African Journal of Biotechnology Vol. 9(33), pp. 5240-5250, 16 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Review

Biotechnological uses of *Azotobacter vinelandii*: Current state, limits and prospects

A. Díaz-Barrera* and E. Soto

Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Chile, Av. Brasil 2147, Casilla 4059, Valparaíso, Chile.

Accepted 23 March, 2010

Azotobacter vinelandii **is a bacterium obligate aerobe able to fix nitrogen and grow under microaerophilic conditions. This microorganism has remarkable cellular machinery with the ability to biosynthesize three molecules of important biotechnological and biomedical applications: the extracellular polysaccharide alginate, the intracellular polyester poly-β-hydroxybutyrate (PHB) and siderophores compounds. Alginate and PHB have been produced in shake flasks and bioreactors (at laboratory scale) under different environmental and nutritional conditions. This mini-review gives a current overview on the application of wild-type and mutant's strains of *A. vinelandii* in bioprocesses to produce alginate or PHB. Scales of production implemented and fermentation strategies used as well as the potential limitations of the use of *A. vinelandii* cells are analyzed. Based on an approach that integrates molecular and bioengineering techniques, new experimental strategies to enhance the productivity of alginate and PHB from *A. vinelandii* are proposed.**

Key words: *Azotobacter vinelandii*, alginate, poly-β-hydroxybutyrate, siderophores, bioprocess, scales of production.

INTRODUCTION

Azotobacter vinelandii is a gram-negative bacterium, obligate aerobe capable of fixing nitrogen and to adapt its metabolism to diverse environmental conditions. *A. vinelandii* forms metabolically dormant cysts after exponential growth or upon induction with specific reagents (Sadoff, 1975). Several years ago, *A. vinelandii* was used as a model for biochemical, physiology and genetic studies, regarding mainly in biological nitrogen fixation studies (Dixon and Kahn, 2004). On this regard, the reduction of N_2 to ammonia (fixation of nitrogen) by nitrogenase enzyme complex is highly sensitive to oxygen, for which *A. vinelandii* have particular physiological mechanisms, such as a high respiration rate (Post et al., 1983) to protect its nitrogenase enzymes from oxygen inactivation.

The most important biotechnological application of *A. vinelandii* cells is their ability to biosynthesize at least three molecules that have important biotechnological and biomedical applications. Under determined nutritional and environmental conditions, this bacterium produces the intracellular polyester poly-β-hydroxybutyrate (PHB), the extracellular polysaccharide alginate and catechol compounds (siderophores). The chemical structure of these molecules is shown in Figure 1.

A. vinelandii excretes siderophores under iron limited conditions (Page and Von Tigerstrom, 1988), which bind iron in strong complexes that are then transported into the cell through highly specific transporters (Page et al., 2003). Recently, it has been found that *A. vinelandii* produces at least five different siderophores: the 2,3-dihydroxybenzoic acid and aminochelin (monocatechols), the azotochelin (bis(catechol)), protochelin (tris(catechol)) and the yellow-green fluorescent pyoverdine-like azotobactin (Kraepiel et al., 2009) (Figure 1a). Some works conducted in shake flasks have evaluated the production of siderophores by *A. vinelandii* and usually the influence of iron and oxygen stress has been studied (Cornish and Page, 1998; Tindale et al., 2000). However, less is known about optimal conditions of production at bioreactor scale

*Corresponding author. E-mail: alvaro.diaz@ucv.cl.
Fax: (56-32) 2273803.

Abbreviations: PHB, poly-β-hydroxybutyrate; **DOT,** dissolved oxygen tension; **OTR,** oxygen transfer rate.

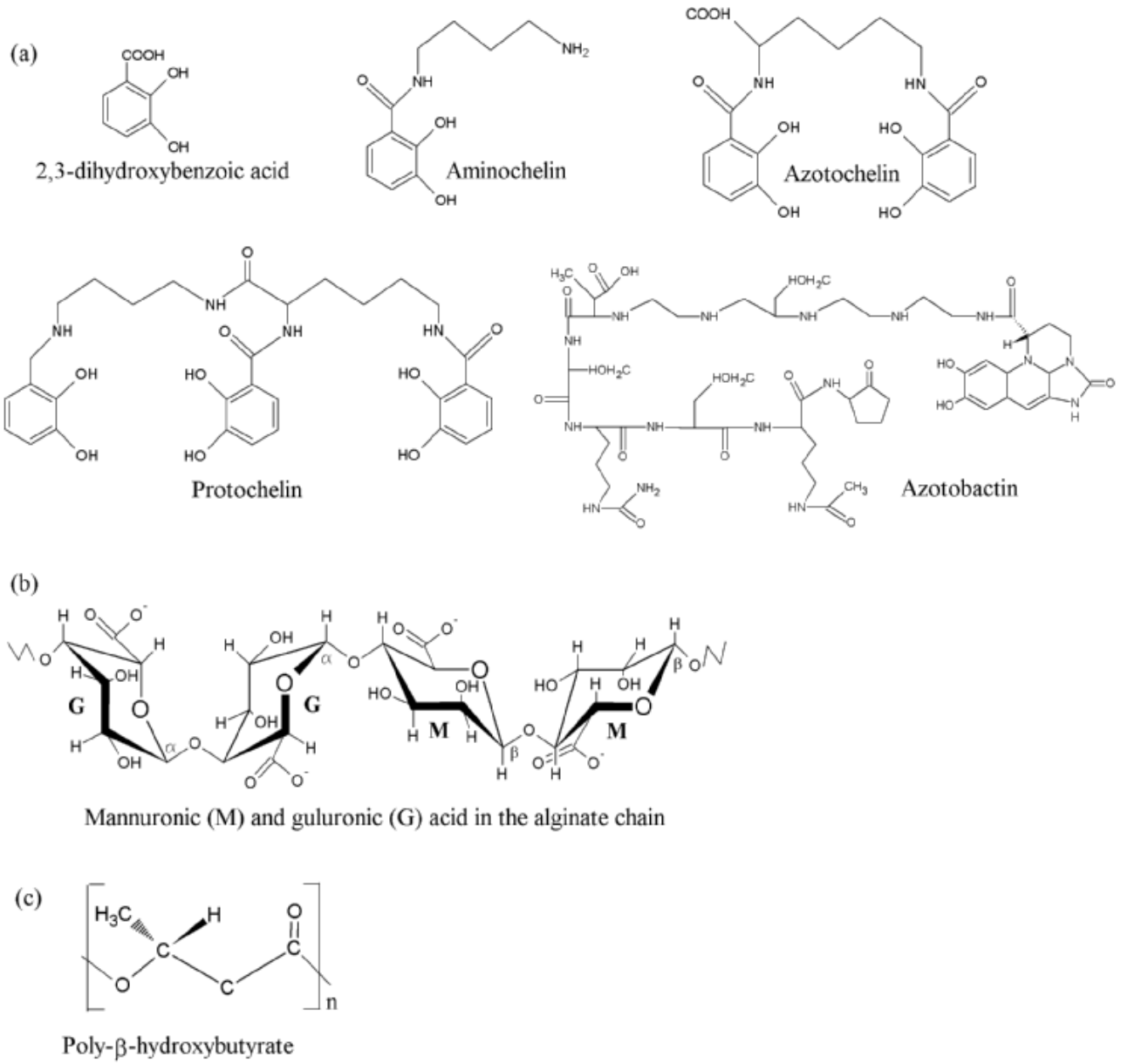

Figure 1. The chemical structures of the molecules produced by *A. vinelandii*. (a) Siderophores compounds, (b) alginate and (c) poly-β-hydroxybutyrate (PHB).

(Fekete et al., 1983). It is known that the main biotechnological applications of siderophores are as drug delivery agents (Möllmann et al., 2009), antimicrobial agents (Upadhyay and Srivastava, 2008) and soil remediation (Braud et al., 2009). Therefore, studies at greater scale would be necessary in order to increase siderophore productivity and evaluate its potential biotechnological/ biomedical application.

Alginates are polysaccharides produced by *Pseudomonas* and *Azotobacter* species (Remminghorst and Rehm, 2006a). Most knowledge about alginate biosynthesis have been particularly well characterized in the opportunistic human pathogen *Pseudomonas aeruginosa*, mainly because alginate plays an important role as a virulence factor during cystic fibrosis (Ramsey and Wozniak, 2005). In contrast to *P. aeruginosa*, *A. vinelandii* is a nonpathogenic bacterium, which has been used for the development of biotechnological process to produce alginate. Alginate is a linear polysaccharide composed of variable amounts of (1-4)-β-D-mannuronic acid and its C-5-epimer α-L-guluronic acid (Figure 1b), which has a wide range of applications such as thickener, stabilizer, gelling agent and emulsifier in food, as well as textile and pharmaceutical industries. Recent biotechnological advances show a promising potential application of alginate in the medical field; for they can be used in the control release

of medical drugs (Yao et al., 2009). It is well-known that their properties depend on the concentration, the molecular weight and, to a lesser extent, on the relative content of the two monomers (mannuronic and guluronic) in alginate molecule (Galindo et al., 2007). Strategies for the biotechnological production of high-quality and/or tailor-made bacterial alginates have been performed by means of the evaluation of the influences of different nutritional and operational conditions (Sabra et al., 2001; Trujillo-Roldán et al., 2004; Díaz-Barrera et al., 2009). Studies at bioreactor laboratory scale (1 or 2 l) have shown the influence of dissolved oxygen tension (DOT) on alginate production (Peña et al., 2000; Sabra et al., 2000; Trujillo-Roldán et al., 2004). In this context, alginate could play a decisive role in protecting the nitrogenase (Sabra et al., 2000) due to higher DOT (20%); alginate forms a capsule on the cell surface, suggesting a possible role as an effective barrier for oxygen.

A. vinelandii also produces the intracellular polyester PHB (polymer of the polyhydroxyalcanoates family), which is a biodegradable and biocompatible thermoplastic used as substitute for bulk plastics such as polyethylene and polypropylene. PHB is polyester that the bacteria accumulate intracellularly as both a carbon and energy reserve material. The production of PHB by fermentation has been normally operated as a two-stage fed-batch process, in which an initial growth phase in nutritionally enriched medium yields sufficient biomass, followed by a PHB formation phase (Chen and Page, 1997). Different strategies have to be addressed in order to make the process economically attractive. In the case of PHB production, as well as alginate production, the construction of recombinant strains to produce more or higher quality polymers and the implementation of different fermentation procedures would be necessary to evaluate the possible development of an attractive bioprocess with scaling-up potential. This possibility, together with the availability of the complete sequence of *A. vinelandii* genome (Setubal et al., 2009) have made of this bacterium an interesting model of study, from both technological and scientific points of view.

A SEARCH OF ARTICLES PUBLISHED

In order to quantify the studies performed on alginate, PHB and siderophores produced by *A. vinelandii*, a search of research articles in the SCOPUS web was carried out (Figure 2). Figure 2a shows the progress of total publications in the last 20 years from 1989 to 2009. The results clearly indicate a higher number of publications regarding alginate (119 in total) as compared to those obtained for PHB (34 publications in total) and siderophores (27 publications in total) production using *A. vinelandii* cells.

An analysis of the publications (from 1989 to 2009) shows that siderophore production by *A. vinelandii* has not been studied with the purpose of developing a bioprocess to greater scale. A sustained increase in the number of publications regarding alginate from *A. vinelandii* was observed from 1989 to 2000; however, the publications showed a decrease from 2003 onwards. This evolution of alginate publications could be explained by the fact that from 2001 onwards, two research groups (W. Sabra and W-D. Deckwer, Germany/F. Clementi and E. Parente, Italy) have not published works with respect to alginate and *A. vinelandii* cells. In particular, Figure 2b shows the evolution of the number of publications regarding alginate, PHB and siderophores production by fermentation (in shake flasks and bioreactors) using *A. vinelandii*. It is interesting to note that although the total publications of *A. vinelandii* and alginate showed a decrease from 2003 (Figure 2a), an increase about twofold in the publications of alginate production by fermentation has been observed between 2004 and 2009 (Figure 2b). Considering that alginate and PHB have been described as the main products of industrial interest from *A. vinelandii* and that there are a higher number of works regarding alginate and PHB production (compared to siderophores) with the purpose of improving the bioprocess, the next sections will be focused on analyzing the biosynthesis pathways, fermentation procedures and scales of production used for the production the these biopolymers.

BIOSYNTHESIS OF ALGINATE AND PHB IN *A. vinelandii*

In this section, a brief review and information update regarding alginate and PHB biosynthesis pathways are presented. A more detailed analysis of alginate biosynthesis pathways and their regulation is given in recent reviews (Remminghorst and Rehm, 2006a; Galindo et al., 2007).

Alginate biosynthesis

The alginate biosynthesis pathway and the knowledge about the genetics of alginate biosynthesis have been widely studied (Loret et al., 1996; Muhammadi and Ahmed, 2007). The alginate biosynthesis pathway can be divided into four different stages (Remminghorst and Rehm, 2006a): (1) Synthesis of precursor substrate (GDP-mannuronic acid) at cytoplasmic level, (2) polymerization and cytoplasmic membrane transfer, (3) periplasmic transfer and modification and (4) export through the outer membrane. Figure 3 shows the biosynthesis of alginate and PHB in *A. vinelandii* cells. It is known that alginate is synthesized from fructose-6-phosphate, which is converted by means of four enzymatic steps involving three enzymes to GDP-mannuronic acid (alginate precursor) (Figure 3a). The transfer of GDP-mannuronic acid that happens through the cytoplasmic membrane and the

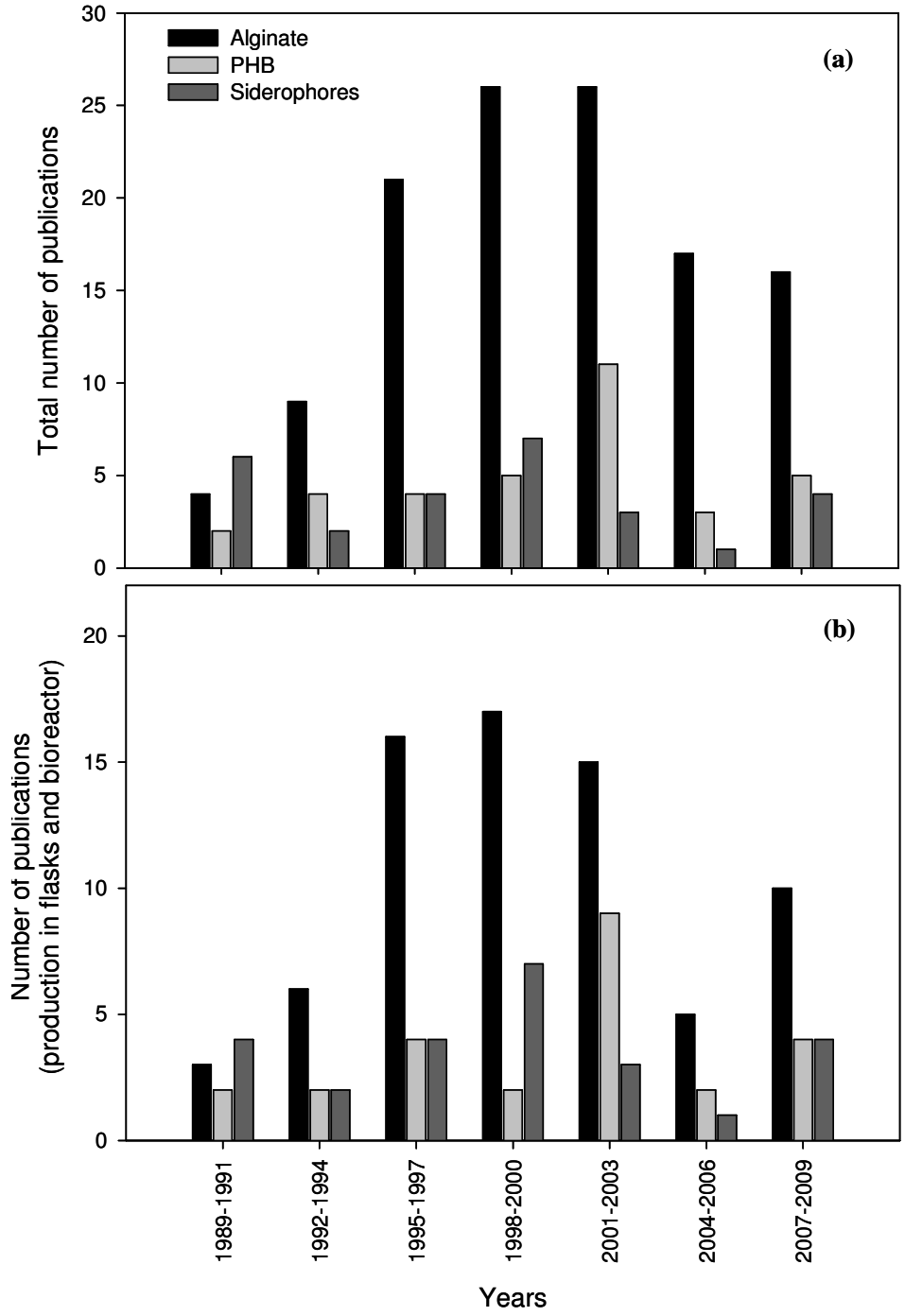

Figure 2. Number of research papers appearing with *A. vinelandii* and 'alginate', or 'PHB', or 'siderophores' in the Scopus database from 1989 to 2009 (database searched 22.10.09). (a) Total publications, (b) publications regarding alginate, PHB and siderophores production in shake flasks and bioreactor.

polymerization of GDP-mannuronic acid (polymannuronic acid) are carried out by a mannuronate polymerase, which is presumably located as a cytoplasmic membrane complex (polymerase complex) (Figure 3b). It has been suggested that AlgG, AlgK, AlgX and AlgL are periplasmic proteins forming a scaffold in the periplasm surrounding the nascent alginate chain. Moreover, the evidence shows that Alg8 and Alg44 are also catalytic subunits of the alginate polymerase (Mejía-Ruiz et al., 1997; Remminghorst and Rehm, 2006b). On t his regard,

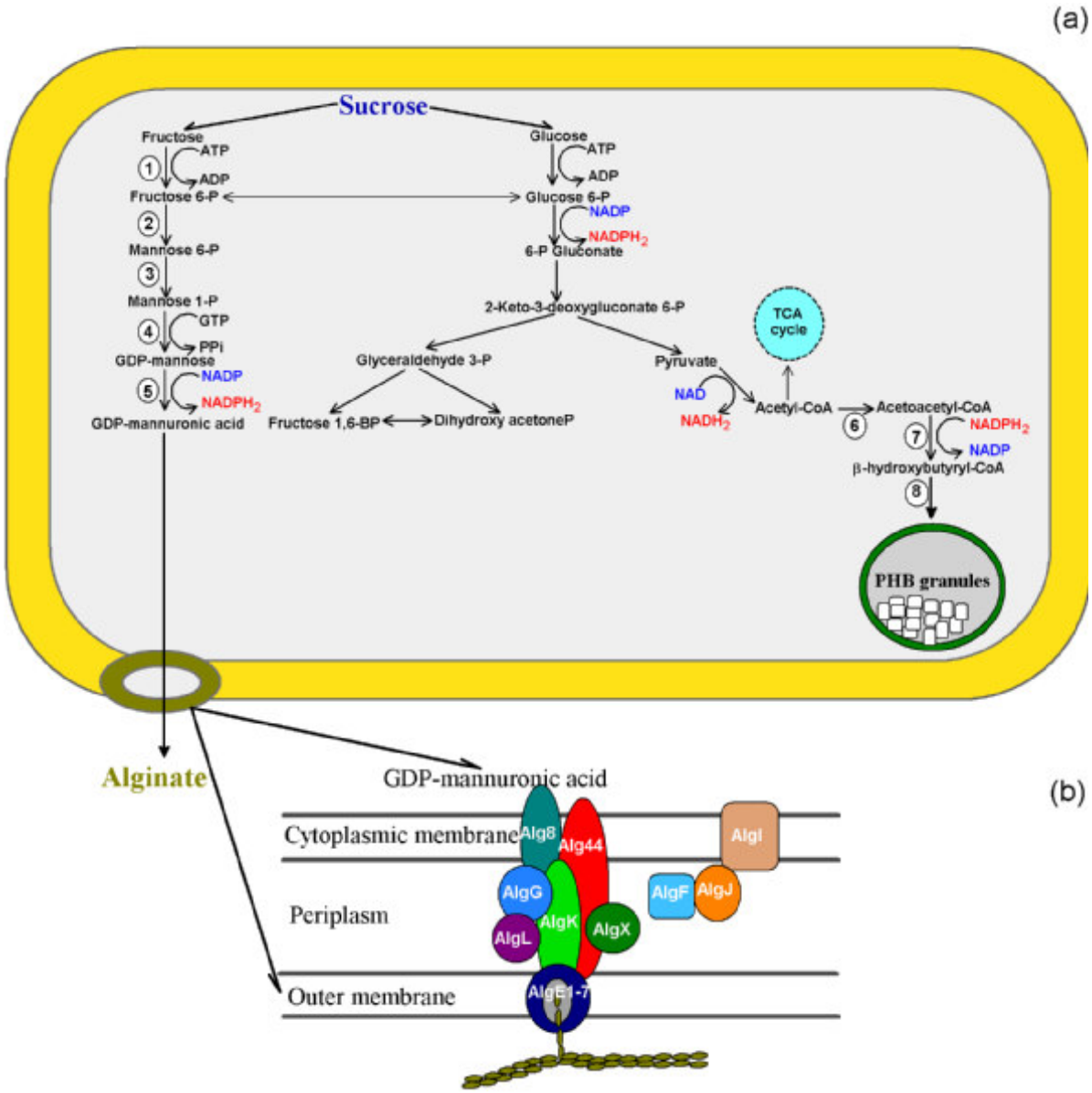

Figure 3. Biosynthesis of alginate and poly-β-hydroxybutyrate (PHB) in *A. vinelandii*. (a) Metabolic pathway to alginate and PHB; (b) model of the multi-protein complex involved in polymerization, modification and export of the alginate. 1 = Fructokinase; 2 = phosphomannose isomerase (*algA*); 3 = phosphomannomutase (*algC*); 4 = GDP-mannose-pyrophosphorylase (*algA*); 5 = GDP-mannose-dehydrogenase (*algD*); 6 = β-Ketothiolase (*phbA*); 7 = acetoacetyl CoA-reductase (*phbB*); 8 = PHB synthase (*phbC*). (Adapted from Remminghorst and Rehm, 2006a; Galindo et al., 2007).

recently, Remminghorst and Rehm (2006b) demonstrated that it is possible to increase in at least 10 times the alginate production of *P. aeruginosa* (a bacterium that also produces alginate) by the incorporation of additional copies of Alg8, suggesting that Alg8 (part of polymerase complex) might be a rate-limiting step of alginate production.

At periplasmic level, polymannuronic acid undergoes chemical modifications comprising acetylation (genes involved *algI*, *algJ*, *algF*) and control of molecular weight by alginate-lyase (AlgL) (Figure 3b), which is encoded by *algL* (LLoret et al., 1996). Different alginate lyases have been characterized in *A. vinelandii* cells; the periplasmic AlgL, the secreted bifunctional mannuronan C5-epimerase and alginate lyase AlgE7 (Kennedy et al., 1992; Ertesvåg et al., 1995; Svanem et al., 2001) and recently, three new *A. vinelandii* alginate lyases have been characterized (Gimmestad et al., 2009). Polymannuronic acid is exported through the outer membrane where the epimerization of mannuronate residues occurs (Figure 3b). *A. vinelandii* have a periplasmic mannuronan C5- epimerase (AlgG) and additionally, the genome of *A. vinelandii* encodes a

family of seven extracellular Ca^{2+} dependend epimerases (AlgE1 - 7) (Ertesvåg et al., 1995; Steigedal et al., 2008). The possibility of manipulating the acetylation degree of polymer, the epimerization pattern and the molecular weight of alginate produced by *A. vinelandii* will allow the production of alginates with different properties.

Poly-β-hydroxybutyrate (PHB) biosynthesis

Under unbalanced growth conditions, *A. vinelandii* produces PHB, a polymer of the polyhydroxyalcanoates family of polyesters. This polymer is accumulated when bacterial growth is limited by depletion of nitrogen, phosphorous or oxygen and when there is an excess amount of a carbon source present (Anderson and Dawes, 1990). It is known that the PHB accumulated serves as a store of carbon and energy and as an electron sink into which excess reducing power can be channeled (Senior and Dawes, 1973). The PHB biosynthesis is a NADPH-dependent pathway (Li et al., 2009). In *A. vinelandii*, the precursor of PHB biosynthesis is acetyl-coenzyme-A (acetyl-CoA) formed from catabolising the carbohydrate through the Entner–Doudoroff pathway (Still and Wang, 1964; Beale and Foster, 1996). It is well known that under oxygen limitation and excess of carbon source, NADH increases, inhibiting the citrate synthase and isocitrate dehydrogenase activities of the tricarboxylic acid cycle and hence acetyl-CoA levels rise, starting the PHB synthesis (Senior and Dawes, 1973). PHB is synthesized by three biosynthetic enzymes from acetyl-CoA. The condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA is catalyzed by a β-Ketothiolase. Acetoacetyl-CoA reductase produce β-hydroxybutyryl-CoA from acetoacetyl-CoA and $NADPH_2$ and the polymerization of β-hydroxybutyryl-CoA with the release of CoASH is catalyzed by PHB synthase (Verlinden et al., 2007) (Figure 3a). The PHB synthesis and its regulation in *A. vinelandii* cells can be revised with more detail in a recent article (Galindo et al., 2007).

Use of mutant's strains to produce alginate and PHB

From a productivity perspective, *A. vinelandii* cells can potentially be used for the production of alginate or PHB. However, it is clear that the synthesis of alginate constitutes a waste of substrate when seeking to optimize PHB production and the synthesis of PHB is undesirable when optimizing alginate production. The difficulty to separate the synthesis of PHB and alginate in a wild-type organism has been pointed out (Martínez et al., 1997). A strategy used by different groups has been blocking the synthesis of either alginate or PHB by mutation. In the case of alginate, different studies have been performed using *A. vinelandii* strains unable to produce PHB (Peña et al., 2002; Segura et al., 2003; Mejía et al., 2010). The main objective, when such strains are been used, has been to increase the alginate production as compared to the use of a wild-type strain. However, the use of mutant *A. vinelandii* strains impaired in PHB has shown opposite results. By comparing mutant decrease in PHB accumulation (named AT268) with the wild-type, Peña et al. (2002) showed that in batch cultures conducted in a bioreactor (at 3% of DOT), alginate production generated by mutants was lower (2.6 g l^{-1}) than the one obtained with the wild-type (3.5 g l^{-1}). Thus, those authors suggested that regulation of the carbon flux is very complex and that carbon source consumed is not necessarily diverted to alginate biosynthesis. It is now clear that alginate and PHB synthesis are under a very complex genetic control (Galindo et al., 2007). Nevertheless, it is possible to change this performance by implementing a different cultivation strategy. In fact, it has been recently shown that the cultivation of the strain AT6 (impaired in PHB) in a two stage fermentation process is potentially useful for the production of alginate, improving the productivity of the polymer (Mejía et al., 2010). In this work, a high efficiency of conversion of sucrose to alginate (0.74 g g^{-1}) and a high alginate concentration (9.5 g l^{-1}) as compared with the wild-type strain (4.8 g l^{-1}) was obtained, which made the process more competitive. In order to compare the alginate production using different mutant's strains of *A. vinelandii*, different results are shown in Table 1. To establish a comparable basis of analysis, the alginate yields ($Y_{p/s}$ and $Y_{p/x}$) and alginate production were normalized, that is, the values obtained using mutant strain were divided by the values using wild-type strain of *A. vinelandii*. As expected, the highest normalized alginate yields and alginate concentration values (above 1) were obtained using the AT6 strain, which was impaired in PHB production.

In the case of PHB, few reports have analyzed the production using a mutant of *A. vinelandii* impaired in the alginate production (Martínez et al., 1997; Segura et al., 2003). Segura et al. (2003) evaluated (in shake flasks cultures) a strain that can not produce alginate, affected in the *algA* gene. The evidence shows an increase in PHB accumulation (between 3 and 10 times), thus, a blockade at the first enzymatic step in alginate biosynthesis (AlgA) improves PHB accumulation. However, the performance of this strain at bioreactor scale has not been evaluated. A summary of normalized PHB yields values ($Y_{p/s}$ and $Y_{p/x}$) using mutant's strains of *A. vinelandii* are shown in Table 2. In these cases, the strategy used has been the construction of mutant's strains of *A. vinelandii* with improved ability to produce PHB (Noguez et al., 2008; Pyla et al., 2009); however, it is interesting note that these strains have not been evaluated at bioreactor scale.

SCALES OF PRODUCTION AND FERMENTATION STRATEGIES USED

Biotechnological process on a large-scale requires the

Table 1. Normalized maximal alginate concentration and alginate yields produced with different mutant's strains of *A. vinelandii.*

Strain	$Y_{p/s}$ normalized	$Y_{p/x}$ normalized	$Alginate_{max}$ normalized	Culture condition	Reference
AT6	3.75	2.73	0.46	Two stage fermentation	Mejía et al., 2010
	2.18	1.19	1.98		
AT6	1.15	2.68	1.16	Flasks	Segura et al., 2003
	1.37	4.18	1.42		
SML2	0.94	0.94	0.94	Batch bioreactor	Trujillo-Roldán et al., 2004
SML2	nd	0.49	0.65	Batch bioreactor	Trujillo-Roldán et al., 2003
CNT26	nd	nd	0.74	Batchbioreactor	Peña et al., 2002
AT268	nd	nd	0.74	Batch bioreactor	Peña et al., 2002
DM	nd	nd	0.74	Batch bioreactor	Peña et al., 2002
LA21	0.09	0.044	0.042	Flasks	Martínez et al., 1997

nd: Not determined. The normalized alginate yields ($Y_{p/s}$ and $Y_{p/x}$) and $alginate_{max}$ corresponds to value obtained using mutant strain divided by the value obtained using wild-type strain (ATCC 9046) under the same culture conditions.

Table 2. Normalized PHB yields produced with different mutant's strains of *A. vinelandii.*

Strain	$Y_{p/s}$ normalized	$Y_{p/x}$ normalized	Culture condition	Reference
△arrF	nd	250	Flasks	Pyla et al., 2009
ptsN-	nd	2.08	Flasks	Noguez et al., 2008
AJ1678	nd	3.4	Plates	Segura and Espin, 2004
JG41	9.67	nd	Flasks	Segura et al., 2003
	1.61	nd		
UWD	nd	2.49	Plates	Page et al., 2001
LA21	3.4	nd	Flasks	Martínez et al., 1997
UWD	2.7	nd	Flasks	Page and Knosp, 1989

nd: Not determined. The normalized PHB yields ($Y_{p/s}$ and $Y_{p/x}$) corresponds to value obtained using mutant strain divided by the value obtained using wild-type strain under the same culture conditions.

development of a suitable technology and economically favorable conditions for the production. Studies of scale-up are necessary to ensure that the optimal production (in the small scale) can be maintained at larger scale (Diaz and Acevedo, 1999; Schmidt, 2005). Although there is an extensive literature about scale-up of bioprocess, there is no common applicable scale-up strategy. Moreover, scale-up of bioprocess is even more complicated when the oxygen demand is high (such as the case of *A. vinelandii* cells), or when the rheological properties of the broth offer a high resistance to the mass transfer, as in alginate production.

There are very few reports of aspects referring to the scale-up of the process for alginate production (Trujillo-Roldán et al., 2001; Reyes et al., 2003) and to our knowledge; no reports are available regarding scale-up strategies for PHB production using *A. vinelandii* cells. In general, the production of alginate at greater scale (at bioreactor laboratory scale) has been realized without considering a scale-up criterion. The available literature shows works regarding scale-up alginate production only from shake flasks to stirred tank of laboratory (1 or 14 l) (Trujillo-Roldán et al., 2001; Reyes et al., 2003; Peña et al., 2008). Studies at pilot scale have not been performed. A typical difficulty found has been the considerable decrease in alginate molecular weight and the viscosity of the broths when the bioprocess is translated to laboratory bioreactor conducted at pH and DOT constant (Peña et al., 1997; Peña et al., 2000; Seáñez et al., 2001). A rigorous and interesting work of scale-up of alginate production by *A. vinelandii* was developed by Reyes et al. (2003). Utilizing as scale-up criterion the initial power drawn, Reyes et al. (2003) demonstrated that this criterion (initial PV^{-1}) did not permit to reproduce both alginate concentration and viscosity profiles of shake flasks in a stirred fermentor of 1 l. Recently, Peña et al. (2008) demonstrated that by simulating the evolution of the actual power input observed in shake flasks in a stirred fermentor, it was possible to produce alginates with a very similar molecular weight (1,800 kDa) and concen-

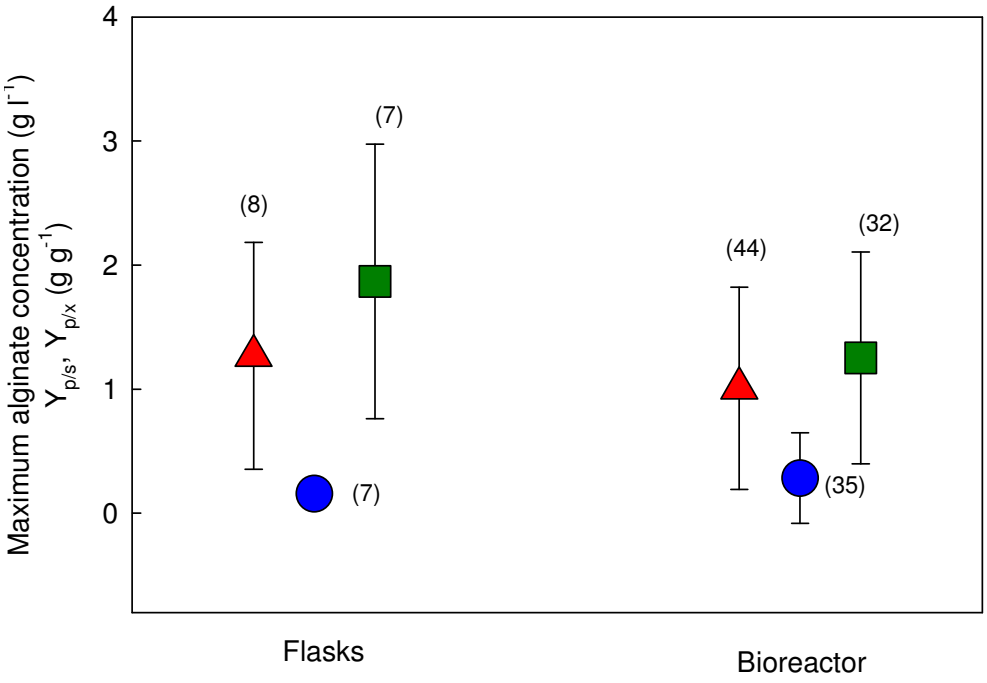

Figure 4. Influence of the scale of production on maximum alginate concentration and alginate yields obtained in *A. vinelandii* cultures using wild-type and mutant's strains. ■, Alginate concentration; ▲, alginate yield per cell basis ($Y_{p/x}$); ●, carbon source yield on alginate ($Y_{p/s}$). Numbers on each symbol indicate the values analyzed.

tration (4.0 g l^{-1}) in a bioreactor of 14 l. A possible explanation to this behavior could be that a similar profile of the DOT and the oxygen transfer rate (OTR) occurred in both shake flasks and bioreactor. These findings open the possibility of evaluating other criterion (example, constant OTR) for scaling-up alginate production, which has been used for the scale-up of the production of xanthan gum (Herbst et al., 1992).

Figure 4 shows a summary of the mean value (and standard deviation) of maximum alginate concentration and alginate yields ($Y_{p/s}$ and $Y_{p/x}$) obtained in *A. vinelandii* cultures conducted under different scale of production. It should be pointed out that the information was collected from very different conditions of cultivation (that is, shake flasks, bioreactor with or without DOT control) and using wild-type and mutant's strains of *A. vinelandii*. Interestingly, the results obtained clearly show that the production of alginate and the alginate yields obtained in shake-flasks cultures cannot be enhanced by increasing the production scale, in particular, laboratory bioreactor scale. Certainly, further study would be necessary to overcome this problem.

In the case of PHB, the studies regarding fermentation strategies (at bioreactor scale) with the aim of increasing PHB production using *A. vinelandii* are scarce (Page and Cornish, 1993; Chen and Page, 1997). In contrast, there is profuse literature regarding PHB production using other microorganisms (Lee, 1996; Reinecke and Steinbüchel, 2009). Using a mutant strain of *A. vinelandii* (named UWD), Chen and Page (1997) designed a fermentation strategy in a 2.5 l bioreactor using higher aeration to promote biomass production (first phase) and a lower aeration using fish peptone as a nitrogen source to promote PHB formation (second phase). An increase up to 36 g l^{-1} in PHB production and a notably improved productivity (1.05 g l^{-1} h^{-1}) was obtained using this approach. Thus, this mutant strain of *A. vinelandii* is attrac-tive since it accumulates PHB up to 85% of dry cell mass (Page and Cornish, 1993).

A recently published article describes two stage fermentation processes (no oxygen-limited and oxygen-limited conditions) for *A. vinelandii* cultures (Mejía et al., 2010). Although this fermentation strategy was designed to improve alginate production, the cultures using the wild-type strain showed an increase in the PHB yield ($Y_{p/s}$) from 0.03 to 0.44 g g^{-1}. In fact, two stage fermentation processes and fed-batch cultures as a strategy to improve the production of PHB have been widely reported (Lee, 1996).

LIMITATIONS OF PRODUCTION TO GREATER SCALE

From an industrial perspective, one potential problem that *A. vinelandii* cultures have is that an optimal alginate production can be obtained when the DOT is accurately controlled in a microaerophilic range (Peña et al., 2000; Sabra et al., 2000). *A. vinelandii* cultures conducted at

low DOT (between 0 and 1%) showed a decrease in alginate production, which was linked to an increase in intracellular storage of PHB (Horan et al., 1983; Díaz-Barrera et al., 2007); whereas at high DOT (about 10%), *A. vinelandii* cells use the carbon source mainly for biomass production (Parente et al., 1998).

On the other hand, it is known that *A. vinelandii* have a high level of respiration (Post et al., 1983), which is important to be considered for their use in industrial bioprocess. Due to this fact, it is possible that biomass of *A. vinelandii* cannot be grown at sufficiently high densities in a typical fermentation strategy at bioreactor scale, which could be a problem when the purpose is to implement the production of alginate or PHB at industrial scale. Despite this potential difficulty, further research is required in order to understand the molecular and biochemical mechanisms involved in alginate and PHB production in *A. vinelandii* cells.

PERSPECTIVES

From a bioengineering perspective and in order to develop a large-scale bioprocess to produce alginate or PHB using *A. vinelandii* cells, it is evident that it is difficult to improve the production of one of these biopolymers, especially if a wild-type strain is used. So far, the findings obtained on alginate and PHB production from *A. vinelandii* cells have only been developed to laboratory scale and therefore, the development of an adequate and efficient bioprocess to greater scale needs further research. As it was indicated by Galindo et al. (2007), the implementation of a multidisciplinary approach integrating molecular and bioengineering strategies is fundamental for optimizing alginate and PHB production using *A. vinelandii*.

A molecular strategy implemented for some years has been to introduce the genes responsible for PHB biosynthesis into *E. coli* (Schubert et al., 1988; Lee, 1998). In particular, the PHB genes of *Ralstonia eutropha* (or *Alcaligenes eutrophus*), one of the most extensively studied microorganisms (Reinecke and Steinbüchel, 2009), have been inserted in *E. coli* (Anderson and Dawes, 1990). As a model organism, recombinant *E. coli* is regarded as a strong candidate for PHB production owing to the fact that it can use several inexpensive carbon sources, the simplicity of extraction and the ease for genetic manipulation (Kim, 2000; Naik et al., 2008; Li et al., 2009). A recent work developed on recombinant *E. coli* strain contains the *pha* biosynthetic genes from an *Azotobacter* sp. strain FA8 (Nikel et al., 2006). In this work, the PHB was efficiently produced from agroindustrial by-products by the recombinant *E. coli* strain grown aerobically in fed-batch cultures and in a laboratory scale bioreactor (5.6 l). This type of strategy becomes an interesting potential application of PHB genes from *Azotobacter sp*. in order to establish an adequate and efficient microbial production process using a recombinant microorganism. Certainly, there is a necessity to continue with the basic research regarding alginate and PHB production in *A. vinelandii* cultures. Also, more research at laboratory bioreactor and pilot scale is required. Based on the analyzed evidence, we believe in the possibility to develop two new strategies in order to increase the concentration of alginate or PHB:

(1) In the case of PHB and considering the possibility of developing an efficient bioprocess to greater scale, we believe that a new recombinant strain could be built, likely, by the introduction of PHB genes from *A. vinelandii*, evaluating at least its performance at bioreactor laboratory and pilot scale. It is known that the PHB biosynthesis is a NADPH-dependent pathway (Li et al., 2009); hence, a recombinant that overproduces NADPH would be interesting to evaluate.
(2) In the case of alginate produced by *A. vinelandii,* we propose the construction of a recombinant strain that overexpress the *alg8* gene (a possibility not yet evaluated) and tests its performance at least in a bioreactor scale. In fact, evaluating the overexpression of *alg8* into mutant strain AT6, which is impaired in PHB production, would be unquestionably interesting. This proposal is based on the fact that alginate production in shake flask cultures of *P. aeruginosa* was at least tenfold higher when additional copies of *alg8* (as part of the polymerase complex) were inserted (Remminghorst and Rehm, 2006b).

There is no doubt that *A. vinelandii* is a remarkable cellular machinery with versatile genetic and metabolic components that has important implications for biotechnological exploitation. Taking into account the current findings, it is necessary to continue evaluating different mutant's strains of *A. vinelandii* and to develop recombinant strains using genes of this microorganism and evaluating their performances at laboratory bioreactor level and at greater scale bioprocess. Overall, the implementation of a new fermentation process at bioreactor scale using mutant's strains is required in order to enhance the productivity and molecular characteristics of alginate and PHB produced.

ACKNOWLEDGEMENT

We thank the Programa Bicentenario de Ciencia y Tecnología (PBCT, Project PSD-081/2008) and DII-PUCV 203.773 for financial support.

REFERENCES

Anderson A, Dawes E (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54: 450-472.

Beale J, Foster J (1996). Carbohydrate fluxes into alginate biosynthesis in *Azotobacter vinelandii* NCIB 8789: NMR investigations of the triose pools. Biochemistry, 35: 4492-4501.

Braud A, Jézéquel K, Bazot S, Lebeau T (2009). Enhanced phytoextraction of an agricultural Cr-and Pb-contaminated soil by bioaugmentation with siderophore-producing bacteria. Chemosphere, 74: 280-286.

Chen GQ, Page W (1997). Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. Biotechnol. Tech. 11: 347-350.

Cornish A, Page W (1998). The catecholate siderophores of *Azotobacter vinelandii:* their affinity for iron and role in oxygen stress management. Microbiology, 144: 1747-1754.

Diaz A, Acevedo F (1999). Scale-up strategy for bioreactors with newtonian and non-newtonian broths. Bioprocess Eng. 21: 21-23.

Díaz-Barrera A, Peña C, Galindo E (2007). The oxygen transfer rate influences the molecular mass of the alginate produced by *Azotobacter vinelandii*. Appl. Microbiol. Biotechnol. 76: 903-910.

Díaz-Barrera A, Silva P, Ávalos R, Acevedo F (2009). Alginate molecular mass produced by *Azotobacter vinelandii* in response to changes of the O_2 transfer rate in chemostat cultures. Biotechnol. Lett. 31: 825-829.

Dixon R, Kahn D (2004). Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. 2: 621-631.

Ertesvåg H, Høidal H, Hals I, Rian A, Doseth B, Valla S (1995). A family of modular type mannuronan C-5-epimerase genes controls alginate structure in *Azotobacter vinelandii*. Mol. Microbiol. 16: 719-731.

Fekete F, Spence J, Emery T (1983). Siderophores produced by nitrogen-fixing *Azotobacter vinelandii* OP in iron-limited continuous culture. Appl. Environ. Microbiol. 46: 1297-1300.

Galindo E, Peña C, Núñez C, Segura D, Espin G (2007). Molecular and bioengineering strategies to improve alginate and polydydroxyalkanoate production by *Azotobacter vinelandii*. Microb. Cell. Fact. 6(7).

Gimmestad M, Ertesvåg H, Heggeset T, Aarstad O, Svanem B, Valla S (2009). Characterization of three new *Azotobacter vinelandii* alginate lyases, one of which is involved in cyst germination. J. Bacteriol. 191: 4845-4853.

Herbst H, Schumpe A, Deckwer WD (1992). Xanthan production in stirred tank fermenters: oxygen transfer and scale-up. Chem. Eng. Technol. 15: 425-434.

Horan N, Jarman T, Dawes E (1983). Studies on some enzymes of alginic acid biosynthesis in *Azotobacter vinelandii* grown in continuous culture. J. Gen. Microbiol. 129: 2985-2990.

Kennedy L, Kenneth McD, Sutherland IW (1992). Alginases from *Azotobacter* species. J. Gen. Microbiol. 138: 2465-2471.

Kim B (2000). Production of poly(3-hydroxybutyrate) from inexpensive substrates. Enzyme Microbial. Technol. 27: 774-777.

Kraepiel A, Bellenger J, Wichard T, Morel F (2009). Multiple roles of siderophores in free-living nitrogen-fixing bacteria. Biometals, 22: 573-581.

Lee SY (1996). Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49:1-14.

Lee SY (1998). Poly(3-hydroxybutyrate) production from xylose by recombinant *Escherichia coli*. Bioprocess Eng. 18: 397-399.

Li ZJ, Cai L, Wu Q, Chen GQ (2009). Overexpression of NAD kinase in recombinant *Escherichia coli* harboring the *phbCAB* operon improves poly(3-hydroxybutyrate) production. Appl. Microbiol. Biotechnol. 83: 939-947.

Lloret L, Barreto R, Campos M, Moreno S, Martínez-Salazar J, Espín G, León R, Soberón-Chávez G (1996). Genetic analysis of the transcriptional arrangement of *Azotobacter vinelandii* alginate biosynthetic genes: identification of two independent promoters. Mol. Microbiol. 21: 449-457.

Martínez P, Guzmán J, Espín G (1997). A mutation impairing alginate production increased accumulation of poly-β-hydroxybutyrate in *Azotobacter vinelandii*. Biotechnol. Lett. 19: 909-912.

Mejía-Ruiz H, Guzman J, Moreno S, Soberón-Chávez G, Espin G (1997). The *Azotobacter vinelandii alg8* and *alg44* genes are essential for alginate synthesis and can be transcribed from an *algD*-independent promoter. Gene, 199: 271-277.

Mejía M, Segura D, Espín G, Galindo E, Peña C (2010). Two-stage fermentation process for alginate production by *Azotobacter vinelandii* mutant altered in poly-β-hydroxybutyrate (PHB) synthesis. J. Appl. Microbiol. 108: 55-61.

Möllmann U, Heinisch L, Bauernfeind A, Köhler T, Ankel-Fuchs D (2009). Siderophores as drug delivery agents: application of the Trojan horse strategy. Biometals 22:615-624.

Muhammadi , Ahmed N (2007). Genetics of bacterial alginate: alginate genes distribution, organization and biosynthesis in bacteria. Curr. Genomics, 8: 191-202.

Naik S, Venu Gopal S, Somal P (2008). Bioproduction of polyhydroxyalkanoates from bacteria: a metabolic approach. W. J. Microbiol. Biotechnol. 24: 2307-2314.

Nikel P, de Almeida A, Melillo E, Galvagno M, Pettinari J (2006). New recombinant *Escherichia coli* strain tailored for the production of poly(3-hydroxybutyrate) from agroindustrial by-products. Appl. Environ. Microbiol. 72: 3949-3954.

Noguez R, Segura D, Moreno S, Hernandez A, Juarez K, Espín G (2008). Enzyme I^{Ntr}, NPr and IIA^{Ntr} are involved in regulation of the poly-β-hydroxybutyrate biosynthetic genes in *Azotobacter vinelandii*. J. Mol. Microbiol. Biotechnol. 15: 244-254.

Page W, Von Tigerstrom M (1988). Aminochelin, a catecholamine siderophore produced by *Azotobacter vinelandii*. J. Gen. Microbiol. 134: 453-460.

Page W, Knosp O (1989). Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 55: 1334-1339.

Page W, Cornish A (1993). Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly-β-hydroxybutyrate. Appl. Environ. Microbiol. 59: 4236-4244.

Page W, Tindale A, Chandra M, Kwon E (2001). Alginate formation in *Azotobacter vinelandii* UWD during stationary phase and the turnover of poly-β-hydroxybutyrate. Microbiology, 147: 483-490.

Page W, Kwon E, Cornish A, Tindale A (2003). The *csbX* gene of *Azotobacter vinelandii* encodes an MFS efflux pump required for catecholate siderophore export. FEMS Microbiol. Lett. 228: 211-216.

Parente E, Crudele M, Aquino M, Clementi F (1998). Alginate production by *Azotobacter vinelandii* DSM576 in batch fermentation. J. Ind. Microbiol. Biotechnol. 20: 171-176.

Peña C, Campos N, Galindo E (1997). Changes in alginate molecular mass distributions, broth viscosity and morphology of *Azotobacter vinelandii* cultured in shake flasks. Appl. Microbiol. Biotechnol. 48: 510-515.

Peña C, Trujillo-Roldán M, Galindo E (2000). Influence of dissolved oxygen tension and agitation speed on alginate production and its molecular weight in cultures of *Azotobacter vinelandii*. Enzyme Microb. Technol. 27: 390-398.

Peña C, Miranda L, Segura D, Núñez C, Espin G, Galindo E (2002). Alginate production by *Azotobacter vinelandii* mutants altered in poly-β-hydroxybutyrate and alginate biosynthesis. J. Ind. Microbiol. Biotechnol. 29: 209-213.

Peña C, Millán M, Galindo E (2008). Production of alginate by *Azotobacter vinelandii* in a stirred fermentor simulating the evolution of power input observed in shake flasks. Process Biochem. 43: 775-778.

Post E, Kleiner D, Oelze J (1983). Whole cell respiration and nitrogenase activities in *Azotobacter vinelandii* growing in oxygen controlled continuous culture. Arch. Microbiol. 134: 68-72.

Pyla R, Kim TJ, Silva J, Jung YS (2009). Overproduction of poly-β-hydroxybutyrate in the *Azotobacter vinelandii* mutant that does not express small RNA ArrF. Appl. Microbiol. Biotechnol. 84: 717-724.

Ramsey D, Wozniak D (2005). Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol. Microbiol. 56: 309-322.

Reinecke F, Steinbüchel A (2009). *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J. Mol. Microbiol. Biotechnol. 16: 91-108.

Remminghorst U, Rehm B (2006a). Bacterial alginates: from biosynthesis to applications. Biotechnol. Lett. 28: 1701-1712.

Remminghorst U, Rehm B (2006b). In vitro alginate polymerization and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 72: 298-305.

Reyes C, Peña C, Galindo E (2003). Reproducing shake flasks perfo-rmance in stirred fermentors: production of alginates by *Azotobacter*

vinelandii. J. Biotechnol. 105: 189-198.
Sabra W, Zeng AP, Lunsdorf H, Deckwer WD (2000). Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase. Appl. Environ. Microbiol. 66: 4037-4044.
Sabra W, Zeng AP, Deckwer WD (2001). Bacterial alginate: physiology, product quality and process aspects. Appl. Microbiol. Biotechnol. 56: 315-325.
Sadoff H (1975). Encystment and germination in *Azotobacter vinelandii*. Bacteriol. Rev. 39: 516-539.
Schmidt FR (2005). Optimization and scale up of industrial fermentation processes. Appl. Microbiol. Biotechnol. 68: 425-435.
Schubert P, Steinbüchel A, Schlegel H (1988). Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. 170: 5837-5847.
Seáñez G, Peña C, Galindo E (2001). High CO_2 affects alginate production and prevents polymer degradation in cultures of *Azotobacter vinelandii*. Enzyme Microb. Technol. 29: 535-540.
Segura D, Guzmán J, Espín G (2003). *Azotobacter vinelandii* mutants that overproduce poly-β-hydroxybutyrate or alginate. Appl. Microbiol. Biotechnol. 63:159-163.
Segura D, Espín G (2004). Inactivation of *pycA*, encoding pyruvate carboxylase activity, increases poly-β-hydroxybutyrate accumulation in *Azotobacter vinelandii* on solid medium. Appl. Microbiol. Biotechnol. 65: 414-418.
Senior P, Dawes E (1973). The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii.* Biochem. J. 134: 225-238.
Setubal J, dos Santos P, Goldman B, Ertesvåg H, Espín G, Rubio L, Valla S, Almeida N, Balasubramanian D, Cromes L, Curatti L, Du Z, Godsy E, Goodner B, Hellner-Burris K, Hernandez J, Houmiel K, Imperial J, Kennedy C, Larson T, Latreille P, Ligon L, Lu J, Mærk M, Miller N, Norton S, O'Carroll I, Paulsen I, Raulfs E, Roemer R, Rosser J, Segura D, Slater S, Stricklin S, Studholme D, Sun J, Viana C, Wallin E, Wang B, Wheeler C, Zhu H, Dean D, Dixon R, Wood D (2009). Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. J. Bacteriol. 191: 4534-4545.
Steigedal M, Sletta H, Moreno S, Mærk M, Christensen B, Bjerkan T, Ellingsen T, Espín G, Ertesvåg H, Valla S (2008). The *Azotobacter vinelandii* AlgE mannuronan C-5-epimerase family is essential for the *in vivo* control of alginate monomer composition and for functional cyst formation. Environ. Microbiol. 10: 1760-1770.
Still G, Wang C (1964). Glucose catabolism in *Azotobacter vinelandii*. Arch. Biochem. Biophys. 105: 126-132.
Svanem BI, Strand WI, Ertesvåg H, Skjåk-Braek G, Hartmann M, Barbeyron T, Valla S (2001). The catalytic activities of the bifunctional *Azotobacter vinelandii* mannuronan C-5-epimerase and alginate lyase AlgE7 probably originate from the same active site in the enzyme. J. Biol. Chem. 276: 31542-31550.
Tindale A, Mehrotra M, Ottem D, Page W (2000). Dual regulation of catecholate siderophore biosynthesis in *Azotobacter vinelandii* by iron and oxidative stress. Microbiology, 146: 1617-1626.
Trujillo-Roldán M, Peña C, Ramírez O, Galindo E (2001). Effect of oscillating dissolved oxygen tension on the production of alginate by *Azotobacter vinelandii*. Biotechnol. Prog. 17: 1042-1048.
Trujillo-Roldán M, Moreno S, Segura D, Galindo E, Espín G (2003). Alginate production by an *Azotobacter vinelandii* mutant unable to produce alginate lyase. Appl. Microbiol. Biotechnol. 60: 733-737.
Trujillo-Roldán M, Moreno S, Espín G, Galindo E (2004). The roles of oxygen and alginate-lyase in determining the molecular weight of alginate produced by *Azotobacter vinelandii*. Appl. Microbiol. Biotechnol. 63: 742-747.
Upadhyay A, Srivastava S (2008). Characterization of a new isolate of *Pseudomonas fluorescens* strain Psd as a potential biocontrol agent. Lett. Appl. Microbiol. 47: 98-105.
Verlinden R, Hill D, Kenward M, Williams C, Radecka I (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. J. Appl. Microbiol. 102: 1437-1449.
Yao B, Ni C, Xiong Ch, Zhu Ch, Huang Bo (2009). Hydrophobic modification of sodium alginate and its application in drug controlled release. Bioprocess Biosyst. Eng. 33: 457-463.