**A circular economy approach for *Cupriavidus necator* DSM 545 biosynthesis of poly hydroxybutyrate**

Silvia Bellini1,2, Tonia Tommasi1, Debora Fino1,2\*

*1Dipartimento di Scienze e Tecnologie Applicate, Politecnico di Torino, Turin, Piemonte, 10129, Italy*

*2Italian Institute of Technology, Turin, Piemonte, 10144, Italy*

*\*Corresponding author E-Mail: debora.fino@polito.it*

**1. Introduction**

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 [5]). 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 [3, 6, 7, 9].

This research is focused on the poly hydroxybutyrate (PHB) biosynthesis, a member of PHAs, through *C. necator* DSM 545 fermentation using two different waste sources as carbon sinks. 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.

**2. Methods**

*Cupriavidus necator* DSM 545 has been grown following the protocol used by Mozumder et al. (2014) [4], i.e. using a medium containing a carbon source (sugar-based waste substrate) and sources of phosphate, sulphate, magnesium, and metals [4]. 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 at different concentrations (2, 3, 4 or 5 g L-1) and hours has been added to the fermentation medium to increase PHB production, after 23 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 H2SO4 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.

**3. Results and discussion**

*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 [2]. 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 [8]. 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* 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 and 6 g L-1 after 15h and 21h), containing glucose and fructose, and acetate at different concentration during the whole fermentation, which lasted for 48h. Acetate feeding has been performed as resumed in the following scheme (figure 1):



**Figure 1.** Scheme of fed-batch feeding using the sugar waste coming from PRIME supply chain and pure acetate at different concentration and time during the whole fermentation in bioreactor (Vw 0,5 L).

Using this strategy, the highest PHB concentration, about 7 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 10 g L-1 concentration at the same hour, as shown in figure 2 (about 70% of PHB content has been achieved).



**Figure 2:** Fermentation in bioreactor (volume 0,5 L) of *C. necator*  DSM 545 using sugar-based waste substrate and acetate at different concentration and hours. About 7 g L-1 of PHB has been obtained after 35 hours in a cell culture biomass of 10 g L-1.

As shown in fig. 2, acetate still remains in the medium after the culture reached the highest PHB concentration, suggesting that the acetate concentration added could be reduced; furthermore, an additional step of sugar waste feeding may be added in order to achieve higher biomass concentration. 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) [4] i.e. the application of exponential feeding and an alkali-addition monitoring strategies, and/or by using a three step C/N ratio approach [1]. 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.

**4. Conclusions**

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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