SCALING UP THE ANTIMICROBIAL POLIHYDROXYALKANOATES PRODUCTION

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The purpose

Among the various biodegradable polymers, polyhydroxyalkanoates (PHA) provide a good alternative to the petrochemically-derived plastics. Due to similarity of physical and material properties with the conventional plastics, PHA's can be recommended for the applications in various areas such as food packaging [1].

The most widely studied poly (3-hydroxybutyrate), [P(3HB)], has been found to exhibit useful properties relative to other PHA's,. PH(3B) and its copolymers can be used to make various biodegradable products including films, coated paper and boards, compost bag, dispensable food service-ware, and molded products such as bottles and razors [2].

C. necator (also called *Wautersia eutropha*, Ra*lstonia eutropha*) has been studied most extensively due to its ability to accumulate large amount of PHB from simple carbon sources, for example, glucose, fructose and acetic acid.

However, PHA has still economic shortcomings that limit their use. The major drawbacks of PHA production are the high cost of raw materials (mainly carbon source), low yield, low productivity and the high cost of the down-stream process [3]. Therefore, much efforts and improvements have to be implemented to reduce the costs of the fermentation and downstream processes. For instance, a volumetric productivity of (6.3-25 g L^{-1} day^{-1}) was reported for fed-batch cultivations of the *C. necator* with pulse feeding of soybean oil [4] or jatropha oil [5].

On the other hand, in the area of active packaging, antimicrobial packaging materials have attracted a great deal of research as they have been envisaged as a promising tool to ensure food quality and safety. Among the most widely used antimicrobials, silver nanoparticles (AgNPs) have emerged as one of the most researched technologies to prevent microbial spoilage and microbial outbreaks in food contact plastics and surfaces. In that connection, the large scale-up development of a new generation of renewable, bio-based, biodegradable and nanostructured materials, incorporating AgNPs as antimicrobial, accompanied by the study of their production parameters and physico-chemical properties, are challenges that will necessarily have to be addressed and are the main objectives of the research proposed here.

The process designed in the home institution is based on the lab scale production of PHB from *C. necator* using sodium gluconate as carbon source and incorporating silver nanoparticles as antimicrobial agent. Although the final material obtained from this process had a high antimicrobial activity against food borne pathogens, it had a lower productivity (0.5g PHB L^{-1}), which is deficient for the manufacture of an active package proof of concept. Therefore, an ambitious idea aimed both at scaling up the PHB production and at reducing the production cost was developed in the present Short Term Scientific Mission.

With this aim in mind, and taking advantage of the wide experience on biotechnological topics and the availability of host institution in terms of fermentation equipment (bioreactors, microbiological facilities to work at sterile conditions) and PHA characterization (HPLC, GC), a feeding strategy, namely, DO-stat mode, was evaluated for the production of PHB with used cooking oil as sole carbon source. Thus, during the STSM in the host institution, the work plan was implemented through the following specific topics:

i) The optimization of bioprocess parameters: the bioreactor operation mode, physical-chemical factors such as temperature, pH, stirring, oxygen demand, the concentration and feeding rate of used cooking oil.

ii) The polyhydroxyalkanoates production in a fed-batch 2L bioreactor operated under DO-stat mode and the scaling up to 10L bioreactor. Study of fermentation kinetics.

iii) The polymer extraction and purification. Estimation of cell dry mass and overall volumetric productivity.

The strategy developed will be used to obtain enough material for the synthesis of antimicrobial AgNPs into polymer matrix and for the preparation of active fibres by electrospinning technique for packaging materials, in which the home institution's researchers are expertise.

The work carried out during the STSM

The microorganism used for the production of PHB was *Cupriavidus necator*. The culture was reactivated from stock cultures kept at -80 ºC by inoculation in solid Luria Bertani (LB) medium. The mineral medium used for inoculum preparation and the bioreactor experiments was made base on nutrient requirements employing $(NH_4)_2HPO_4$, K_2HPO_4, KH_2PO_4,$MgSO_4$ and a micronutrient solution containing Fe, Mn, Co, Ca, Cu, and Zn salts. Used cooking oil (UCO) was used in this study as sole carbon source.

Based on screening studies of bioreactor parameters in order to obtain the best bacterial growth and PHA production, the operating conditions were adjusted as described below. The bioreactor cultivation experiments were performed in 2 L or 10 L bioreactors (BioStat B- 106 Plus, Sartorius, Germany). The inoculum was 10% (v/v) of the initial reactor working volume. It was prepared by inoculating *C. necator* into LB medium and incubation in an orbital shaker, at 30 ºC and 200 rpm, for 24 hours. Afterwards, the culture was transferred into mineral medium

supplemented with UCO, incubated for 42 hours, as described above, and used as inoculum for the bioreactor experiments. The temperature was maintained at 30±1 ºC and the pH was controlled at 6.8±0.2 by the automatic addition of NaOH 2M and/or 25% (v/v) NH_4OH. The dissolved oxygen concentration (DO) was maintained at 30% air saturation. During the first 18-20 hours, the experiments were carried out in batch operation mode, which was followed by a fed-batch phase, wherein the UCO was supplied to the culture. The UCO feeding flow rate was automatically controlled as a function of DO concentration. The pH was initially controlled by the addition of NH_4OH to prolong the exponential growth phase and later with NaOH 5M to change the nitrogen supply conditions.

The growth was followed by absorbance measurements at 600nm. For the gravimetric quantification of the cell dry mass (CDM), a sample was periodically withdrawn from the bioreactor. The biomass was washed with n-hexane (1:1, v/v) and deionized water, centrifuged and then lyophilized.

The biomass was further resuspended in chloroform during 24h at 70°C. The solution thus obtained was precipitated in cold methanol (1:10, v/v) under strong stirring. The polymer was collected by centrifugation, dried at 70°C and stored at room temperature.

The main results obtained during de STSM

The DO-stat mode strategy used in these experiments was based on the online measurement of the DO concentration, which tends to increase upon substrate depletion, thus signaling the automatic feeding of UCO to the culture. Figures 1 and 2 present the results obtained for the fed-batch cultivation of *C. necator* in the 2L bioreactor under the DO-stat mode. As can be observed, the culture grew with a specific growth rate of 0.18 h^{-1} and reached an active biomass of 10.2 g L^{-1} after 26 hours of cultivation.

It is important to note that during the first 20 hours, the pH was controlled with ammonium hydroxide, which served as an additional nitrogen source. Thus, the nitrogen availability during the initial batch phase changed the carbon to nitrogen ratio and promoted a faster cell growth. When this time is up, the DO-stat mode was implemented by starting the substrate feeding as a function of the DO concentration that was set at 30% of air saturation. After that, the culture continued to grow and reached a maximum active biomass of 11.2 g L^{-1}, at 40 hours of cultivation. In the next two hours, no changes were observed either the CDM or absorbance at 600nm (Figure 1 and 2).

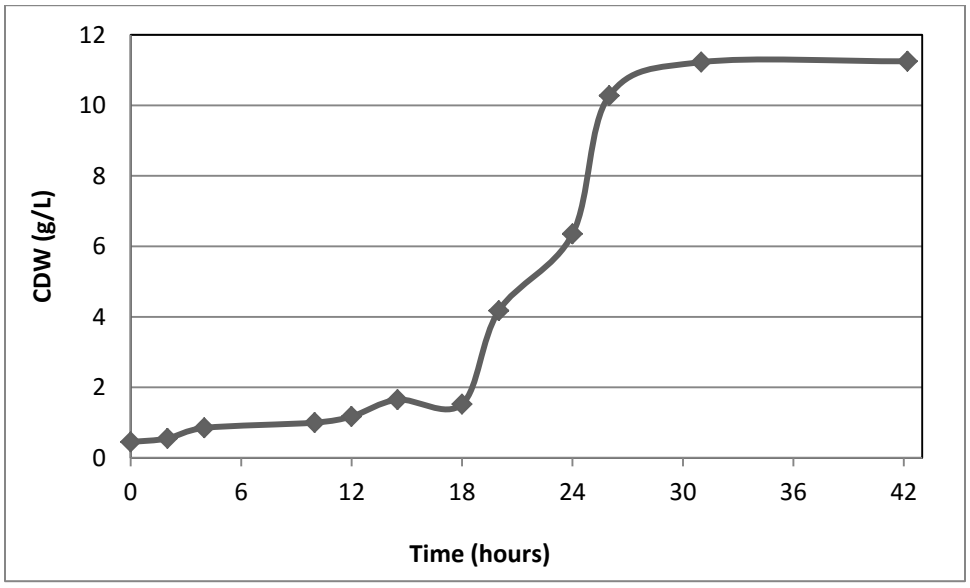

Figure 1. Cell Dry Mass (CDM) vs time for *C, necator* grown in a medium with used cooking oil as sole carbon source

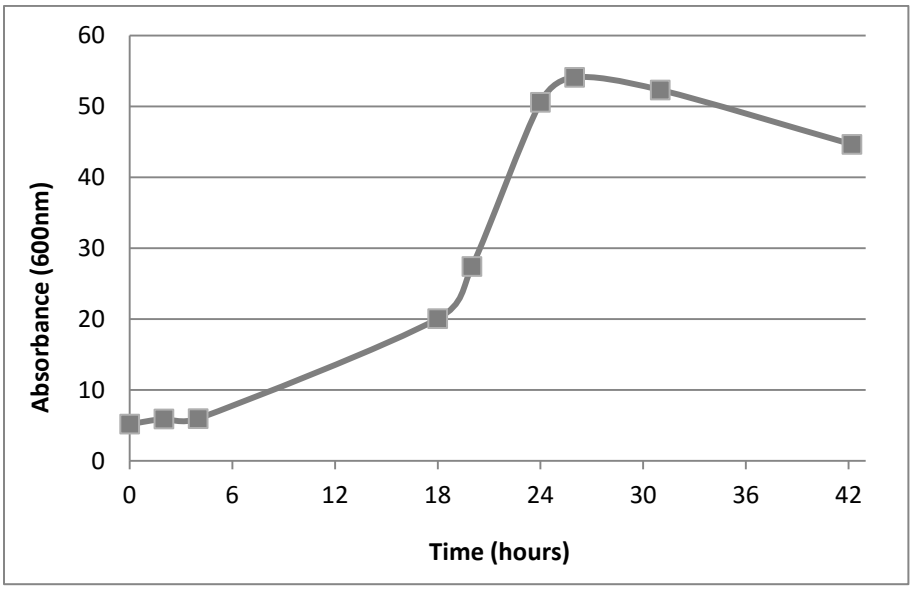

Figure 2. Absorbance at 600 nm vs time for *C, necator* grown in a medium with used cooking oil as sole carbon source

Cell growth obtained during the fermentation process in terms of absorbance at 600nm.

The biomass was the processed as describe above and the PHB extracted was 0.1 g/L, corresponding to an overall volumetric productivity of 1.42 g L-1 day-1. Unfortunately, this value is considerably lower than that obtained in the lab scale procedure developed by the home institution and even than that the reported in the literature.

Because in the 2L bioreactor assay a layer of residual oil was observed in the last three samples withdrawn and considering that this residual oil may be hindering the extraction, a second assay in 10L bioreactor was carried out. In that case, the rate and volume of oil feeding was reduced and also the biomass was washed twice with n-hexane and deionised water to remove residual oil and cell debris that could be interfering with the PHB extraction. However, again, a similar production was achieved.

These results led to the argument that although a high growth is attained with UCO, it does not mean that a PHB production or accumulation of granules inside

the cells can be achieved. Additional research on this topic is required before practical use.

Therefore, for practical reasons, the scaling up of the antimicrobial PHB production from sodium gluconate was the strategy formulated to continue the research. In fact, the first studies are currently being developed also in collaboration with the host institution.

In the medium to long term, future collaborations with the host institution for the characterization of the polymer content, polymer composition and molecular weight of the produced PHB will be done. The obtained material will be used as raw material for the silver nanoparticles addition and for preparation of active fibres by electrospinning techniques by the home institution to make an active package proof of concept and to coat paper. Moreover, the results will be disseminated through participation in the supporting COST action and in others international scientific conferences.

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