J Ind Microbiol Biotechnol (2017) 44:1483–1490
DOI 10.1007/s10295-017-1971-7

BIOENERGY/BIOFUELS/BIOCHEMICALS - ORIGINAL PAPER

Synthesis of citramalic acid from glycerol by metabolically engineered *Escherichia coli*

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Received: 29 March 2017 / Accepted: 18 July 2017 / Published online: 25 July 2017

Abstract Citramalic acid (citramalate) serves as a five-carbon precursor for the chemical synthesis of methacrylic acid. We compared citramalate and acetate accumulation from glycerol using *Escherichia coli* strains expressing a modified citramalate synthase gene *cimA* from *Methanococcus jannaschii*. These studies revealed that *gltA* coding citrate synthase, *leuC* coding 3-isopropylmalate dehydratase, and acetate pathway genes play important roles in elevating citramalate and minimizing acetate formation. Controlled 1.0 L batch experiments confirmed that deletions in all three acetate-production genes (*poxB*, *ackA,* and *pta*) were necessary to reduce acetate formation to less than 1 g/L during citramalate production from 30 g/L glycerol. Fed-batch processes using MEC568/pZE12-*cimA* (*gltA leuC ackA-pta poxB*) generated over 31 g/L citramalate and less than 2 g/L acetate from either purified or crude glycerol at yields exceeding 0.50 g citramalate/g glycerol in 132 h. These results hold promise for the viable formation of citramalate from unrefined glycerol.

Keywords Acetate · Biodiesel · Citramalate synthase · Crude glycerol · Fed-batch

Introduction

The commercial manufacture and use of biodiesel has been rapidly emerging in Europe and US during the last 2 decades. As an alternative to petrochemical fuels, biodiesel is superior in its health and environmental impact, including low sulfur content, lower emission of harmful off-gases, and a better lifecycle of CO_2 [9]. One key challenge in the development and adoption of biodiesel is the low value by-product glycerol, which is generated at about 10% mass ratio from the esterification or transesterification of vegetable oil and animal fats [29]. Fortunately, many microorganisms can naturally utilize glycerol as the sole carbon and energy source, and glycerol is a potential substitute for the traditional carbohydrates such as sucrose or starch in industrial fermentation processes [7, 8, 18]. Glycerol has been evaluated as a raw material for the production of many microbial products, including hydrogen [36], 1,3-propanediol [10], 2,3-butanediol [44], and succinic acid [20].

Methacrylic acid (MAA) is a commodity chemical with an estimated annual global market of about 2.2 million tons, and it is used primarily for the synthesis of poly(methylmethacrylate) [45]. This polyester is widely used as a transparent thermoplastic in construction, furniture, medical material, and display technologies. The most common route for MAA synthesis converts acetone cyanohydrin to methacrylamide sulfate using sulfuric acid [6, 32, 37]. Sulfuric acid regeneration and hazards associated with volatile cyanides are concerns for industrial MAA production, and companies have sought other routes from isobutene, isobutyric acid, and ethylene [6, 32]. Direct microbial production of MAA and acrylate with its reduced hazards has been proposed, but acrylates are extremely toxic to microorganisms such as *Escherichia coli* [2, 39].

Recently, we reported a microbial approach to produce citramalic acid [citramalate, (R)-2-methylmalic acid, (2R)-2-hydroxy-2-methylbutanedioate] from renewable carbohydrates. Citramalate can be directly converted to MAA by base-catalyzed decarboxylation and dehydration [24]. In a fed-batch fermentation, 46.5 g/L citramalate was formed

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with a yield of 0.63 g/g from glucose using an engineered *Escherichia coli* expressing a modified *cimA* gene derived from *Methanococcus jannaschii* coding citramalate synthase [42]. Despite the deletion of citrate synthase (coded by *gltA*) and acetate kinase (*ackA*), about 10 g/L acetate were still formed as an undesirable by-product from glucose. The maximum theoretical yield of citramalate from glycerol in *E. coli* is 0.80 g/g (Fig. 1), and the stoichiometric equation for the biochemical conversion is as follows:

$$2 \text{ glycerol} + 5 \text{ NAD} + 2 \text{ Pi} + 2 \text{ ADP} \rightarrow \text{citramalate} + 5 \text{ NADH} + 2 \text{ ATP} + CO_2.$$

The goal of this study was to examine citramalate formation from glycerol by *Escherichia coli*. In addition to studying whether 5-carbon citramalate can be generated directly from both purified and crude 3-carbon glycerol at high yield, we examined strategies to reduce the formation of acetate as a by-product (see Fig. 1).

Materials and methods

Strain construction

Strains used in this study are shown in Table 1. Gene mutations were transduced into *E. coli* MG1655 from their respective strains in the KEIO collection [5] by the P1 phage method. The Δ*pta* knockout was constructed using the *λ* Red recombination [12]. After completing the Δ*pta* knockout, the *ackA-pta* operon was sequenced confirming that Δ*ackA-pta* was deleted (Eurofins Scientific, Louisville, KY, USA). To knockout multiple genes in single strain, the Kan antibiotic marker was removed using pCP20 [12]. In knockout strains, forward primers external to the target gene and reverse primers within the kanamycin resistance cassette were used to check for proper chromosomal integration. In cured strains, the removal of the markers was verified by PCR. Plasmid pZE12-*cimA* was transformed into all strains for citramalate production [42]. This *cimA* gene from *M. jannaschii* has been evolved to enhance activity and to reduce isoleucine inhibition [3, 23]. For this study, the *E. coli* codon-optimized gene was used (GenScript, Piscataway, USA).

Growth medium

XP medium was composed of (per L): 3.00 g glycerol, 1.00 g/L peptone, 1.44 g KH_2PO_4, 2.11 g K_2HPO_4, 2.00 g K_2SO_4, 3.50 g NH_4Cl, 20.00 mg $Na_2(EDTA)\cdot 2H_2O$, 0.15 g $MgSO_4\cdot 7H_2O$, 20 mg thiamine·HCl, 0.25 mg $ZnSO_4$, 0.125 mg $CuCl_2\cdot 2H_2O$, 1.25 mg $MnSO_4\cdot H_2O$, 0.875 mg

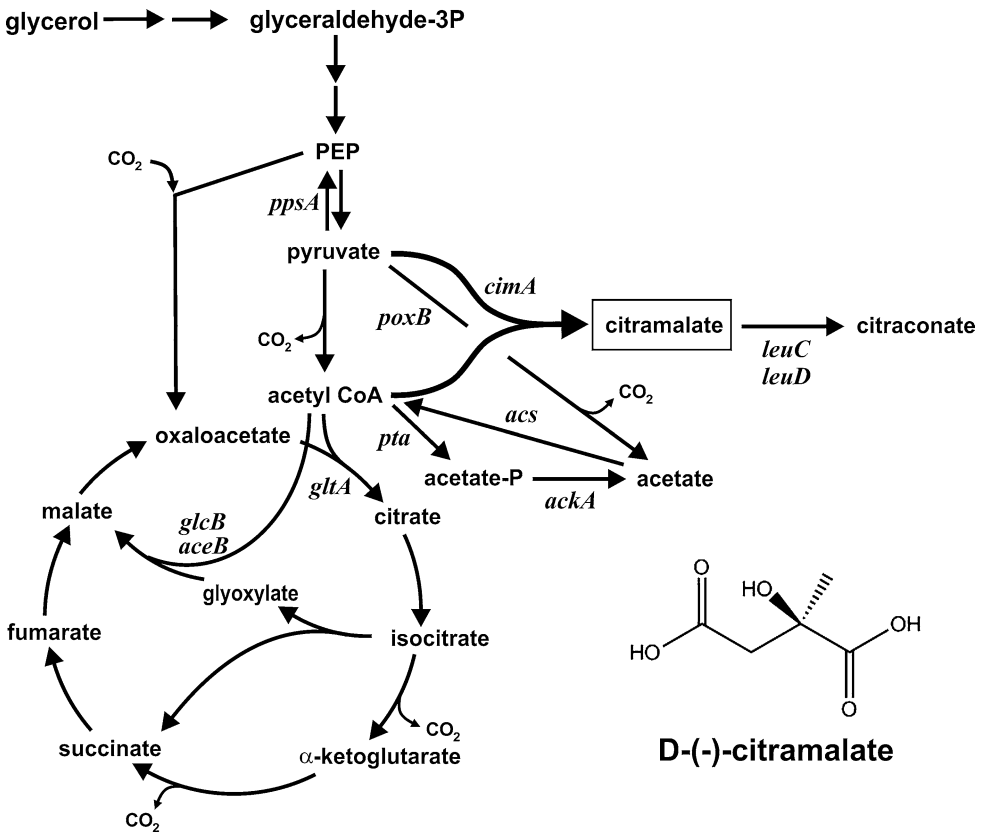

Fig. 1 Biosynthesis of citramalate in *Escherichia coli* expressing the *cimA* gene coding citramalate synthase. Key genes (and coded enzymes) are: *leuC* and *leuD* (3-isopropylmalate dehydratase), *gltA* (citrate synthase), *glcB* and *aceB* (malate synthase), *pta* (phosphotransacetylase), *ackA* (acetate kinase), *poxB* (pyruvate oxidase), and *ppsA* (phosphoenolpyruvate synthetase)

Table 1 Strains used in this study

Strain	Genotype	Notes
MG1655	*E. coli* F-*λ*-*ilvG rfb*-50 *rph*-1	Wild type
MEC480	MG1655 Δ*gltA*770::Kan	[42]
MEC481	MG1655 Δ*aceB*781::Kan	[42]
MEC482	MG1655 Δ*glcB*749::Kan	[42]
MEC485	MG1655 Δ*aceB*781::(FRT) Δ*glcB*749::Kan	[42]
MEC490	MG1655 Δ*gltA*770::(FRT) Δ*leuC*779::Kan	[42]
MEC491	MG1655 Δ*gltA*770::(FRT) Δ*leuD*778::Kan	[42]
MEC498	MG1655 Δ*gltA*770::(FRT) Δ*leuC*779::(FRT)	[42]
MEC499	MEC498 Δ*ackA*778::Kan	[42]
MEC562	MEC498 Δ*ackA*778-*pta*-779::Kan	This study
MEC564	MEC498 Δ*ackA*778-*pta*-779::(FRT) Δ*pps*-776::Kan	This study
MEC566	MEC498 Δ*ackA*778-*pta*-779::(FRT) Δ*acs*-763::Kan	This study
MEC568	MEC498 Δ*ackA*778-*pta*-779::(FRT) Δ*poxB*772::Kan	This study
MEC596	MEC498 Δ*ackA*778::(FRT) Δ*poxB*772::Kan	This study

$CoCl_2 \cdot 6H_2O$, 0.06 mg H_3BO_3, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 5.50 mg $FeSO_4 \cdot 7H_2O$, and 20 mg citric acid. For the growth of strains having *leuC* or *leuD* knockouts, the medium was supplemented with 0.20 g/L L-leucine. For the growth of strains having *gltA* knockouts, the medium was supplemented with 1.00 g/L L-glutamate, since *E. coli* is unable to utilize citrate under aerobic conditions [25]. In addition, 50 mg/L ampicillin and/or 100 mg/L kanamycin were added for plasmid-containing strains or strains having antibiotic resistance. The crude glycerol from biodiesel process was generously provided by a local biodiesel producer (Down To Earth Energy, LLC, Monroe, GA, USA) and was composed of 58.6% w/w glycerol and 0.3% w/w methanol.

Shake flask, batch, and fed-batch processes

To compare various strains for citramalate production in shake flasks, cells were first grown in 3 mL Lysogeny Broth (LB) at 37 °C and 250 rpm (19 mm pitch). After 10–14 h, 0.5 mL was used to inoculate 50 mL of XP medium supplemented with 0.2 mM IPTG in 500 mL baffled shake flasks (in triplicate). After growth at 37 °C and 250 rpm (19 mm pitch) for 24 h, these shake flask cultures were analyzed for citramalate synthase activity, citramalate, and intracellular acetyl-CoA concentration.

To examine citramalate production under controlled bioreactor conditions, cells were first grown as described above in 3 mL LB and then 50 mL XP medium. After 18 h, the shake flask contents were used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 1.0 L modified XP medium with 30 g/L glycerol, 5 g/L peptone, 3 g/L glutamate, and 1 g/L leucine (but otherwise as described above) and 0.2 mM IPTG initially. For duplicate batch and fed-batch processes, the agitation was 400 rpm, and air was sparged at 1.0 L/min, which maintained the dissolved oxygen above 40% of saturation. The pH was controlled at 7.0 using 20% (w/v) NaOH, and the temperature was controlled at 37 °C. For the fed-batch process, an additional 30 g glycerol and 5 g peptone dissolved in 60 mL of water were added when the glycerol concentration decreased below 5 g/L. The calculation of yield is based on the mass of the compound generated divided by the mass of glycerol consumed. Statistical analyses were completed using Student's *t* test (two-tailed, equal variance), and $p < 0.05$ was considered the criterion for significance.

Analytical methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA, USA) was used to monitor cell growth. Extracellular organic acids and glycerol were analyzed by HPLC using a Refractive Index detector as previously described [15]. Glutamate concentration was measured using a glutamate assay kit (Sigma-Aldrich Co., St. Louis, MO, USA). Intracellular acetyl-CoA was analyzed by the previously established method [19].

Cell-free extracts were also used to measure citramalate synthase activity by the generation of free CoA and its reaction product with 5,5′-dithiobis(2-nitrobenzoic acid) detected at a wavelength of 412 nm [23]. One unit of activity is the amount of enzyme that generates 1 µmol of CoA in 1 min at 37 °C.

Results and discussion

Comparison of citramalate and acetate formation by various strains

In *E. coli* expressing citramalate synthase coded by the *cimA* gene, citramalate accumulates as the reaction product of the condensation of pyruvate and acetyl-CoA. In wild-type *E. coli* expressing citramalate synthase (MG1655/pZE12-*cimA*), just over 1 g/L citramalate formed from 3 g/L glycerol, resulting in a citramalate yield of 0.36 g/g (Fig. 2). This wild-type strain expressing citramalate synthase generated substantial acetate in shake flasks, resulting in a yield of 0.033 g acetate/g glycerol (Fig. 2). Since acetyl-CoA and pyruvate are involved in numerous enzyme reactions, we compared citramalate formation from glycerol using several strains having knockouts in genes associated with these metabolites.

Acetyl-CoA is converted to malate via malate synthase coded in *E. coli* by the *glcB* and *aceB* genes [31, 35]. We, therefore, constructed MEC481 (MG1655 *aceB*), MEC482 (MG1655 *glcB*), and MEC485 (MG1655 *aceB glcB*). Compared to MG1655/pZE12-*cimA*, MEC481/pZE12-*cimA* and MEC482/pZE12-*cimA* showed about 28 and 35% higher citramalate accumulation, respectively (Fig. 2). The strain having knockouts in both malate synthase genes, MEC485/pZE12-*cimA*, resulted in only 22% greater citramalate compared to the wild type. Acetyl-CoA is also converted to citrate via citrate synthase coded by the *gltA* gene [14], and we, therefore, examined citramalate production in MEC480 (MG1655 *gltA*) expressing citramalate synthase. MEC480/pZE12-*cimA* grew poorly on XP medium, but growth was restored when the medium was supplemented with 1 g/L glutamate. MEC480/pZE12-*cimA* grown with supplemented glutamate accumulated 0.58 g citramalate/g glycerol, 63% more than MG1655/pZE12-*cimA*. Since MG1655/pZE12-*cimA* grown in XP medium supplemented with 1 g/L glutamate also generated the same yield of citramalate as the same strain without added glutamate (data not shown), we attribute the 63% increase in citramalate formation in MEC480/pZE12-*cimA* to the *gltA* knockout and not to the presence of glutamate. Therefore, media for strains having the *gltA* knockout were henceforth supplemented with 1 g/L glutamate. These strains having knockouts of enzymes associated with the glyoxylate shunt or the TCA cycle (i.e., *aceB*, *glcB*, and *gltA*) accumulated no detectable acetate.

Citramalate could be potentially metabolized in *E. coli* by 3-isopropylmalate dehydratase coded by the *leuC* (large subunit) and *leuD* (small subunit) genes [16, 17]. The two subunits are both required for the activity of isopropylmalate isomerase, an enzyme which is necessary for leucine biosynthesis in *E. coli* [43], and each of these individual deletions was examined by comparing MEC490 (MG1655 *gltA leuC*) and MEC491 (MG1655 *gltA leuD*). With the

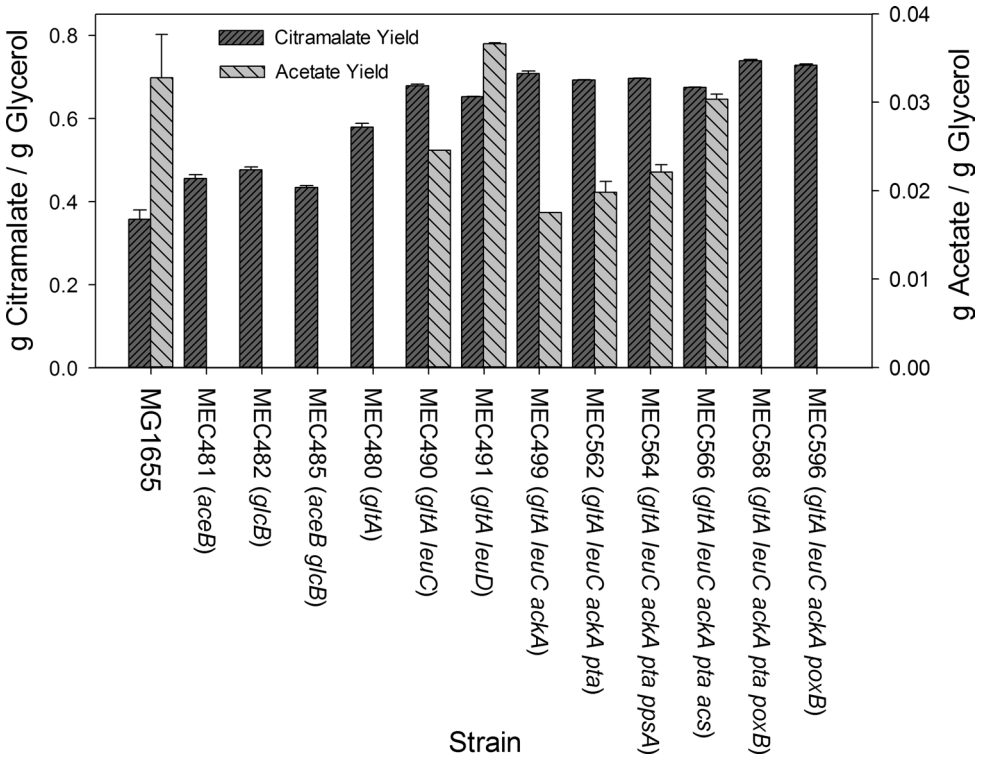

Fig. 2 Comparison of citramalate yield and acetate yield from 3 g/L glycerol in triplicate shake flasks using various knockout strains of *E. coli* expressing the *cimA* gene. The medium used for *leuC* or *leuD* strains was supplemented with 0.2 g/L leucine, while the medium used for *gltA* strains was supplemented with 1 g/L glutamate. The *error bars* represent standard deviations

deletion of either *leuC* or *leuD*, *E. coli* did not grow in XP medium with glycerol as the sole carbon source, despite the presence of peptone (data not shown). Growth was restored by the addition of 0.2 g/L leucine, and MEC490/pZE12-*cimA* accumulated 0.68 g citramalate/g glycerol, 13% greater than MEC480/pZE12-*cimA* (significant at $p < 0.05$), while MEC491/pZE12-*cimA* accumulated 0.65 g citramalate/g glycerol (Fig. 2). MEC490/pZE12-*cimA* and MEC491/pZE12-*cimA* both accumulated a similar concentration of acetate as MG1655/pZE12-*cimA*.

Although MEC490 (*gltA leuC* knockouts) formed significantly more citramalate than MEC480 with only the *gltA* knockout, the additional *leuC* knockout also led to acetate formation from glycerol. To reduce acetate formation in the *E. coli gltA leuC* expressing citramalate synthase, we examined several pathways related to the acetate and pyruvate metabolism. Four enzymes exist in *E. coli* related to acetate and acetyl-CoA. Acetate kinase coded by *ackA* and phosphotransacetylase coded by *pta* are typically considered the primary routes for the conversion of acetyl-CoA to acetyl-phosphate (acetyl-P) and to acetate [26, 30]. Acetyl-P can form acetate via other routes, also, since it can serve as a phosphate donor in gene regulation and protein-dependent transport systems [22, 41]. On the other hand, acetyl-CoA synthetase coded by *acs* functions as an anabolic route and scavenges acetate to acetyl-CoA [28]. Finally, pyruvate oxidase coded by *poxB* can play a role in aerobic growth of *E. coli* and in acetate formation from pyruvate [1]. We also examined phosphoenolpyruvate synthetase coded by *ppsA*, which could affect the intracellular pyruvate and acetyl-CoA pools [33]. We constructed several strains having these knockouts, expressed citramalate synthase, and determined the citramalate and acetate formation in shake flasks (Fig. 2).

The additional deletion in the *ackA* gene or the combination of *ackA* and *pta* genes increased citramalate yield slightly ($p < 0.05$) to 0.71 and 0.69 g/g, respectively. However, both MEC499/pZE12-*cimA* and MEC562/pZE12-*cimA* still formed acetate with yields of about 0.018–0.020 g/g (Fig. 2). Compared to *E. coli gltA leuC ackA-pta* expressing citramalate synthase, an additional *ppsA* deletion did not affect citramalate or acetate formation significantly, while an additional *acs* knockout actually elevated acetate yield to 0.030 g/g. Inexplicably, one previous investigation of an *acs* deletion strain resulted in lower specific acetate formation from glucose [11], while in another study, overexpression of *acs* significantly reduced acetate formation [28]. In our study using strains with additional gene deletions, the increase in acetate formation when *acs* is deleted (in the *ackA-pta* background) suggests that some acetate is formed via pyruvate oxidase, and that acetyl-CoA synthase provides the cells with a means to metabolize that acetate partially. In support of this conclusion, the *poxB* knockout (in the *ackA-pta* background) eliminated acetate formation in the shake flask culture, and increased citramalate yield from glycerol significantly ($p < 0.05$) to 0.74 g/g. To determine whether *poxB* or the combination of *pta poxB* was important to eliminate acetate formation, we also examined MEC596/pZE12-*cimA*, which generated 0.73 g citramalate/g glycerol and no detectable acetate. These results conclusively show that pyruvate oxidase is a key enzyme in the accumulation of acetate during citramalate production in *E. coli*. The deletion of *poxB* has similarly shown reduced acetate formation in an *ackA-pta* strain during the aerobic production of succinate by *E. coli* [27].

Acetyl-CoA is an important substrate for citramalate synthase, and we measured intracellular acetyl-CoA concentration in all triplicate shake flask experiments. These results were used to determine whether any correlation exists between intracellular acetyl-CoA and citramalate yield in the 13 different strains (Fig. 3). The results show that increased citramalate yield correlates with increased acetyl-CoA concentration with a linear correlation of $R^2 = 0.64$ (though any correlation needs not to be linear). The values observed for acetyl-CoA concentration are about 10–20 times greater than those reported during growth on glucose in defined medium, though they are in line with values during stationary phase [38, 40]. In our study, glycerol was exhausted at the time of the 24 h sample during all shake flasks experiments.

Controlled batch citramalate production from glycerol

To determine whether shake flask results were transferable to larger scale, we next examined citramalate production at the 1.0 L scale in controlled bioreactors. In duplicate, we compared six strains expressing citramalate synthase: MG1655, MEC490, MEC499, MEC562, MEC568, or MEC596. To accommodate greater cell growth, the

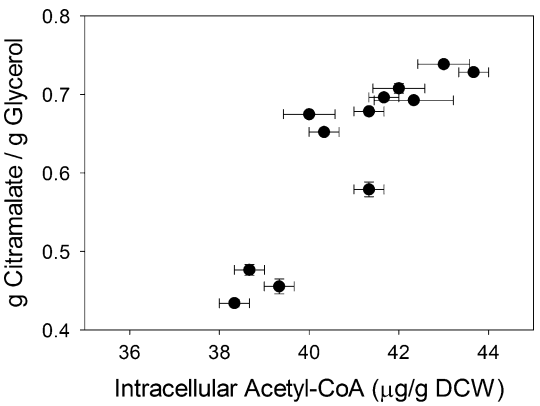

Fig. 3 Relationship between citramalate yield and intracellular acetyl-CoA concentration in shake flasks using various knockout strains of *E. coli* expressing the *cimA* gene (shown in Fig. 2)

medium contained 30 g/L glycerol supplemented with 5 g/L peptone, as well as 3 g/L glutamate and 1 g/L leucine (for strains with *gltA leuC* knockouts). The results of these batch processes are shown in Table 2.

MG1655/pZE12-*cimA* reached an OD of over 20 in 24 h and accumulated 4.3 g/L citramalate (yield of 0.143 g/g) and 0.05 g/L acetate in 30 h (yield of 0.002 g/g). All other strains examined had the *gltA* and *leuC* knockouts which significantly slowed growth despite the presence of glutamate and leucine in the medium, and they generally reached an OD of 10 in 24–30 h. The *gltA leuC* knockouts alone (MEC490/pZE12-*cimA*) resulted in only 5.2 g/L citramalate (yield of 0.175 g/g) and 11.4 g/L acetate (yield of 0.380 g/g). In comparison, the addition of an *ackA* deletion increased citramalate and diminished acetate formation. Nevertheless, the *ackA* deletion was insufficient to prevent acetate formation. The addition of a *poxB* deletion to the *gltA leuC ackA* strain further decreased acetate formation. The lowest accumulation of acetate was observed under controlled batch conditions using the strain with all three acetate pathway knockouts (*gltA leuC ackA-pta poxB*), and MEC568/pZE12-*cimA* also led to the greatest citramalate production (about 17.5 g/L). Typically, the phosphotransacetylase and acetate kinase activities are significant during cell growth, while pyruvate oxidase appears to become important during the stationary phase [13]. Pyruvate oxidase, moreover, bypasses acetyl-CoA formation altogether. The controlled batch experiments contrast with previous shake flask results and demonstrate that shake flask results are weak predictors of larger scale processes. In particular, MEC490, MEC499, MEC596, and MEC568 showed insignificant acetate formation in shake flasks, whereas in the controlled and prolonged batch processes, acetate accumulation was observed for all these strains. In general, the difference between citramalate yields and acetate yields among the six strains were significantly different ($p < 0.05$). The exception to this rule is MEC499/pZE12-*cimA* and MEC562/pZE12-*cimA* for which neither citramalate nor acetate formation could be distinguished, demonstrating that an additional *pta* knockout has much less impact in the *ackA* strain MEC499 on citramalate and acetate formation compared to the additional *poxB* knockout.

During the growth of these strains, succinate, lactate, ethanol, and pyruvate were not detected, and citramalate synthase activity was not affected by the *E. coli* strain genotype (data not shown). The combination of *gltA leuC ackA-pta* and *poxB* knockouts was important to achieve a high yield of citramalate and minimal acetate, and therefore, MEC568 was used for further studies.

Fed-batch production of citramalate

The final concentration of a fermentation product can often be maximized by continuous feeding of the carbon source. We, therefore, next completed duplicate experiments using a fed-batch process in which 30 g glycerol and 5 g peptone were again added to the fermenter once when the glycerol concentration decreased below 5 g/L. MEC568/pZE12-*cimA* was selected for this study, because this strain achieved the greatest citramalate yield in batch processes (Table 2). Like the batch process described above, for these fed-batch processes, the OD reached 10.0 within 36 h at which time the citramalate concentration was 12.5 g/L (Fig. 4). After 132 h, the final citramalate concentration reached an average of 31.4 g/L, corresponding to a yield of 0.52 citramalate g/g glycerol. In addition, only 1.8 g/L acetate was formed as by-product.

Citramalate production using crude glycerol

The rapid growth of the biodiesel industry has resulted in surplus availability of crude glycerol production, which has a purity of 60–80% based on the type of oil used as feedstock [4]. Crude glycerol also often contains 10–15% methanol, 1.5–2.5% ash, and 3.0–5.0% soap as impurities [4]. To determine if *E. coli* could be used to generate citramalate from crude glycerol, we next examined the fed-batch process using unrefined glycerol obtained directly from a local biodiesel manufacturer in place of purified glycerol. In this fed-batch process, about 31 g/L citramalate (0.51 g/g yield) and 1.9 g/L acetate were obtained using MEC568/

Table 2 Summary of citramalate and acetate formation from 30 g/L glycerol in controlled batch bioreactor using various *E. coli* strains expressing citramalate synthase

Strain	Key gene deletions	Time (h)	Citramalate yield (g/g)	Acetate yield (g/g)
MG1655/pZE12-*cimA*	N/A	30	0.143 (0.007)[a]	0.005 (0.001)[a]
MEC490/pZE12-*cimA*	*gltA leuC*	48	0.174 (0.009)[b]	0.381 (0.011)[b]
MEC499/pZE12-*cimA*	*gltA leuC ackA*	60	0.473 (0.001)[c]	0.182 (0.015)[c]
MEC562/pZE12-*cimA*	*gltA leuC ackA-pta*	60	0.482 (0.005)[c]	0.144 (0.011)[c]
MEC596/pZE12-*cimA*	*gltA leuC ackA poxB*	66	0.558 (0.001)[d]	0.085 (0.004)[d]
MEC568/pZE12-*cimA*	*gltA leuC ackA-pta poxB*	60	0.584 (0.006)[e]	0.032 (0.003)[e]

Parentheses indicate standard deviation of measurements, and values in a column with different letters indicate significant difference at $p < 0.05$

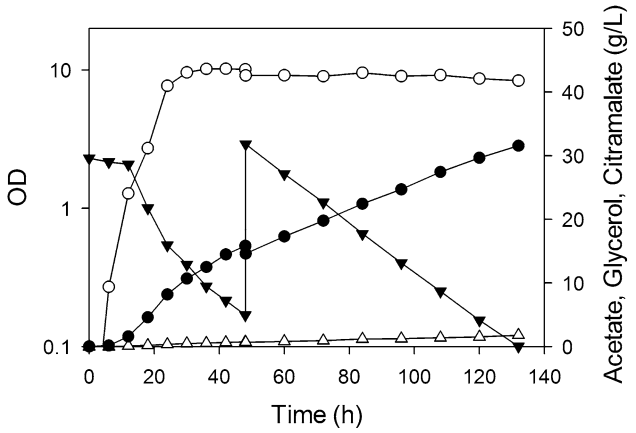

Fig. 4 Citramalate production using pure glycerol in a 1.0 L fed-batch fermentation with MEC568/pZE12-*cimA*: OD (*open circle*), citramalate (*filled circle*), glycerol (*filled downward triangle*), and acetate (*open triangle*). Approximately 30 g purified glycerol and 5 g peptone in 60 mL was added at 48 h

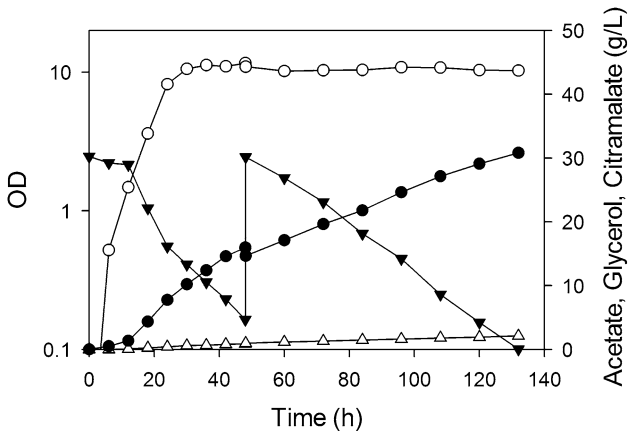

Fig. 5 Citramalate production using crude glycerol in a 1.0 L fed-batch fermentation with MEC568/pZE12-*cimA*: OD (*open circle*), citramalate (*filled circle*), glycerol (*filled downward triangle*), and acetate (*open triangle*). Approximately 30 g crude glycerol and 5 g peptone were added at 48 h

pZE12-*cimA* (Fig. 5). This result is virtually identical to the fed-batch process using purified glycerol, and demonstrates that refining glycerol is not necessary for citramalate production by *E. coli*. Interestingly, the final OD was 22% greater when crude glycerol was used (10.3 vs. 8.4), possibly because of the presence of other unidentified carbon sources in the crude material. Crude glycerol has been used in other studies of biological conversions to value-added chemicals. For example, ethanol formation was similar for purified and unrefined glycerol by a *Klebsiella pneumoniae* mutant [34], and the same 1,3-propanediol concentration was achieved using purified or crude glycerol in a fed-batch fermentation, although the productivity was lower using crude glycerol [21].

Gene knockouts and fermentation optimization improve citramalate production from glycerol and also reduce acetate accumulation. Near elimination of acetate formation necessitates deletions in genes for both pathways associated with acetate formation: *ackA* coding acetate kinase, *pta* coding phosphotransacetylase, and *poxB* coding pyruvate oxidase. Fed-batch fermentations demonstrated that identical citramalate over 30 g/L can be generated from pure or crude glycerol at yield greater than 0.50 g citramalate/g glycerol. This result holds promise that crude glycerol could be used as for citramalate production and ultimately as a source of methacrylate.

Acknowledgements The authors thank Sarah A. Lee and Daniel Geller for the technical assistance.

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