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THE IMPACT OF SUPERHIGH FREQUENCY ELECTROMAGNETIC RADIATION ON THREONINE PRODUCER *Brevibacterium flavum*

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The aim of the research was the impact of non-thermal electromagnetic radiation of superhigh frequencies with waves of millimeter range on threonine amino acid synthesis by bacteria *Brevibacterium flavum* for ordinary (non-mutant) and mutant strains.

The frequencies of millimeter range waves were selected according to previous works as 41.76; 42.2 and 61.0 GHz. The exposition was 10 min. The control samples of bacterial suspension in the flasks were kept under the same conditions as the test ones. Irradiated suspensions were used as inoculum for fermentation on molasses wort at t = +30 °C with aeration. After cultivation for 3 days the samples irradiated with frequency 42.2 and 61.0 GHz gave an increase in colonies forming units, respectively, 1.4 and 1.9 times compared to the control for the non-mutant strain. The quantity of synthesized threonine was determined by thin-layer chromatography on the plates of ciluprevir. A significant increase of the threonine content in the culture fluid was observed for the non-mutant strain (70% compared to control) after the irradiation with frequency 61.0 GHz. The splitting of the sown colonies pigmentation was observed: the control samples were mostly pigmentated, and irradiated bacteria lost this ability immediately after exposure, but after the culturing the irradiated samples restored pigmentation. The pigmentation ability was confirmed by the data on the accumulation threonine in the culture fluid.

The *Brevibacterium flavum* mutant strain did not respond to the irradiation, this influence was negative for generative abilities and accumulation of threonine in the culture fluid.

Key words: *Brevibacterium flavum,* threonine, nonthermal electromagnetic radiation, millimeter range waves.

The producing of amino acids used industrial biotechnological synthesis based on the ability of cultures to form certain essential amino acids in large amounts (lysine, methionine, leucine, isoleucine, tryptophan).

Increasing of amino acid synthesis can be achieved in different ways: by amplification of the range of used substrates, by application of high-productivity strains by auxotrophic and regulatory mutants. Therefore, the main role in the technology holds a biological agent — a strain-producer and its performance for the target amino acid.

The "wild" strains of *Corynebacteria* consume the total precursor in the synthesis of lysine and threonine — aspartic acid semialdehyde — mainly for the synthesis of threonine. This is because the activity of homoserine dehydrogenase is 15 times higher than degidrodipikolinatsinthetase activity (the first enzyme of lysine direction). Really the biosynthesis of lysine begins after the cells have been saturated by threonine, methionine and isoleucine [1, 2].

The ways of increasing of the threonine synthesis can be obtained by using mutant strains in which homoserine dehydrogenase is not sensitive to threonine. And also treonindehydrohenase which controls the synthesis of isoleucine from threonine and degidrodipikolinatsinthetase responsible for the synthesis of lysine — both enzymes are repressed.

Selection and genetic engineering of amino acid producers, search for different ways to improve the biosynthesis of amino acids (particularly by action of physical mutagens) is an actual problem and the methods, which can act on amino acid producers to enhance the synthesis of target products are explored [3 ,4].

One of such methods is the impact of external factors that can regulate cell producers' metabolism, for example, electromagnetic radiation (EMR) of superhigh frequencies (30–300 GHz) or waves of millimeter range with non-thermal power. The possibility to influence on their viability, speed of height, biosynthesis by means of this radiation was shown for many microorganisms, for example, yeasts [5], *E. coli* [6], *Corynebacterium* [7]. It was observed that the irradiation in this frequency range can cause significant effects on living organisms [8, 9]. Unlike the ultraviolet and x-ray, millimeter range is non-ionizing. Usually the effect of a some of selected biologically active frequency bands is examined [10, 11]. Different frequencies can inhibit or increase cell activity [7, 12]. The biological effects of microwave millimeter irradiation action had a sharp resonance character depending on the frequency of exposure [13].

It is believed that non-ionizing electromagnetic radiation is not a mutagenic factor for the cells. However, the experiments indicate that changes in life processes of cells after exposure to millimeter waves persist for several passages of culture [10, 14].

From this point of view it is interesting to compare how the normal culture and mutant strains respond to biologically active radiation. Such data can be useful for finding out of the mechanisms of non-ionizing weak EMR action on expression of genes in cells. In this study we investigated a possibility of using of millimeter microwawes (MMW) for the increase of threonine amino acid synthesis by bacteria *Brevibacterium flavum* on the example of normal (non-mutant) and mutant strains.

Materials and Methods

The experiments were conducted in the laboratory. Here we used source and mutant strains of *Brevibacterium flavum* from Collections of microorganisms and lines of plants for food and agricultural biotechnology of State Institution "Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine" [1, 2].

To prepare the test samples for millimeter EMR, we transferred cultures onto shoals with sloped enriched meat infusion agar and grown for 24 h at t = 30 °C. Based on daily cultures we prepared bacterial suspensions with sterile saline solution (cell titer 10^6–10^7) and distributed in sterile conical flask 100 cm^3, volume of the suspension was 7 cm^3, the layer height — 3.4 mm. From these flasks we selected the control samples, which after serial dilutions we sowed in Petri dishes from infusion peptone agar (IPA) to determine the initial concentration of the bacterial suspension.

Prior to the irradiation exposure we cooled the cell suspension to t = +4 °C, since the lack of nutrients in the medium and cooling increases the sensitivity of cells to the effects of weak external field [11].

Microwave irradiation was carried out at three frequencies which were selected according to published data [7, 10, 15, 16] — 41.76 GHz; 42.2 GHz and 61.0 GHz. The exposition was 10 min according to the method of activation described in [11]. The control samples of suspension were not subjected to radiation, but kept under the same conditions as the test samples.

Fig. 1 shows the scheme of installation for irradiation of cell suspensions. The source of linearly polarized EMR was microwave generator (*1*) G4-141 with working frequency range 37–53 GHz and G4-142 — frequency range 53–78 GHz. The error of setting of the emission frequency was <0.005 GHz. The estimated average power density was 0.07 mW/cm^2. For each sample we set the microwave frequency of microwave electromagnetic radiation. The radiation was submitted through the waveguide (*3*) to the coordinated horn (*2*), to the bottom of the flask (*4*) with cell suspension (*5*).

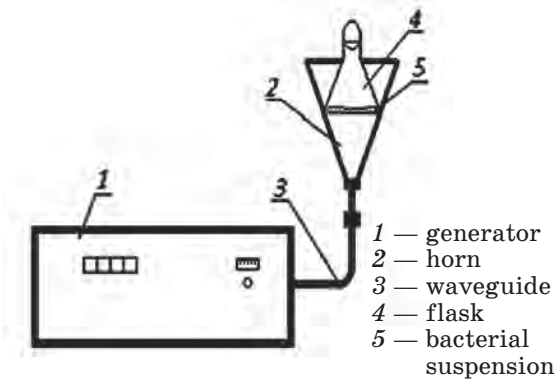

Fig. 1. **Scheme of installation for irradiation of cell suspensions**

After the irradiation of *Brevibacterium flavum* suspension in sterile conditions we prepared a series of successive dilutions in saline to determine the concentration of the bacterial suspension before the fermentation on molasses wort: adding 0.5 cm^3 of culture to 4.5 cm^3 of saline. Last dilutions were grown in IPA in thermostat at t = + 30 °C. The obtained colonies were counted on the third day.

The irradiated suspension was used as inoculum for fermentation on molasses wort. The sowed bacterial culture on the liquid culture wort was cultured at t = + 30 °C in a shaker aeration conditions (vibration frequency 220 min^{-1}).

The quantity of synthesized threonine was determined by thin-layer chromatography (TLC) on the plates of ciluprevir. This technique is designed to measure the mass concentration of threonine in the culture fluid obtained in the threonine biosynthesis (measuring range from 5 g/dm^3 to 120 g/dm^3) and the mass fraction of threonine in concentrated liquid product containing threonine (measuring range from 2% to 20%).

The method is based on the separating of threonine from other amino acids, further coloring of spots in the reaction with ningydrine, elution of obtained colored complex and determining of the optical density of eluate. The optical density is proportional to the content of threonine in eluate.

The mass concentration of threonine in the culture fluid, X, g/dm^3, was determined according to the formula:

$$X = \frac{D_5 - 2D_{2.5} + D}{D_5 - D_{2.5}} \times 2.5 \times n,$$

where $D_{2.5}$, and D_5 — arithmetic mean value of optical density of standard solutions with mass concentration of

2.5 and 5.0 g/dm^3, respectively;

D — the arithmetic mean value of the optical density of the analyzed solution;

2.5 — the mass concentration of standard solution, g/dm^3;

n — the multiplicity of dilution culture fluid sample.

For the final result we took the arithmetic mean of two parallel definitions. The difference between them with respect to their arithmetic mean values did not exceed 10%. The calculations were performed up to the first decimal place.

The statistical processing of the results was performed by calculating the arithmetic mean values, their standard deviations and errors. To determine significant differences between the average values was carried out using Student's criterion. The differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Previous studies with *S. cerevisiae* showed a sharp resonance effect of the millimeter microwaves. Therefore, the series of experiments on irradiation of the samples cultures using generator G4F-141 was carried out for the frequency of 41.76 GHz, identified as stimulating for the culture yeast, and 42.2 GHz (defined as active in [7] and [10]).

We determined the generative ability and the adaptive properties of cultures after the irradiation these frequencies. After reseeding and several dilutions the initial concentration of culture before the growing on molasses wort varied significantly, therefore for the convenience the increasing of CFU was shown compared with the test specimens before fermentation on molasses wort which was determined after the screening of inoculum before fermentation on molasses wort.

Our studies have shown that the irradiated 1-day bacterial suspension after growing in molasses wort for 72 h demonstrated a stimulating and inhibitory effect of electromagnetic radiation, the character of which depends on EMR frequency (Table 1).

It was established that processing by EMR at the frequency of 41.76 GHz of seeding material, which increased generating ability for yeast *Saccharomyces cerevisiae,* on *Corynebacterium* caused a negative effect, but the exposure to irradiation at the frequency of 42.2 GHz stimulated growth processes after fermentation on molasses wort that was determined by CFU after sowing on Petri dishes. It was also noted some reduction of CFU in experimental samples after the exposure, but before the cultivation on molasses wort.

It is known that clones producers of amino acids which are capable to their increased synthesis have the color from yellow to brown and it is possible to evaluate this ability by counting the colonies grown on IPA that have different colors. But the cells become discolored before losing viability. In the view of the information about a certain difference in the impact of microwave electromagnetic radiation on different strains we investigated

Table 1. **Influence of microwawe EMR on generative ability of original (non-mutant) strain *Brevibacterium flavum* after 3-day fermentation of molasses wort**

EMR frequency, GHz	Number of colony forming units, CFU/cm^3		Increasing of colony number, N
	of samples before the fermentation on molasses wort	of sample after the fermentation on molasses wort	
Control (without irradiation)	$(6.0 \pm 0.7)\times10^6$	$(5.1 \pm 0.6)\times10^8$	85.0
41.76	$(3.4 \pm 0.2)\times10^6$	$(1.4 \pm 0.1)\times10^8$	41.2*
42.20	$(2.6 \pm 0.3)\times10^6$	$(3.2 \pm 0.4)\times10^8$	123.1*

Note: hereafter * — $P < 0.05$ to compared with control.

pigmentation of irradiated and non-irradiated original colonies and mutant strains of *Brevibacterium flavum*. It was noted that a mutant strain is auxotrophy, so the impact of the additional exposure may cause unexpected effects.

In this study we used less dilution of the samples before irradiation (Table 2).

However, it should be noted that at high initial concentration of seeding material more pronounced downward trend of irradiated samples was observed that can not be explained only by the inaccuracy of sowing and dilutions, as in the smaller initial concentration. Consequently, there has been some reduction in the viability of the irradiated cells *Brevibacterium flavum* both the non-mutant and the mutant strains.

It should be noted that the irradiated cultures formed different colony sizes and different pigmentation. For the mutant strain of the experimental samples before fermentation on molasses wort a splitting of the size and color of the colonies was observed: to 41.76 GHz frequency — near 5% of small white colonies of the total number of small colonies and 0.04% of large yellow colonies of the total number of yellow colonies; to 42.20 GHz frequency — near 10% of small white colonies of the total number of small colonies and 0.06% of large yellow colonies of the total number of yellow colonies. In the control variant mutant colonies as well as the original strains have yellow color, the number of large colonies — from 40 to 50%. The presence of different sized colonies indicates different adaptive ability of irradiated and non-irradiated cultures; large colonies probably are better adapted to changes and began to develop sooner.

It was observed that in the case of irradiation of more concentrated suspension of bacteria the colonies number after the screening on solid culture medium immediately after irradiation also showed a tendency to decrease on the order compared with the control. This concerned both the original and the mutant strains.

It could be noted that the mutant strain after the fermentation on molasses wort had more divergence of pigmentation. After the fermentation in control samples gave growth by 15% of white colonies; for 41.76 GHz frequency irradiation 33% of colonies were without pigmentation; for 42.2 GHz frequency colonies gave growth by 50% without pigmentation.

Determination of threonine by TLC confirmed the correlation that pigmented colonies are more productive for the content of threonine: threonine content was decreased compared with the control samples after 4 and 5 days of fermentation on molasses wort.

Thus, comparing the effect of frequency radiation, we can say that microwave electromagnetic radiation at the frequency of 42.2 GHz caused more deviations from the control indicators both non-mutant and mutant strain than the frequency of 41.76 GHz. Despite the slight increase of generating ability after the fermentation of non-mutant strain the seeding samples irradiated at the frequency of 42.2 GHz, it can be argued that the overall effect of microwave electromagnetic radiation for the ability of the non-mutant and mutant strains *Brevibacterium flavum* to synthesize increased amounts of threonine is negative. Moreover, the tendency to reduce the number of CFU of the samples after irradiation before fermentation on molasses wort was observed

Table 2. **Impact of EMR on generative ability of the mutant and non-mutant strain *Brevibacterium flavum* after fermentation on molasses molasses wort**

EMR frequency, GHz	Number of colony forming units, CFU/cm^3			
	Sample before the fermentation in molasses wort		Sample after the fermentation in molasses wort	
	Non-mutant strain	Mutant strain	Non-mutant strain	Mutant strain
Control (without irradiation)	$(2.0 \pm 0.1)\times10^8$	$(1.2 \pm 0.1)\times10^8$	$\geq 10^{11}$	$\geq 10^{11}$
41.76	$(3.5 \pm 0.3)\times10^7$	$(4.2 \pm 0.5)\times10^7$		
42.20	$(1.6 \pm 0.2)\times10^7$	$(4.3 \pm 0.3)\times10^7$		

relatively to the control samples that were under the same conditions during the study, but without irradiation, which also confirms generally unfavorable biological effects of irradiation at these frequencies.

From the literature it is known [7] that the impact of irradiation frequencies of 42.2 GHz and 61.0 GHz had a different direction (character) on enzymatic activity of *Corynebacterium tuberculosis*. Therefore, further experiments were carried out, irradiating one-day culture, which was then used as a seeding suspension for fermentation, at the frequency of 61.0 GHz using generator G4-142.

The results of irradiation effects of EMR microwave on the generating ability of the non-mutant and mutant strains *Brevibacterium flavum* are shown in Table 3.

From Table 3 results it can be concluded that irradiation at the frequency of 61.0 GHz did not significantly affect cell viability, both the non-mutant and the mutant strains before the fermentation (with a tendency to increase within the error of the experiment).

As for increasing of the number of CFU, which was determined after the fermentation on molasses wort, then the non-mutant strain under the action of irradiation at the frequency of 61.0 GHz the number of CFU increased almost 1.9 times, the number of CFU of mutant strain both in control and in the experiment remained almost unchanged.

It was also noted that the sowed samples on Petri dishes after irradiation were mainly non pigmented colonies (the percentage pigmented colonies was 0.5–1%) in the populations of the non-mutant and the mutant control samples — only pigmented colonies grew. This means that under the influence of irradiation at the frequency of 61.0 GHz the cells of non-mutant and mutant strains grown on IPA lost the ability to synthesize the pigment. Moreover percentage of non-pigmented colonies of mutant strain was higher.

However, after four days of the fermentation on molasses wort most of the colonies (90%) of *Brevibacterium flavum* irradiated at the frequency of 61.0 GHz were pigmented. So, after the fermentation the cells recovered their properties, had a greater growth rate, low percentage of non pigmented colonies remained in population. But only 50% of colonies from the population of the irradiated mutant strain-producer *Brevibacterium flavum* were pigmented.

Fig. 2 shows the threonine content in the culture fluid after the fermentation on molasses wort bacteria of *Brevibacterium flavum* that were irradiated at the frequency of 61.0 GHz, determined on the second, third and fourth day of cultivation.

Thus, according to diagram in Fig. 2, irradiation at the 61.0 GHz frequency of the non-mutant strain *Brevibacterium flavum* positively impacted on threonine biosynthesis: quantity of this amino acid in the irradiated samples both after fermentation on the second and the third and on the fourth day was bigger than in the control samples. Maximum of threonine content in the culture fluid was observed on the third day of growth for irradiated strain (10.2 g/dm^3), i.e. the increase compared to 6.0 g/dm^3 in the control was 70% ($P < 0.05$). But for the mutant strain such dependence was not noted even after 4 days of the fermentation. On the contrary, a higher threonine content was in the control sample of the mutant *Brevibacterium flavum* strain.

So, the influence of nonthermal EMR MMW on the *Brevibacterium flavum* — strains-producers of threonine at the frequencies of 41.76 GHz, 42.2 GHz 61.0 GHz was investigated. The studies have shown the multidirectional character of the irradiation effect on these frequencies, which agree with previous studies.

Table 3. **Impact of EMR MMW on generating ability of the non-mutant and mutant strain *Brevibacterium flavum* after 4-days fermentation on molasses wort**

EMR frequency, GHz	Number of colony forming units, CFU/cm^3				Increasing of colony number, N	
	Non-mutant strain		Mutant strain			
	Sample before the fermentation in molasses wort	Sample after the fermentation in molasses wort	Sample before the fermentation in molasses wort	Sample after the fermentation in molasses wort	Non-mutant strain	Mutant strain
Control (without irradiation)	$(8.3 \pm 0.8)\times10^6$	$(4.0 \pm 0.3)\times10^8$	$(6.6 \pm 0.7)\times10^6$	$(17.2 \pm 0.9)\times10^8$	48.2	260.6
61.0	$(8.6 \pm 0.8)\times10^6$	$(7.8 \pm 0.8)\times10^8$*	$(7.8 \pm 0.8)\times10^6$	$(20.0 \pm 1.0)\times10^8$	90.7*	256.4

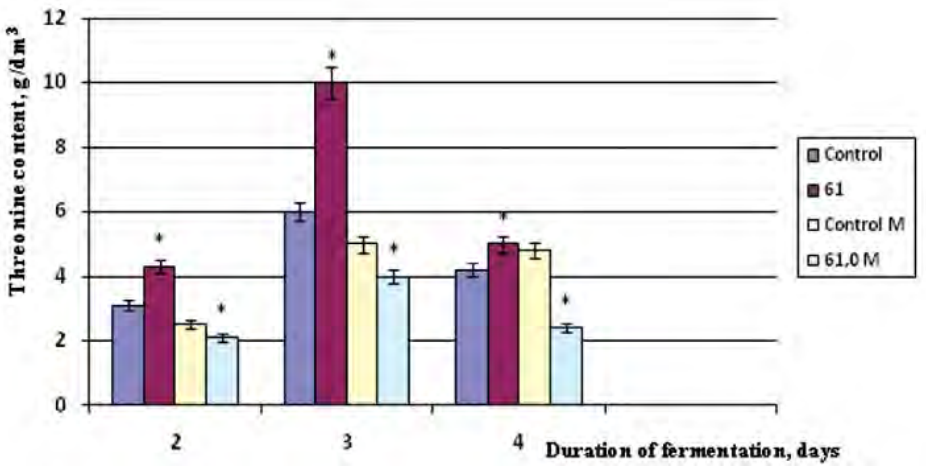

Fig. 2. **Effect of irradiation of *Brevibacterium flavum* at the frequency of 61.0 GHz on threonine productivity after fermentation (M mark — for a mutant strain)**

The stimulatory action of irradiation *Brevibacterium flavum* by the method of [11] at the frequency of 41.76 GHz, which is active for yeast *Saccharomyces cerevisiae* was not confirmed.

There was a tendency to reduce CFU irradiated samples before the fermentation in molasses wort relative to control at the frequencies of 41.76 GHz and 42.2 GHz. As for the 61.0 GHz frequency, this indicator did not change or tended to increase.

After the fermentation on molasses wort for 3 days it was observed an increase in CFU of the samples that irradiated at the frequencies of 42.2 GHz and 61.0 GHz, respectively, 1.4 and 1.9 times compared to the control for the original strain. For the mutant strain, the increase in generative abilities was not found.

There was also the splitting of sown colonies by pigmentation, the control samples were usually pigmented, the irradiated samples to some extent lost this ability immediately after the irradiation, but after the fermentation the irradiated samples recovered pigmentation and/or were probably had a higher growth rate, and the ability to pigmentation was confirmed by the data on threonine content in the wort. The *Brevibacterium flavum* mutant strain did not respond to the frequency of 42.2 GHz and 61.0 GHz or this influence was negative (by splitting pigmentation and accumulation threonine in the culture fluid). A significant increase of threonine content in the culture fluid was observed for the non-mutant strain (70% compared to control) after irradiating at a frequency of 61.0 GHz. We consider that the influence of EMR MMW has epigenetic mechanism and occurs as an adaptive response to irradiation, such as DNA methylation of certain areas of the genome that is reversible. This is confirmed as a short-

term decrease in the coloration of the colonies, which is restored after a certain number of generations after cultivation on a nutrient medium and splitting of the irradiated colonies by pigmentation. This also explains the increase in the synthesis of the target product — threonine — after the fermentation of the non-mutant strain, in contrast to the mutant, for which a certain region of the genome which is responsible for the synthesis of methionine from homoserine already has been blocked.

So, as a result of the work we have received a positive effect of threonine synthesis stimulating of *Brevibacterium flavum* culture, which was irradiated at the frequency of 61.0 GHz. It can be concluded that the way of the irradiation of threonine producers inoculum to improve supersynthesis of this amino acid can have a positive effect, but researches in this direction should be continued.

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ДІЯ ЕЛЕКТРОМАГНІТНОГО ВИПРОМІНЮВАННЯ НАДВИСОКОЇ ЧАСТОТИ НА ПРОДУЦЕНТ ТРЕОНІНУ *Brevibacterium flavum*

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Досліджували вплив нетеплового електромагнітного випромінювання надвисокої частоти на синтез треоніну бактеріями немутантного та мутантного штамів *Brevibacterium flavum*.

Частоти опромінювання було обрано за даними попередніх робіт — 41,76, 42,2 і 61,0 ГГц. Експозиція становила 10 хв. Контрольні зразки бактеріальної суспензії в колбах витримували за таких самих умов, що й дослідні. Опромінені суспензії використовували як інокулят для засівання на мелясне сусло за t = +30 °С з аерацією. Після культивування протягом 3 діб у зразках, які було опромінено на частотах 42,2 та 61,0 ГГц, спостерігали збільшення колонійутворювальних одиниць в 1,4 та 1,9 раза, порівняно з контролем як вихідним штамом. Не підтверджено стимулювальний характер опромінення *Brevibacterium flavum* частотою 41,76 ГГц, що є активною для дріжджів *Saccharomyces cerevisiae*.

Кількість синтезованого треоніну визначали методом тонкошарової хроматографії на силуфолових пластинах. Значне збільшення вмісту треоніну в культуральній рідині виявлено для немутантного штаму (70% порівняно з контролем) після впливу на частоті 61 ГГц. Спостерігалося розщеплення колоній за пігментацією після пересівання: контрольні зразки переважно були забарвлені, а опромінені бактерії втратили цю здатність одразу після опромінення, однак після культивування опромінених зразків пігментація була відновлена. Здатність до пігментації підтверджено даними про накопичення треоніну в культуральній рідині. Мутантний штам *Brevibacterium flavum* не реагував на опромінення або цей вплив був негативним за генеративною здатністю та накопиченням треоніну в культуральній рідині.

Ключові слова: *Brevibacterium flavum*, треонін, нетеплове електромагнітне випромінювання, хвилі міліметрового діапазону.

ВОЗДЕЙСТВИЕ ЭЛЕКТРОМАГНИТНОГО ИЗЛУЧЕНИЯ СВЕРХВЫСОКОЙ ЧАСТОТЫ НА ПРОДУЦЕНТ ТРЕОНИНА *Brevibacterium flavum*

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Исследовали влияние нетеплового электромагнитного излучения сверхвысокой частоты миллиметрового диапазона на синтез треонина бактериями немутантного и мутантного штаммов *Brevibacterium flavum*.

Частоты излучения были выбраны по данным предыдущих работ — 41,76, 42,2 и 61,0 ГГц. Экспозиция составляла 10 мин. Контрольные образцы бактериальной суспензии в колбах выдерживали в тех же условиях, что и опытные. Облученные суспензии использовали в качестве инокулята для засева на мелассное сусло при t = +30 °С с аэрацией. После культивирования в течение 3 сут в образцах, облученных на частотах 42,2 и 61,0 ГГц, наблюдали увеличение колонийобразующих единиц, соответственно в 1,4 и 1,9 раза по сравнению с контролем в качестве исходного штамма. Не подтвердился стимулирующий характер облучения *Brevibacterium flavum* частотой 41,76 ГГц, активной для дрожжей *Saccharomyces cerevisiae*.

Количество синтезированного треонина определяли методом тонкослойной хроматографии на силуфолових пластинах. Значительное увеличение содержания треонина в культуральной жидкости получено для немутантного штамма (70% по сравнению с контролем) после воздействия на частоте 61,0 ГГц. Наблюдалось расщепление колоний по пигментации после пересевания: контрольные образцы в основном были окрашены, а облученные бактерии утратили эту способность сразу после облучения, но после культивирования облученных образцов пигментация была восстановлена. Способность к пигментации подтверждена данными о накоплении треонина в культуральной жидкости. Мутантный штамм *Brevibacterium flavum* не реагировал на облучение либо это влияние было негативным по генеративной способности и накоплению треонина в культуральной жидкости.

Ключевые слова: *Brevibacterium flavum*, треонин, нетепловое электромагнитное излучение, волны миллиметрового диапазона.