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In situ reactive extraction of itaconic acid during fermentation of *Aspergillus terreus*

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ABSTRACT

As a promising value-added chemical from biomass, itaconic acid has great potential in the replacement of petrochemical-based materials and the production of versatile polymers. To integrate itaconic acid recovery in the fermentation process, the applicability of reactive extraction for in situ product removal was investigated. Initially, the biocompatibility of several solvents was assessed based on their influence on the respiratory activity of *A. terreus*. As a result, a mixture of the extractant trioctylamine and the diluent isopropyl myristate was chosen and reactive extraction was successfully integrated into the cultivation of *A. terreus* via solvent addition after 63 h. Thereby, the pH of the culture broth was increased and inhibition by undissociated itaconic acid was reduced. As a consequence, glucose consumption and product formation were considerably improved. In combination with an enhanced amount of glucose, either supplied initially or during the cultivation, this increased the total itaconic acid concentration from 70 to 105 g L^{-1} referred to the absolute amount of itaconic acid divided by the volume of the aqueous culture broth. Simultaneously, volumetric productivity increased from 0.72 to 0.91 g L^{-1} h^{-1}. With reactive extraction, therefore, a highly promising method for in situ removal of itaconic acid could be established.

1. Introduction

With a share of approximately 30–40% of the overall process costs [1], downstream processing has a major impact on the economic feasibility of biotechnological carboxylic acid production. Efficient downstream processing initially requires the selective separation of the carboxylic acid from the aqueous fermentation solution to remove impurities or residual by-products. This can generally be achieved via adsorption, extraction, precipitation, or different membrane processes [2]. Extraction is performed using a liquid phase, which is immiscible with the obtained aqueous phase and, thus, typically consists of organic solvents [3]. As conventional physical extraction is rather inefficient for a transfer of carboxylic acids to a biocompatible organic phase due to the low partition coefficients, so-called reactive extraction is oftentimes favored [4]. Hence, the organic phase contains an extractant, which forms specific complexes, or, in rare cases, chemically reacts with the acid molecules [2]. The addition of organic diluents improves the overall physicochemical properties of the organic phase with regard to e.g. complex solvation or phase separation. Additionally, biocompatibility of inhibiting extractants can be improved by the use of biocompatible diluents [2,5]. Typically, extraction is followed by back-extraction into an aqueous phase and solvent regeneration. Although adsorptive downstream operations currently prevail in industrial carboxylic acid recovery, pilot plant runs have shown the feasibility of citric acid reactive extraction with an amine-based extractant [6,7]. An extensive overview of solvent systems for the extraction of carboxylic acids can be found in the review article of Datta et al. [3].

Microbial production of itaconic acid is commonly achieved using carbohydrate or, less frequently, glycerol feedstocks [8,9]. It was first described by Kinoshita [10], who identified itaconic acid in the culture broth of *Aspergillus itaconicus*. In a subsequent screening of the Northern Regional Research Laboratory (NRRL), strains

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of the fungal species *Aspergillus terreus* proved to be efficient itaconic acid producers [11]. Efforts to increase product formation with these strains involved common medium and process optimization, random mutagenesis, as well as processes with a shift of pH and substrate feeding [12–16]. The latter resulted in yields up to 0.63 g g^{-1} on glucose and a final itaconic acid concentration of 160 g L^{-1}, which is the current maximum in the scientific literature [16]. Aside from *A. terreus* further microbial itaconic acid producers exist, which could, however, not be established in an industrial scale yet. These include species of the genus *Ustilago* such as *U. maydis*, which provides yeast-like, single cell growth as opposed to the filamentous morphology of *A. terreus* [17,18]. Industrially, itaconic acid is purified via repeated cycles of evaporation and crystallization of filtered fermentation broth. Decolorization can be achieved by treatment with activated carbon and subsequent recrystallization [9]. As crystallization can be classified as a means of concentration or purification, primary recovery is technically omitted [2]. Therefore, to improve the purity of the final product, further downstream operations are required [9]. As a highly selective separation technique, reactive extraction of itaconic acid with amine-based or organophosphorus extractants has been the subject of numerous studies. While trioctylamine (TOA) represents the most common extractant [3,19–21], a range of further compounds including Aliquat 336 [22,23], tributyl phosphate [20,22], and trioctylphosphine oxide [24] could be shown to provide similarly efficient complex formation with itaconic acid. As the utilized diluent has a considerable influence on the overall performance of the extraction, the aforementioned extractants were tested in combination with several classes of organic solvents including alcohols, ketones, esters, aromatics, and alkanes [3,19,21,23]. To establish biocompatible solvent mixtures, even sunflower oil was utilized as a diluent [22].

Product inhibition has a major impact on the formation of carboxylic acids, as organic acid biosynthesis is oftentimes associated with product concentrations in the range of 90–240 g L^{-1} [2,25]. In the case of itaconic acid, high final concentrations were achieved without observing negative impact [12,15], however product inhibition is discussed in the older literature [9]. Recent publications show that inhibitory effects can be overcome by increasing the pH during the itaconic acid production phase [15,16]. At low pH values undissociated itaconic acid is prevalent, which can enter the cells via diffusion and dissociate in the cytoplasm. The resulting protons have to be actively transported out of the cells, which requires additional energy [16,26]. In contrast, uptake of the partly dissociated organic acid is difficult due to its polarity [27]. Hence, a change in pH essentially removes a portion of the toxic, undissociated acid from the culture broth. Addition of an alkaline extractant like TOA during the fermentation process combines a favorable pH shift with integrated product recovery. In situ product removal (ISPR) combines two process steps, fermentation and recovery, resulting in shorter overall process times. In some cases, this has the additional positive effect of a reduced base consumption for pH control [2] and lower osmotic pressure. Commonly, ISPR of carboxylic acids is achieved via adsorption, extraction, or membrane processes such as nanofiltration and electrodialysis [2]. Depending on the process, even in situ crystallization can be performed [28]. In situ adsorption was successfully applied for the production of fumaric acid [29], lactic acid [30], and succinic acid [31]. While nanofiltration and electrodialysis can be used for ISPR of lactic and pyruvic acid, membrane fouling can become a major issue for these processes [32–34]. In situ extraction relies solely on liquid streams. Therefore, scale up is easy and high capacities can be realized [35]. However, the biocompatibility of the organic phase is a crucial factor as a positive effect of ISPR could be superimposed by solvent toxicity. Nevertheless, this concept was found to be beneficial for the production of citric acid [36], butyric acid [37], propionic acid [38,39], as well as lactic acid [40].

So far, there are no reports on the selective in situ recovery of itaconic acid during fermentation of *A. terreus*. While Li et al. [41] established itaconic acid extraction in a pertraction module coupled to a fermenter, the obtained filtrate was not recycled to the bioreactor after extraction as the utilized solvent (2-methyltetrahydrofuran) was toxic for *A. terreus*. Hence, the performance of the fermentation was not affected by product recovery. As reactive extraction of itaconic acid is well established, the utilization of this method for ISPR is investigated in this study. First of all, the biocompatibility of several diluents is determined based on their effect on the oxygen transfer rate of *A. terreus* [42]. A suitable solvent system is selected and characterized regarding its extraction efficiency and effect on the cultivation process. The aim of this study is an assessment of the general feasibility of in situ reactive extraction and an initial optimization of the respective conditions in shake flask.

2. Materials and methods

2.1. Microorganism

Experiments were performed with the strain *Aspergillus terreus* DSM 23081. This strain was selected as a highly potent itaconic acid producer by Kuenz et al. [14]. Fungal spore suspensions were prepared using Czapek-Dox agar medium containing 30 g L^{-1} sucrose, 3 g L^{-1} $NaNO_3$, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 1 g L^{-1} K_2HPO_4, and 13 g L^{-1} agar. The pH was adjusted to 7.2 with 1 M HCl and the medium was sterilized at 121 °C. 1 mL of a sterile-filtered trace element solution was added to 1 L of medium to allow fast sporulation. The trace element solution contained 15 g L^{-1} EDTA, 4.5 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.84 g L^{-1} $MnCl_2 \cdot 2H_2O$, 0.3 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.3 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.4 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 4.5 g L^{-1} $CaCl_2 \cdot 2H_2O$, 3 g L^{-1} $FeSO_4 \cdot 7H_2O$, 1 g L^{-1} H_3BO_3, and 0.1 g L^{-1} KI. 90 mm Petri dishes with 10 mL of the final medium were inoculated with 50 µL of a spore suspension stored in a chest freezer (Forma 700 series, Thermo Fisher Scientific, Waltham, USA) at −80 °C in 32% (v/v) glycerol. The spore suspension was distributed evenly with a Drigalski spatula and the agar plates were incubated at 30 °C for 5–7 days. Spores were harvested with 5 mL of sterile NaCl solution (9 g L^{-1}) and counted in a Neubauer improved counting chamber to determine the spore concentration.

2.2. Cultivation of Aspergillus terreus

Cultivations were performed in a minimal medium according to Kuenz et al. [14] and Hevekerl et al. [43]. If not stated otherwise, the medium contained 180 g L^{-1} glucose, 0.8 g L^{-1} KH_2PO_4, 3 g L^{-1} NH_4NO_3, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.00167 g L^{-1} $FeCl_3 \cdot 6H_2O$, 0.008 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.015 g L^{-1} $CuSO_4 \cdot 5H_2O$ and 0.000001 g L^{-1} $MnCl_2 \cdot 4H_2O$. The medium was completed from stock solutions which, except for the $CaCl_2$, $FeCl_3$ and $MnCl_2$ solutions, were adjusted to pH 3.1 with 1 M H_2SO_4. All solutions were sterilized at 121 °C for 20 min. Shake flask cultivations were conducted at 33 °C in unbaffled 250 mL Erlenmeyer flasks with a filling volume of 20 mL. Upon inoculation of the medium with a defined spore concentration of $5 \cdot 10^6$ spores per mL, the flasks were placed in an orbital shaker with a shaking diameter of 50 mm and a shaking frequency of 300 min^{-1}. Similar to the experimental procedure described by Wewetzer et al. [44], parallel cultivations in separate shake flasks were performed for each cultivation condition as visualized in Fig. A1. Therefore, cultivation data presented in this work result from cultivations in at least six individual shake flask for each investigated condition. To monitor the respiratory activity of the microorganisms during the cultivation, a Respiration Activity Monitoring System (RAMOS), fabricated in-house, was used [45].