Bioelectrochemistry 117 (2017) 40–47

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Enhancement of anaerobic lysine production in *Corynebacterium glutamicum* electrofermentations

Nikolaos Xafenias *, Cathleen Kmezik, Valeria Mapelli

Division of Industrial Biotechnology, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg 41296, Sweden

ARTICLE INFO

Article history:
Received 21 March 2017
Received in revised form 30 May 2017
Accepted 3 June 2017
Available online xxxx

Keywords:
Anthraquinone-2-sulfonate
Biocathodes
Corynebacterium glutamicum
Electrofermentations
Lysine

ABSTRACT

It has been suggested that application of electric potential can affect lysine producing fermentations, although experimental evidence is lacking. To study this hypothesis we used the lysine producer *Corynebacterium glutamicum* ZW04, and we exposed it to 12 different conditions regarding anaerobic gas environment, applied electrode potential (cathodic, open circuit, anodic), redox mediator and nitrate presence. The gas environment was found to play a major role, with CO_2 leading to double the lysine concentrations and yields when compared to N_2. Electrode potentials also played a major role, with reductive conditions doubling the titers and increasing the yields of lysine up to 4 times. Addition of the redox mediator anthraquinone-2-sulfonate (AQ2S) under the presence of CO_2 and reductive conditions led to additional doubling of the titers, although the yields were not altered considerably. This study demonstrates for the first time that cathodic electrode conditions combined with CO_2 and AQ2S as a redox mediator can significantly improve both the yields and the titers of lysine production of a *C. glutamicum* lysine producing strain, reaching levels that have only been achieved under aerobic conditions.

1. Introduction

Lysine is an amino acid with considerable industrial importance and market value, important in both human and microbial nutrition. Lysine is nowadays produced aerobically by industrial strains of *Corynebacterium glutamicum*, in an aeration-intensive and sensitive process [1]. *C. glutamicum* is a facultative anaerobe that can utilize alternative electron acceptors in addition to oxygen (e.g. nitrate and humic acids) [1–4], and possesses a capability for marginal growth in the absence of an external electron acceptor, in particular when CO_2 is supplied [5]. Anaerobic lysine production could be an attractive alternative option to the aerobic process [1], and from a microbial ecology point of view this is a very interesting topic as *C. glutamicum* are soil bacteria often present in mixed microbial systems [6]. Even though the proof-of-concept has been demonstrated for some lysine producing strains of *C. glutamicum* [1], an efficient strategy for anaerobic lysine production has not yet been developed.

C. glutamicum has been extensively studied and engineered for producing lysine [7], aiming, among others, in modifying the central carbon metabolism, the terminal pathways, and the redox co-factor regeneration systems which are playing a major role [6]. In particular, regeneration of NADPH is a crucial obstacle as 4 mol of NADPH are required to produce 1 mol of lysine from glucose [8]. Intracellular redox balance could be potentially achieved by electrochemically assisted fermentations (electrofermentations), a concept where microbes use electrodes to dispose of or receive electrons from [9]. Although this is a relatively old concept, it has recently regained interest mainly because of "green" electricity expansion and the lower electricity prices that make the grid an attractive source of electrons. Incorporating electrodes as energy source to lysine fermentations could result in cost savings and better market conditions [10], which is an additional reason why we need to learn more about the conditions needed for this strategy to succeed.

Kracke and Krömer recently demonstrated by elementary mode analysis that both anode and cathode electrodes can increase lysine yields from glucose [11]. While anodic electron sinks are expected to result in a proton gradient that will eventually drive ATP synthesis, cathodes are expected to have a higher impact by producing more reduced redox factors (NADPH) [11]. *Corynebacterium* spp. are the main lysine producers and also capable of extracellular electron transfer [12], and therefore they are potential candidates for electrofermentations. In fact this concept has been demonstrated by Hongo and Iwahara, who first showed that there is a potential for increasing glutamate yields from glucose by 10–15% when neutral red is mediating electrons from cathodes used as additional energy sources [13,14]. Recently, Sasaki et al. reported an increase of the lactate yield from glucose by 20% when anthraquinone-2,6-disulfonate was added as a redox mediator in an oxygen-supplemented cathode [15]. In our previous study with a wild type strain of *C. glutamicum* we showed a glucose consumption up to 6 times faster under strongly reductive conditions in the presence of the redox mediator anthraquinone-2-sulfonate (AQ2S) [3].

* Corresponding author.
E-mail address: xafenias@chalmers.se (N. Xafenias).

http://dx.doi.org/10.1016/j.bioelechem.2017.06.001

In this study we aimed at identifying and understanding the conditions under which lysine production by *C. glutamicum* can be electrochemically supported with the use of polarized electrodes. To achieve this aim we tested *C. glutamicum* ZW04, a lysine overproducing strain [16,17] engineered in a similar way as reported elsewhere for the generation of the AHP-3 strain [18–20]: *lysC* (T311I), hom (V59A), and *pycA* (P458S) [16]. To study lysine production under anaerobic electrofermentation conditions we subjected this strain to 12 different conditions in relation to the gas environment (N_2 and CO_2), the electrode potentials (reductive, open circuit, oxidative), the presence/absence of a soluble electron acceptor (with and without nitrate), and the presence/absence of a soluble redox mediator (with and without AQ2S). This study demonstrates for the first time the importance and different roles of CO_2, reductive potentials, and redox mediator conditions in lysine electrofermentations. This knowledge is necessary to expand the use of bioelectrochemical systems to the production of fine chemicals, and to consider "green" electricity produced from sustainable sources as an alternative electron source.

2. Materials and methods

2.1. Reactor assembling and electrochemical control

H-type bioelectrochemical reactors of a total 720 mL volume were constructed by joining two 360 mL borosilicate bottles together (Adams and Chittenden Scientific Glass, USA). A Nafion® N117 (Ion Power Inc., USA) proton exchange membrane was placed in between each pair of bottles to separate the working electrode (WE) from the counter electrode (CE) chamber. Pretreatment of the proton exchange membrane and assembling of the reactor was performed as previously described [3]. Each bioelectrochemical reactor consisted of a three-electrode setup where the WE and CE were made of graphite felt (SIGRATHERM, SGL Carbon Ltd., UK) with a total projected surface area of 38 cm^2 (5.0 cm × 3.0 cm × 0.5 cm). These were pretreated and bonded to graphite rods (4.5 cm × ∅ 0.3 cm; 40,765, Alfa Aesar®) and Ti wires as described elsewhere [3]. WE, CE, and reference electrodes (RE; Ag/AgCl: 3 M NaCl, RE-5B, BASi, USA) were connected with two-channel potentiostats (MLAB, Bank Elektronik-Intelligent Controls GmbH; Germany) to apply the desired potentials and record the current produced, every 1 min.

2.2. Chemicals

High purity chemicals purchased from Sigma Aldrich (Sweden) were used in this study and all solutions were made using milli-Q water. Pre-cultures of *Corynebacterium glutamicum* were grown in a rich, autoclaved (121 °C, 20 min) growth medium (pH 7.2) consisting of (g/L): bacto peptone (10.0), yeast extract (5.0), glucose (5.0) and NaCl (5.0). The medium used for the WE solution (pH 7.0) was adapted from another study [21] and consisted of (g/L): glucose (10.0), NaCl (1.0), $CaCl_2 \cdot 2H_2O$ (0.06), $MgSO_4 \cdot 7H_2O$ (0.20), $(NH_4)_2SO_4$ (15.0), K_2HPO_4 (10.7), KH_2PO_4 (5.25), $FeSO_4 \cdot 7H_2O$ (20.0×10^{-3}), biotin (0.5×10^{-3}), thiamine·HCl (1.0×10^{-3}), 3,4-dihydroxybenzoic acid (30.0×10^{-3}; dissolved in 950×10^{-6} L of milli-Q water mixed with 50×10^{-6} L of 4 M NaOH), $FeCl_3 \cdot 6H_2O$ (2.0×10^{-3}), $MnSO_4 \cdot H_2O$ (2.0×10^{-3}), $ZnSO_4 \cdot 7H_2O$ (0.5×10^{-3}), $CuCl_2 \cdot 2H_2O$ (0.2×10^{-3}), $Na_2B_4O_7 \cdot 10H_2O$ (0.2×10^{-3}), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.1×10^{-3}). Prior to mixing the media components, stock solutions were prepared and sterilized separately to avoid precipitation: salt solution (NaCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$), $(NH_4)_2SO_4$ solution, buffer solution (K_2HPO_4, KH_2PO_4; pH 7.0) and $FeSO_4 \cdot 7H_2O$ solution (pH 1.0) were autoclaved separately. The vitamin solution (biotin and thiamine·HCl), the 3,4-dihydroxybenzoic acid solution, and the trace elements solution ($FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; pH 1.5) were filter sterilized (0.2 μm) separately and mixed when the autoclaved solutions had cooled down. An autoclaved cell washing solution (pH 7.0) was also used and consisted of: 5.8 g/L NaCl, 6.0 g/L K_2HPO_4, and 2.0 g/L KH_2PO_4. This was also used as a CE solution. When mentioned, a nitrate solution (252.0 g/L KNO_3) was added to the WE medium to give concentrations of $557 \pm 72 \times 10^{-3}$ g-NO_3^- N/L. Also the redox mediator AQ2S ($E' = -0.45$ V at pH 7.0 [3], supplemented in the oxidative form) was added in the WE chamber when mentioned, at a final concentration of 0.1 mM (equivalent to an electric charge of 5C), because this particular compound was shown to enhance glucose fermentation by *C. glutamicum* [3]. Adjustments of pH were made using filter sterilized (0.2 μm) HCl and NaOH solutions, whichever appropriate.

2.3. Setup and operation

Twelve different conditions were tested in duplicate (24 reactors in total) to test the effect of the gas environment (CO_2 or N_2), the electrode potential (reductive, open circuit, oxidative), the presence of nitrate as electron acceptor, and the presence of AQ2S as redox mediator. Because the focus of this work is on lysine production and lysine was poorly produced under a N_2 gas environment independently of the electrochemical conditions, all subsequent experiments were performed under CO_2 sparging. The reactors were assembled and filled with milli-Q water prior to autoclaving. After autoclaving the setting up of the reactors was done under sterile conditions in a laminar flow cabinet; the milli-Q water was discarded, the WE chamber was filled with 270 mL of the WE medium, and the CE chamber was filled with 280 mL of the CE solution. Ethanol-sterilized reference electrodes were inserted into the WE chamber in close proximity to the WE. Autoclaved spargers were inserted into the WE chamber and immersed into the solution. Reactors were sparged with filter-sterilized (0.2 μm) CO_2 or N_2 gas depending on the setup. Mixing was performed by magnetic flees on magnetic stirrer plates. The pH in the WE and CE was re-adjusted manually 2–3 times a day to an average daily value of 7; in the case of N_2 sparging and reductive conditions, adjustments were made 4–6 times a day. All experiments were performed at room temperature (20 ± 1 °C).

2.4. Corynebacterium glutamicum ZW04 cultivation

Glycerol stocks (50% v/v glycerol) of *Corynebacterium glutamicum* ZW04 were made as described elsewhere [3] and kept at −80 °C prior to cultivation. For pre-cultures 10×10^{-6} L of stock culture was added in rich growth medium in Erlenmeyer flasks and incubated under aerobic conditions in shakers (KS 4000 I control, IKA®) at 180 rpm and 30 °C. After two days the cells were harvested by centrifugation (Allegra™ 25R Centrifuge, BECKMAN COULTER™) of the fermentation broth for 20 min (5000 ×*g*, 4 °C). Cells were then washed as outlined in a previous study [22] prior to inoculation of the WE reactor chambers. Cells were then suspended in 10×10^{-3} L of WE medium and added into 270 mL of medium already in the WE chamber, resulting in an initial OD_{600} of 1.2.

2.5. Analytical methods and calculations

Sampling was performed daily by extracting 1.0×10^{-3} L of working chamber solution using sterile syringes. Samples were first analyzed for pH and OD_{600} and then centrifuged (5 min, 21,100 ×*g*) and filtered (0.2 μm) prior to further analyses. A high-performance liquid chromatographer (HPLC; Dionex® Ultimate 3000, Dionex Corp., USA) equipped with a Rezex™ ROA-Organic Acids H^+ column (8%, 300 mm × 7.8 mm, Phenomenex Inc., Denmark), a refractive index detector (RI-101; Dionex Corp., USA) and a variable wavelength detector (VWD 3100; Dionex Corp., USA) was used to monitor glucose and organic acid concentrations (lactate, acetate, succinate). Lysine concentrations were determined electrochemically with an ion chromatographer (IC; Dionex® ICS-5000, Dionex Corp., USA) equipped with a Dionex AminoPac™ PA-10 column (250 mm × 2 mm; Dionex Corp., USA). HPLC and IC methods