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OPTIMALIZATION OF WHEY MEDIUM COMPOSITION FOR BIOSYNTHESIS OF VITAMIN B_{12} BY PROPIONIBACTERIA

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Key words: propionibacteria, whey, Vitamin B_{12}.

The main component of the medium for biosynthesis of vitamin B_{12} was deproteinized, whey supplemented with a cheap source of nitrogen and mineral salt: ammonium lactate, molasses and rapeseed meal extract, in addition of kefir whey was used as a factor stimulating the activity of propionic acid bacteria. Using a mathematical method for optimalization the composition for high yield of biosynthesis more than 30 mg corrinoids per litre medium has been established.

INTRODUCTION

In the cultivation of proprionibacteria for biosynthesis of vitamin B_{12} semi-synthetic and natural media can be used. The yield obtained on these media range from 3 to 16 mg corrinoids per litre depending on the strain used, medium composition and the conditions of fermentation. Various by-products or wastes of the agricultural and food industry are more and more frequently used as main components of the medium e.g. potato stillage, sugar beet molasses [11], whey [4, 9, 12, 14].

Leviton and Hargrove [9] who applied a two-stage processes of lactic and propionic fermentations obtained good results in the intensification of biosynthesis. A stimulating effect of the filtrate from the culture of lactic bacteria on the activity of propionibacterium strains was found also by Lutskova [10] and Czarnocka-Roczniakowa [5, 6]. In recent years attention has been paid to employing mixed cultures of propionibacteria with yeast and lactic fermentation bacteria (e.g, with kefir microflora), micrococci and other microorganisms [5, 6].

This paper deals with futher attempts to intensify biosynthesis of vitamin B_{12} by selection of cheap waste components of the medium (e.g.

deproteinized whey), exploitation of stimulating effect of kefir microflora on biochemical activity of propionibacteria and by improving (optimalization) of the medium composition.

EXPERIMENTAL

Strains. A strain T 112 of *Propionibacterium petersonii* from the collection of the Institute of Food Engineering and Biotechnology, The Olsztyn Academy of Agriculture and Technology was used. The strain was selected from among 30 strains belonging to 14 species of *Propionibacterium* [1]. It was grown in a lactate medium under relatively anaerobic conditions at 30°C for 3 days. Inoculum was prepared on whey supplemented with 0.5% yeast extract and 0.15% NaCl. Whey medium was inoculated with a three-day culture in the amount of 5% (v/v), incubated at 30°C for 3 days adjusting every day pH to some 7 with a saturated solution of Na_2CO_3.

Medium components. A basic medium component was deproteinized whey as a by-product in the production of cottage cheese. Whey proteins were precipitated with $CaCl_2$. Deproteinized whey was sterilized at 112°C for 5 minutes.

Proteinous extract from rapeseed meal, sugar beet molasses, ammonium lactate and kefir whey (whey fermented using kefir grains) were used as supplementary components.

Extract from rapeseed oil meal was used mainly as a source of simple protein compounds and amino acids substituting whey proteins. The extract was prepared in the manner described by Pokorny [13]. Accordingly, the rapeseed meal was trated with a ten-fold amount of the solvent (0.3% aqueous solution of NaOH), shaken for 40 minutes, filtrated through linen, centrifugated, and then sterilized by using Berkefeld's filters. The obtained filtrate was transferred to sterilized flasks, in portions for single use, and cold stored at — 18°C.

Molasses was used chiefly as a source of carbon and mineral salts; ammonium lactate — as a source of nitrogen and carbon. These compounds were used in the form of 50% aqueous solutions and sterilized at 121°C for 20 minutes.

Kefir whey constituted a source of growth elements and stimulators of biosynthesis. It was obtained by culturing and separating kefir grains. Accordingly, the deproteinized whey was inoculated with kefir grains in the proportion of 5%, at 25°C for 24 hrs. After grains were separated, kefir whey was freezed in portions for single use and cold stored at — 18°C.

Medium was prepared just before inoculation by adding sterily supplementary components, to the whey, and $CoCl_2$ in the proportion of 5 mg/l medium.

Culture conditions and methods of determination. Cultures were grown in 100 ml flasks containing 75 ml medium, which ensured relatively anaerobic conditions. The prepared medium was inoculated with 10% (v/v) of the culture grown on whey and incubated at 28 to 30°C for 11 days at pH 6.2 to 7.2. To adjust pH sterile saturated Na_2CO_3 solution was used.

Corrinoids (vitamin B_{12} and its analogues) were determined by the plate method employing cylinder technique and *E. coli* mutant plate test [7]. Corrinoid-protein complexes were cracked by cyanolysis [8]. Dilutions of the samples and standard solutions of vitamin B_{12} and the calculation of results were made as suggested by Borensztajn and Kuryłowicz [2]. Percentage share of vitamin B_{12} in biosynthesis products was determined using bioautography after chromatographic resolution of corrinoids [15].

RESULTS AND DISCUSSION

Optimalization of medium composition *). In this study an experimental design "response-surfaces" given by Box and Hunter [3] was applied. Four variable factors (supplementary medium components) were studied: proteinous extract from rapeseed oil meal, molasses, ammonium lactate and kefir whey. Levels applied and symbols for the components are given in Table 1.

Table 1. Parameters of variables studies in part I of optimalization

Parameters	Symbols	Levels		
		minimum −1 %	maximum 1 %	medium 0 %
Rapeseed oil meal extract	A	0	20.0	10.0
Ammonium lactate	B	0.5	2.5	1.5
Molasses	C	0.5	2.0	1.25
Fermented whey	D	0	20.0	10.0

Optimalization was performed in two series. The programme of the first series comprised 16 (24) unrepeated combinations of factors present at minimum and maximum levels and 8 parallel central tests in which all the parameters were present at mean levels (arithmetical means of minimum and maximum levels). Combinations of media and the results obtained are presented in Table 2.

*) Programing of experiments and mathematical interpretation of results were performed in the Institute for Applied Mathematics and Mathematical Statistics, Warsaw University Agriculture under the guidance of Professor Zygmunt Nawrocki

Table 2. Optimalization of whey medium compositon; results of the first series of experimental

Medium No.	Parameter combinations	Components of media, %				Corrinoids mg/l
		rapesed oil meal extract	ammonium lactate	molasses	kefir whey	
		A	B	C	D	
1	$A_{-1}B_{-1}C_{-1}D_{-1}$	0	0.5	0.5	0	0.1
2	$A_{-1}B_{-1}C_{-1}D_{1}$	0	0.5	0.5	20	18.2
3	$A_{-1}B_{-1}C_{1}D_{-1}$	0	0.5	2.0	0	0.041
4	$A_{-1}B_{-1}C_{1}D_{1}$	0	0.5	2.0	20	12.52
5	$A_{-1}B_{1}C_{-1}D_{-1}$	0	2.5	0.5	0	11.2
6	$A_{-1}B_{1}C_{-1}D_{1}$	0	2.5	0.5	20	16.32
7	$A_{-1}B_{1}C_{1}D_{-1}$	0	2.5	2.0	0	0.04
8	$A_{-1}B_{1}C_{1}D_{1}$	0	2.5	2.0	20	0.00775
9	$A_{1}B_{-1}C_{-1}D_{-1}$	20	0.5	0.5	0	1.02
10	$A_{1}B_{-1}C_{-1}D_{1}$	20	0.5	0.5	20	0.034
11	$A_{1}B_{-1}C_{1}D_{-1}$	20	0.5	2.0	0	0.114
12	$A_{1}B_{-1}C_{1}D_{1}$	20	0.5	2.0	20	0.048
13	$A_{1}B_{1}C_{-1}D_{-1}$	20	2.5	0.5	0	0.052
14	$A_{1}B_{1}C_{-1}D_{1}$	20	2.5	0.5	20	0.33
15	$A_{1}B_{1}C_{1}D_{-1}$	20	2.5	2.0	0	0.22
16	$A_{1}B_{1}C_{1}D_{1}$	20	2.5	2.0	20	0.041
	Central combinations					
1	$A_{0}B_{0}C_{0}D_{0}$	10	1.5	1.25	10	mean of eight determinations 3.5
2	"	"	"	"	"	
3	"	"	"	"	"	
4	"	"	"	"	"	
5	"	"	"	"	"	
6	"	"	"	"	"	
7	"	"	"	"	"	
8	"	"	"	"	"	

The corrinoid yield in the studied series of media varied from 0.008 to 18.2 mg per 1. The highest yield of biosynthesis was found in medium No. 2 that contained 20% kefir whey (maximum level), 0.5% molasses and 0.5% ammonium lactate (minimum levels) High yields ranging from 11.2 to 16.3 mg/l were also obtained in media nos. 4, 5 and 6. The amount of corrinoids in the other media combinations was very small and did not exceed 1 mg/1. In the parallel "central" tests 3.5 mg corrinoids per 1, on an average, were obtained. A high scatter of results obtained in the group of 16 unrepeated basic combinations pointed to the need of narrowing minimum and maximum levels of certain constituents. That is why the optimalization called for programming of the second degree. A starting

point in planning the second series of experiments was medium na 2 in which the highest biosynthesis yield was obtained.

The plan for further optimalization included 24 unrepeated combinations of media and 7 parallel "central" tests. The applied levels of parameters are given in Table 3. The composition of respective media and the yield of biosynthesis obtained in them are presented in Table 4. In the second series of experiments a very high yield of corrinoids was obtained; it amounted to 38.5 mg per litre culture. This yield was obtained in medium no 17 (called additional combinations) that consisted of 0.75% molasses and 20% kefir whey. Approximate yields were also obtained in the other two media from the group of combinations (37.1 mg/l in no. 21 and 36.6 mg/l in medium No. 24). In the group of "central" combinations an average yield was 33.8 mg/l, varying slightly from 28.8 to 37.8 mg/l. It must be emphasized that in the group of "central" and "additional" combinations similar yields were obtained. The lowest and most differentiated yields were noted in so-called basic combinations (nos. 1-16). In this group the highest amount of corrinoids, 25.28 mg/l, was found in the culture on medium No. 16, where all the supplementary components were at maximum levels.

Table 3. Parameters of variables studied in part II of the optimalization

Parameters	Symbols	Levels, %				
		-2	-1	0	1	2
Rapeseed oil meal extract	A	0	0.25	0.5	0.75	1.0
Ammonium lactate	B	0.25	0.5	0.75	1.0	1.25
Molasses	C	0	0.25	0.5	0.75	1.0
Fermented whey	D	10.0	15.0	20.0	25.0	30.0

The presented results were a basis to establish optimum levels of the supplementary components of the whey medium designed for the biosynthesis of vitamin B_{12} by Propionibacteria. The following levels were determined by digital computer:

rapeseed oil meal extract	0.6012%
ammonium lactate	0.7426%
molasses	0.4071%
kefir whey	21.4594%

The maximum amount of corrinoids possible to obtain in the whey medium containing the above proportions of components is, according to the statistical method, 34.8 mg/l. In repeated tests with the various portions of whey similar results were obtained and whey varied from 32 to 40 mg/l. In single cases higher corrinoid levels were found that exceded 50 mg/l, which seems to be an exceptional phenomenon requiring further

Table 4. Optimilization of whey medium composition; results of the second series of experiments

Medium No.	Parameter combinations	Components of media, %				Corrinoid mg/l
		rapeseed oil meal extract	ammonium lactate	molasses	kefir whey	
		A	B	C	D	
1a	$A_{-1}B_{-1}C_{-1}D_{-1}$	0.25	0.5	0.25	15	12.20
2a	$A_1B_{-1}C_{-1}D_1$	0.25	0.5	0.25	25	10.50
3a	$A_{-1}B_{-1}C_1D_{-1}$	0.25	0.5	0.75	15	9.60
4a	$A_{-1}B_{-1}C_1D_1$	0.25	0.5	0.75	25	11.40
5a	$A_{-1}B_1C_{-1}D_{-1}$	0.25	1.0	0.25	15	12.96
6a	$A_{-1}B_1C_{-1}D_1$	0.25	1.0	0.25	25	14.98
7a	$A_{-1}B_1C_1D_{-1}$	0.25	1.0	0.75	15	9.20
8a	$A_{-1}B_1C_1D_1$	0.25	1.0	0.75	25	9.60
9a	$A_1B_{-1}C_{-1}D_{-1}$	0.75	0.5	0.25	15	23.04
10a	$A_1B_{-1}C_{-1}D_1$	0.75	0.5	0.25	25	24.40
11a	$A_1B_{-1}C_1D_{-1}$	0.75	0.5	0.75	15	13.56
12a	$A_1B_{-1}C_1D_1$	0.75	0.5	0.75	25	14.72
13a	$A_1B_1C_{-1}D_{-1}$	0.75	0.0	0.25	15	18.20
14a	$A_1B_1C_{-1}D_1$	0.75	1.0	0.25	25	22.05
15a	$A_1B_1C_1D_{-1}$	0.75	1.0	0.75	15	9.00
16a	$A_1B_1C_1D_1$	0.75	1.0	0.75	25	25.28
17b	$A_{-2}B_0C_0D_0$	0.0	0.75	0.5	20	38.52
18b	$A_2B_0C_0D_0$	1.0	0.75	0.5	20	28.40
19b	$A_0B_{-2}C_0D_0$	0.5	0.25	0.5	20	32.40
20b	$A_0B_2C_0D_0$	0.5	1.25	0.5	20	27.30
21b	$A_0B_0C_{-2}D_0$	0.5	0.75	0.0	20	37.12
22b	$A_0B_0C_2D_0$	0.5	0.75	1.0	20	23.40
23b	$A_0B_0C_0D_{-2}$	0.5	0.75	0.5	10	31.92
24b	$A_0B_0C_0D_2$	0.5	0.75	0.5	30	36.60
		Central combinations				
1c	$A_0B_0C_0D_0$	0.5	0.75	0.5	30	37.80
2c	,,	,,	,,	,,	,,	32.40
3c	,,	,,	,,	,,	,,	37.44
4c	,,	,,	,,	,,	,,	36.04
5c	,,	,,	,,	,,	,,	28.08
6c	,,	,,	,,	,,	,,	28.16
7c	,,	,,	,,	,,	,,	36.80

studies. On the basis of bioautography it was shown that vitamin B_{12} synthetized in the elaborated medium produces on an average, 35% total corrinoids.

On account of using natural and various medium components and obtained high yield, complementary studies were made to determine the medium effect on the results of analyses.

The amount of corrinoids found in the centrifugated and washed biomass was some 10% lower than that obtained in the direct culture. The medium that remained after the biomass was centrifugated and the water after its washing had a neglegible effect on growth promotion of the strain (trace zone). This strain responded similary to the complete medium and whey. Complementary studies on growth promotion in the test strain by pure methionine and its various combinations with the medium showed that this amino acid had no bearing on the results, which is in accordance with the findings of other authors [7, 12].

A comparision of the presented results with the yield of corrinoids obtained by other authors may raise doubts on account of employing different analytic methods. However, taking into consideration only those works where whey was used in corrinoid determinations by the plate method, the proposed medium for the biosynthesis of vitamin B_{12} must be considered highly useful. It gives a high yield and, besides, unlike the whey media used hitherto [10, 12] makes it possible to utilize whey waste that remains after precipitating proteins so valuable in human nutrition.

The cited papers of other authors and also our results show that there are great possibilities of intensifying the biosynthesis of vitamin B_{12}. No doubt, an increased activity of propionibacteria is obtained in mixed cultures with lactic bacteria and yeast. Moreover, the use of the mathematical method for medium optimalization offers high possibilities to intensify biosynthesis. The presented results entirely confirm the usefulness of that method shown earlier by Majchrzak and al. [11] in the intensification of microbiological processes.

The results (2 series of experiments) show that the quantitative proportion of the particular components of a medium has a decisive effect on yield of the studied process.

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OPTYMALIZACJA SKŁADU POŻYWKI SERWATKOWEJ DO BIOSYNTEZY WITAMINY B_{12} PRZEZ BAKTERIE PROPIONOWE

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Streszczenie

Badania obejmowały ustalenie optymalnego składu pożywki serwatkowej dla bakterii propionowych, przeznaczonej do biosyntezy witaminy B_{12}. Składnikami pożywki były głównie produkty uboczne przemysłu spożywczego: odbiałczona serwatka, melasa buraczana, śruta rzepakowa i mleczan amonu. Stosowano ponadto dodatek „serwatki kefirowej" zawierającej żywe komórki bakterii mlekowych i drożdży, co wpłynęło stymulująco na biosyntezę korynoidów. Posługując się matematyczną metodą optymalizacji ustalono następujący skład pożywki produkcyjnej: wyciąg ze śruty rzepakowej — 0,60%, mleczan amonu — 0,74%, melasa — 0,41%, serwatka kefirowa — 21,46%. Wyliczona wydajność możliwa do uzyskania na tej pożywce wynosi 34,8 mg korynoidów w litrze hodowli. Praktycznie w wielokrotnie prowadzonych hodowlach otrzymywano wydajności zbliżone, a w sporadycznych przypadkach wyższe.