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# INFLUENCE OF $FE^{++}$ ON METABOLISM OF PROPIONATE BACTERIA

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**Abstract**

*As a result of the carried out researches it was established, that activated propionate bacteria cultures have high antimutagenic and adhesive properties, synthesize a significant quantity of corrinoids and heme-containing enzymes. It was found out that together with Fe-strengthening there was acceleration of synthetic process of exocellular metabolites, which facilitated adaptation of cultures to a metal. Optimum process parameters of getting caseic phosphopeptides were determined. It was proved that they have high ability to solubilize ferrous iron. There was established the interplay between Fe-percentage and solubilization level. It was noted that Fe chelated with caseic phosphopeptides persists in the bivalent form during a long period of storage.*

**Key words:** *propionate bacteria, catalase, peroxydase, superoxide dismutase, caseic phosphopeptides, Fe-solubilization.*

## 1. INTRODUCTION

The concept of the optimum nutrition assumes as one of the major conditions of health preservation adequate providing of human's organism with both macro- and micronutrients, including essential microelements, in particular Fe. Iron-deficient state still continues to be a relevant and in many ways not solved problem of modern medicine. Deficit of Fe in an organism results in negative health outcome, such as asiderotic anemia [1].

Considering the fact that in daily life a person consumes Fe as part of products of plant and animal origin and that amino acids, peptides and proteins of animal origin promote the better fixation of this microelement, human diet should be enriched with organic Fe-forms. In our opinion the most convenient object for biotechnological preparation of Fe in its organic form are propionate bacteria, which can synthesize a significant quantity of heme-containing enzymes and corrinoids, that improve fixation of Fe in the organism [2].

It is known, that Fe in the organism can be soaked only as $Fe^{2+}$. However bivalent Fe is exposed to the fast chemical oxidation passing into a solventless indigestible for the organism trivalent form. For preservation Fe-biovailability chelating agents are important, which promote solubilization of minerals, keeping them in the same time in their solvable state. Example of these chelators are caseic phosphopeptides (CPPs). CPPs are phosphopeptides resulting from caseins of cow milk at digestion of them with alimentary proteinases [3]. It is important to note that caseic phosphopeptides are till now poor explored both as chelating agents for minerals and as potential nutraceuticals in human's nourishment. Besides there are not any data in the literature for influence of CPPs on solubilization of Fe. Therefore research of iron-binding capacity of CPPs is of great interest.

The Object of this research was to study out the influence of various concentrations of ferrous sulphate on growth and biosynthesis of exocellular metabolites with propionate bacteria, and also research of chelated properties of caseic phosphopeptides.

## 2. MATERIALS AND METHODS

*2.1. Bacteria and cultivation conditions*

The subject of research were propionate bacteria cultures (PBC): strains *Propionibacterium freudenrichii subsp. shermanii AC-2503, Propionibacterium freudemrichii subsp. fredenreichii AC-2500, Propionibacterium cyclohexanicum Kusano AC-2260 u Propionibacterium cyclohexanicum Kusano AC-2259,* which were received from the fund of All-Russian microorganisms collection of Institute of Biochemistry and Microbial Physiology (Moscow) and which were activated by a unique biotechnological way, that was developed at East-Siberian State University of Technology. As a sourse of Fe bivalent sol ($FeSO_4$) was used. Cultivation of propionate bacteria was carried out in serum-supplemented medium with addition of growth-stimulating factors [4]. As inoculum circadian culture was used, which was cultivated on the base of separated milk. Ferrous sulphate was added into the growth-stimulating media in strength 0,25-0,55 mg/ml. Cultivation of propionate bacteria with presence of ferrous sulphate had been carrying out during 24 hours at 30ºC. Kinetics of cultures growth was analyzed with standard methods.

*2.2. Analytic methods*

Process of iron-binding was observed in amount of the derived chelated $Fe^{2+}$ (% of Fe in its bivalent form kept from the base dose). The quantity of iron ($Fe^{2+}$) was determined with a reference method [5]. The quantity of iron ($Fe^{3+}$) was defined with a spectrophotometric method. The technique is developed according to All-Union standard 34-70-953.4-88. The subject method is based on interreacting of dissolved iron with sulphosalicylic acid and on optical density measurement of color solutions resulting thereat.

Determination of exocellular metabolites was carried out at the end of exponential phase. Catalase activity was defined with colorimetric method [6], peroxydase activity – spectrophotometrically with o-dianisidine reagent [7], superkiddismutase activity was determined at hemisine autoxidation [8].

Antimutagenic activity was found out with the help of Ames test [2]; adhesion properties were researched on formalinized erythrocytes by the developed method of Brilis V.I., adhesiveness of othe strain was determined by index of adhesiveness of microorganisms (IAM) [9]; concentration of exopolysaccharides - through the antronov method [10]; content of cobalamin $B_{12}$ – spectrophotometrically [11].

Solution of caseic phosphopeptides was found out through zymohydrolysis of sodium caseinate. It is known that metal-binding ability of CPPs depends on phosphorylation degree. With the purpose to produce hydrolysate with the maximal content of low-molecular phospholpeptides and free amino acids, which are capable from then on to give soluble Fe-complexes, we cpecified technological parameters of separation of CPPs. At this separation we applied the circuit of one-stage hydrolysis of sodium caseinate together with pepsin and trypsin at different hydrolysis time. Molecular-mass distribution of peptides as part of aqueous solution of caseic phosphopeptides was estimated with size-exclusion chromatography of mean pressure on the column TSK GEL (0,8/30sm). In tables statistically-valid differences are shown at $p<0,05$.

## 3. RESULTS

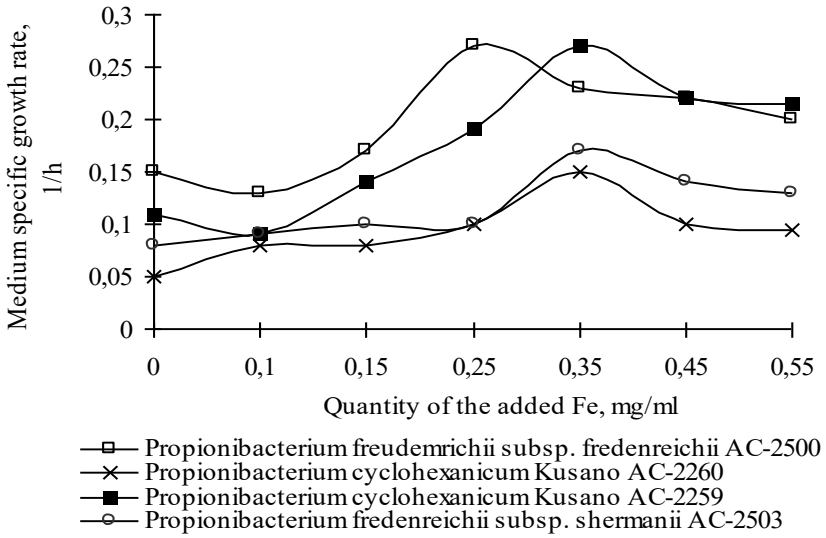
*3.1. Studying influence of ferrous sulphate on growth and biosynthesis of exocellular conditioning factors of propionibacteria*

Exocellular metabolites, which are synthesized by microorganisms and which regulate their activity, were called autoregulators. It is important to emphasize that among numerous functions of

autoregulators the factors, providing adaptation of microorganisms to adverse physico-chemical medium conditions, are extremely poor investigated.

In this connection in the further researches we explored influence of ferrous sulphate on synthesis of exometabolites with propionibacteria. It is known that biological effect of cooperation of microorganisms with metals is determined through the metal concentration, its toxic property and metabolic functions of microorganisms [12].

Results of our researches (Picture 1) showed that ferrous sulphate up to the certain concentration (0,25 mg/ml for P. freudemrichii subsp. fredenreichii AC-2500 и 0,35 mg/ml for all other strains) increases specific growth rate of propionibacteria, that indicates importance of Fe for the standard cytometabolism. Further densification in $FeSO_4$ results in growth-rate inhibition. Thus the quantity of living cells remains on a high level ($10^{11}$ кfu(colony-forming unit)/$sm^3$). It is necessary to note that surplus content of metal inhibits the metabolism, in this case nocifensors appear, which compensate adverse metal effect.




Picture. 1 - Influence of ferrous sulphate on growth rate of propionibacteria

At research of biotechnological potential it was established that propionibacteria synthesize a significant amount of heme-containing enzymes [13]. As their synthetic process and activity depend on iron ions content, further researches are derected on studying influence of $FeSO_4$ on biosynthesis of catalase, peroxydase and superoxide dismutase (SOD). Research results are tabulated 1.

Analysis of the tabled data 1 shows that with increase of iron dose at all stains increase of enzymes activity takes place, such as catalase and SOD. Densification of ferrous sulphate up to 0,45-0,55 мg/мl leads to increase of catalase activity (upon the average) in 1,5 times, and SOD – in 1,7-1,85 times. Concerning peroxydase its activity in all control samples changed only slightly. It must be explained through concentration of exclusively intracellular enzyme. Correlation dependence between activity of enzymes (Y) and concentration of ferrous sulphate is established:

$Y_1 = -38{,}90x^2 + 40{,}61x + 19{,}40$ - SOD;

$Y_2 = -0,115x^2 + 0,861x+0,514$ - Catalase.

Correlation coefficients $R_{1,2}$ give 0,990 and 0,898 appropriately.

Table 1. Influence of ferrous sulphate on activity of antioxidative enzymes, which are synthesized with propionibacteria

| Strain | Fe- content, mg/ml | Enzyme strength | | |
|---|---|---|---|---|
| | | Catalase (mkat/ml) | Peroxydase (nmol/(min· mg of protein)) | SOD, unit/mg of protein |
| P. freudenrichii subsp. fredenreichii AC-2500 | 0 | 1280,0 | 1,573 | 1,02 |
| | 0,25 | 1290,5 | 1,572 | 1,77 |
| | 0,35 | 1300,9 | 1,572 | 1,77 |
| | 0,45 | 1492,5 | 1,570 | 1,78 |
| | 0,55 | 1490,6 | 1,571 | 1,78 |
| P. cyclohexanicum Kusano AC-2260 | 0 | 1712,2 | 0,905 | 1,03 |
| | 0,25 | 1802,5 | 0,890 | 1,85 |
| | 0,35 | 1895,3 | 0,853 | 1,86 |
| | 0,45 | 1907,4 | 0,850 | 1,86 |
| | 0,55 | 1912,3 | 0,853 | 1,86 |
| P. cyclohexanicum Kusano AC-2259 | 0 | 1561,9 | 1,118 | 1,01 |
| | 0,25 | 1807,0 | 1,125 | 1,83 |
| | 0,35 | 1991,1 | 1,122 | 1,83 |
| | 0,45 | 2007,0 | 1,119 | 1,83 |
| | 0,55 | 2091,3 | 1,119 | 1,84 |
| P. fredenreichii subsp. shermanii AC-2503 | 0 | 2318,6 | 1,113 | 1,17 |
| | 0,25 | 2554,8 | 1,112 | 1,98 |
| | 0,35 | 2789,3 | 1,113 | 1,99 |
| | 0,45 | 2954,3 | 1,112 | 2,01 |
| | 0,55 | 2952,3 | 1,113 | 2,01 |

It is necessary to note that increase of activity of catalase and SOD greatly increases oxidizing power of propionibacteria to defend from oxidative stress, because exactly these enzymes can take superoxide radicals from cells.

From literature it is known that defence from toxic concentration of metal at microorganisms is shown in appearing of various agents, which bind metal in form of low-toxic compounds. In this connection further researches were devoted to analysis of influence of ferrous sulphate on synthesis of exocellular conditioning factors of bacteria. Research results are tabulated 2.

Analysis of the tabled data 2 shows that addition of iron ions into the grow-supporting microenvironment stimulates synthesis of exocellular metabolites. So, it was established that with increase of $FeSO_4$ content higher antimutagen activity of propionibacteria was observed, that specifies induction of antimutagenesis. Increased biosynthesis of exopolysaccharides (EPC) with addition of Fe demonstrates nonenzymatic protection of bacteria, when EPC interrupt entry of excessive iron into the cell because of circumfluence of this cell. Upgrading adhesion is explained not only through defense reaction of cultures against metal, but also through literary data, which say that be- and trivalent

cations in the medium lead to thinning of dipole layers afloat in aqueous media, that promotes adhesion due to thickness electrostatic repulsive forces.

Research of morphology of propionibacteria, which are cultivated at different iron concentrations, showed that with increase of $FeSO_4$ content up to 0,55 mg/ml deme (cohesion) was observed. Probably cells in the intercellular structure support their viability through cell aggregation.

Table 2. Influence of ferrous sulphate on synthesis of exocellular metabolites

| Strain | Fe- content, mg/ml | Index | | | |
|---|---|---|---|---|---|
| | | Adhesive activity | EPC (exopolysaccharides) mkg/ml | Inhibition (antimutagenic activity), % | Quantityof vitamine $B_{12}$, mkg/ml |
| P. freudenrichii subsp. fredenreichii AC-2500 | 0 | 4,0 | 29,81 | 43,6 | 31,0 |
| | 0,25 | 4,0 | 29,96 | 44,2 | 32,0 |
| | 0,35 | 4,2 | 30,05 | 44,8 | 32,5 |
| | 0,45 | 4,6 | 35,50 | 48,9 | 34,0 |
| | 0,55 | 5,1 | 36,80 | 48,6 | 34,5 |
| P. cyclohexancum Kusano AC-2260 | 0 | 3,7 | 31,85 | 46,2 | 22,0 |
| | 0,25 | 3,8 | 32,56 | 48,9 | 26,0 |
| | 0,35 | 3,9 | 36,98 | 48,7 | 27,0 |
| | 0,45 | 4,4 | 37,20 | 48,6 | 29,0 |
| | 0,55 | 4,7 | 48,30 | 57,9 | 28,0 |
| P. cyclohexancum Kusano AC-2259 | 0 | 2,8 | 36,65 | 44,8 | 18,0 |
| | 0,25 | 3,1 | 36,90 | 46,2 | 18,0 |
| | 0,35 | 3,6 | 36,99 | 47,5 | 18,0 |
| | 0,45 | 4,2 | 38,70 | 52,8 | 19,0 |
| | 0,55 | 4,6 | 44,78 | 54,2 | 19,5 |
| P. fredenreichii subsp. shermanii AC-2503 | 0 | 5,4 | 41,30 | 47,7 | 33,0 |
| | 0,25 | 5,4 | 44,52 | 49,6 | 35,0 |
| | 0,35 | 5,8 | 49,56 | 50,1 | 35,5 |
| | 0,45 | 6,1 | 50,20 | 51,2 | 36,0 |
| | 0,55 | 6,3 | 56,58 | 57,3 | 36,0 |

These results testify that synthesis of exometabolites promotes adaptation of propionibacteria to iron ions. The detected regularities not only give an insight into principium of metabolic structure of propionibacteria, but they are scientific basis for creation of nutritional supplements, containing iron cin its organic bioaccessible form.

*3.2. Influence of caseic phosphopeptides on Fe-solubilization in the grow-supporting microenvironment*

Experimental researches showed that at content of iron in the medium 0,45 mg/ml and more color of concentrates changes and residuum falls, that testifies appearing of solventless $Fe^{3+}$ ions. In this connection in further researches we studied the influence of caseic phosphopeptides ($CPP_s$) on Fe-solubilization (chelation) in the grow-supporting microenvironment. CPPs are phosphopeptides from caseins of cow milk at reboiling with digestive proteinases.

It is known that metal-binding ability of CPPs depends on phosphorylation degree. To get hydrolysate with the highest content of low-molecular phosphopeptides and free amino acids, which can further make soluble complexes with iron, we specified technological parameters of allocation of CPPs. At producing CPPs we applied the circuit of one-stage hydrolysis of sodium caseinate with pepsin and tripsin at different hydrolysis time. Research results are tabulated 3.

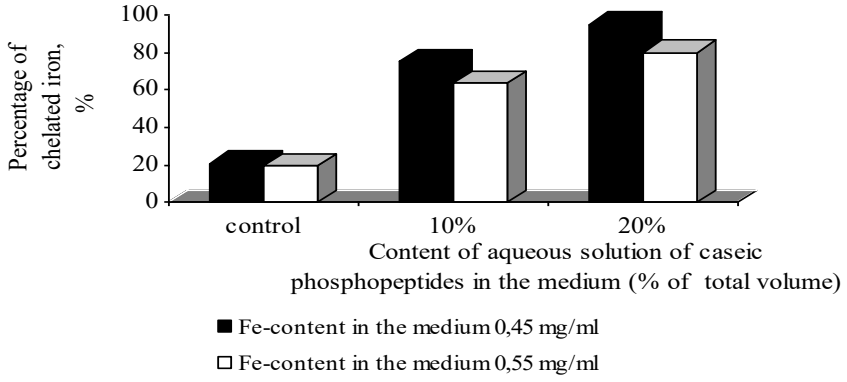
Table 3. Molecular-mass distribution of peptide fractions

| Hydrolysis time, min | Range of molecular masses, kD | Content of fractions (%) within the range of molecular masses depending on applied enzyme | | | |
|---|---|---|---|---|---|
| | | pepsin 1% | pepsin 3% | tripsin 1% | tripsin 3% |
| 40 | more than 138 | 1,8 | 1,6 | 1,7 | 2,0 |
| | 138-10,5 | 5,4 | 5,6 | 3,2 | 4,0 |
| | 10,5-5,1 | 9,5 | 10,3 | 7,8 | 6,2 |
| 60 | more than 138 | 2,1 | 1,8 | 1,7 | 2,1 |
| | 138-10,5 | 6,3 | 5,6 | 4,2 | 4,3 |
| | 10,5-5,1 | 9,8 | 11,0 | 7,8 | 6,4 |
| 120 | more than 138 | 2,2 | 2,3 | 2,2 | 2,1 |
| | 138-10,5 | 7,1 | 11,2 | 6,2 | 6,4 |
| | 10,5-5,1 | 11,9 | 18,7 | 10,3 | 10,8 |
| 240 | more than 138 | 1,8 | 1,9 | 1,7 | 1,7 |
| | 138-10,5 | 9,5 | 16,5 | 10,4 | 4,5 |
| | 10,5-5,1 | 12,9 | 18,1 | 10,9 | 10,4 |

Analysis of the tabled data 3 shows that at hydrolysis of sodium caseinate with 3% pepsin we can reach within 120 min the highest content of low molecular (10,5-5,1 kD) structures: 18,7%. The further increase of hydrolysis time does not result in noticeable change of low-molecular fractions. Besides these data allow to assert, that hydrolysis with pepsin is much more effective in comparison with tripsin. so, at the same concentration of enzyme (3%) we can get in 1, 7 times more low molecular peptide fractions at hydrolysis with pepsin, than at hydrolysis with tripsin.

As a result taking into account determination of molecular mass distribution of peptide fractions we chose optimum technological parameters of allocation of caseic phosphopeptides.

At the following stage of researches different doses of aqueous solution of CPPs and ferrous sulphate were added. The process of iron-binding was watched by quantity of the derived chelated $Fe^{2+}$ (% of Fe, in its bivalent form kept from the base dose). Research results (average indexes at all analyzed strains) are shown on picture 2.




Picture 2. Dependence between content in the media with digestible (chelated) iron and content of aqueous solution of caseic phosphopeptides

According to the data on this picture caseic phosphopeptides in the medium promote solubilizatin of iron and keep it in the form of $Fe^{2+}$ ions. The maximum quantity of soluble Fe-complexes (90-95%) appears when we add 20% aqueous solution of caseic phosphopeptides and 0,45 mg/ml ferrous sulphate. It is interesting that with increase of $FeSO_4$ content the degree of Fe-solubilization reduces. It is probably explained through that CPPs can bind only the limited number of Fe-molecules, i.e. quantity of the ionized mineral should not overgrow the quantity of available anionic water receptive areas of amino acids of caseic phosphopeptides.
There is an opinion that synthetic chelate forms of minerals collapse and lose their strength in storage, therefore they surrender natural salts of these elements. In this connection we researched preservation of iron, which was chelated with caseic phosphopeptides, in its bevalent form during a long period of storage. Research results are shown in the table 4.

Table 4. Influence of CPPs on Fe-solubilization in storage

| Strain | Content of CPPs, % | Content of $Fe^{2+}$ in the medium in storage (% of the base dose), d | | | |
|---|---|---|---|---|---|
| | | 30 | 60 | 90 | 120 |
| P. freudenrichii subsp. fredenreichii AC-2500 | control | 19,0 | 19,0 | 19,5 | 18,5 |
| | 10 | 58,0 | 62,0 | 62,5 | 60,0 |
| | 20 | 88,0 | 88,0 | 88,5 | 88,0 |
| P. cyclohexanicum Kusano AC-2260 | control | 30,0 | 29,5 | 30,0 | 28,5 |
| | 10 | 69,0 | 70,5 | 70,0 | 69,0 |
| | 20 | 94,5 | 95,0 | 95,0 | 94,5 |
| P. cyclohexanicum Kusano AC-2259 | control | 32,0 | 32,0 | 30,5 | 29,0 |
| | 10 | 60,0 | 60,5 | 60,0 | 59,5 |
| | 20 | 75,0 | 75,0 | 75,5 | 75,0 |
| P. fredenreichii subsp. shermanii AC-2503 | control | 22,0 | 25,0 | 25,5 | 19,0 |
| | 10 | 66,0 | 67,0 | 66,0 | 63,5 |
| | 20 | 95,0 | 96,0 | 96,0 | 95,0 |

Tabulated data show that content of chelated iron in concentrates with CPPs only slightly changes during storage. At the same time in control the significant reduce of content of soluble $Fe^{2+}$ ions was observed.

All obtained data show that caseic phosphopeptides are very important chelating agents if we must get new bioaccessible forms of iron. As a result of reserches optimum doses of $FeSO_4$ and aqueous solution of $CPP_s$ were sorted out, which promote maximum content of the solubilized iron.

**RESUME**

1. It was established that activated cultures of propionibacteria synthesize heme-containing enzymes (catalase, SOD, peroxydase), that opens wide prospects for practical application of them.

2. Optimum doses of ferrous sulphate were chosen, which promote active growth and high quantity of living cells of propionibacteria.

3. It was noted that addition of Fe-ions into the grow-supporting microenvironment stimulates synthesis of exocellular metabolites, which promote adaptation of propionibacteria to metal.

4. According to molecular-mass distribution of peptide combinations at hydrolysis casein the most effective way of getting caseic phosphopeptides was chosen.

5. It was established that caseic phosphopeptides have high ability to solubilize Fe and support it in its bivalent form during a long period of storage.

6. The structure of grow-supporting microenvironment was optimized through addition of $FeSO_4$ and aqueous solution of CPPs, which promotes high bioavailability of Fe.

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