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ORIGINAL ARTICLE

Production of polyhydroxyalkanoates from cheese whey employing *Bacillus megaterium* CCM 2037

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Abstract Poly(3-hydroxybutyrate) (PHB) is a polyester belonging to the family of polyhydroxyalkanoates, which accumulate in a wide variety of bacterial strains. PHB appears to be a biodegradable alternative to traditional petrochemical polymers such as polypropylene and polyethylene. In this work, we tested direct conversion of cheap waste cheese whey into PHB employing the bacterial strain *Bacillus megaterium* CCM 2037. Optimization of medium composition improved PHB yields about 50 fold (biomass and PHB yields 2.82 and 1.05 g l^{-1}, respectively) as compared to none-optimized whey. Furthermore, PHB yields were improved by about 40% by introducing 1% ethanol into the medium at the beginning of the stationary phase of growth (biomass 2.87 g l^{-1}, PHB 1.48 g l^{-1}). According to the results of experiments carried out in Erlenmeyer flasks, *B. megaterium* CCM 2037 can be considered a candidate for direct PHB production from waste cheese whey. Nevertheless, experiments in laboratory-scale and semi-productive fermentors are needed to test performance under high cell density cultivation.

Keywords *Bacillus megaterium* · Polyhydroxyalkanoate · Poly(3-hydroxybutyrate) · Cheese whey · Exogenous stress

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Introduction

Polyhydroxyalkanoates (PHA) are biopolymers produced and accumulated in the form of intracellular granules by a number of bacterial strains. Of the large PHA family, a homopolymer of 3-hydroxybutyrate, poly(3-hydroxybutyrate) (PHB), is the most widespread in nature and the best characterised PHA compound. PHB has aroused much interest in industry and research thanks to its biocompatible, biodegradable, thermoplastic and piezoelectric properties. Nowadays, PHB is considered to act as an alternative to common plastics derived from petrol (Kadouri et al. 2005).

The high production cost is one of the main factors preventing wider use of PHB. Analysis and economic evaluation of bacterial PHB production has suggested that the cost of substrate (mainly carbon source) contributes most (up to 50%) to the overall production costs (Choi and Lee 1997). Therefore, PHB could be produced more economically using cheap waste substrates.

Cheese whey is the major by-product from the manufacture of cheese and casein, representing 80–90% of the volume of transformed milk. Cheese production in the European Union produces a total of approximately 40,462,000 tons of whey per year. Most of this whey is used for production of lactose and in animal feed, but an annual amount of 13,462,000 tons of whey per year, containing about 619,250 tons of

lactose, constitutes a surplus product (Koller et al. 2008). For this reason, cheese whey represents a promising substrate for cheap production of PHB in large amounts.

Although biotechnological production of PHB from different sugars via condensation of acetyl-CoA units stemming from hexose catabolism is well described (Kessler and Wilholt 1999), only a limited number of bacterial strains directly convert lactose into PHB. A few reports are available on PHB production from lactose and whey by recombinant *Escherichia coli* (Wong and Lee 1998; Ahn et al. 2000). *Methylobacterium* sp. ZP24 (Yellore and Desai 1998; Nath et al. 2007) and the thermophillic bacterium *Thermus thermophilus* HB8 (Pantazaki et al. 2009) are also able to utilize whey lactose for PHA biosynthesis. Recently, Pandian et al. (2010) reported PHA production from a mixture of rice bran, non-specified diary waste and sea water employing a Gram-positive bacterium isolated from brackish water. Based on morphological and physiological properties and the nucleotide sequence of its 16S rRNA, it was suggested that the isolate was closely related to *Bacillus megaterium* (Pandian et al. 2010). *Pseudomonas hydrogenovora* was also used for PHA production using cheese whey, but this bacterial strain is not able to utilize lactose directly, requiring hydrolysis of whey lactose prior to cultivation (Koller et al. 2008).

We have previously reported that *Bacillus megaterium* CCM 2037 is able to utilize lactose and accumulate PHB (Obruca et al. 2008). In this study, we focused on optimization of the whey medium in order to enhance PHB and biomass yields. Moreover, application of exogenous stress was studied as a potential strategy to enhance PHB biosynthesis in cells.

Materials and methods

Microorganism, media and growth conditions

Bacillus megaterium CCM 2037 was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic).

CCM Bacillus medium, consisting of peptone 5 g l^{-1}, yeast extract 3 g l^{-1}, $MnSO_4$ 0.01 g l^{-1} and agar 20 g l^{-1}, was used for maintaining the culture. Mineral medium (MM) was used for inoculum preparation. MM contained lactose 8 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, Na_2HPO_4 2.5 g l^{-1}, KH_2PO_4 2.5 g l^{-1}, $MgSO_4$ 0.2 g l^{-1} and $MnSO_4$ 0.01 g l^{-1}. The initial pH of the medium was adjusted to 7.0. Inoculum was developed in 250-mL Erlenmeyer flasks containing 100 ml medium. MM medium was inoculated with bacterial culture and cultivated at an agitation speed of 150 rpm, at 30°C for 24 h. Subsequently, 5 ml of the culture was inoculated into a 250-ml Erlenmeyer flask containing 100 ml whey medium.

Whey was obtained from the cheese manufacturer Pribina Pribyslav (Pribyslav, Czech Republic). The whole whey was treated in order to remove excess proteins. Whey was acidified to pH 4.0 with 1.0 M H_2SO_4 and heated (100°C for 20 min), cooled and centrifuged at 8,000 rpm for 5 min. The treated whey was used in experiments after adjusting the pH to 7.0 with 1.0 M NaOH and MM medium components (except lactose), and 0.1 g l^{-1} yeast extract were added at the concentration used in MM unless otherwise indicated.

Analytical methods

Cell growth was monitored by measuring the absorbance of culture broth at 630 nm on a Helios α instrument (Unicam, Leeds, UK) after suitable dilution with distilled water. Cell biomass was calculated using a calibration curve at $A_{630\ nm}$ and dry cell mass. For dry cell mass determination, harvested cells (centrifugation: 8,000 rpm, 10 min) were dried at 105°C to constant weight. The supernatant obtained by centrifugation of the culture broth at 8,000 rpm for 10 min was used for analysis of residual lactose by the Somogyi-Nelson method (Deng and Tabatabai 1994).

The PHB content in dried cells was determined by gas chromatography (Finnigan Trace GC Ultra, Austin, TX, column DB-WAX 30 m by 0.25 mm) with mass spectrometry detection (Finnigan Trace DSQ) according to Brandl et al. (1988).

Analysis of treated whey

Concentration of dry matter was estimated after drying (105°C) 10 ml whey to constant weight. Ash content was determined as the weight of solids after incubation of 2 ml whey at 800°C for 2 h. The phosphorus content was measured by the molybdenum blue colorimetric method ($A_{610\ nm}$) using whey ash. The concentration of soluble protein was performed using the biuret method with bovine serum albumin as a standard. Whey sugar content was estimated by HPLC (pump LCP 4020, thermostat LCO 101, degasser DG-1210, refractometric detector RIDK 102; Ecom, Czech Republic) with a ZOBRAX NH_2 column (150 cm×4,6 mm, 5 μm; Chromservis, Czech Republic), chromatographic conditions were: 25°C; acetonitrile: water 75:25; mobile phase flow 1.0 ml/min.

Media optimization using Placket-Burman experimental design

The dilution of whey (carbon source), and concentrations of nitrogen source [$(NH_4)_2SO_4$], mineral salts and yeast

Table 1 Range of factors studied in the Placket-Burman experiment

Factor	Name	Level	
		1	−1
A	Whey	Not-diluted (Lactose 40 g l^{-1})	Diluted (Lactose 40 g l^{-1})
B	$(NH4)_2SO_4$	5 g l^{-1}	1 g l^{-1}
C	Na_2HPO_4; KH_2PO_4 (1:1)	5 g l^{-1}	1 g l^{-1}
D	$MgSO_4$	0.2 g l^{-1}	0.04 g l^{-1}
E	Yeast autolysate	1 g l^{-1}	0.1 g l^{-1}

extract were tested using a Placket-Burman experimental design. Each parameter was tested at two levels, high (+1) and low (−1) (Table 1). A design of 12 experiments was formulated for five factors using Minitab software. The experiments were performed in 250-ml Erlenmeyer flasks containing 100 ml whey medium at 150 rpm, 30°C for 50 h in duplicate. Response was measured in terms of (1) biomass production, (2) PHB accumulation in cells (% w/w), and (3) PHB yields.

Results and discussion

Analysis of whey composition

Used cheese whey contains about 6.8% of dry matter, composed mainly of sugars and salts. Moreover, some soluble proteins are still present in whey even after the treatment. In spite of the high concentration of salts (expressed as ash), which could result in high osmotic pressure influencing bacterial growth negatively, the tested waste cheese whey seems to be a promising complex substrate for PHB production. Whey sugars (lactose and glucose; galactose was not detected) could be utilized by the bacterial culture as carbon sources, soluble proteins could serve as a complex carbon and nitrogen sources, and a low concentration of potential phosphorus sources was also observed (Table 2). In addition, the whey could be expected to contain some minor components such as free amino acids and vitamins (not analyzed), which are likely to support bacterial growth.

Table 2 Composition of cheese whey. Results given are average ± standard deviation; each analysis was performed in triplicate

Substance	Concentration
Water	93%
Dry matter	68 g l^{-1}
Ash	27.10±0.30 g l^{-1}
Lactose	39.60±0.45 g l^{-1}
Glucose	0.35±0.02 g l^{-1}
PO_4^{3-}	63.0±1.2 mg l^{-1}
Soluble proteins	2.0±0.1 g l^{-1}

Supplementation of whey by salts

In order to test whether whey itself contains all necessary elements for bacterial growth, a culture of *Bacillus megaterium* was inoculated into whey with and without added salts according to MM (Fig. 1). Biomass and PHB yields were analyzed after 48 h.

In whey medium without added salts, bacterial growth and PHB production were rather low. Conversely, addition of salts promoted the growth of bacterial culture and also improved PHB production (almost 10 times). An explanation could be that, despite excess salts, whey itself lacks nitrogen, phosphorus, magnesium and manganese. Therefore, these components must be added in the form of mineral salts.

Optimization of whey supplementation

For multivariable processes such as biotechnological systems, in which numerous potentially influential factors are involved, it is not always obvious to determine which are the most important. Hence, it is

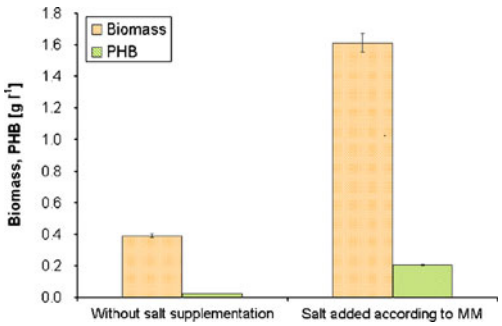

Fig. 1 Biomass and poly(3-hydroxybutyrate) (PHB) yields by *Bacillus megaterium* on whey with and without addition of salts

Table 3 Experimental design and responses of Placket-Burman study

	Whey	$(NH_4)_2SO_4$ [g l^{-1}]	PO_4^{2-} [g l^{-1}]	$MgSO_4$ [g l^{-1}]	Yeast Extract [g l^{-1}]	Biomass [g l^{-1}]	PHB [%]	PHB [g l^{-1}]
1	None-dilution	1.0	5.0	0.04	0.10	0.90	2.06	0.02
2	None-dilution	5.0	1.0	0.20	0.10	0.76	3.20	0.02
3	Diluted	5.0	5.0	0.04	1.00	2.56	16.26	0.42
4	None-dilution	1.0	5.0	0.20	0.10	0.92	1.90	0.02
5	None-dilution	5.0	1.0	0.20	1.00	0.77	5.08	0.04
6	None-dilution	5.0	5.0	0.04	1.00	0.75	1.95	0.01
7	Diluted	5.0	5.0	0.20	0.10	2.40	27.36	0.66
8	Diluted	1.0	5.0	0.20	1.00	2.02	12.72	0.26
9	Diluted	1.0	1.0	0.20	1.00	1.95	13.51	0.26
10	None-dilution	1.0	1.0	0.04	1.00	1.01	2.17	0.02
11	Diluted	5.0	1.0	0.04	0.10	2.47	35.48	0.87
12	Diluted	1.0	1.0	0.04	0.10	2.99	35.49	1.06

necessary to submit the process to an initial screening design prior to optimization. Plackett–Burman methodology could be a tool for this initial screening, because it makes it possible to determine the influence of various factors with only a small number of trials (Khanna and Srivastava 2005).

We used Placket-Burman methodology to optimize the composition of whey medium for PHB production. Five factors were selected for optimization, and each factor was tested at two levels, high and low (Table 1). PHB and biomass yields were analyzed after 50 h. The experiment was designed by Minitab software.

According to the results of the Placket-Burman study (results summarized in Tables 3, 4), the concentrations of $(NH_4)_2SO_4$, PO_4^{3-} and $MgSO_4$ are not statistically important either for biomass or for PHB production ($P>0.05$). Nevertheless, in previous experiments addition of salts had strongly enhanced biomass and PHB production; therefore, in subsequent experiments salts were added at the concentration used as the lower level (−1) in the Placket-Burman analysis. The addition of yeast extract, which was tested as a potential source of vitamins, amino acids etc., had a statistically significant negative impact on PHB production. This is probably due to the fact that yeast extract can serve as a nitrogen source, and PHB biosynthesis is much more pronounced under nitrogen-limiting conditions (Kessler and Wilholt 1999). Therefore, in subsequent experiments only a low concentration (0.1 g l^{-1}) of yeast extract was added to the whey medium. Conversely, whey dilution seems to be crucial for biomass as well as for PHB production ($P<0.05$). A high concentration of salts and lactose in undiluted whey medium probably caused high osmotic pressure, which consequently inhibited bacterial growth and PHB biosynthesis (t values<0). High concentrations of lactose could also induce substrate growth inhibition, hence whey dilution was optimized in order to achieve maximal biomass and PHB yields (Fig. 2).

The highest biomass and PHB yields were obtained when whey was diluted to a lactose concentration of 20 g l^{-1}. Thereafter, biomass and PHB yields were 2.51 g l^{-1} and 0.79 g l^{-1}, respectively.

The growth and PHB production course of *B. megaterium* in optimized whey medium were also determined. Growth was accompanied by lactose utilization throughout the whole cultivation. Biomass formation

Table 4 The results of data analysis (t-values and P values) for the effect of medium components on growth and poly(3-hydroxybutyrate) (PHB) production

	Biomass [g l^{-1}]		PHB [%]		PHB [g l^{-1}]	
	t value[a]	P value	t value[a]	P value	t value[a]	P value
Whey concentration	-11.46	0.000	-7.53	0.000	-6.40	0.001
$(NH_4)_2SO_4$	-0.10	0.923	1.30	0.241	0.73	0.493
PO_4^{2-}	-0.48	0.647	-1.98	0.095	-1.70	0.139
$MgSO_4$	-2.31	0.051	-1.79	0.123	-2.17	0.073
Yeast extract	-1.69	0.142	-3.26	0.017	-3.09	0.021

[a] t value is statistically significant only if $P<0.05$

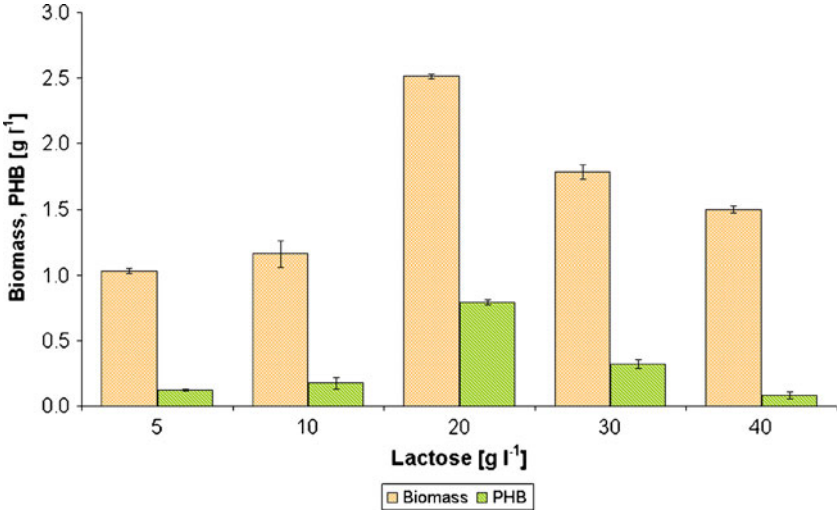

Fig. 2 The effect of whey dilution (expressed as lactose concentration) on PHB and biomass production

reached a maximum after 28 h of cultivation; thereafter, stationary phase occurred. This lasted until the 50th hour of cultivation, when biomass concentration started to decrease. The highest PHB yields were observed at the 50th hour (see Fig. 3).

PHB production under stress conditions

We have recently reported that the stress response of *Cupriavidus necator* H16 to ethanol and hydrogen peroxide is accompanied by enhanced PHB accumulation. However, the stress has to be applied at the beginning of the stationary phase, and at an optimized level. This strategy could be used in biotechnology as a simple, cheap and effective tool to enhance total PHB yields (Obruca et al. 2010a, b). In order to study whether this strategy could also be used in *Bacillus megaterium* cultivated on cheap whey medium, we decided to apply different concentrations of ethanol and hydrogen peroxide at the 25th hour of cultivation (Table 5).

Both hydrogen peroxide and ethanol increased PHB biosynthesis in *B. megaterium* cells. The most effective strategy was the application of 1% ethanol, which enhanced PHB yields about 41% as compared to the control culture (Table 5). In contrast, application of hydrogen peroxide enhanced PHB yields only very slightly.

The reason why ethanol enhances PHB yield is that ethanol is metabolized via oxidation to acetyl-CoA. During these reactions, reduced coenzymes NAD(P)H stimulating flux of acetyl-CoA into PHB biosynthetic pathway are formed, and free CoA, which inhibits PHB biosynthesis, is used to build acetyl-CoA. Moreover, acetyl-CoA, as the final product of ethanol metabolism, is the initial substrate of the PHB biosynthetic pathway (Obruca et al. 2010b).

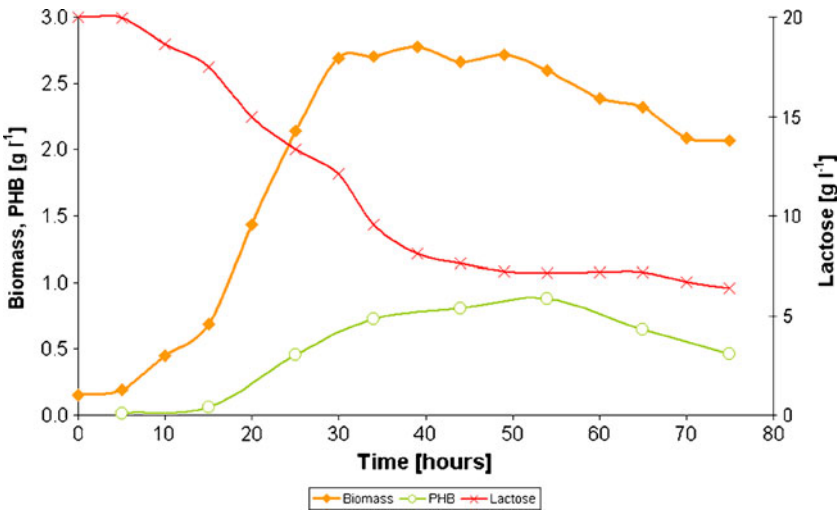

Fig. 3 Growth and production characteristics of *Bacillus megaterium* in optimized whey medium

Table 5 PHB and biomass yields after stress factor application at the 25th hour of cultivation. Results given are average ± standard deviation; each cultivation was performed and analyzed in triplicate

	Biomass [g l^{-1}]	PHB [g l^{-1}]	PHB [%]
Control	2.82±0.11	1.05±0.05	37.23
EtOH 0.5%	2.79±0.08	1.43±0.07	51.23
EtOH 1 %	2.87±0.08	1.48±0.08	51.57
EtOH 1.5 %	2.68±0.03	1.22±0.12	45.52
H_2O_2 1 mM	2.62±0.12	1.18±0.16	45.04
H_2O_2 3 mM	2.75±0.06	1.09±0.03	39.61
H_2O_2 5 mM	2.77±0.09	1.15±0.05	41.53

Table 6 summarizes yields of PHA production employing wild type bacterial strains from cheese whey that have been reported recently in the literature. Total PHB yields obtained in this work are relatively low as compared to those obtained in fermentor in fed-batch mode (Nath et al. 2007; Koller et al 2007). This is due predominantly to the relatively low growth of bacterial culture in Erlenmeyer flasks. On the other hand, *Pseudomonas hydrogenovora* was employed for PHA production from whey in fermentors in fed-batch mode with highest PHA yields of 1.4 g l^{-1} (Koller et al. 2008). This is comparable to the yield reached in batch mode in our flasks experiments (1.5 g l^{-1}).

Optimization of medium composition as well as controlled introduction of stress factors significantly enhanced PHB content in cells to relatively high levels, even in comparison with results reported in the literature (Table 6). This is beneficial in terms of total PHB yields but, furthermore, is also likely to reduce the cost of PHA recovery because the PHB content in cells strongly affects the efficiency and cost of downstream processing (Lee and Choi 1999). *Bacillus megaterium* CCM 2032 is a Gram-positive strain that could also facilitate simple and environmentally friendly recovery of PHA using lytic enzymes such as lysozyme or mutanolysin. Finally, Gram-negative bacteria, which are currently the only commercial sources of PHA, accumulate lipopolysacharides that co-purify with PHA and cause immunogenic reactions. This complicates application of PHA in medicine. On the other hand, Gram-positive bacterial strains, such as *Bacillus megaterium* CCM 2037, lack lipopolysacharides, which makes them a more attractive source of PHA (Valappil et al. 2007).

Further experiments should focus on fermentor cultivation to reach high cell density and improve PHB yields. Nevertheless, we have proved that application of controlled stress conditions (ethanol) is a promising strategy for improving the process of PHB production from cheese whey using *B. megaterium*.

Conclusions

In this work, we tested *Bacillus megaterium* CCM 2037 as a bacterial strain able to utilize waste cheese whey and produce PHB. Because supplementation of cheese whey medium with salts is necessary to reach higher PHB yields, optimization of whey media composition was performed. In our experiments, PHB production was enhanced about 50 times by optimization of cheese medium as compared to cheese whey alone. Furthermore, even higher PHB yields can be obtained if the bacterial culture is exposed to 1% ethanol as an exogenous stress factor applied at the beginning of stationary phase. This novel strategy enhanced PHB production by about 41%. Our results indicate the potential of *Bacillus megaterium* for industrial PHB production from cheap whey substrate. Nevertheless, further experiments carried out in laboratory and semi-productive bioreactors are needed to obtain high cell density and improve production parameters still further.

Table 6 Biomass and polyhydroxyalkanoate (PHA) yield from cheese whey reported in the literature

Reference	Biomass [g l^{-1}]	PHA [%]	PHA [g l^{-1}]	Microorganism	Cultivation device
Nath et al. 2007			3.9	*Methylobacterium* sp. ZP24	Fermentor
Koller et al. 2008		12	1.4	*Pseudomonas hydrogenovora*	Fermentor
Koller et al. 2007		12	1.3	*Pseudomonas hydrogenovora*	Fermentor
Koller et al. 2007		40	2.7	*Hydrogenophaga pseudoflava*	Fermentor
Koller et al. 2007		50	5.5	*Haloferax meditarranei*	Fermentor
Yellore and Desai 1998	9.9	59	5.9	*Methylobacterium* sp. ZP24	Flasks
Pantazaki et al. 2009	1.6	35	0.5	*Thermus thermophilus* HB8	Flasks
This work	2.9	51	1.5	*Bacillus megaterium* CCM 2037	Flasks

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