A polyhydroxybutyrate producing mutant of *Azotobacter vinelandii* lacking the regulatory proteins RsmA and Enzyme IIA^{Ntr} utilizes the carbon source more efficiently

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1. Introduction

The polyhydroxyalkanoates (PHAs) are natural polyesters produced by several bacteria and archaea as a reserve of carbon and energy (Anderson, Dawes, 1990). These biopolymers have industrial applications because they have thermoplastic properties similar to those of the synthetic plastics and elastomers obtained from oil, with the advantage of being fully biodegradable, biocompatible and renewable (Lee, 1996; Streinbuchel, 2001). For this reason, some PHAs are actually produced at an industrial scale for the production of biodegradable plastics (Chen, 2009), and they are also being used in products with medical application (Wu et al., 2009).

Azotobacter vinelandii is a bacterium producing polyhydroxybutyrate (PHB), a PHA made up of hydroxybutyrate units. This bacterium has characteristics that make it interesting for PHB production, such as its capacity to grow on many substrates (Chen, Page 1994, 1997; Page, 1992; Page, Cornish, 1993) and to accumulate this polymer up to 90% of its dry cell weight. It also results interesting because it can produce PHB of a high molecular weight, of up to 4 million Daltons (Ashby et al., 1999; Chen, Page, 1994).

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In A. *vinelandii*, the PHB is synthesized from acetyl-CoA in three enzymatic steps. The first one is the condensation of two molecules of acetyl-CoA catalyzed by the enzyme β-ketothiolase, producing acetoacetyl-CoA, which is reduced by the acetoacetyl-CoA reductase NADPH dependent, producing β-hydroxybutyryl-CoA, that is polymerized by the PHB synthase (Manchak, Page, 1994). The genes coding for these enzymes are encoded in the *phbBAC* operon (Peralta-Gil et al., 2002; Segura et al., 2000, 2003).

The amount of PHB produced by *A. vinelandii* depends on diverse factors, such as the culture conditions and the genetic regulatory mechanisms that control the expression of the biosynthetic genes *phbBAC*. Several regulatory systems are involved in the control of the expression of these genes. The regulator PhbR acts is a transcriptional activator of the *phbBAC* operon (Hernández-Eligio et al., 2011; Peralta-Gil et al., 2002). The sigma factor RpoS participates in the transcription of both the regulatory gene *phbR,* and the biosynthetic genes *phbBAC* (Peralta-Gil et al., 2002). Also the regulator called CydR has been reported as a transcriptional regulator of *phbBAC* (Wu et al., 2001).

In addition, there are two global regulatory systems controlling the expression of the genes *phbBAC,* The first one is formed in turn by two systems: The global two-component system, composed by the GacS sensor kinase and its response regulator GacA (Castañeda *et al*., 2000, 2001), and the posttranscriptional regulatory system RsmA-RsmZ/Y. The control of PHB synthesis in this case is exerted by the RsmA protein, that binds to the mRNA of the biosynthetic operon *phbBAC*, repressing its translation and promoting its degradation. In turn, the GacA response regulator, phosphorylated by GacS, activates the transcription of the small regulatory RNAs named RsmZ/Y (Hernández-Eligio et al., 2012; Manzo et al., 2011). These small RNAs bind RsmA, counteracting its repressor activity allowing the expression of *phbR* and *phbBAC* (Hernández-Eligio et al., 2012).

The other global regulatory system controlling the synthesis of PHB is the nitrogen-related phosphotransferase system (PTS^{Ntr}). This system is formed by the EI^{Ntr}, Npr, and IIA^{Ntr} proteins. It regulates the production of PHB through a phosphoryl transfer chain from phosphoenolpyruvate to EI^{Ntr} (Segura, Espín, 1998), to Npr, and then to IIA^{Ntr} (Noguez et al., 2008). The protein IIA^{Ntr}, in its non-phosphorylated form, acts as a negative regulator on the expression of *phbR* and the biosynthetic operon *phbBAC* through a yet unknown mechanism (Noguez et al., 2008).

The PTS^{Ntr} system in *A. vinelandii*, besides regulating PHB synthesis, participates in the respiratory protection of the nitrogenase, which consists in the generation of a high respiratory rate under conditions of nitrogen fixation and high oxygen concentration to create an intracellular environment adequate for the nitrogenase enzyme, which is oxygen sensitive (Segura, Espín, 1998).

In the two genetic regulatory systems described there are two negative regulators of PHB synthesis, the proteins RsmA and IIA^{Ntr}. This has allowed obtaining mutant strains overproducing PHB through the inactivation of *ptsN* (Noguez et al., 2008; Peña et al., 2013), or *rsmA* (Hernández-Eligio et al., 2012).

To construct an *A. vinelandii* strain with higher PHB production capacity, a double mutant was constructed where the two negative regulators were inactivated. This mutant, named OPNA, is able to accumulate more PHB than the parental strains (unpublished results).

The culture conditions considerably affect the amount of PHB produced by *A. vinelandii*. In this bacterium the concentration of oxygen in the culture has a strong influence on the synthesis of PHB and a low oxygen concentration induces the production of PHB (Galindo et al., 2007; Page, Knosp, 1989; Senior, Dawes, 1972, 1973). When *Azotobacter* grows under conditions of oxygen limitation, a lower activity of the tricarboxylic acids cycle is observed. This seems to be due to the accumulation of NADH induced by the lack of the terminal electron acceptor in the respiratory chain. This allows for the use of acetyl-CoA for the production of the polymer, which acts as an electron sink (Page, Knosp, 1989; Senior et al., 1972). This effect of oxygen limitation on PHB synthesis has been reported in several strains like UW (Page & Knosp, 1989), ATCC 9046 (Peña et al., 2011) and UWD (Page, Knosp, 1989). In fact, changes in the oxygen level have been used as strategy to increase the production of PHB in fermentors (Chen, Page, 1997; Page et al., 2001).

The aim of this study was to analyze the effect of the inactivation *ptsN* and *rsmA* on the response to different oxygen concentrations of *A. vinelandii* strain OP.

2. Materials and methods

2.1 Bacterial strains and culture conditions

In this study, the strains used were *A. vinelandii* OP (Bush & Wilson, 1959) and its mutant derivative OPNA. This mutant has inactivated the genes *rsmA* and *ptsN*, which code for two negative regulators of PHB synthesis. The effect of three different oxygen concentrations on growth, PHB production and utilization of the carbon source was tested.

The strains were grown in 10 mL microreactors of a Micro-24 MicroReactor System (Pall Life Sciences), each one equipped with an air injection port, with fluorescent sensors for pH, dissolved oxygen and with independent control of temperature, pH, and oxygen. A working volume of 4 mL was used. The experiments were done at 30 °C and three different Dissolved Oxygen Tensions (DOT) were tested: 1, 5 and 10% of saturation, through the control of air injection and using an agitation of 500 rpm. The culture medium was PYS (peptone, 5 g/L; yeast extract 3 g/L; sucrose, 20 g/L, at pH 7). Antifoam Sigma was used at a final concentration of 0.001%.

2.1.1 Analytical methods

The experiments were done in triplicate and the data shown represent the mean of all three experiments. The temperature, dissolved oxygen and pH of the microreactors were continuously monitored on line.

Growth of *A. vinelandii* in the microreactors was analyzed by quantifying the protein concentration in the cultures by the method of Lowry et al., (1951), using a standard curve of bovine serum albumin, and also by measuring the optical density at 600 nm. The cellular PHB content was determined by gas chromatography (GC-FID) of the methanolysed polyester. Methanolysis was carried out by suspending 3 mg of lyophilized cells in 2 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 incubation at 100 °C for 4 h. After cooling, 1 mL of demineralized water was added, and the organic phase, containing the methyl esters, was analyzed by GC (Lageveen et al., 1988).

Sucrose concentration was quantified by chromatography (HPLC), using a IR detector at a temperature of 40 °C with a Rezex ROA-Organic Acid H^+ (8%) column, operated at 60 °C with an isocratic flux of 0.5 mL/min of H_2SO_4 7mM as the mobile phase.

3. Results and discussion

The production of PHB is affected by the culture conditions and the fermentation strategy used. The DOT is among the fermentation parameters more relevant for the synthesis of this polymer in *A. vinelandii* (Chen, Page, 1997; Page et al., 2001; Page, Knosp, 1989; Peña et al., 2011). In addition, the modification of the genetic systems regulating the synthesis of PHB also affects its production capacity. In this work we decided to evaluate the growth and PHB production capacities of the *A. vinelandii* mutant OPNA under different dissolved oxygen conditions. With the aim to achieve a good control of the different oxygen conditions to be tested and to have results scalable to fermentations at a higher volume, a microreactor system with the capacity to monitor and control the dissolved oxygen, pH and temperature was used. In Figure 1, the results obtained of cell growth, measured as optical density and protein, are shown. The increase in the DOT had a negative effect on cellular growth, reaching the highest biomass under a DOT of 1 %. This response was similar for both strains tested.

The observed behavior could be related with the so-called respiratory protection of the nitrogenase. This constitutive phenomenon, present in bacteria of the genus *Azotobacter*, consists in the generation of a high respiratory rate when the oxygen levels in the medium are high. This increase in the respiratory rate allows for the removal of intracellular oxygen, producing an anoxic environment adequate for nitrogen fixation, because the nitrogenase (enzyme responsible for this process) is sensitive to oxygen (Gallon, 1992). In this way, the concentration of oxygen present has a profound effect on the activity of the respiratory chain in *A. vinelandii*. This change of activity is

achieved expressing alternative components of the respiratory chain, which are very active but only partially coupled to the generation of energy (Bertsova et al., 2001). The use of these alternative components lowers the intracellular oxygen concentration, but produces five times less ATP than the fully coupled chain (Bertsova et al., 1998). The observed response of lower growth in the microreactors with increasing DOT could be related with the activation of the respiratory protection. When growth was measured as optical density (Fig. 1A), *A. vinelandii* OP showed a higher growth rate, although the biomass produced at 36 h was similar between both strains for the three DOT levels; however, when the growth was estimated quantifying the protein (Fig. 1B), the mutant OPNA showed maximal protein concentrations 29 to 34 % higher than those obtained with the wild type strain under the three DOT tested.

The difference between the two methods could be due to the fact that the turbidity of the culture is affected by the presence of PHB that accumulates as insoluble intracellular inclusions. For two cultures with the same amount of cells, the one with a lower PHB production has a lower turbidity. When the amounts of PHB produced by the two strains under the three DOT tested were compared (Fig. 2A), a negative effect of oxygen was observed. The highest accumulation of the polymer was reached in the cultures where the DOT was 1 %, where the PHB content was around 90 % of the total dry cell weight in the wild type strain and 83 % in the mutant. On higher DOTs the PHB contents were lower, reaching only 39% in the wild type and 36 % in the mutant in the cultures with a DOT of 10 %. This result is similar to those of previous reports showing that PHB synthesis occurs under oxygen limiting conditions. These results have been explained considering an inhibition of enzymes of the tricarboxylic acid cycle due to the accumulation of NADH produced by the limitation of the final electron acceptor in the respiratory chain, which in turn stimulates PHB synthesis (Manchack, Page, 1994; Page, Knosp, 1989; Peña et al., 2011). It is important to note that, in opposition to what was expected considering the mutations introduced in the OPNA mutant, the accumulation of PHB in the condition of 10 % DOT in the wild type strain was 19% higher than that in the OPNA mutant; however, it was similar at 1 % DOT. At 5 % DOT, the mutant produced a considerably higher production (47 %) than the wild type (Fig. 2A). These results show that the mutations introduced improve the production of

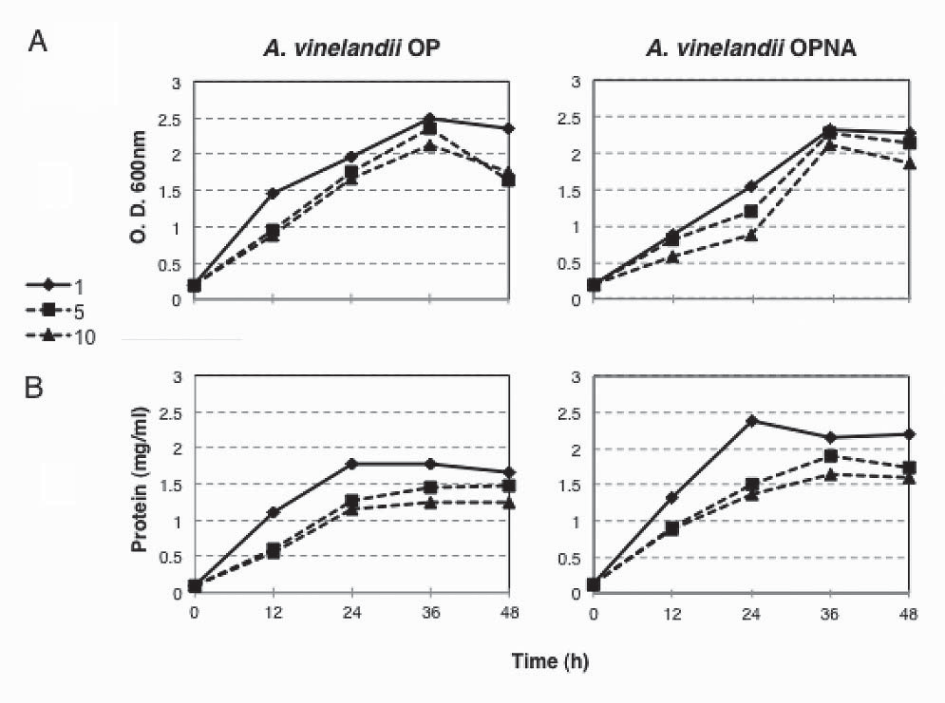

Figure 1. Growth kinetics of the *A. vinelandii* wild type strain OP and its mutant OPNA, in microreactors under three different conditions of DOT (1, 5 and 10%). Growth was estimated as optical density at 600 nm (A), or as protein (B).

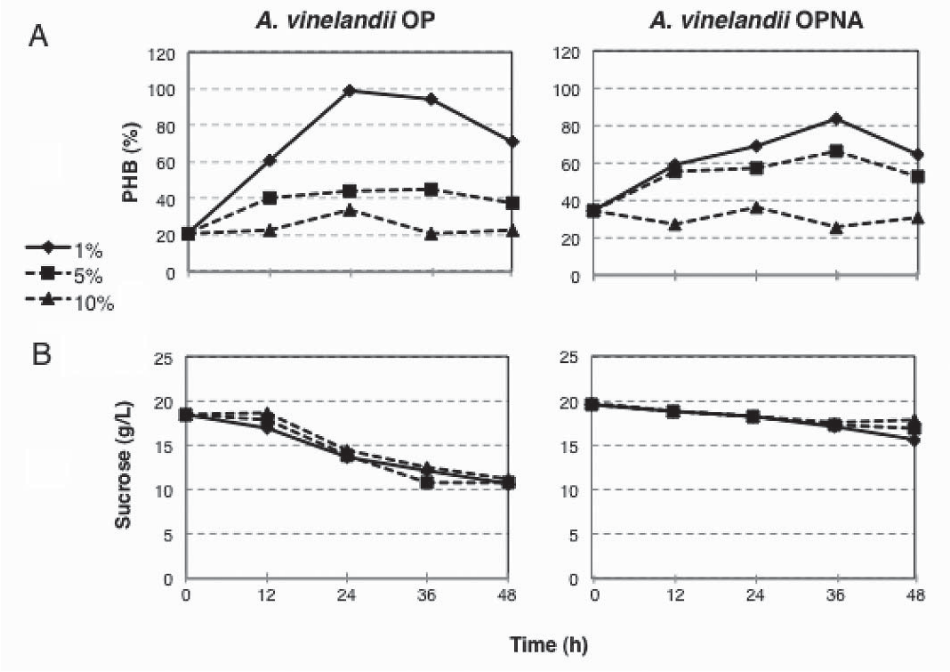

Figure 2. PHB production (A) and sucrose consumption (B), of the *A. vinelandii* wild type strain OP and its mutant OPNA in microreactors under three different conditions of DOT (1, 5 and 10%). PHB production is presented as percentage of the total cell dry weight.

PHB, as the previous reports had shown (Hernández-Eligio et al., 2012; Noguez et al., 2008; Peña et al., 2013), but only under certain conditions of DOT in the cultures.

A very interesting difference between the two strains used that could have important repercussions for PHB production, is in the sucrose consumption rates (Fig. 2B). Whereas the wild type strain consumed between 6 and 7.7 g/L of the carbon source at 36 h, a time where the highest growth and PHB production had been reached, the mutant consumed only between 2.2 and 2.7 g/L, depending on the DOT condition tested. Considering the biomass produced at 36 h, it can be seen that the mutant OPNA uses the carbon source provided more efficiently, because the biomass yield for the mutant was of 0.7 grams per gram of sucrose consumed, whereas for the wild type strain was of only 0.3 for the condition of 1% DOT. A similar difference between the two strains was obtained for the other two conditions of DOT (data not shown).

The putative metabolic differences between the two strains were also seen in the measurements of dissolved oxygen (Fig. 3A). In the cultures of both strains under the three DOT conditions tested, the oxygen levels wanted were maintained only for the first hours of the culture. During the exponential growth phase the oxygen requirements were higher than the oxygen transfer capacity of the microreactors system, and the DOT levels dropped to less than 0.1 %. Although three DOT was close to cero under the three conditions tested, the respiratory activity, evaluated through the DOT profiles, was very different, especially for the conditions of 1 and 10 %. For example, in the case of the cultures set at 10 %, the dissolved oxygen dropped faster during the first hours of the culture (12 h), both for the wild type and the mutant strains, suggesting a faster consumption of oxygen. Besides, the periods of oxygen limitation (oxygen close to cero) were longer for the cultures of the wild type strain at 1 % and 5 % DOT, in comparison to the mutant OPNA. The highest oxygen consumption, observed for both strains at 10 % DOT, supports the hypothesis that under high oxygen conditions the bacterium activates the alternative respiratory protection.

Another indication for the existence of metabolic differences between the two strains was observed in the pH profiles throughout the culture (Fig. 3B). While the pH of the cultures of the wild type strain *A. vinelandii* OP

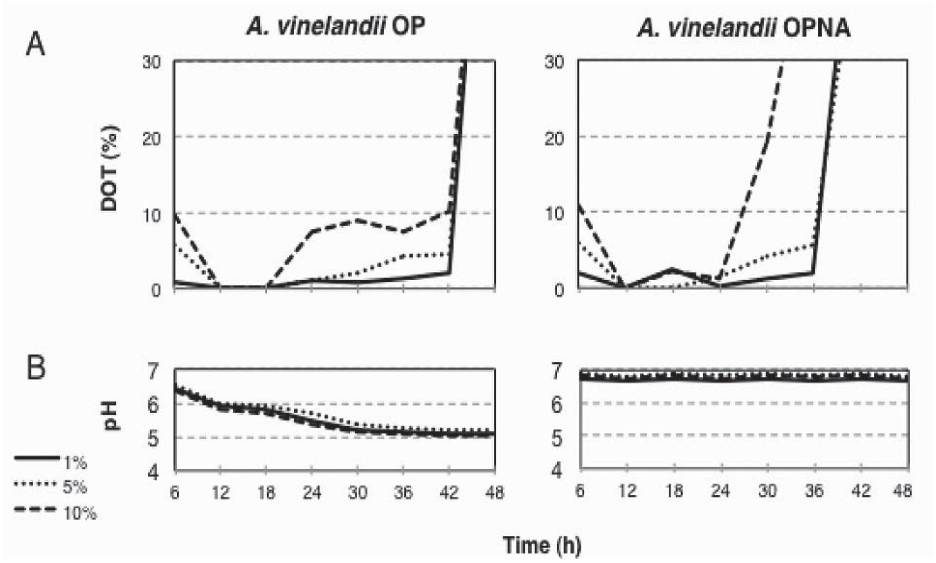

Figure 3. Evolution of the dissolved oxygen (A) and pH (B), during growth of *A. vinelandii* strains OP and OPNA in microreactors under three different conditions of DOT (1, 5 and 10%).

dropped to a value close to 5, that of the mutant was maintained close to 6.8. This drop in the pH values of the wild type strain could be due to the secretion of organic acids produced in the metabolism.

The observed differences between *A. vinelandii* OP and its mutant OPNA could be explained if the mutations introduced affected the respiratory activity. A lower expression (or activity) of the alternative respiratory chains responsible for the respiratory protection, could lead to a more efficient use of the carbon source, through the use of the fully coupled respiratory chains that generate five times more ATP than those involved in the respiratory protection (Bertsova et al., 1998). This would allow the higher yield of biomass seen in the cultures of the OPNA mutant, apart from the effect of the *ptsN* inactivation on the expression of the PHB biosynthetic genes. These respiratory changes would be positive under conditions where nitrogen fixation is not needed because a nitrogen source is provided in the medium, such as the one used in this study. In fact, the inactivation of the enzyme I (EI^{Ntr}), another component of the PTS^{Ntr} regulatory system where the product of *ptsN* also participates, produces changes in the respiratory protection of the nitrogenase and such mutant strain is unable to fix nitrogen under high oxygen conditions, unless a high concentration of the carbon source is provided (Segura, Espín, 1998).

With respect to the *rsmA* mutation, homologs of the regulatory system RsmA- RsmZ/Y have been shown to regulate central carbon metabolic pathways, such as glycolysis and gluconeogenesis in diverse bacteria (Romeo *et al*., 2012), and it has been reported that the attenuation of activity of CsrA, a homolog of RsmA, affects the growth efficiency on a broad range of carbon sources, including compounds utilized by the Entner-Doudoroff pathway in *E. coli* strain Nissle 1917 (Revelles et al., 2013), so in *A. vinelandii* it could have a similar role. The possibility that the PTS^{Ntr} system and/or RsmA- RsmZ/Y could be controlling the carbon or energy metabolism in this bacterium remains to be investigated.

3. Conclusions

The DOT in cultures of *A. vinelandii* OP considerably affects growth and PHB production. High oxygen concentrations have a negative effect on growth and in the accumulation of the polymer, whereas oxygen-limiting conditions favor growth and PHB production, as previously reported. The mutations *ptsN* and *rsmA* introduced in strain OPNA

did not modify this response to oxygen under the three DOTs tested, although they improved PHB production at intermediate oxygen concentrations (5 %), so these genetic modifications favor a higher accumulation of the polymer, as previously reported, but only under specific oxygen conditions.

The genetic modifications of strain OPNA had a noticeable effect on the sucrose consumption rate and doubled the biomass yield (g/g of sucrose consumed), so the OPNA mutant uses the carbon source more efficiently than the wild type strain.

Although the DOT was not properly controlled in the cultures of *A. vinelandii* in the microreactors (due to the high respiratory rate of this bacterium), the use of this system allowed to establish different oxygen conditions and to monitor the dissolved oxygen present throughout the culture, facilitating the interpretation of the respiratory activity under reproducible conditions, with the possibility to run several replicas simultaneously for each condition.

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