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Ester production from bio-based dicarboxylates via direct downstream catalysis: Succinate and 2,5-furandicarboxylate dimethyl esters

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Abstract

Two culture broths, containing succinate produced *de novo* by *Corynebacter glutamicum* and 2,5-furandicarboxylate by whole cell biotransformation of 5-(hydroxymethyl)furfural (HMF) by a recombinant *Pseudomonas putida,* were used for dimethyl ester production. For anion exchange, they were characterized for competing organic anions (i.e., other carboxylates) and inorganic anions (phosphate, sulfate and chloride). These affect capturing of the target dicarboxylate via sorption. For the analysis of the sorption process, independent multicomponent column experiments using mimicked mixtures of the respective target building block with organic anions, inorganic anions and actual fermentation broth were performed. In the case of succinate, breakthrough profiles and column capacities showed that α-ketoglutarate, malate and other fermentation impurities reduced sorption capacity. For 2,5-furandicarboxylate the effect of impurities in sorption was less pronounced, with residual HMF eluting without any apparent ionic interaction. After sorption, upgrading via alkylation from mimicked and bio-based broth was successfully carried out producing the respective succinate and 2,5-furandicarboxylate dimethyl esters. Yield towards dimethyl succinate was reduced from 0.98 to 0.66 mol ester/mol carboxylate due to the presence of fermentation impurities, which were also esterified in good yields. Dimethyl 2,5-furandicarboxylate final yield ranged between 0.75-0.77 mol ester/mol carboxylate for both pure and raw bio-based sorbed furandicarboxylate. Esterification kinetics correlate well with the acidity of the carboxylates and impurities.

Introduction

Many carboxylates can be produced conveniently by fermentation, but their recovery is not straightforward.[1] A new option has recently been proposed.[2] Upon sorption of aqueous carboxylates from fermentation broth using quaternary anion-exchange resins, the sorbed carboxylate may be converted in dimethyl carbonate as solvent and reactant. This may lead to methyl carboxylate and regenerated resin in one step. This integrated direct downstream catalysis has proven to be successful when using synthetic sodium succinate solutions. However, it is not clear if it can be applied with real fermentation broth, containing many impurities, and with other carboxylates.

In this study, the proposed integrated method will be studied using succinate from *C. glutamicum* broth[3] and 2,5-furandicarboxylic acid (FDCA) from *P. putida* broth.[4] The resulting dimethyl esters are potentially interesting as building blocks.[5, 6] In particular, dimethyl 2,5-furandicaboxylate is a more attractive monomer for the production of polyethylene 2,5-furandicarboxylate (PEF), a drop-in replacement of polyethylene terephthalate (PET), than its diacid precursor.[6]

Materials and methods

Materials

All chemicals used were analytical grade. A strong anion exchange resin, Dowex Marathon MSA (macroporous), was obtained in the chloride form and converted to the bicarbonate form prior to utilization. Samples of bio-based succinate and 2,5-furandicarboxylate in crude fermentation broth were obtained from Research Centre Jülich[3] and Bird Engineering[4] (now Corbion), respectively. The used succinate broth was produced by a batch fermentation using *C. glutamicum* BOL-1/pAN6-pycP458S and a similar fermentation protocol and cultivation media as Litsanov et al.[3] Briefly, both cell cultivation and anaerobic succinate production were combined in a single fermentation via a controlled dissolved oxygen (D.O.) ramp. Cells were grown aerobically during 10 h until 30% D.O. was reached, followed by a linear ramp to anaerobic conditions (0% D.O.) for 6 h. Total cultivation time was 62 h and final succinate titer was 26 g/L. The harvested broth was centrifuged, filtered, frozen and shipped and subsequently thawed just before its use in these studies. Moreover, a mimicked mixture using the major carboxylate by-products found in a high producing succinate fed-batch fermentation using the strain BOL-3/pAN6-gap was prepared based on the composition reported by Litsanov et al.[3] In the case of FDCA, the raw mixture was prepared by dissolving a partially purified FDCA sample with a declared purity of 67%, containing HMF and HMF acid as impurities. Pure and partially purified FDCA were recovered and purified based on existing methods.[4]

For comparison purposes, a dimethyl 2,5-furandicarboxylate (dmFDCA) standard was synthetized via Fischer esterification adapted from an existing protocol.[7] Pure FDCA from Bird Engineering (3 g, 19.2 mmol) was reacted with excess of methanol (32 g, 1 mol) using hydrochloric acid (1.5 mL) as a catalyst. The reaction was carried out under reflux

for 18 h. After reaction, the catalyst was neutralized by addition of 50 mL of 0.5 mol/L methanolic KOH solution. Methanol was evaporated and the solid product dissolved in chloroform. The solution was filtered and washed with deionized water. Subsequently traces of water were removed using brine and magnesium sulfate. The filtered solution was evaporated and the solids recrystallized from acetone obtaining white crystals. Yield: 74%. Purity was >99% according to absence of contaminant peaks in HPLC and NMR. ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm): 3.93 (s, 6H), 7.22 (d, 2H). ^{13}C-NMR (400 MHz, $CDCl_3$, δ, ppm): 52.36 (OCH_3), 118.48 (furan ring C3 and C4), 146.62 (furan ring C2 and C5), 158.31 (C=O).

Fermentation broth characterization

Samples of succinate and FDCA fermentation broths were characterized in physical appearance and composition of relevant impurities. Succinate broth was obtained free of suspended solids whereas raw FDCA broth contained cells and other solids which were removed by centrifugation (5000 rpm, 20 min) and a sequence of filtration steps. Using water, broth was diluted according to the column size, and thus its theoretical capacity, to be able to analyze the sorption profile. No further additional pretreatment was done prior to sorption experiments. Further broth characterization comprised the determination of organic anionic by-products and inorganic anions from nutrient salts.

Dicarboxylate recovery by column sorption

Dynamic sorption experiments were carried out in a similar way as in previous studies [8] Briefly, a Bio-Rad column (1 cm internal diameter x 27 cm height) was packed with anion exchange resin, resulting in a 21 mL bed volume corresponding to 16 g wet resin (5.7 g dry resin). Carboxylate solutions were pumped at a 2 mL/min flow at 25 °C and 1 mL fractions were collected for analysis. Furandicarboxylate sorption was done in a shorter column (1 cm internal diameter x 6.2 cm height) with a 5 mL column bed corresponding to a 3.8 g wet resin (1.4 g dry resin).

Ester formation by O-alkylation experiments

Main ester formation experiments were performed as reported previously [2]. Typically, in a stirred autoclave reactor, 30 g of dimethyl carbonate were added and 1 g of dry loaded resin was held in the solid addition device until reaction temperature (100 °C) was reached.

After releasing of the resin into the vessel, samples were taken periodically for ester quantification. Ester formation experiments for individual impurities were performed in agitated glass closed tubes heated using an oil bath. A given amount of resin in the bicarbonate form was loaded batch-wise with carboxylate, then washed and dried, finally placed in the agitated tube and reacted with dimethyl carbonate.

Analytical methods

Organic acids present in succinate broth, mimicked mixture and column sorption eluent were determined by an established ion exchange HPLC method.[8] Concentrations of relevant organic impurities present in FDCA broth and column sorption eluent were determined using a RP-HPLC method based on the method developed by Koopman et al.[4] Inorganic anions such as phosphate, sulfate and chloride were measured spectrophotometrically using respective commercial cuvette tests from Hach-Lange.

Methyl esters of succinate and other carboxylates from succinate fermentation broth and mimicked mixture were determined by gas chromatography as reported before[2] using anisole as internal standard and commercial methyl ester standards from Sigma. dmFDCA was determined using the same RP-HPLC method as for the acid, properly adjusting the running time and using the synthesized diester, as described above, as the quantification standard. The identity of dmFDCA produced by alkylation was confirmed by ^{1}H-NMR and ^{13}C-NMR as previously described.[7, 9]

Results and discussion

Fermentation broth characterization and definition of mimicked mixtures

Succinate fermentation broth[3] had a translucent purple to brown appearance without the presence of any solids. The color is attributed to the presence of protocatechuic acid (3,4-dihydroxybenzoic acid), used as a micronutrient for *C. glutamicum*. The broth containing 2,5-furandicarboxylate had a very dark brown color and contained solids in suspension, which were effectively removed by centrifugation and filtration.

Succinate and FDCA raw broths may contain other carboxylates that may decrease the sorption capacity towards the target dicarboxylate, which indicates the importance of their identification and quantification prior to recovery using sorption. As mentioned in the previous section, the reported final composition of a fed-batch cultivation for a similar

succinate fermentation[3] was chosen to compose the mimicked mixture used in the current study. Table 1 summarizes the composition of the three cases.

Table 1: Composition of a representative succinate fermentation final broth and the mimicked mixture used in sorption experiments. All broths were adjusted to neutral pH.

	Reported composition[3]		Mimicked mixture	Diluted fermentation broth
Component	mM	g/L	g/L	g/L
Succinic	1135	134.0	10.0	10.0
Pyruvic	6	0.53	0.04	0.12
Acetic	20	1.20	0.09	0.10
α-Ketoglutaric	35	5.11	0.38	0.05
Malic	33	4.42	0.33	---
Fumaric	13	1.51	0.11	0.02

In the case of 2,5-furandicarboxylate, the final broth contained several HMF-related compounds co-produced during the biotransformation (Table 2). The partially purified sample, used in this case as the equivalent mimicked mixture, and broth were diluted as done in the succinate case. Given the very low concentrations of residual furanic compounds in the partially purified compounds, all of them were below quantification limits after dilution and thus reported as not detected.

Table 2: Composition of a representative raw and diluted FDCA fermentation broth

	Reported composition[a]		Mimicked mixture[b]	Diluted biotransformation broth
Component	mM	g/L	g/L	g/L
FDCA	472.42	73.74	9.3	10.0
HMF acid[c]	142.10	4.53	n.d.	0.60
FFA[d]	140.09	2.92	n.d.	n.d.
HMF	12.11	8.34	n.d.	1.10

n.d.: Not detected. Limit of detection < 0.5 g/L
[a] Data provided by Bird Engineering
[b] Prepared using a partially purified FDCA sample of 67%
[c] 5-Hydroxymethyl-2-furancarboxylic acid
[d] 5-Formyl furoic acid

Inorganic anions, present from unconsumed salts in the medium, will influence the carboxylate sorption equilibrium. For succinate production, most of the salts used in the medium were sulfate salts, especially ammonium sulfate as nitrogen source. Phosphates and chlorides were expected in lower amounts. Whole cell bioconversion of HMF to FDCA was done using culture conditions based on a described protocol[4] in which a phosphate buffer system was used and ammonium sulfate as nitrogen source. Other micronutrient salts contribute with additional sulfate and chloride anions. The concentration of such inorganic anions in the raw broths was determined for each case and presented in Table 3. The levels encountered for both cases are in line with the values expected on basis of the initial medium composition.

Table 3: Main inorganic anions present in succinate and FDCA final broths

Fermentation broth	**PO_4^{3-}**	**SO_4^{2-}**	**Cl^-**
	g/L	g/L	g/L
Succinate	0.88±0.02	11.53±0.07	0.09±0.01
FDCA	2.09±0.02	5.30±0.30	0.22±0.01

Upgrading of bio-based succinate via direct downstream catalysis

Part 1: Dynamic sorption

Sorption studies were done using the mimicked mixture and diluted fermentation broth. Although the succinate titers are low, the molar ratio between the different components was kept, resulting in an experiment that is intended to reflect the real concentrated case in terms of component separation.

Figures 1 and 2 show the elution profile of the carboxylates as breakthrough curves for both cases. Normalized outlet concentrations allow a better analysis of the system behavior. As expected, monocarboxylic acids (acetate and pyruvate) are less retained than dicarboxylic acids. As ion exchange is the main interaction mechanism present, selectivity rules according to anion valence apply. In the mimicked case, Figure 1, succinate breakthrough occurred at approximate 76 mL and showed a narrow mass transfer zone saturating the column until 208 mL. Later in the run, α-ketoglutarate and fumarate eluted slowly and did not reach the feed concentration by the end of the experiment after 640 mL

(30 bed volumes, BV, 5.3h). The elution profiles of the dicarboxylates are consistent with trends observed in several studies[1] where the selectivity order was established as fumarate > succinate ≥ malate for strong anion exchangers.

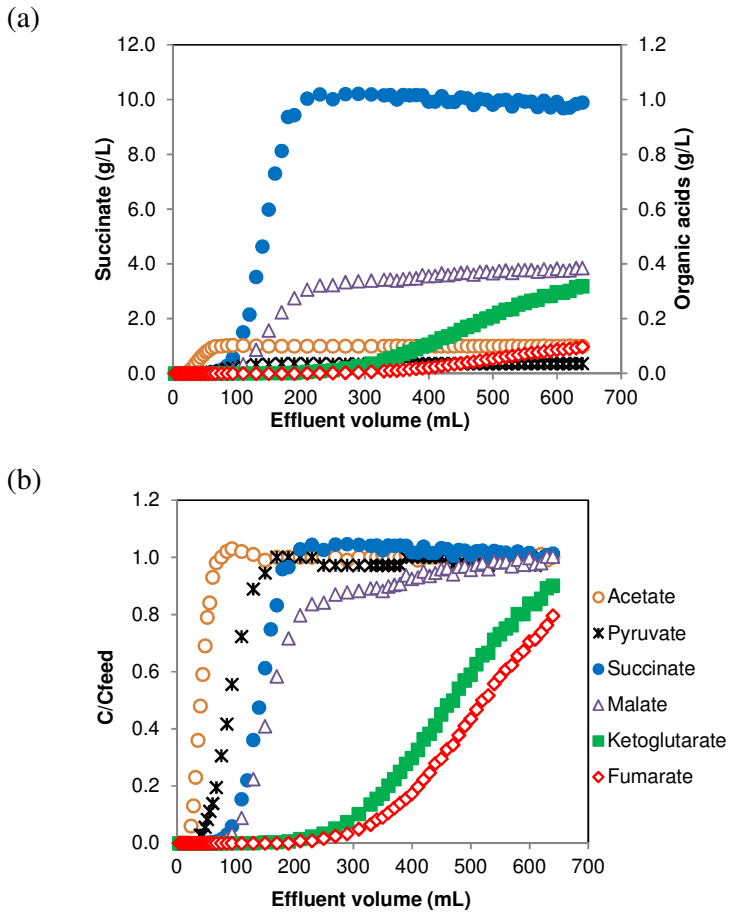


Figure 1: Multicomponent column sorption experiments for succinate using mimicked mixture (a). Normalized profiles for each component are presented in (b). The elution profiles were constructed by overlaying two sets of data from independent experiments.

Acetate, pyruvate and succinate showed chromatographic peaking resulting in elution concentrations higher than feed concentrations. The reason of this can be attributed to the high affinity towards α-ketoglutarate and fumarate, which compete for exchange sites already occupied resulting in desorption of those species. In a longer run it is expected than the inlet and outlet concentrations will be equal, meaning that the column is exhausted and in equilibrium with the feed solution.

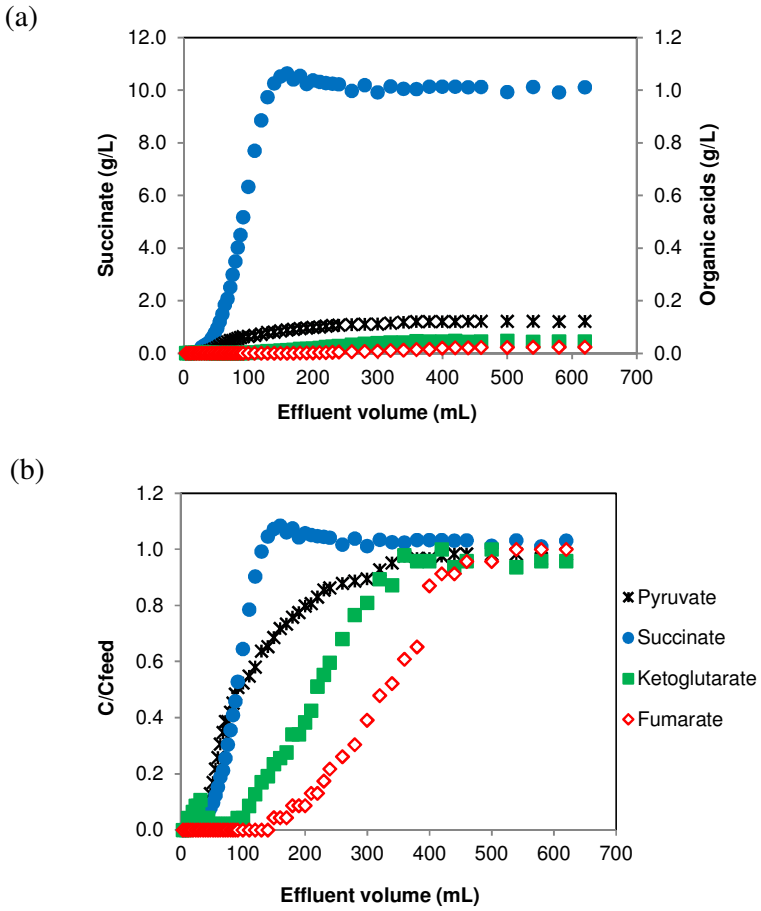


Figure 2 Multicomponent column sorption experiments for bio-based succinate using diluted fermentation broth. Normalized profiles for each component are presented in (b). The elution profiles were constructed by overlaying two sets of data from independent experiments. Profiles for malate and acetate present in fermentation broth were not determined as they were not properly resolved by HPLC.

Similar behavior was observed in the dynamic sorption runs with fermentation broth presented in Figure 2. An earlier succinate breakthrough at 25 mL was observed reaching succinate saturation after 132 mL. All other species started to elute faster when compared to the mimicked mixture. A cause for this could be the presence of inorganic anions, competing for exchange sites. Chromatographic peaking was less pronounced in this case though, resulting in less succinate desorption.

As all the carboxylates adsorb to a certain extent, the column capacity towards succinate was reduced. Sorption capacities for each component were evaluated by integral analysis at succinate saturation and at the end of the run. The results of these calculations are presented in table 4.

Table 4: Sorption capacities for organic anions at succinate saturation and at the end of the column run.

	Sorption capacity (g carboxylate/g dry resin)			
	Mimicked mixture		Fermentation broth	
Component	Succ. Sat.	End	Succ. Sat.	End
Succinate	0.210	0.184	0.120	0.108
α-Ketoglutarate	0.013	0.031	0.009	0.002
Malate	0.009	0.012	---	---
Fumarate	0.008	0.011	5.0×10^{-4}	0.001
Pyruvate	5.0×10^{-4}	5.0×10^{-4}	0.0016	0.003
Acetate	4.0×10^{-4}	4.0×10^{-4}	---	---

In both studied cases, a reduction in succinate capacity compared with the single component case (0.24 g succinate/g dry resin) was observed. For the fermentation broth, the decrease is as much as 50%. During such run, darkening of the resin was noticed as an indication that other species might be interfering. Although it is not clear whether such colored compounds are bound by ion exchange, as they were only desorbed by acid treatment (not by salt displacement), they are likely to influence the sorption by fouling the resin impeding accessibility to functional sites.

Part II: Ester formation by O-alkylation

After loading using either mimicked mixture or fermentation broth, the resin was used in alkylation experiments with dimethyl carbonate as solvent and alkylating agent. The reaction mechanism is expected to follow the reaction stoichiometry proposed previously,[2] where sorbed carboxylates are methylated in the presence of water yielding a methyl or dimethyl ester in the case of mono and dicarboxylates, respectively. An additional feature of the reaction is the regeneration of the anion exchange resin to the bicarbonate form and methanol formation. Figure 3 shows the obtained yield profiles for both cases. In a first instance (Fig. 3a), dimethyl succinate, dimethyl fumarate and methyl acetate were produced in good yields. Dimethyl succinate formation kinetics resembles the case in which succinate is present as the only counter-ion in the resin, seen in previous studies.[2] In the case of the sorbed succinate from fermentation broth (Fig. 3b), only partial conversion towards dimethyl succinate was found. The reason for this incomplete conversion was not

clear, and might be related to other species being sorbed, and their influence on the catalytic activity of quaternary ammonium site. Therefore, the conversion of other carboxylates was determined and interestingly, a high yield of methyl acetate was determined and traces or dimethyl fumarate were observed.

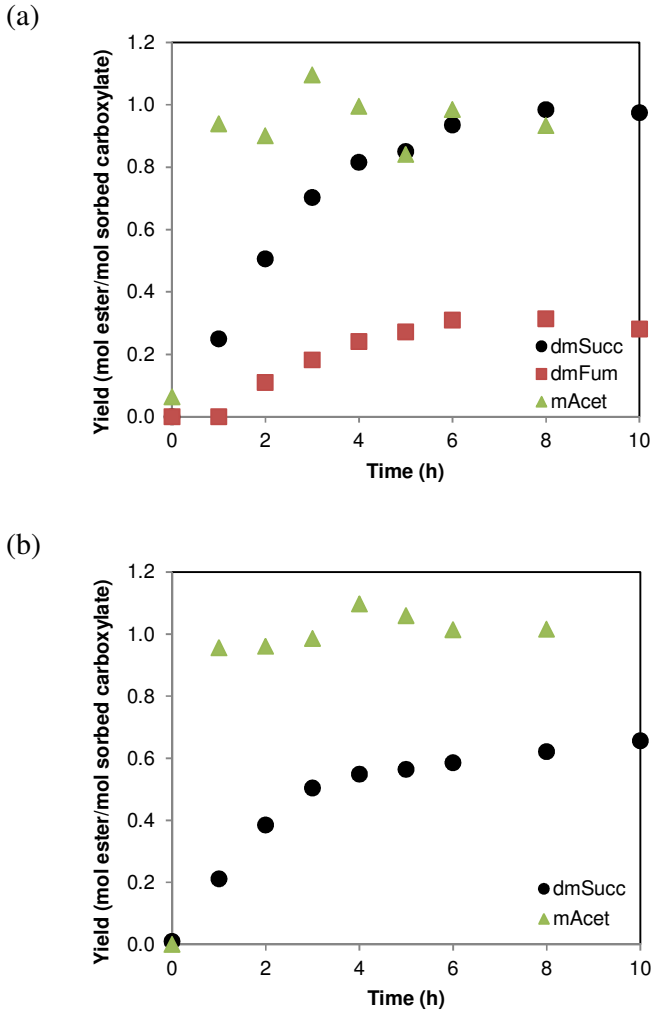


Figure 3: Formation of esters (dmSucc, dimethyl succinate; dmFum, dimethyl fumarate; mAcet, methyl acetate) from sorbed carboxylate species using final loaded resin from mimicked mixture (a) and fermentation broth (b).

In none of the experiments, dimethyl α-ketoglutarate, dimethyl malate and methyl pyruvate were detected as products. To elaborate a feasible hypothesis about this observation, experiments where the resin was loaded solely with the carboxylate impurities were carried out. Table 5 shows that, as expected, esters of succinate (control experiment), acetate and fumarate were produced in good yield. Esters of the ketoglutarate and pyruvate were not

detected and led to a dark coloration of the resin after reaction, indicating a possible decomposition of those carboxylates at the tested reaction conditions. Interestingly, *malate* was mainly converted to *dimethyl fumarate* rather than the expected dimethyl malate. Malic acid is an alpha-hydroxy acid that can undergo dehydration at the alpha carbon yielding fumaric acid. Although it is known that esters are better substrates for such reaction, it occurs in the presence of an acid as catalyst. The mechanism of reaction in our particular case is not understood. It was noted that such reaction might be of industrial interest if the same mechanism would prevail in the case of lactate, for which methyl acrylate would be obtained. However, preliminary experiments pointed at formation of oligomers of unclear composition rather than at methyl acrylate.

Table 5: Resin loading and ester yield for carboxylate impurities tested individually. Reaction conditions 100 °C, 500 rpm, 10 h

Component	**Resin loading**	**Ester yield**
	g/g dry resin	mol ester/mol sorbed carboxylate
Succinate	0.16	1.03
Acetate	0.11	1.00
Fumarate	0.19	0.44
Malate	*0.18*	*0.10 (0.33[a])*
Pyruvate	0.17	0.00
α-Ketoglutarate	0.23	0.00

[a]Yield for dimethyl fumarate based on sorbed malate

Observed reaction yields and rates are correlated. Their magnitude can be partly attributed to the strength of the interaction carboxylate-quaternary amine, being higher for more acidic carboxylic acids (Table 6). The presence of other impurities, and even the carboxylate concentration in the resin bead could also be affecting factors.

Table 6: Acid dissociation constant of selected carboxylates.[10]

Acid name	p$K_{a,1}$	p$K_{a,2}$
Acetic	4.75	---
Succinic	4.16	5.61
α-Ketoglutaric	3.90	
Malic	3.40	5.11
Fumaric	3.03	4.44

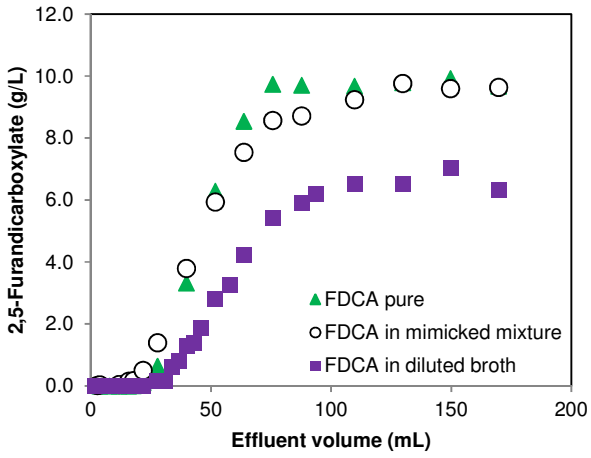
Pyruvic	2.50	---
Furandicarboxylic[11]	2.69	4.13

Upgrading of bio-based 2,5-Furandicarboxylate via direct downstream catalysis

Part 1: Dynamic sorption

Similarly to the succinate case, column loading experiments were carried out using three different 2,5-furandicarboxylate feeds. Figure 4a shows the sorption comparison between breakthrough curves for $FDCA^{2-}$ pure, in the mimicked mixture based on a partially purified sample and in diluted bioconversion broth. A certain reduction can be observed in FDCA loading capacity as the feed mixture complexity is increased. Table 7 summarizes the calculated capacities for the three cases, in which a maximum capacity of 0.30 g $FDCA^{2-}$/g dry resin is achieved in the pure case, dropping 17% in the case of the diluted bio-based FDCA. Such reduction in capture capacity is less pronounced than in the succinate case (50%), however it cannot be justified by the sorption of the main furanic-related impurities. Figure 4b shows normalized breakthrough curves for FDCA, HMF acid and HMF, being the former initially sorbed but almost fully desorbed by competition with FDCA, seen as chromatographic peaking. HMF eluted at the empty bed volume and was not sorbed at the run conditions, indicating no major ionic or hydrophobic interactions between the compound and the resin.

(a)



(b)

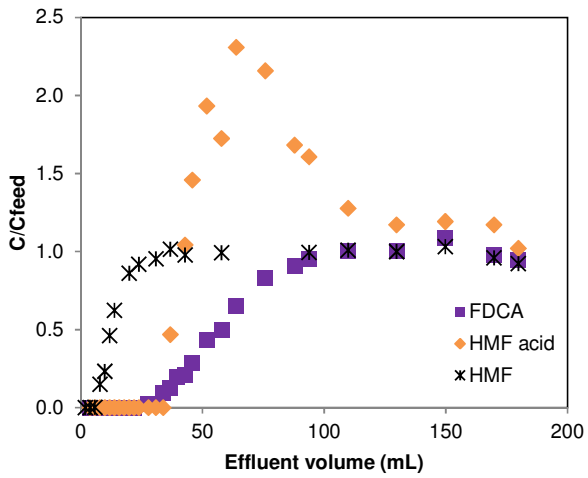


Figure 4: 2,5-Furandicarboxylate breakthrough curves from different mixtures (a) FDCA breakthrough from pure, mimicked mixture and diluted biotransformation broth. (b) Normalized multicomponent breakthrough of FDCA and related impurities in diluted biotransformation broth.

The reduction seen for the diluted broth is likely to be caused by sorption of competing inorganic anions and fouling of the resin by other compounds not determined in this study. These sorption results are promising for FDCA, and may indicate a greater feasibility of ion exchange sorption as primary recovery.

Table 7. Sorption capacities for FDCA and related impurities at the end of the column run for the three cases studied.

Component	**Sorption capacity** (g carboxylate/g dry resin)		
	FDCA pure	Mimicked mixture	Diluted bioconversion broth
FDCA	0.30	0.29	0.25
HMF acid	--	--	1.0×10^{-4}
HMF	--	--	Not sorbed

Part II: Ester formation by O-alkylation

As demonstrated before, succinate and other carboxylates can be upgraded to esters via *O*-alkylation. However, it was also seen that stronger carboxylates are alkylated at a slower reaction rate. To demonstrate the feasibility of this transformation for FDCA, a more acidic dicarboxylic acid, alkylation experiments with dimethyl carbonate were carried out in a

similar manner as for succinate, using FDCA-loaded resins from the three mentioned mixtures. The reaction stoichiometries for the sorption and upgrading of FDCA are assumed to be analogous to those suggested for the succinate case and are presented in Figure 5a and 5b, respectively. Briefly, during sorption at neutral pH the divalent furandicarboxylate anion will be captured, occupying two exchange sites and subsequently alkylated producing the respective dimethyl ester. As a result of both processes, the resin is regenerated to the bicarbonate form with stoichiometric amounts of the respective bicarbonate salt and methanol as by-products. As discussed in previous studies, such by-products can be recycled within an integrated process comprising fermentation and upgrading.[2]

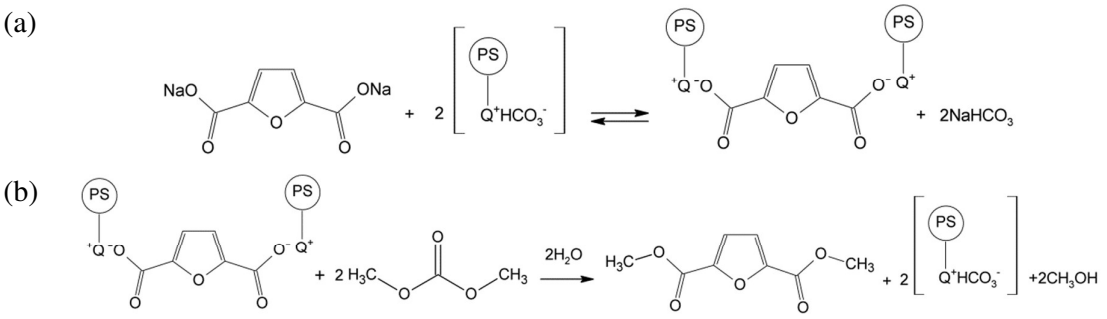


Figure 5. Proposed overall stoichiometry for the formation of dmFDCA via direct downstream catalysis. (a) FDCA sorption stoichiometry. (b) Production of dmFDCA by *O*-alkylation using dimethyl carbonate as alkylating agent. PS is polystyrene resin, Q is quaternary ammonium.

After initial short reaction trials, the reaction product was analyzed in RP-HPLC by comparing the retention time of the compound, corresponding well with dmFDCA standard prepared by Fischer esterification. Further identity verification was done using NMR after work up of the product of direct downstream catalysis.

Sorbed 2,5-furandicarboxylate from the three cases evaluated was used to determine the rate of formation of its dimethyl ester. As can be seen in Figure 6, alkylation of FDCA from a purified mixture yielded 0.77 mol dmFDCA/mol FDCA after 40 h. Compared to succinate, the reaction kinetics are very slow, in agreement with the discussed pK_a reasoning in the previous section. In contrast to the results seen in the case of succinate, ester formation kinetics and yields were not significantly decreased when raw bio-based solutions were used, suggesting the importance of optimizing fermentation conditions towards residual salt concentration and by-products. Higher temperatures would make the

process more attractive for this carboxylate, resin stability being a major hurdle for this improvement.

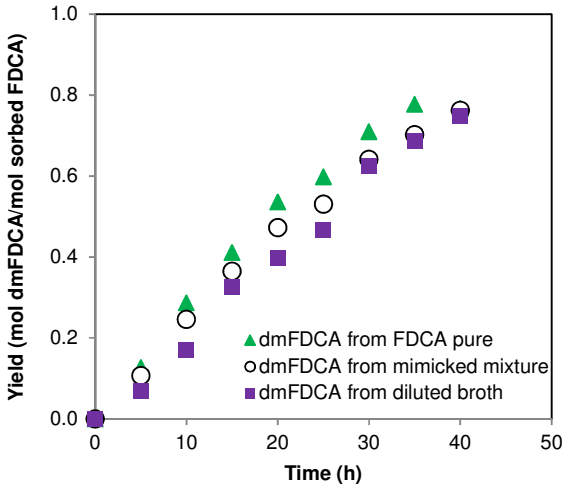


Figure 6: Dimethyl furandicarboxylate (dmFDCA) formation yield from sorbed FDCA from pure solution, mimicked mixture and diluted bioconversion broth.

Conclusions

The application of the direct downstream catalysis concept to bio-based succinate and 2,5-furandicarboxylate is promising and a potential processing alternative for carboxylates produced by fermentation, resulting in the production of diesters without the need of prior carboxylate purification. Ion exchange as a capturing step for raw dicarboxylates can be efficient, as demonstrated for 2,5-furandicarboxylate for which a capacity of 0.3 g $FDCA^{2-}$ /g dry resin was achieved. The presence of competing anions, which reduce the sorption capacity, has to be minimized by optimization of fermentation conditions.

The main characteristics of the reaction system used for *O*-alkylation, described initially for succinate, were also applicable to 2,5-furandicarboxylate and appear to be general to other carboxylates. A reaction feature such as dehydration of certain alpha-hydroxyacids, such as malic acid, during ester formation was observed and could be of important relevance if extended to other substrates. In general, the reaction rate is relatively slow, especially for carboxylic acids with a low pK_a. Further evaluation of this integration concept for bio-based carboxylates could pave the development of fully sustainable building blocks produced by biological transformations.

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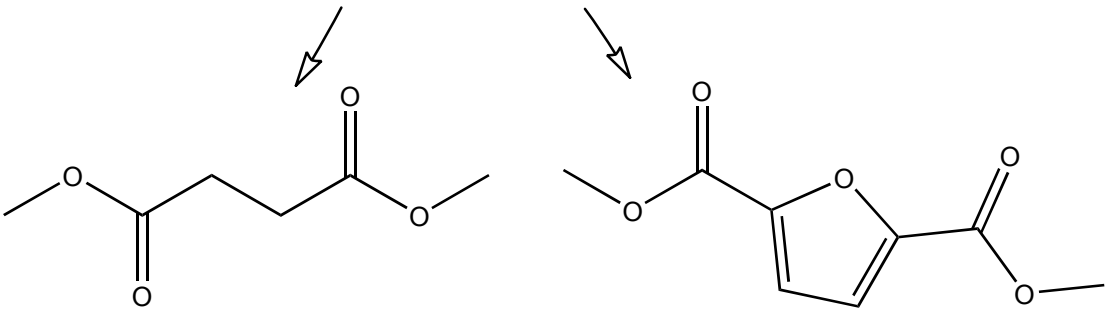
fermentation broths



dimethyl succinate dimethyl furan-2,5-dicarboxylate