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Research Article

Integrated process development of a reactive extraction concept for itaconic acid and application to a real fermentation broth

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Itaconic acid (IA) has a high potential to be used as a bio-based platform chemical and its biocatalytic production via fermentation has significantly improved within the last decade. Additionally downstream processing using reactive extraction (RE) was described, potentially enabling a more efficient sustainable bioprocess producing IA. The bottleneck to overcome is the connection of up- and downstream processing, caused by lack of biocompatibility of the RE systems and direct application to fermentation broth. Within this study, a biocompatible RE system for IA is defined (pH dependency, extraction mechanism) and used for direct application to a fermentation broth. By optimizing the biocatalyst, the production medium, and the extraction system in an integrated approach, it was possible to define critical parameters that enabled a tuning of the overall bioprocess. With an extraction yield of $Y_{IA} = 0.80 \pm 0.03$, IA could be produced as sole carboxylic acid ($b_{IA,0}^{aq} = 0.490 \text{ mol/kg}^{aq}$) using a RE system consisting of ethyl oleate as organic solvent and tri-*n*-octylamine as extractant ($b_{T-C8}^{org} = 0.6 \text{ mol/kg}^{org}$). This work is a proof of concept and demonstrates that by joint consideration of up- and downstream processing, optimized bioprocesses can be developed.

Keywords: Biocatalysis / Downstream processing / Itaconic acid / Reactive extraction / *Ustilago maydis*

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1 Introduction

Organic acids are receiving increasing attention as renewable, biomass-derived substitutes for traditional petroleum-based products. Among these, itaconic acid (IA) has been identified as a promising platform compound for the production of bio-based polymers, chemicals, and fuels [1]. Therefore, this di-carboxylic acid was chosen as a desired product within this study. Its market volume rose from 15.000 t/a to 80.000 t/a between 2001 and

2004 [1, 2]. The estimated world market for IA in 2014 was valued at U.S.\$ 126.4 million with further potential to increase [3]. It is described by the U.S. Department of Energy as one of the 12 most promising valuable bio-based platform chemicals or building blocks derived from biocatalytic origin [1], although in a later revision IA was removed from the list with the argument that too little research was presented [4]. There are several organisms known for the biocatalytic production of IA. The most used organism, described well in open literature, is the filamentous fungus *Aspergillus terreus* [5–7]. In this study, the IA is produced via fermentation with the yeast-like fungus *Ustilago maydis* as biocatalyst [8–10]. *U. maydis* is well suited for the production of IA due to its unicellular growth pattern and insensitivity toward medium impurities or organic solvents compared to the filamentous *A. terreus* [10]. The state-of-the-art downstream process for IA is a multistep combination of filtration, centrifugation, and crystallization (with crystallization being the recovery and polishing step) often leading to problems with respect to purity or yield caused by the high polarity of the acid [1, 3, 11]. A

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Abbreviations: D-C6, di-*n*-hexylamine; D-C8, di-*n*-octylamine; EO, ethyl oleate; FTIR, Fourier-transformed infrared; IA, itaconic acid; RE, reactive extraction; SA, succinic acid; T-C6, tri-*n*-hexylamine; T-C8, tri-*n*-octylamine; T-C12, tri-*n*-dodecylamine

downstream process using crystallization as recovery and polishing operation is unfavorable because of its high demand in energy and costly equipment [3].

In bioprocess engineering, downstream unit operations are often developed separately from the biocatalytic upstream process. Although new and innovative downstream concepts are constantly being developed, these concepts are often suboptimally implemented, since their interactions with the upstream biocatalysis are often neglected at an early stage of overall process design where high-impact decisions have to be made. This results in a cost- and energy-intensive recovery of the envisioned product [12], which may ultimately hinder novel biocatalytic applications. An integrated approach for the process development, in which up- and downstream are considered in parallel, has enormous potential for process intensification. The downstream processing (stand-alone) via reactive extraction (RE) has been identified as a promising concept for the purification of carboxylic acids from complex aqueous solutions [3, 13–17]. The RE of IA from aqueous solutions was already investigated by Wasewar et al. [18, 19] and López-Garzón et al. [20], using the amine extractant mixture Aliquat 336. Aşçi and İnci [21], Uslu and Datta [22], and Kaur and Elst [23] investigated RE of IA with the triamine tri-*n*-octylamine (T-C8, or mixtures containing T-C8) as extractant using bio-incompatible organic solvents. Within the mentioned studies, RE was shown to be applicable for IA and hence offers a solid starting point for the presented integrated process development approach. RE has the advantages of high capacity and easy scale-up, if right solvents (biocompatible) can be developed and the missing link to the fermentation system can be overcome. In comparison to the state-of-the-art downstream concept for IA using crystallization as recovery and polishing step, the RE is a very promising alternative for the initial recovery step [3, 13].

This study presents the development of a bioprocess for IA considering up- and downstream processing, laying the focus to the recovery of the acid from fermentation broth by RE. The RE is used for IA purification from real fermentation broth, starting with a systematic screening for the best-performing amine extractant embedded in a biocompatible organic solvent. An analysis of the pH dependency and the underlying mechanism allows for the definition of process boundaries of the RE. Identification of critical process parameters (e.g., nutrient salts, byproducts) led to iterative optimization rounds between up- and downstream processing in order to streamline the complete process, demonstrating the advantages of an integrated process development for the production of IA (Fig. 1).

2 Materials and methods

2.1 Chemicals for reactive extraction

Ethyl oleate (EO), di-*n*-hexylamine (D-C6), tri-*n*-hexylamine (T-C6), and T-C8 were purchased from AlfaAesar (Karlsruhe, Germany). Di-*n*-octylamine (D-C8) was bought from TCI (Tokyo, Japan). Tri-*n*-dodecylamine (T-C12) was bought from Acros Organics (Geel, Belgium). 1-Dodecanol, magnesium sulfate, D(+)-glucose, IA, and succinic acid (SA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium

Figure 1. Principle scheme of the systematic integrated approach for the development of a production concept for IA.

chloride and sulfuric acid were bought from Prolabo (Radnor, PA, USA). Sodium hydroxide and hydrochloric acid were bought from Merck (Darmstadt, Germany). Monopotassium dihydrogen phosphate was purchased from VRW Chemicals (Darmstadt, Germany).

2.2 Chemicals for fermentation

D(+)-glucose, ammonium sulfate, ammonium chloride, magnesium sulfate heptahydrate, ferric II-sulfate-heptahydrate, and potassium dihydrogen phosphate were purchased from Carl Roth (Karlsruhe, Germany). Yeast extract was purchased from Sigma–Aldrich.

2.3 Fermentation of itaconic acid

Batch cultivation (fermentation) of *U. maydis* MB215 wildtype (itaconate, malate, and 2-hydroxyparaconate producer [10]) and *U. maydis* $\Delta cyp3$ P_{etef} *ria1* (itaconate producer without malate and 2-hydroxyparaconate [24]) were performed either in batch medium containing 200 g/L glucose and 4 g/L ammonium chloride or in screening medium containing 50 g/L glucose and 0.8 g/L ammonium chloride as described by Geiser et al. [8], unless stated otherwise. Culture broth was harvested after 214 h by centrifugation for 5 min at 5000 rpm and filtrated with Rotilabo syringe filters (CA, 0.20 μ m, $\varnothing$ 15 mm).

2.4 Reactive extraction experiments

The RE experiments were performed in 15 mL centrifugal tubes with sealed screw caps manufactured by VWR International (Radnor, PA, USA). The total mass of each experiment was 9 g with a phase ratio of 1:2 aqueous to organic ($m_{aq}:m_{org}$). Each aqueous and organic phase was prepared separately via weighing the single compounds and then transferred into a centrifugal tube. Sufficient mixing was ensured by shaking the tubes in a ThermoMixer from Eppendorf (Hamburg, Germany) for 30 min at 1000 rpm. The ThermoMixer also guaranteed the temperature of every experiment was held constant to $T = 25^\circ\text{C}$ for the whole time. None of the tested systems formed a stable

emulsion. After equilibration, the 15 mL tubes were centrifuged in a Centrifuge 5804 R Eppendorf (Hamburg, Germany) equipped with an A4-44 rotor at 5000 rpm and a temperature of 25°C. Within the range considered (20–35°C in case of fermentation), temperature only showed a minor influence on RE performance (results not shown) and was thus not further varied. After RE, the samples were analyzed as described in Section 2.5.

The concentration of IA for the initial screening experiments was always $c_{IA}^{aq} = 75$ g/L ($b_{IA}^{aq} = 0.62$ mol/kg^{aq}), the concentration of the single di-carboxylic acid within the different fermentation broths varied and will be given for each experiment in Section 3. The pH of a solution containing $c_{IA}^{aq} = 75$ g/L ($b_{IA}^{aq} = 0.62$ mol/kg^{aq}) IA was measured to be 2.2 ± 0.1 . For the investigation of pH-dependent RE and RE from fermentation broth, the pH of the aqueous phase was adjusted with aqueous hydrochloric acid or sodium hydroxide solution. The organic solvent EO served as standard organic phase for RE experiments including an amine as extractant at different concentrations. The concentration of the amine extractant will be given for each experiment in Section 3.

The RE conversion $X_{RE,i}$ is calculated using Eq. (1), in which $n_{i,0}^{aq}$ is the amount of the respective carboxylic acid in the aqueous phase before RE and n_i^{aq} is the amount of the respective carboxylic acid after RE.

$$X_{RE,i} = \frac{n_{i,0}^{aq} - n_i^{aq}}{n_{i,0}^{aq}} = \frac{n_i^{org}}{n_{i,0}^{aq}} \quad (1)$$

The amount of the single carboxylic acid in the organic phase n_i^{org} was calculated via mass balance of the carboxylic acid in the aqueous phase. Each experiment was performed twice; the error for the single experiment was calculated by Gaussian error propagation. The term $\Delta X_{RE,i}$ is the absolute average deviation of the two experiments. The extraction selectivity S_i is calculated by Eq. (2), where n_i^{org} is the amount of a carboxylic acid i in the organic phase and $\sum_i n_i^{org}$ is the molar sum of all extracted carboxylic acids in the organic phase.

$$S_i = \frac{n_i^{org}}{\sum_i n_i^{org}} \quad (2)$$

The term ΔS_i was calculated as it was described for $\Delta X_{RE,i}$. The term Y_i as product of $X_{RE,i}$ and S_i gives the extraction yield for a specific carboxylic acid i and is calculated using Eq. (3).

$$Y_i = X_{RE,i} \cdot S_i \quad (3)$$

The term ΔY_i was calculated as it was described for $\Delta X_{RE,i}$.

2.5 Analytical methods

Concentration analysis of the aqueous phase was performed via HPLC using an Agilent 1200 Series HPLC (Santa Clara, CA, USA) and a Nucleogel Sugar 810 H, 7.8 · 300 mm column including the corresponding guard column (both by Macherey-Nagel, Düren, Germany). The temperature was kept constant at 35°C. The eluent at a volume flow of 0.6 mL/min was a pure 5 mM sulfuric acid solution. A Refractive Index Detector was used for detection. The injected sample amount was always 5 µL. The sample of the aqueous phase was taken by punching a cannula

Table 1. Results of the initial amine screening for RE of IA

Amine	$X_{RE,IA}$	Third phase
D-C6	0.84 ± 0.04	Yes
D-C8	0.96 ± 0.04	Yes
T-C6	0.89 ± 0.03	Yes
T-C8	0.89 ± 0.04	No
T-C12	0.87 ± 0.03	No

Tested amine extractants: D-C6, D-C8, T-C6, T-C8, and T-C12. EO was used as organic solvent. The concentration of each amine was always $b_i^{org} = 0.5$ mol/kg^{org}. $X_{RE,i}$ is calculated using Eq. (1).

through the bottom of the 15 mL centrifugal tube after centrifugation. All aqueous samples were diluted with eluent in a ratio of 1:5 ($V_{aq}:V_{eluent}$) and transferred into two HPLC vials by syringe filtration (polyethylene sulfone membrane 0.4 µm) by VWR International.

3 Results and discussion

3.1 Definition of a reactive extraction system for itaconic acid

Prior to a first iteration cycle using the real fermentation medium, an applicable and biocompatible RE system for the recovery of IA from aqueous solutions at the highest possible conversion was defined using a model solution. An initial screening was performed for a range of water insoluble amines (D-C6, D-C8, T-C6, T-C8, T-C12) as extractants. The selection of amines and the biocompatible organic solvent (EO, ester) was done based on previous studies [17]. The initial amine concentration in the organic phase was set to a value of $b_i^{org} = 0.5$ mol/kg^{org}. The aqueous model solution consisted of pure IA dissolved in water ($c_{IA}^{aq} = 75$ g/L). The pH of a solution containing $c_{IA}^{aq} = 75$ g/L IA was measured to be 2.2 ± 0.1 . The results of these experiments are summarized in Table 1. For comparison, the solubility of IA in the organic solvent EO corresponds to a conversion of 0.05 ± 0.04 .

All amines investigated (D-C6, D-C8, T-C6, T-C8, and T-C12) allow for extraction conversions of $X_{RE,IA} \geq 0.8$ with D-C8 having the highest conversion of 0.96 ± 0.04 . An RE of IA with D-C6, D-C8, and T-C6 led to the formation of a third phase located between the aqueous and the organic phase, but wetted with the organic phase. A microscopic analysis of this phase showed that it is not an emulsion phase. Potentially, this third phase is composed of aggregates of the amine IA complex [25–28]. A direct recovery of the acid from this third phase is not possible and thus formation needs to be avoided since it has a direct negative influence onto the yield of the overall process. It was shown that the formation of the third phase can be prevented if the polarity of the organic phase is increased by the addition of a polar modifier (1-dodecanol within this study) [17]. Besides preventing a third phase, the polarity of the organic phase has an influence on the RE conversion [22, 29]. The three most promising amines D-C8, T-C6, and T-C8 were chosen for optimization of amine and modifier concentration under consideration of increased conversion ($X_{RE,IA} > 0.90$).

Table 2. Reactive extraction systems for the extraction of IA

Amine	b_i^{org} (mol/kg ^{org})	$w_{1-\text{dodecanol}}^{\text{org}}$ []
D-C8	0.4	0.15
T-C6	0.5	0.075
T-C8	0.6	0

The three systems led to a conversion of $X_{\text{RE,IA}} > 0.90$ without a formation of a third phase for RE of IA from pure water. EO was used as organic solvent.

for pure IA from water) and the prevention of a third phase (Table 2).

In order to investigate the selectivity for an RE of IA from a fermentation broth that may contain other organic acid byproducts, experiments were performed applying a biologically relevant amount of IA ($c_{\text{IA}}^{\text{aq}} = 75 \text{ g/L}$) along with SA ($c_{\text{SA}}^{\text{aq}} = 12.5 \text{ g/L}$) in pure water. Within these experiments, T-C8 showed the highest selectivity for IA ($S_{\text{IA}} = 0.92 \pm 0.03$) and was thus chosen for all following experiments.

The presented RE system using T-C8 can be compared to the systems published by Uslu and Datta ($b_{\text{IA},0}^{\text{aq}} = 0.62 \text{ mol/kg}^{\text{aq}}$, $b_{\text{T-C8}}^{\text{org}} = 1.946 \text{ mol/kg}^{\text{org}}$ in octan-1-ol, given extraction efficiency = 94.8%, $\text{pH} < \text{pK}_{\text{a},1}$) [22] and Kaur and Elst ($b_{\text{IA},0}^{\text{aq}} = 0.525 \text{ mol/kg}^{\text{aq}}$, $b_{\text{T-C8}}^{\text{org}} = 1 \text{ mol/kg}^{\text{org}}$ in octan-1-ol, given extraction efficiency = 97–99%, $\text{pH} < \text{pK}_{\text{a},1}$ (pH values are not given but were measured within this work)) [23], who also used T-C8 as extractant. Apart from differing concentrations for IA and T-C8, all three systems allow for IA recovery of 90% and higher. Higher extraction rates (extraction conversions) for both published systems [22, 23] can be explained by the higher physical extraction potential of pure octan-1-ol for IA (38.7% [23]) compared to EO (5%). Aşçi and İnci [21] described mixtures of T-C8 and T-C12 in different bio-incompatible organic solvents with recovery rates of the IA from pure water of over 90%. The systems of Wasewar et al. [18, 19] and López-Garzón et al. [20], using the amine extractant mixture Aliquat 336, are based on ion exchange [20] and are thus not directly comparable. Taking all published systems into account, the system developed in this work allows for high extraction conversion at lowest T-C8 concentration in a biocompatible organic solvent. Biocompatibility was already proven in previous works (results not shown) and solves a crucial problem of RE as purification step for IA from biocatalytic origin [3].

3.2 pH dependency

One of the most critical parameters during RE of carboxylic acids using amine extractants is the pH dependency [17, 30–32]. This dependency was investigated for the respective RE system considered within this work by adjusting the pH of the aqueous model solution (pure IA and water) using aqueous hydrochloric acid and sodium hydroxide solutions in a range of 1.8–12.9. The results are illustrated in Fig. 2.

For pH values < 3 , the extraction conversion $X_{\text{RE,IA}}$ is constant at > 0.90 . In the range of $3 < \text{pH} < 6$, the extraction conversion $X_{\text{RE,IA}}$ decreases from 0.91 ± 0.04 to 0.05 ± 0.04 , re-

Figure 2. RE of IA in dependency of the pH with T-C8 as extractant (left axis, black dots) and distribution of IA species (right axis, black line = undissociated IA (H_2A), dark gray = first dissociation of IA (HA^-), light gray = second dissociation of IA (A^{2-})). EO was used as organic phase without the addition of any modifier ($w_{1-\text{dodecanol}}^{\text{org}} = 0$). The concentration of the T-C8 in EO was $b_{\text{T-C8}}^{\text{org}} = 0.6 \text{ mol/kg}^{\text{org}}$. The two pK_{a} values of IA are $\text{pK}_{\text{a},1} = 3.84$ and $\text{pK}_{\text{a},2} = 5.55$.

maining constant for pH values > 6 . A conversion of 0.05 ± 0.04 corresponds to the solubility of IA in the used organic solvent EO. As expected, the conversion $X_{\text{RE,IA}}$ strongly correlates with the expected concentration of fully protonated IA. As soon as the second $\text{pK}_{\text{a},2}$ of the acid ($\text{pK}_{\text{a},1} = 3.84$, $\text{pK}_{\text{a},2} = 5.55$) is exceeded, the extraction conversion remains at the level of physical extraction into the organic solvent EO. The published systems of Uslu and Datta [22] and Kaur and Elst [23], which are also using T-C8 as extractant are lacking in pH-dependent measurements, but both investigations were performed at $\text{pH} < \text{pK}_{\text{a},1}$. This supports the presented trend in Fig. 2. It was shown that the decreasing performance is attributable to the H-bond mechanism of the RE. With increasing pH, IA is dissociated and no H-bond is formed with the central nitrogen atom of the amine resulting in the presented drop for $X_{\text{RE,IA}}$ [16, 31]. To verify the H-bond mechanism for the RE of IA Fourier-transformed infrared (FTIR) measurements while RE were performed. FTIR measurements showed that IA is extracted in its undissociated (full protonated) form into the organic phase validating the H-bond mechanism (Supporting information).

3.3 Integrated process optimization of the process

After identification of a promising RE system as well as critical process parameters, a first iteration between up- and downstream was performed using a real fermentation system. A pH-controlled batch fermentation of *U. maydis* MB215 wildtype was performed in batch medium without optimization as described by Geiser et al. [8]. For the presented work, each fermentation broth was filtered and the pH of the solid-free aqueous solution was set to 2.5 using high-concentrated hydrochloric acid solution. Hydrochloric acid was chosen to allow comparisons with the initial screening experiments and due to the small amounts

Figure 3. RE of IA from fermentation broth. pH-controlled batch fermentation of *U. maydis* MB215 wildtype was performed in batch medium as described by Geiser et al. [8]. The conversion of the RE is given as $X_{\text{RE,i}}$ in gray bars, the expected conversion $X_{\text{theor,IA}}$ for IA is given as crosshatched bar and the selectivity S_i for the specific acid as dark gray bars. Organic RE phase: EO and T-C8 in a concentration of $b_{\text{T-C8}}^{\text{org}} = 0.6 \text{ mol/kg}^{\text{org}}$. The fermentation broth was adjusted to a pH of 2.5. Concentration analysis before RE: IA $b_{\text{IA,0}}^{\text{aq}} = 0.188 \text{ mol/kg}^{\text{aq}}$, SA $b_{\text{SA,0}}^{\text{aq}} = 0.030 \text{ mol/kg}^{\text{aq}}$, hydroxyparaconic acid (HPA) $b_{\text{HPA,0}}^{\text{aq}} = 0.076 \text{ mol/kg}^{\text{aq}}$, and malic acid (MA) $b_{\text{MA,0}}^{\text{aq}} = 0.813 \text{ mol/kg}^{\text{aq}}$.

needed to change the pH to a value less than $\text{p}K_{\text{a,1}}$. Figure 3 presents the results of this experiment.

Using the RE system previously defined (EO, T-C8 in a concentration of $b_{\text{T-C8}}^{\text{org}} = 0.6 \text{ mol/kg}^{\text{org}}$), all carboxylic acids present in the real fermentation broth are extracted. The extraction yield is the highest for IA ($Y_{\text{IA}} = 0.36 \pm 0.02$), followed by malic acid ($Y_{\text{MA}} = 0.04 \pm 0.01$), hydroxyparaconic acid ($Y_{\text{HPA}} = 0.01 \pm 0.01$), and SA ($Y_{\text{SA}} = 0.004 \pm 0.001$). The expected conversion achieved for the RE of IA from a pure aqueous solution ($X_{\text{theor,IA}} = 0.92 \pm 0.03$, $X_{\text{RE,IA}} = 0.64 \pm 0.04$) was not achieved. Two aspects lead to a decreased extraction conversion for IA. First, it was reported before that salts and substrate can have an influence on the extraction conversion $X_{\text{RE,i}}$ [23,33–36]. Second, as other carboxylic acids are co-extracted beside IA, less T-C8 is available for the formation of a complex with IA in comparison to the aqueous model solution [25,31,37–39].

To address the first aspect, RE of IA from aqueous solutions containing fermentation salts and substrate was performed. With the known fermentation recipe of Geiser et al. [8], the influence of the substrate glucose and three salts (highest concentration in the fermentation broth, which is ammonium chloride (NH_4Cl), monopotassium dihydrogen phosphate (KH_2PO_4), and magnesium sulfate (MgSO_4)) was investigated prior to a second iteration cycle. Aqueous IA solutions ($c_{\text{IA}}^{\text{aq}} = 75 \text{ g/L}$) were spiked with differing glucose concentrations in the range of 50–200 g/L, corresponding to the common concentrations within a real fermentation broth [8], and used for RE experiments. The performance of the RE was independent on the concentration of glucose (data not shown). For the following experiments, the glucose concen-

Figure 4. Decrease of conversion for IA $X_{\text{RE,IA}}$ (gray bars, left axis) after addition of different fermentation salts (black squares, right axis). All presented experiments contain glucose in a concentration of $c_{\text{Glucose}} = 50 \text{ g/L}$. Organic RE phase: EO and T-C8 in a concentration of $b_{\text{T-C8}}^{\text{org}} = 0.6 \text{ mol/kg}^{\text{org}}$. The complexity of the aqueous model solution increases from left to right with the first aqueous solution containing only IA and glucose, and the last aqueous solution containing IA, glucose, ammonium chloride (NH_4Cl), monopotassium dihydrogen phosphate (KH_2PO_4), and magnesium sulfate (MgSO_4).

tration was held constant at 50 g/L ($c_{\text{IA}}^{\text{aq}} = 75 \text{ g/L}$), while typical concentrations of NH_4Cl , KH_2PO_4 , and MgSO_4 [8] were added subsequently. The results are shown in Fig. 4. The complexity of the RE system increases from left to right with the first aqueous solution containing only IA and glucose, and the last aqueous solution containing IA, glucose, NH_4Cl , KH_2PO_4 , and MgSO_4 . The pH of each RE was set to 2.5 using high-concentrated hydrochloric acid solution.

The results show that NH_4Cl and KH_2PO_4 have a noticeable impact on the extraction conversion $X_{\text{RE,IA}}$. After addition of all salts, the overall conversion $X_{\text{RE,IA}}$ decreased from 0.92 ± 0.03 (glucose) to 0.83 ± 0.04 (glucose, NH_4Cl , KH_2PO_4 , MgSO_4). The negative influence of chloride and phosphate salts on the extraction performance of an RE of carboxylic acids was observed before. As acids are formed by the anions of the salts and H^+ of the carboxylic acid, the extraction conversion $X_{\text{RE,i}}$ decreases. These acids compete with the carboxylic acid and thus lower the achievable extraction conversion [33,35,36]. It was described before that T-C8 dissolved in an ester, such as EO is favorable to avoid a high influence of salts on the RE performance [23]. Including its biocompatibility, the used solvent EO is a recommendable choice for an organic solvent in a RE system for carboxylic acids.

In consideration of these results, a subsequent fermentation was performed, using both an optimized screening medium with 0.99 g/L ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) instead of 0.8 g/L NH_4Cl and an optimized strain *U. maydis* $P_{\text{etef}} \text{ rla1 } \Delta \text{cyp3}$. This optimized strain no longer produces the byproduct 2-HPA, and also overexpresses the IA production pathway, leading to increased itaconate production and decreased malate production [9,24]. In this system, IA was the only detectable carboxylic acid and the broth contained IA at a concentration of

Figure 5. Proposed process concept for the production of IA. ORG, organic solvent; WSA, water-soluble amine; N₂, nitrogen.

$b_{\text{IA},0}^{\text{aq}} = 0.490 \text{ mol/kg}^{\text{aq}}$ after addition of low amounts of high concentrated hydrochloric acid solution to a pH of 2.5. IA was extracted with a yield of $Y_{\text{IA}} = 0.80 \pm 0.03$. In contrast to the yield of $Y_{\text{IA}} = 0.36 \pm 0.02$ achieved before this clearly shows the benefits of the integrated approach. Comparing the results with the RE from the artificial aqueous phase it is clear that other substances in the fermentation broth also lead to a decrease in the apparent extraction conversion. The reduced conversion and as direct consequence the reduced yield for fermentation broths in comparison to artificial systems are assumed to be caused by substances such as other byproducts than carboxylic acids. Additionally, pilot experiments indicate that the treatment and storage of the broth are critical in achieving higher yields. An investigation of this effect is part of our ongoing studies, but it underlines the need for process integration and in situ extraction of the IA.

3.4 Proposed process concept

The results of this work in combination with previous results shown by Gorden et al. [40] allow for the proposal of a process concept for the production and purification of IA using RE as initial purification step (Fig. 5).

Within this concept, pure solid IA is recovered after the final purification by evaporation-based crystallization. After RE using T-C8, the organic phase containing the IA from fermentation is transferred into the re-extraction step. The re-extraction is realized using a water-soluble amine as proposed before. Previous work has shown that concentration of water-soluble amine can be set to a value allowing for a back extraction rate of 90% and higher without any water-soluble amine detectable in the organic phase, not affecting initial RE and thus ensuring the required recycle of the organic phase [40]. The IA is extracted into the aqueous re-extraction phase; the organic phase is recycled into the in situ product removal. Furthermore, the re-extraction using a water-soluble amine also ensures a recycle of the aqueous re-extraction phase. A stripping using N₂ ($T = 90^\circ\text{C}$) is proposed as final purification step leading to an evaporation-based crystallization, avoiding inefficient and cost-intensive salt processing within pH-based crystallization steps. This concept does require a production host that can produce IA at a pH below 3.8. The current optimal production strain *U. maydis* MB215 P_{etef} *ria1* Δ *cyp3* only produces IA efficiently at pH > 5. However, other

Ustilaginaceae such as *U. cynodontis* are more pH-tolerant [8], enabling the operation of the proposed process, if the optimizations described for *U. maydis* are transferred to this host. Alternatively, this process could also be applied for *A. terreus*, which produces IA efficiently at pH < 3.8 [7]. A next step will be the direct application of the proposed concept onto a fermentation system in which aspects such as the mutual influence of the fungi on the RE and vice versa will be the key aspects.

4 Concluding remarks

A system using EO as biocompatible organic solvent, containing T-C8 in a concentration of $b_{\text{T-C8}}^{\text{org}} = 0.6 \text{ mol/kg}^{\text{org}}$ as extractant, led to conversions of $X_{\text{RE,IA}} > 0.90 \pm 0.04$ for pure IA from water. The investigation of the pH dependency and the performed FTIR measurements showed that the IA / T-C8 complex is formed via H-Bond.

The subsequent iteration cycles between up- and downstream and the first application of a RE system for IA to a real fermentation broth led to the following general conclusions. A comparison of the RE from fermentation broth demonstrates that the RE performance is not only influenced by the presence of byproducts but also by the composition of the fermentation broth. The fermentation medium should avoid salts, such as NaCl, that are able to form an acid with the H⁺ of the carboxylic acids (i.e., product, byproduct(s)). Nevertheless, examination of the salt influence on the RE of IA showed that the combination of the biocompatible organic solvent EO and T-C8 is a recommendable combination for RE systems in which the carboxylic acid is recovered from a fermentation broth. Additionally, an extractant (amine) for RE will not exclusively extract a single carboxylic acid as byproducts are often very similar in structure. Nevertheless, specific amine/carboxylic acid combinations can affect selectivity. This can be ensured via an initial screening as it was presented before.

Under consideration of the given aspects, it was possible to produce only itaconic acid by fermentation (without the formation of other carboxylic acids) with an optimized biocatalyst *U. maydis* P_{etef} *ria1* Δ *cyp3*. Upon elimination of organic acid byproducts, the subsequent RE led to the yield of $Y_{\text{IA}} = 0.80 \pm 0.03$ ($b_{\text{IA},0}^{\text{aq}} = 0.490 \text{ mol/kg}^{\text{aq}}$). Likely, other components not investigated within this study decrease the conversion and by that the yield of the RE. The presented results allowed the

proposal of a process concept that allows for further investigation of the RE as downstream process for itaconic acid. Compared to the state-of-the-art downstream for itaconic acid, based on crystallization steps for recovery and purification, the presented concept can overcome the disadvantage of high energy demand for the recovery step of the itaconic acid. The use of technical grade EO as cost-efficient and biocompatible organic solvent, which also allows for highest yields applied in RE, potentially allows the direct application of the RE onto a fermentation system. The close cooperation of the involved disciplines in this study demonstrates the potential of an integrated approach for the development of biocatalytic production processes.

Practical application

State-of-the-art production of most chemicals is a production from limited natural resources, such as petroleum. An alternative is platform chemicals produced from renewable biomass, such as itaconic acid (IA). The bottleneck toward a broader industrial application of biocatalytic produced IA is the connection between the production and purification. A promising possibility for the purification of IA from biocatalytic origin having the potential to overcome the described bottleneck is reactive extraction. In this work, we describe a biocompatible reactive extraction system, comparing it to published systems, investigating the mechanism, and directly apply it to a fermentation broth. In an integrated approach, it was possible to define critical process parameters and optimize the bioprocess. On the basis of the results, it was possible to propose a process concept for biocatalytic-produced IA. The study demonstrates as a proof of concept the benefits of an integrated process development approach.

Nomenclature

<i>b</i>	[mol/kg]	Molality
<i>w</i>	[-]	Weight fraction
<i>X</i>	[-]	Conversion
<i>S</i>	[-]	Selectivity
<i>Y</i>	[-]	Yield
<i>c</i>	[g/L]	Concentration
<i>pK_a</i>	[-]	Acid constant
<i>n</i>	[mol]	Amount
<i>V</i>	[L]	Volume
<i>m</i>	[g]	Mass
<i>T</i>	[°C]	Temperature

Greek symbol

Δ	[-]	Deviation
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Indices

org	[-]	Organic phase
aq	[-]	Aqueous phase
0	[-]	Prior to reactive extraction

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