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EFFECT OF IRON SULFATE ON BIOSYNTHESIS OF EXTRACELLULAR METABOLITES OF PROPIONIC ACID BACTERIA

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Abstract: In the work, activated cultures of propionic acid bacteria were found to exhibit high antimutagenic activity and adhesion properties, synthesize considerable amount of corrinoids and heme-containing enzymes. Increase of iron concentration in the medium was shown to intensify synthesis of extracellular metabolites promoting adaptation of the culture to the metal. Optimal technological parameters for isolation of casein phosphopeptides were determined. Ability of phosphopeptides to efficiently solubilize divalent iron was confirmed. Relationship between iron concentration and extent of solubilization was established. Iron chelated with casein phosphopeptides was noted to stay in divalent form for prolonged period.

Keywords: propionic acid bacteria, catalase, peroxidase, superoxide dismutase, casein phosphopeptides, iron solubilization

INTRODUCTION

The concept of optimal nutrition implies adequate organism supply with both macro- and micronutrients, including the essential microelements, particularly iron, as a key prerequisite for preservation of human health. Iron-deficient conditions remain a topical and untreated issue of modern medicine. Lack of iron in the organism leads to many negative consequences. One of them is the development of iron deficiency anemia [1].

Taking into account that man consumes iron in vegetable and animal products in everyday life and the presence of amino acids and peptides, as well as proteins of animal origin, promotes intake of the microelement, enrichment of diets with organic forms of iron seems reasonable. In our opinion, propionic acid bacteria, which possess the ability to synthesize considerable amounts of heme-containing enzymes and corrinoids thus increasing iron uptake, are the most convenient object for development of biotechnological production of iron in organic form [2].

Iron is known to be consumed only in the form of Fe^{2+}. However, divalent iron undergoes chemical oxidation to an insoluble, nonassimilable trivalent form. To preserve bioavailability of iron, role of chelating agents, which promote solubilization of minerals preserving their soluble state, is of interest. Casein phosphopeptides (CPPs) are among the representatives of the chelators. CPPs are phosphorylated peptides formed from caseins of cow milk upon digestion by proteases [3–5]. Casein phosphopeptides are still poorly studied as both chelating agents and potential nutriceutics for human nutrition. Besides, there are no data in literature on the effect of CPPs on iron solubilization. Therefore, studies on iron-binding capacity of CPPs are of interest.

The aim of the work was to study the effect of various concentrations of iron sulfate on growth and biosynthesis of extracellular metabolites by propionic acid bacteria, as well as the study on chelating properties of casein phosphopeptides.

MATERIALS AND METHODS

Bacteria and culturing conditions. Cultures of the following propionic acid bacteria (PABs) strains were subject of the study: *Propionibacterium freudenrichii* subsp. *shermanii* AC-2503, *Propionibacterium freudenrichii* subsp. *freudenrichii* AC-2500, *Propionibacterium cyclohexanicum* Kusano AC-2260, and *Propionibacterium cyclohexanicum* Kusano AC-2259, all obtained from the All-Russian Collection of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms (Moscow) and activated by a unique biotechnology method developed in the East Siberian State University of Technology and Management. Divalent salt ($FeSO_4$) was used as iron source. Propionic acid bacteria were cultured in serum medium supplemented with growth factors [6]. One-day culture grown on low-fat milk was used as an inoculate. Iron sulfate was added to the growth medium at concentration of 0.25–0.55 mg/mL. Propionic acid bacteria were cultured in the presence of iron sulfate for 24 h at 30°C. Culture growth kinetics was calculated according to a custom method.

Analytical procedures. The process of iron binding was followed by the amount of chelated Fe^{2+} (% iron remaining in divalent form to the total initial dose). Content of Fe^{2+} was determined using a reference method [7]. Content of Fe^{3+} was determined by spectrophotometry. The technique was developed according to the Industry-Specific Standard 34-70-953.4-88. The method is based on the interaction of dissolved iron with sulfosalicylic acid and measurement of optical density of the colored solutions thus formed.

Determination of extracellular metabolites was performed in the end of the exponential growth phase. Catalase activity was determined using a colorimetric technique [8], peroxidase activity, by spectrophotometry using the *o*-dianisidine reagent [9], and that of superoxide dismutase, by autoxidation of adrenalin [10].

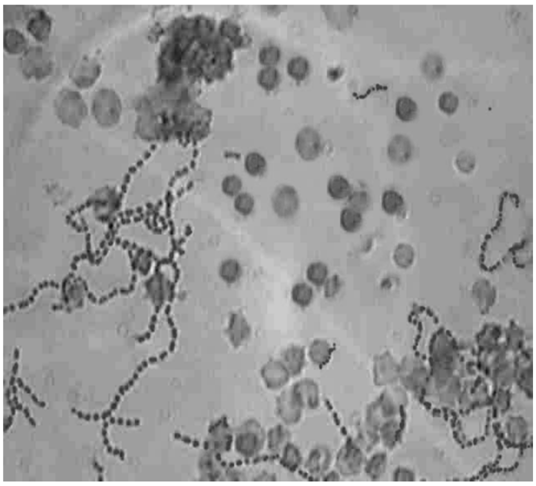
Antimutagenic activity was determined by the Ames test [2]; adhesion properties were studied on formalinized erythrocytes according to the in-depth Brilis technique; strain adhesiveness was estimated according to the index of microorganism adhesiveness (IMA) [11]; concentration of exopolysaccharides was estimated with anthrone reagent [12]; and vitamin B_{12} content was determined by spectrophotometry [13].

Solution of casein phosphopeptides was obtained by enzymatic hydrolysis of sodium caseinate. Metal-binding ability of CPPs depends on the extent of phosphorylation. To obtain hydrolysate with the maximal content of low-molecular weight phosphorylated peptides and free amino acids capable of formation of soluble complexes with iron, we redefined process parameters of CPP isolation. One-stage hydrolysis of Na caseinate with pepsin and trypsin with varying hydrolysis time was used. Molecular weight distribution of peptides in the aqueous solution of casein phosphopeptides was evaluated by moderate pressure size exclusion chromatography on a TSK GEL (0.8/30 cm) column. Chelated iron content was determined by mass spectrometry. Tables discuss statistically significant differences at $p < 0.05$.

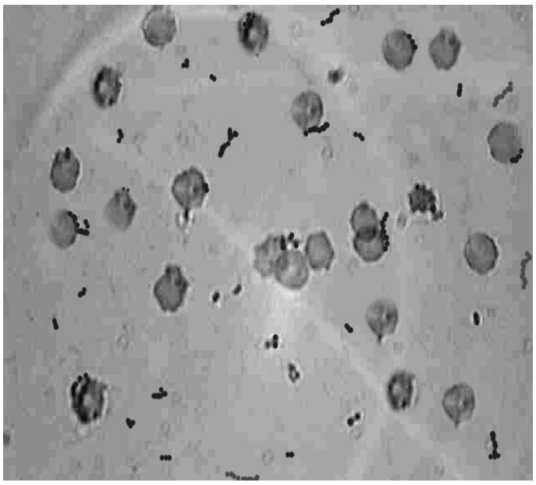
RESULTS AND DISCUSSION

Adhesion properties of propionic acid bacteria

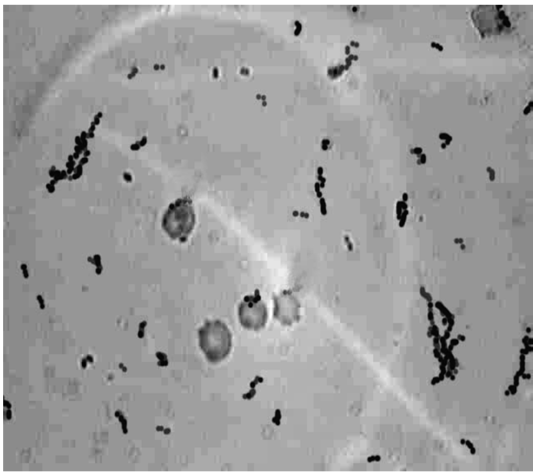
One of the current areas of modern microbiology studies adhesion process in various microorganisms. Adhesion is a intercellular interaction manifested through tight attachment of cells to a substrate. Concerning the propionic acid bacteria (PABs), we did not find any information on their adhesion properties in the literature.



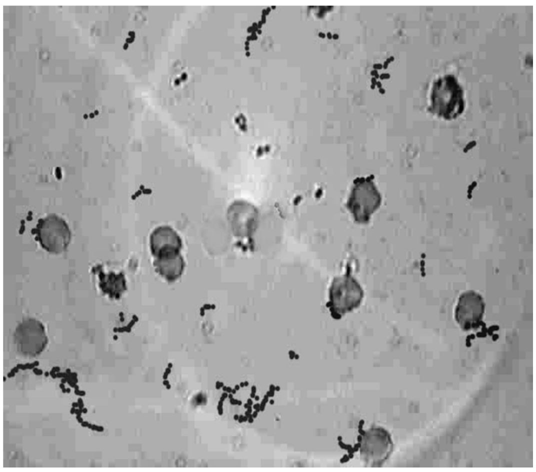
(a) *P. fredenreichii* subsp. *shermanii* AC-2503



(c) *P. freudemrichii* subsp. *fredenreichii* AC-2500



(b) *P. cyclohexanicum* Kusano AC-2259



(d) *P. cyclohexanicum* Kusano AC-2260

Fig. 1. Interaction of propionic acid bacteria with erythrocytes.

It should be noted that composition, stability, and protective properties of the macroorganism microflora largely depend on its adhesion properties. In this connection, we studied adhesion properties of various strains of propionic acid bacteria. Formalinized erythrocytes were chosen as model macroorganism cells. Process of propionic acid bacteria adhesion on erythrocytes is presented in Fig. 1.

Analysis of the data presented in Fig. 1 shows that propionic acid bacteria possess varying capability for adhesion on erythrocytes. Some strains were found to adhere in the form of individual bacterial cells (Figs. 1 b–d) or aggregates that cover erythrocyte surface almost completely (Fig. 1a).

Adhesive properties of cultures were evaluated by the average adhesion index (AAI), erythrocyte participation factor (EPF); adhesiveness was judged by the index of adhesiveness of microorganisms (IAM). According to the technique, microorganisms were considered non-adhesive at IAM values below 1.75, low adhesive, from 1.76 to 2.5; moderately adhesive, from 2.51 to 4.0, and highly adhesive, at IAM above 4.0. The results are presented in Table 1.

Table 1. Adhesiveness of propionic bacteria

Strain	AAI	EPF, %	IAM (M ± m)	Adhesiveness
P. freundenreichii subsp *freudereichii* AC-2500	3.2	79	4 ± 1.5	moderately adhesive
P. cyclohexanicum Kusano AC-2260	3.9	82	3.7 ± 1.2	moderately adhesive
P. freudereichii subsp. *shmanii* AC–2503	4.6	85	5.4 ± 1.1	highly adhesive
P. cyclohexanicum Kusano AC-2259	3.3	80	3.1 ± 1.8	moderately adhesive

As follows from Table 1, propionic bacteria possess relatively pronounced adhesion properties. Of all studied cultures, *Propionibacterium fredenreichii* subsp. *shermanii* AC-2503 is highly adhesive, which is evidenced by adhesiveness index value (IAM = 5.4), as well as AAI (4.6) and EPF (85%) values. Consequently, the strain will attach to bowel cells better than others, creating a protective barrier. Other strains exhibited moderate adhesiveness according to all tested parameters.

Effect of iron sulfate on growth and biosynthesis of extracellular adaptation factors in propionic acid bacteria

Extracellular metabolites synthesized by microorganisms and regulating their activity are called autoregulators. It is important to stress that among the multiple functions of autoregulators factors ensuring adaptation of microorganisms to unfavorable physicochemical environmental conditions are poorly studied.

In this connection, in further studies the effect of iron sulfate on synthesis of exometabolites by propionic acid bacteria was studied. Biological effect of microorganism interaction with metals is known to be determined by concentration of the metal, its toxicity, and metabolic potential of the microorganism [14].

Our data (Fig. 2) show that below certain concentration (0.25 mg/mL for *P. freudenrichii* subsp. *freudenrichii* AC-2500 and 0.35 mg/mL, for the rest of the strains) iron sulfate increases specific rate of propionic acid bacteria growth, which evidences iron importance for normal cell metabolism. Further increase in $FeSO_4$ concentration in the medium leads to growth slowdown. The number of viable cells remains high (10^{11} CFU/cm^3). It should be noted that excess metal content inhibits metabolism, turning on protective mechanisms compensating for the negative effects o the metal.

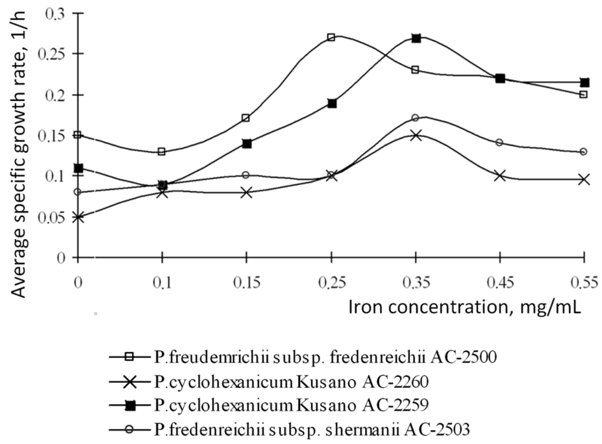


Fig. 2. Effect of iron sulfate on the growth rate of propionic acid bacteria.

Table 2. Effect of iron sulfate on the activity of antioxidant enzymes synthesized by propionic acid bacteria

Strain	Iron content, mg/mL	Enzyme activity		
		catalase, mcat/mL	peroxidase, nmol/(min mg protein)	SOD, units/mg 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	2554.6	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

Studying biotechnology potential, we found that propionic acid bacteria synthesize considerable amount of heme-containing enzymes [15]. Since heme-containing enzyme synthesis and activity depend on the content of iron ion, we studied the effect of $FeSO_4$ on biosynthesis of catalase, peroxidase, and superoxide dismutase. The results are presented in Table 2.

Analysis of the data presented in Table 2 shows that increase in iron concentration led to increase in the activities of catalase and SOD in all studied strains. Increase in iron sulfate concentration in the medium up to 0.45–0.55 mg/mL led to 1.5-fold increase in catalase activity and 1.7–1.85-fold increase in SOD acitivity (on average). As for peroxidase, its activity in all experimental samples practically did not change. Probably, this may be explained by accumulation of the endoenzyme solely. Correlation between the enzymes' activity (*Y*) and iron sulfate concentration was established:

$Y_1 = -39.90x^2 + 40.61x + 19.40$ for SOD and

$Y_2 = -0.115x^2 + 0.861x + 0.514$ for catalase.

Correlation coefficient values $R_{1,2}$ are 0.990 and 0.898, respectively.

It should be noted that increase in catalase and SOD activities considerably exceeded the capacity of propionic acid bacteria to protect themselves from oxidative stress since these very enzymes are responsible for superoxide radicals removal from cells.

As follows from the literature data, protection from toxic metal concentration in microorganisms is manifested through formation of substances capable of metal binding in the form of low-toxicity compounds. Therefore, we studied the effect of iron sulfate on synthesis of bacteria extracellular adaptation factors. The results are presented in Table 3.

Table 3. Effect of iron sulfate on synthesis of extracellular metabolites

Strain	Iron content, mg/mL	Parameters			
		Adhesion activity (IAM)	EPS, µg/mL	Inhibition (antimutagenic activity), %	Vitamin B_{12} concentration, µg/mL
P. freudenrichii subsp. *fredenrei chii* AC-2500	0	4.0	29.81	43.6	13.0
	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

Data presented in Table 3 evidence that the addition of iron ions to nutrient medium for PAB cultivation stimulated synthesis of extracellular metabolites. For example, higher antimutagenic activity of PABs was noted with the increase in $FeSO_4$ concentration, which indicates antimutagenesis induction. Increased biosynthesis of exopolysaccharides (EPS) upon the addition of iron is a manifestation of bacterial non-enzymatic protective mechanisms, when EPS prevent excess iron penetration in cells through coating of the bacterium surface. Increased adhesion is explained by not only protective response of cultures to the metal, but also the fact that, according to the literature data, the presence of di- and trivalent cations leads to shrinking of charged double layers on surfaces in aqueous media, which promotes adhesion through decrease in electrostatic repulsion.

When studying morphology of propionic acid bacteria cultured at various iron concentrations, cell aggregates (cohesion) were noted upon increase of $FeSO_4$ dose to 0.55 mg/mL. Probably, cells managed to maintain viability under conditions of intercellular contacts in aggregates.

The results evidence that synthesis of exometabolites promotes adaptation of propionic acid bacteria to iron ions. The tendencies reveled allow understanding of the principle of metabolic organization in propionic acid bacteria and form scientific basis for development of biologically active supplements containing iron in organic bioavailable form.

The effect of casein phosphopeptides on iron solubilization in the nutrient medium

When conducting experimental studies, we noted that at iron concentration of 0.45 mg/mL and above color of the concentrate changes and precipitate is formed, which evidences formation of insoluble Fe^{3+} ions. In this connection, we studied the effect of casein phosphopeptides (CPPs) on solubilization (chelating) of iron in the nutrient medium. CPPs are phosphorylated peptides formed from cow milk upon their digestion by proteases.

Table 4. Molecular-weight distribution of fermentolysis fractions

Molecular weight limits, kDa	Size of peptide fractions in hydrolysates, nm	Enzymes		
		pepsin	trypsin	chymosin
>20	>10	10.5	—	20.5
20.1–18.7	7–10	9.2	—	22.6
18.7–12.5	5–7	7.6	5.7	18.4
12.5–11.0	4–5	15.7	15.4	16.7
11.0–5.1	3–4	19.5	13.2	11.8
5.1–2.8	~3	14.4	17.0	9.4
2.8–1.0	1–2	11.7	26.6	—
<1	<1	10.1	22.1	—

Table 5. Effect of iron sulfate concentration and proteolytic enzymes on chelated iron content

Iron sulfate introduced, mg/mL	Chelated iron content in aqueous solutions of casein phosphopeptides, mg			
	pepsin hydrolysis	trypsin hydrolysis	chymosin hydrolysis	chymotrypsin hydrolysis
1	0.51	0.87	0.48	0.71
2	0.88	1.99	0.98	1.12
3	1.47	2.67	1.25	2.52
4	1.99	3.13	1.87	3.25
5	2.10	4.98	2.12	4.18
6	2.89	5.25	2.58	5.16
7	2.99	6.96	2.98	6.45
8	3.58	7.27	3.15	7.15
9	4.12	8.12	4.12	8.45
10	4.69	7.72	5.12	6.89

Metal-binding capacity of CPPs is known to depend on the extent of phosphorylation. To obtain hydrolysate with the maximum content of low-molecular weight phosphorylated peptides and free amino acids that are able to form soluble complexes with iron we redefined the technological parameters of CPP isolation. A one-stage sodium caseinate hydrolysis with proteolytic enzymes was used to prepare CPPs. The results are presented in Fig. 4.

Data presented in Tables 4 and 5 and Figs. 3–7 evidence that casein phosphopeptides form nano-size chelate complexes with iron ions. These particles should easily bind cell surface, efficiently carry iron ions across the intestine wall, and protect the mineral from interactions with other components of the stomach.

As a result of the studies reported herein, technological scheme of casein phosphopeptide isolation was modified (see Fig. 8).

There is an opinion that artificial chelated forms of minerals are destroyed upon storage and lose their efficiency, therefore they are inferior to natural organic salts of the elements. For this reason, we studied preservation of iron chelated with casein phosphopeptides in divalent form upon prolonged storage. The results are presented in Table 6.

Mass spectra of hydrolysates before and after introduction of iron

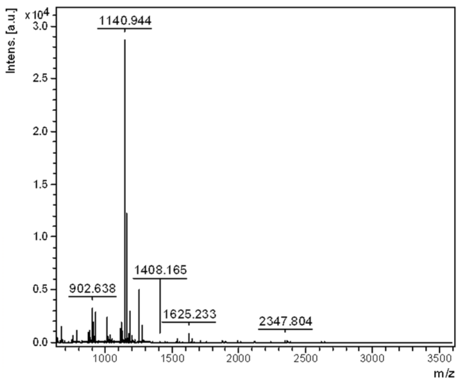


Fig. 3. Hydrolysate before the addition of iron.

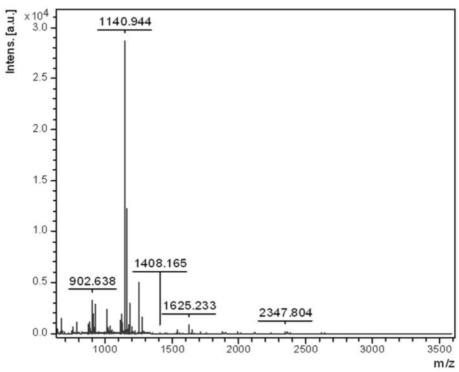
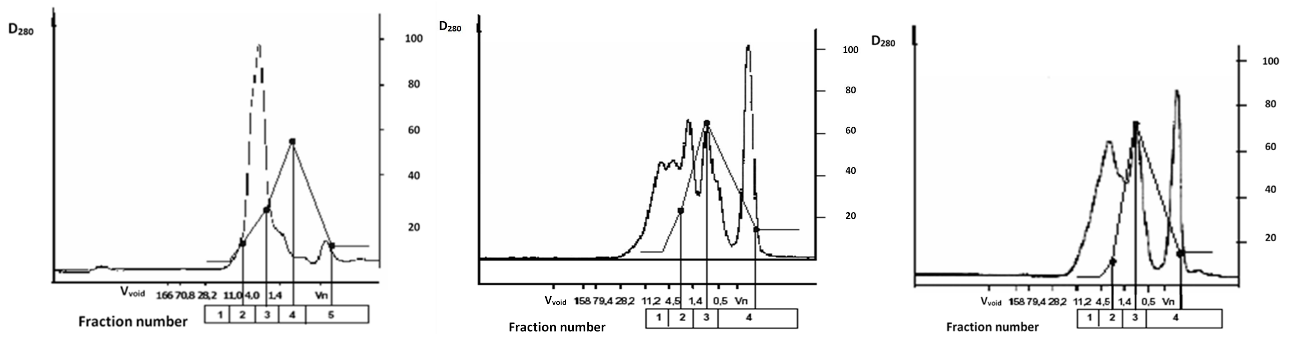


Fig. 4. Hydrolysate after the addition of iron.

Table 6. Effect of CPPs on the process of iron solubilization upon storage

Strain	CPPs content, %	Content of Fe^{2+} in storage medium (% to initially introduced dose), days			
		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



Figs. 5–7. Content of iron in chromatography fractions of iron complexes with trypsin, pepsin, and chymotrypsin hydrolysates of sodium caseinate, respectively (left to right).

Sodium caseinate
↓
Endoprotease digestion
↓
Hydolysis (acidification pH 4.6)
↓
Removal of non-peptide material (centrifugation)
↓
Precipitation with 15% TCA
↓
Centrifugation
↓
Introduction of $FeSO_4$ → Solution enriched with CPPs
↓
Nanofiltration
↓
Solution of CPPs with chelated iron

Fig. 8. Modified technological scheme of casein phosphopeptide preparation.

Data presented in Table 6 indicate that in the process of storage, chelated iron content in concentrated CPP-containing solutions practically did not change, while considerable decrease in soluble Fe^{2+} ion content was observed in control.

Altogether, the data indicate that casein phosphopeptides are promising chelating agents to obtain new bioavailable iron forms. Optimal doses of $FeSO_4$ and aqueous solution of CPPs providing for the maximum amount of solubilized iron were determined.

CONCLUSIONS

1. Activated cultures of propionic acid bacteria were found to synthesize heme-containing enzymes (catalase, SOD, and peroxidase), which opens new perspectives for their practical application.
2. Optimal doses of iron sulfate providing for the active bacteria growth and high number of viable cells of propionic acid bacteria were determined.
3. Addition of iron ions to the nutrient medium was found to stimulate synthesis of extracellular metabolites that promote adaptation of propionic acid bacteria to the metal.
4. Molecular-weight distribution and order of peptide fractions in casein phosphopeptides were studied at nanolevels.
5. The method of isolation of casein phosphopeptides was optimized to provide for the maximum yield of low-molecular weight peptide nanostructures with characteristic size of 1–10 nm capable of chelating maximum amount of iron (up to 7 mg/mL).
6. Complexes of casein phosphopeptide with microelements were studied, mechanism of the mineral ion binding with peptide fractions in the complexes was characterized, and the specific content of chelated mineral in the complexes was determined.

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