Process Biochemistry 48 (2013) 995–1003

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Oxygen supply strongly influences metabolic fluxes, the production of poly(3-hydroxybutyrate) and alginate, and the degree of acetylation of alginate in *Azotobacter vinelandii*

Tania Castillo[a], Elmar Heinzle[b], Susanne Peifer[b], Konstantin Schneider[b], Carlos F. Peña M[a,*]

[a] *Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Post. 510-3 Cuernavaca, 62250 Morelos, Mexico*
[b] *Biochemical Engineering Institute, Saarland University, Campus A1.5, D-66123 Saarbrücken, Germany*

ARTICLE INFO

Article history:
Received 29 October 2012
Received in revised form 20 April 2013
Accepted 22 April 2013
Available online 10 May 2013

Keywords:
A. vinelandii
Metabolic flux
Alginate
Acetylation
PHB

ABSTRACT

The aim of this study was to evaluate carbon flux in *Azotobacter vinelandii* using metabolic flux analysis (MFA) under high and low aeration conditions to achieve an improved understanding of how these changes could be related to alginate acetylation and PHB production. Changes in oxygen availability had a considerable impact on the metabolic fluxes and were reflected in the growth rate, the specific glucose consumption rate, and the alginate and PHB yields. The main differences at the metabolic flux level were observed in three important pathways. The first important difference was consistent with respiratory protection; an increase in the flux generated through the tricarboxylic acid (TCA) cycle for cultures grown under high aeration conditions (up to 2.61 times higher) was observed. In the second important difference, the fluxes generated through pyruvate dehydrogenase, phosphoenol pyruvate carboxykinase and pyruvate kinase, all of which are involved in acetyl-CoA metabolism, increased by 10, 43.9 and 17.5%, respectively, in cultures grown under low aeration conditions compared with those grown under high aeration conditions. These changes were related to alginate acetylation, which was 2.6 times higher in the cultures with limited oxygen, and the changes were also related to a drastic increase in PHB production. Finally, the glyoxylate shunt was active under both of the conditions that were tested, and a 2.79-fold increase was observed in cultures that were grown under the low aeration condition.

1. Introduction

Alginates are polysaccharides that are produced by brown algae, such as *Laminaria hyperborea*, and bacteria of the genera *Azotobacter* and *Pseudomonas*. Alginates are linear unbranched copolymers of β-D-mannuronic acid (M) and its C5 epimer, α-L-guluronic acid (G). Bacterial alginates may also contain O-acetyl groups located at O-2 and/or O-3 of mannuronate residues [1,2]. Alginates are widely used in the food and pharmaceutical industries because of their viscosifying, stabilizing and gelling properties [2]. The chemical structure of alginates including the G/M relation, the degree of acetylation and the mean molecular weight (MMW) determines their functional properties. Although alginate can be obtained from brown algae, the main advantage of the alginates produced by bacteria is that their chemical composition can be modified by changes in the culture conditions [3].

In the case of alginate acetylation, the presence of O-acetyl groups affects the physical properties of alginates by changing the conformation of the polymer [4], reducing the interactions with divalent cations and increasing the solubility in aqueous solutions [5]. It has been observed that small changes in the degree of acetylation have important effects on the viscosity of alginate solutions, independent of the MMW [6]. The swelling capabilities of acetylated alginates are also improved, yielding softer and more hydrated alginate gels [5].

Alginate acetylation also has important biological functions in *Azotobacter vinelandii* and *Pseudomonas*. For example, *Pseudomonas aeruginosa* is an opportunistic pathogen that requires acetylated alginate to form stable biofilms during infection processes; in addition, the acetylation of alginates enhances the resistance of this bacterium to complement-mediated and opsonic antibody-mediated phagocytosis [7,8]. On the other hand, *A. vinelandii* is capable of differentiating into cysts under conditions of desiccation, and alginate is an important component of the mature cyst structure [9]. Vázquez et al. [9] reported that a mutant strain of *A. vinelandii* (AJ34), which produces unacetylated alginates, had

* Corresponding author. Tel.: +52 777 329 16 17; fax: +52 777 317 23 88.
E-mail addresses: tmarenco@ibt.unam.mx (T. Castillo), e.heinzle@mx.uni-saarland.de (E. Heinzle), susanne.peifer@mx.uni-saarland.de (S. Peifer), ko.schneider@mx.uni-saarland.de (K. Schneider), carlosf@ibt.unam.mx (C.F. Peña M).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.04.014

an important reduction (5-fold) in its encystment efficiency. The mutant strain's resistance to desiccation (measured as viability) after 35 days was also lower with respect to the wild type [9]. Additionally, it has been reported that the acetyl group protects the mannuronate residues from guluronate epimerization [4] and protects the polymer from alginate lyase activities [9].

In *Azotobacter*, the main pathway for glucose catabolism is the Entner–Doudoroff (ED) pathway [10]. Glucose is internalized by an inducible Glu-1 permease [11] and phosphorylated by glucokinase to form glucose-6-phosphate (G-6-P). G-6-P is transformed into 6-phosphogluconate by the combined action of glucose-6-phosphate dehydrogenase [12] and 6-phosphogluconolactonase [13]. Then, 6-phosphogluconate enters the ED pathway, where it is converted into 2-keto-3-deoxyphosphogluconate (KDPG) by 6-phosphogluconate dehydratase (Edd). Finally, KDPG is hydrolyzed into glyceraldehyde-3-phosphate (GAP) and pyruvate (PYR) by 2-keto-3-deoxy-6-phosphogluconate aldolase [13].

For alginate biosynthesis, GAP is directed to begin gluconeogenesis and forms fructose-6-phosphate (F-6-P) [14]. F-6-P is isomerized by the bifunctional enzyme phosphomannose isomerase/guanosine diphosphomannose pyrophosphorylase (AlgA) to produce mannose-6-phosphate (M-6-P). Phosphomannomutase (AlgC) transforms M-6-P into mannose-1-phosphate (M-1-P), which is converted into GDP-mannose by the enzyme AlgA. GDP-mannose dehydrogenase (AlgD) oxidizes GDP-mannose into GDP-mannuronic acid [14]. All of the above reactions take place in the cytoplasm of the bacteria. GDP-mannuronic acid is then polymerized by the inner membrane proteins Alg8 and Alg44 [14]. In the periplasmic space alginate is susceptible to acetylation, and it is finally exported out of the cell [14].

Acetyl-Coenzyme A (acetyl-CoA) is the primary acetyl donor during acetylation processes catalyzed by a wide number of acetyl transferases, and acetyl-CoA has been proposed as the acetyl donor for alginate acetylation [15]. The O-acetylation of alginates takes place in the periplasmic space of *A. vinelandii* by the action of a protein complex composed of AlgI, AlgV and AlgF [9]. These proteins show high sequence identities with the AlgI, AlgJ and AlgF proteins, respectively, in *P. aeruginosa* [9]. In *P. aeruginosa*, it has been proposed that the AlgI and AlgJ proteins are responsible for the transport of O-acetyl groups from their cytoplasmic precursors across the inner membrane, while AlgF transfers the acetyl groups to the alginate [15].

A. vinelandii also produces poly(3-hydroxybutyrate) (PHB), a typical bacterial reserve of carbon and energy, which has potential industrial and commercial uses as a substitute for oil-derived plastics [14]. In *Azotobacter* species, PHB biosynthesis and its regulation are well known [16,17]. Acetyl-CoA is the key precursor during PHB biosynthesis, which begins with the condensation of two acetyl-CoA molecules by the enzyme β-ketothiolase. This condensation is positively regulated by acetyl-CoA, and it is competitively inhibited by free Coenzyme A (CoA) [17].

The role of the oxygen supply in the production of alginate and PHB has been widely studied [3,14]. In the case of alginate, it has been observed that oxygen availability affects not only yields of the polymer but also its chemical structure, primarily its MMW and degree of acetylation [3]. On the other hand, although the process of PHB accumulation in several microorganisms is promoted under conditions of essential nutrient limitation (i.e., NH_3 or PO_4) with an excess of a carbon source, in *Azotobacter* vegetative cells, PHB accumulation mainly occurs under oxygen limitation, and it is promoted preferentially during the stationary growth phase [16,17].

A. vinelandii has been widely studied for several decades, not only because of its potential to carry out oxygen-sensitive processes even in the presence of oxygen, particularly nitrogen fixation [18], but also due to its ability to produce alginate and PHB [14,18]. However, currently there are no studies utilizing metabolic flux analyses (MFAs) in *A. vinelandii*. MFA aids the understanding of intracellular carbon fluxes and their regulation [19,20]. MFA also provides a flux map describing the reactions involved in metabolism and an estimate of the steady-state rate at which every reaction occurs [19,20]. In addition, MFA could be used to evaluate the rigidity or flexibility of metabolic branch points according to changes in growth conditions, such as oxygen availability [19,20].

Due to the biological and commercial relevance of *A. vinelandii* and its products, the carbon flux distribution in this bacterium was evaluated under high and low aeration conditions to elucidate how these changes could be related to alginate and PHB production.

2. Materials and methods

2.1. Microorganism

Experiments were carried out using wild type *A. vinelandii* ATCC 9046 (American Type Culture Collection). This strain was maintained through monthly subculture on Burk's agar slopes and stored at 4 °C.

2.2. Growth conditions

Two growth conditions (high and low aeration) were evaluated in shaken flasks. The first condition was achieved using conventional 500 mL Erlenmeyer flasks filled with 100 mL of media, and the second condition was studied using 500 mL baffled flasks filled with 50 mL of media. The dissolved oxygen tension (DOT) was monitored with PRESENS optical sensors [21]. The cultures under both conditions were incubated at 30 °C at 200 rpm and were monitored for 64 h.

2.3. Culture medium

Burk's media was used [22] with no nitrogen source and with the following composition: 20 g L^{-1} glucose, 0.66 g L^{-1} K_2HPO_4, 0.16 g L^{-1} KH_2PO_4, 1.42 g L^{-1} MOPS, 0.05 g L^{-1} $CaSO_4{\cdot}2H_2O$, 0.2 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4$, 0.0029 g L^{-1} $Na_2MoO_4{\cdot}2H_2O$ and 0.027 g L^{-1} $FeSO_4{\cdot}7H_2O$. The initial pH was adjusted to 7.2 using NaOH (1 N) before autoclaving.

2.4. Cultures

For each condition, three replicates were performed to quantify the growth, degree of alginate acetylation and production of alginate and PHB. Each experimental flask was inoculated with 10% of its total volume. Pre-cultures were grown for 15 h under high aeration conditions in 500 mL baffled flasks with 50 mL of culture media. Under this growth condition, cells did not accumulate PHB. Samples were taken every 1 or 2 h during the exponential growth phase and every 4 or 6 h during the stationary phase. All samples were immediately centrifuged at 13,000 rpm and 4 °C to separate the biomass from the supernatant. The samples were stored at −20 °C for future analyses.

2.5. Cell growth

Microbial growth was evaluated by optical density (O.D.) at a wavelength of 560 nm. Measurements of absorbance were performed in duplicate at each time point. In addition, dry weight measurements were carried out using 1 mL of culture. The samples were placed in 1.5 mL Eppendorf tubes that had been previously dried and weighed. Then, samples were centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatant was recovered for alginate and glucose quantification. Cells were washed twice with distilled water and dried at 80 °C until they reached a constant weight. The optical density and dry weight were correlated to produce a standard curve.

2.6. Glucose and organic acids quantification

Quantification of the glucose levels in the supernatant was performed by HPLC using an Aminex HPX-87H column (300 mm × 7.8 mm) (Biorad, Hercules, CA, USA). The eluent was H_2SO_4 (7 mM) and was eluted at a flow rate of 0.8 mL min^{-1}. Glucose was detected using a refractive index (RI) detector (ERC-7515A, ERC Inc., Altegolfsheim, Regensburg, Germany) [19]. The supernatant was also tested for the presence of organic acids, using the same chromatographic method, followed by UV absorption at 210 nm using a photodiode array detector (ERC Inc., Altegolfsheim, Regensburg, Germany) [19]. However, under both aeration conditions evaluated, no organic acids were detected in the supernatant.

2.7. Alginate quantification

Alginate was quantified using a modified carbazole method [23]. Uronic acids were quantified directly from the supernatant. The volume of H_2SO_4-borate used was 1.2 mL instead of the 6 mL that was used in the original technique, and 140 μL