



## Regular article

# Production of poly(3-hydroxybutyrate) by simultaneous saccharification and fermentation of cereal mash using *Halomonas boliviensis*



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## ABSTRACT

The production of biodegradable polymer poly(3-hydroxybutyrate) (PHB) by the halophilic bacterium *Halomonas boliviensis*, using cereal mash as the carbon source, was carried out in this study. Simultaneous saccharification and fermentation (SSF) methodology, commonly used in first generation (1G) biorefineries, was applied in order to evaluate the integration of PHB production in a bioethanol plant. Separate hydrolysis and fermentation (SHF) was compared to the SSF process based on the results of overall mass balance and productivities. The highest PHB production ( $26 \text{ g L}^{-1}$ ) was obtained by applying the SSF configuration, which was 60% higher than the value reached with the SHF mode. Moreover, the application of the SSF mode permitted a 47% reduction in the overall processing time. This study demonstrates, for the first time, the feasibility of applying an SSF process for producing PHB from a real cereal mash and its integration within a 1G ethanol biorefinery.

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## 1. Introduction

Plastics are essential materials for contemporary society. In 2014, 59 Mt of plastic was manufactured in Europe, of which 25.8 Mt was discharged as waste. Only 69.2% of the total plastic waste was recovered through recycling or used for energy production [1], with the remaining fraction posing a major environmental issue. A recent study estimated that 250 kt of plastic is floating adrift in the oceans [2], thereby directly affecting marine fauna, as such debris is known to entangle or be ingested by marine life [3]. These types of materials should also be considered as hazardous wastes due to their pollutant sorption ability [4]. To overcome such issues, our consumer ethics and consumption rates must change, especially to reduce the generation of plastic waste. Moreover, conventional petroleum-based plastics must be substituted with environmentally friendly biopolymers in several applications.

One such biopolymer is the family of polyhydroxyalkanoates (PHAs), which are produced and stored intracellularly by many microorganisms as an energy and carbon reserve. The homopolymer, poly(3-hydroxybutyrate) (PHB) is one of the most extensively

studied PHAs, since its mechanical properties are similar to those of petroleum-based thermoplastics, such as polyethylene or polypropylene, and it is the PHA that is most commonly produced by wild type bacteria [5].

A promising producer of PHB is *Halomonas boliviensis*, which is a moderate halophile that produces high PHB yields (50–80 wt.%) when cultivated on different kinds of carbon sources, such as glucose [6,7], sucrose [8], volatile fatty acids [9], and hydrolysed starch [10]. Due to its high salt requirements for growth, an open unsterile fermentation process can be successfully developed [11]. Concomitantly, if no sterilization is required, a cheaper bioreactor material can be used and the cost related with the energy consumption is reduced [12].

Despite the attractive advantages of bioplastics, such as biodegradability and biocompatibility, PHAs production costs still cannot compete with those of petroleum-based plastics [13]. The costs of PHAs production are mainly determined by the price of the carbon source used in the fermentation process and the downstream step [14]. Therefore, the development of fermentation strategies and the search for cheap carbon sources are of special importance.

Agricultural residues were studied as cheap carbon sources for PHA production by Van-Thuoc et al. [15]. However, in that study, wheat bran and potato residues gave a low final PHB concentration, of only  $4 \text{ g L}^{-1}$ . Cereal crops, which are rich on starch and are also

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relatively cheap carbon sources, have not yet been used with *H. boliviensis* to produce PHB. Only synthetic starch hydrolysate has been tested with this bacterium [10] and, in comparison with Van-Thuoc et al. [15], the PHB content was not improved. Koutinas et al. [16] obtained better results (51 g L<sup>-1</sup> of PHB) when they studied the production of PHA from wheat by applying a two-step process for hydrolysis and fermentation using *Ralstonia eutropha*.

Of the various cereals used in the European bioethanol industry, corn kernel is the most used [17] and was therefore chosen to study the PHB production with *H. boliviensis*. During the bioethanol process, after the milling of the grain and the gelatinization of the cereal mash at high temperature, a two-step hydrolysis process is applied. First, a thermostable  $\alpha$ -amylase is added during the liquefaction step at 70–80 °C for quick and random hydrolysis of the  $\alpha$ -1,4 bonds. This step greatly reduces the size of the starch polymer. Secondly, glucoamylase converts the liquefied starch into glucose. Hydrolysis with glucoamylase can either be performed together with fermentation in a single-step process known as simultaneous saccharification and fermentation (SSF), or in a two-step process called separate hydrolysis and fermentation (SHF).

The main advantage of SHF is that each step can be performed using the optimal conditions for the enzyme or the microorganism, while with SSF, a compromise must be found. However, a major drawback of SHF is the end-product inhibition of glucoamylase by glucose. SSF was first performed by Takagi et al. [18] and was shown to be superior to SHF in several cases [19–21]. The advantages of the SSF process stem from the continuous and slow production of glucose. This reduces both the risk of microbial contamination and the initial osmotic stress due to the low glucose concentration at the beginning of the fermentation, and is generally more energy-efficient [22].

In addition to ethanol, various bioproducts, such as butanol [23], monosodium glutamate, lactic and succinic acids [24,25], were obtained using the SSF technique. Dahman and Ugwu [21] evaluated the production of PHB from wheat straw by *Ralstonia eutropha* by applying the SHF and SSF process. However, the application of an SSF process to produce PHA from corn mash and its integration in a first generation biorefinery scheme has not been previously evaluated.

The aim of this study is to evaluate the feasibility of PHB production from corn mash by *H. boliviensis* using an SSF process and the integration of the biopolymer production within a 1G-biorefinery platform.

## 2. Materials and methods

### 2.1. Bacterial strain and maintenance

The Gram-negative bacterium *H. boliviensis* LC1 (ATCC<sup>®</sup> BAA-759<sup>™</sup>) was used in the present study. It was maintained at 4 °C on solid HM medium [26], containing (per L): NaCl, 45 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.25 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.09 g; KCl, 0.5 g; NaBr, 0.06 g; peptone, 5 g; yeast extract, 10 g; glucose, 1 g; and granulated agar, 20 g. The pH of the medium was adjusted to 7.5 with 5 mol L<sup>-1</sup> NaOH.

### 2.2. Bioreactor scale: SSF and SHF experiments

SHF and SSF cultivations were performed in a Biostat<sup>®</sup> MD bioreactor (B. Braun Biotech International) equipped with pH, dissolved oxygen, temperature and foam probes, using a working volume of 2 L. The temperature was maintained at 30 °C by recirculating water from an external thermostated bath through the vessel jacket. The initial aeration and agitation rates were 1 L min<sup>-1</sup> and 400 rpm, respectively. For all the experiments, the airflow rate and agitation speed were increased gradually when the dissolved oxygen fell

below 60%. The maximum airflow rate and agitation speed attained were 5 L min<sup>-1</sup> and 650 rpm, respectively. The pH was kept at 7.5 by the automatic addition of 5 mol L<sup>-1</sup> NaOH or HCl. Foam was controlled through the addition of antifoam A (Sigma), when required. The phosphate concentration was monitored and maintained in the range of 0.7–3.7 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> to avoid limiting concentrations of this nutrient.

Corn kernel mash, after the liquefaction step, was obtained from Bioetanol Galicia SA (Teixeiro, A Coruña, Spain) and stored at –20 °C until use. The thawed mash was centrifuged at 2370g for 10 min before fermentation to prevent the high solid content (29.6% wt/wt) of the cereal mash from interfering with PHB downstream separation and biomass quantification. Solids removed can be directed to the DDGS (Distiller's Dried Grains with Solubles) production stream before fermentation instead of after, as occurs during the bioethanol process.

In the case of the SHF experiment, centrifuged cereal mash, hereafter called cereal juice, was saccharified in an incubator shaker (Innova 4000, New Brunswick Sci., Co, USA) before fermentation, through treatment with 146 mg of a commercial glucoamylase solution per L of cereal juice for 72 h at 50 °C, with rotary shaking at 200 rpm. The enzyme is obtained from *Aspergillus niger* and the commercial solution (Spirizyme, Novozymes A/S, Denmark) presents a density of 1.13 g mL<sup>-1</sup> and an activity of 100 U L<sup>-1</sup>, where one unit of glucoamylase (U) is defined as the amount of enzyme that releases 1 g L<sup>-1</sup> of glucose from starch in 1 h at 30 °C and pH 4.8. After this step, a stream called corn syrup, with a glucose concentration of 271 ± 37 g L<sup>-1</sup>, was obtained. This value is the mean concentration (± standard deviation) obtained after carrying out saccharification of the cereal juice three times. Two inoculation stages were needed for inoculum preparation. First, a *H. boliviensis* culture from an agar plate was inoculated into 20 mL of seed medium in a 100-mL flask and, after 18 h of incubation at 30 °C, this pre-inoculum was transferred into 200 mL of seed medium in a 1-L flask. Seed medium contains (per L): 10 g glucose from corn syrup; 45 g NaCl; 1.4 g MgSO<sub>4</sub>·H<sub>2</sub>O; 0.55 g K<sub>2</sub>HPO<sub>4</sub>; 2.3 g NH<sub>4</sub>Cl; 0.005 g FeSO<sub>4</sub>·7H<sub>2</sub>O; 3 g monosodium glutamate (MSG) and 15 g Tris(hydroxymethyl)aminomethane. The latter seed culture was incubated at 30 °C for 22 h with rotary shaking at 200 rpm. The initial medium for the bioreactor was composed (per L) of: 20 g glucose from corn syrup; 45 g NaCl; 2.8 g MgSO<sub>4</sub>·H<sub>2</sub>O; 2.2 g K<sub>2</sub>HPO<sub>4</sub>; 4.0 g NH<sub>4</sub>Cl; 0.005 g FeSO<sub>4</sub>·7H<sub>2</sub>O; and 20 g MSG. The glucose concentration was monitored during the fed-batch experiments through off-line analysis and maintained in the range of 16–24 g L<sup>-1</sup> by adding a feeding solution (FS) based on corn syrup (100% v/v) and composed of (per L): 45 g NaCl; 2.8 g MgSO<sub>4</sub>·H<sub>2</sub>O and 0.125 g FeSO<sub>4</sub>·7H<sub>2</sub>O.

In the case of the SSF experiment, the same two-stage inoculum preparation was performed. After the first pre-inoculum, the bacterial culture was grown in Erlenmeyer flasks with 100 mL of seed medium. This seed medium was composed of 50% v/v corn juice and 50% v/v water, the same amount of all the salts added to the seed medium used in the SHF process, and 146 mg of glucoamylase per L of corn juice. Seed culture was incubated at 30 °C for 22 h with a rotary shaking at 200 rpm.

Implementation of SSF for PHB production was based on the common process used for bioethanol production, as described by Pena [27]. Thus, 80% of the working bioreactor volume was composed of cereal juice, the rest was composed of inoculum (5%), glucoamylase enzyme solution (5%) and salt solution required for *H. boliviensis* growth (10%). The bioreactor filling sequence (Fig. 1) and the overall saccharification and fermentation time (72 h) were adapted from the standard bioethanol process. The minimum volume of 700 mL of corn juice (35% of the total working volume), necessary to cover the probes, was filled into the bioreactor, which was then sterilized in an autoclave at 121 °C for 20 min. The concen-

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