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Anodic respiration of *Pseudomonas putida* KT2440 in a stirred-tank bioreactor

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ABSTRACT

Anodic batch production of *para*-hydroxybenzoic acid (*p*HBA) from citric acid with a genetically modified *Pseudomonas putida* KT2440 strain was studied in a bio-electrochemical system (BES) based on a standard lab-scale stirred-tank bioreactor at fully controlled anaerobic reaction conditions. Electron transfer to the anode was mediated by addition of $K_3Fe(CN)_6$ to the medium. Effects of varying anode surface areas (graphite rod, felt and brush), power input (stirrer speed) and mediator concentrations were investigated. The obligate aerobic *P. putida* grew anaerobically with mediated anodic respiration and *p*HBA production was observed. Anodic respiration was best applying the graphite rod electrode which showed a maximal current density of 12.5 mA cm^{-2}. This is the highest measured for non-porous electrodes in BES until now. Increasing the power input to 2.93 W L^{-1} (700 rpm) and online control of the redox potential E_{Medium} at 225 mV (vs. Ag/AgCl) in the medium by controlled addition of mediator resulted in a maximal *p*HBA yield of 9.91 $mmolC_{pHBA}$ $molC^{-1}{}_{citrate}$ which exceeds *p*HBA yields in the aerobic batch process by 69 % (5.87 $mmolC_{pHBA}$ $molC^{-1}{}_{citrate}$).

1. Introduction

Innovative fermentation processes are necessary for cost-effective bio-production of chemicals [1]. Fermentation processes are operated under aerobic or anaerobic conditions. Aerobic processes have a high energy demand for intensive aeration of the fermentation broth. Anaerobic processes can be operated with lower energy input but show formation of unwanted by-products [2]. Microorganisms form by-products to balance their redox metabolism [3] which reduces yields and can cause product inhibition [4–6]. An alternative may be offered by electro-fermentation, where an electrode provides an electron source or sink to overcome bottlenecks or drive fermentations towards particular desired products [7,8].

In such bio-electrochemical processes the redox metabolism can be balanced by external electron transfer between the microorganism and an electrode as electron acceptor (anodic process) or donor (cathodic process) under anaerobic conditions. Electro-fermentation has shown to be effective at increasing the synthesis of several products including ethanol, n-butanol and succinate with a variety of microorganisms such as *Saccharomyces cerevisiae*, *Clostridium acetobutylicum* or *Actinobacillus succinogenes* [9–12]. In contrast to all other electron acceptors or donors that can be utilised by microorganisms, electrodes cannot be depleted. Thus they offer the possibility of developing anaerobic production pathways that do not need to be stoichiometrically balanced with respect to the oxidation states of substrates and products [13].

Para-hydroxybenzoic acid (*p*HBA) is a high value intermediate in the shikimate pathway which is used for the production of poly-crystal polymers [14]. So far *p*HBA has been produced under aerobic conditions from glucose in recombinant strains of *Escherichia coli* [15], *Saccharomyces cerevisiae* [16,17] and *Pseudomonas putida* [18,19]. Maximal *p*HBA yields of 49 $mmolC_{pHBA}$ $molC^{-1}{}_{glucose}$ were achieved with *P. putida* in a chemostat cultivation [19]. *P. putida* may be a preferred production host for *p*HBA because of its high tolerance to aromatic compounds [20,21].

Bio-electrochemical systems in which microbial redox reactions are performed consist of an anodic and a cathodic chamber that are separated by a membrane [22]. When the oxidation of organic matter by microorganisms takes place in the anodic chamber, the process is referred to as anodic respiration. Anodic respiration requires electron transfer from the microorganism towards an external acceptor. External electron transfer, the central feature of electrogenic microorganisms, can occur by two different

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mechanisms [23]. Firstly, microorganisms can be directly attached to electrode surfaces forming electro-active biofilms. The direct electron transfer can occur via redox proteins in the membrane or pili-like structures, the so called nanowires. Secondly, redox mediators dissolved in the aqueous phase can shuttle electrons from the microorganism towards the electrode. Natural redox mediators can be produced by the microorganism or artificial mediators can be added. The major advantage of mediators is the enlargement of the reaction space so that electrochemical reactions can take place not only at the electrode surface but also in the bulk medium. It was previously observed that ferricyanide can serve as electron acceptor during the oxidation of nicotinic acid by *Pseudomonas fluorescens* [24] and the oxidation of glucose by *Pseudomonas putida* F1 [25].

Bio-electrochemical reactor systems described so far in literature vary strongly with respect to geometries, were not well mixed and important state variables (e.g. pH) were not controlled [26]. Modification of stirred-tank bioreactors in order to establish bio-electrochemical systems could be beneficial for mediated bio-electrochemical processes because stirred-tank bioreactors can easily be scaled-up and are very well established in industry.

This paper deals with the application of a commercially available standard stirred-tank bioreactor for mediated anodic respiration with all modifications being reversible. Anodic batch production of *p*HBA from citric acid with *Pseudomonas putida* KT2440 with overexpression of the chorismate-pyruvate lyase gene and knock-out of the 4-hydroxybenzoate 3-monooxygenase gene will be studied at fully controlled reaction conditions. Investigations will be focused on potentially limiting process variables such as electrode surface area, power input (stirrer speed) and mediator concentrations (potassium ferricyanide). The bio-electrochemical process performance of the recombinant *Pseudomonas putida* KT2440 will be compared to the aerobic batch process in a standard stirred-tank bioreactor.

2. Materials and methods

2.1. Microorganism and strain construction

Pseudomonas putida KT2440 was engineered to produce *p*HBA by knocking out *pobA* encoding for 4-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2). This disrupts the conversion of *p*HBA to 3,4-dihydroxybenzoic acid and thus prevents *p*HBA degradation via the ortho-cleavage pathway. Furthermore, the strain was transformed with the vector pSEVA2345 for overexpression of the gene *ubiC,* which encodes the enzyme chorismate-pyruvate lyase converting chorismate to *p*HBA and pyruvate.

2.2. Strain construction

The *ubiC* gene was artificially synthesized based on the amino acid sequence from *Escherichia coli* K-12 substr.W3110 (CAA40681.1) and expression was induced by IPTG. Genetic engineering was applied using the pEMG/pSW-2 system [27].The 500bp–800 bp upstream and downstream sequences of the gene *pob*A were amplified with the primer pairs KTKO PobA P1/KTKO PobA P3 and KTKO PobA P4/KTKO PobA P6. In the following the primer pair KTKO PobA P2/KTKO PobA P5 was used to fuse the gene upstream and downstream of the *pobA* sequence.

The resulting fusion gene was digested with EcoR I/BamH I, and inserted into the vector pEMG resulting in the plasmid pMEG-Δ*pobA*, which was further transformed into *P. putida* KT2440. The plasmid integrated into the chromosome which was confirmed by colony PCR. Then the second plasmid containing an I-SceI endonuclease was introduced into the resulting strain and induced with 15 mM 3-methylbenzoate to remove the pEMG backbone. The resulting strains were identified using colony PCR.

The construction of overexpression plasmid was done by a restriction enzyme digest and ligation approach. The gene *ubiC* was inserted into EcoR I/Hind III of the vector pSEVA234 [28], resulting in the plasmid pSEVA234-*ubiC*.

All constructions were confirmed by sequencing (Australian Genome Research Facility, St Lucia, Australia). The expression plasmid pSEVA234-*ubiC* was electro-transformed into the mutant *P. putida* Δ*pob*A following Choi's protocol [29], generating the strain of *P. putida* Δ*pob*A/pSEVA234-*ubiC*.

2.3. Medium, chemicals and reagents

LB medium with 10 g L^{-1} peptone (Appli Chem, Darmstadt, Germany), 5 g L^{-1} yeast extract (Deutsche Hefewerke, Nuremberg, Germany), 10 g L^{-1} NaCl (Carl Roth, Karlsruhe, Germany) and 50 μg mL^{-1} kanamycin (Carl Roth, Germany) was used for the cultivation of *Pseudomonas putida* KT2440 Δ*pobA*/pSEVA-*ubiC* in test tubes. A defined medium was applied in shake flasks and in fully controlled stirred-tank bioreactors with 5 g L^{-1} citrate (Carl Roth, Germany), 6 g L^{-1} Na_2HPO_4 (neoLab, Heidelberg, Germany), 3 g L^{-1} KH_2PO_4 (Carl Roth, Germany), 1 g L^{-1} NH_4Cl (neoLab, Germany), 0.1 g L^{-1} $MgSO_4{\cdot}7H_2O$ (Carl Roth, Germany), 0.015 g L^{-1} $CaCl_2{\cdot}2H_2O$ (Merck, Darmstadt, Germany), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Carl Roth, Germany), 50 μg mL^{-1} kanamycin (Carl Roth, Germany) and 1 mL L^{-1} of a trace elements stock solution containing 1.5 g L^{-1} $FeCl_3{\cdot}6H_2O$ (Merck, Germany), 0.15 g L^{-1} H_3BO_3 (Merck, Germany), 0.03 g L^{-1} $CuSO_4{\cdot}5H_2O$ (Merck, Germany), 0.18 g L^{-1} KI (Merck, Germany), 0.12 g L^{-1} $MnCl_2{\cdot}4H_2O$ (VWR, Radnor, PA, USA), 0.06 g L^{-1} $Na_2MoO_4{\cdot}2H_2O$ (Merck, Germany), 0.12 g L^{-1} $ZnSO_4{\cdot}7H_2O$ (Merck, Germany), 0.15 g L^{-1} $CoCl_2{\cdot}6H_2O$ (Merck, Germany), 10 g L^{-1} EDTA (acid form) (Carl Roth, Germany), 0.023 g L^{-1} $NiCl_2{\cdot}6H_2O$ (Merck, Germany). The mediator potassium ferricyanide (Alfa Aesar, Karlsruhe, Germany) was applied at an initial concentration of 3 mM in electro-fermentations if not stated otherwise. Media were sterilised at 121 °C for 20 min. $CaCl_2{\cdot}2H_2O$, $K_3Fe(CN)_6$, IPTG and kanamycin were filter sterilised and added after sterilisation. Traces of oxygen were stripped with nitrogen gas for at least 12 h before inoculation of electro-fermentations. The electrolyte for cathode chamber and glass bridge tube was the defined medium without citrate.

All restriction enzymes, ligase and polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Plasmid extraction and PCR product purification was done using GeneJET Plasmid Miniprep Kit and GeneJET PCR Purification Kit, respectively (Thermo Fisher Scientific, Australia). All DNA manipulation was followed according to the suppliers' recommendation.

2.4. Stirred-tank based bio-electrochemical reactor

A 1.8 L autoclavable stirred-tank bioreactor made of glass (Labfors, Infors, Bottmingen, Switzerland) was modified to operate the reactor as an electrically insulated bio-electrochemical cell. The stainless steel lid with all openings for probes, agitator shaft, sampling and fluid connectors was electrically isolated from the metal rack by a silicone ring. All electrically conductive connections between the lid and the reaction medium were replaced by non-conductive materials. The stainless steel agitator shaft was replaced by an agitator shaft made from polyether-etherketone (PEEK) (diameter 12 mm). This shaft was equipped with two Rushton turbines (Fig. 1). The original stainless steel immersion tube for the temperature probe was replaced by an immersion tube made out of PEEK. The original stirred tank baffles (stainless steel) did not reach the lid and were therefore used without modification. Visually, no corrosion effects were observed over a period of 1.5 years of