Journal of Bioscience and Bioengineering
VOL. 109 No. 4, 418–421, 2010

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NOTE

Production of polyhydroxyalcanoates (PHAs) using milk whey and dairy wastewater activated sludge
Production of bioplastics using dairy residues

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Received 24 July 2009; accepted 14 October 2009
Available online 8 November 2009

The production of polyhydroxyalcanoates (PHAs), which are biodegradable plastics, was studied using milk whey and dairy wastewater activated sludge to define a suitable C/N ratio, the pre-treatments required to reduce the protein content, and the effect of pH correction. The results show good production of PHAs at a C/N = 50 and without pH correction. The use of dairy wastewater activated sludge has the advantage of not requiring aseptic conditions.

[**Key words:** Polyhydroxyalcanoates (PHAs); Activated sludge; Whey; Microbial consortium; C/N ratio]

Bioplastics, in particular polyhydroxyalcanoates (PHAs), are widely studied as biodegradable materials for substitution of oil-derived polymers. At present, however, they have not had a wide application mainly because of their production costs, which are relatively higher than for traditional plastics.

A cost reduction in the biosynthesis process could be obtained either by searching for new cheap substrates (1), new fermentative strategies (2), and new recovery and purification steps (3) or by using microorganisms capable of synthesizing and accumulating PHAs to high concentrations (4). The production medium must contain low-molecular-weight organic compounds; the best yields, reported in the literature, are obtained in the presence of pure compounds, such as mono- or disaccharides and organic acids (5).

Selected or recombinant PHA-producing microorganisms that give higher yields require a sterile process (from medium to product formation). The solvents and their final disposal increase the final product price. A number of studies have considered the use of residues and by-products as substrates: in particular, materials derived from the food industry, due to their composition of readily degradable organic molecules. In these cases, the PHA yield is lower than with pure substrates, but with the advantage of reduced medium costs (1).

Similar considerations can be addressed for the microorganisms: the selected and specific microorganisms, including the recombinant ones, can produce higher yields, but they require a sterile process. On the other hand, with mixed cultures (consortia), the medium sterilization phase can be avoided, removing one of the operative costs. Few studies about PHA production with mixed microorganisms and residue-derived substrates have been carried out. As a potential food industry residue and by-product, milk whey has received attention for its composition, suitable for the biological process in PHA production. It contains lactose, proteins and fats as its main constituents, which are all useful in different ways to the fermentation process.

Production of PHAs from whey has been described in different works. Marangoni et al. (6) and Koller et al. (7) produced PHAs from hydrolyzed whey permeate with *Ralstonia eutropha* DSM545 and *Pseudomonas hydrogenovora*, respectively. In the work of Nikel et al. (8) a recombinant *Escherichia coli* strain was used with whey supplemented with corn steep liquor. *Hydrogenophaga pseudoflava* DSM1034 (9) and *Methylobacterium* sp. ZP24 (10) have been reported as examples of wild-type microorganisms able to synthesize PHAs directly from lactose. In all of the above-mentioned examples of PHA production with whey, the use of a pure culture, recombinant or not, required aseptic conditions. Recently, Pantazaki et al. (11) described the production of a heteropolymer from whey by *Thermus thermophilus* HB8, and a sterile process was also necessary in this case.

In the present work, a further improvement was added in order to better utilize milk whey for PHA production: using activated sludge derived from a dairy wastewater treatment plant as the inoculum. Another novel aspect introduced in the present study is the definition of a suitable medium to be used by a consortium of microorganisms that, given their origin, are already acclimatized to the milk whey environment.

The PHA production process was defined through a series of steps, taking into account and optimizing the operative parameters involved. The steps involve the evaluation of the influence of sludge conservation on enrichment, the definition of the treatment to reduce the protein concentration, the comparison of PHA yield obtained with two different carbon/nitrogen (C/N) ratio of the medium and the evaluation of the influence of pH correction.

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1389-1723/$ - see front matter
doi:10.1016/j.jbiosc.2009.10.012

To have comparable trials, a commercial whey powder was used (Molkolac®, Milei GmbH) with the following composition: lactose 84%, protein 4%, ashes 6%, lipids 1% and moisture 5% (w/w). To diminish whey protein content, three different pre-treatments were applied on a 120.48 g/l Molkolac® solution (equivalent to lactose 100 g/l): (i) whey solution was heated to 74 °C for 15 min, cooled and centrifuged at 8000 *g* for 15 min at 4 °C; (ii) whey solution was autoclaved at 121 °C for 15 min, cooled, centrifuged at 8000 *g* for 15 min at 4 °C, and then filtered with a glass fiber filter (Whatman GF/C 0.47 μm pore size); (iii) whey solution was prepared as in the pre-treatment step (ii) and then ultrafiltered in an Amicon apparatus equipped with a YM10 membrane (cut-off 10 kDa).

Enrichment of PHA producers was carried out in a synthetic medium with acetic acid (20 g/l) as the sole carbon source (12), and the pH value was set to 7.0 and controlled at 8.0 ± 0.3 with fed-batch addition of fresh medium (pH 7.0). PHA production tests were carried out in two different mineral media: MR medium described by Ahn et al. (13), and Molkolac® solution (K-molk medium) according to Khardenavis et al. (12). For both media, pre-treated whey powder solution represented the sole carbon source, and the initial lactose concentration was 40 g/L and 20 g/L for MR and K-molk media, respectively. K-molk medium was tested at two different C/N ratios: 50, as reported by Khardenavis et al. (12), and 20 (the same value for the MR medium) where the C/N was changed while maintaining a constant *N* value.

Biomass for PHA production was enriched from dairy plant activated sludge (with an average Mixed Liquor Suspended Solids content equal to 5.9 g/l). Activated sludge, fresh or frozen (-25 °C), was used as inoculum (10% v/v): 20 ml of activated sludge was added to 180 ml of biomass enrichment medium. Enrichment was carried out for about 500 h for fresh inoculum, and for about 100 h for the frozen one. Biomass for PHA production was collected from the enrichment culture after 100 h and used as inoculum (10% v/v). Enriched activated sludge biomass (20 ml) was added to 180 ml of MR or K-molk media, with Molkolac® as a carbon source. All the cultures were carried out in 500 ml Erlenmeyer flasks and incubated on a rotary shaker at 120 rpm and at 30 °C. Whole cultures were sacrificed at different times and analyzed for pH, biomass and PHAs.

Cell growth was monitored during fermentation tests by measuring the optical density (OD) at 620 nm (HP 8452A Diode Array Spectrophotometer). At the end of the experiments, biomass was measured in terms of the dry weight after drying a washed cell sample obtained by centrifugation (18,000 rpm, 15 min, 4 °C) at 105 °C for 24 h. Biomass was pelletized by centrifugation (15,000 rpm, 10 min, 10 °C) in pre-weighed glass tubes and then dried at 60 °C for 48 h. The polymer was extracted from dried biomass according to the method of chloroform-hypochlorite dispersion described by Hahn et al. (14). The PHA content, defined as the ratio of PHA concentration to cell concentration, is given as a percentage.

In order to monitor lactose consumption by activated sludge, samples (2 ml) were taken from the growing cultures at different time intervals and filtered with a fiberglass filter (Whatman GF/C 0.47 μm pore size). The cell-free supernatant was used to determine the residual lactose using a commercial kit (Boehringer Mannheim, Roche n° 10 986 119 035).

The enrichment in PHA producers was carried out with acetic acid as a carbon source according to Khardenavis et al. (12). In order to evaluate the influence of sludge conservation, two different kinds of inocula were used: fresh and frozen activated sludge. On the basis of the resulting values of optical density and pH, the following observations can be made: the lag phase is very similar for the two cases and lasts about 20 h; the exponential phase is very similar just for the first period that lasts about 30 h, then the frozen sludge grows more slowly; the fresh sludge reaches the stationary phase after about 55 h, with an optical density value that is reached by the frozen one at 100 h; in both conditions, the consumption of acetic acid produces a basification of the medium with an increase in pH to over 8. To neutralize the pH, after 173 h, 100 ml of the exhausted medium was substituted with fresh medium. Since no differences in culture behavior exist between fresh and frozen inocula until 100 h of enrichment, all of the following runs were carried out with frozen sludge to avoid the problems caused by using fresh whey. In cultures inoculated with fresh and frozen activated sludge, PHA production was determined at different fermentation times (from 100 to 500 h of fermentation) during the stationary phase: a mean value of 10% (w/w) was obtained.

In milk whey, protein concentration is on the order of 4–7 g/l, resulting in a low carbon to nitrogen ratio (C/N) unsuitable for the PHA production process (15). A study by Suresh Kumar et al. (16) demonstrated that the PHA yield from sludge microorganisms increases with the C/N ratio, up to C/N = 144. A reduction of protein content is required to increase the C/N ratio. In order to reduce protein content, the previously described treatments, namely (i), (ii) and (iii) were applied to the Molkolac® solution used as a carbon source in cultures where the inocula were derived from sludge enrichment. MR-medium described by Ahn et al. (13) was used for the saline components. The obtained results show the absence of a lag phase: this means that the microbial consortium is able to use the lactose as a carbon source.

The cultures submitted to the treatments (ii) and (iii) show very similar optical density profiles; the trends demonstrate a higher growth rate than that of the culture whose protein content had been reduced by a mild heating. The runs have a very similar pH behavior: in all cultures, the medium acidity increases due to ammonium uptake. This result was also confirmed by the PHA production value at 120 h of fermentation. PHA content (g PHAs/g dry mass) and PHA concentration (g/l) are, respectively: (i) 8.6 % and 0.24 g/l, (ii) 11.1 % and 0.42 g/l, (iii) 7.5 % and 0.34 g/l. It is evident that the best results were obtained with the culture using the whey solution submitted to the treatment (ii). Consequently, it was decided that this treatment can be considered as the reference treatment for protein content reduction, and it was used for all the following tests. A similar result was presented by Yellore and Desai (10): in cultures with *Methylobacterium* sp. ZP24 working in milk whey, higher PHA production was noted when the milk whey had been partially deproteinized than with raw whey.

The influence of C/N ratio on PHA yield was studied to compare, and eventually define, an optimized medium composition in order to enhance both microbial growth and PHA production. The previous cultures used the formulation MR-medium given by Ahn et al. (13) as saline components and the enriched sludge derived from the culture formulation given by Khardenavis et al. (12). This condition implies that microorganisms must adapt themselves to a new environment when they are used in PHA production cultures, with a subsequent lag phase. On this basis, it was decided to test PHA production cultures with sludge enriched according to the saline formulation given by Khardenavis et al. (12) and Molkolac® as a carbon source. Two possibilities were considered: (i) a formulation with C/N = 50, i.e., the ratio of Khardenavis medium (K-molk 50); (ii) another with C/N = 20, i.e., the ratio C/N used in the MR-medium (K-molk 20). The MR medium was used as a control. All the runs showed similar exponential phases; however, the K-molk cultures reached the stationary phase at lower optical density values, meaning their biomass content was lower than for the MR medium. Comparison done on the basis of the obtained PHA concentration shows the data presented in Fig. 1. The highest values of PHAs, measured both by culture volume and the dry biomass, is that of the K-molk 50 medium: an explanation of this can be given considering the lactose concentration at the end of the runs since a C/N = 20 (K-molk 20 medium) is under the detection limit, i.e., the lactose was completely

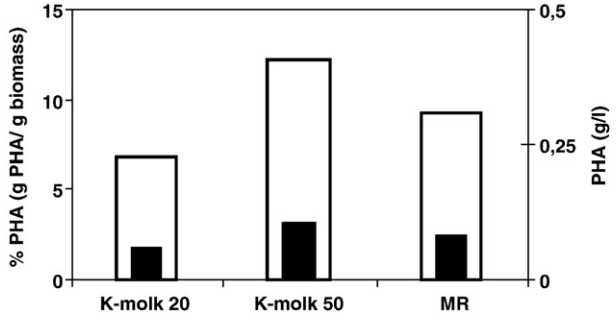

FIG. 1. PHA production in the tested formulation medium. Symbols: open, %PHA; closed, PHA.

consumed. Two hypotheses can be proposed: the lack of the carbon source could have contributed to partial PHA degradation, or the carbon absence could have stopped the PHA production.

In the other runs, the final lactose concentrations were 1.95 g/l and 3.29 g/l for K-molk 50 and MR-medium, respectively. Though the biomass content in the K-molk 50 cultures was lower than that in the MR cultures, in the first case, PHA content and concentration were higher, probably due to the different C/N ratio of the media, as described by Suresh Kumar et al. (16). Residual lactose concentration (higher in MR cultures) seems to have no effect on PHA content in MR culture; an explanation may be the synergic effect of C/N ratio and lactose concentration. Different authors observed a similar influence of substrate concentration on PHA content (17, 18), and a fed-batch strategy was suggested. From these results, one can conclude that the ratio C/N = 50 in Khardenavis medium is suitable for the PHA production process, and it can be considered as an optimized formulation for future studies.

According to different authors (13, 17), in fermentation carried out with lactose, pH control represents a critical point in PHA production. Since a significant pH decrease was measured during the fermentation process, it was decided to investigate the influence of pH correction, and a series of runs was carried out both with the new formulation medium (K-molk 50, C/N = 50) and with the MR reference one (C/N = 20), with and without pH correction. When the pH value was lower than 6.0, it was returned to the initial value (7.0) by adding a NaOH solution (12 N). The correction was done after 20, 27 and 43.5 h. Fig. 2 shows the optical density values obtained with and without pH correction. It is evident that when neutralization is done in MR-medium cultures, the optical density always has higher values with respect to those without correction; in K-molk medium, this trend works for just about 50 h, then the optical density becomes higher for the runs without pH corrections. In general, the optical density of

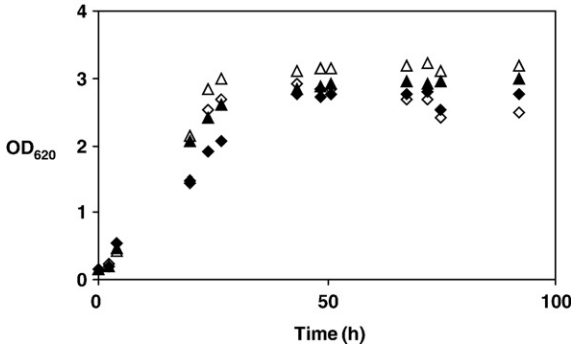

FIG. 2. Influence of pH correction on biomass growth. Symbols: closed rhombuses, K-molk (pH not corrected); open rhombuses, K-molk (pH corrected); closed triangles, MR (pH not corrected); open triangles, MR (pH corrected).

TABLE 1. Influence of pH correction on PHAs production.

Sample	% PHAs (g PHAs/g biomass)	PHAs (g/l)
K-molk (pH correction)	5.10	0.072
K-molk (no pH correction)	13.82	0.224
MR-medium (pH correction)	6.44	0.284
MR-medium (no pH correction)	8.67	0.227

MR-medium cultures is higher than the K-molk medium ones. PHA production, obtained after 92 h of fermentation and reported in Table 1, indicates the highest value for the culture in medium K-molk without pH correction.

At the end of the fermentation process, the lactose concentration in the samples was determined. In the medium K-molk with pH correction, it was under the detection limit, which could explain the lower (5.10 g PHAs/g biomass) PHA value obtained in the test. In the medium without pH correction, final lactose concentration was 6.17 g/l, and consequently, PHA content was higher (13.82%). In order to clarify the real effect of pH value on PHA production, a bioreactor test with a control of pH value may be necessary.

While in all the reports in the literature, the use of a pure culture, recombinant or not, required aseptic fermentation processes, the present work showed that the application of a consortium enriched from dairy plant activated sludge did not required a sterile environment. Experimental tests carried out with enriched biomass, obtained from fresh or frozen activated sludge, showed a similar growth curve and reached very comparable PHA values during the stationary phase, suggesting the possibility of activated sludge storage. Among the different methods tested, the best results (high PHA values) for whey pre-treatment were obtained with thermal treatment followed by microfiltration when pre-treated whey was added to the saline medium at a C/N ratio value of 50. Unlike similar reports in the literature, pH correction during fermentation was disadvantageous for PHA production.

The results of this report demonstrate the production of PHAs from whey in the presence of an enriched biomass able to directly utilize the lactose in a non-sterile fermentation process without pH control.

ACKNOWLEDGMENTS

The authors wish to acknowledge the REGIONE PIEMONTE for the Grant C.I.P.E. 17/2003 “Produzione di plastiche biodegradabili da scarti agroalimentari.”

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