CHEMICAL ENGINEERING TRANSACTIONS Volume 24, 2011
Editor Sauro Pierucci

 ISBN 978-88-95608-15-0 ISSN 1974-9791
DOI: 10.3303/CET1124082

Application of SBB Staining Technique for Monitoring PHA Production in Milk Whey Fermentation

Riccardo A. Carletto*, Alessandro Chiadò, Luca Gillio, Francesca Bosco, Luca Marmo

Dipartimento di Scienza dei Materiali ed Ingegneria Chimica, Politecnico di Torino
Corso Duca degli Abruzzi 24, 10129, Torino
riccardo.carletto@polito.it

Agro-industrial raw materials are becoming increasingly important in the field of biotechnological fermentation: they could be used as fermentation media, usually after the proper pretreatment, and in that way are usefully exploited. Milk whey is an important by-product of the dairy industry, nutrient-rich and potentially used as a growth medium for the production of commercial products. Many fermentation processes can be applied to whey, from using recombinant bacteria to mixed consortium. The latter often requires careful monitoring.
Monitoring and control of biotechnological processes based on complex media is often expensive and tricky, and usually involve only physical-chemical parameters. When a mixed culture is involved, microbiology and molecular biology can provide a more complete view of biological variation within the bioreactor.
In this work, a new monitoring technique was applied to an enriched mixed culture in a milk whey fermentation. The presence of the desired product PHA (polyhydroxyalkanoate), a biodegradable biopolymer, was evaluated throughout the SBB (Sudan Black B) staining. This cheap and easy method, usually employed in isolation of PHA storing bacteria, was useful in monitoring the fermentation process, not only to identify the recovery time but also for the assessment of the percentage of useful microorganisms present.

1. Introduction

Materials made from synthetic polymers are not biodegradable and are often improperly discarded. These materials are typically derived from petroleum-based plastics. Rapid progress in materials science technology has created new plastic products with favorable mechanical integrity and excellent durability. Nevertheless, plastic products usually have single-use applications, especially in food packaging and medical materials. Because these plastic products are not biodegradable, they are extremely persistent and accumulate in the ecosystem, resulting in a significant burden on solid waste management. For this reason European countries are trying to reduce oil consumption for plastic bags production, bringing it from the 100 to 60 million barrels/year (www.plasticnews.com).

Please cite this article as: Carletto R.A., Chiado A., Gillio L., Bosco F. and Marmo L., 2011, Application of sbb staining technique for monitoring pha production in milk whey fermentation, Chemical Engineering Transactions, 24, 487-492
DOI: 10.3303/CET1124082

Polymers have a wide range of properties, which make them suitable for many uses and at the same time very difficult to replace. One of the most promising alternatives to oil produced polymers is the development of new biodegradable polymers ones.
During recent years these materials have become more and more important on the worldwide market: the PlasticsEurope research group is expecting a yearly 16.6% increase in USA demand for biopolymers, for a total expected demand of $1.48 \cdot 10^5$ tons in 2014 (www.plasticseurope.com).
Among the various types of biodegradable plastics, PHAs are the most studied, being recognized as completely biosynthetic and biodegradable, with zero toxic waste, and completely recyclable into organic waste. They are microbial polyesters produced by a wide range of microorganisms, under unbalanced growing conditions, mostly as intracellular storage compounds for energy and carbon. Their properties span a wide range, including materials that imitate thermoplastic properties and others that possess electrometric properties. Their biggest downside is represented by their production cost, usually higher than the oil produced polymers one.
Nowadays PHA price is affected mainly by fermentation medium, extraction and purification costs (Bosco and Chiampo 2010). Many papers have addressed this issue in recent years (Lee 1996, Masani al. 2008, Loo et al. 2007). One of the most interesting methods to reduce process costs is to use a low cost fermentation medium. Many agro-industrial by-products, like milk whey, have been considered as an effective fermentation medium. Milk whey is now a high BOD waste but, since it has the proper composition for PHA production its use will serve a double purpose: to lower PHA production costs and to reduce the amount of waste produced by the cheese industry (Panesar et al. 2007, Jelen 2003, Kemp et al. 1989).
Another way of reducing PHA production costs is to use mixed cultures instead of single strain cultures one. This will remove the need for bioreactor and feedstock sterilization, on the other hand, it will become necessary to develop a method to control which colonies are effectively producing PHA.
Staining is the oldest and probably the cheapest and fastest technique available to isolate PHA producing microorganisms. Nile Red, Nile Blue A and Sudan Black B (SBB) are the most used dyes for selective staining of PHA granules (De lima et al. 1999, Burdon 1946, Redzwan et al 1997, Alias and Tan 2005, Ostle and Holt 1982, Williamson and Wilkinson 1958, Zakaria et al 2010).
The application of SBB staining was never applied to monitoring PHA content during a fermentation process, but only to confirm its presence in reactor grown biomass (Bengtsson 2009).
In this work, PHA production was carried out both in controlled and uncontrolled STR fermentation tests. A new monitoring approach was tested: the investigation through SBB staining technique of the PHA production.

2. Materials and methods

Biomass for PHA production was taken from a dairy plant activated sludge and enriched in a synthetic medium (Khardenavis et al. 2007). Biomass in exponential

growth phase (about 75 hours) was collected and used as inoculum (10% v/v) for PHA production cultures in a 10 l bioreactor.
K-mol fermentation medium (Bosco and Chiampo, 2010) with added deproteinized milk whey powder (Molkolac®, Milei GmbH) containing an initial lactose concentration of 20 g/l was employed. Tests were conducted either in uncontrolled and controlled conditions (30°C, pH 7±0,02). Cell growth was monitored by measuring the optical density (OD) at 620 nm (HP 8452A Diode Array Spectrophotometer). Culture broth samples were taken at different times, biomass was harvested by centrifugation (18000 rpm, 10 min, 4°C), in pre-weighted glass tubes and dried to constant weight at 60°C for 48 hours. The polymer was extracted from dried biomass according to the chloroform-hypochlorite method described by Hahn et al. (1994). PHA content (weight percentage) was defined as PHA concentration to cell concentration ratio. Plates were made starting from different fermentation time samples (10ml every 24 hours). Serial dilutions, 10^{-5} or 10^{-6}, were prepared using 0,9% NaCl solution; 100µl of diluted samples were spread on malt agar plates containing: malt extract 20g/l, D-glucose 20g/l, peptone 2g/l, agar 20g/l. After two to seven days incubation at 30°C, potential PHA producers were detected by SBB staining of the colonies. According to de Lima et al. (1999), a 0.02% SBB solution in ethanol 97% was gently spread over the plates, completely soaking them, incubated at room temperature for 30-60 min, then discarded and washed with ethanol 97%. PHA producers tend to be stained dark-blue or black, while negative PHA accumulators remains white or light-blue. Counting of the different kind of colonies (white and coloured) was performed.

3. Results and Discussion

The aim of this work is the evaluation of SBB staining as an effective method to optimize process parameters of PHA production fermentations. Staining was applied to uncontrolled and controlled STR batch fermentations (30°C, pH 7 ± 0,02); various tests were carried out with different initial lactose concentration and C/N ratio; in the current tests lactose concentration was 20 g/L and C/N ratio value was 50.
OD analysis on uncontrolled and controlled fermentations showed different trends: faster growth of controlled culture is clear, stationary phases were comparable but reached at different times, 70 hours and 20 hours respectively (Figure 1).
The lactose concentration, in fact, decreased significantly in the first 24 hours of controlled fermentation, reaching a value of 5.62 g/l and 1.3 g/l after 22 and 27 hours respectively, whereas was still 18,17 g/l at 28 hours of uncontrolled fermentation, becoming 10,72 g/l at 96 hours (Rella 2009 master thesis).
Trends of ammonium consumption were also significantly different; its depletion occurred at 27 hours of controlled fermentation, while in uncontrolled one at 96 hours ammonium concentration was still 0,107 g/l.
The maximum polymer yield, as well as the maximum concentration of biomass, obtained is much higher in controlled fermentation (41.20% g $_{PHA}$/g $_{dried\ biomass}$; 1.8 g/l) (Figure 2) than in uncontrolled one (38,01% g $_{PHA}$/g $_{dried\ biomass}$; 1.04 g/l) and is constant over time after reaching the stationary phase.

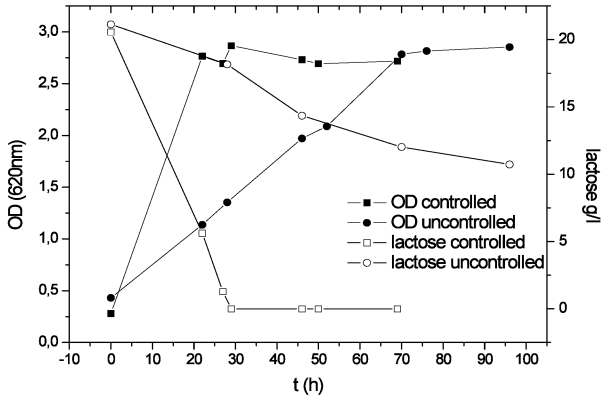

Figure 1: OD and lactose of uncontrolled and controlled cultures

To investigate the percentage of PHA producers, in each trial, microbiological analysis were performed. Through SBB staining is possible to make a cheap and easy qualitative estimate of the biopolymer content during fermentation.
To evaluate PHA producers percentage present in the biomass during fermentation, samples were withdrawn at different times, plated on malt agar and incubated 2 to 7 days at 30°C. After SBB staining, coloured colonies counting returned the percentage of PHA producers. The plates presented different kind of colonies with distinct morphology and growth rate, roughly representative of the producers and non-producers population inside the mixed culture. During the culture's stationary phase, when PHA content was the highest and the carbon source was in excess respect to the nitrogen one, only half biomass in the reactor seemed constituted by biopolymer producers. Moreover, by plotting these percentage along with PHA percentage yields, and concentration, was possible to observe a good correlation between curves (Figure 2). This result confirms the hypothesis reported in a previous paper (Bosco et al. 2008).
From controlled SBR fermentation plates, it's possible to remark that the ratio of PHA producers obtained in the stationary phase is different form the one obtained in the enrichment phase. This could suggest the presence of a limiting step either during the fermentation or the enrichment phase. The enrichment phase selectivity could be enhanced. Although initial pH is adjusted to 7.0, it isn't corrected during the preculture step: in this phase pH usually increases, selecting a biomass adapted to higher pH. Furthermore, a different carbon source was used in the enrichment and in the SBR tests (acetic acid and lactose, respectively) forcing microorganisms to metabolise different substrates, with different yields.
In order to better evaluate these findings further investigation will be necessary.
Comparing controlled and uncontrolled SBR plated samples, it becomes clear that same percentages of PHA-producers were achieved earlier in controlled SBR tests (46 and 70 hours respectively); the same can be said about nutrients consumption and maximum OD value.

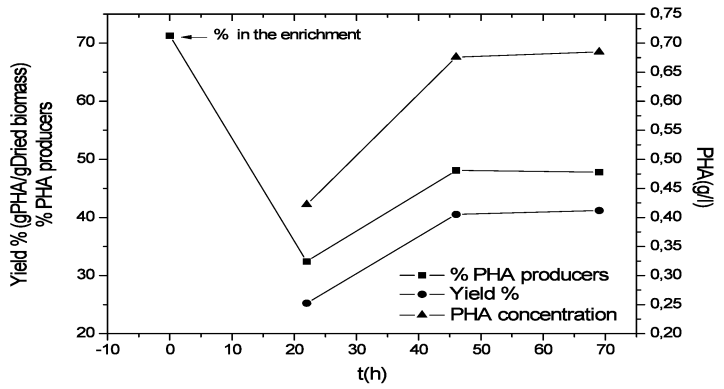

Figure 2: %PHA producers, Yield % and PHA concentration of controlled fermentation

These values obtained by SBB staining reflect the similarity between yield values obtained with controlled and uncontrolled runs. The advantage of controls is that biomass concentration is higher, leading to a higher polymer production.

4. Conclusions

In this work were carried out two types of fermentation: uncontrolled and controlled one. Starting with the same medium composition (lactose 20 g/l, C/N 50) temperature and pH were set at 30°C and 7 respectively in controlled fermentation while only initial pH was set in uncontrolled one. In controlled fermentation stationary phase was reached at 20 hours, nutrients consumption depletion occurred earlier and yield was higher (41,2%) than in uncontrolled trials; from these results comes out that controlled process gives advantages in terms of shorter fermentation times. During the tests OD, pH, lactose, ammonium concentration were monitored; together with them, SBB staining technique was applied in order to investigate the trend of PHA producers presence. Exploiting this parameter allowed us to determine the moment with the highest PHA concentration. The application of SBB staining technique was successful, showing that PHA producers trend matches with PHA yield and concentration ones.

References

Alias Z., Tan I.K.P., 2005, Isolation of palm oil utilising, polyhydroxyalkanoate (PHA)-producing bacteria by an enrichement technique, Bioresource Technology, 96, 1229-1234.

Bengtsson S., 2009, The utilization of glycogen accumulating organisms for mixed culture production of polyhydroxyalkanoates, Biotechnology and Bioengineering, 104, 698-708.

Bosco F., Chiampo F., Chimirri F., Marchese F., 2008, Scarti dell'industria alimentare, potenziale utilizzo per la produzione di bioplastiche, Industrie alimentari, 485, 1112-1119.

Bosco, F. Chiampo, F., 2010, Production of polyhydroxyalcanoates (PHAs) using milk whey and dairy wastewater activated sludge. Production of bioplastics using dairy residues, Journal of Bioscience and Bioengineering, 109, 418–421.

Burdon K.L., 1946, Fatty acid material in bacteria and fungi revealed by staining dried, fixed, slide preparations, Journal of Bacteriology, 52, 665-678.

De Lima T. C. S., Grisi B. M., Bonato M. C. M., 1999, Bacteria isolated from a sugarcane agroecosystem: their potential production of polyhydroxyalcanoates and resistance to antibiotics, Revista de Microbiologia, 30, 214-224

Jelen, P., 2003, Whey processing, Encylcopedia of dairy sciences, Vol. 4, Eds. Roginski H., Fuquay J. W., Fox P. F., Academic Press, London, United Kingdom.

Khardenavis A. A., Suresh Kumar M., Mudliar S.N., Chakrabarti T., 2007 Biotechnological conversion of agro-industrial wastewaters into biodegradable plastic, poly β-hydroxybutyrate, Bioresource Technolology, 98, 3579-3584.

Kemp, D.L., Quickenden, J. 1989, Whey processing for profit–a worthy alternative, Resource and Application of Biotechnology–The New Wave, Eds. R. Greenshield, Macmillan Ltd, Basingstoke, United Kingdom.

Lee S.Y., 1996, Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria, Trends in Biotechnology, 14, 431-438.

Loo C. Y., Sudesh K., 2007 Polyhydroxyalkanoates: bio-based microbial plastics and their properties, Malaysian Polymer Journal, 2, 31-57.

Masani, A.M.Y., Parveez G. K. A., Ho C. L., 2008, Transgenic plants producing polyhydroxyalkanoates, Asia Pacific Journal of Molecular Biology and Biotechnology, 16 , 1-10.

Ostle, A.G., Holt, J.G., 1982, Nile Blue A as fluorescent stain for poly-β-hydroxybutyrate, Applied Environonmental Microbioogy, 44, 238-241.

Panesar, P. S., Kennedy J. F., Gandhi D. N., Bunko K., 2007, Bioutilisation of whey for lactic acid production, Food Chemistry, 105, 1–14.

Plasticnews, 2010, <www.plasticnews.com> accessed 16.10.2010.

Plasticseurope, 2010, <www.plasticseurope.com> accessed 20.11.2010.

Redzwan G., Gan S.N., Tan I.K.P., 1997, Short communication: isolation of polyhydroxyalkanoate-producing bacteria from an integrated-farming pond and palm-oil mill effluent ponds, World Journal of Microbiology and Biotechnology, 13, 707-709.

Rella F. G. S., 2009, Produzione di biopolimeri da scarti dell'industria agroalimentare, Tesi di laurea, Dipartimento di scienze dei materiali e ingegneria chimica, Politecnico di Torino, Torino, Italia.

Williamson, D.H., Wilkinson, J.F. 1958 The isolation and estimation of poly-b-hydroxybutyrate inclusions of Bacillus sp., Journal of General Microbiology, 19, 198-209.

Zakaria M. R., Tabatabaei M., Ghazali F. M., Suraini A. A., Shirai Y., Hassan M. A., 2010, Polyhydroxyalkanoate production from anaerobically treated palm oil mill effluent by new bacterial strain Comamonas sp. EB172, World Journal of Microbiology and Biotechnology, 26, 767–774.