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Effect of ultrasonic treatment on metabolic activity of *Propionibacterium shermanii*, cultivated in nutrient medium based on milk whey

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Abstract. Utilization of milk whey still remains one of the most serious problems of the modern world dairy industry. Because of high biological value of whey it can be used as nutrient media in techniques of direct microbiological synthesis of complex high-molecular substances, for example the B_{12} vitamin, which participate in various biocatalytic reactions in organism providing normal functioning of the brain, nervous and hematogenic systems.
The main industrial producer of B_{12} vitamin is *Propionibacterium shermanii* species, which can use lactose as main carbon source and can develop directly in milk whey. However, common ways of vitamin B_{12} microbiological production can't be applied on the food plants and there must be used alternative safe methods, such as ultrasonic treatment of the cell culture, which can carry out the direct modulation of metabolic activity of bacteria for increasing of a yield of B_{12} vitamin.
For the definition of ultrasonic processing influence on metabolism of *Propionibacterium freudenreichii ssp. shermanii* industrial strain it was cultivated in the nutrient media based on milk whey and treated by low intensity ultrasound at a frequency of 20 kHz within 10 and 20 min each 24 h. Received results allow to suggest that ultrasonic modulation of propionic bacteria metabolic activity can be used for an intensification of B_{12} vitamin biotechnological obtaining and manufacturing of fermented food products based on milk whey and enriched with B_{12} vitamin.

Key words: ultrasound, *Propionibacterium shermanii*, B12, cobalamine, milk whey.

INTRODUCTION

The one of the most important vitamins for the human health is the B_{12} vitamin which involves several kinds of specific forms of cobalt-containing compounds, known as cobalamins. The main industrial form of vitamin is cyanocobalamin due to its high stability. Cobalamines serve as cofactors for various important enzymes participating in maintenance of normal DNA synthesis, regeneration of methionine and other biocatalytic reactions providing normal functioning of the brain and nervous system, blood formation, etc. Deficiency of these compounds in diet can lead to different neurological diseases, vascular damage, thrombosis and even to serious form of anemia called Addison's pernicious anemia (Green & Miller, 2010).

Naturally they are synthesized only by microorganisms and contain only in food of an animal origin. The lack of these compounds leading to various diseases often can be observed in the diet through various gastric disorders, such as chronic gastritis that lead to malabsorption of vitamin, or inadequate dietary intake, for example in vegetarian diet. Deficiency of B_{12} can be corrected by introduction special food products enriched with high amounts of vitamin which can provide adequate B_{12} intake even through a passive diffusion mechanism that allows assimilate only of 1–2% of vitamin containing in food. That type of food products also can be used as medical food for people who can't absorb vitamin B_{12} via the normal physiologic route. The recommended dietary allowance of B_{12} for males and females is 2.4 µg per day and higher, so such products should contain at least 240 µg kg^{-1} of vitamin.

However nowadays B_{12} vitamin is one of the most expensive vitamins and it is used mostly as medical agent for injections and due to its high cost it can't be used as simple food additive.

Vitamin B_{12} is produced exclusively by biosynthetic way. The main industrial producer of B_{12} vitamin is *Propionibacterium freudenreichii ssp. shermanii* which was recently used mainly as a ripening culture in Swiss-type cheeses. These microorganisms synthesize exclusively biologically active forms of vitamin and able to use a variety of carbon substrates, including lactose and lactic acid, that allows them to grow in milk whey (Falentin et al., 2010).

Milk whey contains from 4.2 to 7.5% of solids, most of which are disaccharide lactose (3.5–5.2%), a large number of organic and inorganic compounds containing essential chemical elements such as potassium, calcium, magnesium, sodium and the phosphorus in natural proportions, water- and fat-soluble vitamins from milk such as A, B_1, B_6, C, E, and nitrogenous substances like whey proteins and free amino acids.

All that allow considering whey as a valuable nutrient media for propionic bacteria. The highest vitamin B_{12} yield for *Propionibacterium freudenreichii* cultivated on milk whey was 15 µg mL^{-1} (Berry & Bullerman, 1966) and that shows high application potential for enriching fermented products with natural vitamins.

Profitable production of various functional products with the use of milk whey enriched with B_{12} vitamin based on propionic fermentation can be arranged economically on the dairy factories. This can partially solve the problem of profitable utilization of milk whey which still remains one of the most serious problems of the modern world dairy industry.

Traditional ways of vitamin B_{12} microbiological production demand long-time multistage process of synthesis of the precursor and the vitamin itself, high-temperature processing for vitamin releasing from the cells, and also using of dangerous substances, as cyanides and the phenol for stabilization and extraction of vitamin, application of which at the food factories is inadmissible due to their high toxicity. Economically effective alternative to the common techniques of vitamin obtaining and stabilization can be ultrasonic treatment of the Propionibacterium cell culture developing directly in food product for the purpose of B_{12} synthesis intensification and soft destruction of bacteria cells without product overheating.

Low-frequency ultrasound was proved itself as a powerful and economic tool for intensification of biochemical reactions by means of increasing an exchange between cells and a nutrient medium (Christi, 2003), reducing duration of enzymatic hydrolysis and maintaining activity of some enzymes during cavitation (Rokhina et al., 2009).

For example ultrasound treatment with 20 kHz frequency which is widely used in food industry ultrasonic equipment increases growth rate and overall biomass of the cyanobacteriae cells (Francko et al., 1994). Treatment on this frequency is also used for stimulation of anaerobic digestion processes in wastewater treatment plants (Dewil et al., 2006). Therefore low intensity ultrasound treatment of liquid medium which cause acceleration and initiation of chemical reactions in the sonicated medium can change the metabolic activity of bacteria cells (Akopyan & Ershov, 2005). By means of ultrasonic treatment it is possible to carry out the direct modulation of metabolic activity of bacteria for B_{12} vitamin yield increase without increasing in duration of production process using no special devices (Suchkova & Shershenkov, 2013).

MATERIALS AND METHODS

For the definition of ultrasonic processing influence on industrial strain of *Propionibacterium freudenreichii ssp. shermanii* metabolism bacteria cells were cultivated during 96 h in the nutrient media based on milk whey with concentration of lactose of 5% and adding of 0.002% $CoCl_2 \cdot 6H_2O$ and 0.001% $MgSO_4$ according to Zalashko (1990). The strain used for vitamin B12 production was industrial strain *Propionibacterium freudenreichii I-63,* which is a gram-positive, non-spore forming, rod-shape, and aerotolerant anaerobic bacterium producing vitamin B_{12} intracellularly.

The major problem in vitamin B_{12} production using *Propionibacterium* is the growth inhibition of the cell due to the accumulation of inhibitory metabolites such as propionic acid and acetic acid. Control of pH at approximately 7.0 is critical for reaching high vitamin B_{12} yields (Ye et al., 1996). Therefore pH was kept at level about 6.9 by means of periodic neutralization of the nutrient media during the cultivation by calcium carbonate.

Cell culture was treated by low intensity ultrasound at operating frequency of typical ultrasonic homogenizer 20 kHz within 10 and 20 min each 24 h.

First several cultivation cycles were performed in anaerobic conditions during all 96 h. Since oxygen is required for the biosynthesis of the lower ligand of active form of vitamin B_{12}, 5, 6-dimethylbenzimidazole, main product of this cultivation was vitamin analogs called corrinoids, which lack of nucleotide.

Next cultivation cycles were performed with continuous aeration of fermented media by means of sparging by sterile air during the second stage of cultivation after 48 h of anaerobic cultivation.

Measurement of titratable acidity of milk whey by 0.1N NaOH solution for definition of amount of organic acids synthesized by bacteria as primary metabolites was performed for research of metabolic activity of bacteria during the cultivation. Results are presented and compared in degrees of Turner (Table 1). Comparative photometric definition of corrinoid compounds concentration in the milk whey after cultivation was also made.

RESULTS AND DISCUSSION

It was found out that increase in duration of low intensity ultrasound treatment cause about 1–4% increase of total titratable acidity in all 20 minute treated samples and it depends upon duration of ultrasound treatment.

Table 1. Average total titratable acidity of fermented milk whey probes in various treatment conditions

Cultivation time, hours	Duration of treatment, min					
	0		10		20	
	ºT	%	ºT	%	ºT	%
0	3.1	100.0	3.1	100.0	3.1	100.0
24	40.6	100.0	41.6	102.5	39.5	97.3
48	74.9	100.0	74.9	100.0	74.9	100.0
72	108.9	100.0	111.0	101.9	113.0	103.8
96	140.7	100.0	140.4	99.8	146.4	104.0

That points on acceleration of fermentation processes and bacteria activity increase. Also changes in morphology of the bacteria cells subjected to ultrasound treatment and reduction of their sizes in comparison with untreated culture were observed. Average linear size of cells without treatment was 1.0–1.5 μm; linear size of treated cells in both experiments was 0.5–0.8 μm (Fig. 1).

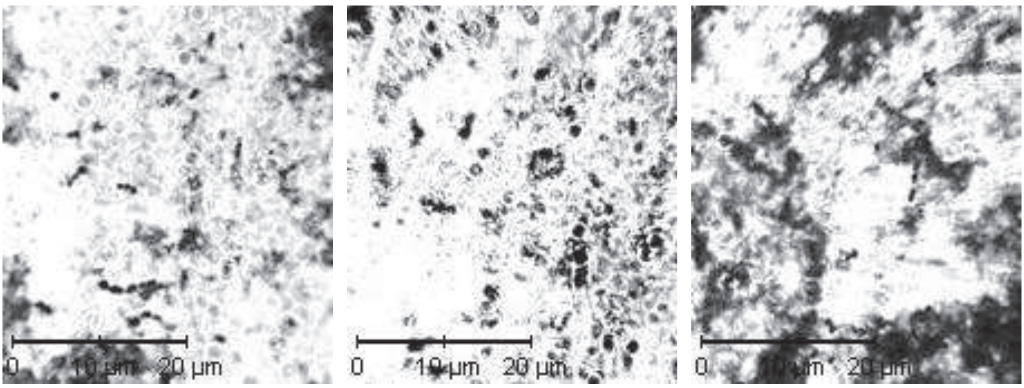

Figure 1. Microscope slides of fermented whey samples after anaerobic cultivation (untreated culture; 10 min treatment; 20 min treatment respectively).

Photometric analysis of fermented media was made after completion of fermentation process, thermal destruction of cells and filtration of the media. Received results are the same both for anaerobic cultivation and mixed anaerobic-aerobic cultivation cycles (Figs 2, 3).

During cultivation propionic bacteria use Co (II) from solution and turn it into corrin ring of cobalamines. Formed corrinoids have light absorption maximum which varies from 351 to 375 nm for various forms (Dolgov et al., 2004). Due to this fact it is possible to estimate their concentration in the fermented media and reveal the tendency of ultrasound impact on bacteria metabolic activity.

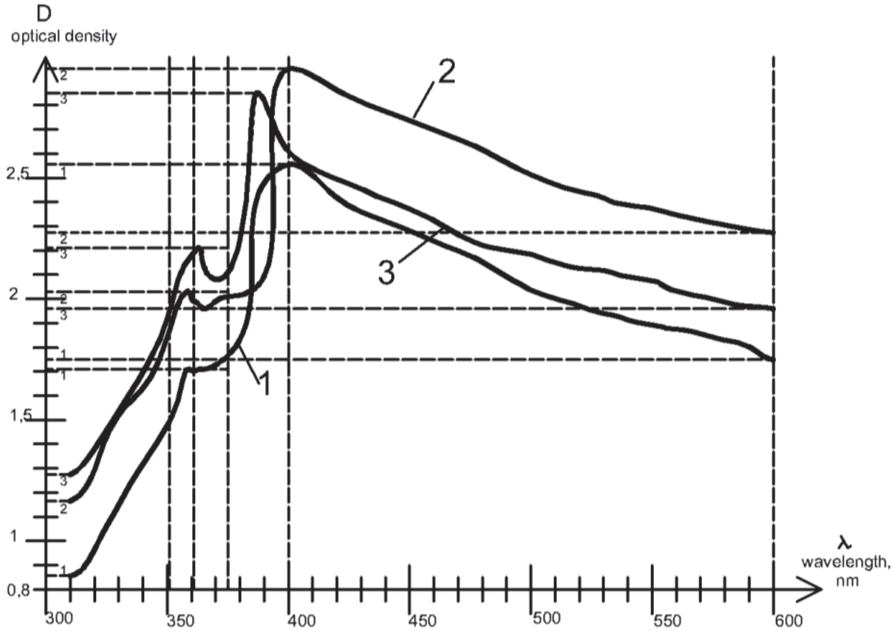

Figure 2. Absorption spectrum of fermented whey samples received after 96 h of anaerobic cultivation (1 is for untreated culture; 2 is for 10 min treatment; 3 is for 20 min treatment).

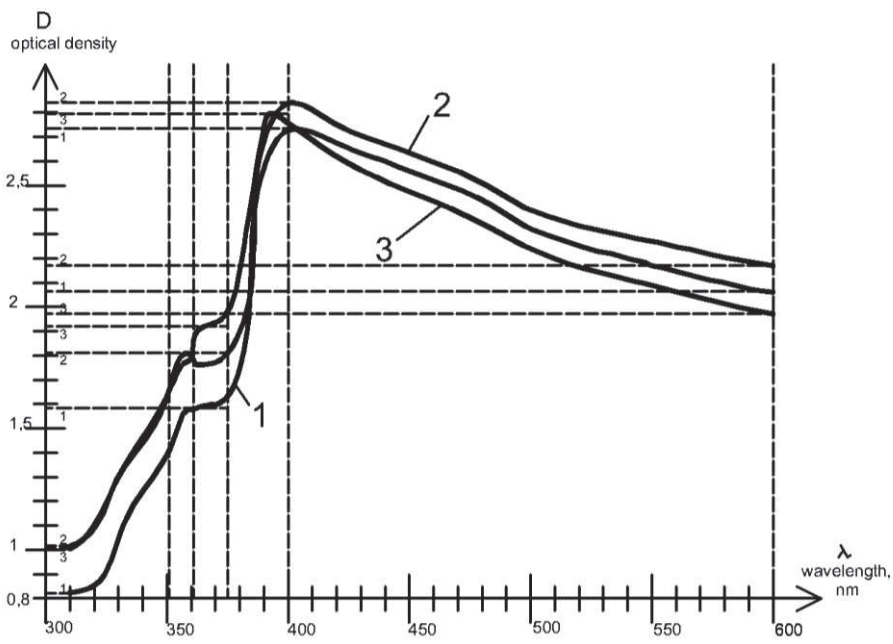

Figure 3. Absorption spectrum of fermented whey samples after 48 h of anaerobic and 48 h of aerobic cultivation (1 is for untreated culture; 2 is for 10 min treatment; 3 is for 20 min treatment).

For comprehensive analysis of corrinoid concentration graphical method based on additivity principle was used. For that purpose was measured difference between levels of 351–375 nm optical density peaks of averaged absorption spectra, received by comparing spectra of multiple probes, and overall optical density of probe averaged spectra without peak (Fig. 4). Results are presented and compared in relative photometrical units (Table 2).

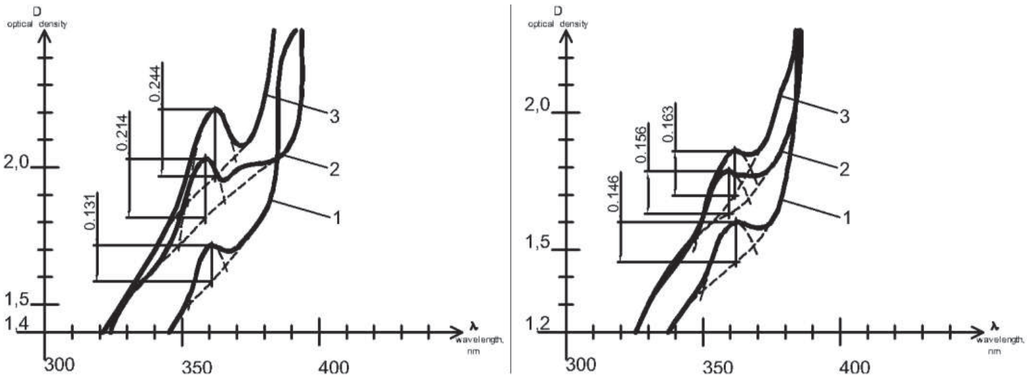

Figure 4. Graphical method for comparation of corrinoid concentration on fragments of absorption spectra of fermented whey samples.

Table 2. Comprehensive analysis of corrinoid concentration in fermented milk whey probes in various treatment conditions

Treatment duration, min	With aeration			Without aeration		
	0	10	20	0	10	20
Absorption maximum, nm	361	359	362	362	360	361
Overall optical density	1.717	2.032	2.211	1.601	1.786	1.860
Optical density without peak	1.585	1.817	1.967	1.455	1.630	1.697
Optical density difference	0.132	0.215	0.244	0.146	0.156	0.163
Content of corrinoids, photometrical units (D·100)	13.2	21.5	24.4	14.6	15.6	16.3
Relative content of corrinoids, %	100.0	162.9	184.8	100.0	106.8	111.6

Results show that formation of corrinoids is minimal without treatment and increase with increasing duration of ultrasound treatment from 10 to 20 min respectively.

Overall affect of the low-intensity ultrasound on metabolism of *Propionibacterium* can be noticed by comparing relations of left and right sides of different spectra and needs further investigation.

CONCLUSION

Received results show that ultrasonic modulation of a metabolism of propionic bacteria is the perspective direction for an intensification of B_{12} vitamin and its analogs obtaining and it can be also used in production of the various fermented food products enriched with B_{12} vitamin.

Received enriched milk whey can be used as an additive for production of the various dairy products enriched with the vitamin. Also there is an interest in the probiotic activity of *Propionibacterium freudenreichii* which can produce bifidogenic compound 1,4-dihydroxy-2-naphthoic acid and stimulates growth of bifidobacteria (Hugenshmidt et al., 2010). This effect can be also used in various fermented dairy products.

Enriched whey can be used for production of other enriched non-dairy like bakery products and confectionery for the purpose of ensuring the intake of daily requirement of B_{12} vitamin.

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