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High production of poly-β-hydroxybutyrate (PHB) by an *Azotobacter vinelandii* mutant altered in PHB regulation using a fed-batch fermentation process

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ABSTRACT

A mixed fermentation strategy based on exponentially fed-batch cultures (EFBC) and nutrient pulses with sucrose and yeast extract was developed to achieve a high concentration of PHB by *Azotobacter vinelandii* OPNA, which carries a mutation on the regulatory systems PTSNtr and RsmA-RsmZ/Y, that negatively regulate the synthesis of PHB. Culture of the OPNA strain in shake flaks containing PY-sucrose medium significantly improved growth and PHB production with respect to the results obtained from the cultures with the parental strain (OP). When the OPNA strain was cultured in a batch fermentation keeping constant the DOT at 4%, the maximal growth rate ($0.16\,h^{-1}$) and PHB yield ($0.30\,g_{PHB}\,g_{Suc}{}^{-1}$) were reached. Later, in EFBC, the OPNA strain increased three fold the biomass and 2.2 fold the PHB concentration in relation to the values obtained from the batch cultures. Finally, using a strategy of exponential feeding coupled with nutrient pulses (with sucrose and yeast extract) the production of PHB increased 7-fold to reach a maximal PHB concentration of $27.3 \pm 3.2\,g\,L^{-1}$ at 60 h of fermentation. Overall, the use of the mutant of *A. vinelandii* OPNA, impaired in the PHB regulatory systems, in combination with a mixed fermentation strategy could be a feasible strategy to optimize the PHB production at industrial level.

1. Introduction

Poly-3-hydroxybutyrate (PHB) is an intracellular polyester of the family of polyhydroxyalkanoates (PHAs), produced by numerous bacteria, including *Azotobacter vinelandii*. This polyester is composed of 3-hydroxybutyrate monomers in which the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer and it has properties similar to those found in conventional plastics [1,2]. The PHB is a biodegradable and biocompatible thermoplastic, which can be processed to create consumer products, including plastics, films, and fibers [3]. Recently new applications of PHB in the biomedical area have been found, where this polymer could be used as scaffold for tissue engineering or as nano-/micro-beads for the delivery of drugs [2,4].

Currently there are more than 300 species of bacteria producing PHB; however, only a few of them have been used at industrial scale to produce the polymer. These include *Cupriavidus necator*, *Azohydromonas lata* and *A. vinelandii* [5]. These bacteria can accumulate up to 90% of PHB on its dry weight. Biosynthesis of PHB in *A. vinelandii* has been widely described by several authors [6–9]. Two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA. This product is subsequently reduced to produce (R)-β-hydroxybutyryl-CoA, which finally is polymerized by a PHB synthase to form the polymer. The genes coding for these enzymes are contained in the PHB biosynthetic operon *phbBAC* [7–9].

Regarding to the regulation of PHB synthesis in *A. vinelandii*, it has been reported that the nitrogen-related phosphotransferase system (PTS^{Ntr}), formed by the proteins EI^{Ntr}, Npr, and IIA^{Ntr}, affects the expression of the PHB biosynthetic operon [10,11]. It is known that this protein acts as negative regulator of PHB synthesis in its non-phosphorylated state [11]. Also, the two-component global regulatory system formed by the sensor kinase GacS [12] and its corresponding response regulator GacA is required for synthesis of PHB. GacA activates transcription of the small RNAs called RsmZ/Y, which together with a protein called RsmA constitute a postranscriptional regulatory system where RsmA binds the mRNAs of the *phbBAC* biosynthetic operon and of the *phbR* activator, repressing their translation and the production of PHB. The RsmZ/Y RNAs bind RsmA, counteracting its repressor activity, allowing PHB synthesis [13].

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The production of PHB by fermentation using bacteria such as *A. vinelandii* as well as *A. lata* could be a feasible strategy for the synthesis of this polymer. Recent reports on PHB production using *A. lata* have been published [14–17]. In one of these studies the optimization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) production process by using *A. lata* MTCC 2311 was carried out. By using a genetic algorithm on artificial neural network, a maximum predicted PHB production of 5.95 g L^{-1} was found, using 35.2 g L^{-1} of sucrose and 1.58 g L^{-1} of urea [14]. The same authors reported that using propionic acid together with cane molasses allowed the synthesis of copolymer P (3HB-co-3HV) in maximal concentrations of 7.2 g L^{-1} in shake flasks and 6.7 g L^{-1} in 3 L bioreactor [15]. More recently, an integrated model was used for optimization of the production of PHB with tailor-made molecular properties in *A. lata* bacteria. A single-shot feeding strategy with fresh medium free of nitrogen was designed and experimentally tested. Using this strategy, a maximal concentration of PHB of 11.84 g L^{-1} was obtained, equivalent to polymer content equal to 95 wt % of DCW [17].

In the case of *A. vinelandii*, the PHB concentrations in batch cultures reported so far in the literature are low [6–8]. This problem is due to the poor growth of *A. vinelandii* when it is grown in batch cultures, in comparison with other bacteria such as *Escherichia coli* [18–20]. It is important to point out that due to the high respiratory rates of *A. vinelandii*, oxygen limitations could be occurring at early stages of the fermentation in cultures where the dissolved oxygen tension (DOT) is not controlled [21]. Therefore, the dissolved oxygen plays a critical role in the culture. It is known that a low DOT (<1%), promotes the PHB synthesis; whereas at high values of DOT (10%), the bacterium uses the carbon source mainly for cell growth [22,23].

One strategy to improve the growth of *A. vinelandii* consists in the use of fed-batch cultures, where a high biomass concentration can be achieved, in order to take advantage of the higher specific production capacities of mutant strains of *A. vinelandii* [8,19]. These processes have been used for the production of PHB using bacteria such as *A. lata* and *C. necator* [24,25]. In the case of *Azotobacter* strains, Kim and Chang [26] reported that *A. chroococcum* in fed-batch cultures produced a maximal concentration of PHB of 25 g L^{-1} from starch; whereas Page and Cornish [27] reported 25 g L^{-1} of PHB in fed-batch cultivations of the *A. vinelandii* mutant strain UWD and Chen and Page [18], using a two-stage process with the UWD mutant of *A.vinelandii*, reported a maximal concentration of 36 g L^{-1}.

The aim of the present study was to improve the PHB production, in terms of the volumetric concentration and yield, by a mutant of *Azotobacter vinelandii* (OPNA) altered on the regulatory systems PTSNtr and RsmA-RsmZ/Y, and using a fed-batch fermentation process.

2. Materials and methods

2.1. Microorganisms

Azotobacter vinelandii strains used in this investigation were OP [28] and its mutant derivative named OPNA (*ptsN*::*Km*^{r} and *rsmA*::*Sp*^{r}). Mutant strain OPNA carries mutations that inactivate the genes coding for the IIA-PTSNtr and RsmA proteins that negatively regulate the PHB synthesis [10,11,13]. For the construction of this mutant, competent cells of strain OPN, a *ptsN*::Km^r mutant derived from strain OP [29], with total DNA from *A. vinelandii* *rsmA*::Sm^r/Sp^r mutant SOP1 were transformed [13]. After 24 h of growth in medium with no antibiotic, the transformed cells were plated in selective medium with spectinomycin and kanamycin. Ten transformants, resistant to kanamycin and spectinomycin, were selected and five were confirmed by PCR analysis using flanking primers, to carry the *rsmA*::Sm^r/Sp^r inactivation, resulting from a double crossover recombination. One of these transformants was isolated and named OPNA. The cells of strain OPNA were cryopreserved at −70 °C in 40% glycerol solution and maintained by monthly subculture on Burk' agar slopes containing the appropriate antibiotic and stored at 4 °C [30].

2.2. Culture medium

A. vinelandii strains were grown in PY sucrose medium with the following composition (in g L^{-1}): sucrose 20, yeast extract (Difco) 3 and peptone (Difco) 5. The pH was adjusted to 7.2 with a concentrated 2 N NaOH solution.

2.3. Preparation of inoculum

The inoculum was prepared as follows: *A. vinelandii* cells were grown at 29 °C in 500 mL Erlenmeyer flasks, containing 100 mL of PY medium during 24 h at 200 rpm. Flasks were incubated in a New Brunswick G-25 shaker (NJ, USA) until they reached a biomass concentration of 1.0 g L^{-1} (measured by dry weight). The biomass obtained was collected by centrifugation at 15,500 × *g*, during 10 min and re-suspended in 150 mL of fresh PY liquid medium. This suspension was inoculated into the bioreactor containing 1350 mL of fresh culture medium.

2.4. Batch cultures

Batch cultures were carried out in a 3.0 L Applikon bioreactor (The Netherlands) using 1.5 L of working volume. This was operated at 700 rpm and 29 °C with airflow of 1 L min^{-1} (0.66 vvm). The pH was measured with an Ingold probe (Applikon, ADI 1010) and controlled by an on/off system using a peristaltic pump and a 4 N NaOH solution. Dissolved oxygen (DO) was measured with an Ingold polarographic probe and controlled at 4% of the air saturation value (by gas blending) using a system based on a PID control which has been previously described [31].

2.5. Exponentially fed-batch cultures (EFBC)

Exponentially fed-batch cultures (EFBC) were conducted in the way described by Priego-Jiménez et al. [32]. The bioreactor used was the same as described above. EFBC were started in batch mode. After 12 h, 550 mL of PY medium was fed exponentially (during 15 h), increasing the feeding rate from 0.2 to 1.53 mL min^{-1} until the final volume (2.05 L) was reached. The culture was then run in batch mode for another 10 h. The exponential feed rate of PY medium with sucrose was added to the bioreactor with a precision peristaltic pump (Masterflex L/S Model 758-18) from a sterile reservoir containing the medium. Sucrose profiles were designed to yield pseudo-steady state specific growth rates of 0.16 h^{-1}. The pH was controlled at 7.2 and the DOT at 4%, as previously was described for batch cultures.

2.6. Exponentially fed-batch cultivations with nutrient pulses

For these cultivations, an initial period of EFBC was coupled to nutrient pulses of 100 mL of a solution of sucrose (30 g L^{-1}) and yeast extract (20 g L^{-1}) at different times of the cultivation (38 and 48 h). The pH was controlled at 7.2 and DO at 4% in the same way as for the batch culture.

2.7. Mathematical analysis

2.7.1. Batch culture

The specific growth rate (μ) for the batch fermentation was calculated considering the growth from 3 h to 16 h of the cultivation, period at which the culture was growing exponentially.