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# EFFECT OF IRON IONS AND 5.6-DIMETHYLBENZIMIDAZOLE ON BIOSYNTHESIS OF CORRINOIDS BY *PROPIONIBACTERIUM SHERMANII* 1 ON CHEESE-WHEY MEDIUM *)

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Effect of different doses of iron ions $FeSO_4 \cdot 7\ H_2O$ and 5,6-dimethylbenzimidazole (DMB) on biosynthesis of corrinoids by *P. shermanii* 1 on cheese-whey medium was studied DMB was added to culture after 3, 6 and 8 days of fermentation. The addition of iron had a positive effect on the corinoid biosynthesis yield. The highest yield increase (about 190%) was achieved through an addition of 5-10 mg $FeSO_4 \cdot 7H_2O$ per liter of culture. In these samples the proportional share of vitamin $B_{12}$ was also the highest. No significant differences in yields of corrinoid, were observed when DMB was added in portions of 10 to 30 mg per 1 liter culture introduced after 3 and 6 days of fermentation. A nearly 2-fold increase of corinoid biosynthesis yield was achieved when adding DMB (10, 16 and 30 mg/l culture) after 8 days of fermentation.

Biosynthesis of corrinoids by propionic acid bacteria can only take place when ions of cobalt and iron as well as 5.6-dimethylbenzimidazole, a precursor of Vit. $B_{12}$, are present in medium. In spite of a relatively high level of iron [1, 18] in chesse-whey it is not utilized by propionic acid bacteria, or the demand for iron is much greater. It was observed that in the biomass of cells obtained from cheese-whey cultures with added cobalt ions only the level of corrinoids was rather limited. Insufficiency of iron in medium results in inhibition of corrinoids and increase of the quantity of porphyrins; the growth of cell biomass is also reduced [10, 4]. Quantity and quality of the synthesized corrinoids also depend on addition of 5.6-dimethylbenzimidazole.

The aim of the present study was to determine the optimal dosage of iron ions and 5.6-dimethylbenzimidazole for biosynthesis of corrinoids

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by *P. shermanii* on cheese-whey medium cultures. The biosynthesis is conducted to obtain biologically highly active forms of Vit. $B_{12}$ preparations to be widely applied in diverse technological processes. Preparations of this type may constitute a potential substitute of crystalline Vit. $B_{12}$.

## MATERIALS AND METHODS

The strain of *Propionibacterium shermanii* 1 *) used in the experiment originated from our own collection. It was cultivated on a yeast-lactate medium according to the method described by Pędziwilk [12]. The acid-rennet whey used for preparation of the experimental medium was from a local dairy plant.

The whey composition: dry matter 5.62% protein (N×6.25) 0.65% (11.66% dry matter), lactose 4.2% (75.1% dry matter), pH 4.3. Fresh whey was filtrated and poured into flasks. 12 mg $CoSO_4 \cdot 7\ H_2O$/1 liter culture was added; pH was brought to 6.8. The experimental doses of iron were introduced at 0, 5, 10, 15 and 30 mg $FeSO_4 \cdot 7\ H_2O$/1 liter medium under sterile condition. The precursor of Vit. $B_{12}$, 5.6-dimethylbenzimidazole (DMB) from Koch-Light Co., England, was introduced in three different doses — 10, 16 and 30 mg/1 after 72, 144 and 192 hours of cultivation. The medium was sterilized in the Koch apparatus for 45 min twice every 24 hrs. The experimental 500 ml cultures were kept in conical 750 ml flasks in relatively anaerobic conditions at 30°C; growth period — 8 to 9 days. During fermentation pH of the medium was kept at 6.8 by means of concentrated $NH_4OH$. When the cultivation was terminated the biomass was centrifuged at 3000 g.

Lactose in the whey and post-fermentation liquids was determined after Hostettler et al. [7] with 3.5-dinitrosalicylic acid. Isolation of corrinoids was carried out according to the method given by Janicki and Pędziwilk [8].

The contents of corrinoids after being converted to the dicyjanoform was measured spectrophometrically at 580 nm. Extinction coefficient: $E^{1\%}_{1cm} = 105$. Absorbancy measurements for ultraviolet and visible light were made with Spekord UV-VIS Zeiss and Spektronom 202 spectrophometer.

Vitamin $B_{12}$ was also determined microbiologically with *Escherichia coli* 113-5 [5]. The electrophoretic separation of the isolated corryne compounds was performed after Holdworth [6]. Volatile fatty acids — acetic and propionic — were determined in a gas chromatograph Willy Giede GCHF 13 including a flame ionization detector [9, 14].

*) The bacteria nomenclature used here follows the 7th edition of Bergey's Manual of Determinative Bacteriology.

## RESULTS AND DISCUSSION

Already in the course of incubation of *P. shermanii* 1 in the whey with cobalt ions added in the form of $CoSO_4 \cdot 7H_2O$ it was possible to observe a strong pink-violet colouring of the culture. The post-centrifuging liquid was also intensively coloured. Traces of corrinoids observed in the post-fermentation liquid could not, therefore, be the cause of the colouring. It is quite likely that the colouring was produced by porphyrins, which undergo synthesis on a relatively large scale when insufficiency of iron ions occurs. Pawełkiewicz and Zodrow [10] reported quite early that *P. shermanii* on a semi-synthetic medium without iron ions produces porphyrins to a level of 94 mg/l culture. Also Giec et al. [4], who studied the effects of protein-free whey on synthesis of corrinoids and porphyhins, observed that when iron and cobalt ions are added the corrinoid synthesis is partially increased. Results given in Table 1 indicate that insufficiency of iron inhibits the growth of propionic acid bacteria, as evidenced by a reduced increment of biomass and by the reduced productivity of corrinoid synthesis. The cultures devoid of iron contained much less corrinoids in comparison with the ones enriched with it. Following the electrophoretic separation of isolated corrinoids the electroneutral fraction corresponding to Vitamin $B_{12}$ in ironless cultures was present in 71% while in those enriched with iron its presence ranged from 90.68% to 96.35%.

The cultures containing iron ions were observed to display a considerable growth of the propagated cell dry matter content. Addition of iron also improves the degres of lactose fermentation. It is, therefore, possible to say that 5 to 15 mg $FeSO_4 \cdot 7H_2O$ per 1 liter medium doses are optimal for biosynthesis of corrinoids and a better utilization of the whey lactose by *P. shermanii* 1. The propionic acid bacteria possess a limited capacity for synthesizing the benzimidazole ring required for building the nucleotide group of Vit. $B_{12}$. According to Perlman et al. [11], propionic acid bacteria are capable of utilizing a number of derivates of benzimidazole from medium. If 5,6-dimethylbenzimidazole is added to medium then propionic bacteria synthesize cobalamin (Vit. $B_{12}$), both in aerobic and unaerobic conditions. No unequivocal information on DMB dosages and at what phase of bacteria incubation they should be added to obtain their full utilization and possibly the highest output of cyjanocobalamin in cheese-whey medium cultures are available. Table 2 presents the results of an experiment on determination of the most adequate DMB dose and time of its introduction to the *P. shermanii* 1 culture on whey medium. The dose used in the experiment was adopted from the routine analyses: 16 mg/1 liter culture as well as 10 and 30 mg/1 liter culture. Every dose was applied at three time intervals — after 72, 144, 192 hrs of cultivation. It was observed that the most decisive factor was

Table 1. Effect of iron on biosynthesis of corynoids by *Propionibacterium shermanii* 1.*)

| Dose of $FeSO_4 \cdot 7H_2O$ mg/l culture | Dry mass of cells biomass g | Quantity of**) unused lactose % | Total quantity of microbiologically determined corynoids from *E. coli* 113—3 | | Total quantity of physico-chemically determined corynoids | | Composition of corynoid fractions % | | Fatty acids, mg/ml culture | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | μg/ml culture | μg/g dry mass | μg/ml culture | μg/g dry mass | electro-neutral fraction ($B_{12}$) | electro-positive fraction | acetic acid | propionic acid |
| 0 | 5.45 | 0.98 | 7.4 | 678 | 5.63 | 516 | 71.00 | 29.00 | 5.5 | 15.9 |
| 5 | 6.56 | 0.23 | 14.0 | 1067 | 8.17 | 623 | 95.08 | 4.92 | 6.3 | 18.1 |
| 10 | 6.11 | 0.24 | 13.0 | 1063 | 6.50 | 531 | 96.35 | 3.65 | 6.9 | 17.7 |
| 15 | 6.55 | 0.25 | 11.5 | 877 | 8.66 | 661 | 91.73 | 8.27 | 6.1 | 17.1 |
| 30 | 6.46 | 0.23 | 11.5 | 889 | 8.97 | 693 | 90.68 | 9.32 | 5.9 | 16.3 |

*) The culture was kept on a whey medium added with $COSO_4 \cdot 7H_2O$ (12 mg/l culture) and DMB (16 mg/l culture). The 500 ml cultures were grown for 8 days at 30°C

**) The whey lactose contents was 3.9%

Table 2. Biosynthesis of corynoids by *Propionibacterium shermanii* 1 as dependent on different doses DMB and time of their introduction to cultures*)

| DMB added to medium mg/1 l culture | Time of DMB introduction counted from the start of culture, hrs | Culture dry mass g | Quantity of**) unused lactose % | Total quantity of microbiologically determined corynoids from *E. coli* 113—3 | | Percentage of corynoid fractions | | Volatile fatty acids, mg/ml culture | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | µg/ml culture | µg/g dry mass | electro-neutral ($B_{12}$) | electro-positive | acetic acid | propionic acid |
| 10 | 72 | 6.28 | 0.08 | 5.8 | 420 | 94.76 | 5.24 | 5.7 | 16.3 |
| 16 | 72 | 6.35 | 0.13 | 5.6 | 412 | 76.85 | 23.15 | 6.7 | 19.4 |
| 30 | 72 | 6.60 | 0.09 | 6.1 | 537 | 88.26 | 11.74 | 6.3 | 18.6 |
| 10 | 144 | 6.66 | 0.11 | 8.0 | 581 | 83.56 | 16.44 | 6.5 | 19.4 |
| 16 | 144 | 6.73 | 0.10 | 7.95 | 573 | 83.36 | 16.64 | 6.7 | 19.2 |
| 30 | 144 | 6.36 | 0.17 | 7.95 | 616 | 64.04 | 35.96 | 7.1 | 19.8 |
| 10 | 192 | 6.23 | 0.11 | 16.40 | 1254 | 65.81 | 34.19 | 6.7 | 20.0 |
| 16 | 192 | 6.36 | 0.15 | 12.60 | 918 | 72.67 | 27.33 | 6.7 | 19.6 |
| 30 | 192 | 6.27 | 0.11 | 16.80 | 1246 | 74.65 | 25.35 | 6.7 | 19.8 |

*) The 500 ml culture were grown on a whey medium for 9 days at 30°C

**) The whey lactose contents was 3.9%

not the quantity of the precursor (between 10 and 30 mg/1 liter culture) but the time it was put into the fermentation medium. This had a primary effect on quantities of synthesized corrinoids.

As Table 2 indicates, the highest volume of corrinoids was obtained when DMB was added after 192 hours of cultivation, that is, 24 hours before the end of fermentation. It must also be mentioned that neither the quantity of the precursor not the time of its infusion had any influence on the degree of fermentation of lactose in medium and on production of propionic acid in the analyzed cultures of *P. schermanii* 1. It is necessary to stress, however, that there are certain empirical (unpublished) data indicating the possibility of substantial shortening of propionic bacteria incubation period retaining similar levels of corrinoid yields. In this case it is essential to introduce precursor of Vit. $B_{12}$ (DMB) into the medium 24 hrs before the end of incubation.

## CONCLUSIONS

1. Insufficiency of iron inhibits the growth of propionic acid bacteria as evidenced by reduced increments of biomass and lower yield of the synthesised corrinoids.

2. 5 to 15 mg $FeSO_4 \cdot 7H_2O$/1 liter medium is the optimum dose of iron ions for the synthesis of corrinoids and better utilization of whey lactose by *P. shermanii*.

3. The yield of corrinoids synthesized by *P. shermanii* 1 on cheese-whey medium does not depend on dosage of 5,6-dimethylbenzimidazole but on introduction time of precursor into cultures. Maximum synthesis of corrinoids was obtained when DMB was added 24 hrs before the end of fermentation.

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WPŁYW ŻELAZA I 5,6-DWUMETYLOBENZIMIDAZOLU NA BIOSYNTEZĘ KORYNOIDÓW PRZEZ *PROPIONIBACTERIUM SHERMANII* 1 NA PODŁOŻU SERWATKOWYM

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Streszczenie

Badano wpływ różnych dawek jonów żelaza ($FeSO_4 \cdot 7H_2O$) oraz 5,6-dwumetylobenzimidazolu (DMB) na wydajność biosyntezy korynoidów przez *Propionibacterium shermanii* 1 na podłożu serwatkowym; DMB wprowadzano do hodowli po 3, 6 i 8 dniach fermentacji. Dodatek żelaza wpływał korzystnie na wydajność biosyntezy korynoidów. Najwyższy wzrost wydajności (ok. 190%) uzyskiwano przy dodatku 5-10 mg $FeSO_4 \cdot 7H_2O$ na litr hodowli. W próbach tych był również najwyższy procentowy udział witaminy $B_{12}$.

Nie stwierdzono znaczniejszych różnic w wydajności korynoidów przy dodatku DMB w dawkach 10-30 mg na litr hodowli wprowadzonego po 3 i 6 dniach fermentacji. Natomiast przeszło 2-krotny wzrost wydajności biosyntezy korynoidów uzyskiwano, gdy DMB (10, 16 i 30 mg/l hodowli) wprowadzono po 8 dniach fermentacji.