A circular economy approach for poly hydroxybutyrate biosynthesis through *C. necator* DSM 545

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Since their first invention, the petrol chemical plastics have been rapidly and widely diffused worldwide. The huge rising in plastic demand and utilization have resulted in a proportional increasing of plastic pollution (about 6.2 million tonnes (MT) of macro-plastics and 3MT of micro-plastics were lost to the environment out of the 322 MT of plastics produced globally in 2015 (Ryberg et al., 2019)). In this scenario, there is the need of valid, sustainable, harmless, economically competitive, and biodegradable polymers, dealing with circular economy principles. The so called "green polymers", including polyhydroxy alkanoates (PHAs), polylactic acid (PLA) and polybutylene succinate (PBS), are nowadays intensively studied for their applications and as replacement of conventional plastics. With respect to the synthetic and enzymatic polymerization of lactic and succinic acid of PLA and PBS respectively, PHAs polymerization can be mainly performed by a wide repertoire of bioplastics producers bacteria, which are able to store PHAs as carbon sink usually under restricted and nutrient shortage growth conditions (Kourmentza et al., 2017; Shang et al., 2003; Singh Saharan et al., 2014; Verlinden et al., 2007).

This work has been promoted by Regione Piemonte and Novamont® and it is included in the regional and circular economy-based project "PRIME" (Processi e pRodotti Innovativi di chiMica vErde), having the aim to study and develop advanced chemistry and biorefineries processes to produce new biomaterials and products belonging to several economic sectors (agriculture, automotive, textile, food, cosmetic, etc). The first waste substrate, the sugar waste, is furnished by Sedamyl S.p.A, a factory involved in PRIME project.

This research is focused on the poly hydroxybutyrate (PHB) biosynthesis, a member of PHAs, through *Cupriavidus necator* DSM 545 fermentation using two different waste sources as carbon sinks. *C. necator* has the extraordinary capability to accumulate up to 90% of PHB per cell dry weight (CDW), a polymer consisting of only short-chain-length (SCL) monomers, guaranteeing both high biomass and biopolymer yields (Hanisch et al., 2006; Uchino and Saito, 2006). The modified strain *C. necator* DSM 545 owns a constitutive expression of the gene codifying for glucose-6-phosphate dehydrogenase (G-6-PDH), resulting in increased NADPH molecule production, an important cofactor of acetoacetyl-CoA reductase (*phaB*), one of the three enzymes involved in PHB biosynthetic pathway. In this research work, the biopolymer biosynthesis has been induced two different wastes as carbon sinks. The first one is a waste sugar substrate, demineralized and isomerized, containing mainly glucose and fructose (about fifty percent each). This waste substrate has been used combined with another waste substrate, the consumed medium of *Acetobacterium woodii* autotrophic fermentation, whose carbon sink is acetate. This medium has been supplied to *C. necator* DSM 545 during the PHB accumulation phase.

C. necator DSM 545 has been grown following the protocol used by Mozumder et al. (2014), i.e. using a medium containing a carbon source (sugar-based waste substrate) and sources of phosphate, sulphate, magnesium, and metals (Mozumder, Heleen De Wever, 2014). Fermentation has been carried out by keeping pH at 6.8 in a bioreactor (Sartorius®, working volume 0.5 L) firstly using the sugar base waste substrate coming from PRIME supply chain, then acetate from the *Acetobacterium woodii* autotrophic fermentation has been added to the fermentation medium to increase PHB production, after 30 hours. During the test, samples have been taken at 12, 15, 21, 24, 36, 40, 44 and 48 hours from the beginning of fermentation and the biomass has been sampled (total volume of 3 mL) by centrifugation (5 min at 10000 rpm) and derived supernatant, filtered through 0.2-µm filters in PES, analyzed at HPLC using a Resex18 column using H_2SO_4 5 mM in distillated water. The biomass has been dried at 80 °C for 15-20 h. PHB extraction has been performed digesting dried biomass in pyrex tubes using 1 mL of 96% sulphuric acid, in a silicon oil bath at 90°C for 1 h under stirrer agitation. The resulting solution was diluted 1:1000 to be then filtered through 0.2-µm filters in PES and analyzed by HPLC to check the production of PHB.

C. neator can better grow on fructose rather than other sugars, but this modified strain can perform a glucose fermentation too, therefore the waste substrate containing both glucose and fructose (fifty percent each) can be used to ferment this strain. The fed-batch fermentation has been performed supplying and modulating the sugar substrate (12 g L^{-1} at the beginning of fermentation, 5 g L^{-1} after 15h, 24h and 36h, 2 g L^{-1} after 18h, 21h, 42h, and 44h), containing glucose and fructose, and acetate (2 g L^{-1}) after 30h during the late phase of fermentation, which lasted for a total of 48h.

Using this strategy, the highest PHB concentration, about 10 g L^{-1}, has been reached after 35 hours of fermentation at 30°C, at a vvm of 0,5 h^{-1} (using air and a Rushton impeller to oxygenate and mix the culture, and the biomass reached 12 g L^{-1} concentration at the same hour, as shown in figure 2 (about 83% of PHB content has been achieved).

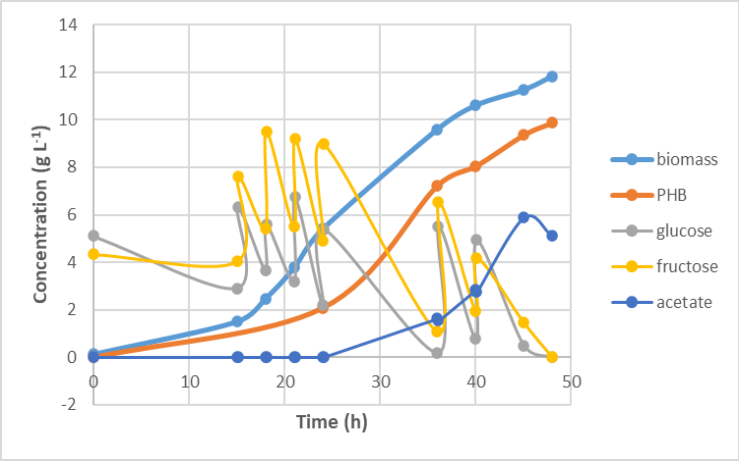

Fig. 1: Fermentation in bioreactor (volume 0,5 L) of *R. eutropha* DSM 545 using sugar-based waste substrate and acetate at different concentration and hours. About 10 g L^{-1} of PHB has been obtained after 48 hours in a cell culture biomass of 12 g L^{-1}.

As shown in fig. 1, acetate still remains in the medium after the culture reached the highest PHB concentration, suggesting that the acetate concentration added could be reduced. Some other strategies to enhance the biopolymer accumulation could be the optimization of the operative conditions, such as those studied by Mozumder et al. (2014) i.e. the application of exponential feeding and an alkali-addition monitoring strategies, and/or by using a three step C/N ratio approach (Garcia-gonzalez and Wever, 2018). This fermentation strategy could be implemented by using both sugar waste substrate and acetate to apply a pH-stat fed-batch feeding strategy in combination with an additional Dissolved Oxygen (DO)-dependent feed.

The development of fermentation based on wastes utilization, in agreement with circular economy perspective, can lead to a production process which can potentially reduce both environmental impact and production costs. Sugar waste and acetate utilization results in a good PHB production in *C. necator* DSM 545; still further studies must be done in order to improve PHB production, by also applying pH-stat and/or DO-stat feeding strategy, and monitoring C/N ratio.

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The authors declare no conflict of interest.

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