Riboflavin, also known as vitamin B<sub>2</sub>, is an essential vitamin for humans, and has been selected by the World Health Organization (WHO) as one of the six key indicators for assessing human growth, development and nutritional status [1]. Riboflavin is involved in various reducing-oxidizing reactions, and is an essential component of the main metabolism, as well as an important nutrient and growth factor in humans, animals, plants and microorganisms. Riboflavin is a component of two major coenzymes, the flavin mononucleotide (FMN; also known as riboflavin-5-phosphate) and the flavinadenine dinucleotide (FAD).

Riboflavin is not synthesized in humans and animals, and it must be obtained with food, so riboflavin is widely used as an additive in the food and feed industry [2, 3].

Riboflavin deficiency or “faulty transport” of riboflavin may cause various disorders and even various diseases [4–6]. Nowadays there is some deficiency of riboflavin, which raises serious health concerns in both developing and developed countries [7].

On an industrial scale, riboflavin has long been produced by chemical synthesis. At present, on an industrial scale, riboflavin is mainly (97%) produced by microbial biosynthesis, and common microorganisms are fungi and bacteria. The main strains for the industrial production of riboflavin are the yeast-like fungi *Eremothecium ashbyii* and *Ashbya gossypii*, which accumulate riboflavin in the mycelium, as well as the bacterium *Bacillus subtilis* [8, 9].

Obtaining vitamins, in particular riboflavin (vitamin B<sub>2</sub>), using microbiological synthesis is a commercially successful and promising method. The issues of economical consumption of carbon substrate, reduction of costs for the cultivation process, as well as the most complete transformation of the substrate into target metabolites are particularly relevant for industrial technology. The solution to these problems requires the use of new high-yielding
strains of microorganisms — producers that are able to accumulate the target products extracellularly. The study of physiological and biochemical properties of new strains and principles of biosynthesis regulation makes it possible to implement biotechnological processes most effectively [9–11].

Compared with the fermentation of fungi, the bacterial one has a number of advantages for the biosynthesis of riboflavin, such as the relatively short duration of cultivation, simple cultivation environment and the possibility of applying genetic engineering technology to prokaryotic bacteria [12, 13].

Bacteria of the species _B. subtilis_ have unique properties, and they produce a number of metabolites: enzymes, vitamins, and amino acids. Compared to _E. ashbyi_, bacteria of the species _B. subtilis_ produce riboflavin extracellularly, i.e. accumulation occurs in the culture fluid, which simplifies the technology. In addition, the strains of _B. subtilis_ have a high degree of tolerance, i.e. for their vital activity it is possible to use a wide range of values of temperature, pH and other important technological parameters [14, 15].

The aim of the study was to establish optimal cultivation conditions for the producer strain of _B. subtilis_ IMB B-7797 to increase the accumulation of riboflavin.

Materials and Methods

The object of the study was the domestic strain of _B. subtilis_ IMB B-7797 [16] from the “Collection of strains of microorganisms and plant lines for food and agricultural biotechnology” of the State Enterprise “Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine”. The culture was stored in tubes with bevelled L-agar.

Cultivation conditions and media. For the cultivation of the strains producing riboflavin a nutrient medium of the following composition (L-agar) was used: yeast extract — 5.0 g; sodium chloride — 5.0 g; peptone — 5.0 g; agar — 25.0 g; distilled water — up to 1.0 dm³, pH 7.2 ± 0.1. Colonies of microorganisms were grown at a temperature of 38 ± 1 °C during three days. All colonies grown on solid media were selected for cultivation and testing for riboflavin accumulation. To obtain single colonies the method of boundary dilutions was used [17].

To grow the inoculum a medium of the following composition was used: molasses — 30.0 g, ammonium sulphate — 4.0 g, magnesium sulphate — 0.5 g, corn extract — 15.0 g, tap water — was added to the mark of 1.0 dm³.

For the cultivation of _B. subtilis_ an enzymatic medium of the following composition was used: glucose — 120.0 g; pressed baker’s yeast — 40.0 g; corn extract — 10.0 g; magnesium sulphate — 0.5 g; distilled water — added to the mark of 1.0 dm³; it was addwd phosphate buffer 0.5 dm³ per 100 dm³ medium as well. The cultivation was performed during 24 h in a shaker-incubator BIOSAN ES-20 (Latvia) at a temperature of 38 °C and at a speed of 240 rpm.

To study the effect of a carbon source on the accumulation of riboflavin at the initial stage the glucose, fructose and sucrose at a concentration of 60 g/dm³ were used. To increase the accumulation of riboflavin and to determine the optimal sugar content, the glucose was subsequently added to the enzymatic medium at a concentration of 30.0, 50.0, 80.0, 120.0 and 150.0 g/dm³.

The cultivation was performed during 96 h in a shaker-incubator BIOSAN ES-20 (Latvia) at a temperature of 38 °C and at a speed of 240 rpm. The samples were taken at intervals of 12 h. The growth of strains producing riboflavin on solid nutrient media (Fig. 1) was determined after 96 hours of cultivation using the photocolorimeter “KFK-2” (RF) with a green light filter, wavelength 533 nm, cuvette volume 0.01 dm³. The source medium was used as a standard for comparison. The number of microorganisms in 0.01 dm³ medium was determined on a graduated curve, which was built on the basis of determining the units of optical density of bacterial suspensions. The pH of the medium was measured using a digital pH meter (pH meter 150, RB). The microscopy was performed using the microscope “Laboval 4” (Carl Zeiss, FRG). The photos were taken with Canon PowerShot A640 (Japan). The amount of accumulated riboflavin was determined by the fluorimetric method using the fluorimeter “EF-3MA” (RF) [19].

The statistical data were processed using Microsoft Excel. All experiments were performed in three replicates. The difference between the two mean values was considered significant at _P_ < 0.05 (t-Student test). To determine the reliability of the data, calibration graphs of solutions of riboflavin and bacterial biomass on buffer and enzymatic medium were determined with the determination of their adsorption in...
10 replicates, and the determination of the standard deviation. The values obtained for the studies in triplicate and their standard deviation were compared with these graphs. If the difference between the standard deviations of the graph and the study was less than 0.05, then this value was considered reliable [18].

**Results and Discussion**

For the cultivation of microorganisms, the medium composition and technological conditions that would provide high productivity of the accumulation of the necessary metabolites, in this case — riboflavin, are important. It is known that the state of the *B. subtilis* inoculum significantly affects the accumulation of the target product during cultivation [20]. The culture shall be in the active phase of growth during inoculation, so the effect of different time of the inoculum cultivation on the accumulation of riboflavin and biomass during fermentation was studied (Table 1).

The data obtained show that the use of the inoculum in the late exponential growth phase (20–24 h) has led to an increase in the accumulation of bacterial biomass, but also to a decrease in the accumulation of riboflavin compared with the use of inoculum with a cultivation period of 16–20 h.

It was determined that the use of the inoculum with a cultivation period of 16 hours was optimal for the accumulation of riboflavin. The concentration of riboflavin after cultivation was 4.4 g/dm³. That can be explained by the fact that at that time most cells were in the exponential growth phase, which could be seen during the cytological examination (Fig. 2).

As the Fig. 2 shows, most cells had an elongated shape, spores were not formed, waste products were contained in small quantities (*a*), compared with riboflavin crystals at the 48th hour of cultivation (*b*), so this state precedes further cell division.

However, not only the term of inoculum cultivation affects the accumulation of the target product, but also its concentration. The effect of the amount of applied seed on the accumulation of biomass and riboflavin during 72 hours of cultivation was studied (Table 2).

It was shown that the greatest accumulation of riboflavin at the 72nd hour of cultivation was with the introduction of 10% of inoculum. In case of the introduction of 5% of inoculum, the amount of riboflavin accumulated at the 72nd hour of cultivation was 3.40 g/dm³. The introduction of 1% inoculum led to the accumulation of 0.6 g/dm³ of riboflavin at the 72nd hour of cultivation, which can be explained by the accumulation of mainly biomass under these conditions. Increasing the inoculum concentration to 20% inhibited the accumulation of biomass and riboflavin. Therefore, the optimal inoculum concentration was 10% of the enzyme medium. In further studies, the inoculum was used at the concentration of 10%.

Substances that are used as an energy source are needed to support the life of microorganisms and the production of...
important compounds, including riboflavin. The influence of carbohydrates — glucose, fructose and sucrose on the accumulation of riboflavin and biomass was studied (Table 3).

Table 3 shows that glucose was the best source of carbon in the enzymatic medium for the accumulation of riboflavin (5.2 g/dm³).

The accumulation of riboflavin with the use of sucrose and fructose was lower — 4.9 and 4.8 g/dm³, respectively.

In further studies, glucose was used as a carbon source. However, not only the carbon source but also its concentration influenced the accumulation of biomass and the target product [21]. The effect of different glucose concentrations on the accumulation of riboflavin and biomass of *B. subtilis* culture IMB B-7797 was studied (Table 4).

Table 4 shows that the change in glucose concentration in the culture medium influenced the accumulation of riboflavin. Increasing the concentration of glucose in the enzymatic medium from 30 to 120 g/dm³ led to the accumulation of biomass and riboflavin, which increased proportionally. The introduction of glucose into the enzymatic medium with the concentration of more than 120 g/dm³ almost did not increase the accumulation of riboflavin and inhibited the growth of biomass. The largest accumulation of riboflavin (7.2 g/dm³) was at the concentration of 120 g/dm³.

One of the important factors influencing the growth of microorganisms and their physiological activity is the temperature of cultivation [15]. The accumulation of riboflavin by strain IMB B-7797 at different cultivation temperatures was studied (Fig. 3).
It is shown that the accumulation of riboflavin increased to 8 g/dm³ with an increase in the cultivation temperature from 28 °C to 39 °C, a further increase in temperature led to the decrease in the accumulation of riboflavin (3.86 g/dm³). The optimal cultivation temperature to increase the accumulation of riboflavin strain IMB B-7797 was 38 °C. Further studies were performed at this temperature.

The pH of the medium has a significant effect on the accumulation of biomass and the target product [22]. Fig. 4 shows the effect of pH of the enzymatic medium on the accumulation of riboflavin strain IMB B-7797.

It was shown that the maximum concentration of accumulated riboflavin was 8.9 g/dm³. The change in pH from 6.5 to 7.5 did not affect the accumulation of riboflavin. Subsequent studies were performed at pH 7.0.

One of the economic factors of the technological process of fermentation is its duration. To determine the optimal time of cultivation, the dynamics of riboflavin accumulation was studied. The cultivation was

| Table 3. Effect of carbon source on biomass and riboflavin accumulation* |
|---------------------------------------------------------------|
| Carbone source | Accumulation after cultivation, g/dm³ |
|----------------|--------------------------------------|
|                | biomass | riboflavin |
| Glucose        | 14.6 ± 0.4 | 5.20 ± 0.12 |
| Sugar          | 15.8 ± 0.5 | 4.90 ± 0.10 |
| Fructose       | 12.8 ± 0.4 | 4.8 ± 0.10  |

| Table 4. Effect of glucose concentration on biomass and riboflavin accumulation* |
|-------------------------------------------------------------------------------|
| Concentration, g/dm³ | glucose | biomass | riboflavin |
|----------------------|--------|--------|-----------|
| 30                   | 12.3 ± 0.4 | 3.40 ± 0.14 |
| 50                   | 13.8 ± 0.4 | 5.60 ± 0.18 |
| 80                   | 15.8 ± 0.4 | 6.80 ± 0.21 |
| 120                  | 16.1 ± 0.5 | 7.20 ± 0.16 |
| 150                  | 14.9 ± 0.5 | 6.80 ± 0.18 |

Fig. 3. Effect of cultivation temperature on riboflavin accumulation by IMB B-7797
* — P < 0,05 relative to control (enzyme medium was taken as control)

Fig. 4. Effect of enzymatic medium pH on the accumulation of riboflavin by strain IMB B-7797
* — P < 0,05 relative to control (enzyme medium was taken as control)
performed during 96 hours, the accumulation of riboflavin (concentration) was measured every 12 h (Fig. 5).

It was found that the optimal cultivation period to increase the accumulation of riboflavin strain IMB B-7797 was 68 hours. The increase in the cultivation period did not lead to a further increase in the accumulation of riboflavin in the culture fluid.

**Conclusion**

The accumulation of riboflavin in *B. subtilis* culture can be increased by selecting appropriate culture conditions. As a result of optimization of technological parameters of cultivation of the strain IMB B-7797 (cultivation period of the inoculum — 16 hours, quantity of the brought inoculum — 10%, glucose in concentration — 120 g/dm³, temperature of cultivation — 38 °C, pH — 7.0, cultivation period — 72 hours) the accumulation of riboflavin was increased more than 2 times (from 4.3 g/dm³ to 8.9 g/dm³), compared with unoptimized cultivation conditions. The results of the study showed that the strain IMB B-7797 accumulated almost 9 g/dm³ of riboflavin, which can be compared with the results of the accumulation of riboflavin presented in [23, 24] using recombinant strains of overproducers.

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ОПТИМІЗАЦІЯ УМОВ КУЛЬТИВУВАННЯ ШТАМУ-ПРОДУЦЕНТА РИБОФЛАВІНУ

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Мета дослідження полягала у встановленні оптимальних умов культивування для збільшення накопичення рибофлавіну штамом-продуцентом Bacillus subtilis IMB В-7797. Об’єктом дослідження був штам B. subtilis IMB В-7797 із «Колекції штамів мікроорганізмів та ліній рослин для харчової і сільськогосподарської біотехнології» ДУ «Інститут харчової біотехнології і геноміки НАН України».

Визначено відсоток (10%) та термін культивування (16 годин) інокуляту, потрібного для накопичення рибофлавіну. Вивчено вплив джерела вуглецю на накопичення рибофлавіну та показано, що найбільше накопичення (5,2 г/дм3) було за використання глюкози. Наведено динаміку накопичення рибофлавіну та визначено оптимальний термін культивування (68 год). Визначено оптимальні умови культивування (концентрація глюкози — 120 г/дм3, температура — 38 С, рН середовища — 7,0), за якими збільшилося накопичення рибофлавіну в культуральній рідині більш ніж у 2 рази. Зроблено висновок, що накопичення рибофлавіну можна підвищити за допомогою зміни умов культивування.

Ключові слова: штам-продуцент, рибофлавін, мікробіологічний синтез, Bacillus subtilis.

ОПТИМІЗАЦІЯ УСЛОВІЙ КУЛЬТИВИРОВАНИЯ ШТАММА-ПРОДУЦЕНТА РИБОФЛАВИНА

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Цель исследования — установление оптимальных условий культивирования для повышенного накопления рибофлавина штаммом-продуцентом Bacillus subtilis IMB В-7797. Объектом исследования был штамм Bacillus subtilis из «Коллекции штаммов микроорганзмов и линий растений для пищевой и сельскохозяйственной биотехнологии» ГУ «Институт пищевой биотехнологии и геномики НАН Украины».

Определен процент (10%) и срок культивирования посевного материала (16 часов), необходимого для накопления рибофлавина. Изучено влияние источника углерода на накопление рибофлавина и показано, что наибольшее накопление (5,2 г/дм3) было за использование глюкозы. Показана динамика накопления рибофлавина и определен оптимальный срок культивирования (68 часов). Подобраны оптимальные условия культивирования (концентрация глюкозы — 120 г/дм3, температурный режим — 38 С и рН среды — 7,0), при которых увеличилось накопление рибофлавина в культуральной жидкости более чем в 2 раза. Сделан вывод, что накопление рибофлавина можно увеличить с помощью изменений условий культивирования.

Ключевые слова: штам-продуцент, рибофлавин, микробиологический синтез, Bacillus subtilis.