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Co-synthesis of medium-chain-length polyhydroxyalkanoates and CdS quantum dots nanoparticles in *Pseudomonas putida* KT2440

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ABSTRACT

Microbial polymers and nanomaterials production is a promising alternative for sustainable bioeconomics. To this end, we used *Pseudomonas putida* KT2440 as a cell factory in batch cultures to coproduce two important nanotechnology materials– medium-chain-length (MCL)-polyhydroxyalkanoates (PHAs) and CdS fluorescent nanoparticles (*i.e.* quantum dots [QDots]). Due to high cadmium resistance, biomass and PHA yields were almost unaffected by coproduction conditions. Fluorescent nanocrystal biosynthesis was possible only in presence of cysteine. Furthermore, this process took place exclusively in the cell, displaying the classical emission spectra of CdS QDots under UV-light exposure. Cell fluorescence, zeta potential values, and particles size of QDots depended on cadmium concentration and exposure time. Using standard PHA-extraction procedures, the biosynthesized QDots remained associated with the biomass, and the resulting PHAs presented no traces of CdS QDots. Transmission electron microscopy located the synthesized PHAs in the cell cytoplasm, whereas CdS nanocrystals were most likely located within the periplasmic space, exhibiting no apparent interaction. This is the first report presenting the microbial coproduction of MCL-PHAs and CdS QDots in *P. putida* KT2440, thus constituting a foundation for further bioprocess developments and strain engineering towards the efficient synthesis of these highly relevant bioproducts for nanotechnology.

1. Introduction

The microbial fermentation of various feedstocks is one of the most sustainable systems for producing chemicals at an industrial scale. This process can yield a wide variety of biochemical compounds, such as polymers, fuels, proteins, amino acids, and, more recently, nanomaterials (Nielsen et al., 2013; Lee and Kim, 2015; Becker et al., 2015; Narayanan and Sakthivel, 2010). Bacterial *Pseudomonas* strains are employed as cell factories for the synthesis of polyhydroxyalkanoates (PHAs) (Poblete-Castro et al., 2014; Prieto et al., 2016). The mechanical and physical properties of PHAs are similar to conventional plastics, thus making this sort of biopolymer one the most promising alternatives for replacing oil-based plastics in the near future (Chen, 2009).

Polyhydroxyalkanoate production is promoted in the cell when the nutritional environment is unbalanced towards high carbon concentrations and the limitation of an inorganic nutrient (*e.g.* O_2, N, or P) (Madison and Huisman, 1999). *Pseudomonas putida* strains, in particular, can naturally produce medium-chain-length-PHAs (MCL-PHAs) from different carbon sources, including glucose, glycerol, fatty acids, and waste materials (Poblete-Castro et al., 2012a). Additionally, *P. putida* is highly versatile metabolically (Santos VAP et al., 2004; Poblete-Castro et al., 2017), a trait that allows this bacterium to cope with adverse growth conditions caused by abiotic factors, including high/low temperatures, elevated pressure, or high metal concentrations (Srivastava et al., 2008; Miller et al., 2009; Follonier et al., 2012). The metal-coping and −conversion abilities of *P. putida* are of particular interest since the wide distribution of heavy metals in the environment, as a result of anthropogenic activities, has risen public and ecological concern due to their high degree of toxicity. Consequently, *Pseudomonas* spp. strains have long been used for bioremediation purposes (Pieper and Reineke, 2000; Wasi et al., 2013). Building on the ability of *Pseudomonas* spp. to diminish metal toxicity in the cell by reducing heavy

Abbreviations: MCL, medium-chain-length; PHA, polyhydroxyalkanoate; QDots, quantum dots

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metal ions, these bacterial strains have recently been exploited to synthesize cadmium-based quantum dots (QDots) (Gallardo et al., 2014). Fluorescent nanoparticles QDots have a wide array of applications, ranging from optical imagining to drug-delivery platforms (Zrazhevskiy et al., 2010). They have initially been used in biomedicine and biotechnology where target organic molecules are labeled with QDots in order to be tracked and differentiated from other molecules in a complex system like live cells. As they display a broadband absorption spectrum than standard fluorophores, this feature enables them to have a long fluorescent lifetime allowing to separate their signal (unique color) from that of shorter lived species (Michalet et al., 2005). QDots nanoparticles have many applications today, most importantly in displays, lighting, photovoltaic, sensor and life science. Market analysts forecast that the market value of QDots devices and component will be about 4 billion USD by 2020.

The current industrial production of QDots is mostly carried out in nonpolar organic solvents, where the temperature used in the process can modulate the size and shape of the nanocrystals (de Mello Donegá et al., 2006). These processes do not address several environmental aspects such as the use of expensive and toxic organic solvents, which can account for up to 90% of the total production costs, and the high input energy needed within the manufacturing process (Jacob et al., 2016). The microbial production of QDots has opened new avenues for a more sustainable synthesis route compared to chemical production. The bio-based synthesis is more energy efficient, requires a lower input of toxic reagents, and generates nanoparticles with increased biocompatibility and tolerance to harsh conditions (Wegner and Hildebrandt, 2015; Ulloa et al., 2016). They can be purified by using discontinuous sucrose density gradient followed by several centrifugation steps (Park et al., 2016). Furthermore, the size and shape of the resulting nanocrystals can be defined by the culture conditions (*e.g.* pH, reducing agents, and temperature) (Sweeney et al., 2004; Bai et al., 2009). Stable nanoparticles can also be coated with polymers and ligands, thus preventing aggregation, toxic effects, and enzymatic degradation (Pandian et al., 2011). A great deal of attention has recently focused on using biopolymers as matrices for encapsulating fluorescent nanoparticles. This emphasis is due to a growing need to target specific cells under *in vivo* conditions (Hezinger et al., 2008; Tomczak et al., 2013). Biochemical coproduction using microbial strains shows promise as a more cost-competitive option to processes that separately synthesize target compounds (Wang et al., 2012; Hara et al., 2014). The simultaneous production of PHAs and rhamnolipids is possible using the human and plant pathogens *Pseudomonas aeruginosa* and *Thermus thermophilus* HB8 (Pantazaki et al., 2011). Cosynthesis is also possible for polyhydroxybutyrate and 5-aminolevulinic acid in a metabolically engineered *Escherichia coli* strain (Li et al., 2016). Notable, several studies report that bacteria capable of producing PHAs exhibit improved survival and stress tolerances, especially when challenged by oxidative stress (Kadouri et al., 2005). Considering the relevant, increasing need for viable coproduction alternatives of biopolymers, the aim of the present study was to co-synthesize MCL-PHAs and CdS QDots in batch cultures using *P. putida* KT2440 as a cell factory. Focus was given to assessing the effects of cadmium on biomass, PHA yields, and the monomer composition of the biopolymer, as well as to several properties of the synthesized QDots and localization of each compound within the cell.

2. Materials and methods

2.1. Strain and growth conditions

The wild-type *P. putida* KT2440 (DSM 6125, DSMZ, Braunschweig, Germany) was used in this study. Cells were kept as frozen stocks in 25% glycerol at −80 °C. To obtain single colonies, it was plated onto Luria Bertani agar plates after one day incubation at 30 °C. Inoculum was prepared by picking up a single colony from the plate and inoculating it into a 50 mL shake flask containing 10 mL of the defined minimal medium (M9) consisting of 12.8 $g{\cdot}L^{-1}$ $Na_2HPO_4{\cdot}7H_2O$, 3 $g{\cdot}L^{-1}$ KH_2PO4, 0.5 $g{\cdot}L^{-1}$ NH_4Cl, 0.5 $g{\cdot}L^{-1}$ NaCl. This medium was autoclaved and subsequently supplemented with 0.12 $g{\cdot}L^{-1}$ of $MgSO_4{\cdot}7H_2O$, trace elements (6.0 $FeSO_4{\cdot}7H_2O$, 2.7 $CaCO_3$, 2.0 $ZnSO_4{\cdot}H2O$, 1.16 $MnSO_4{\cdot}H_2O$, 0.37 $CoSO_4{\cdot}7H_2O$, 0.33 $CuSO_4{\cdot}5H_2O$, 0.08 H_3BO_3) ($mg{\cdot}L^{-1}$) (filter-sterilized), and 20 mM of decanoate as the unique carbon source. The cells were grown under aerobic conditions at 30 °C in an Ecotron incubator shaker (INFORS HT, Switzerland) set at 160 rpm. By taking a calculated volume of the overnight-grown cell suspension (to begin the PHA-accumulating process with an initial OD_{600} of 0.05), the cells were then inoculated into 500 mL baffled Erlenmeyer flasks with 100 mL of culture medium and cultivated in a rotary shaker as described above.

2.2. Minimal inhibitory concentration (MIC)

MIC-$CdCl_2$ of *P. putida* KT2440 was determined over a wide range of metal concentrations (300–1000 $\mu g{\cdot}mL^{-1}$) in LB liquid and minimal medium supplemented with decanoate 5 mM. Each culture was inoculated with a defined volume of grown cells to achieve an initial OD_{600} value of 0.05 in shaking flask (50 mL). Subsequently cells were grown under aerobic conditions at 30 °C in an Ecotron incubator shaker (INFORS HT, Switzerland) set at 160 rpm for 24 h. The MIC value for cadmium was determined as the value for colony forming units (CFU) showing 99% decrease in bacterial growth when compared with the controls, measured by streaking a series of dilutions of *P. putida* cells onto LB agar plates and incubated in an oven at 30 °C for 24 h.

2.3. Biosynthesis of nanoparticles

180 or 360 ($\mu g{\cdot}mL^{-1}$) of $CdCl_2$ were added to the flask culture at 48 or 68 h of cultivation. To induce the biosynthesis of CdS nanoparticles in *P. putida* KT2440, cysteine was then supplemented to the culture (to a final concentration of 1 mM) at 72 h, samples were collected every hour to evaluate CdS QDots formation *via* fluorescence emission.

2.4. Purification of intracellular nanoparticles

50 mL of culture producing QDots were concentrated by centrifugation at 8000 × *g* for 5 min and washed twice with 50 mM Tris-HCl pH 7.4. Cell lysis was carried out using glass beads (MP-Biomedicals) for 3 cycles of homogenization. The suspension was then further sonicated (10 cycles, 15 s on and 30 s off) to achieve full cell disruption. Subsequently, the resulting solution was centrifuged 10 min at 14,000 x *g* and the supernatant was loaded on a Sephadex column G75 for gel filtration. Subsequently, fluorescent fractions were stored at 4 °C for further characterization.

2.5. Zeta potential analysis of the biosynthesized CdS QDots

The mean superficial charge of the different formulations of nanoparticles was determined by Zeta potential (Zetasizer NanoS90, Malvern Instrument, U.K.). The measurements were carried out at 25 °C using cuvettes DTS1070. To resolve the given values three consecutive measurements were performed (Program Zetasizer version 7.11). In each measure the cuvettes were rinsed with MiliQ grade water and dried. Nanoparticles were diluted 10 times for each measure.

2.6. Size distribution of the purified CdS nanocrystals

The mean size of nanoparticles was determinate for nanoparticle using the tracking acquisition (NTA) method (NanoSight NS300, Malvern Instrument, U.K.). The measurements were carried out at 25 °C and processed with the NanoSight software (NTA 3.2 Dev Build 3.2.16). Between each measurement the plate was rinsed ten times in an equal