Brief Report

Syngas obtained by microwave pyrolysis of household wastes as feedstock for polyhydroxyalkanoate production in *Rhodospirillum rubrum*

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Summary

The massive production of urban and agricultural wastes has promoted a clear need for alternative processes of disposal and waste management. The potential use of municipal solid wastes (MSW) as feedstock for the production of polyhydroxyalkanoates (PHA) by a process known as syngas fermentation is considered herein as an attractive bio-economic strategy to reduce these wastes. In this work, we have evaluated the potential of *Rhodospirillum rubrum* as microbial cell factory for the synthesis of PHA from syngas produced by microwave pyrolysis of the MSW organic fraction from a European city (Seville). Growth rate, uptake rate, biomass yield and PHA production from syngas in *R. rubrum* have been analysed. The results revealed the strong robustness of this syngas fermentation where the purity of the syngas is not a critical constraint for PHA production. Microwave-induced pyrolysis is a tangible alternative to standard pyrolysis, because it can reduce cost in terms of energy and time as well as increase syngas production, providing a satisfactory PHA yield.

Introduction

The development of a bio-based industry requires the production of chemicals and biomaterials to be settled on the exploitation of non-fossil carbon resources. Municipal solid wastes (MSW) and other organic waste resources, such as agricultural residues and sewage sludge contain significant reusable carbon fractions suitable for eco-efficient valorization processes. These resources take advantage over fossil sources as they are abundantly available, do not require additional production costs and are substrates that do not compete with human nutrition chain.

In this context, thermochemical conversion technologies of wastes, other than combustion, such as pyrolysis and gasification are becoming widely accepted as suitable valorization alternatives for non-fossil resources (Arena, 2012; Tanigaki et al., 2013). For instance, the gasification of organic wastes produces syngas (CO+H₂) that can be used as chemical platform for the production of bulk chemicals such as ethanol, butanol, acetic acid or butyric acid (Munasinghe and Khanal, 2010). In this regard, the microbial bioconversion of thermochemically processed wastes presents a highly attractive potential nowadays (Koutinas et al., 2014; Drzyzga et al., 2015) (Fig. 1). Of special interest is the development of the chemical industry devoted to produce bio-based and biodegradable plastics, such as polyhydroxyalkanoates (PHA), with many applications including biomedical uses (Reddy et al., 2003; Chen and Wu, 2005; Madbouly et al., 2014; Drzyzga et al., 2015). These bioplastics represent an alternative to oil-derived plastics and are degraded by many microorganisms, having gained importance in the last years (Keshavarz and Roy, 2010; Nikodinovic-Runic et al., 2013).

The production of syngas from biomass and organic wastes is mainly accomplished by means of gasification...
processes (Mahinpey and Gomez, 2016), although new emerging thermal conversion technologies, such as the microwave-induced pyrolysis (MIP) has been reported as an effective and greener way to increase the waste conversion to H2 and CO (Budarin et al., 2011; Beneroso et al., 2013, 2014, 2015a,b,c). The attractiveness behind MIP is the fact that microwaves are able to induce an instantaneous and volumetric heating within the samples and thus, overcoming heat transfer constraints as a result of the low thermal conductivity of biomass and bio-wastes (Beneroso et al., 2015b; Beneroso and Fidalgo, 2016a). In addition to syngas and tars, a carbonaceous solid fraction (known as char) is produced during MIP. This fraction has a low value owing to its high ash content, although this can be used as a solid fuel or for soil amendment purposes. Beneroso et al. (2016b) has recently reported the potential use of char as an additive to soils, and particularly, the char obtained from MSW can be added to soil for carbon sequestration without negatively affecting the soil fertility. Therefore, MIP is an attractive alternative within thermochemical technologies for syngas production (Beneroso et al., 2015b).

Despite the toxicity of CO for most organisms, several microorganisms can use CO as source of carbon and/or energy for growth. Rhodospirillum rubrum, which is the type strain for the Rhodospirillaceae family, is able to grow under a broad variety of conditions, including syngas (Do et al., 2007). This bacterium can utilize CO under anaerobic conditions as a sole carbon and energy source in the presence or absence of light (Kerby et al., 1995; Revelles et al., 2016a,b). It is important to emphasize that R. rubrum has a photosynthetic apparatus involved in the photosynthesis on anaerobic conditions. When exposed to CO, both a CODH and a CO-insensitive hydrogenase are induced catalysing the oxidation of CO into CO2 and H2. Approximately, 40% of the carbon fraction of syngas (CO2 and CO) is assimilated into biomass. Although R. rubrum is a highly versatile bacterium that possess the Calvin–Benson–Bassham cycle, it has been recently shown that other carboxylases than Rubisco are actively incorporating CO2 from syngas into biomass (Revelles et al., 2016a,b). Furthermore, R. rubrum is an attractive syngas utilizer for its ability to produce PHA as an energy storage molecule during the fermentation process. We recently reported around 20% of poly(3-hydroxybutyrate) (PHB) production on this microorganism during syngas fermentation both in darkness and/or light (Revelles et al., 2016a,b). Other studies reported the heterologous overexpression of different genes encoding pyridine nucleotide transhydrogenases (pntAB, udhA) and acetooacetyl-CoA reductases (PhaB) in R. rubrum S1, during the synthesis of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(HB-HV) from syngas (Heinrich et al., 2015). This engineered bacterium was pointed out as a promising production strain for syngas-derived, second-generation biopolymers, increasing the potential of this strain for industry application.

The utilization of MSW-derived syngas for the sustainable production of PHB is shown in this work. Of particular interest is the innovative use of MIP to achieve this goal, as this technology provides a syngas able to induce an efficient growth of R. rubrum, reducing cost in terms of energy and time at a much higher syngas productivity compared with syngas from conventional pyrolysis technologies.

**Results and discussion**

**Growth of R. rubrum in microwaving versus synthetic syngas**

An organic fraction from a MSW provided by ABENGOA BIOENERGÍA from a landfill in Seville (Spain) was used in this study. The MSW fraction was subjected to removal of moisture and inert solids, such as glass or metals. After this pre-treatment, the fraction size was

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reduced to 1–3 mm. The characterization of the sample can be found somewhere else (Beneroso et al., 2015a). To prepare syngas, the waste was subjected to MIP in a microwave oven (Beneroso et al., 2015a). The char from previous pyrolysis experiments was used as microwave receptor to induce the pyrolysis due to the low capacity of organic wastes to absorb microwaves. When the microwaves pass through the sample, they are absorbed by the receptor and the temperature increases, allowing the heat to be conducted to the waste to reach a temperature high enough to start the pyrolysis. As the pyrolysis proceeds, the waste is carbonized and is then able to absorb microwaves, so that from that point on it can be directly heated by microwave radiation. The detailed methodology has been described previously (Beneroso et al., 2015a). Table 1 shows the syngas composition of the MIP compared with the standard synthetic syngas used for bacterial fermentation (Revelles et al., 2016a,b).

Starter cultures of *R. rubrum* (ATCC 11170) were grown under anaerobic conditions on SYN medium (Revelles et al., 2016a) supplemented with 15 mM fructose at 30°C during 48 h until stationary phase (OD600 1.5). This culture was used as pre-inoculum for syngas fermentation.

Syngas experiments were done in bottles of 100 mL containing 20 mL of SYN medium supplemented with 10 mM acetate as we had previously demonstrated that the presence of acetate during syngas fermentation favours PHB accumulation (Revelles et al., 2016a,b). Before adding syngas, the closed degasified serum vials were subjected to 1 min vacuum-purge and the atmosphere was further saturated with syngas, either synthetic or microwave-derived syngas, to 1 atm of pressure. This procedure for syngas feeding was repeated every day.

*R. rubrum* is a highly versatile bacterium that can ferment syngas either in dark or in light. On anaerobic conditions when the cells are exposed to light, the photosynthesis becomes active being this an extra energy source. Therefore, the potential impact of light on cell physiology during syngas fermentation was also evaluated. A syngas sample obtained from the MIP process and the synthetic syngas were used as substrates for syngas fermentation in parallel cultures of SYN medium supplemented with 10 mM of acetate in darkness and in light. A very detailed protocol for culturing *R. rubrum* in syngas both in darkness and in light can be found in Revelles et al. (2016a,b).

Table 2 shows the fermentation kinetic parameters, such as growth rate, $\mu$; acetate uptake rate, $Q_s$; and biomass dry weight production yield. None difference in the growth rate $\mu$ was observed among the two different syngas tested in light or darkness, i.e., 0.030 h$^{-1}$ and 0.022 h$^{-1}$ respectively. In both syngas, a higher growth rate was detected in light. It is worthy to stress that light has a positive impact in the growth rate, likely due to the contribution of the photosynthesis in the energetic status of the cell. The small presence of hydrocarbons (methane, ethylene and ethane) within the MIP-derived syngas (Table 1) does not have a harmful effect on bacterial growth. By high-performance liquid chromatography analysis, the presence of extracellular products such as malate, succinate, propionate, pyruvate and/or formate were measured, but none of them were detected in the supernatant. Interestingly, when MIP-derived syngas was used in the fermentation process, 1.5- to 2-fold higher acetate uptake rate was found compared with synthetic syngas in light and darkness respectively (Table 2). This effect was observed in the yield of biomass from acetate as well, being 1.5-fold higher in MIP-derived syngas than in synthetic syngas (Table 2).

### CO consumption and PHB production during the syngas fermentation

CO assimilation in *R. rubrum* is associated to the CODH enzyme that catalyses the oxidation of CO into CO$_2$. When the cells are growing on syngas plus acetate, the carbon fraction of syngas is incorporated into biomass and PHB by the activity of different carboxylases of the central carbon metabolism (Revelles et al., 2016a,b). Acetate assimilation involves two steps: the ferredoxin-dependent pyruvate synthase (PFOR) enzyme and the ethylmalonyl-CoA pathway (Revelles et al., 2016a,b). The use of acetate during syngas fermentation is critical to achieve PHB accumulation, where approximately 40% of PHB total carbon backbone comes from the carbon

| Gases          | Synthetic | MIP     |
|----------------|-----------|---------|
| CO             | 40        | 27      |
| CO$_2$         | 10        | 6       |
| H$_2$          | 40        | 37      |
| N$_2$          | 10        | 26      |
| CH$_4$+C$_2$H$_4$+C$_2$H$_6$ | 0       | 4       |

*a.* Synthetic syngas was provided by Air Liquide (Air Liquide, www.airliquide.com). Composition of microwaving syngas was determined in a gas chromatograph (GC, Agilent 7890A) equipped with a TCD and two columns connected in series (80/100 Porapak Q and 70/80 Molesieve 13X). The initial oven temperature was 30°C, which was maintained with an isothermic step of 5 min. It was then programmed with a rate of 25°C min$^{-1}$ until reached 180°C. The injector and detector temperatures were 150 and 250°C respectively. Helium (Air Liquide, www.airliquide.com) was used as carrier gas. Prior to the measurements, the gas analyser was calibrated by a standard gas and a calibration curve (Airliquide.com) was used as carrier gas. Before the measurements, the gas analyser was calibrated by a standard gas and a calibration curve (Airliquide.com) was used as carrier gas.

The synthetic gas was balanced with N$_2$ while in the mixture of gases from MIP, the N$_2$ content is due to the N$_2$ used as carrier gas but not released in the pyrolysis process.
fraction of syngas and the remaining fraction comes from acetate (Revelles et al., 2016a,b). PHB is produced by a biosynthetic pathway consisting of three different enzymatic reactions from acetyl-CoA. The first step is the condensation of two acetyl-CoA molecules into acetoacetyl-CoA by 3-ketothiolase (catalysed by the enzyme PhaA). Then, acetoacetyl-CoA reductase (PhaB) allows the reduction of acetoacetyl-CoA by NADH to 3-hydroxybutyryl-CoA. Finally, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB synthase (PhaC).

The CO consumption at the end of the growth curve for each different syngas fermentation process was monitored. As no difference was registered between light and darkness, the CO consumption was analysed on light. A sample (1 mL) from the head-space of the culture bottle was taken at time zero and after 48 h of incubation in light. The differences in the final CO values were measured using a gas chromatograph equipped with a thermal conductivity detector (TCD). The results on CO consumption as percentage of gas converted and the uptake of CO are shown in Tables 2 and 3 respectively. The CO conversion registered was of about 40% in both synthetic and MIP-derived syngas. Although in both cases the % of CO conversion does not differ, the final amount of metabolized CO is half for MIP syngas than synthetic syngas (Table 3, 0.19 versus 0.44 mmoles of CO respectively). The lower CO uptake (see Table 2) determined for MIP syngas is compensated with a higher acetate uptake (Qs) observed in this syngas to keep a constant growth rate.

PHB production was quantified from cells harvested from cultures grown in syngas by centrifugation at 8000 g for 15 min at 4°C (Eppendorf Centrifuge 5810R). Cells were then washed twice in distilled water and lyophilized in Cryodos-50 (Telstar, Terrasa, Spain) at –56°C and 10⁻² mbar. Furthermore, PHB monomer composition and cellular PHB content were determined by gas chromatography (GC) of the methanolysed polyester. Methanolysis was carried out by suspending 5–10 mg of lyophilized cells in 0.5 mL of chloroform and 2 mL of methanol containing 15% sulfuric acid and 0.5 mg mL⁻¹ of 3-methylbenzoic acid (internal standard), followed by an incubation at 80°C for 7 h. After cooling, 1 mL of demineralized water and 1 mL of chloroform were added and the organic phase containing the methyl esters was analysed as described previously (de Eugenio et al., 2010). A standard curve from 0.5 to 2 mg of PHB (Cat: 36,350-2; Sigma, San Luis, Misuri, United States) was used to interpolate sample data.

Table 2 shows the PHB content of cells of R. rubrum from MSW-derived syngas versus synthetic syngas. When light was used as an extra source of energy, the percentage of PHB detected remained similar regardless of the source of syngas (16 ± 5% and 20 ± 1% respectively). But, a small reduction was detected in cells grown on darkness (28 ± 10% and 10 ± 1% respectively). Furthermore, granules of PHB can be clearly observed in R. rubrum grown in MIP-derived syngas by transmission electronic microscopy during syngas fermentation when using MIP-derived syngas (Fig. 2).

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In this work, it has been shown that the use of wastes-derived syngas did not affect the growth rate of \( R. \ rubrum \) being of \( \sim 0.02 \ \text{m}^{-1} \), but an increase in the acetate uptake rate was found indicating a higher consumption of acetate that will compensate the lower CO consumption observed in MIP syngas, although CO conversion does not change. The lower CO consumption could be explained by the lower percentage of CO presence in MIP syngas. Interestingly, the accumulation of PHB observed in cells grown with MIP-derived syngas was similar to that found on synthetic syngas, especially when they were exposed to light. These results are a proof of concept of the potential of pyrolysed MSW as feedstock for the production of bioplastics. Furthermore, the robustness and versatility of \( R. \ rubrum \) to accumulate PHB regardless of the composition of the syngas increases the industrial interest of this bacterium to be used in syngas fermentation without introducing a previous costly step for syngas purification. The capability of this microorganism to synthesize other polymers different from PHA of industrial interest from different carbon sources has been recently shown in an engineering strain (Heinrich \textit{et al.}, 2015) broadening its potential to be used in biopolymers production industry. In summary, this work represents the first experimental demonstration that MIP of MSW followed by syngas fermentation can become a valuable environmentally friendly strategy for waste treatment rendering high value-added products such as bioplastics.

**Conflict of interest**

The authors declare no conflict of interest.

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