Polyhydroxyalkanoates (PHAs)

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Polyhydroxyalkanoates (PHAs) are promising biodegradable and biocompatible polymers that can be obtained through microbial fermentation of agro-industrial byproducts, e.g., milk and cheese whey by using both microbial consortia and pure bacterial cultures.

Keywords: Polyhydroxyalkanoates ; biopolymers ; carbon-rich milk

1. Introduction

Petrol-derived plastic polymers are recalcitrant to biodegradation and persist in natural environments. Approximately 50% of all plastics are bottles and packaging materials, which are improperly disposed and floating (an average of 270,000 tons) on the surface of the sea [1]. Through UV light exposure and other weathering processes, floating plastic wastes form microparticles and nanoparticles that can be ingested by marine turtles, large cetaceans, seabirds, fish, thus reaching human beings through the food chain [2]. These aspects, together with the increasing cost of oil extraction, have prompted researchers to investigate renewable polymers.

PHAs are high molecular weight (about 105 Da) polyesters, produced by bacterial anabolism, that exhibit several features similar to oil-derived plastics with the added value of biodegradability and biocompatibility [3][4]. These advantages result from the fact that the monomeric units of these polyesters are always in the R(-) configuration, due to the stereo-specificity of PHA synthases [5][6]. Therefore, many microorganisms can degrade PHAs using depolymerizing enzymes (e.g., *Pha*Z).

More than 150 naturally biosynthesized PHAs have been identified; however, chemical modification of naturally occurring PHAs [7], as well as the use of engineered bacteria [8] to produce higher performing PHAs, can account for a larger number of biopolymers. Among the different kinds of PHAs, the most interesting from a commercial viewpoint are poly(3-hydroxybutyrate) (P(3HB)) and poly(3-hydroxybutyrate)-co-(3-hydroxyvalerate) co-polymer (P(3HB-co-3HV)) whose physical and mechanical properties are very similar to those of traditional plastics [9].

Currently, PHAs are not competitive with oil-derived polymers because of their high cost [10]. However, the use of inexpensive carbon sources as PHA carbon feedstock, such as industrial and agriculture byproducts, can contribute to a reduction of about 40% of the overall production costs. PHA production using vegetable oils [11], swine wastewater [12], molasses [13], wheat and rice bran [14][15] and activated sludge wastewaters enriched with milk whey [16] has been proposed in the past two decades.

The huge amounts of carbon-rich milk whey make this industrial byproduct one of the most promising [17]. Milk whey is the watery portion after the precipitation of casein and fat from whole milk, and it is rich in lactose, lactoglobulins and lactoalbumins, minerals, and vitamins [18]. Part of the whey produced by the dairy sector is transformed in nutritional supplements or animal feed, while the rest is disposed as waste, causing environmental problems owing to its high biochemical oxygen demand (BOD, 40,000–60,000 ppm) and chemical oxygen demand (COD, 50,000–80,000 ppm) [19]. To increase lactose utilization, milk whey is generally pre-treated to decrease residual lipid and protein concentration [16]. A different way to exploit the caseification supernatant is to cook milk whey to produce ricotta. Ricotta is a low-fat casein-free food, which still contains albumins, globulins, and lactoferrin as its main proteins. Once ricotta has been separated, a supernatant enriched in salts and organic acids called scotta is obtained.

Due to all these considerations, the screening of natural bacteria able to grow on dairy byproducts has attracted and still attracts the attention of several research groups (see the extensive review by Amaro et al. [20]). The use of mixed microbial cultures (MMCs), although they have been associated with lower yields of PHA production, has two advantages: (i) MMCs do not require sterile conditions and (ii) they are able to adapt to changing industrial waste complex substrates (26, 27). In fact, studies on MMCs have shown that they consist of diverse bacterial genera, which change according to the tested fermentation carbon source (28). A very promising approach is the use of open mixed cultures, such as those

from activated sludges, since these bacteria intrinsically display plasticity and versatility to fast changing conditions [21][22]. To date, the use of pure cultures for efficient production of PHA from whey is challenging, because good PHA producers have displayed poor growth on lactose, whereas good lactose utilizers only direct a small part of their metabolism to PHA production [20]. Nevertheless, in spite of these considerations, a strain of *Alcaligenes latus* (Gram-negative) has been described as a good PHA producer when grown on whey [23]. It is evident that the use of pure cultures of lactic acid bacteria (LAB) could support better metabolic performances on whey because LAB have evolved in the milk ecological niche and are therefore well adapted to this environment. [24]. However, their use for PHA production from whey has been poorly explored so far.

2. Discussion

The microbial world can provide a huge number of sustainable alternatives to petroleum-derived chemicals, including plastic polymers [25]. Several bacterial species can accumulate PHAs as carbon and energy storage material. High levels of PHAs can be obtained by *Methylobacterium organophilus* [26], *A. latus* [27], *Rhodopseudomonas palustris* [28], and especially *Cupriavidus necator* (originally called *Alcaligenes eutrophus* and later *Ralstonia eutropha*) [11]. Very recently, PHA production by halophilic archea has been proposed as well [29][30]. However, not all of these microorganisms are able to synthesize sufficient amounts of PHA for large-scale production [31]. PHA's main metabolic role is as an energy reserve molecule, present in the cell as spherical inclusion bodies or granules [32]. Recently, PHAs' additional functions in microbial physiology have also been reported, for example, their role in protecting bacteria from hydroxyl-radical attack [33] [34][35]. Therefore, these molecules do not need to be abundant in the bacterial cell.

On the other hand, bio-based and biodegradable polymers constitute a real advantage only if they can be produced from renewable resources, including industrial byproducts and not food-competing sources [36][37]. *C. necator* is the most studied PHA producing model because of its ability to synthesize large amounts of P(3HB) from simple carbon substrates such as glucose, lactic acid, acetic acid, and P(3HB-co-3HV) from n-alkanoates [38]. Nevertheless, it is unable to metabolize more complex low-cost substrates such as molasses, starchy wastes, or whey. For this reason, mixed cultures of lactic acid producing bacteria such as *Lactococcus lactis* [39] or *Lactobacillus delbrueckii* [40] and *C. necator* have been used to bypass this bottleneck. The microbial ability to use lactose as a low cost carbon source is strictly dependent on the presence of β-galactosidase, a glycoside hydrolase enzyme that catalyzes the hydrolysis of lactose into its monosaccharide components, glucose, and galactose through the breaking of a glycosidic bond [17]. A recent review [20] showed that several microorganisms are able to convert whey lactose into PHA with productivity varying from 0.0035 g/L/h for MMC culture to 5.2 g/L/h for engineered *E. coli* strain. In the work of Raho et al. [41], a multi-step fractionation was used to recover a RCEW fraction containing 12.6% (*w*/*v*) of lactose that was enzymatically hydrolyzed. PHA yields obtained with *Haloferax mediterranei* in bioreactor tests were in the range 7–10 % (*w*/*w*), lower than those obtained in the present work (52% *w*/*w*).

The work of Berwig et al. [23] showed that *A. latus* is able to convert whey lactose into PHA with a productivity of 0.11 g/L/h. Although the yields are not comparable to those obtained in conventional media, discovering new bacterial species that can directly produce high levels of PHA from lactose is a promising step to obtaining economically competitive production of PHAs from whey. On the other hand, using an unknown mixed microbial community to produce PHAs can raise questions regarding biocompatibility when PHAs are to be used in medical applications. Furthermore, approaches exploiting wild-type bacteria can also circumvent the use of genetically engineered strains that requires more controlled production plants.

In the present study, a microbial consortium derived from an activated sludge plant was investigated for its ability to grow on scotta and pre-treated Toma cheese whey and to produce satisfactory amounts of PHAs. Both byproducts supported PHA production by MMC. However, the best result in term of PHA yield (0.52 g/g) and productivity (0.037 g/L/h) was obtained in bioreactor fermentation with scotta whey medium at a controlled pH value. Scotta can be considered as a complete medium for PHA production, without the need for any addition of other salts or substances. The application of MMC in PHA production could represent an advantage from an economic point of view, since sterility is not necessary. Moreover, the use of MMCs represents an interesting option for identifying new PHA producing microorganisms, as described in the previous paragraph [20].

We found that one of the most active and abundant PHA-producing bacterial population was *L. mesenteroides*. Since the microbial consortium was grown in scotta, the real origin of this bacterium is questionable. From one side we can speculate that this strain belongs to the rich multispecies microbiota present in the activated sludge. However, it is also reasonable to assume that it can belong to the scotta microflora, because no sterilization treatment was performed on it prior to fermentation, since when a mixed microbial population is used no selective condition is needed. The viability of

Leuconostoc sp. in non-dairy environments such as activated sludges has been described by Lee et al. [42], along with its attitude to colonizing the milk ecosystem [43]. The most probable hypothesis is that the dairy environment of scotta has promoted a positive selection on the *Leuconostoc* population among the other non-lactic microorganisms. Moreover, possible syntrophic events occurring between activated sludge microbiota and scotta microbial population may have supported improved performances. In the present study, pure cultures of *L. mesenteroides* in synthetic media containing glucose or lactose as a carbon/energy source demonstrated that, in spite of the low cell number, good PHA yield was produced. In glucose-supplemented medium (C/N ratio about 107), the average PHA concentration was 0.09 g/L, with an average yield of 48%. In lactose-supplemented medium (C/N ratio about 112) the average PHA concentration was 0.06 g/L, with an average yield of 38%. This result is consistent with the higher energy expenditure required for galactose (in case of extracellular lactose hydrolysis occurs) or lactose (if hydrolysis occurs inside the cell) internalization and processing, including either permease-based proton symport systems or antiport systems involving galactose extrusion [44]. In the latter case, only half of the carbon substrate is available for intracellular metabolism. Furthermore, the synthesis of the beta-galactosidase enzyme also requires energy. Finally, the original ecological niche in which *L. mesenteroides* was selected (activated sludges) suggests that lactose cannot be the preferred sugar substrate for this strain.

Despite the high growth rates observed in both glucose and lactose-supplemented media, *L. mesenteroides* displayed poor biomass yield. This probably occurred because of a too high medium acidification, owing to the relative sensitivity of this bacterial genus to acidic pH. Actually, being an obligate heterofermenter, *L. mesenteroides,* besides producing lactic acid, also converts part of the monosaccharide substrate into gluconic acid and, in this case, acetic acid, which can lower the pH far below the natural tolerance of this genus (optimal pH around 5.5–5.8) [45]. A low tolerance of this genus to pH lower than 4.3 was previously reported [46]. However, in this study the real cellular damage hindering growth only occurred below pH 3.8. Furthermore, this problem can be easily overcome by performing cultures with regulated pH or by using complex media that support other elements, possibly buffering the environment. This is the case of scotta and whey, media rich in minerals and vitamins that exactly respond to this requirement.

To date, to the best of our knowledge, PHA synthesis in *L. mesenteroides* has never been reported. It would be interesting to outline the possible biosynthetic pathway for PHA production in this strain, but any effort in this direction is merely speculative, and out of the scope of the present investigation.

Considering that the building blocks for PHA generation are common metabolites (i.e., acetate and 2-oxobutyrate) available in almost all cellular systems, *L. mesenteroides* might have acquired the capability to produce esters from these very simple compounds by horizontal gene transfer. Recombination events, promoting enhanced metabolic and biosynthetic capabilities, are especially frequent in both the habitat of the activated sludge and in the whey ecological niche, where mixed and diversified bacterial populations exist. A possible strategy to better elucidate the biosynthetic route for PHA production in this strain is to compare protein expression profiles in control conditions and during PHA synthesis, by using intracellular gel-free proteomics. This approach will give reliable results and, therefore, deserves further investigations in the future to improve knowledge about *L. mesenteroides* biology and biochemistry.

References

1. Eriksen, M.; Lebreton, L.C.M.; Carson, H.S.; Thiel, M.; Moore, C.J.; Borerro, J.C.; Galgani, F.; Ryan, P.G.; Reisser, J. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLoS ONE 2014, 9, e111913.
2. Bouwmeester, H.; Hollman, P.C.H.; Peters, R.J.B. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. Environ. Sci. Technol. 2015, 49, 8932–8947.
3. Rehm, B.H.A. Bacterial polymers: Biosynthesis, modifications and applications. Nat. Rev. Microbiol. 2010, 8, 578–592.
4. Philp, J.C.; Bartsev, A.; Ritchie, R.J.; Baucher, M.-A.; Guy, K. Bioplastics science from a policy vantage point. New Biotechnol. 2013, 30, 635–646.
5. Philip, S.; Keshavarz, T.; Roy, I. Polyhydroxyalkanoates: Biodegradable polymers with a range of applications. J. Chem. Technol. Biotechnol. 2007, 82, 233–247.
6. Byrom, D. Polyhydroxyalkanoates. In Plastics from Microbes: Microbial Synthesis of Polymers and Polymer Precursors; Mobley, D.P., Ed.; Car Hanser Verlag: Munich, Germany, 1994; pp. 5–33.
7. Kim, D.Y.; Hyung, W.K.; Moon, G.C.; Young, H.R. Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. J. Microbiol. 2007, 45, 87–97.

8. Escapa, I.F.; Morales, V.; Martino, V.P.; Pollet, E.; Avérous, L.; García, J.L.; Prieto, M.A. Disruption of β-oxidation pathway in Pseudomonas putida KT2442 to produce new functionalized PHAs with thioester groups. Appl. Microbiol. Biotechnol. 2011, 89, 1583–1598.
9. Sudesh, K.; Abe, H.; Doi, Y. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Prog. Polym. Sci. 2000, 25, 1503–1555.
10. Salehizadeh, H.; van Loosdrecht, M. Production of polyhydroxyalkanoates by mixed culture: Recent trends and biotechnological importance. Biotechnol. Adv. 2004, 22, 261–279.
11. Fukui, T.; Doi, Y. Efficient production of polyhydroxyalkanoates from plant oils by Alcaligenes eutrophus and its recombinant strain. Appl. Microbiol. Biotechnol. 1998, 49, 333–336.
12. Choi, J.-I.; Lee, S.Y. High-Level Production of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) by Fed-Batch Culture of Recombinant Escherichia coli. Appl. Environ. Microbiol. 1999, 65, 4363–4368.
13. Omar, S.; Rayes, A.; Eqaab, A.; Vos, I.; Steinbüchel, A. Optimization of cell growth and poly(3-hydroxybutyrate) accumulation on date syrup by a Bacillus megaterium strain. Biotechnol. Lett. 2001, 23, 1119–1123.
14. Huang, T.-Y.; Duan, K.-J.; Huang, S.-Y.; Chen, C.W. Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by Haloferax mediterranei. J. Ind. Microbiol. Biotechnol. 2006, 33, 701–706.
15. Van-Thuoc, D.; Quillaguamán, J.; Mamo, G.; Mattiasson, B. Utilization of agricultural residues for poly(3-hydroxybutyrate) production by Halomonas boliviensis LC1. J. Appl. Microbiol. 2007, 104, 420–428.
16. Bosco, F.; Chiampo, F. Production of polyhydroxyalcanoates (PHAs) using milk whey and dairy wastewater activated sludge: Production of bioplastics using dairy residues. J. Biosci. Bioeng. 2010, 109, 418–421.
17. Koller, M.; Bona, R.; Chiellini, E.; Fernandes, E.G.; Horvat, P.; Kutschera, C.; Hesse, P.; Braunegg, G. Polyhydroxyalkanoate production from whey by Pseudomonas hydrogenovora. Bioresour. Technol. 2008, 99, 4854–4863.
18. Guimarães, P.M.; Teixeira, J.; Domingues, L. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnol. Adv. 2010, 28, 375–384.
19. Kim, H.O.K.; Wee, Y.J.; Kim, J.N.; Yun, J.S.; Ryu, H.W. Production of lactic acid from cheese whey by batch and repeated batch cultures of Lactobacillus sp. RKY2. Appl. Biochem. Biotechnol. 2006, 131, 694–704.
20. Amaro, T.M.M.M.; Rosa, D.; Comi, G.; Iacumin, L. Prospects for the Use of Whey for Polyhydroxyalkanoate (PHA) Production. Front. Microbiol. 2019, 10, 992.
21. Chua, A.S.; Takabatake, H.; Satoh, H.; Mino, T. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: Effect of pH, sludge retention time (SRT), and acetate concentration in influent. Water Res. 2003, 37, 3602–3611.
22. Lemos, P.; Serafim, L.; Reis, M.A. Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. J. Biotechnol. 2006, 122, 226–238.
23. Berwig, K.H.; Baldasso, C.; Dettmer, A. Production and characterization of poly(3-hydroxybutyrate) generated by Alcaligenes latus using lactose and whey after acid protein precipitation process. Bioresour. Technol. 2016, 218, 31–37.
24. Pessione, E. Lactic acid bacteria contribution to gut microbiota complexity: Lights and shadows. Front. Cell. Infect. Microbiol. 2012, 2, 86.
25. Akaraonye, E.; Keshavarz, T.; Roy, I. Production of polyhydroxyalkanoates: The future green materials of choice. J. Chem. Technol. Biotechnol. 2010, 85, 732–743.
26. Kim, S.W.; Kim, P.; Lee, H.S.; Kim, J.H. High production of Poly-?-hydroxybutyrate (PHB) from Methylobacterium organophilum under potassium limitation. Biotechnol. Lett. 1996, 18, 25–30.
27. Wang, F.; Lee, S.Y. Poly(3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Culture of Alcaligenes latus under Nitrogen Limitation. Appl. Environ. Microbiol. 1997, 63, 3703–3706.
28. Carlozzi, P.; Sacchi, A. Biomass production and studies on Rhodopseudomonas palustris grown in an outdoor, temperature controlled, underwater tubular photobioreactor. J. Biotechnol. 2001, 88, 239–249.
29. Mitra, R.; Xu, T.; Xiang, H.; Han, J. Current developments on polyhydroxyalkanoates synthesis by using halophiles as a promising cell factory. Microb. Cell Factories 2020, 19, 1–30.
30. Ben Abdallah, M.; Karray, F.; Sayadi, S. Production of Polyhydroxyalkanoates by Two Halophilic Archaeal Isolates from Chott El Jerid Using Inexpensive Carbon Sources. Biomolecules 2020, 10, 109.
31. Chanprateep, S. Current trends in biodegradable polyhydroxyalkanoates. J. Biosci. Bioeng. 2010, 110, 621–632.

32. Grage, K.; Jahns, A.C.; Parlane, N.; Palanisamy, R.; Rasiah, I.A.; Atwood, J.A.; Rehm, B. Bacterial Polyhydroxyalkanoate Granules: Biogenesis, Structure, and Potential Use as Nano-/Micro-Beads in Biotechnological and Biomedical Applications. Biomacromolecules 2009, 10, 660–669.
33. Koskimäki, J.J.; Kajula, M.; Hokkanen, J.; Ihantola, E.-L.; Kim, J.H.; Hautajärvi, H.; Hankala, E.; Suokas, M.; Pohjanen, J.; Podolich, O.; et al. Methyl-esterified 3-hydroxybutyrate oligomers protect bacteria from hydroxyl radicals. Nat. Chem. Biol. 2016, 12, 332–338.
34. Batista, M.B.; Teixeira, C.S.; Sfeir, M.Z.T.; Alves, L.P.S.; Valdameri, G.; de Oliveira Pedrosa, F.; Sassaki, G.L.; Steffens, M.B.R.; De Souza, E.M.; Dixon, R.; et al. PHB Biosynthesis Counteracts Redox Stress in Herbaspirillum seropedicae. Front. Microbiol. 2018, 9, 472.
35. Obruca, S.; Sedlacek, P.; Slaninova, E.; Fritz, I.; Daffert, C.; Meixner, K.; Sedrlova, Z.; Koller, M. Novel unexpected functions of PHA granules. Appl. Microbiol. Biotechnol. 2020, 104, 4795–4810.
36. Bugnicourt, E.; Cinelli, P.; Lazzeri, A.; Álvarez, V. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. Express Polym. Lett. 2014, 8, 791–808.
37. Bugnicourt, E.; Schmid, M.; Nerney, O.M.; Wildner, J.; Smykala, L.; Lazzeri, A.; Cinelli, P. Processing and Validation of Whey-Protein-Coated Films and Laminates at Semi-Industrial Scale as Novel Recyclable Food Packaging Materials with Excellent Barrier Properties. Adv. Mater. Sci. Eng. 2013, 2013, 1–10.
38. Ryu, H.W.; Hahn, S.K.; Chang, Y.K.; Chang, H.N. Production of poly(3-hydroxybutyrate) by high cell density fed-batch culture of Alcaligenes eutrophus with phospate limitation. Biotechnol. Bioeng. 1997, 55, 28–32.
39. Tanaka, K.; Katamune, K.; Ishizaki, A. Fermentative production of poly(β-hydroxybutyric acid) from xylose viaL-lactate by a two-stage culture method employing Lactococcus lactis IO-1 and Alcaligenes eutrophus. Can. J. Microbiol. 1995, 41, 257–261.
40. Patnaik, P.R. Perspectives in the Modeling and Optimization of PHB Production by Pure and Mixed Cultures. Crit. Rev. Biotechnol. 2005, 25, 153–171.
41. Raho, S.; Carofiglio, V.E.; Montemurro, M.; Miceli, V.; Centrone, D.; Stufano, P.; Schioppa, M.; Pontonio, E.; Rizzello, C.G. Production of the Polyhydroxyalkanoate PHBV from Ricotta Cheese Exhausted Whey by Haloferax mediterranei Fermentation. Foods 2020, 9, 1459.
42. Lee, E.Y.; Yang, H.S.; Choi, J.E.; Lee, M.R.; Park, J.H. A Study of sewage sludge solubilization by activity of Lactic acid bacteria. Jpn. Soc. Mater. Cycles Waste Manag. 2008, 19, 315.
43. Marion, D.; Sylvie, P.; Veronique, R.; Yann, D. Evolution of the Raw Cow Milk Microflora, Especially Lactococci, Enterococci, Leuconostocs and Lactobacilli over a Successive 12 Day Milking Regime. Int. J. Dairy Sci. 2008, 3, 117–130.
44. Vaughan, E.E.; David, S.; de Vos, W.M. The lactose transporter in Leuconostoc lactis is a new member of the LacS subfamily of galactoside-pentose-hexuronide translocators. Appl. Environ. Microbiol. 1996, 62, 1574–1582.
45. Hemme, D.; Foucaud-Scheunemann, C. Leuconostoc, characteristics, use in dairy technology and prospects in functional foods. Int. Dairy J. 2004, 14, 467–494.
46. Sánchez, J.I.; Martínez, B.; Rodríguez, A. Rational selection of Leuconostoc strains for mixed starters based on the physiological biodiversity found in raw milk fermentations. Int. J. Food Microbiol. 2005, 105, 377–387.