I. Electrochemical activation in the practice of medicine / in: 2nd International Symposium "Electrochemical Activation". Proc. rep. and brief reports. – Moscow. 1999. Vol. 1. P. 15–23.

15. Toropkov V.V., Altshul E.B., Peresypkin O.I. Therapeutic efficacy of anolyte and ANC on the mucous membranes of the oral cavity / in: 2nd International Symposium "Electrochemical Activation". – Moscow. 1999. Vol. 1. P. 93–95.

16. Prilutsky V.I., Bakhir V.M. Electrochemically activated water: Anomalous properties, Mechanism of biological action. – Moscow: VNIIIMT, 1997. Vol. 1. 124 p. [in Russian].

17. Babtsova N.F., Komarov I.F. Experience of using STELS in the surgical department / in: 2nd International Symposium. Electrochemical activation. Proc. and brief reports. 1999. Vol. 1. P. 131–132.

18. Suzuki T., Itakura J., Watanabe M., Ohta M., Sato Y., Yamata Y. Inactivation of staphylococcal Enterotoxin-A with an electrolyzed anodic solution // Journal of Agricultural and Food Chemistry. 2002. Vol. 50. P. 230–234.

19. Edwards A., Edwards S. Survival and inactivation of classical swine fever virus // Veterinary Microbiology. 2000. Vol. 73. P. 175–181.

20. Karadzov S., Atanasov A., Ivanova E., Mosin O.V., Ignatov I. Mathematical models of electrochemical aqueous sodium chloride solutions (anolyte and catolyte) as types of water. Study of the effects of anolyte on the virus of classical swine fever virus // Journal of Health, Medicine and Nursing. 2014. Vol. 5. P. 30–55.

21. Sands J.A., Landin P., Auperin D. Enveloped virus inactivation by fatty acid derivates // Antimicrob. Agents Chem. 1979. Vol. 15. P. 134–136.

22. Springthorpe U.S., Sattar S.A. Chemical disinfection of virus-contaminated surfaces // Crit. Rev. Environ. Control. 1990. Vol. 20. P. 169–229.

23. Stoner G.E., Cahen G.L. Jr., Sachyani M., Gileadi E. The mechanism of low frequency a.c. electrochemical disinfection // Bioelectrochemistry and Bioenergetics. 1982. Vol. 9(3). P. 229–24.

24. Zinkevich V., Beech I.B., Tapper R., Bogdarina I. The effect of super-oxidized water on *Escherihia coli* // Journal of Hospital Infection. 2000. Vol. 46. P. 153–156.

25. Kumar S.V., Ezeike G.O., Hung Y.C., Doyle M.P. Efficacy of electrolyzed oxidizing water for inactivating *Escherihia coli O157:H7*, *Salmonela enteritidis*, and *Lusteria monocytogenes* // Applied and Environmental Microbiology. 1999. Vol. 23. P. 4276–4279.

26. Atanasov A., Karadzhov S., Ivanova E., Mosin O.V., Ignatov I. Study of the effects of electrochemical aqueous sodium chloride solution (anolite) on the virus of classical swine fever virus. Mathematical models of anolyte and catolyte as types of water // Journal of Medicine, Physiology and Biophysics. 2014. Vol. 4. P. 1–26.

27. Antonov A. Research of the Nonequilibrium Processes in the Area in Allocated Systems. Diss. thesis doctor of physical sciences. – Sofia: Blagoevgrad, 1995. P. 1–255.

28. Ignatov I., Mosin O.V. Structural mathematical models describing water clusters // Journal of Mathematical Theory and Modeling. 2013. Vol. 3. № 11. P. 72–87.

29. Luck W., Schiöberg D., Ulrich S. Infared investigation of water Structure in desalination membranes // J. Chem. Soc. Faraday Trans. 1980. Vol. 2. № 76. P. 136–147.

30. Ignatov I., Mosin O.V. Methods for measurements of water spectrum. Differential non-equilibrium energy spectrum method (DNES) // Journal of Health, Medicine and Nursing. 2014. Vol. 6. P. 50–72.

31. Keller W. Safety and immunogenicity of an inactivated influenza A/H5N1 vaccine given with or without aluminum hydroxide to healthy adults: Results of a phase I-II randomized clinical trial // J. Infect. Dis. 2008. Vol. 198. № 9. P. 1309–1316.

32. Ignatov I., Karadzhov S., Atanasov A., Ivanova E., Mosin O.V. Electrochemical aqueous sodium chloride solution (anolyte and catholyte) as types of water. Mathematical models. Study of effects of anolyte on the virus of classical swine fever virus // Journal of Health, Medicine and Nursing. 2014. Vol. 8. P. 1–28.

УДК 631.531.027: 635.63

Электрохимически активированные растворы воды: Биофизические и биологические эффекты католита и анолита

[1*] Георгий Глухчев
[2] Игнат Игнатов
[3] Стоил Караджов
[4] Георгий Милошев
[5] Николай Иванов
[6] Олег Викторович Мосин

[1] Институт информации и коммуникационных технологий
Болгарская Академия Наук, Болгария
Доцент, Ph. D.
1113, София, Акад. Г. Бончева ул., 2
*E-mail: gluhchev@iinf.bas.bg
[2] Научно-исследовательский центр медицинской биофизики (РИЦ МБ), Болгария
Профессор, доктор наук Европейской академии естественных наук (ФРГ), директор НИЦ МБ.
1111, София, ул. Н. Коперника, 32/6
E-mail: mbioph@dir.bg
[3] Болгарская ассоциация активированной воды, Болгария
Профессор, D. Sc.
1619, София, Кутузов бульв., 39
[4] Институт молекулярной биологии
Болгарская Академия наук, Болгария
Доцент, Ph. D.
1113, София, Акад. Г. Бончева ул., 2
[5] Институт информации и коммуникационных технологий
Болгарская Академия Наук, Болгария
Доцент, диплом. инженер
1113, София, Акад. Г. Бончева ул., 2
[6] Московский государственный университет прикладной биотехнологии, Российская Федерация
Старший научный сотрудник кафедры биотехнологии, канд. хим. наук
103316, Москва, ул. Талалихина, 33
E-mail: mosin-oleg@yandex.ru

Аннотация. В статье описываются результаты антимикробного действия электрохимически активированных водных растворов (анолит/католит), полученных в анодной и катодной камере электролитической ячейки. В лабораторных условиях культура клеток суспензии вируса свиного гриппа была обработана анолитом. После прививки их с культурами клеток присутствие вируса (наличие вирусного антигена) было измерено с использованием иммунопероксидазного метода. Было обнаружено, что анолит не влияет на рост культуры клеток РК-15; вирусной рост при заражении клеточного монослоя замедлялся вирусом в наибольшей степени при разведении анолита в пропорции 1:1 и менее в других

разведениях; в то время как вирусный рост при инфекции клеточной суспензии с вирусом замедлялся анолита в наибольшей степени в разведении 1:1, и менее в других разведениях; вирусный рост при инфекции вирусом суспензии клеток монослоя зависел от присутствия анолита во всех разведениях. Неожиданно сильный биоцидный эффект католита наблюдался при обработке штамма *E. coli DH5* анолитом и католитом соответственно. Для получения дополнительных данных о противовирусной активности электроактивированных растворов воды, а также о структурных изменениях, были измерены неравновесный энергетический спектр (НЭС) и дифференциальный неравновесный энергетический спектр (ДНЭС) анолита и католита.

Ключевые слова: анолит; католит; *E. coli DH5;* вирус свиного гриппа; НЭС; ДНЭС.

Published in the Russian Federation
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN 2409-1332
Vol. 7, Is. 1, pp. 27-36, 2015

DOI: 10.13187/ejmb.2015.7.27
www.ejournal8.com

UDC 631

Uses Semi–quantitative and Relative Quantity Methods to Analysis Gene Expression of *DGAT1* Gene Responsible for the Olive Diacylglcerol Acyltransferases in 10 Cultivars of Olive (*Olea europaea*. L)

Ali Saeed Atiyah AL-Janabi

University of Kufa, Najaf, Iraq
PhD Plant Biotechnology (fruit) / Molecular genetic
Horticulture and Landscape gardening Department, Faculty of Agriculture
E-mail: alialjenaby@yahoo.com

Abstract

In this study gene expression for *DGAT1* gene was analyzed. Diacylglycerol acyltransferases (DGATs) catalyze the final step of the triacylglycerol (TAG) biosynthesis of the Kennedy pathway. Two major gene families have been shown to encode DGATs, DGAT1 (type-1) and DGAT2 (type-2). Gene expression were analyzed for 10 Olive cultivars (*Olea europaea* L.) (Khaderi, Qaysi, Manzenillo, Baashiqi, Arabqween, Nabali, Labeeb, Dahkan, Shami and Sorani). Different plant organs as plant materials (mature leaves, mesocarp and seeds for drups) used for analysis. Two methods for analysis gene expression were used, first method was called semi – quantitative and second method was called relative – quantitative, used in relative method (Real time PCR and Actine gene as Housekeeping gene). On the other hand chemical analysis was used on fruits like moisture % and oil % of dry and fresh weigh. The results revealed the following: *DGAT1* gene expression in leaves, mesocarp and seeds by two methods (semi- quantitative and relative quantity) were the convergent results and clear, also if this results compared with chemical analysis shows that the best cultivars were Arabqween, Khaderi, Qaysi and Labeeb. The cultivars Shami and Khaderi then were contain in fruits desirable qualities of olive oil, low moisture and high oil percentages ratios. While Nabali, Manzanello, and Sorani cultivars middle desirable quantity, and Baashiqi and Dahkan cultivars had undesirable because of low oil quantity and high moisture in contain fruits. Some cultivars have low intensity in semi- quantitative and little fold in relative quantity but it have high oil in contain fruits that may be indicate that these cultivars were complete gene expression and begin to accumulation and save oil in tissue. Therefore particular emphasis was given to the temporal regulation of olive DGATs during drupe development. In olive fruit, TAGs are formed and stored in both the mesocarp and the seed .Two drupe compartments that have different physiological functions and roles and also display difference in the mode of TAG accumulation. DGATI share an overlapping expression pattern after 28 WAF, suggesting that they probably function at those stages. However, following maximal mRNA levels at 22 WAF, DGAT1 transcription declined substantially.

Keywords: olive; *DGAT1;* diacylglycerol acyltransferases; gene expression.

Introduction

The olive tree *(Olea europaea* L.) is an evergreen species that ranks sixth in the world's production of vegetable oils [1]. Since ancient times it has been cultivated in the Mediterranean Basin, where ~2600 cultivars have been identified based on morphological traits [2]. In more recent years, interest in olive oil production has been extended to countries outside the Mediterranean region such as Argentina, the USA, Mexico, South Africa, western Africa, Australia, Azerbaijan, China and Japan [3, 4]. Olive trees lack dormancy, and are sensitive to low temperatures [5].

Plant lipids contain polyunsaturated fatty acids, mainly linoleic and α-linolenic acids, which play crucial roles in plant metabolism as storage compounds mainly in the form of triacylglycerols (TAG), as structural components of membrane lipids, and as precursors of signaling molecules involved in plant development and stress response [6,7]. Linoleic acid, together with oleic acid, is a major fatty acid in vegetable oils and its content greatly affects the technological properties such as their oxidative stability [8] and nutritional characteristics [9].

A number of plants accumulate large amounts of triacylglycerols (TAGs) in their seeds as storage reserves for germination and seedling development. Key points in the accumulation of TAGs are the early events of fatty acid biosynthesis and the last and critical events of TAG [10, 11, 12, 13]. There are few fruit crops that deposit most of the oil in the mesocarp tissues to attract animals for seed dispersal. Among them, olive is of predominant economic importance because its oil is ideal for direct consumption. It is therefore of great importance to elucidate the key-points in the olive oil biosynthesis pathway and storage. Such knowledge could speed up the breeding programs aimed at selecting clones with superior fatty acid composition and is also essential for selecting high oil-yielding genotypes more efficiently and rapidly, thus improving decision-making processes. Nevertheless, the molecular basis of gene regulation underlying olive oil production is far from complete. There is a significant amount of information concerning the regulation of several genes involved in fatty acid synthesis and modification [2,14,15], but much less is known about the cellular mechanisms governing the transfer of fatty acids into storage TAGs not only in olive but generally in plants [16].

TAG biosynthesis is principally accomplished by membrane-bound enzymes that operate in the endoplasmic reticulum (ER) through the glycerol-3-phosphate or so called Kennedy pathway [17, 18]. The first step in the process involves the acylation of glycerol-3-phosphate (GP) at the sn-1 position to produce lysophosphatidic acid (LPA) by GP acyltransferase (GPAT). LPA is further acylated at the sn-2 position by LPA acyltransferase (LPAT) resulting in the formation of phosphatidic acid (PA). PA is dephosphorylated to produce diacylglycerol (DAG), which is further acylated to produce TAG by diacylglycerol acyltransferase (DGAT), the only enzyme in the pathway that is thought to be exclusively committed to TAG synthesis. As much as DGAT catalyses the final and most critical step for TAG synthesis, it has been suggested that it may constitute a rate-limiting factor in TAG bioassembly in developing seeds [19, 20, 13]. However, TAGs could also be produced via the transfer of acyl groups from phospholipids to diacylglycerols, an acyl-CoA-independent reaction catalyzed by the enzyme phospholipid: diacylglycerol acyltransferase (PDAT) [21, 22, 23]. TAGs are not only produced in seeds or mesocarps. Both TAG accumulation and DGAT activity have been reported in several other organs such as flowers, developing siliques, germinating seeds, young seedlings, and senescing leaves of *Arabidopsis* [24, 25], and in stems, flowers, roots, and leaves of tobacco [26]. Based on those observations it has been suggested that TAG may also be implicated in physiological roles other than as a carbon or energy source [27, 28]. Two major unrelated gene families have been shown to encode DGATs, namely *DGATI* (type-I) and *DGAT2* (type-2) both of which are ER-localized. *DGATI* genes have been cloned from several plant species, including olive [29]. *DGAT2* genes have been cloned from diverse eukaryotes, including the oleaginous fungus *Mortierella ramanniana* [30], human [31], and the plant species *Arabidopsis* [30], castor bean [32], and tung tree [16]. A third member of the DGAT family (type-3), highly unrelated to the previously reported was identified in peanut that possesses a cytosolic localization [33]. Accumulating data suggest that DGAT activity may have a substantial effect on carbon flow into seed oil of *Brassica napus* [34, 35], *Arabidopsis thaliana* [24, 11], and maize [36]. In an attempt to gain the further insight into the role(s) of DGATs in plant lipid biosynthesis the expression patterns of *DGATl* in several other organs/tissues of the olive tree indicated that genes

are differentially regulated to fulfill the needs for TAG accumulation at certain points of growth and development.

Materials and methods

Plant Material

Used the mature leaves (ML), mesocarp fruit (ME) and seeds (S) included emberyo and endosperm of 10 olive cultivers (*Olea europaea*) were: (Khaderi, Qaysi, Manzenillo, Baashiqi, Arabqween, Nabali, Labeeb, Dahkan, Shami and Sorani) trees grown in Iraqi orchard near Mosul, and chilled in liquid nitrogen, and stored at 80℃.

Total RNA Extraction and cDNA Synthesis

Total RNA for samples were isolated by uses (SV Total RNA Isolation kit/Promega.USA). The quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis. DNA was removed from RNA samples using the DNase I Mix/Promega USA (DNase I, MnCl2, yellow core buffer). First-strand cDNA was synthesized from (16 µl) of total RNA using the (power cDNA Syntheses kit/IntronBio.Inc. USA) with Oligo (dT) 15 primer, following the manufacturer's instructions and quantified using gel electrophoresis.

Semi- Quantitative Real Time PCR

Analysis were carried out by change PCR program respectively with Primer for gene-specific (*DGAT1*) amplification and cDNA Syntheses from 25th to 35th cycle. PCR products were also checked for purity by 1 % agarose gel electrophoresis and the following program shows the method.

Cycle	Time	Tm	Stage	
1	5 min	95	Pre - Denaturation	1
25	45 sec	95	Denaturation	2
	45 sec	55	Annealing	3
	45 sec	72	Extension	4
1	10 min	72	Final extension	5
Cycle	Time	Tm	Stage	
1	5 min	95	Pre - Denaturation	1
35	45 sec	95	Denaturation	2
	45 sec	55	Annealing	3
	45 sec	72	Extension	4
1	10 min	72	Final extension	5

*Tm (melting temperature)

Quantitative Real Time PCR (qRT -PCR)

Gene expression analysis was performed by qRT-PCR using an Mini Opticon System real-time PCR and GO Taq Master Mix SYBR Green kit Q-PCR/ IntronBio.Inc.USA. Primers for gene specific amplification were designed to generate a product of 100-200 bp and to have a Tm (melting temperature) of 60 ℃. PCR reactions were carried out in duplicate in plate. Reaction mix (22.5 µl per well) contained 12.5 µl, Master Mix SYBR Green, 2.5 µl forward and reverse primers,7.5 µl DEPC-D.W and 2.5 µl of cDNA. The thermal cycling conditions consisted of an initial denaturation step of 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 30 s, 60 ℃ for 1 min, and 72 ℃ for 30 s. The specificity of the PCR amplification was monitored by melting curve analysis following the final step of the PCR products were also checked for purity by agarose gel electrophoresis. The housekeeping olive Actin gene (*OeActin*) was used to normalize as endogenous reference. The real-time PCR data were analyzed by GeneX program. The gene of the olive tree, used for the design of primers are available in Gen Bank (www.ncbi.nih.nlm.gov/ Gen Bank/EMBL/DDBJ. the primers sequences (*DGAT1*) for QRT-PCR amplification were two parts forward (5-TTGGCTGAATATATTAGCGGAACTTC-3) and reverse (5- CTCATCATAAAAATGTCCACATCC-3) and Actin gene primer were forward (5-ACCACCTCAGCCGAACGGGA-3) and reverse (5-TGCTGGGAGCCAAGGC AG TG-3).

Chemical analysis

Uses Soxhlet method to determine oil percentage (%) in fresh and dry flesh of 10 olive cultivars.

Results

Figure 1 showed the results of isolated total RNA of the mature leaves (ML) , flesh mesocarp fruit (ME) and seeds (S) of the studied olive cultivars manner filters and then migrated to agar gel 1 %, voltage 100 V for 20 minutes noting the success of the method to isolate RNA from this plant parts.

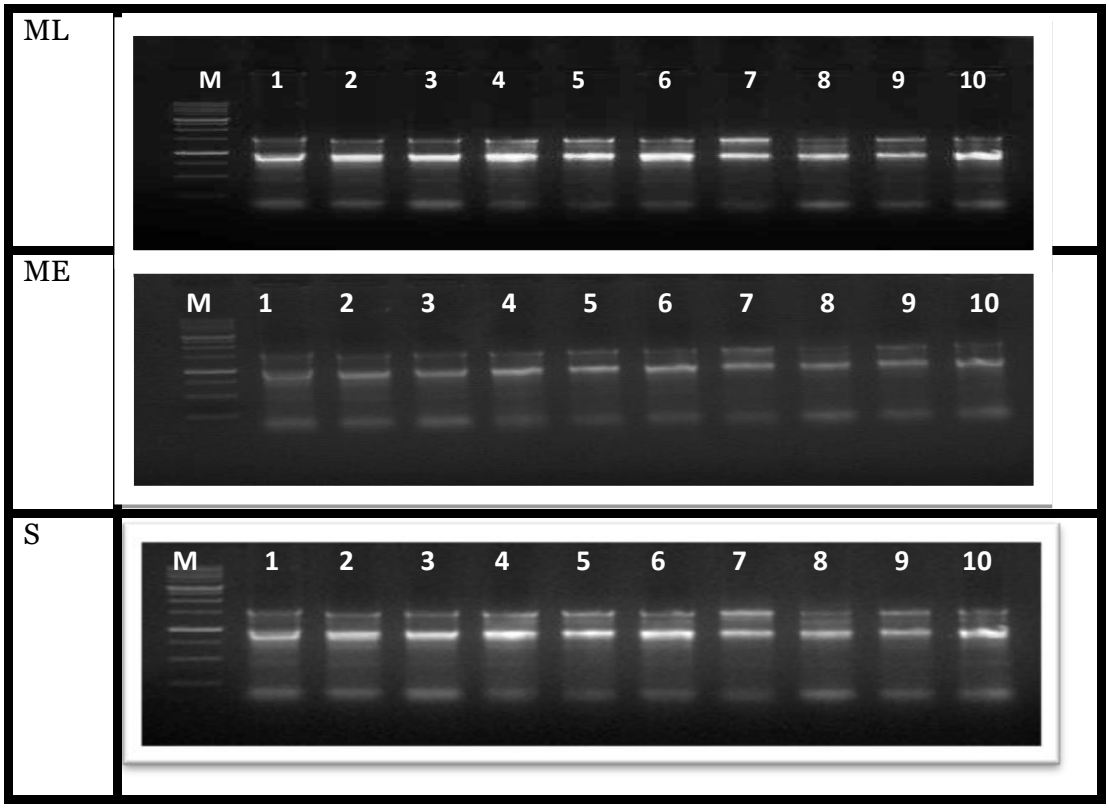
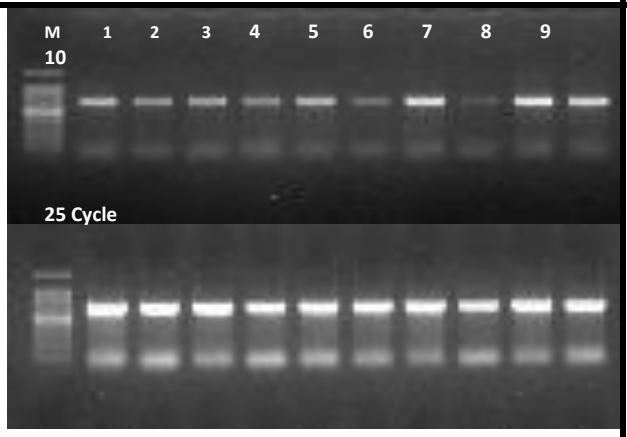
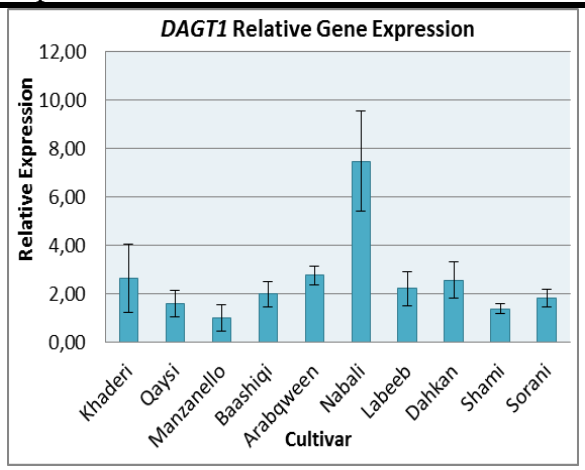
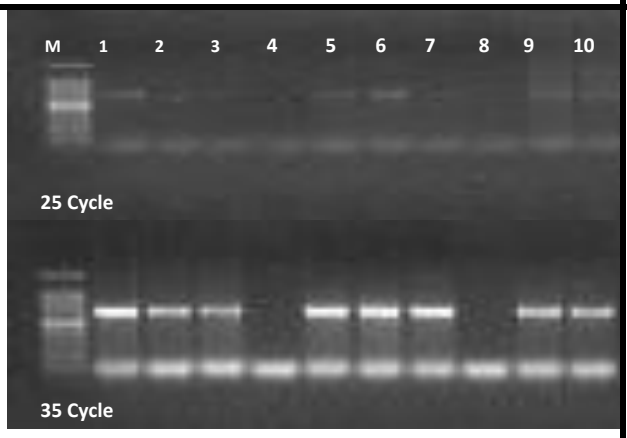
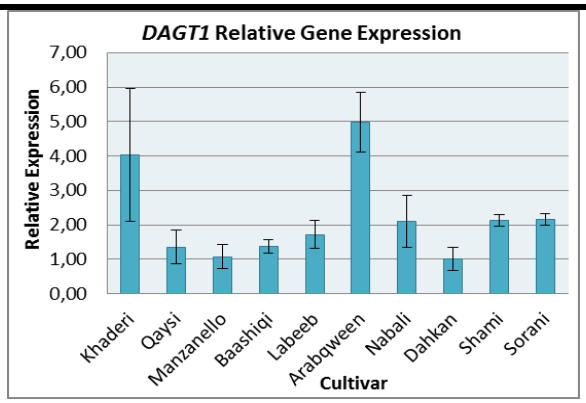


Figure (1) represents the isolated total RNA of the mature leaves(ML), mesocarp(MS) and seeds(S) of ten olive cultivars on agarose gel (1%) and voltage (100 V) for (20 minutes). M = Marker Leader: 1 = Khaderi, 2 = Qaysi 3 = Manzenillo, 4 = Baashiqi, 5 = Arabqween, 6 = Nabali, 7 = Labeeb, 8 = Dahkan, 9 = Shami and 10 = Sorani.

The semi-quantitative measurement of gene expression of the gene *DGATl* in the mature leaves (ML) of cultivars (Figure 2. ML) refers to the emergence of clear on bands and high intensity in some cultivars and a few intensity in other cultivars at the 25th cycle of the PCR as shine bands cultivars were Labeeb, Shami and Sorani but the cultivars Khaderi, Oaysi, Manzenillo and Arabqween were medium intensity bands, either cultivars less intensity were at cultivars Baashiqi, Nabali and Dahkan, but when 35th cycle that all cultivars were similar and equal approximately. Analysis relative gene expression of the gene *DGATl* in the mature leaves (ML) of cultivars were different then show Nabali variety the highest level gene expression nearly75 fold, but another cultivars were equals in gene expression approximately.

The process of analyzing the cultivars mesocarp (ME) showed clear differences among cultivars. Figure 2.ME refer to supremacy Khaderi, Arabqween, Nabali, Shami and Sorani cultivars which have clear bands in 25th cycle, but in 35th cycle less cultivars intensity were at cultivars Baashiqi and Dahkan if compared with the other cultivars. Notes from the analysis of relative amount of the gene *DGATl* in fruit mesocarp of olive cultivars under study shows that cultivars Khaderi and Arabqween had a higher level of expression of gene after 28 weeks of full bloom and it reached approximately 4 and 5 fold respectively, and either lower the level of gene expression was Dahkan cultiver which amounted to 1 folde approximately.

In seed, analysis cultivars gene expression *DGATl* showed clear differences among cultivars then analysis semi-quantitative in the 25thcycle of the PCR program that for cDNA cultivars appeared Baashiqi and Dahkan cultivars weak intensity bands, on the other hand this cycle (25th) showed high intensity of clear bands in Arabqween, Nabali, Labeeb, Shami and Sorani cultivars while another cultivars were has middle intensity (Figure 2.S). In 35th cycle of the PCR program of gene expression to gene *DGAT1* in the seeds of olive cultivars that clear bands for all cultivars especially Khaderi, Manzenillo, Arabqween, Nabali and Labeeb cultivars, except Baashiqi and Dahkan cultivars had weak intensity. Notes from the analysis of relative amount of the *DGAT1* gene in seed (Figuare 2.S) showed Shami, Arabqween and Manzenillo cultivars had high relative gene expression reach (79,51 and 35 fold) respectively. While Khaderi, Qaysi, Sorani and Baashiqi cultivars were less relative gene expression, while Nabali, Labeeb and Dahkan were middle in relative gene expression.

	Semi-quantitative Gene expression	Relative quantitative (realtime PCR) Gene expression
ML		
ME		

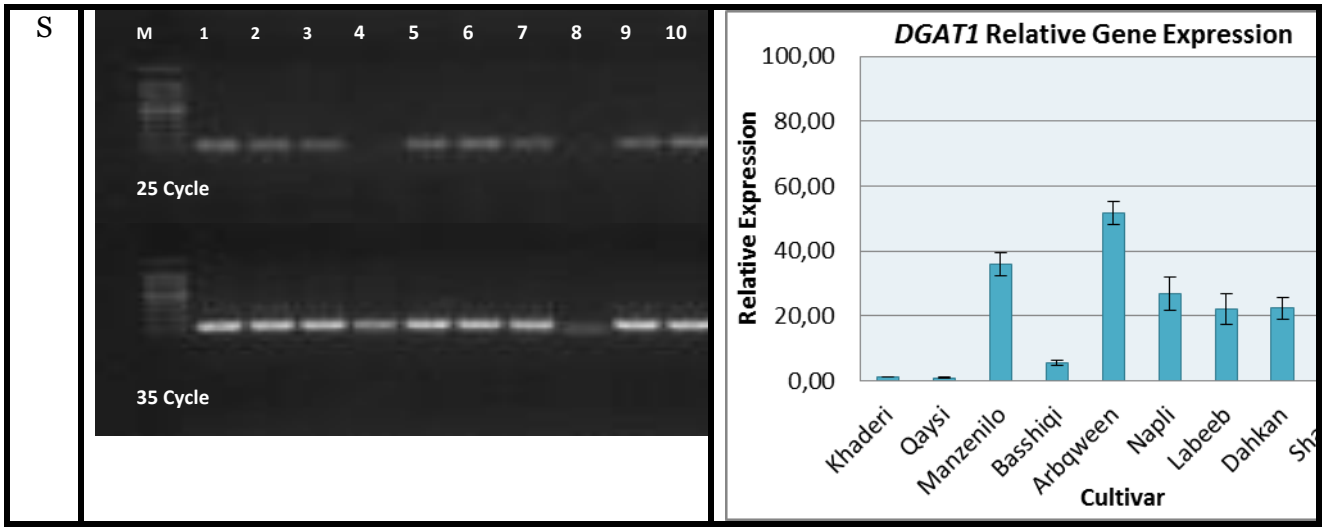


Figure (1) represents the Semi-quantitative measurement and Relative quantitative of *DGATI* Gene expression to the mature leaves(ML), mesocarp(MS) and seeds(S) of ten olive cultivars semi-quantitative were on agarose gel (1%) and voltage (100 V) for (20 minutes). M = Marker Leader: 1 = Khaderi, 2 = Qaysi 3 = Manzenillo, 4 = Baashiqi, 5 = Arabqween, 6 = Nabali, 7 = Labeeb, 8 = Dahkan, 9 = Shami and 10 = Sorani.

Chemical analysis result

Chemical analysis of the fruits of olive cultivars showed highest moisture percentage in Dahkan variety fruits with significant difference reached (71.3 %), while the lowest moisture percentage in Khaderi variety fruit. Arabqween variety fruit contained high percentage of oil in dry weight reached (57.5 %) and Baashiqi fruit had low percentage of oil dry weight reached (15.5 %). Percentage of oil in fresh weight showed which highest significant value in Khaderi variety which was (37.4 %), while the lowest percentage in Iraqi cultivars (Baashiqi and Dahkan) reached (4.9 and 6.6 %) respectively. Table (l).

Table (l) analysis moisture and oil in olive fruits cultivars

Cultivars	Moisture (%)	Oil.dw (%)	Oil.fw (%)
Khaderi	30.4 f	53.7 c	37.4 a
Qaysi	36.4 e	56.3 ab	35.4 b
Manzanello	38.8 de	38.9 e	23.8 e
Baashiqi	68.4 b	15.5 g	4.9 f
Arabqween	41.6 c	57.5 a	33.6 c
Nabali	39.5 d	49.8 d	30.1 d
Labeeb	39.6 d	55.8 b	33.7 c
Dahkan	71.3 a	23.2 f	6.6 f
Shami	37.8 e	49.8 d	31.5 d
Sorani	38.8 de	38.6 e	24.2 e

***Similar letters refer to nonsignificant difference.**
***Dissimilar letters refer to significant difference.**
***This analysis of variance and means were separated by Duncan's multiple range test at the 5% level.**

Discussion

In this study (Figure 1) appear *DGAT1* gene expression in leaves, mesocarp and seeds by two methods (semi- quantitative and relative quantity) were the convergent results and clear, and if these results compared with chemical analysis (Table 1) shows that the best cultivars were Shami, Arabqween, Khaderi, Qaysi and Labeeb. Tables (1) indicates that the cultivars Shami and Khaderi then were contain in fruits desirable qualities of olive oil, low moisture and high oil percentages ratios. While it were Nabali, Manzanello, and Sorani cultivars middle desirable qualities wheres cultivars Baashiqi and Dahkan were undesirable because of low oil quantity and high moisture in fruits. Some cultivars have little intensity in semi- quantitative and little fold in relative quantity but it's have high oil contain in fruits that maybe indicate to that cultivars were complete gene expression and begin to accumulation and save oil in tissue. Therefore particular emphasis was given to the temporal regulation of olive DGATs during drupe development. In olive fruit, TAGs are formed and stored in both the mesocarp and the seed. Two drupe compartments that have different physiological functions and roles and also display difference in the mode of TAG accumulation. Storage TAGs in seeds are proposed to provide energy for germination. They are present in small 0.5-2 mm diameter subcellular-oil bodies complete covered by oleosins to prevent them from coalescence [37]. The fleshy olive mesocarp possesses much larger (about 30mm diameter) lipid particles TAGs which are devoid of surface oleosins [38, 39]. Accumulation of TAG in olive seeds is relatively fast, if compared with the mesocarp being completed within a relatively short period [40]. Although massive TAG storage in seeds starts at about 11 WAF, coinciding with endocarp lignification, DGATI transcripts were present as early as 5 WAF, albeit at low levels. By contrast, DGAT2 transcripts were almost undetectable until 11 WAF, pointing to a principal role of DGATI in early TAG accumulation in olive drupes, especially in the seed [41]. As the drupe grows further, the rate of oil synthesis in seed tissues accelerates reaching a plateau at about 22 WAF [40]. The pattern of oil deposition in seeds correlates well with DGATI regulation both in embryo and endosperm. The bell-shaped expression pattern of DGATl coincides well with the relative expression of the olive oleosin gene in seed tissues [39]. Similarly, in oilseed species, a transient increase of DGATs activity occurs at the stage of active oil accumulation, but when the lipid content reaches plateau the activity decreases markedly [42, 43]. The present results suggest a prominent role of DGATI in seed olive oil accumulation. This is in contrast to oleogenic seed crops that contain unusual fatty acids where, DGAT2 may play a more central role than DGAT1 in oil production [44, 16, 20, 45]. Olive oil does not contain unusual fatty acids and olive is one of the few exceptions of commercially important oil producing crops in that most of the oil is produced in the mesocarp. Oil accumulation in the mesocarp follows a typical sigmoidal curve [40]. The major proportion of oil generally starts to accumulate at 16-19 WAF and reaches a plateau at about 28 WAF. However, the pattern of accumulation may vary due to environmental conditions, different agricultural practices and/or the olive variety [46]. DGATI share an overlapping expression pattern after 28 WAF, suggesting that they probably function at those stages. However, following maximal mRNA levels at 22 WAF, DGAT1 transcription declined substantially.

The reference [47] showed that oil bodies, primarily composed of steryl esters and triacylglycerols were abundant in the Physcomitrella photosynthetic vegetative gametophyte. In this study, relatively high levels of transcription of OeDGATl were detected in olive leaves of cultivars, where the regulation of expression was clearly developmentally regulated. Accumulating data, as stated above, suggest that DGATl gene also play roles other than its 'classical' role to synthesize TAGs in the storage organs [23, 41, 48]. The present results point to a differential contribution of each DGAT gene in various organs in a temporal-related manner.

Conclusion

In conclusion DGATI share overlapping but distinct transcription patterns during vegetative growth, suggesting that they are differentially regulated in a developmental and cellular manner. They probably have similar functions but they also serve different purposes. Distinct expression patterns of DGATI were observed between the leave, seed and mesocarp, with DGATI contributing most of the TAG deposition in seeds, reflecting the large differences in the mode of TAG accumulation among the cultivars fruit compartments. Important differences between the expression profiles of the gene were also apparent during drupe ripening.

References

1. Alagna, F.; D'Agostino, N.; Torchia, L.; Servilli, M.; Rao, R.; Pietrella, M.; Giuliano, G.; Chiusano, M.L.; Baldoni, L. and Perrotta, G. 2009. Comparative 454 Pyrosequencing of transcripts from two olive genotypes during fruit development. BMC Genomics 10, 399.

2. Hatzopoulos, P.; Banilas, G.; Giannoulia, K.; Gazis, F.; Nikoloudakis, N.; Milioni and Haralampidis, K. 2002. Breeding molecular markers and molecular biology of the Olive tree. European Journal of Lipid Science and Technology, 104 : 547-586.

3. Rugini, E.; Biasi, R. and Muleo, R. 2000. Oliv. (*Olea europaea* L.) var. sativa) transformation in: Moahn, JS, Minocha Sc, eds. Molecular biology of woody plants. Dordrocht: Kluwer Academic Publishers: 245-279.

4. Mannina, L.; Fortanazza, G.; Patumi, M. Ansanelli, G. and Segre, A. 2001. Italian and Argentine olive oils: A NMR and gas chromatographic study. Grasas Y Aceites, 52 : 380-388.

5. D'Angeli, S.; Malho`, R. and Altamura MM. 2003. Low-temperature sensing in olive tree: calcium signalling and cold acclimation. Plant Science 165: 1303–1313.

6. Ohlrogge, J. and Browse J. 1995. Lipid biosynthesis. The Plant Cell 7: 957–970.

7. Weber, H. 2002. Fatty acid – derived signals in plants. Trends Plant Sci. 7 : 217-224.

8. Marqez-Ruiz, G.; Garces, R.; Leon-Camacho, M. and Mancha, M. 1990. Thermoxidativ stability of triacylglcerols from mutant sunflower seeds. J. Am. Oil Chem. Soc. 76:1169-1174.

9. Cunnane, S. 2003. Problems with essential fatty acids: time for a new paradigm? Prog. Lipid Res. 42, 544-568.

10. Bao, X. and Ohlrogge, J. 1999. Supply of fatty acid is one limiting factor in the accumulation of triacylglycerol in developing embryos. Plant Physiology 120: 1057–1062.

11. Jako, C.; Kumar, A.; Wei, YD.; Zou, JT.; Barton, DL.; Giblin, EM.; Covello, PS. and Taylor, DC. 2001. Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiology 126: 861–874.

12. Weselake, R.J. 2005. Storage lipids in: Murphy, D.J. ed. Plant Lipids: biology, Utilization, and manipulation. Oxford: Blackwell Publishing: 162-225.

13. Lung, SC. and Weselake, RJ. 2006. Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis. Lipids 41:1073–1088.

14. Doveri, S. and Baldoni, L. 2007. Genome mapping and molecular breeding in plants. Volume 4: Olive fruits and Nuts: 253-264. Springer – Verlag, Berlin Heidelberg.

15. Banilas, G. and Hatzopoulos, P. 2009. Developmental perplexity of oil biosynthesis gene expression in olive. In: Berti, L. Maury, J. eds. Advances in olive resources. Kerala, India: Transworld Research Network, 1–22.

16. Shockey, JM.; Gidda, SK.; Chapital, DC.; Kuan, JC.; Dhanoa, PK.; Bland, JM.; Rothstein, SJ.; Mullen, RT. and Dyer, JM. 2006. Tung (*Vernicia fordii*) DGAT1 and DGAT2 possess different affinities for eleostearic acid-containing substrates and are localized to different subdomains of the endoplasmic reticulum. The Plant Cell 18: 2294–2313.

17. Kennedy, EP. 1961. Biosynthesis of complex lipids. Federation Proceedings 20: 934–940.

18. Browse, J. and Somerville, C. 1991. Glycerolipid synthesis: biochemistry and regulation. Annual Review of Plant Physiology and Plant Molecular Biology 42: 467–506.

19. Ichihara, K.; Takahashi, T. and Fujii S. 1988. Diacylglycerol acyltransferase in maturing safflower seeds: its influences on the fatty acid composition of triacylglycerol and on the rate of triacylglycerol synthesis. Biochimica et Biophysica Acta 958: 125–129.

20. Burgal, J.; Shockey, J.; Lu, C.; Dyer, J.; Larson. T.; Graham; I.and Browse J. 2008. Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotechnology Journal 6, 819–831.

21. Dahlqvist, A.; Stahl, U.; Lenman, M.; Banas, A.; Lee, M.; Sandager, L.; Ronne, H. and Stymne, S. 2000. Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyses the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proceedings of the National Academy of Sciences, USA 97: 6487–6492.

22. Stahl, U.; Carlsson, AS.; Lenman, M.; Dahlqvist, A.; Huang, B.; Banas, W.; Banas, A.and Stymne, S. 2004. Cloning and functional characterization of a phospholipid: diacylglycerol acyltransferase from Arabidopsis. Plant Physiology 135, 1324–1335.

23. Zhang, M.; Fan, J. Taylor, DC. and Ohlrogge, JB. 2009. DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. The Plant Cell 21: 3885–3901.

24. Zou, J.; Wei, Y.; Jako, C.; Kumar, A.; Selvaraj, G. and Taylor, DC. 1999. The *Arabidopsis thaliana* TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene. The Plant Journal 19: 645–653.

25. Kaup, MT. Froese, CD. and Thompson, JE. 2002. A role for diacylglycerol acyltransferase during leaf senescence. Plant Physiology 129: 1616–1626.

26. Zhang, FY.; Yang, MF. and Xu, YN. 2005. Silencing of tobacco causes a reduction in seed oil content. Plant Science 169: 689–694.

27. Lu, C. and Hills MJ. 2002. Arabidopsis mutants deficient in diacylglycerol acyltransferase display increased sensitivity to abscisic acid, sugars, and osmotic stress during germination and seedling development. Plant Physiology 129:1352–1358.

28. Lu, CL.; de Noyer, SB.; Hobbs, DH.; Kang, J.; Wei, Y.; Krachtus, D. and Hills, MJ. 2003. Expression pattern of diacylglycerol acyltransferase-1, an enzyme Involved in triacylglycerol biosynthesis, in Arabidopsis thaliana. Plant Molecular Biology 52: 31–41.

29. Giannoulia K, Haralampids K, Poghosyan Z, Murphy DJ, Hatzopoulos P. 2000. Differential expression of diacylglycerol acyltransferase (DGAT) genes in olive tissues. Biochemical Society Transactions 28: 695–697.

30. Lardizabal, KD.; Mai, JT.; Wagner, NW.; Wyrick, A.; Voelker, T. and Hawkins, DJ. 2001. DGAT2 is a new diacylglycerol acyltransferase gene family: purification, cloning, and expression in insect cells of two polypeptides from Mortierella ramanniana with diacylglycerol acyltransferase activity. Journal of Biological Chemistry 276: 38862–38869.

31. Cases, S.; Stone, SJ.; Zhou, P.; Yen, E.; Tow, B.; Lardizabal, KD.; Voelker, T.and Farese Jr RV. 2001. Cloning of DGAT2 a second mammalian diacylglycerol acyltransferase, and related family members. Journal of Biological Chemistry 276: 38870–38876.

32. Kroon, JT.; Wei, W.; Simon, WJ. and Slabas, AR. 2006. Identification and functional expression of a type 2 acyl-CoA: diacylglycerol acyltransferase (DGAT2) in developing castor bean seeds which has high homology to the major triglyceride biosynthetic enzyme of fungi and animals. Phytochemistry 67: 2541–2549.

33. Saha, S.; Enugutti, B.; Rajakumari, S. and Rajasekharan, R. 2006. Cytosolic triacylglycerol biosynthetic pathway in oilseeds: molecular cloning and expression of peanut cytosolic diacylglyerol acyltransferase. Plant Physiology 141:1533–1543.

34. Perry, HJ.; Bligny, R.; Gout, E. and Harwood, JL. 1999. Changes in Kennedy pathway intermediates associated with increased triacylglycerol synthesis in oil-seed rape. Phytochemistry 52:799–804.

35. Weselake, RJ.; Shah, S. and Tang, M. 2008. Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (*Brassica napus*) to increase seed oil content. Journal of Experimental Botany 59, 3543–3549.

36. Zheng, P.; Allen, WB. and Roesler, K. 2008. A phenylalanine in DGAT is a key determinant of oil content and composition in maize. Nature Genetics 40: 367–372.

37. Hsieh, K. and Huang, AHC. 2004. Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. Plant Physiology 136, 3427–3434.

38. Murphy, D.J. 2001. The biogenesis and functions of lipid bodies in animals, plants, and microorganisms. Progress in Lipid Research. 40: 325-438.

39. Giannoulia, K.; Banilas, G. and Hatzopoulos, P. 2007. Oleosin gene expression in olive. Journal of Plant Physiology 164: 104–107.

40. Sanchez, J. 1994. Lipid photosynthesis in olive fruit. Progress in Lipid Research 33: 97–104.

41. Banilas, G.; Karampelias, M.; Makariti, I.; Kourti, A. and Hatzopoulos, P. 2011. The olive DGAT2 gene is developmentally regulated and shares overlapping but distinct expression patterns with DGAT1. Journal of Experimental Botany, 62.(2): 521-532.

42. Tzen, JTC.; Cao, YZ.; Laurent, P.; Ratnayake, C. and Huang, AHC. 1993. Lipids, proteins, and structure of seed oil bodies from diverse species. Plant Physiology 101:267–276.

43. Weselake, RJ.; Pomeroy, MK.; Furukawa, TL.; Golden, JL.; Little, DB. and Laroche, A. 1993. Developmental profile of diacylglycerol acyltransferase in maturing seeds of oilseed rape and safflower and microspore-derived cultures of oilseed rape. Plant Physiology 102:565–571.

44. Yu, K.; McCracken, CTJ.; Li, R. and Hildebrand, DF. 2006. Diacylglycerol acyltransferases from Vernonia and Stokesia prefer substrates with vernolic acid. Lipids 41: 557–566.

45. Li, R.; Yu, K. and Hildebrand, DF. 2010. DGAT1, DGAT2, and PDAT expression in seeds and other tissues of epoxy and hydroxy fatty acid accumulating plants. Lipids 45: 145–157.

46. Connor, D.J. and Fereres, E. 2005. The physiology of adaptation and yield expression in olive. Hort. Rev. 31: 155-229.

47. Huang, CY.; Chung, CI.; Lin, YC.; Hsing, YI. and Huang, AH. 2009. Oil bodies and oleosins in Physcomitrell a possess characteristics representative of early trends in evolution. Plant Physiology 150: 1192–1203.

48. Desouky, M.; Laila, F.H.; Abd El-Miggeed, M.M. and El-Hady, E.S. 2010. Changes in some physical and chemical fruit properties during fruit development stage of some olive oil cultivars. American Eurasian, J. Agric. And Environ. Sci., 7 (1) : 12-17.

Published in the Russian Federation
European Journal of Molecular Biotechnology
Has been issued since 2013.
ISSN: 2310-6255
E-ISSN 2409-1332
Vol. 7, Is. 1, pp. 37-52, 2015

DOI: 10.13187/ejmb.2015.7.37
www.ejournal8.com

UDC 579.871.08+577.385.4.08

The Biosynthesis of Deuterium Labeled Amino Acids Using a Strain of Facultative Methylotrophic Bacterium *Brevibacterium Methylicum* 5662 With RuMP Cycle of Carbon Assimilation

[1] Oleg Mosin
[2] Ignat Ignatov
[3] Dmitry Skladnev
[4] Vitaly Shvets

[1] Moscow State University of Applied Biotechnology, Russian Federation
Senior research Fellow of Biotechnology Department, Ph. D. (Chemistry)
103316, Moscow, Talalihina ulitza, 33
E-mail: mosin-oleg@yandex.ru
[2] The Scientific Research Center of Medical Biophysics (SRC MB), Bulgaria
Professor, D. Sc., director of SRC MB
1111, Sofia, N. Kopernik street, 32
E-mail: mbioph@dir.bg
[3] Scientific Center of Russian Federation Research Institute for Genetics and Selection of Industrial Microorganisms “Genetika”, Russian Federation
Professor, D. Sc (Biology), leading scientist of “Genetika”
117545, Moscow, 1st Dorozhniy proezd, 1
E-mail: genetika@genetika.ru
[4] M.V. Lomonosov Moscow University of Fine Chemical Technology, Russian Federation
Academician of Russian Academy of Sciences, D. Sc. (Chemistry), head of the department of biotechnology and nanobiotechnology
119571, Moscow, Vernadskogo avenue, 86
E-mail: mitht@mitht.ru

Abstract

We used Gram-positive aerobic facultative methylotrophic bacterium, *Brevibacterium methylicum*, L-phenylalanine producer with ribulose-5-monophosphate (RuMP) cycle for carbon assimilation for microbiological preparation of [^{2}H]phenylalanine via conversion of low molecular weight substrates ([U-^{2}H]MeOH and ^2H_2O). For this purpose, the cells of the methylotroph with improved growth characteristics were used on minimal salt media M9 supplemented with 2 % (v/v) [U-^{2}H]MeOH and increasing gradient of ^2H_2O concentration from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O. L-phenylalanine was isolated from the growth medium after adding 5 M ^{2}HCl (in ^2H_2O), pH = 2,0 by extraction with isopropanol and subsequent crystallization in ethanol (output 0,65 g/l). Alanine, valine, and leucine/isoleucine were produced and accumulated exogenously in amounts of 5–6 μmol in addition to the main product of biosynthesis. The method allows to obtain [^{2}H]amino acids with different levels of deuterium enrichment, depending on ^2H_2O concentration

in growth media, from 17 atom% ^2H (2 deuterium atoms) (on the growth medium with 24,5 % (v/v) ^2H_2O) up to 75 atom% ^2H (6 deuterium atoms) (on the growth medium with 98 % (v/v) ^2H_2O) with introduction of deuterium to benzyl $C_6H_5CH_2$-fragment of molecule that is confirmed with the data of electron impact (EI) mass spectrometry analysis of methyl ethers of N-5-dimethylamino(naphthalene)-1-sulfochloride [^2H]amino acids after the separation by reverse-phase HPLC.

Keywords: *Brevibacterium methylicum;* [U-^2H]MeOH; heavy water; biosynthesis; [^2H]amino acids; EI mass spectrometry; HPLC.

Introduction

Labeling of amino acid molecules with deuterium is becoming an essential part for various biochemical and biomedical studies with ^2H-labeled molecules and investigation of certain aspects of their metabolism [1]. Tendencies to preferable using of stable isotopes in clinical diagnostics in comparison with their radioactive analogues are caused by absence of radiation danger and possibility of localization of a label in a molecule by highly resolution methods as NMR spectroscopy and mass spectrometry [2]. Development of these methods for detecting stable isotopes for last years has allowed to improve considerably carrying out numerous biological studies with amino acids de novo, and also to study the pathways of their metabolism. ^2H-labeled amino acids are applied in medical diagnostics and biochemical research [3], as well as in chemical synthesis of various isotopically labeled compounds on their basis, for example, [^2H]phenylalanine – in synthesis of peptide hormones and neurotransmitters. Isotopically labeled analogues of L-phenylalanine find the increasing application in diagnostic purposes, for example, for detecting hereditary phenylketonuria (PKU) – a metabolic genetic disorder characterized by a mutation in the gene of the hepatic enzyme phenylalanine hydrolase (PAH), making it nonfunctional and connected with disorders of phenylalanine metabolism in organism [4]. For biomedical diagnostic of PKU is essential to study distribution of isotope label in end-products of metabolism of this amino acid by introducing isotopically labelled phenylalanin into organism [5]. Therefore it is important to develop new biotechnological approaches for preparation of natural isotopically labelled analogues of phenylalanine, including its deuterated analogues. Advantages of biotechnological methods for synthesis of isotopically labelled amino acids in comparison with chemical synthesis consist in high exits of synthesised products and that they possess a natural L-configuration.

For introduction of deuterium into amino acid molecules either chemical or biosynthetic methods may be used. Chemical synthesis of ^2H-labeled compounds has one significant limitation; it is a very laborious and costly multistep process resulting in obtaining a racematic mixture of D,L-enantiomers [6]. Although chemomicrobiological synthesis overcomes this problem [7], the amounts of purified enzymes, required is prohibitive [8]. The biosynthesis of ^2H-labeled amino acids usually involves growth of an organism on selective growth media containing the labeled substrates: e.g., growth of algae autotrophically on media with 96 % (v/v) ^2H_2O, is a well established method for biosynthesis of highly deuterated biochemicals including amino acids [9]. Though this method, being generally applicable, is still limited due to lower resistance of plant cells towards ^2H_2O along with the expense of the mixture of ^2H-labeled amino acids which is isolated from hydrolysates of biomass [10].

Alternatively inexpensive sources for microbiological synthesis of [^2H]amino acids seem methylotrophic bacteria, capable to oxidize methanol (MeOH) and other one-carbon compounds containing methyl CH_3-group to formaldehyde via ribulose-5-monophosphate and serine pathways of carbon assimilation [11]. Interest to use methylotrophic bacteria in biotechnology has recently increased owing to development of new perspective technologies for chemical synthesis of methanol. Owing to 50 % level of bioconversion of methanol (at efficiency of methanol conversion 15,5–17,3 gram of dry biomass for 1 gram of consumed substrate) methlotrophic bacteria are considered as cheap sources of deuterated protein and irreplaceable amino acids, whose technological expenses for their preparation are defined, basically by cost of ^2H_2O and [^2H]methanol. These bacteria have shown a great practical advantage in terms of its industrial usage because of their ability to produce and accumulate in culture liquid large quantities of ^2H-labeled amino acids during its growth on media supplemented with ^2H_2O and [U-^2H]MeOH and is a cost effective process due to lower price of [U-^2H]MeOH [12]. The traditional approach for this aim is cultivation of strain-producers of amino acids on growth media containing [^2H]methanol

and heavy water (^2H_2O) with subsequent fractionation of liquid cultural media for isolation of [^{2}H]amino acids [13].

In the past few years, a certain progress was observed in terms of isolation of methylotrophs wherein RuMP cycle operates. However still for such studies, limited research is carried out with methylotrophs due to poor growth characteristics obtained on ^2H_2O containing media. Realizing this, the production of ^{2}H-labeled amino acids by obligate methylotrophs *Methylobacilus flaggellatum* for carbon assimilation was conducted by 2-keto 3-deoxy 6-phospho-6-gluconate aldolase/transaldolase (KDPGA/TA) variant of RuMP cycle which involves the bacterial growth on salt medium supplemented with approx. 75 % (v/v) ^2H_2O [14]. In the present study, a mutant strain of Gram-positive aerobic facultative methylotroph, *Brevibacterium methylicum*, with NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of RuMP cycle for carbon assimilation was selected, which seems more convenient for the preparation of ^{2}H-labeled amino acids because of its ability to grow on minimal salt media M9 with 98 % (v/v) ^2H_2O as compared to the obligate methylotroph *M. flagellatum* [15].

The main aim of the present study was to study the biosynthesis of deuterium labeled amino acids using Gram-positive aerobic facultative methylotrophic bacterium *B. methylicum*, L-phenylalanine producer, adapted to high level of ^2H_2O in growth media.

Material and methods

Chemicals

^2H_2O (99,9 atom% ^{2}H) was purchased from JSC "Isotope" (Sankt Petersburg, Russia), [U-^{2}H]MeOH (97,5 atom% ^{2}H) (Biophysics Center, Pushino, Russia). Dansylchloride (DNSCl) of sequential grade was from Sigma Chemicals Corp. (USA). Diazomethane (DZM) was prepared from N-nitroso-methylurea (N-NMU) ("Pierce Chemicals Corp.", USA). Inorganic salts were crystallized in 99,9 atom% ^2H_2O; ^2H_2O was distilled over $KMnO_4$ with the subsequent control of isotopic enrichment by ^{1}H-NMR spectroscopy on Brucker WM-250 device ("Brucker Corp.", USA) (working frequency – 70 MHz, internal standard – Me_4Si).

Bacterial Strain

A Gram-positive parental strain of L-leucine auxotroph of facultative methylotrophic bacterium *Brevibacterium methylicum* 5662, producer of L-phenylalanine used in this research was obtained from Russian State Scientific Center for Genetics and Selection of Industrial Microorganisms GNIIGENETIKA. The parental strain was modified by adaptation to ^2H_2O *via* plating the cells onto 2 % (w/v) agarose media with an increasing gradient of ^2H_2O concentration from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O and the subsequent selection of separate colonies resistant to ^2H_2O, capable to produce L-phenylalanine while grown on liquid growth media with the same isotopic content.

Adaptation and Growth Conditions

The parent strain was adapted to deuterium via plating the cells to individual colonies followed by subsequent selection of cell colonies which were resistant to deuterium on solid 2 % (w/v) agarose media M9 with 2 % (v/v) MeOH/[U-^{2}H]MeOH containing a step-wise increasing gradient of ^2H_2O concentration from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O. Minimal salt medium M9 (g/l): KH_2PO_4 – 3; Na_2HPO_4 – 6; NaCl – 0,5; NH_4Cl – 1, with MeOH (2 %, v/v) as a carbon and energy source and L-Leu (100 mg/l) was used for bacterial growth. Selection of separate colonies was performed by their resistance to ^2H_2O. Cell colonies grown on the growth media M9 with a low gradient of ^2H_2O concentration were transferred onto the growth media with higher gradient of ^2H_2O concentration; this was repeated up to 98 % (v/v) ^2H_2O. At a final stage of this technique on growth medium with 98 % (v/v) ^2H_2O were isolated the separate cellular colonies representing posterity of one cell, resistant to the action of ^2H_2O. Then cells were transferred to the liquid growth medium M9 with the same deuterium content, and grew up during 3–4 days at temperature 34 ^oC. The adaptation was monitored by formation of individual colonies onto the surface of solid agarose media with ^2H_2O and also by values of optical density (OD) of cell suspension in liquid media, determined in a quartz cuvette (light path 10 mm) at λ = 620 nm using a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA). The bacterium adapted to ^2H_2O was grown on the maximally deuterated M9 medium with 2 % (v/v) [^{2}H]methanol and 98 % (v/v)

^2H_2O in 250 ml Erlenmeyer flasks containing 50 ml of growth medium at 32–34 °C and vigorously aerated at 200 rpm on orbital shaker Biorad 380-SW ("Biorad Labs", Poland). The exponentially growing cells (cell density 2,0 at absorbance 620 nm) were pelleted by centrifugation (1200 g for 15 min), the supernatant was lyophilized in vacuum and used for chemical derivatization.

Determination of L-Phenylalanine

The amount of *L*-phenylalanine was determined for 10 μl aliquots of liquid minimal salt medium M9 by TLC method with solvent mixture iso-PrOH–ammonia (7 : 3, v/v) using pure commercially available *L*-phenylalanine as a standard. The spots were treated with 0,1 % (w/v) ninhydrin solution (prepared in acetone), and was eluted with 0,5 % (w/v) $CdCl_2$ solution prepared in 50 % (v/v) EtOH (in H_2O) (2 ml). Absorbance of the eluates was measured on spectrophotometer Beckman DU-6 ("Beckman Coulter", USA) at λ = 540 nm, the concentration of *L*-phenylalanine in samples was calculated using a standard calibration curve.

Isolation of [^{2}H]Phenylalanine from Growth Medium

Deutero-biomass of *B. methylicum* obtained after growth in maximally deuterated medium M9 with 2 % (v/v) [U-^{2}H]MeOH and 98 % (v/v) ^2H_2O, was separated using centrifugation ("Heraues Sepatech", Model T-24, Germany) at 1200 g for 15 min. Supernatant thus obtained was evaporated under vacuum. To 5 g of the lyophilized growth medium M9; 30 ml of iso-PrOH was added, and thus acidified to pH = 2,0 using 5 M ^{2}HCl (in ^2H_2O) and keep at room temperature for 4 h. The salts were removed through centrifugation and the supernatant was evaporated. Phenylalanine (0,65 g/l) was recrystallized from EtOH: $[\alpha]_D^{20}$ = 35° (EtOH); UV-spectrum (0,1 *M* HCl): λ_{max} = 257,5 nm, $\varepsilon_{257.5}$ = 1,97·10^2 M^{-1}·cm^{-1}.

Synthesis of Methyl Esters of N-DNS-[^{2}H]Amino Acids

To 200 mg of lyophilized growth media M9 in 5 ml 2 *M* $NaHCO_3$ (0.002 mol) solution, pH = 9–10, 320 mg (1,2 mol) DNSCl in 5 ml of acetone was added. A mixture was kept at 40 °C under vigorous stirring for 1 h. After the reaction was completed, the solution was acidified by 2 *M* HCl till pH = 3,0, and extracted with ethyl acetate (3×5 ml). The combined extracts were dried over sodium sulphate and evaporated under vacuum. The further derivatization to methyl esters of N-DNS-[^{2}H]amino acids was performed using a standard chemical procedure with DZM. To, 20 ml of 40 % (w/v) KOH in 40 ml of diethyl ether 3 g of N-NMU was added and mixed at 120 rpm on a water bath cum stirrer at 4 °C for 15–20 min. After intensive evolution of gaseous DZM ether layer was separated and washed out by cold water till pH = 7,0 was achieved, then dried over sodium sulphate for further processing and analysis.

High-Performance Liquid Chromatography

Analytical separation of methyl esters of N-DNS-[^{2}H]amino acids was performed by a reversed-phase HPLC on liquid chromatograph Knauer ("Knauer GmbH", Germany), supplied with UV-detector and integrator C-R 3A ("Shimadzu", Japan). Methyl esters of N-DNS-[^{2}H]amino acids were detected at λ = 254 nm by UV-absorbance. As a motionless phase was used Separon SGX C_{18} ("Kova", Czech Republic), 22 °C, 18 μm, 150×3,3 mm. The mobile phase composed of a mixture of solvents: (A) – acetonitrile–trifluoroacetic acid (20:80 %, v/v) and (B) – acetonitrile (100%, v/v). The gradient started from 20 % A to 100 % B for 30 min; at 100 % B for 5 min; from 100 % B to 20 % A for 2 min; at 20 % A for 10 min.

EI Mass-Spectrometry

EI MS was performed for the determination of deuterium enrichment levels using methyl esters of N-DNS-[^{2}H] amino acids on Hitachi MB 80 mass spectrometer ("Hitachi", Japan) at an ionizing energy of 70 eV, with an accelerating electrical voltage of 8 kV, and an ion source temperature of 180–200 °C. Each measurement was repeated at least three times. Calculation of deuterium enrichment of [^{2}H]amino acids was performed on a parity of contributions of molecular ion ($M^{+\cdot}$) peaks of N-DNS-[^{2}H]amino acid methyl esters obtained in isotopic experiments relative to the control in protonated minimal M9 medium. Statistical processing of experimental data was carried out with using the program of statistical package, STATISTICA 6.

Results and discussion

Studying phenylalanine biosynthesis by the strain B. methylicum in ^2H_2O containing media.

As is known, the majority of the microorganisms distributed in nature cannot serve as good producers of aromatic amino acids, owing to the presence in a cell effective mechanism of regulation of their biosynthesis, though this ability is known for a number of their mutant forms [16] (Mosin et al., 1996). Effective microbial producers of *L*-phenylalanine are, as a rule, the mutants, which do not have negative control over such key enzymes of biosynthesis of this amino acid as prephenate dehydratase (EC 4.2.1.51), chorismate mutase (EC 5.4.99.5) and 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54). Phenylalanine is synthesized in most bacteria *via* shikimic acid pathway from prephenic acid, which through formation of phenylpyruvate that turns into phenylalanine under the influence of cellular transaminases [17]. The precursors for the biosynthesis of phenylalanine are phosphoenolpyruvate (PRP) and erythrose-4-phosphate (ERP). The latter compound is an intermediate in the pentose phosphate (PenP) pathway and, in some methylotrophs, during the RuMP cycle of carbon assimilation [18]. The basic metabolic transformation of phenylalanine in animals is enzymatic hydroxilation of this amino acid with formation of tyrosine [19].

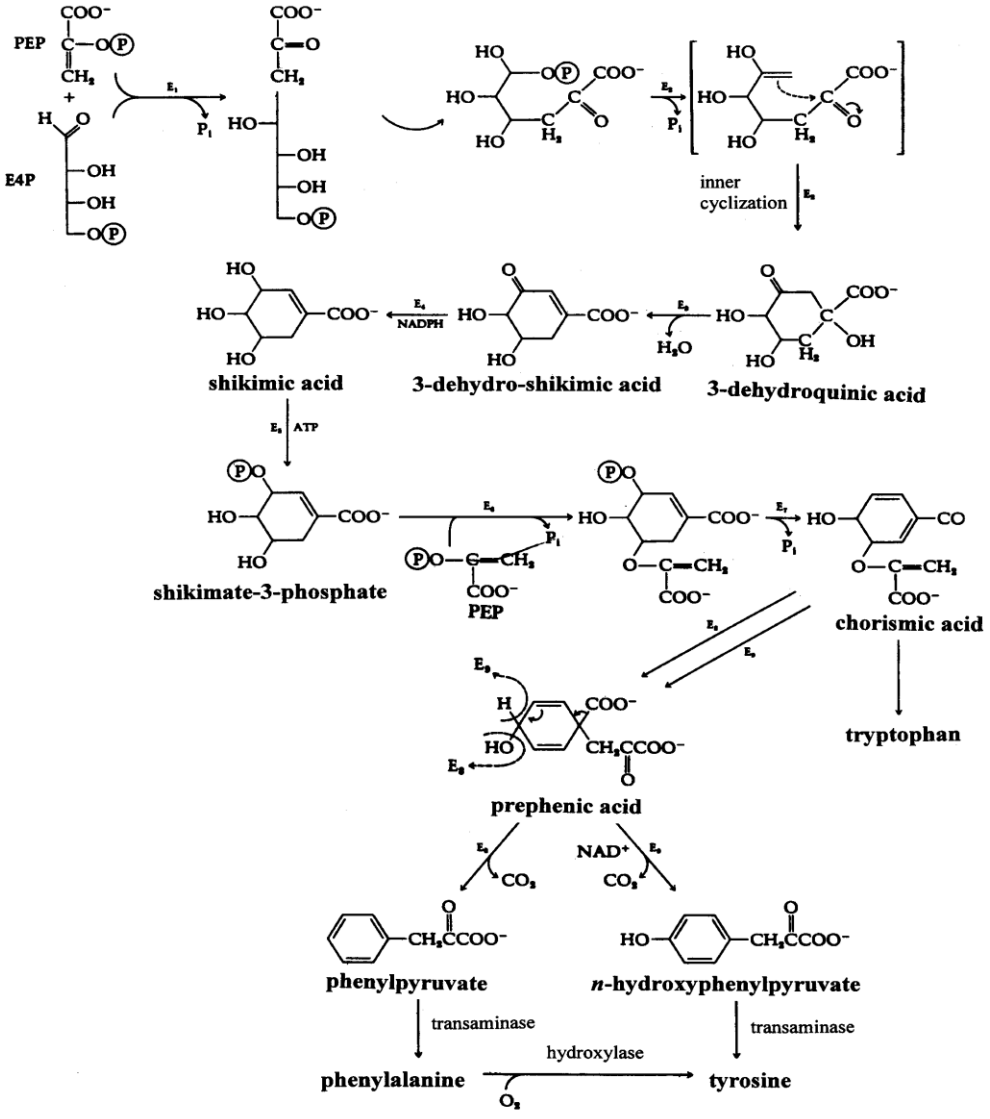


Figure 1. Scheme of biosynthesis of aromatic amino acids – phenylalanine, tyrosine and tryptophan from the general predecessors – phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (adapted from R.C. Bohinski [19])

Phenylalanine is synthesized in most organisms *via* shikimic acid pathway from the common aromatic amino acid precursors as erythrose-4-phosphate (E4P) formed through formation stages of shikimic acid, chorismic acid and prephenic acid (Fig. 1). The basic intermediate products of phenylalanine catabolism and metabolically related tyrosine in various organisms are fumaric acid, pyruvate, succinic acid, acetaldehyde etc. At hereditary penylketonuria disease the hepatic enzyme phenylalanine hydroxylase (PAH), which is necessary to metabolize phenylalanine to tyrosine is nonfunctional, that caused accumulation of phenylalanine and its metabolites (phenylpiruvate, phenyllactate, phenylacetate, *ortho*-hydroxyphenyl acetate), superfluous amounts of which negatively affect development of nervous system [20].

The native bacterial strains can not be the potential producers of phenylalanine owing to their effective mechanisms in its metabolic regulation, although certain bacterial mutants with mutations in their metabolic machinery such as in their of prephenate dehydrogenase (EC 1.3.1.12), prephenate hydratase (EC 4.2.1.51), chorismate mutase (EC 5.4.99.5), 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54) and in other enzymes which thereby proves to be an active producers of this amino acid [21]. The best phenylalanine producing strains once selected were the mutants partially or completely dependent on tyrosine or tryptophan for growth. The reports about other regulative mechanisms for phenylalanine biosynthesis in bacterial cells are quite uncommon, though it is known that RuMP cycle operates in certain number of auxotroph mutants of methylotrophs, covers numerous steps in aromatic amino acid biosynthesis [22].

A certain practical interest represents the research of the ability to produce L-phenylalanine by a leucine auxotroph of Gram-negative aerobic facultative methylotrophic bacterium *B. methylicum* realizing the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation – convenient, but insufficiently explored object for biotechnological usage. Unlike other traditional producers of L-phenylalanine, which do not have negative control of prephenate dehydratase (EC 4.2.1.51) or 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54), the uniqueness of this strain consists in that it requires L-leucine for growth. The initial stage of biochemical research with this strain of methylotrophic bacteria was connected with obtaining auxotrophic mutants, which in majority cases are characterized by the limited spectrum of mutant phenotypes and, besides that the high level of reversions. The initial L-leucine dependent strain *B. methylicum*, producer of L-phenylalanine was obtained *via* selection at previous stage of research after processing of parental strain by nitrozoguanidin. Screening for resistant cell colonies was carried out by their stability to the analogue of phenylalanine – *meta*-fluoro-phenylalanine (50 μg/ml). The analogue resistant mutants allocated on selective media were able to convert methanol and accumulate up to 1 gram per 1 liter of L-phenylalanine into growth media. Comparative analyses (TLC, NMR) showed, that L-phenylalanine, produced by this strain of methylotrophic bacteria is identical to the natural L-phenylalanine.

Further attempts were made to intensify the growth and biosynthetic parameters of this bacterium in order to grow on media M9 with higher concentration of deuterated substrates – [U-^{2}H]MeOH and ^2H_2O. For this, deuterium enrichment technique *via* plating cell colonies on 2 % (w/v) agarose media M9 supplemented with 2 % (v/v) [U-^{2}H]MeOH with an increase in the ^2H_2O content from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O, combined with subsequent selection of cell colonies which were resistant to deuterium. The degree of cell survive on maximum deuterated medium was approx. 40 %. The data on the yield of biomass of initial and adapted *B. methylicum*, magnitude of lag-period and generation time on protonated and maximum deuterated M9 medium are shown in Figure 2. The yield of biomass for adapted methylotroph (*c*) was decreased approx. on 13 % in comparison with control conditions (*a*) at an increase in the time of generation up to 2,8 h and the lag-period up to 40 h (Figure 2). As is shown from these data, as compared with the adapted strain, the growth characteristics of initial strain on maximally deuterated medium were inhibited by deuterium.

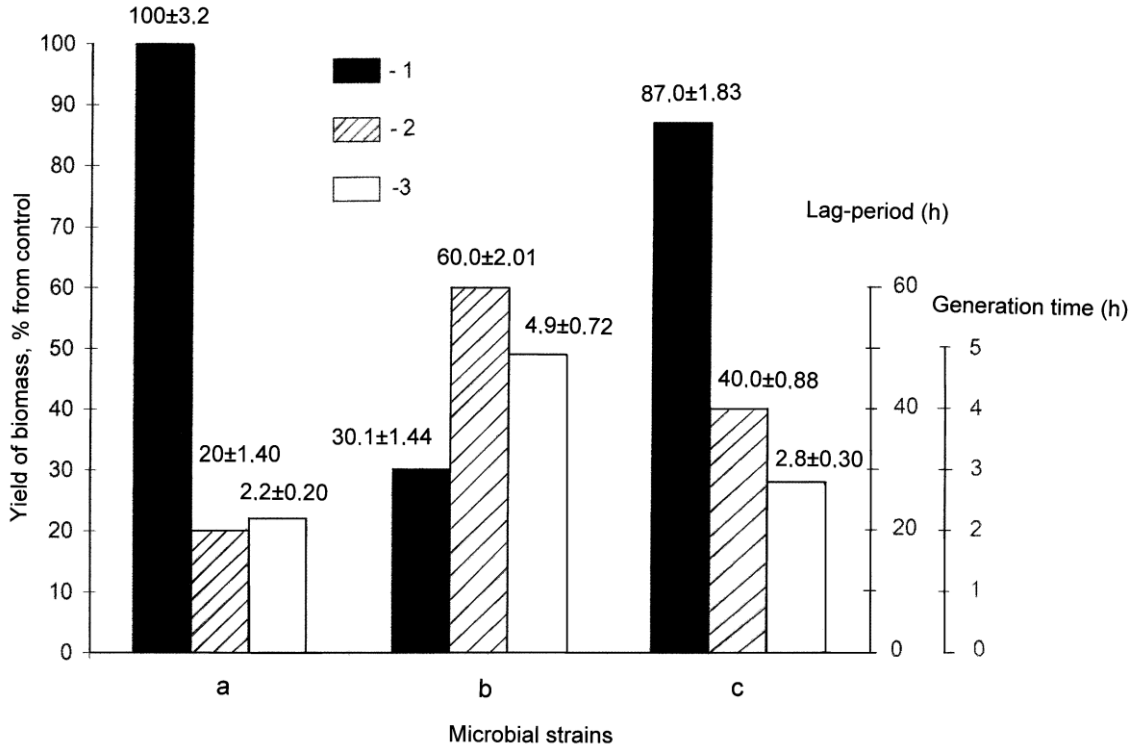


Figure 2. Yield of microbial biomass of *B. methylicum*, magnitude of lag-period and generation time in various experimental conditions: initial strain on protonated M9 medium (control) with water and methanol (*a*); initial strain on maximally deuterated M9 medium (b); adapted to deuterium strain on maximally deuterated M9 medium (c): 1 – yield of biomass, % from the control; 2 – duration of lag-period, h; 3 – generation time, h.

Experimental conditions are given in Table 1 (expts. 1–10) relative to the control (expt. 1) on protonated medium M9 and to the adapted bacterium (expt. 10'). Various compositions of [U-^{2}H]MeOH and ^2H_2O were added to growth media M9 as hydrogen/deuterium atoms could be assimilated both from MeOH and H_2O. The maximum deuterium content was under conditions (10) and (10') in which we used 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]MeOH. The even numbers of experiment (Table 1, expts. 2, 4, 6, 8, 10) were chosen to investigate whether the replacement of MeOH by its deuterated analogue affected growth characteristics in presence of ^2H_2O. That caused small alterations in growth characteristics (Table 1, expts. 2, 4, 6, 8, 10) relative to experiments, where we used protonated methanol (Table 1, expts. 3, 5, 7, 9). The gradual increment in the concentration of ^2H_2O into growth medium caused the proportional increase in lag-period and yields microbial biomass in all isotopic experiments. Thus, in the control (Table 1, expt. 1), the duration of lag-period did not exceed 20,2 h, the yield of microbial biomass (wet weight) and production of phenylalanine were 200,2 and 0,95 gram per 1 liter of growth medium. The results suggested, that below 49 % (v/v) ^2H_2O (Table 1, expts. 2–4) there was a small inhibition of bacterial growth compared with the control (Table 1, expt. 1). However, above 49 % (v/v) ^2H_2O (Table 1, expts. 5–8), growth was markedly reduced, while at the upper content of ^2H_2O (Table 1, expts. 9–10) growth got 3,3-fold reduced. With increasing content of ^2H_2O in growth media there was a simultaneous increase both of lag-period and generation time. Thus, on maximally deuterated growth medium (Table 1, expt. 10) with 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]MeOH, lag-period was 3 fold higher with an increased generation time to 2,2 fold as compared to protonated growth medium with protonated water and methanol which serve as control (Table 1, expt. 1). While on comparing adapted bacterium on maximally deuterated growth medium (Table 1, expt. 10') containing 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]MeOH with non adapted bacterium at similar concentration showed 2.10 and 2.89 fold increase in terms of phenylalanine production and biomass yield due to deuterium enrichment technique, while, the lag phase as well as generation time also got reduced to 1,5 fold and 1,75 fold in case of adapted bacterium.

Table 1: Effect of variation in isotopic content (0–98 % ^2H_2O, v/v) present in growth medium M9 on bacterial growth of B. methylicum and phenylalanine production

Exp. number	Media components, % (v/v)				Lag-period (h)	Yield in terms of wet biomass (g/l)	Generation time (h)	Phenylalanine production (g/l)
	H_2O	^2H_2O	MeOH	[U-^{2}H] MeOH				
1 (control)	98,0	0	2	0	20,2±1,40	200,2±3,20	2,2±0,20	0,95±0,12
2	98,0	0	0	2	20,3±1,44	184,6±2,78	2,4±0,23	0,92±0,10
3	73,5	24,5	2	0	20,5±0,91	181,2±1,89	2,4±0,25	0,90±0,10
4	73,5	24,5	0	2	34,6±0,89	171,8±1,81	2,6±0,23	0,90±0,08
5	49,0	49,0	2	0	40,1±0,90	140,2±1,96	3,0±0,32	0,86±0,10
6	49,0	49,0	0	2	44,2±1,38	121,0±1,83	3,2±0,36	0,81±0,09
7	24,5	73,5	2	0	45,4±1,41	112,8±1,19	3,5±0,27	0,69±0,08
8	24,5	73,5	0	2	49,3±0,91	94,4±1,74	3.8±0.25	0,67±0,08
9	98,0	0	2	0	58,5±1,94	65,8±1,13	4,4±0,70	0,37±0,06
10	98,0	0	0	2	60.1±2.01	60,2±1,44	4,9±0,72	0,39±0,05
10’	98,0	0	0	2	40.2±0.88	174,0±1,83	2,8±0,30	0,82±0,08

Notes:
* The date in expts. 1–10 described the growth characteristics for non-adapted bacteria in growth media, containing 2 % (v/v) MeOH/[U-^{2}H]MeOH and specified amounts (%, v/v) of ^2H_2O.
** The date in expt. 10’ described the growth characteristics for bacteria adapted to maximum content of deuterium in growth medium.
***As the control used exprt. 1 where used ordinary protonated water and methanol

The adapted strain of *B. methylicum* eventually came back to normal growth at placing over in protonated growth medium after some lag-period that proves phenotypical nature of a phenomenon of adaptation that was observed for others adapted by us strains of methylotrophic bacteria and representatives of other taxonomic groups of microorganisms [23-25]. The effect of reversion of growth in protonated/deuterated growth media proves that adaptation to ^2H_2O is rather a phenotypical phenomenon, although it is not excluded that a certain genotype determines the manifestation of the same phenotypic trait in the growth media with different isotopic compositions. On the whole, improved growth characteristics of adapted methylotroph essentially simplify the scheme of obtaining the deutero-biomass which optimum conditions are M9 growth medium with 98 % ^2H_2O and 2 % [^{2}H]methanol with incubation period 3–4 days at temperature 35^{o}C.

Literature reports clearly reveal that the transfer of deuterated cells to protonated medium M9 eventually after some lag period results in normal growth that could be due to the phenomenon of adaptation wherein phenotypic variation was observed by the strain of methylotrophic bacteria. The effect of reversion of growth in protonated/deuterated growth media proves that adaptation to ^2H_2O is a phenotypical phenomenon, although it cannot be excluded that a certain genotype determined the manifestation of the same phenotypic attribute in media with high deuterium content. The improved growth characteristics of the adapted bacterium essentially simplify the obtaining of deutero-biomass in growth medium M9 with 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]methanol.

Adaptation, which conditions are shown in experiment 10’ (Table 1) was observed by investigating of growth dynamics (expts. 1*a,* 1*b,* 1*c*) and accumulation of L-phenylalanine into growth media (expts. 2*a,* 2*b,* 2*c*) by initial (*a*) and adapted to deuterium (*c*) strain *B. methylicum* in maximum deuterated growth medium M9 (Fig. 3, the control (*b*) is obtained on protonated growth medium M9). In the present study, the production of phenylalanine (Fig. 3, expts. 1b, 2b, 3b) was studied and was found to show a close linear extrapolation with respect to the time up to exponential growth dynamics (Fig. 3, expts. 1a, 2a, 3a). The level of phenylalanine production for non-adapted bacterium on maximally deuterated medium M9 was 0,39 g/liter after 80 hours of growth (Fig. 3, expt. 2b). The level of phenylalanine production by adapted bacterium under those growth conditions was 0,82 g/liter (Fig. 3, expt. 3b). Unlike to the adapted strain the growth of initial strain and production of phenylalanine in maximum deuterated growth medium were inhibited. The important feature of adapted to ^2H_2O strain *B. methylicum* was that it has kept its ability to synthesize and exogenously produce L-phenylalanine into growth medium. Thus, the use

of adapted bacterium enabled to improve the level of phenylalanine production on maximally deuterated medium by 2.1 times with the reduction in the lag phase up to 20 h. This is an essential achievement for this strain of methylotrophic bacteria, because up till today there have not been any reports about production of phenylalanine by leucine auxotrophic methylotrophs with the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation. This makes this isolated strain unique for production of phenylalanine.

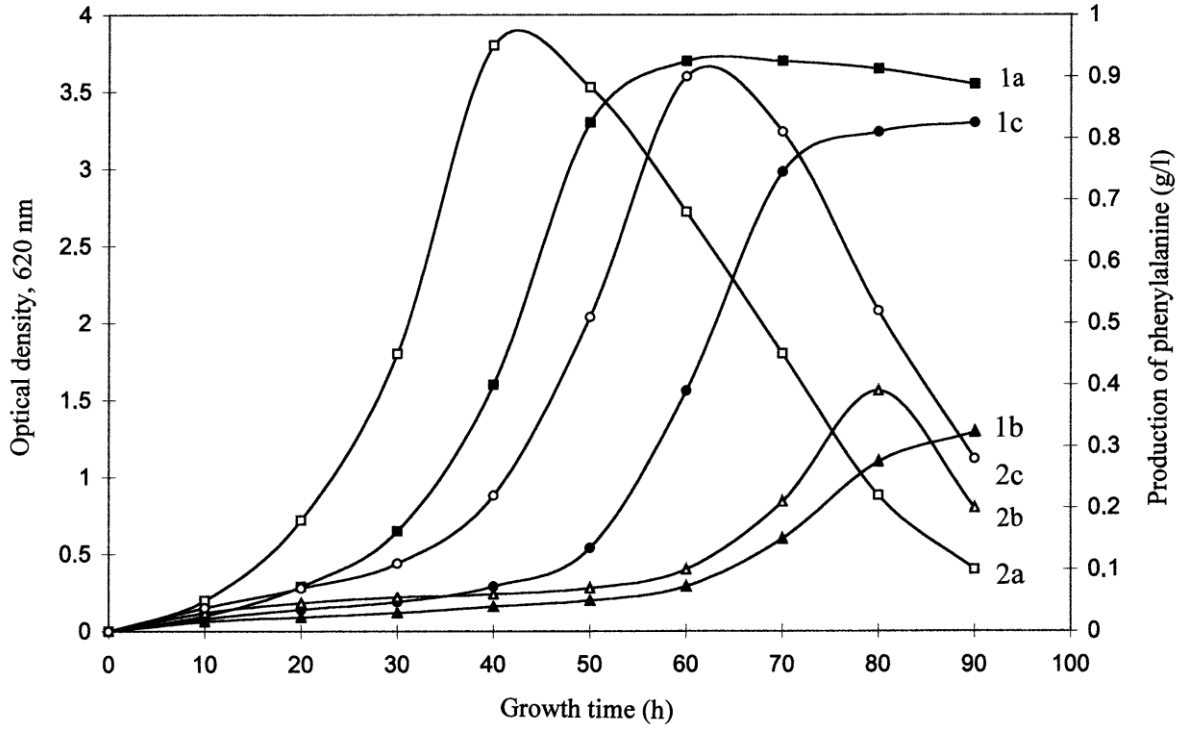


Figure 3. Growth dynamics of *B. methylicum* (1a, 2a, 3a) and production of phenylalanine (1b, 2b, 3b) on media M9 with various isotopic content: 1a, 1b – non-adapted bacterium on protonated medium (Table 1, expt. 1); 2a, 2b – non-adapted bacterium on maximally deuterated medium (Table 1, expt. 10); 3a, 3b – adapted bacterium on maximally deuterated medium (Table 1, expt. 10')

The general feature of phenylalanine biosynthesis in $H_2O/^2H_2O$-media was increase of its production at early exponential phase of growth when outputs of a microbial biomass were insignificant (Figure 3). In all the experiments it was observed that there was a decrease in phenylalanine accumulation in growth media at the late exponential phase of growth. Microscopic research of growing population of microorganisms showed that the character of phenylalanine accumulation in growth media did not correlate with morphological changes at various stages of the cellular growth. Most likely that phenylalanine, accumulated in growth media, inhibited enzymes of its biosynthetic pathways, or it later may be transformed into intermediate compounds of its biosynthesis, e.g. phenylpyruvate [26]. It is necessary to notice, that phenylalanine is synthesised in cells of microorganisms from prephenic acid, which through a formation stage of phenylpiruvate turns into phenylalanine under the influence of cellular transaminases. However, phenylalanine was not the only product of biosynthesis; other metabolically related amino acids (alanine, valine, and leucine/isoleucine) were also produced and accumulated into growth media in amounts of 5–6 μmol in addition to phenylalanine. This fact required isolation of [^{2}H]phenylalanine from growth medium, which was carried out by extraction of lyophilized LC with iso-PrOH and the subsequent crystallization of [^{2}H]phenylalanine in EtOH. Analytical separation of [^{2}H]phenylalanine and metabolically related [^{2}H]amino acids was performed using a

reversed-phase HPLC on Separon SGX C_{18} Column, developed for methyl esters of N-DNS-[^{2}H]amino acids with chromatographic purity of 96–98 % and yield of 67–89 %.

Study of deuterium inclusion into molecules of secreted amino acids

For evaluation of deuterium enrichment by EI MS method, methyl esters of N-DNS-[^{2}H]amino acids were applied because the peaks of molecular ions ($M^{+\cdot}$) allow to monitor the enrichment of multicomponential mixtures of [^{2}H]amino acids in composition with growth media metabolites. Furthermore, EI MS allows detect amino acid samples with concentrations 10^{-9}–10^{-10} mol. This method in combination with reversed-phase HPLC has proved to be better for research studies on deuterium enrichment levels of [^{2}H]amino acids in composition of their multicomponental mixtures of lyophilized growth media, and can be used for the analysis of amino acids of various natural sources. N-DNS-amino acids were obtained through the derivatization of lyophilized growth media with DNSCl. The reaction was carried out in the alkaline environment in the presence of 2 M $NaHCO_3$ (pH = 9–10) in water-organic solvent (acetone) in the mass ratio of DNSCl – the amino acid, (2:1, w/w). The volatility of N-DNS-amino acids in the mass spectrometry analysis may be increased by additional derivatization on carboxyl group (esterification) by diazomethane. The choice of DZM as esterification reagent was caused by the necessity of carrying out the reaction of esterification in soft conditions excluding isotopic (^{1}H–^{2}H) exchange in an aromatic fragment of the phenylalanine molecule. However, with DZM treatment derivatization occurs on αNH_2-group in molecules, so that their N-methylated derivatives could be formed in addition to the methyl ester of N-DNS-amino acids.

The control over deuterium inclusion into phenylalanine molecule due to the conversion of [U-^{2}H]MeOH at growth of the bacterium on medium M9, containing H_2O and 2 % (v/v) [U-^{2}H]MeOH (Table 1, expt. 2) has shown insignificant amount of deuterium detected in molecule. The enrichment level was calculated on intensity of a peak ($M^{+\cdot}$) at *m/z* 413 minus the contribution of peak of an impurity of a natural isotope (no more than 5 %). This testified about delution of deuterium label *via* biochemical processes connected with disintegration of [U-^{2}H]MeOH during its assimilation by a cell, as well as reactions of isotopic (^{1}H–^{2}H) exchange and dissociation in ^2H_2O. Thus, from 4 deuterium atoms in molecule $C^2H_3O^2H$, only deuterium at hydroxyl O^2H-group is the most mobile and easily dissociates one deuterium atom in water with formation of C^2H_3OH. Three remained deuterium atoms in C^2H_3OH are included into a cycle of biochemical oxidation of methanol leading to formation of substances more oxidized than methanol, *e.g*. formaldehyde. In particular, this confirms the classical scheme of biochemical oxidations of methanol to formaldehyde by methylotrophic bacteria, after that formaldehyde assimilates by this strain of methylotrophic bacteria *via* RuMP cycle of carbon assimilation.

EI mass spectra of methyl esters of N-DNS-amino acids obtained from growth media M9 where used 0; 49,0; 73,5 and 98 % (v/v) ^2H_2O with 2 % (v/v) [U-^{2}H]MeOH (Table 1, expts. 1, 6, 8, 10) are shown in consecutive order in Fig. 4–7. The fragmentation pathways of methyl esters of N-DNS-amino acids by EI MS method lead to the formation of distinguished peaks of molecular ions ($M^{+\cdot}$) from which the fragments with smaller *m/z* ratio further are formed. The peak of amino fragment A at *m/z* 353 generally has a low intensity, while and peak of aminoacyl fragment B at *m/z* 381 has the lowest intensity in EI mass spectra, or ever absent (see as example Fig. 4 corresponds to expt. 1, Table 1). A high continuous left background region in EI mass spectrum at *m/z* 100–200 is associated with peaks of contaminant metabolites and products of derivatization of metabolites of growth medium with DNSCl and DZM, and peaks at *m/z* 250, 234, 170 are fragments of further decay of dansyl fragment to N-(dimethylamino)naphthalene. A right region in EI mass spectra contains four peaks of molecular ions ($M^{+\cdot}$) of N-DNS-amino acid methyl esters: Phe at *m/z* 412; Leu/Ile at *m/z* 378; Val at *m/z* 364; Ala at *m/z* 336 (Fig. 4), which fragmentation by the method of EI mass spectrometry will allow to carry out mass spectrometry monitoring of [^{2}H]amino acids in composition of intact LC of the strain-producer, containing mixes of amino acids and other metabolites of growth media before their chromatographic separation. However, since the value of ($M^{+\cdot}$) for Leu is the same as for Ile, these two amino acids could not be clearly estimated by EI MS method.

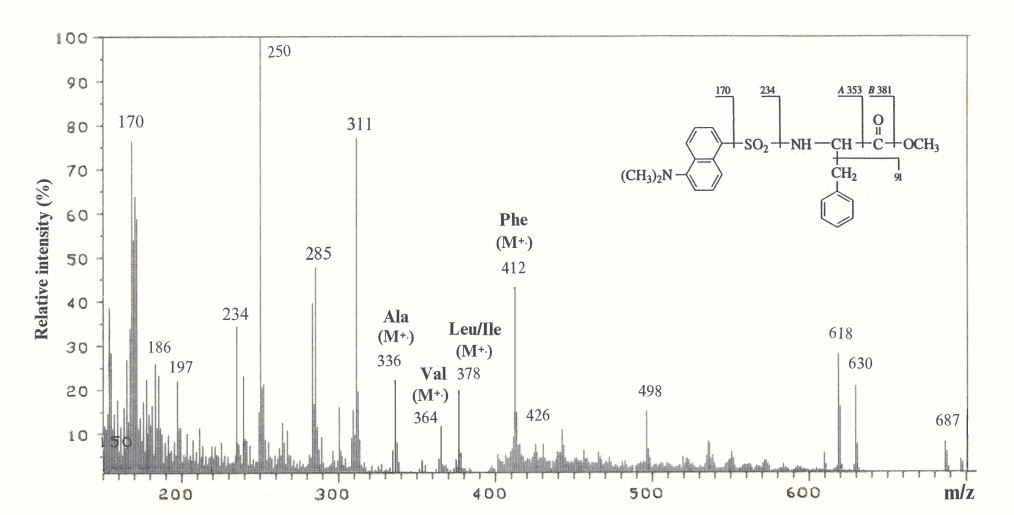


Figure 4. EI Mass spectrum of methyl esters of N-DNS-amino acids from protonated growth medium M9 (expt. 1, Table 1) after processing by DNSCl and DZM, and fragmentation of methyl ester of N-DNS-phenylalanine by EI MS method. Symbols of amino acids refer to the peaks of molecular ions ($M^{+\cdot}$) of methyl esters of N-DNS-amino acids

The results confirmed the character of labeling of [^{2}H]amino acids as heterogeneous, judging by the presence of clusters of adduct peaks in molecular ions ($M^{+\cdot}$) in EI mass spectra; the species of molecules with different numbers of deuterium atoms were detected. Therefore, the peaks of a molecular ion of amino acid derivatives ($M^{+\cdot}$) were split polymorphously on separate clusters with an impurity of molecules having a statistical set of mass numbers *m/z* with the various contribution to a total level of deuterium enrichment of the molecule. The most abundant peak ($M^{+\cdot}$) in each cluster (the peak with the greatest contribution to level of deuterium enrichment), was a peak with an average *m/z* ratio registered by mass spectrometer in each experimental condition, relative to which the deuterium enrichment of each individual [^{2}H]amino acid was calculated. Thus, in experiment (expt. 6, Table 1) shown in Figure 5 where was used 49 % (v/v) ^2H_2O deuterium enrichment of Phe molecule was 2 (27,5 % ^{2}H), calculated by ($M^{+\cdot}$) at *m/z* 414 (instead of ($M^{+\cdot}$) at *m/z* 412 for non-labeled compound); Leu/Ile – 5 (50 % ^{2}H) (($M^{+\cdot}$) at *m/z* = 383 instead of ($M^{+\cdot}$) at *m/z* = 378)); Val – 4 (50 % ^{2}H) (($M^{+\cdot}$) at *m/z* = 368 instead of ($M^{+\cdot}$) at *m/z* = 364)); Ala – 3 (50 % ^{2}H) deuterium atoms (($M^{+\cdot}$) at *m/z* = 339,2 instead of ($M^{+\cdot}$) at *m/z* = 336)). The area of EI mass spectrum with values *m/z* = 90–300 corresponds to products of derivatization of metabolites of growth media by dansylchloride and diazomethane. The law intensity peak at *m/z* = 431 detected in EI mass spectra in all isotopic experiments, corresponds to a product of additional methylation of [^{2}H]phenylalanine on α-NH-(Dns)-group. The peak at *m/z* = 400 (Fig. 5) corresponds to a product of chip off methyl CH_3-group from deuterated methyl ester of N-Dns-[^{2}H]phenylalanine.

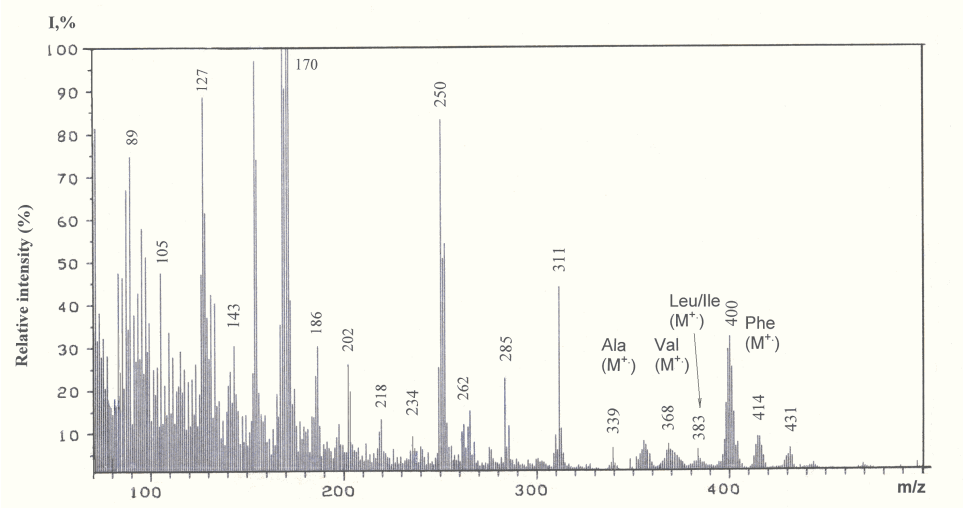


Figure 5. EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from growth medium M9 containing 2 % (v/v) [U-^{2}H]MeOH and 49,0 % (v/v) ^2H_2O (expt. 6, Table 1)

The similar result on proportional specific increase of levels of deuterium enrichment in [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids was observed in all isotopic experiments wherein used increasing concentration ^2H_2O in growth media. With increasing ^2H_2O content in growth media M9, the levels of deuterium enrichment in [^{2}H]amino acids varied proportionally. As shown in Fig. 6 in experiment (expt. 8, Table 1) where in 73,5 % (v/v) of ^2H_2O, the deuterium enrichment of Phe was 4 (50 % ^{2}H) ($(M^{+\cdot})$ at m/z = 416 instead of m/z = 412 ($M^{+\cdot}$)); Leu/Ile – 5 (50 % ^{2}H) ($(M^{+\cdot})$ at m/z = 383 instead of ($M^{+\cdot}$) at m/z = 378); Val – 4 (50 % ^{2}H) ($(M^{+\cdot})$ at m/z = 368 instead of ($M^{+\cdot}$) at m/z = 364; Ala – 3 (50 % ^{2}H) deuterium atoms ($(M^{+\cdot})$ at m/z = 339 instead of ($M^{+\cdot}$) at m/z = 336). Evidently, the deuterium atoms at carbon backbone of [^{2}H]amino acid molecules were synthesized *de novo* from deuterated substrates. Easily exchanged protons (deuterons), *e.g.* protons at heteroatoms in NH_2- and COOH-groups of amino acids could be replaced by deuterium due to dissociation in $H_2O/^2H_2O$.

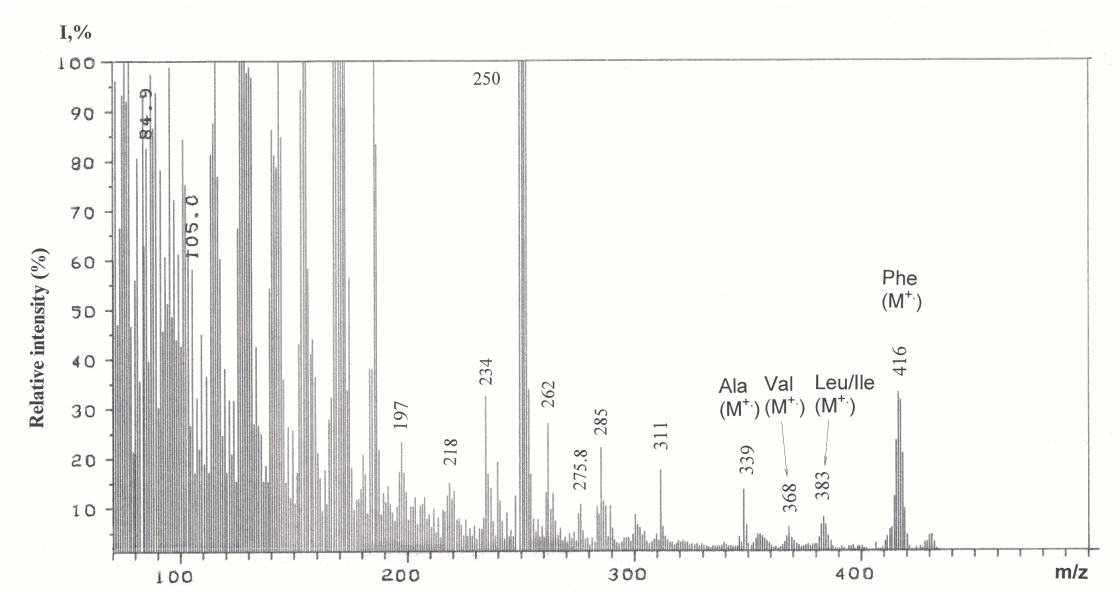


Figure 6. EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from growth medium M9 containing 2 % (v/v) [U-^{2}H]MeOH and 73,5 % (v/v) ^2H_2O (expt. 8, Table 1).

The used methylotrophic bacterium was leucine auxotroph with leucine being added to growth media in protonated form; therefore the levels of deuterium enrichment in [^{2}H]amino acids of pyruvic acid family as alanine, valine and leucine were less than for phenylalanine (phenylalanine related to the family of the aromatic amino acids synthesized from shikimic acid). This is distinctly visible on the maximally deuterated M9 medium. As shown in Fig. 7 in experiment (expt. 10, Table 1) wherein 98 % (v/v) ^2H_2O was used the deuterium enrichment of Phe molecule was 6 (75 at.% ^{2}H) ($(M^{+\cdot})$ at m/z = 418 instead of ($M^{+\cdot}$) at m/z = 412); Leu/Ile – 5 (50 % ^{2}H) ($(M^{+\cdot})$ at m/z = 383 instead of m/z = 378 ($M^{+\cdot}$)); Val – 5 (62,5 % ^{2}H) ($(M^{+\cdot})$ at m/z = 369 instead of m/z = 364 ($M^{+\cdot}$)); Ala – 3 (50 % ^{2}H) deuterium atoms ($(M^{+\cdot})$ at m/z = 339 instead of m/z = 336 ($M^{+\cdot}$)). The peak at m/z = 432, detected in EI mass spectrum of methyl esters of N-DNS-amino acids in Figure 7 corresponds to a product of additional methylation of [^{2}H]phenylalanine on α-NH_2-group. Additionally, in EI mass spectrum is detected a peak of deuterium enriched benzyl $C_6H_5CH_2$-fragment of phenylalanine molecule at m/z = 97 (instead of m/z = 91 in the control), that specifies sites of localization of 6 deuterium atoms in [^{2}H]phenylalanine at positions C1–C6 of aromatic protons in benzyl $C_6H_5CH_2$-fragment. EI MS data suggest that at other concentration of ^2H_2O deuterium atoms also incorporated into the aromatic ring of the [^{2}H]phenylalanine molecule as metabolism of adapted to ^2H_2O bacterium does not undergo essential changes in ^2H_2O. The obtained result on distribution of atoms of deuterium into [^{2}H]phenylalanine molecule is important for its further use in medical diagnostics where it is necessary to use [^{2}H]amino acids with high levels of isotope enrichment.

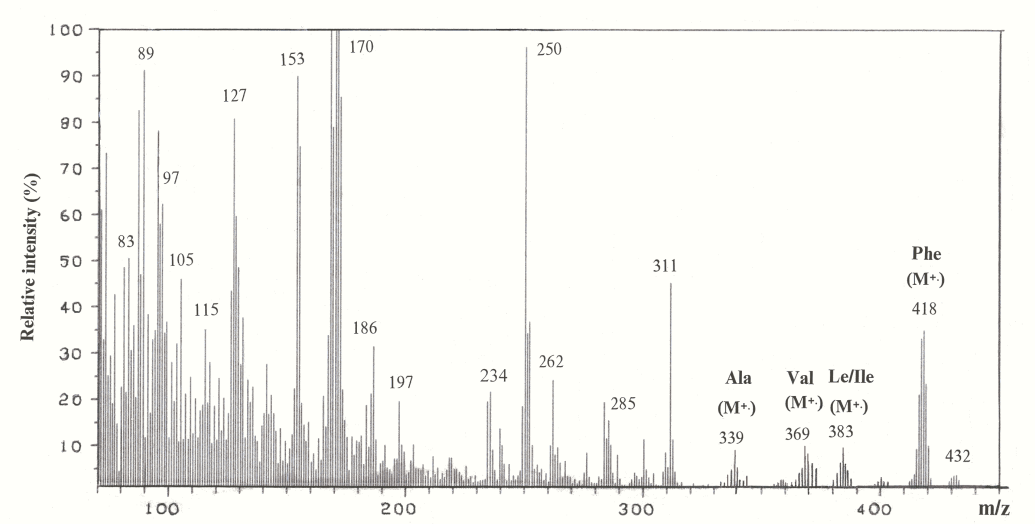


Figure 7. EI mass spectrum of methyl esters of N-DNS-[^{2}H]amino acids from growth medium M9 containing 2 % (v/v) [U-^{2}H]MeOH and 98 % (v/v) ^2H_2O (expt. 10, Table 1).

The similar result on proportional specific increase of levels of deuterium enrichment into [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids (alanine, valine and leucine/isoleucine) was observed in all isotopic experiments where used increasing concentration ^2H_2O in growth media (Table 2). Predictably, enrichment levels of [^{2}H]phenylalanine related to the family of aromatic amino acids synthesised from shikimic acid and metabolically related [^{2}H]amino acids of pyruvic acid family – alanine, valine and leucine at identical ^2H_2O concentration in growth media are correlated among themselves. Such result is fixed in all isotope experiments with ^2H_2O (Table 2). Unlike [^{2}H]phenylalanine, deuterium enrichment levels in accompanying [^{2}H]amino acids – Ala, Val and Leu/Ile keep a stable constancy within a wide interval of ^2H_2O concentration: from 49 % (v/v) to 98 % (v/v) ^2H_2O (Table 2). Summarizing these data, it is possible to draw a conclusion on preservation of minor pathways of the metabolism connected with biosynthesis of leucine and metabolic related amino acids of pyruvic acid family – alanine and valine, which enrichment levels were in correlation within identical concentration of H_2O in growth media (phenylalanine is related to the family of aromatic amino acids synthesized from shikimic acid). Since leucine was added into growth media in protonated form, another explanation of this effect, taking into consideration the various biosynthetic pathways of Leu and Ileu (Ileu belongs to the family of aspartic acid, while Leu belongs to the pyruvic acid family), could be cell assimilation of protonated leucine from growth media. Since Leu and Ileu could not be clearly estimated by EI MS method, nothing could be said about possible biosynthesis of [^{2}H]isoleucine. Evidently, higher levels of deuterium enrichment can be achieved by replacement of protonated leucine on its deuterated analogue, which may be isolated from hydrolysates of deuterated biomass of this methylotrophic bacterium.

Table 2: Effect of deuterium enrichment levels (atom%) in the molecules of [^{2}H]amino acids excreted by *B. methylicum**

[^{2}H]amino acid	Concentration of ^2H_2O in growth media, % (v/v)**			
	24,5	49,0	73,5	98,0
Alanine	24,0±0,70	50,0±0,89	50,0±0,83	50,0±1,13
Valine	20,0±0,72	50,0±0,88	50,0±0,72	62,5±1,40
Leucine/isoleucine	20,0±0,90	50,0±1,38	50,0±1,37	50,0±1,25
Phenylalanine	17,0±1,13	27,5±0,88	50,0±1,12	75,0±1,40

Notes:

* At calculation of enrichment levels protons (deuterons) at COOH- and NH_2-groups of amino acids were not considered because of dissociation in H_2O (^2H_2O).

** The data on enrichment levels described bacteria grown on minimal growth media M9 containing 2 % (v/v) [U-^{2}H]MeOH and specified amounts (%, v/v) of ^2H_2O.

Conclusions

As a result of the selection approach used in this research it was possible to adapt L-phenylalanine producer strain of aerobic Gram-positive facultative methylotrophic bacterium *B. methylicum* to maximal concentration of deuterated substrates for microbiological preparation of [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids (alanine, valine and leucine/isoleucine) with various levels of deuterium enrichment. Advantages of this methylotroph for synthesis of [^{2}H]amino acids are improved growth and biosynthetic characteristics on maximally deuterated growth medium, which was achieved by adaptation to ^2H_2O. By using the adapted methylotroph it was possible to obtain 0,82 g/liter of [^{2}H]phenylalanine (75 % ^{2}H), which was isolated from growth medium by extraction with iso-PrOH and the subsequent crystallization in EtOH. [^{2}H]phenylalanine was also isolated from growth medium by reversed-phase HPLC as methyl ester of N-DNS-[^{2}H]phenylalanine with a yield of 85 % and a purity of 97 %. The method is suitable for preparation of other [^{2}H]amino acids produced by methylotrophic bacteria. It should be noted, however, that higher levels of deuterium enrichment into [^{2}H]amino acid molecule can be achieved via replacement of protonated leucine on its deuterated analogue.

References:

1. LeMaster D.M. Deuterium labeling in NMR structural analysis of larger proteins / D.M. LeMaster // Quarterly Reviews of Biophysics. 1990. V. 23. P. 133–174.

2. Mosin O.V. Mass-spectrometric determination of levels of enrichment of ^{2}H and ^{13}C in molecules of amino acids of various bacterial objects / O.V. Mosin, D.A. Skladnev, T.A. Egorova, V.I. Shvets // Bioorganic Chemistry. 1996. V. 22. № 10–11. P. 56–869.

3. Kushner D.I. Pharmacological uses and perspectives of heavy water and deuterated compounds / D.I. Kushner, A. Baker, T.G. Dunstall // Canadian Journal of Physiology and Pharmacology. 1999. V. 77. № 2. P. 79–88.

4. Surtees R. The neurochemistry of phenylketonuria / R. Surtees, N. Blau // European Journal of Pediatrics. 2000. V. 169. P. 109–113.

5. Dewick P.M. Medicinal Natural Products: A Biosynthetic Approach / P/M. Dewick, ed. – New York: John Wiley & Sons Ltd, 2009, P. 137–186.

6. Blomquist A.T. Synthesis of highly deuterated amino acids / A.T. Blomquist, R.J. Cedergren, B.F. Hiscock, S.L. Tripp, D.N. Harp // PNAS. 1966. V. 55. № 3. P. 453–456.

7. Faleev N.G. Preparation of α-deuterated amino acids by E. coli cells containing tryptophanase / N.G. Faleev, S.B. Ruvinov, M.B. Saporovskaya, V.M. Belikov, L.N. Zakomyrdina // Izv. Akad. Nauk. USSR Ser. Khim. 1989. V. 10. P. 2341–2343 [in Russian].

8. Walker T.E. An efficient chemomicrobiological synthesis of stable isotope-labeled L-tyrosine and L-phenylalanine / T.E. Walker, C. Matheny // Journal of Organic Chemistry. 1986. V. 51. P. 1175–1179.

9. Cox J. Stable isotope labeled biochemicals from microalgae / J. Cox, D. Kyli // Trends in Biotechnology. 1988. V. 6. P. 279–282.

10. Mosin O.V. Isotope effects of deuterium in bacterial and microalgae cells at growth on heavy water (D_2O) // O.V. Mosin, I. Ignatov // Water: Chemistry and Ecology. 2012. V. 3. P. 83–94 [in Russian].

11. Antony C. Bacterial oxidation of methane and methanol / C. Antony // Advances in Microbial Physiology. 1986. V. 27. P. 113–210.

12. Karnaukhova E.N. Biosynthetic production of stable isotope labeled amino acids using methylotroph *Methylobacillus flagellatum* / E.N. Karnaukhova, O.V. Mosin, O.S. Reshetova // Amino Acids. 1993. V. 5. № 1. P. 125–126.

13. Mosin O.V. Studying of microbic synthesis of deuterium labeled L-phenylalanine by methylotrophic bacterium *Brevibacterium methylicum* on media with different content of heavy water / O.V. Mosin, V.I. Shvets, D.A. Skladnev, I. Ignatov // Russian Journal of Biopharmaceuticals. 2012. V. 4. P. 11–22 [in Russian].

14. Skladnev D.A. Methylotrophic bacteria are sourses of isotopically labelled ^{2}H- and ^{13}C-amino acids / D.A. Skladnev, O.V. Mosin, T.A. Egorova, S.V. Eremin, V.I. Shvets // Biotechnologija. 1996. V. 5. P. 25–34 [in Russian].

15. Mosin O.V. Biosynthesis of ^{2}H-labeled phenylalanine by a new methylotrophic mutant *Brevibacterium methylicum* / O.V. Mosin, D.A. Skladnev, V.I. Shvets // Bioscience, biotechnology and biochemistry. 1998. V. 62. № 2. P. 225–229.

16. Mosin O.V. Studying of biosynthesis of amino acids by a strain of *Brevibacterium methylicum* at growth on media, containing heavy water and deuteron-methanol / O.V. Mosin, D.A. Skladnev, T.A. Egorova, A.M. Yurkevitch, V.I. Shvets // Biotechnologija. 1996. V. 3. P. 3–12 [in Russian].

17. Conn E.E. Recent Advances in Phytochemistry. The Shikimic Acid Pathway / E.E. Conn. 2nd ed. – New York: Plenum Press, 1986, P. 20–22.

18. Lindstom M.E. Methylotrophs: genetics and commercial applications / M.E. Lindstom, D.I. Stirling // Annual Review of Microbiology. 1990. V. 4. P. 27–58.

19. Bohinski R.C. Modern Concepts in Biochemistry / R.C. Bohinski. – Boston, London, Sydney, Toronto, Newton, Massachusetts: Allyn & Bacon Inc., 1983, P. 376–378.

20. Michals K. Phenylalanine metabolites, attention span and hyperactivity / K. Michals, R. Matalon // American Journal of Clinical Nutrition. 1985. V. 42. № 2. P. 361–365.

21. Wendisch V.F. Amino Acid Biosynthesis – Pathways, Regulation and Metabolic Engineering /F. Volker, ed. – Helderberg: Sprinfer Verlag, 2007, P. 237–240.

22. de Boer L. Phenylalanine and tyrosine metabolism in the facultative methylotroph *Nocardia sp. 239* / L. de Boer, W. Harder, L. Dijkhuizen // Archives of Microbiology. 1988. V. 149. P. 459–465.

23. Mosin O.V. Microbial synthesis of ^{2}H-labelled L-phenylalanine with different levels of isotopic enrichment by a facultive methylotrophic bacterium *Brevibacterium methylicum* with RuMP assimilation of carbon / O.V. Mosin, V.I. Shvets, D.A. Skladnev, I. Ignatov // Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry. 2013. V. 7. № 3. P. 249-260.

24. Mosin O.V. Studying of the biosynthesis of ^{2}H-labeled inosine by a Gram-positive chemoheterotrofic bacterium *Bacillus subtilis B-3157* on heavy water ($^{2}H_2O$) medium / O.V. Mosin, I. Ignatov // Chemical and Process Engineering Research. 2013. V. 15. P. 32–45.

25. Mosin O.V. Microbiological synthesis of ^{2}H-labeled phenylalanine, alanine, valine, and leucine/isoleucine with different degrees of deuterium enrichment by the Gram-positive facultative methylotrophic bacterium *Brevibacterium methylicum* / O.V. Mosin, I. Ignatov // International Journal of Biomedicine. 2013. V. 3. № 2. P. 132–138.

26. Maksimova N.P. Regulation of phenylalanine biosynthesis in the obligate methylotroph *Methylobacillus M75* / N.P. Maksimova, E.V. Dobrozhinetskaia, Iu.K. Fomichev // Mol. Gen. Microbiol. Virusol. 1990. V 10. P. 28–30.

УДК 579.871.08+577.385.4.08

Биосинтез дейтерий-меченных аминокислот штаммам факультативных метилотрофных бактерий *Brevibacterium methylicum* # 5662 с РМФ циклом ассимиляции углерода

[1] Олег Викторович Мосин
[2] Игнат Игнатов
[3] Дмитрий Анатольевич Складнев
[4] Виталий Иванович Швец

[1] Московский государственный университет прикладной биотехнологии, Российская Федерация
Старший научный сотрудник кафедры биотехнологии, канд. хим. наук
103316, Москва, ул. Талалихина, 33
E-mail: mosin-oleg@yandex.ru
[2] Научно-исследовательский центр медицинской биофизики (РИЦ МБ), Болгария
Профессор, доктор наук Европейской академии естественных наук (ФРГ)
1111, София, ул. Н. Коперника, 32/6
E-mail: mbioph@dir.bg

[3] Государственный научно-исследовательский институт генетики и селекции промышленных микроорганизмов ГосНИИГенетика, Российская Федерация
Профессор, доктор биол. наук, ведущий научный сотрудник ГосНИИГенетика
117545, Москва, 1-й Дорожный пр., 1.
E-mail: genetika@genetika.ru
[4] Московский государственный университет тонких химических технологий им. М.В. Ломоносова, Российская Федерация
Академик РАН, доктор химических наук
117571, Москва, Вернадского просп., 86
E-mail: mitht@mitht.ru

Аннотация. Мы использовали штамм грамположительных аэробных факультативных метилотрофных бактерий Brevibacterium, methylicum, продуцент L-фенилаланина с рибулозо-5-монофосфатным (РМФ) циклом ассимиляции углерода для микробиологического получения [^{2}H]фенилаланина за счет биоконверсии низкомолекулярных субстратов (([U-^{2}H]MeOH и ^2H_2O). Для этой цели были использованы клетки метилотрофа с улучшенными ростовыми характеристиками на минимальное солевой среде М9 с добавлением 2 % (об/об) (([U-^{2}H]MeOH и ступенчато увеличивающимся градиентом концентрации ^2H_2O от 0; 24,5; 49,0; 73,5 до 98 % (об/об) ^2H_2O. L-фенилаланин, был выделен из ростовой среды после добавления 5 М 2HCl (в ^2H_2O), pH = 2,0 экстракцией изопропанолом и последующей кристаллизацией в этаноле (выход 0,65 г / л). Аланин, валин, и лейцин/изолейцин накапливались в ростовой среде экзогенно в количестве 5-6 ммоль в дополнение к основному продукту биосинтеза. Метод позволяет получить [^{2}H]аминокислоты с разными уровнями обогащения дейтерия, в зависимости от концентрации ^2H_2O в питательных средах М9, от 17 ат.% ^{2}H (2 атома дейтерия) (на питательной среде с 24,5 % (об/об) ^2H_2O) до 75 ат.% ^{2}H (6 атомов дейтерия) (на питательной среде с 98 % (об/об) ^2H_2O) с включением дейтерия к бензильный $C_6H_5CH_2$-фрагмент молекулы, что подтверждено данными масс-спектрометрии электроного удара (ЭУ) метиловых эфиров N-5-диметиламино(нафтален)-1-сульфонил хлоридных производных [^{2}H] аминокислот после их разделения обращенно фазовой ВЭЖХ.

Ключевые слова: *Brevibacterium methylicum;* [U-^{2}H]MeOH; тяжелая вода; биосинтез; [^{2}H]амино кислоты; масс спектрометрия ЭУ; ОФ ВЭЖХ.