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Fate of β-hexachlorocyclohexane in the mixed microbial cultures (MMCs) three-stage polyhydroxyalkanoates (PHA) production process from cheese whey

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HIGHLIGHTS

- PHA production could valorize contaminated waste from food/agriculture industry.
- β-HCH did not cause interference with the performance of investigated process steps.
- The PHA production process remove about 99.9% of initial β-HCH content.
- The case of β-HCH can be transferred to other recalcitrant pesticide.

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ABSTRACT

This work aimed to study the fate and effect of β-hexachlorocyclohexane (β-HCH) during several steps of PHA production and purification, by using an artificially contaminated cheese whey (CW) as the feedstock. Most of β-HCH (around 90%) was adsorbed on CW solids and it was removed after the acidogenic fermentation step, when residual CW solids are separated along with anaerobic biomass from the liquid-phase. Purification steps also contributed strongly to the removal of residual β-HCH; overall, the PHA production process removed about 99.9% of initial β-HCH content. Moreover, it has been shown that β-HCH has neither detrimental effect on acidogenic fermentation nor on PHA accumulation, that were performed by using unacclimated mixed microbial cultures.

1. Introduction

The production of biodegradable polymers and the replacement of their petroleum-derived counterparts is one of the goals that should be fulfilled by the sustainable chemical industry. This can be achieved via restructuring current industrial sectors (e.g. food industry) through valorization of by-product and waste streams (Reis et al., 2011).

The biological polyesters known as polyhydroxyalkanoates (PHA) are mainly produced by microbial fermentation processes, and a major challenge is to reduce their production costs. Due to the large impact of the carbon source price, one approach to reduce costs is to use wastes and by-products, given their large availability and diversity, as raw material for PHA production processes. Several studies have been focusing on the use of food and agriculture industries wastes/by-products, such as olive mill wastewater (Campanari et al., 2014; Ntaikou et al., 2014), paper mill wastewater (Jiang et al., 2012), palm oil mill effluent (Din et al., 2012), dairy wastewater (Duque et al., 2014), sugar cane molasses (Bengtsson et al., 2010). It is noteworthy to mention that PHA, when compared to conventional plastics, is biodegradable and biocompatible (Castilho et al., 2009); however, biocompatibility is a crucial point when the PHA is produced from food/agriculture wastes because of the use of commercial chemicals in agriculture, as pesticides, particularly intensified on global scale after World War II (Fischer et al., 2011). These "pioneer" chemicals were organochlorines (OCs), also classified as "persistent organic pollutants" (POPs), due to their lipophilicity and resistance to biodegradation (Luzardo et al., 2012). For these reasons, they accumulate in the biosphere and, although they had been banned in many European countries already in the 1970s, measurable levels may still be found in many foods, especially milk and fat-rich dairy products, such as butter, cheese, etc. Among OCs, the γ-hexachlorocyclohexane (γ-HCH), known as

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lindane, has been used earlier as organochlorine insecticide worldwide. Commercial formulations of hexachlorocyclohexane (HCH) generally contain α, β, γ and δ isomers, which are persistent organic pollutants (Vijgen et al., 2011) with relative toxicity in the order γ > α > δ > β. Lindane is extremely toxic to humans and harmful for environment (Chaurasia et al., 2013). Many papers reported the presence of lindane and its isomers, together with other pesticides, in fresh and pasteurized cow's milk, butter, fresh white cheese, feta cheese, yoghurt, casein, butter-oil, and whey powder. The range of HCH isomer concentration (as sum of each isomer) is extremely wide (0.001–4.0 mg L^{-1}) probably because the samples were collected from different dairy products coming from different countries, both in and outside Europe (Luzardo et al., 2012; Zidan et al., 1994; Mallatou et al., 2002; John et al., 2001; Radzyminska et al., 2008; Ilori et al., 2008; Salem et al., 2009; Georgescu et al., 2011; Kampire et al., 2011). Only few studies demonstrated that HCH isomer concentration could be affected by effects of processing and storage of dairy products (Zidan et al., 1994; Mallatou et al., 2002). Furthermore, there is no study suggesting that HCH, present in raw material (especially coming from food/dairy industry) used as feedstock for biopolymer production, could migrate to final PHA product, in mixed microbial culture (MMCs) based processes.

For these reasons the β-hexachlorocyclohexane (β-HCH) isomer was chosen as representative of the OCs pesticides used in agriculture, and the present paper aims to investigate its effect and its fate during several steps of a typical PHA production process, by using cheese whey as carbon source. β-HCH was chosen for its physical–chemical properties, which make it the most recalcitrant isomer to the biological activity (Xiao et al., 2004). Aerobic HCH biodegradation was investigated in several batch and continuous studies by using pure and mixed microbial populations in soil, slurry, sludge, and culture media (Bhatt et al., 2009). Accordingly, in most of the experiments, the β-HCH was found to be the most recalcitrant for degradation. Some cases reported the complete degradation of β-HCH, with pure culture of microorganisms: it was demonstrated that *Sphingomonas paucimobilis*, *Pseudomonas aeruginosa*, *Pseudomonas paucimobilis* and *Sphingobium* had specific genes responsible for the degradation of β-HCH in liquid culture as well as contaminated soils, under aerobic conditions (Bhatt et al., 2009; Nagata et al., 2005; Ito et al., 2007). Moreover, Quintero et al. (2005, 2006) quantified the degradation of γ, α, β, δ-HCH in liquid and slurry cultures via reductive dehalogenation, assessing the efficiency of an anaerobic reactor. The most recalcitrant isomers were β- and δ-HCH, which were removed up to 90% only after 50 days of operation at the best process condition of the anaerobic reactor. The presence of intermediate metabolites was also observed: pentachlorocyclohexane (PCCH), tetrachlorocyclohexene (TCCH), tri-, di- and mono-chlorobenzenes.

Present study was performed by taking into account the well known 3-step production process based on using open microbial communities (Reis et al., 2011; Dias et al., 2006). For the sake of simplicity, only two steps of the biological process were investigated here, i.e. the cheese whey fermentation (first stage) and the PHA accumulation (third stage). Furthermore, the β-HCH fate was also followed during the downstream processing: NaClO treatment for PHA extraction, lyophilization and $CHCl_3$ polymer purification.

2. Methods

2.1. Addition of β-HCH to cheese-whey and evaluation of its distribution between solid and liquid phases

Cheese whey solution (CW) was prepared by re-hydrating cheese whey powder (CWP) in tap water (at 20 g L^{-1}); the CWP had been supplied by Estação Agronómica Nacional Edificio IBET/ITQB, Oeiras – Portugal. Subsequently, 400 μL of a standard solution of β-HCH in toluene (β-HCH PESTANAL® 33376, Sigma–Aldrich) was added to CW in order to obtain a β-HCH initial concentration of 1000 μg L^{-1}, maintaining the solution under mixing with magnetic stirrer for 1 day at room temperature.

β-HCH analytical determination in cheese whey was carried out as follows:

- 1.0 mL of cheese whey was sampled under intense stirring and directly used for analysis of total β-HCH concentration (β-HCH_{TOT});
- 5.0 mL of cheese whey were sampled and centrifuged (8500 rpm or 8079*g*, for 20 min); then 1.0 mL of supernatant was sampled and used to quantify β-HCH dissolved fraction (β-HCH_{cent});
- Residual supernatant was filtered (cellulose-acetate filter; 0.45 μm porosity) and 1.0 mL of filtered solution was used to determine β-HCH filtered fraction (β-HCH_{filt}).

2.2. Fermentation batch tests

The CWP was dissolved in tap water at final concentration of 15 g L^{-1} (25.5 g COD L^{-1}). β-HCH solution was prepared in toluene at final concentration of 8.0 g β-HCH L^{-1} and added to different aliquots of cheese whey solution, in order to obtain initial β-HCH concentration ranged from 0 to 2000 μg L^{-1}.

Fermentation batch tests were carried out in 500 mL glass bottles with perforated screw caps and latex under-caps. They were filled with 350 mL of CW (15 g L^{-1}; 25.5 g COD L^{-1}) and 50 mL of anaerobic inoculum. The inoculum was obtained from a mesophilic anaerobic digester of wastewater treatment plant in the North Italy (volatile suspended solids, VSS, 5.7 g L^{-1}). In order to set and maintain anaerobic conditions, each bottle was fluxed with N_2 (at the beginning of the test as well as after each sampling). The initial pH of each test was equal to 7.3. Four fermentation tests (two replicates for each test) were performed simultaneously for 4 weeks, by using cheese whey at different initial β-HCH concentration: 0 μg L^{-1} (A), 400 μg L^{-1} (B), 1000 μg L^{-1} (C) and 2000 μg L^{-1} (D).

During the fermentation test, all bottles were kept at 25 °C in thermostatic bath. For β-HCH analysis, 6.0 mL of anaerobic slurry were sampled in triplicate, at the beginning of the test, and once a week until the end. The samples were processed as previously described for the determination of β-HCH concentration in the slurry, in the centrifuged supernatant, and in the filtrate.

For volatile fatty acid (VFA) analysis, 2 mL of slurry was sampled twice a week; all samples were filtered on 0.45 μm glass-fiber filters and the filtrates analyzed by gas-chromatography. After each sampling, the bottles were opened for the pH control; CaO was added to maintain pH values around 6.0. Finally, each bottle was maintained under N_2 flux for 20 min to re-establish anaerobic conditions. At the end of the fermentation tests, the bottles of the same series were mixed and centrifuged; then the supernatant was collected to be used for the following accumulation tests.

2.3. Batch PHA accumulation tests with fermented cheese whey (FCW)

The supernatant of centrifuged FCW from the series A and D was used in two PHA accumulation tests, that were performed in parallel in two batch reactors of 500 mL. The batch reactors were inoculated with an aerobic biomass which was cultivated in parallel in a Sequencing Batch Reactor (SBR) (Valentino et al., 2014).

In the accumulation test with β-HCH, 120 mL of SBR biomass was mixed with 25 mL of tap water, 200 mL of aerobic mineral medium (Valentino et al., 2014) and 150 mL of supernatant of centrifuged FCW (series D); 400 μL of β-HCH standard solution (β-HCH 1.30 g L^{-1} in ethanol) were added in order to obtain a β-HCH initial