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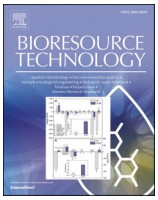

From molasses to purified α-ketoglutarate with engineered *Corynebacterium glutamicum*

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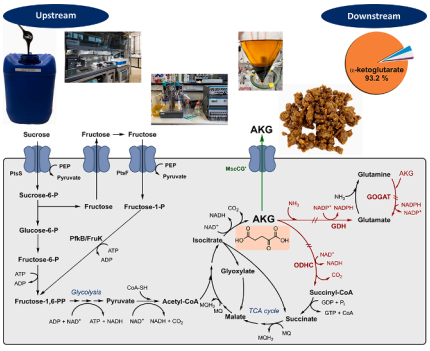
HIGHLIGHTS

- Production of α-ketoglutarate using engineered *Corynebacterium glutamicum.*
- Utilization of molasses, a side stream from sugar beet processing.
- Fed-batch bioreactor process on molasses with a conversion yield of 0.64 g g^{-1}.
- Downstream processing by liquid–liquid extraction with ethyl acetate.
- Extraction efficiency of 87 % and product purity of > 93 %.

GRAPHICAL ABSTRACT



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ABSTRACT

α-ketoglutarate (AKG) is a valuable dicarboxylic acid with multiple applications in the food, pharmaceutical, and chemical industries. Its chemical synthesis is associated with toxic by-products, low specificity, and high energy input. To create a more environmentally friendly and sustainable alternative, a microbial production process for AKG was developed. Four potential producer strains were generated by metabolic engineering of *Corynebacterium glutamicum* and characterized on defined glucose/sucrose media as well as molasses, a side stream from sugar beet processing. While strain *C. glutamicum* P_{O6}-*iolT*1 Δ*gdh* Δ*gltB mscCG*' Δ*odhA* was not able to grow on defined media it outperformed all predecessor variants on molasses. Successful scale-up into a fed-batch bioreactor process with molasses yielded 96.2 g AKG with a conversion yield of 0.64 g g^{-1}. Finally, downstream processing by liquid–liquid extraction with ethyl acetate enabled product purification with an extraction efficiency of 87 % and an AKG purity of > 93 %.

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1. Introduction

The intermediate of the tricarboxylic acid (TCA) cycle 2-oxoglutarate, also known as α–ketoglutarate (AKG), is a key metabolite for the synthesis of the proteinogenic amino acids of the glutamate family and for ammonia homeostasis. In addition to its direct application as a dietary supplement and therapeutic agent in pharmaceuticals and cosmetics, AKG is used as a building block for polymers and for the synthesis of the platform chemical ethene (Brewitz et al., 2021; Legendre et al., 2020). Bio-based AKG therefore has the potential to support the transition to a sustainable circular economy, especially in the field of petroleum-based polymer chemistry.

However, the majority of AKG and its derivatives are produced following multi-step chemical synthesis route, which requires a high energy input, leads to the accumulation of toxic waste (by)products, but only low yields (Brewitz et al., 2021; Chopra et al., 2022; Stottmeister et al., 2005). The development of economically efficient and ecologically sustainable production processes for AKG and its precursors is therefore of great interest (Legendre et al., 2020; Li et al., 2016; Tenhaef et al., 2021).

In early studies on AKG production, several bacterial hosts such as *Pseudomonas fluorescens*, *Serratia marcescens*, and *Bacillus* species were studied for their ability to produce AKG as well as different process parameters that promote its secretion and accumulation (Asai et al., 1955; Koepsell et al., 1952; Lockwood and Stodola, 1946). Under defined glucose conditions, *P. fluorescens* produced up to 45 g L^{-1} AKG with a yield of 0.45 g g^{-1} (Koepsell et al., 1952). High AKG titers of up to 195 g L^{-1} were achieved with the yeasts *Yarrowia lipolytica* and *Candida lipolytica* on fossil *n*-alkanes (Maldonado et al., 1973; Weissbrodt et al., 1988) and rapeseed oil (Förster et al., 2006). However, these carbon sources cannot be easily utilized at an industrial scale, and the reported process times were often long. The first metabolic engineering approach to improve AKG production in bacteria was described in the model organism *Corynebacterium glutamicum*. Through inactivation of glutamate dehydrogenase, the *C. glutamicum* strain accumulated about 5 g L^{-1} AKG in the supernatant (Verseck et al., 2009). Applying biotin- and nitrogen-limiting conditions, allowed for a product titer of 45.6 g L^{-1} AKG and a yield of 0.34 g g^{-1} with a glutamate dehydrogenase-deficient *C. glutamicum* strain on glucose medium supplemented with glutamate and soybean protein hydrolysate (Li et al., 2016). A further modified *C. glutamicum* strain with additional deletion of genes encoding for the glutamate synthase and isocitrate lyase was cultivated in an ammonium-limited fed-batch process with the feeding of a glucose/ molasses mixture that resulted in a final AKG titer of 51.1 g L^{-1} (Lee et al., 2013). Noteworthy, the fermentation medium contained glucose, molasses, glutamate, and soybean protein hydrolysate rendering the medium quite expensive. Hence, in order to enable sustainable and potentially economical production of AKG, cheaper starting materials, a higher product yield and suitable product separation are required.

In this study, *C. glutamicum* AKG producer strains were constructed and step-wise rational metabolic engineering was performed to eliminate competing pathways and increase product synthesis and product export. Growth and production performance of engineered strains were assessed on defined (glucose, sucrose) and complex media (molasses). The strain that performed best on molasses was used to develop a lab-scale fed-batch process. Finally, a downstream process was established to purify AKG from the molasses fermentation broth, which closes the loop from waste stream to value-added product.

2. Materials and methods

2.1. Construction of strains and plasmids

The strains and plasmids utilized in this study are shown in Table 1. Oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany) and are listed in Supplementary Table S1. All enzymes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). PCR, gel electrophoresis, DNA restriction, and further standard cloning work were performed as described previously (Sambrook and Russell, 2001). Chromosomal DNA fragments, which were required for plasmid constructions, were amplified by PCR using isolated genomic DNA of *C. glutamicum* ATCC 13032. Plasmids for homologous recombination were constructed by Gibson Assembly (Gibson et al., 2009). Correct plasmid construction was verified by colony PCR and subsequent DNA sequencing at Eurofins Genomics (Ebersberg, Germany). *Escherichia coli* DH5α, used for cloning purposes only, was routinely transformed by heat shock, and *C. glutamicum* was transformed by electroporation followed by an additional heat shock (Eggeling and Bott, 2005; Hanahan, 1983). In-frame deletions of genes and gene truncations were performed by two-step homologous recombination using pK19*mobsacB* (Schäfer et al., 1994).

2.2. Cultivation media and conditions

E. coli DH5α was routinely cultivated at 37 °C on a rotary shaker (130 rpm) in 500 mL baffled shake flask with 50 mL Lysogeny Broth (LB) medium (Bertani, 1951) or on LB agar plates. *C. glutamicum* strains were cultivated aerobically at 30 °C in brain heart infusion (BHI) medium using reaction tubes (170 rpm), 500 mL baffled shake flasks (130 rpm), or BHI agar plates. For characterization and bioreactor cultivations, defined CGXII medium supplemented with 2 % (w v^{-1}) carbon source was used (Keilhauer et al., 1993). When necessary, kanamycin (*E. coli:* 50 μg mL^{-1}; *C. glutamicum*: 25 μg mL^{-1} for cultivations and 15 μg mL^{-1} for colony selection after transformation) was added to agar plates and liquid media.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or Reference
E. coli		
DH5α	F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA- argF*)U169 *recA1 endA1 hsdR17* (rK-, mK +) *phoA supE*44λ- *thi*-1 *gyrA96 relA1*	Invitrogen (Karlsruhe, Germany)
C. glutamicum		
ATCC 13032 (WT)	Biotin-auxotrophic wild type	(Abe et al., 1967)
P_{O6}-*iolT1*	Derivative of *C. glutamicum* ATCC 13032 with two mutations in the promoter of *iolT1* (cg0223) relative to the start codon at position −113 (A to G) and −112 (C to G)	(Brüsseler et al., 2018)
P_{O6}-*iolT1* Δ*gdh* (AKG-1)	Derivative of *C. glutamicum* ATCC 13032 P_{O6}-*iolT1* with in-frame deletion of *gdh* (cg2280)	This work
AKG-1 *mscCG'* (AKG-2)	Derivative of *C. glutamicum* AKG-1 with C-terminal truncation of 345 bp in *mscCG* (cg1434)	This work
AKG-2 Δ*gltB* (AKG-3)	Derivative of *C. glutamicum* AKG-2 with in-frame deletion of *gltB* (cg0229)	This work
AKG-3 Δ*odhA* (AKG-4)	Derivative of *C. glutamicum* AKG-3 with in-frame deletion of *odhA* (cg1280)	This work
Plasmids		
pK19*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 $oriV_{Ec}$ *sacB* lacZα)	(Schäfer et al., 1994)
pK19*mobsacB*-Δ*gdh*	pK19*mobsacB* derivative for in-frame deletion of *gdh* (cg2280)	This work
pK19*mobsacB*-Δ*gltB*	pK19*mobsacB* derivative for in-frame deletion of *gltB* (cg0229)	This work
pK19*mobsacB*-Δ*mscCG'*	pK19*mobsacB* derivative for C-terminal truncation of 345 bp in *mscCG* (cg1434)	This work
pK19*mobsacB*-Δ*odhA*	pK19*mobsacB* derivative for in-frame deletion of *odhA* (cg1280)	(Brüsseler et al., 2018)

2.3. Microbioreactor cultivation

For characterization of *C. glutamicum* strains small-scale cultivations were performed in the microbioreactor system BioLector (Beckman Coulter Life Sciences, CA, USA). 48-well FlowerPlates (Beckman Coulter Life Sciences, CA, USA) of the category BOH-1 (with optodes) and a filling volume of 1000 µL medium were applied. Transient ("sacrifice") sampling was realized with the Mini Pilot Plant technology, which integrates the BioLector into a liquid handling platform Tecan Freedom EVO 200 (Tecan Group, Männerdorf, Switzerland) and provides specialized workflows for high-throughput microbial phenotyping (Hemmerich et al., 2019; Unthan et al., 2015). This combination enables automated harvesting of MTP cultivation wells in a time-dependent manner, separation of cells from the fermentation broth by centrifugation, and direct storage of the supernatant on a 4 °C cooling carrier on the platform. The supernatant samples were filtered with a 96-well AcroPrep filter plate (Pall Cooperation, NY, USA) and stored at -20 °C until they were thawed, diluted with MilliQ (factor 0.5 to 0.1) and measured with the HPLC as described below.

2.4. Lab-scale bioreactor cultivation

For lab-scale bioreactor cultivations with the selected production strain *C. glutamicum* AKG-4, four 1 L bioreactors (Eppendorf AG, Jülich, Germany) were assembled, pH electrodes were calibrated, and the vessels were filled with 820 mL CGXII base solution and autoclaved. Trace elements, biotin, protocatechuic acid and molasses (1:2 diluted, 876 mM sucrose) were mixed under sterile conditions and 157 mL of the mixture were added to the reactor through a syringe port after autoclaving. The final sucrose concentration in the reactor was 117 mM, which is twice as high as the concentration in the microbioreactor experiment. As preculture, *C. glutamicum* AKG-4 was grown in four 500 mL baffled shake flasks, each containing 50 mL CGXII medium and 58.5 mM sucrose in molasses. Cells were washed twice with 0.9 % (w v^{-1}) sterile NaCl solution and pooled. With 20 mL of each preculture, all four reactors were inoculated to a starting OD_{600} of 0.25. During the process, the pH was regulated at 7.0 via addition of H_3PO_4 and NH_4OH. A DO-dependent cascade was defined, in which the lower limit for the DO was set to 30 %. The cascade for the stirrer speed started with 400 rpm and could be increased to a maximum of 1,400 rpm at 80 % of the controller output. The air flow was set to 30 L h^{-1}. For offline analytics, samples of 7 mL volume were taken regularly from the reactor vessels after mixing the sampling tube several times.

2.5. Substrate and by-product analysis

During lab-scale bioreactor cultivation the cell dry weight (CDW) was determined in triplicates using 2 mL of the samples from the bioreactor cultivations. After centrifugation for 10 min at 12,500 g in a tabletop centrifuge the supernatant was removed, and the cell pellet was resuspended in 1 mL 0.9 % (w v^{-1}) sterile NaCl solution. Following a second centrifugation step, the supernatant was discarded, and the cell mass was dried in a drying oven at 80 °C for 12–24 h. Samples were cooled down in an excavator and weighed immediately after drying.

The remaining 6 mL supernatant samples from the 2 mL triplicates were sterile-filtered with a syringe through a cellulose acetate filter (pore size 0.2 µm, DIA-Nielsen, Düren, Germany), and then diluted 1 to 10 with high purity water.

Glucose, sucrose, and AKG concentrations of small-scale and lab-scale bioreactor samples were determined via HPLC on an Agilent 1260 Infinity system (Agilent Technologies, Santa Clara, CA, USA). A polymer-based column for sugars and organic acids (Metab-AAC, 300 x 7.8 mm, BF-series, particle size 10 µm, Isera, Düren, Germany) was used as the stationary phase and 10 mM H_2SO_4 as the mobile phase. The flow rate was 0.6 mL min^{-1} at 80 bar, the column temperature was set to 30 °C and the injection volume was 20 µL. As a reference, a dilution series of each component standard starting from 50 mM was prepared in seven 1 to 2 steps to 0.39 mM. The eight standards and all samples were measured in triplicates and averaged.

The optical density (OD_{600}) was measured with a spectrophotometer (Shimadzu UV-1800, dual-beam device) at a wavelength of 600 nm in a 2 mL cuvette. The samples were diluted with 0.9 % (w v^{-1}) NaCl solution. The NaCl solution also served as a reference for these measurements. The samples were diluted until the measured OD_{600} was in the range between 0.05 and 0.3.

The composition of the solid extract was determinded by GC-ToF analytics using an Agilent 8890N double SSL gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a L-PAL3-S15 liquid autosampler and coupled to a LECO GCxGC HRT + 4D high resolution time of flight mass spectrometer (LECO, Mönchengladbach, Germany). The system was controlled by the LECO ChromaToF software. Sample processing and method setting were used as described in (Paczia et al., 2012).

2.6. AKG purification

For the purification of AKG from the fermentation broth a liquid–liquid extraction with ethyl acetate was established based on a recently published method (Zeng et al., 2018). In the first step, the AKG-containing fermentation broth of the bioreactor cultivation was centrifuged in a refrigerated centrifuge (6 x 1 L tube, JLA-8.1000 rotor, Avanti JXN-26, Beckmann Coulter, Brea, CA, USA) for cell separation. The remaining supernatant was filtered with a pleated filter (pore size 5–8 µm, ROTILABO Typ 113P, Roth Werke GmbH, Dautphetal-Buchenau, Germany). To protonate the dicarboxylic acid (pK_a 2.47, 4.68), the filtrate was titrated with hydrochloric acid to a pH value of 1. The acidified solution was then mixed with ethyl acetate as organic solvent (four different approaches) in a separating funnel for 3 min with gentle shaking and venting six times for 30 s each. To separate the two phases, the separating funnel was suspended in the stand (10 min) and the two phases that formed during this time were separated. The aqueous phase was mixed again with fresh ethyl acetate and the separation procedure was repeated up to three rounds. After each round a sample of 1 mL from the aqueous phase was measured on the HPLC to quantify the remaining amount of AKG after each extraction step. The organic solvent of all extraction rounds was pooled and concentrated on a rotary evaporator (Rotavapor R-100, BÜCHI Labortechnik GmbH, Essen, Germany). The solution was heated in a 60 °C water bath and a vacuum of 300 mbar was applied to the system. Finally, the concentrate was cooled to 4 °C and the precipitate was separated. This solid precipitate was measured using GC-ToF-MS.

3. Results and discussion

3.1. Engineering of C. glutamicum for AKG production

As a basis for the rational engineering of *C. glutamicum* to produce AKG, strain *C. glutamicum* P_{O6}-*iolT1* was chosen (Brüsseler et al., 2018). This strain carries two point mutations in the repressor binding site of the promoter of the gene *iolT1*, which leads to its deregulated expression. The non-phosphorylating *myo*-inositol/proton symporter IolT1 mediates the uptake of the polyol *myo*-inositol (Klaffl et al., 2013) but has been shown to enable the import of different hexoses and pentoses such as fructose, glucose, and xylose (Bäumchen et al., 2009; Brüsseler et al., 2018; Ikeda et al., 2011).

As expected, the initial strain *C. glutamicum* P_{O6}-*iolT1* grew rapidly in defined CGXII medium with 20 g L^{-1} glucose as sole carbon and energy source, but no AKG accumulation was detectable in the supernatant over the course of the cultivation (Fig. 2A).

To initiate AKG accumulation in *C. glutamicum* P_{O6}-*iolT1,* a reduced formation of the amino acid glutamate was targeted (cf. Fig. 1). Therefore, the gene encoding glutamate dehydrogenase was deleted,

yielding the strain *C. glutamicum* AKG-1. Glutamate dehydrogenase catalyzes the synthesis of glutamate by reductive amination of AKG under high ammonia conditions (Börmann et al., 1992). The single deletion mutant showed significantly slower growth and lower final biomass under glucose conditions, but accumulation of 1.92 mM AKG in the supernatant could be observed (cf. Fig. 2A).

With regard to AKG export, several non-specific dicarboxylate transporters in *C. glutamicum* have been described (Fukui et al., 2019), but no specific AKG exporter is known. The native mechanosensitive glutamate transporter MscCG (Ncgl1221, also called YggB) has been intensively studied over the past decades and several conditions have been discovered to enhance glutamate secretion via this channel, including biotin limitation, addition of ß-lactam antibiotics, and addition of specific detergents (Nakamura et al., 2007). It was also found that various C-terminal truncations of *mscCG* promote glutamate export without induction. A recent study has shown that MscCG partially exports 2-hydroxyglutarate, a direct derivative of AKG (Kataoka et al., 2023). Consequently, the *mscCG* gene was truncated to evaluate whether the deregulated MscCG-transporter also facilitates constitutive AKG export in our engineered strain variant (*C. glutamicum* AKG-2). This variant showed a further reduction in growth rate and final biomass but a pronounced increase in AKG titer and AKG yield by 67 %, respectively, showing that the truncated MscCG-transporter is indeed capable of transporting AKG across the cell membrane (cf. Fig. 2A).

In a next step, the gene encoding the large subunit GltB of the enzyme glutamine-2-oxoglutarate aminotransferase (GOGAT) was deleted (cf. Fig. 1), resulting in the variant *C. glutamicum* AKG-3. In a coupled reaction with glutamine synthetase (GS), GOGAT catalyzes the formation of glutamate from AKG and glutamine (Ertan, 1992). Although the GS/GOGAT system plays a minor role in glutamate formation under standard cultivation conditions (excess of nitrogen), it has been reported to be upregulated in *C. glutamicum* in response to *gdh* deletion (Kholy et al., 1993). The additional deletion of *gltB* resulted in a further increase in AKG titer (3.5 mM) under glucose conditions (cf. Fig. 2A), which emphasizes the effectiveness of the restricted glutamate formation. It is noteworthy that these gene deletions do not render the respective strains glutamate-auxotrophic, which is probably due to the presence of several glutamate-dependent transaminases in *C. glutamicum* (Marienhagen et al., 2005).

Finally, to reduce the conversion of AKG along the TCA cycle, the gene encoding the subunit *odhA* of the 2-oxoglutarate dehydrogenase complex (ODHC) was deleted to evaluate the possibility of interrupting the TCA cycle, yielding strain *C. glutamicum* AKG-4. Interestingly, this strain failed to grow under glucose conditions, and the production of AKG was greatly reduced to 1.5 mM (cf. Fig. 2A).

3.2. AKG production from defined sucrose media

It should be noted that the deregulated promoter of P_{O6}-*iolT1* should not have an enhancing effect on sucrose uptake and is therefore not necessary for the production of AKG from sucrose. However, for the comparison of AKG production on the different carbon sources tested in this study and to exclude other possible influencing factors, it was decided to use the identical strains and inoculate the main cultures in parallel from the same preculture.

On defined sucrose media, the basic strain *C. glutamicum* P_{O6}-*iolT1* showed a similar growth phenotype as on glucose and no AKG accumulation was detected here either (Fig. 2B). In contrast, the strain variants AKG-1 to AKG-3 accumulated considerably higher AKG titers of 6.3 to 22.0 mM, but also showed a further corresponding reduction in final biomass by 16.1 to 24.7 % compared to glucose conditions (cf. Fig. 2B).

The different carbon fluxes into the biomass and the product AKG could be explained by the way sucrose is utilized by the cells. Sucrose enters the cell via the sucrose-specific phosphotransferase system (PtsS) with simultaneous phosphorylation to sucrose-6-phosphate (Krahn et al., 2021) (cf. Fig. 1). The latter is then spontaneously cleaved to glucose-6-phosphate and fructose. While glucose-6-phosphate is readily metabolized through glycolysis, fructose must be exported and re-imported through PtsF in order to be activated by phosphorylation as fructose-1-phosphate. Fructose-1-phosphate enters glycolysis via ATP dependent phosphorylation to fructose-1,6-bisphosphate by 1-phosphofructokinase (PfkB/FruK). As the oxidative pentose phosphate pathway (oxPPP) is initiated via glucose-6-phosphate, the oxPPP flux is significantly reduced in cells grown with fructose (Krahn et al., 2021). In addition, fructose-6-phosphate and glyceraldehyde-3-phosphate are required for the operation of the reductive PPP in the reverse direction via transketolase, whereby the availability of the first reactant might also be limited by the operation of

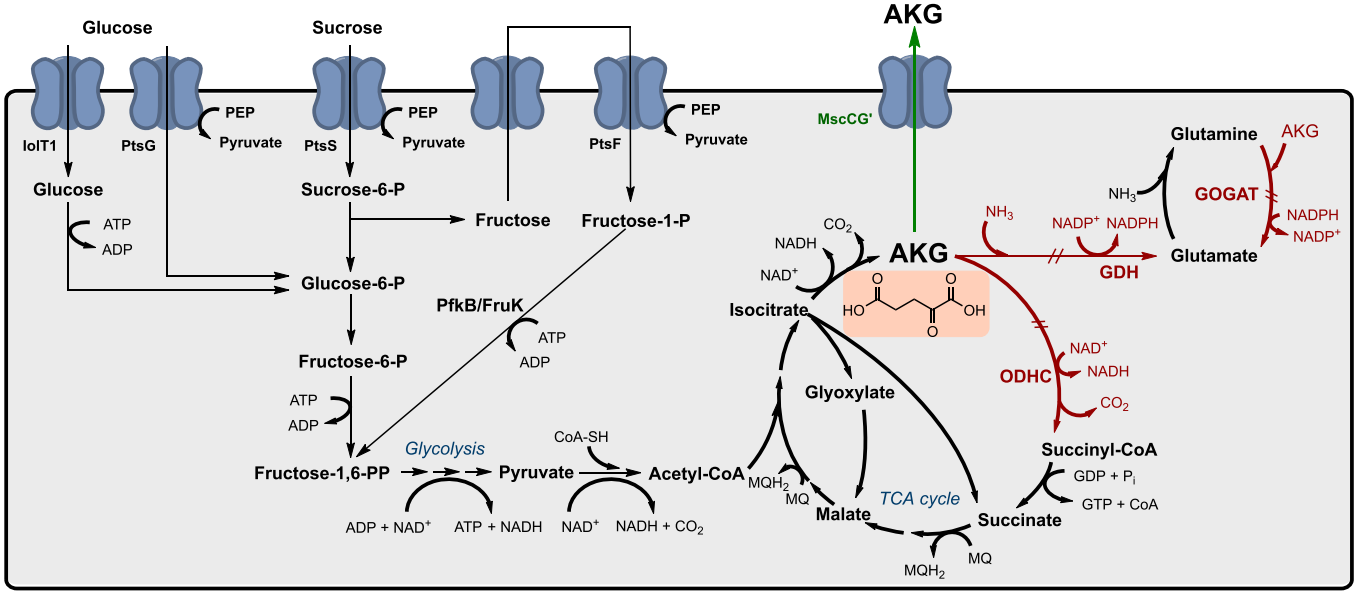

Fig. 1. Schematic overview of the metabolic pathways in *C. glutamicum* leading to AKG production from sucrose-containing feedstocks. GDH: glutamate dehydrogenase; GOGAT: glutamine-2-oxoglutarate aminotransferase/ glutamate synthase; IolT1: *myo*-inositol/proton symporter; MscCG: mechanosensitive glutamate transporter; ODHC: 2-oxoglutarate dehydrogenase complex; PfkB/ FruK: 1-phosphofructokinase; PtsF: fructose-specific phosphotransferase system; PtsG: glucose-specific phosphotransferase system; PtsS: sucrose-specific phosphotransferase system.

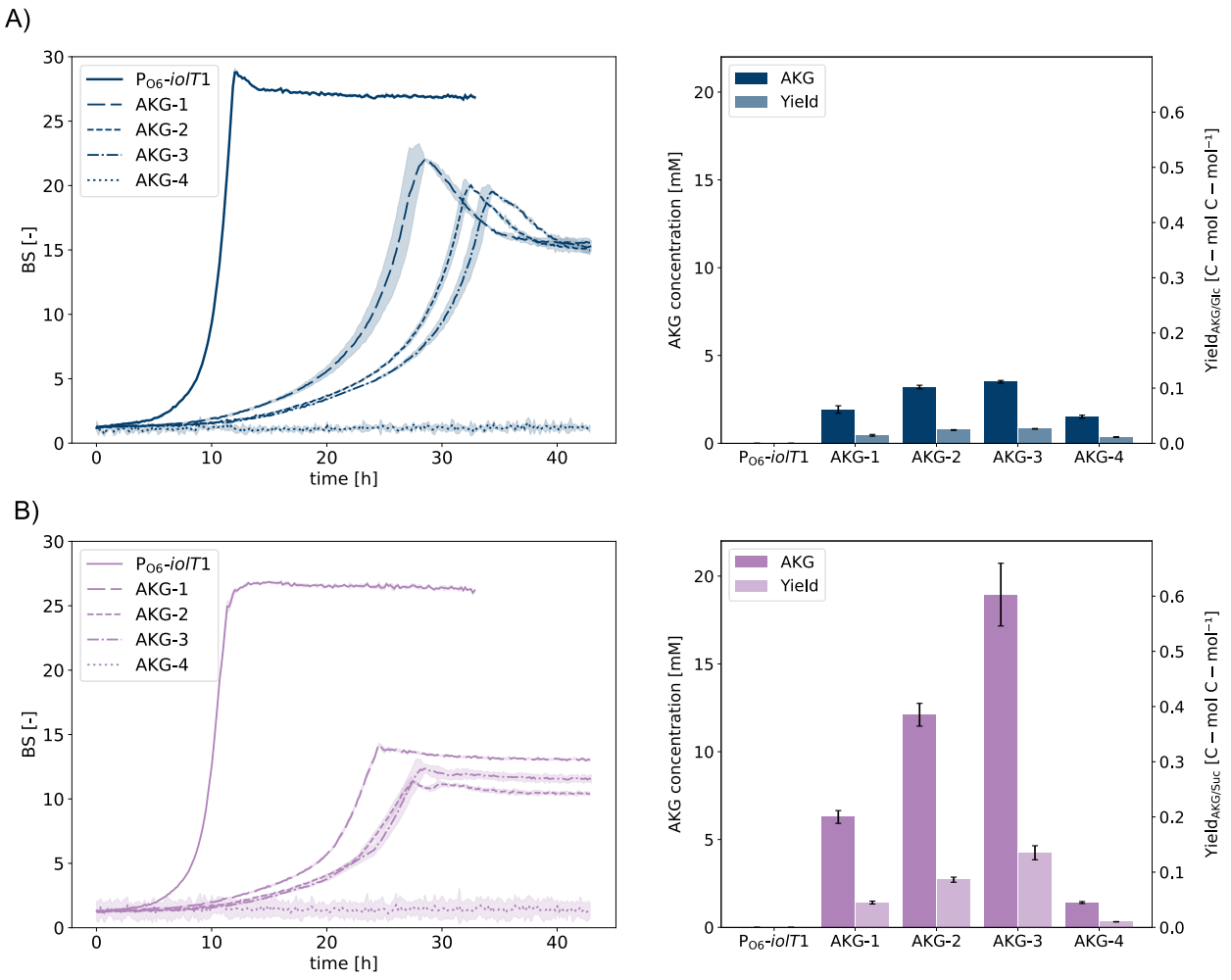

Fig. 2. Growth and AKG production of engineered *C. glutamicum* in defined media. All strains were grown in a BioLector system using a 48-well FlowerPlate containing defined CGXII media with 20 g L^{-1} of A) glucose or B) sucrose as sole carbon and energy source, respectively. The filling volume was 1 mL, the shaking frequency was set to 1,400 rpm and a backscatter gain of 3 was used. Endpoint sampling was carried out to determine AKG titers. Mean values and standard deviations were calculated from triplicate cultures.

the gluconeogenetic enzyme fructose-1,6-bisphosphatase, which is up-regulated under fructose conditions (Noack et al., 2017). Consequently, the metabolic routes supplying NADPH and the precursors erythrose-4-phosphate and ribose-5-phosphate are reduced and less biomass can be formed. Conversely, the comparatively higher conversion of fructose to fructose-1,6-bisphosphate leads to a higher carbon flux along lower glycolysis and towards the oxidative TCA cycle allowing for the accumulation of the target product AKG.

As expected, strain *C. glutamicum* AKG-4, which lacked ODHC activity, also failed to grow under sucrose conditions, and the AKG titer of 1.4 mM was comparably low (cf. Fig. 2B).

3.3. AKG production from residual feedstock

The applied molasses feedstock has a sucrose content of more than 60 % and contains several different nitrogen sources (for details on composition see (Helm et al., 2023). Therefore, it is well suited as a feedstock for *C. glutamicum*, and the phenotyping on defined media showed the potential of sucrose in connection with AKG production.

Indeed the reference strain *C. glutamicum* P_{O6}-*iolT1* as well as the engineered producer strains AKG-1 to AKG-3 showed comparable growth on molasses (Fig. 3A). However, the AKG production was significantly lower compared to sucrose, i.e. 3.9 mM for the best performing variant AKG-3, indicating a preferential (and well-balanced) utilization of available carbon and other essential nutrients in molasses for biomass rather than product synthesis.

In contrast, the highest engineered strain AKG-4, which was unable to grow on either glucose or sucrose in defined media, also showed poor growth performance on molasses (cf. Fig. 3A and 2B). Surprisingly, the AKG titer of 82.7 ± 1.2 mM and the corresponding yield of $Y_{P/S} = 0.59$ C-mol C-mol^{-1} were increased by more than 325 % compared to the best AKG production achieved on sucrose media. The genetic modifications introduced in AKG-4 (Δ*gdh*, Δ*gltB*, Δ*odhA*) interfere with ammonium fixation and flux through the TCA cycle. Possible deficits could be compensated for by the presence of other nitrogen sources (amino acids, NO_3^-) in molasses and the potential activation of the glyoxylate shunt by the available acetate (5 mM). Although the addition of glutamate led to a slight increase in the growth performance of strains AKG-1 to AKG-3, growth could not be restored to the level of the reference strain *C. glutamicum* P_{O6}-*iolT1* (Fig. S1). For strain AKG-4, the supplementation of glutamate had no effect on growth under any of the conditions tested. To obtain a clear understanding on the underlying flux distributions in this strain, more advanced experiments including proteomics and fluxomics are required, which were out of the scope of this study.

In a following scale-up experiment the best performing strain AKG-4 was cultivated in a 1 L lab-scale bioreactor under fed-batch conditions and molasses was used as the sole source of carbon and energy

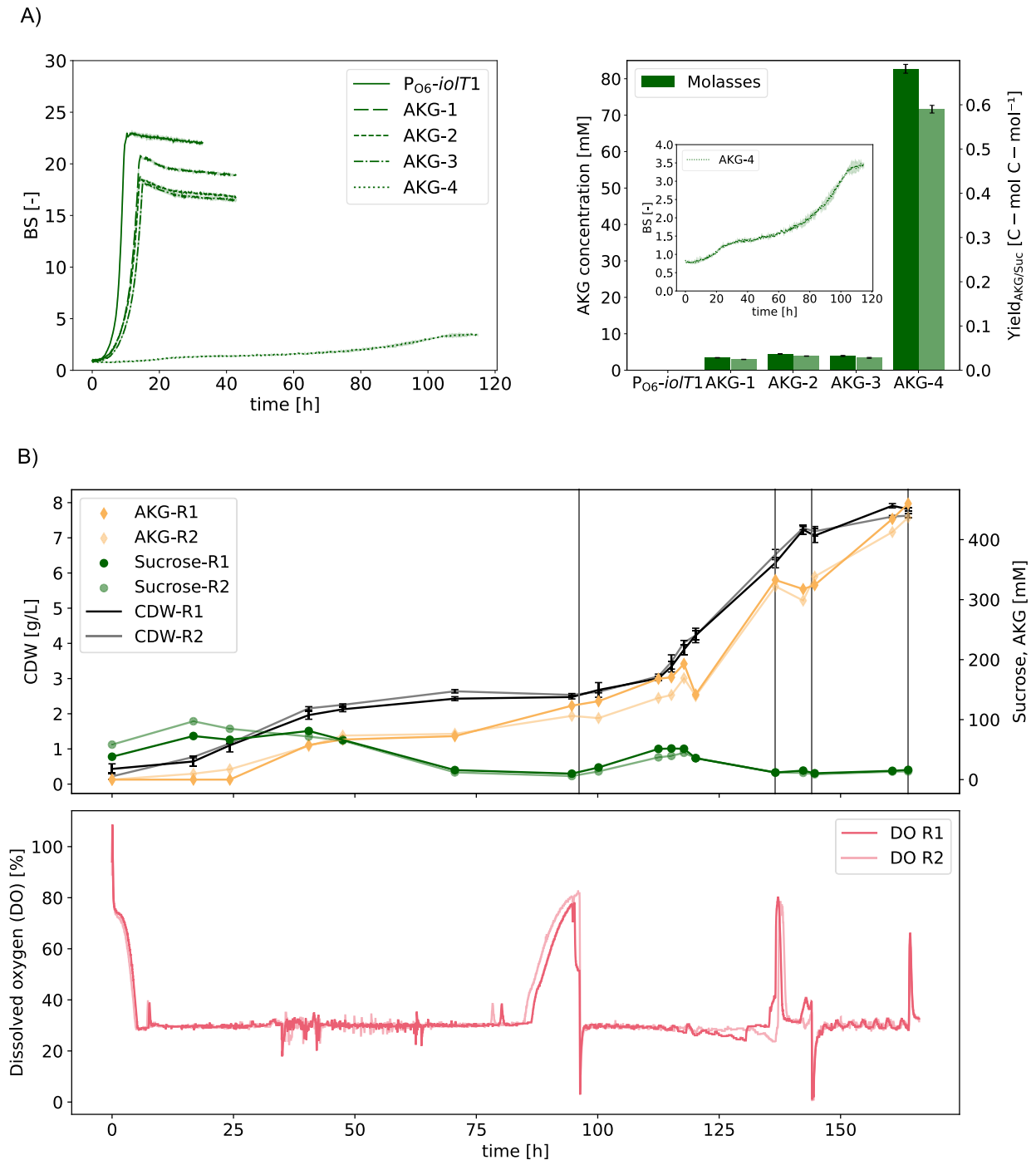

Fig. 3. Growth and AKG production of engineered *C. glutamicum* in molasses media. A) BioLector batch cultivations on molasses. Strains were grown as triplicates in a 48-well FlowerPlate containing defined CGXII media with molasses at a final concentration of 20 g L^{-1} sucrose. The filling volume was 1 mL, the shaking frequency was set to 1,400 rpm and a backscatter gain of 3 was used. Endpoint sampling was carried out to determine AKG titers. Mean values and standard deviations were calculated from triplicate cultures. B) Bioreactor fed-batch cultivation of *C. glutamicum* P_{O6}*.iolT1* Δ*gdh* Δ*gltB* Δ*odhA mscCG'* (AKG-4). Cells were cultivated in two parallel 1 L bioreactors (R1, R2) on CGXII media containing molasses with a final sucrose concentration at the start of the cultivation of 40 g L^{-1}. The feed solution contained molasses with a concentration of 300 g L^{-1} sucrose and no additional CGXII salts.

throughout the process. During the batch phase the initially supplied amount of 40 g L^{-1} molasses was converted into 2.5 g L^{-1} biomass and 131 mM (19.1 g L^{-1}) AKG (Fig. 3B). The observed slow biomass formation and high growth-coupled AKG production confirmed the results from the microbioreactor experiments with this engineered strain (cf. Fig. 3A). After about 80 h the dissolved oxygen (DO) signal started to increase, indicating limitation of the available carbon sources from molasses. In contrast to the sudden peaks of 90–100 % within a few minutes that are known for defined glucose media, the DO in this process rose to around 80 % within 15 h. This leads to the conclusion that the cells were still metabolically active, which is also reflected in the sucrose, which had not yet been fully utilized at the time of the DO peak (cf. Fig. 3B). Upon subsequent feeding, the DO immediately decreased and cell growth and AKG accumulation were observed. The initial high accumulation of sucrose indicates moderate overfeeding at the beginning until the cells grew back into carbon limitation after feeding was

stopped 42 h later. Interestingly, the DO peak observed here was more abrupt than at the end of the batch phase. This can be attributed to the higher cell mass, which consumes correspondingly more oxygen. After the sudden increase in the DO, it also fell again abruptly, which indicates further metabolic activity without feeding. At the beginning of the second feeding phase, the DO again fell abruptly below 30 %, and cell growth and AKG accumulation were observed for 20 h, analogous to the first feeding phase.

A total of 659 mmol (96.2 g) of AKG was produced and a conversion yield of 0.63 C-mol C-mol^{-1} (0.64 g g^{-1}) was achieved in each reactor. The resulting space time yield was 2.7 mmol L^{-1} h^{-1}. Compared to previously described production processes using *E. coli* with a maximum space time yield of 3.7 mmol L^{-1} h^{-1} and product yield of 0.32 g g^{-1} (Chen et al., 2020), the overall productivity with strain AKG-4 was 27 % lower, but the AKG yield was increased by a factor of two. To our knowledge, this is the highest AKG titer and yield produced by *C. glutamicum* and the first AKG production from the molasses waste stream described to date. Further optimization potential for this process lies in a higher inoculation density, which should lead to a faster conversion of the substrate at an approximately constant specific production rate of 1.2 mmol g_{CDW}^{-1} h^{-1}. Since cell growth is very slow, this is an obvious way to increase the current space time yield of 2.7 mmol L^{-1} h^{-1}. In addition, it should be possible to shorten the batch phase by 25 h, as substrate uptake is almost stagnant from 70 to 95 h and only 2.1 g sucrose was consumed by the cells.

3.4. Purification of AKG from fermentation broth

To purify AKG from the molasses fermentation broth a liquid–liquid extraction method with ethyl acetate was established. In a first step, cells and suspended solids were separated by centrifugation and filtration (Fig. 4A). The resulting supernatant was then acidified with hydrochloric acid to a pH of about 1 to ensure that the hydronium concentration below the pK_a of 2.47 was high enough to protonate both carbonyl functions of the dicarboxylic acid. Subsequently, up to five times the volume of ethyl acetate was added to one volume of the acidified solution in a separating funnel. After repeated mixing by swirling, three phases were formed during separation. The aqueous phase was collected and mixed again with ethyl acetate in a second and third round, the viscous phase was discarded and the organic phase with the extracted product was stored. The latter phase was concentrated on a rotary evaporator and the evaporated solvent was recycled. Finally, the concentrate was cooled to 4 °C and the AKG was precipitated by crystallization.

Four different approaches were used to test the influence of the number of extraction rounds (mixing of fresh ethyl acetate with the sample) and the volume ratio of sample to solvent on the extraction efficiency (Fig. 4B). The comparison of the extraction methods showed that the multiple addition of fresh ethyl acetate (approach 2 and 3) led to a higher extraction efficiency. As a final result, AKG was purified from the fermentation broth with an extraction efficiency of 87.1 %, and an overall process yield for AKG from sucrose in molasses of 0.55 C-mol C-mol^{-1} (0.56 g g^{-1}).

The purity of AKG after crystallization was determined as 93.2 % by GC-ToF-MS (Fig. 4C). The remaining impurity consisted mainly of succinate and glycerol. The other detected components (marked as “unident”) could not be assigned.

The presented method offers a simple approach for the purification of AKG produced in fed-batch with *C. glutamicum* AKG-4. To further increase product purity, the remaining succinate (2.7 %) should be

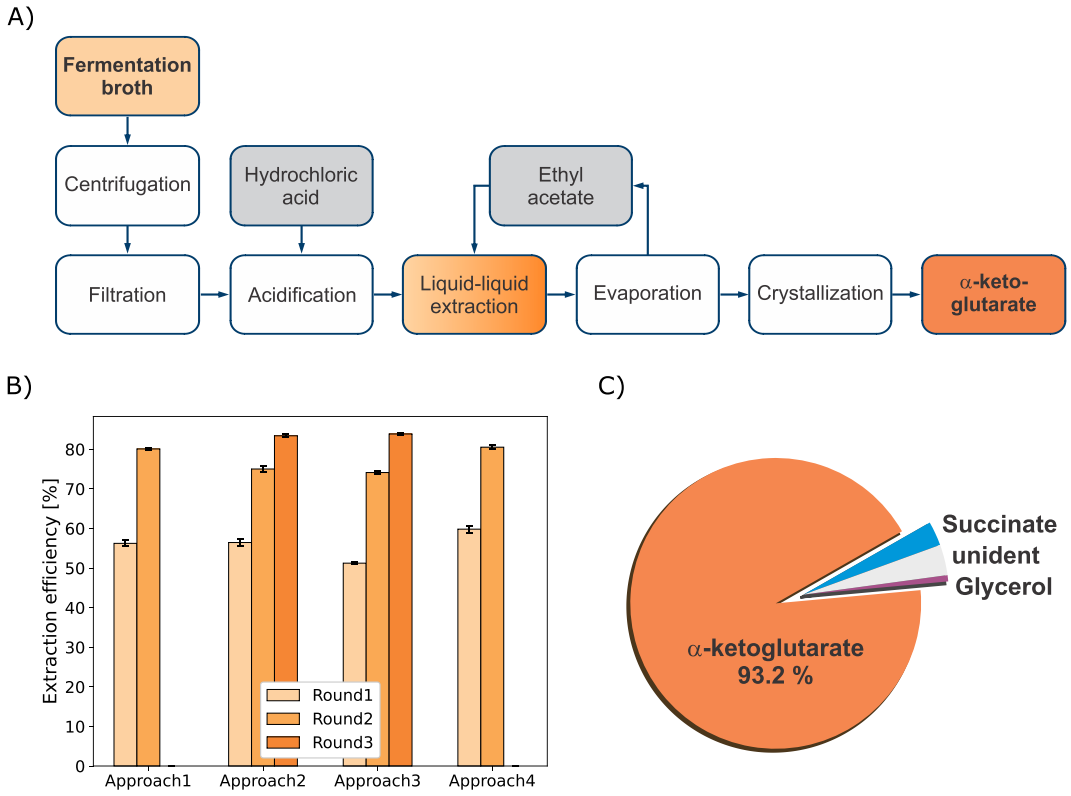

Fig. 4. Purification of bio-based AKG from molasses fermentation broth applying multistep liquid–liquid extraction with ethyl acetate as solvent. A) Flow-chart of purification process from AKG-containing fermentation broth to final crystallization of the dicarboxylic acid. B) Four different approaches for cross-current liquid–liquid extraction. First and fourth approach with only two extraction steps (sample-solvent-ratios 1:4, 1:4 and 1:5, 1:3). Second and third approach with three extraction steps (sample-solvent-ratios 1:4, 1:2, 1:2 and 1:3, 1:3, 1:2). The total amount of ethyl acetate was always eight times the sample volume. C) Composition of precipitate after purification procedure measured with GC-ToF-MS.

separated. This could be achieved by introducing an additional step in the downstream processing. By remixing the organic phase with water at a pH of 3, AKG remains favorably in the aqueous phase due to the different pK_a values (2.47 and 4.68) compared to succinate (4.16 and 5.61). Other unidentified uncharged molecules as well as succinate should remain in the organic phase. However, this would be associated with a loss of product. Noteworthy, since eight times the volume of ethyl acetate must be used to extract AKG from one volume of fermentation broth, it is difficult to transfer this purification concept to pilot or industrial scale. A possible alternative would be continuous purification using a counter-current extraction process. Here, ethyl acetate would have to be fed through the fermentation broth from below, drain of at the top, be concentrated by evaporation, and the condensed ethyl acetate fed back at the bottom. Succinate could be separated from the concentrate and the product decolorized with activated carbon if necessary.

4. Conclusions

Conclusively, the developed AKG process represents a sustainable and cost-effective alternative to chemical synthesis routes for AKG production. The space time yield should be further optimized for industrial applications. As the cells only grow slowly and AKG production is linked to growth, a repetitive batch mode or a set-up, which enables continuous operation would be preferable. For large-scale supply of bio-based AKG, i.e. as monomer for biopolymers, purification should be carried out as a continuous process due to the necessary steps of the cross-current liquid–liquid extraction required.

CRediT authorship contribution statement

Lars Halle: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniela Höppner:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Marvin Doser:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Christian Brüsseler:** Writing – review & editing, Conceptualization. **Jochem Gätgens:** Formal analysis, Data curation. **Niclas Conen:** Writing – review & editing, Validation, Conceptualization. **Andreas Jupke:** Supervision, Funding acquisition. **Jan Marienhagen:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Stephan Noack:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131803.

Data availability

Data will be made available on request.

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