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Production of PHA from Whey by Indigenous Microflora and Activated Sludge: Preliminary Investigation

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Milk whey was employed to produce polyhydroxyalcanoates (PHA), using as inoculum either whey indigenous microflora or enriched activated sludge deriving from a dairy wastewater treatment plant.
The aim of the present study was to test the capability of mixed consortia to produce PHA directly from lactose. The experimental runs demonstrated that milk whey lactose can constitute a cheap carbon source to synthesize PHA in fermentations where the microorganisms are represented by indigenous microorganisms or enriched activated sludge: this result constitutes an evident advantage since whey pre-treatment and aseptic conditions are not required. Tests with commercial whey powder (Molkolac®) and on raw milk whey showed a PHA production around 10 % (by weight on dry biomass).

1. Introduction

In the last two decades, a broad number of studies was related to the production of biodegradable plastics from milk whey.
For the European dairy industry, it is estimated an annual production of 75 million tons of whey from cheese makers, and about 40% of this amount is discarded and managed as waste.
From an economical and environmental point of view, whey disposal represents a serious problem, whereas its use in fermentation processes (e.g. bioplastic production) may be advantageous not only for environment but also for economy (Jambunathan and Zhang, 2016; Ienczak *et al.*, 2013). Biotechnological production of polyhydroxyalcanoates (PHA) from lactose was described in different works (Marangoni *et al*., 2002; Nikel *et al*., 2005; Ienczak *et al.*, 2013); because of few wild type microorganisms were able to directly convert lactose to PHA (Koller et al., 2007), different strains of r*E. coli* were used on whey.
The use of recombinant strain was reported in many works: Kim (2000) described PHA production with r*E. coli* GCSC6576 in fed-batch oxygen limited culture; Ahn *et al*. (2001) reported polyhydroxybutyrate (PHB) production with r*E. coli* GCSC 4401 in highly concentrated whey solution and a pH-stationary fed-batch solution; Nikel *et al.* (2005) applied a statistical optimization on cultural medium containing whey powder and corn steep liquor in presence of different r*E. coli* strains.
In all the aforesaid examples of PHA production on whey, the use of a pure culture, recombinant or not, required aseptic condition with additional operations and costs.
The opposite approach to PHA production was the use of mixed cultures, e.g. activated sludge derived from food processing wastewater treatment plant (Suresh Kumar *et al.*, 2004).
In the work of Khardenavis *et al.,* (2007), wastewater from food processing industry (potato cheeps, wafers and sweets) was used as substrate for PHB production and the value obtained after 48 hours of incubation in filtered wastewater was 39.1 % (on dry weight basis).
The present study was carried out in order to verify the PHA production capability of mixed cultures derived either from whey or from dairy wastewater activated sludge. The study was based on a series of steps aimed to evaluate the influence of medium composition on biomass growth and PHA production and to identify, if possible, an optimized composition for PHA production.
The first step was the study of the treatment to reduce the whey protein content, in order to reduce nitrogen concentration and medium turbidity.

Low protein content whey and a synthetic medium with glucose or galactose as carbon source were used for indigenous whey biomass growth in order to identify the presence of PHA-producing microorganisms. Indigenous whey biomass and a microbial consortium, enriched from dairy activated sludge, were used in PHA production tests carried out on raw whey and commercial whey powder (Molkolac®), respectively.

2. Methods

2.1 Pre-treatment of raw whey

To reduce protein content, two different methods were tested:

- method 1: whey was heated to 74 °C for 15 min, cooled and centrifuged at 10,000 g for 15 min at 4 °C (Yellore and Desai, 1998);
- method 2: whey was autoclaved at 121 °C for 15 min and centrifuged at 11,000 g for 15 min to remove aggregates (Ahn *et al.*, 2000).

Both methods work in acidic condition (pH = 4.5). In this study the raw whey samples were not acidified due to their low pH values, usually around 4.5.
The methods were also tested at neutral pH as initial pH value of fermentation. Moreover, the influence of the storage temperature was evaluated (+4 °C and -30 °C).

2.2 Indigenous whey microorganisms culture and growth conditions

Indigenous microflora of raw whey was grown on 4 different media, as summarized in Table 1.

Raw milk whey was used as inoculum (10 % v/v). Microbial growth was carried out in Erlenmeyer flasks, containing 200 mL of medium, incubated on a rotary shaker at 120 rpm and 30 °C, till the stationary phase was reached.

Table 1: Medium and carbon source used to grow indigenous microflora of raw milk whey.

No. medium	Medium	Carbon source
1	Whey	Lactose
2	Whey with MR medium salts (Ahn *et al.*, 2000)	Lactose
3	Glucose with MR medium salts	Glucose (10 g/L)
4	Galactose with MR medium salts	Galactose (10 g/L)

2.3 Biomass determination

During fermentation tests, cell growth was monitored as optical density at 620 nm (OD_{620}) by a HP 8452A Diode Array Spectrophotometer.
At the end of each run, the sample was centrifuged (15,000 rpm, 10 min, 10 °C) and biomass was measured in terms of dry weight after drying at 60 °C.

2.4 Detection of potential PHA producers

Biomass derived from the 4 tested media was collected after 24 hours, diluted and grown on Malt Agar plates at 30 °C.
After 4 days, colonies of potential PHA producers were detected by Sudan Black B (SBB) staining. The colonies were spread with 0.02 % SBB solution in 96 % ethanol for 60 minutes, then washed with 96 % ethanol (de Lima *et al.*, 1999; Carletto et al., 2011). PHA-producing colonies (dark blue) were enumerated.

2.5 Enrichment of PHA producers

A dairy plant activated sludge was enriched as biomass for PHA production (the average Mixed Liquor Suspended Solids content was equal to 5.9 g/L) in a synthetic medium with acetic acid (20 g/L) as sole carbon source (Khardenavis et al., 2007). The cultures were incubated on a rotary shaker at 120 rpm and 30° C, pH was initially set at 7.0 and controlled at 8.0 ± 0.3 with fed-batch addition of fresh medium. Enrichment was carried out for about 30 days; when pH value increased to around 8.4-8.5, spent supernatant was replaced with fresh medium (pH = 7.0).

2.6 PHA production medium

For PHA production tests, two carbon sources were used, namely:

- commercial whey powder (Molkolac® Milei, GmbH, Leutkirch**,** Germany)
- raw milk whey.

Both media were pretreated with method 2 at unmodified pH to reduce protein content.

Enriched biomass was cultivated on MR medium (Ahn *et al.*, 2000) adding commercial whey powder to obtain a final lactose concentration equal to 40 g/L.
As regard indigenous whey biomass, it was cultivated on pretreated milk whey in the same condition of lactose concentration.
Initial pH of each medium was adjusted to 7.0 ± 0.1 in all the experiments.

2.7 Culture conditions

Indigenous whey microorganisms or enriched activated sludge (10% v/v) biomass were added, respectively, to 180 mL of milk whey or Molkolac® in Erlenmeyer flasks and incubated on a rotary shaker at 120 rpm and 30 °C.
Each culture was entirely sacrificed at different time to analyse biomass and PHA content.

2.8 Isolation and determination of PHA

Biomass was pelletized by centrifugation (15,000 rpm, 10 min, 10 °C) and then dried at 60°C for 48 hours. The polymer was extracted from dried biomass according to the chloroform-hypochlorite dispersion method described by Hahn *et al.* (1994). The PHA content was defined as the percentage of the PHA weight to the biomass one.

2.9 FT-IR analysis

Spectra were collected by a Bruker Equinox 55 spectrometer, equipped with a Germanium crystal.
PHB sample was placed in the instrument without any further preparation. A total of 64 scans were averaged for each sample and the spectra were recorded in the range 600-4000 cm^{-1} (resolution = 2 cm^{-1}). The background was recorded before every analysis and subtracted automatically from the sample spectrum.

3. Results and discussion

3.1 Pre-treatment of raw whey

In order to reduce proteins content and turbidity, raw whey samples were processed according to the methods reported in the section **Methods – Pre-treatment of raw whey**. Table 2 reports the results for protein concentration before and after treatment, at unmodified pH or at neutral pH.

About lactose concentration, both the thermal treatments gave similar results showing a limited reduction of this parameter (reduction percentage was in the range 4-5 %). This result is fundamental to ensure a constant value of lactose concentration in a whey-based medium for PHA production, as also found by Yellore and Desai (1998).
As regard to reduction of protein content, the application of method 2 gave better results, as demonstrated in Table 2 where the maximum value of percentage reduction (56.35 %) was obtained in the sample at pH = 4.5.
Moreover, the effect of whey temperature storage on protein removal was also evaluated. The tests reported in Table 2 (samples maintained at 4 °C) were also carried out on the same whey samples maintained at -30 °C for six months. No substantial differences in protein content reduction were observed between cold-storage and freeze-storage samples. In case of long-term storage, frozen represented the best preservative method because microbial activity was completely inhibited and steady chemical properties of whey were assured.
Method 2 at unmodified pH was also applied on Molkolac®, and the obtained results were comparable to those reported for milk whey both for lactose and for protein concentration. Considering the economy of the process, method 2 is the most profitable because the operation allows to a simultaneous of protein content and sterilization of the whey samples (if required). Given this finding, the method 2 was used to treat the whey used in all the following experimental tests.

Table 2: Lactose and protein concentration in whey samples maintained at 4 °C.

	Before treatment	After treatment	% reduction
Protein (g/L) method 1 unmodified pH	7.61	4.84	36.40
Protein (g/L) method 1 pH = 7.0	7.61	4.62	39.29
Protein (g/L) method 2 unmodified pH	6.14	2.68	56.35
Protein (g/L) method 2 pH = 7.0	6.14	3.13	49.02

3.2 Indigenous whey microorganism cultures

In order to evaluate the growth capability of whey indigenous microorganisms on lactose and its hydrolysis products (glucose and galactose), cultures with whey and MR synthetic medium (Ahn et al., 2000) with glucose and galactose were carried out, respectively.
Figure 1 shows the results of the microbial growth, as OD_{620}, in the 4 tested media and with fresh inoculum, as summarized in Table 1.
About milk whey, the biomass growth is very similar with or without MR salts in the first part of the run; then, it is higher for the whey without MR salts.
About the synthetic media, to say glucose or galactose with MR salts, they behave in the same way for the first part of the run (about 24 hours), where the curves have similar shape; then, the growth in the culture with glucose continues and the OD_{620} values (about 2.5-2.6) obtained in whey runs are reached. Vice versa, the growth in galactose medium stops.
Figure 1 also shows, as comparison, the data obtained in previous runs with whey indigenous microflora grown in whey without MR salts and with a diluted inoculum (1% v/v). It is evident that the stationary phase is reached at the same absorbance values but in a longer period.
Very similar behaviours are shown when frozen inoculum was used; in this case, the maximum OD_{620} value was comparable to that of fresh inoculum, even if reached in a longer time. Probably, this is due to a lower microbial activity of the frozen whey indigenous microflora.
These results show the capability of indigenous whey biomass, either cold-stored or frozen, to use all different carbon sources present in whey: lactose or glucose and galactose that derive from hydrolysis of lactose.

3.3 Potential PHA producers

The experimental results suggested the possibility to use whey as complex medium for PHA-producing microorganisms. In order to verify the presence of microorganisms able to synthetize PHA, samples of all the tested media, inoculated with indigenous microorganisms contained in fresh or frozen whey, were submitted to Sudan Black B staining (for details, see **Methods** - **Detection of potential PHA producers**). The results confirm that the whey indigenous microorganisms are able to accumulate PHA only in the medium constituted of whey with MR salts. Probably, the composition of the MR medium promotes the PHA accumulation.
A possible explanation for this result could be due to different values of total organic carbon to nitrogen ratio (C/N), that for the tested media is, respectively:

- C/N = 27 for whey
- C/N = 12 for whey with MR salts
- C/N = 5 for glucose or galactose with MR salts.

About glucose or galactose, the C/N ratio is probably too low for the PHA-producers microorganisms; this idea finds support in other studies on PHB production, such as that by Suresh Kumar *et al*. (2004) on activated sludge from food industry wastewater plant that showed an increase of PHB content (till 33 % of biomass) when the C/N ratio was increased from 24 to 144.

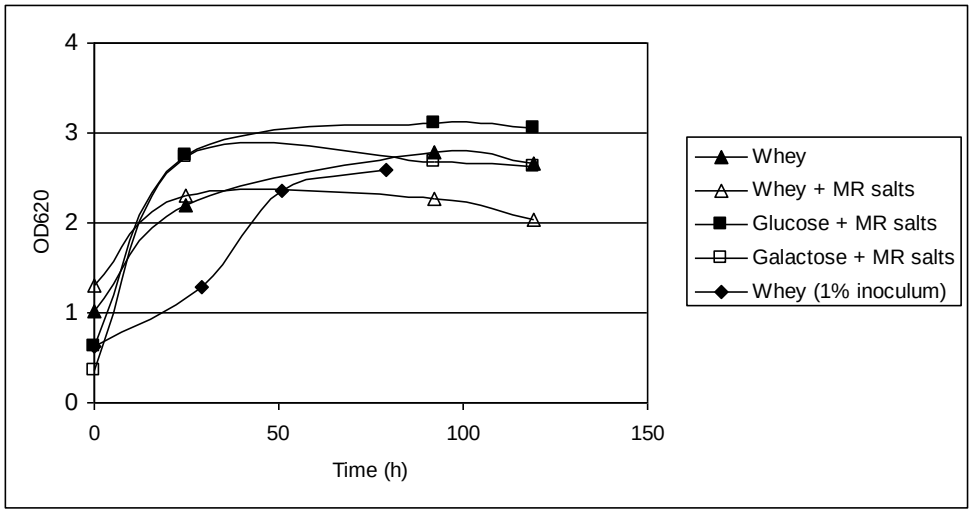

Figure 1: Biomass production with inoculum derived from fresh whey.

As far as the storage temperatures of indigenous whey microorganisms is concerned, the capability of PHA production is not influenced by the type of inoculum preservation, since in both the tested storage methods (fresh and frozen), colonies of PHA producers appeared.

3.4 Enrichment of PHA-producing biomass from activated sludge

Fresh activated sludge from a dairy processing wastewater treatment plant was collected and enriched in a synthetic medium with acetic acid (20 g/L) as carbon source (Khardenavis *et al.*, 2007).
Increase in biomass (as OD_{620}) and pH were estimated on culture samples. Results are reported in Figure 2, where they show an initial lag phase (about 20 hours) followed by an exponential one (about 30 hours). Stationary phase began when pH reached a value of 7.8-8.0.
PHA production was evaluated at different time: the maximum value of 25 % (by weight on dry biomass) was obtained at about 550 hours and pH = 8.3.

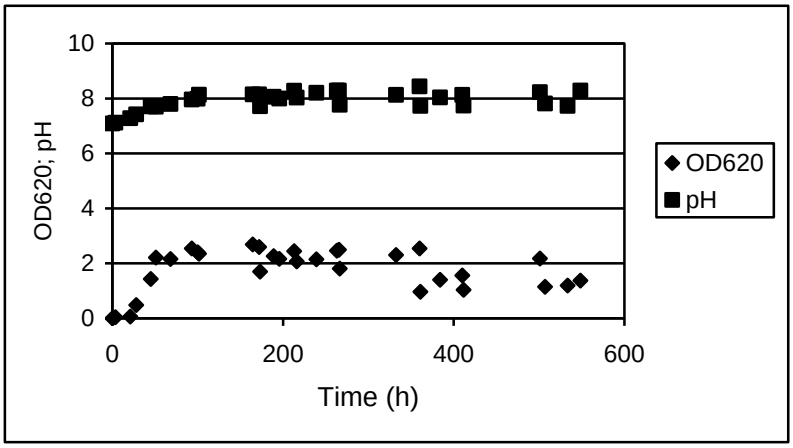

Figure 2: Enrichment of PHA-producing biomass.

3.5 Production of PHA with Molkolac® and enriched activated sludge

The production of PHA was obtained in MR medium with Molkolac® as substrate, at a concentration equivalent to 40 g/L lactose, and enriched activated sludge as inoculum. In Figure 3, time course of biomass, PHA production and pH values are reported.

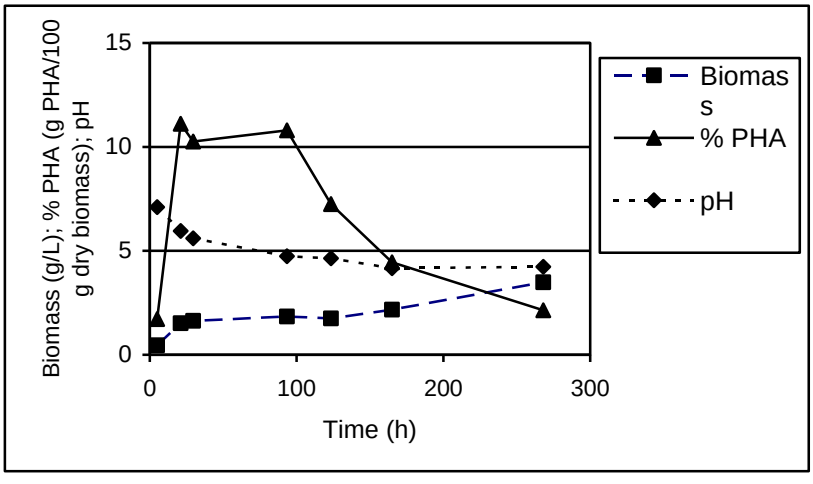

Figure 3: PHA production, biomass and pH in fermentation using Molkolac® as substrate and enriched activated sludge as inoculum.

After 21 hours of fermentation, when biomass reached stationary phase, a PHA concentration around 11 % (by weight on dry biomass) was obtained, then this same value was maintained 72 hours. Afterwards, PHA

content diminished (from 7 % after 124 h to 2 % after 268 h); on the contrary, biomass weight increased (from 1.5 to 3.5 g/L). The different behavior of PHA content and biomass could be related to the depletion of carbon source in the medium as described in the work of Ahn et al. (2001).PHA samples were examined by FT-IR spectroscopy and compared to commercial PHB. The findings showed the same C-H and carbonyl stretching bands as standard PHB, located at 2,900 and 1,730 cm^{-1}, respectively.
About PHA production in culture with raw milk whey with MR medium salts, the experimental runs showed that after 25 hours the % PHA was around 9 % (by weight on dry biomass).

4. Conclusion

This preliminary study confirmed the PHA production capability of mixed cultures, derived either from raw whey or from dairy plant activated sludge, on raw whey or commercial whey powder (Molkolac®) as carbon source. In both media it is required:

- To reduce the whey protein concentration and maintain a high C/N ratio, by thermal treatment
- To add MR salts to the medium.

The obtained results suggest the possibility of PHA synthesis with both the tested biomasses and media.
Further studies are required to improve the culture conditions.

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