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Engineering *Escherichia coli* for Efficient Aerobic Conversion of Glucose to Malic Acid through the Modified Oxidative TCA Cycle

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Abstract: Malic acid is a versatile building-block chemical that can serve as a precursor of numerous valuable products, including food additives, pharmaceuticals, and biodegradable plastics. Despite the present petrochemical synthesis, malic acid, being an intermediate of the TCA cycle of a variety of living organisms, can also be produced from renewable carbon sources using wild-type and engineered microbial strains. In the current study, *Escherichia coli* was engineered for efficient aerobic conversion of glucose to malic acid through the modified oxidative TCA cycle resembling that of myco- and cyanobacteria and implying channelling of 2-ketoglutarate towards succinic acid via succinate semialdehyde formation. The formation of succinate semialdehyde was enabled in the core strain MAL 0 (Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*) by the expression of *Mycobacterium tuberculosis kgd* gene. The secretion of malic acid by the strain was ensured, resulting from the deletion of the *mdh*, *maeA*, *maeB*, and *mqo* genes. The *Bacillus subtilis pycA* gene was expressed in the strain to allow pyruvate to oxaloacetate conversion. The corresponding recombinant was able to synthesise malic acid from glucose aerobically with a yield of 0.65 mol/mol. The yield was improved by the derepression in the strain of the electron transfer chain and succinate dehydrogenase due to the enforcement of ATP hydrolysis and reached 0.94 mol/mol, amounting to 94% of the theoretical maximum. The implemented strategy offers the potential for the development of highly efficient strains and processes of bio-based malic acid production.

Keywords: 2-ketoglutarate decarboxylase; *Escherichia coli*; glucose; malic acid; succinate semialdehyde

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1. Introduction

Malic, or 2-hydroxybutanedioic, acid is an important dicarboxylate that can serve as a precursor of many value-added products, including in the food, pharmaceutical, and chemical industries [1–5]. Along with the structurally related dicarboxlylic acids, succinic and fumaric, malic acid was rated among the top 12 valuable building block chemicals by the US Department of Energy [6]. Currently, most malic acid is produced via the hydration of maleic anhydride, which, in turn, is obtained from petroleum-derived benzene or butane [6,7]. Nevertheless, as an intermediate of the central metabolism of a variety of living organisms, malic acid can potentially be produced biosynthetically from renewable carbon sources, e.g., plant biomass-derived sugars.

Although malic acid is not generally secreted by the vast majority of known microorganisms, several fungi of the genus *Aspergillus*, such as *A. flavus*, *A. niger*, and *A. oryzae*, are naturally capable of producing this dicarboxylate during utilisation of glucose and other carbon sources. The ability to produce malic acid from glucose was first observed for *A. flavus*, which converted the substrate to the target product with a yield of 0.78 mol/mol [8]. The corresponding yield has further been improved through the optimisation of the biosynthetic process conditions and reached the maximum value of 1.26 mol/mol [9]. However, the secretion of carcinogenic aflatoxins by *A. flavus* severely

limited the potential application of this fungus for the industrial bio-production of malic acid. A number of *A. oryzae* strains have also been found to convert glucose to malic acid with relatively high efficiency. The production of this acid from glucose with a yield of 0.9 mol/mol and a titre of 30.3 g/L has been demonstrated using the strain NRRL 3488 [10], while the strain DSM 1863 was able to accumulate up to 58.2 g/L malic acid, converting substrate to the target product with a yield of 0.68 mol/mol [11]. At the same time, the total l-malic acid production of 178.0 g/L has been achieved with the strain DSM 1863 using repeated-batch cultivation [12]. The *A. niger* strains ATCC 9142 and ATCC 10577 synthesised malic acid by utilising thin stillage containing 3.4 g/L glucose, 17.1 g/L glycerol, and 15.8 g/L lactic acid, accumulating in the medium up to 20 g/L of the corresponding dicarboxylate [13].

Several metabolic engineering approaches have been applied to improve the biosynthetic performance of wild-type fungal malic acid producers. Most of them were aimed at the enhancement of the reductive formation of the target product from oxaloacetate (OAA) via the malate dehydrogenase-catalysed reaction upon the intensification of the formation of the corresponding substrate from the glycolytic precursors, pyruvic acid and phosphoenolpyruvate (PEP). Thus, the yield of malic acid synthesised by the strain NRRL 3488 was increased up to 1.38 mol/mol resulting from the overexpression of the native genes *pyc*, *mdh3*, and *c4t318*, which code for cytosolic pyruvate carboxylase, malate dehydrogenase, and the four-carbon dicarboxylates transporter [14]. In the case of *A. niger*, overexpression in the strain of the same genes, along with the concomitant inactivation of oxaloacetate acetylhydrolase, ensured the production of malic acid from glucose by the corresponding strain S575, with a yield increased to 1.27 mol/mol [15].

In addition to *Aspergillus* species, in recent years, industrially relevant microorganisms such as *Saccharomyces cerevisiae* and *Escherichia coli* have successfully been engineered to produce malic acid from carbohydrate carbon sources. Various attempts have been made to ensure an efficient reductive formation of the target product in the recombinant strains. The production of malic acid from glucose with a yield of 0.42 mol/mol by the *S. cerevisiae* strain RWB525 was achieved resulting from the overexpression of the native pyruvate carboxylase and malate dehydrogenase genes in the parent strain CEN.PK 113-7D lacking pyruvate decarboxylase and, therefore, deficient in ethanol formation [16]. A similar yield was achieved upon the overexpression in the strain of the corresponding genes from *R. oryzae* and *A. flavus* [17].

E. coli possesses a complete set of enzymes involved in the functioning of the entire reductive branch of the TCA, which results in the formation of succinic acid under anaerobic conditions. Thus, to allow the reductive formation of malic acid in this bacterium, fumarases, which convert the target product to fumaric acid, need to be primarily inactivated. The inactivation of the mixed-acid fermentation pathways, competing with malic acid biosynthesis for reducing equivalents and key precursor metabolites, is another obligatory requirement. These are generally achieved through the deletion of the *fumA*, *fumB*, and *fumC* genes encoding fumarases and the *ackA*, *pta*, *poxB*, *ldhA,* and *adhE* genes coding for the enzymes catalysing the major reactions of acetic acid, lactic acid, and ethanol formation [18–20]. Additional metabolic engineering interventions aimed at the improvement of the characteristics of *E. coli* strains synthesising malic acid anaerobically comprise the prevention of pyruvate to acetyl-CoA conversion by the inactivation of pyruvate formate lyase [18,19], increasing the intracellular OAA availability by the overexpression of genes encoding anaplerotic enzymes [20,21], targeting of enzymes responsible for OAA and malic acid formation to both the periplasm and cytoplasm [22], and the integration of synthetic modules of CO_2 fixation and mitigation [23]. The data concerning the effect of manipulation of the expression of malic enzymes that catalyse the interconversion of pyruvic and malic acids are controversial. Both concomitant inactivation of native NADH- and NADPH-dependent malic enzymes [18] and overexpression of the heterologous gene encoding NADPH-dependent enzyme from *Arabidopsis thaliana* [19] have been shown to be favourable for malic acid synthesis by recombinant *E. coli* strains, while the overexpression

of *E. coli* NADH-dependent malic enzymes led to increased production of malic acid by *Zymomonas mobilis* [24]. The most efficient anaerobic conversion of carbohydrate substrate to the target product to date was achieved with an *E. coli* strain XZ658 (ATCC 8739 Δ*ldhA*, Δ*ackA*, Δ*adhE*, Δ*pflB*, Δ*mgsA*, Δ*poxB*, Δ*frdBC*, Δ*sfcA*, Δ*maeB*, Δ*fumB*, Δ*fumAC*), which was engineered by combining most of these approaches and was able to synthesise malic acid from glucose with a yield of 1.42 mol/mol [18].

As a facultative anaerobe, *E. coli* can also synthesise malic acid aerobically via the oxidative TCA cycle and/or in the reactions of the glyoxylate shunt (GS). The operation of GS leads to the formation of one molecule of malic acid and one molecule of succinic acid, which can further be converted to malic acid by the sequential action of succinate dehydrogenase and fumarase. Indeed, Trichez et al. recently reported the engineering of *E. coli* for aerobic biosynthesis of malic acid from glucose resulting from the combined action of the oxidative TCA cycle and GS [25]. The notable secretion of malic acid by the engineered strain during aerobic glucose utilisation was achieved primarily by preventing the conversion of the target product to OAA or pyruvic acid by the inactivation of the malate dehydrogease, malate-quinone oxidoreductase, and malic enzymes due to the deletion of *mdh*, *mqo*, *maeA* and *maeB* genes. The operation of GS was ensured through the inactivation of the transcriptional regulator IclR, which represses the expression of the *aceBAK* operon genes encoding GS enzymes. The gene *arcA* was deleted in the strain to avoid the effect of oxygen limitation, causing ArcA-mediated repression of genes coding for the enzymes implicated in respiratory metabolism and NADH accumulation. Finally, malic acid-insensitive phosphoenolpyruvate carboxylase, Ppc^{K620S}, and NADH-insensitive citrate synthase, $GltA^{R164L}$, were expressed in the strain to improve the channelling of PEP, OAA, and acetyl-CoA toward the initial reactions of the oxidative TCA cycle. The resulting strain was capable of converting glucose to malic acid with a yield of 0.82 mol/mol. However, metabolic flux analysis demonstrated that most of the malic acid was synthesised by the strain via GS, while the flux through the reactions of the oxidative TCA downstream of isocitrate dehydrogenase was insignificant. The main reason for the prevalent participation of GS in the synthesis of the target product appears to be that the expression of 2-ketoglutarate dehydrogenase in *E. coli* is rather low during aerobic growth in rich and glucose-containing media [26], thus limiting the functioning of the entire oxidative TCA cycle. In another study aimed at the engineering of *E. coli* for the production of malic acid through GS, Gao et al. implemented a similar strategy to create the core-producing strain B0013-44 (B0013 Δ*adhE*, Δ*ackA-pta*, Δ*ldhA*, Δ*maeA*, Δ*maeB*, Δ*mdh*, Δ*iclR*, Δ*arcA*), which was able to convert glucose to the target product with a yield of about 0.45 mol/mol [27]. The yield of malic acid was significantly improved and reached 0.85 mol/mol upon the direct overexpression and optimisation of the transcription in the B0013-44-derived strain B0013-47 of the native and heterologous genes encoding enzymes that catalyse pyruvate carboxylation, *pycA* from *A. flavus*, initial reactions of the TCA cycle leading to isocitrate formation, *E. coli* genes *gltA* and *acnB*, and key GS reactions, *aceA* from *E. coli* and *aceB* from *Streptomyces coelicolor*.

We previously implemented a novel design for the engineering of *E. coli* strains for efficient production of succinic and fumaric acids, the direct precursors of malic acid, from glucose through the modified oxidative TCA cycle instead of GS [28,29]. The corresponding design implied the artificial channelling of 2-ketoglutarate towards succinic acid via intermediate succinate semialdehyde (SSA) formation, thus bypassing the 2-ketoglutarate dehydrogenase-catalysed reaction in a manner similar to that of the variant TCA cycle found in myco- [30] and cyanobacteria [31]. In the present study, we report the successful application of an appropriate design to create an *E. coli* strain capable of efficient aerobic conversion of glucose to malic acid.

The previously engineered *E. coli* strain MSG1.0 Δ*aceBAK* Δ*glcB* (MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*) [28], devoid of all mixed-acid fermentation pathways, GS reactions, and possessing a modified system of glucose transport and phosphorylation, was selected as a chassis for the construction of the malic

acid-producing strain. Initially, the *mdh*, *maeA*, *maeB*, and *mqo* genes were sequentially deleted in the strain to ensure the secretion of malic acid as a notable end-product of aerobic glucose utilisation. The *Bacillus subtilis pycA* gene, encoding pyruvate carboxylase, was expressed in the strain to enable the carboxylation of pyruvic acid to OAA. The formation of SSA from 2-ketoglutarate was allowed, resulting from the expression of the *Mycobacterium tuberculosis kgd* gene coding for 2-ketoglutarate decarboxylase (EC 4.1.1.71), which is absent in *E. coli*, while the native cellular succinate semialdehyde dehydrogenase Sad was responsible for further oxidation of SSA to succinic acid. To stimulate glucose consumption by the engineered strain and to further improve its biosynthetic characteristics, the activity of ATPase was finally enhanced by the deletion of *atpFH* genes. As a result, the engineered *E. coli* strain was able to convert glucose to malic acid aerobically with a yield value of 0.94 mol/mol, which exceeded those reported previously for aerobic processes and was close to the corresponding stoichiometric maximum.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* K-12 strain MG1655 was used as the parent for the construction of all mutants described in the study.

Table 1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Genotype	Reference
E. coli strains		
MG1655	Wild-type *E. coli* K-12 strain VKPM B-6195	VKPM
MAL 0	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*	[28]
MAL M	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*, Δ*mdh*	This study
MAL 3M	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*, Δ*mdh*, Δ*maeA*, Δ*maeB*	This study
MAL 4M	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*, Δ*mdh*, Δ*maeA*, Δ*maeB*, Δ*mqo*	This study
MAL 4MP	MG1655 Δ*ackA-pta*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*, Δ*mdh*, Δ*maeA*, Δ*maeB*, Δ*mqo*; *poxB*::P_L-$pycA^{Bs}$	This study
MAL 4MPA	MG1655 Δ*ackA-pta*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*aceBAK*, Δ*glcB*, Δ*mdh*, Δ*maeA*, Δ*maeB*, Δ*mqo*; *poxB*::P_L-$pycA^{Bs}$; Δ*atpFH*	This study
Plasmids		
pKD46	pINT-ts, *bla*, P_{araB}-λ*gam-bet-exo*	[32]
pMW118-(λ*attL*-Cm-λ*attR*)	pSC101, *bla*, *cat*, λ*attL-cat*-λ*attR* cassette	[33]
pMWts-Int/Xis	pSC101-ts, *bla*, P_R-λ*xis-int*, cIts857	[34]
pMW-*kgd*	pMW119 with cloned *M. tuberculosis* 2-ketoglutarate decarboxylase gene (*kgd*)	[35]

Chromosomal modifications were performed using the lambda Red recombineering technique [32]. The primers that were used are listed in Table S1. Linear DNA fragments, comprising the chloramphenicol acetyltransferase (*cat*) gene flanked by λ*attR* and λ*attL*, which were used for the inactivation of the *mdh*, *maeA*, *maeB*, *mqo*, and *atpFH* genes, were obtained with PCR using primer pairs P1, P2; P5, P6; P9, P10; P13, P14; and P17, P18 as well as the pMW118-(λ*attL*-Cm-λ*attR*) plasmid [33] as a template. The PCR products were integrated separately into the chromosome of the *E. coli* MG1655 strain carrying the pKD46 [32] helper plasmid. The deletions of the target genes in the chromosomes of the obtained strains were verified by PCR using the locus-specific primers P3, P4; P7, P8; P11, P12; P15, P16; and P19, P20. The integration of the *B. subtilis pycA* gene encoding pyruvate carboxylase into the chromosome instead of the *poxB* gene under the control of

the strong constitutive promoter P_L of phage lambda and the native ribosome binding site was performed as described previously [29].

The modifications were first obtained individually and then combined in the chromosomes of the target strains by P1-mediated transductions. Excision of the antibiotic resistance marker from the chromosomes of the strains was performed using the pMWts-Int/Xis plasmid, as described previously [34]. The transformation of the strains with the pMW-*kgd* plasmid carrying the *Mycobacterium tuberculosis kgd* gene encoding 2-ketoglutarate decarboxylase [35] was performed according to a standard procedure.

E. coli cells were grown in Luria-Bertani (LB), SOB, SOC, and M9 media. Ampicillin (100 μg/mL) and/or chloramphenicol (30 μg/mL) were added as needed.

2.2. Culturing of the Engineered Strains for Aerobic Production of Malic Acid

For malic acid production, the engineered strains were initially grown overnight in M9 medium containing 2 g/L glucose at 37 °C. Five millilitres of the overnight culture was diluted ten times with 45 mL of M9 medium containing 10 g/L (55.5 mM) glucose, 10 g/L yeast extract, and 2.5 g/L $NaHCO_3$. The resulting cultures were incubated aerobically in 750 mL flasks with vented plugs at 37 °C on a rotary shaker at 250 rpm for 8 or 19 h. After 2.5 h of incubation, isopropyl-β-D-thiogalactoside (IPTG) was added to the medium at a final concentration of 1 mM to induce the expression of the *kgd* gene.

Two-stage fermentation was performed as follows. For the initial biomass accumulation, cells were grown as described above and harvested after 8 h of flask cultivation and appropriate induction of *kgd* gene expression. The cell suspensions were centrifuged for 15 min at ~2000× *g* and 4 °C. The cell pellets were resuspended in 50 mL of modified M9 medium containing 10 g/L glucose and 2.5 g/L $NaHCO_3$, but lacking $NH4^+$ ions, to an OD_{600} of ~6.5 (DCW ~2.6). The cultures were further incubated in 750 mL flasks with vented plugs at 37 °C on a rotary shaker at 250 rpm for 19 h.

All experiments were performed in biological triplicates, and the media were supplemented with 100 mg/L ampicillin to maintain plasmid stability when necessary.

2.3. Analytical Techniques

Fermentation samples were freed from biomass by centrifugation for 10 min at 15,000× *g* and the supernatants were used for further analysis.

The concentrations of organic acids and glucose in the culture media were measured by high-performance liquid chromatography using a Waters HPLC system (Waters, Milford, MA, USA). For the organic acid measurements, a Rezex ROA-Organic Acid H+ (8%) ion-exclusion column (300 mm × 7.8 mm, 8 μm, Phenomenex, Torrance, CA, USA) was used with detection at 210 nm. An aqueous solution of sulphuric acid (2.5 mM) was used as the solvent at a flow rate of 0.5 mL/min. For the glucose measurements, a Waters HPLC system equipped with a Waters 2414 refractive index detector and a Spherisorb-NH2 reversed-phase column (4.6 mm × 250 mm, 5 μm, Waters, Milford, MA, USA) was used. The mobile phase contained acetonitrile/water in a ratio of 75/25 (*v*/*v*) at a flow rate of 1.0 mL/min. Samples were identified by comparing the retention times with those of the corresponding standards.

3. Results and Discussion

3.1. Construction and Evaluation of an Engineered E. coli Strain Capable of the Aerobic Production of Malic Acid from Glucose

The *E. coli* strain MSG1.0 Δ*aceBAK* Δ*glcB* [28], designated as MAL 0 (Table 1), was selected as a chassis to construct a strain capable of producing malic acid from glucose under aerobic conditions. This strain was previously engineered to serve as a platform for the development of efficient producers of valuable 2-ketoglutarate derivatives. The competing pathways for acetyl-CoA and pyruvate utilisation leading to acetic acid, lactic acid, and ethanol formation were inactivated in the strain by the deletion of the *ackA*, *pta*, *poxB*, *ldhA*, and *adhE* genes, encoding key enzymes catalysing the respective reactions.

The increased intracellular availability of PEP for OAA biosynthesis was ensured in the strain due to PEP-independent glucose transport and phosphorylation resulting from the inactivation of the main *E. coli* glucose permease *ptsG* gene upon the enhanced expression of the *galP* and *glk* genes, which encode the H+-symporter of galactose and ATP-dependent glucokinase. The functioning of GS was precluded in the strain through the deletion of *aceBAK* operon genes, which encode isocitrate lyase, malate synthase A, and bifunctional kinase/phosphatase of isocitrate dehydrogenase, as well as the *glcB* gene encoding malate synthase G (Figure 1a).

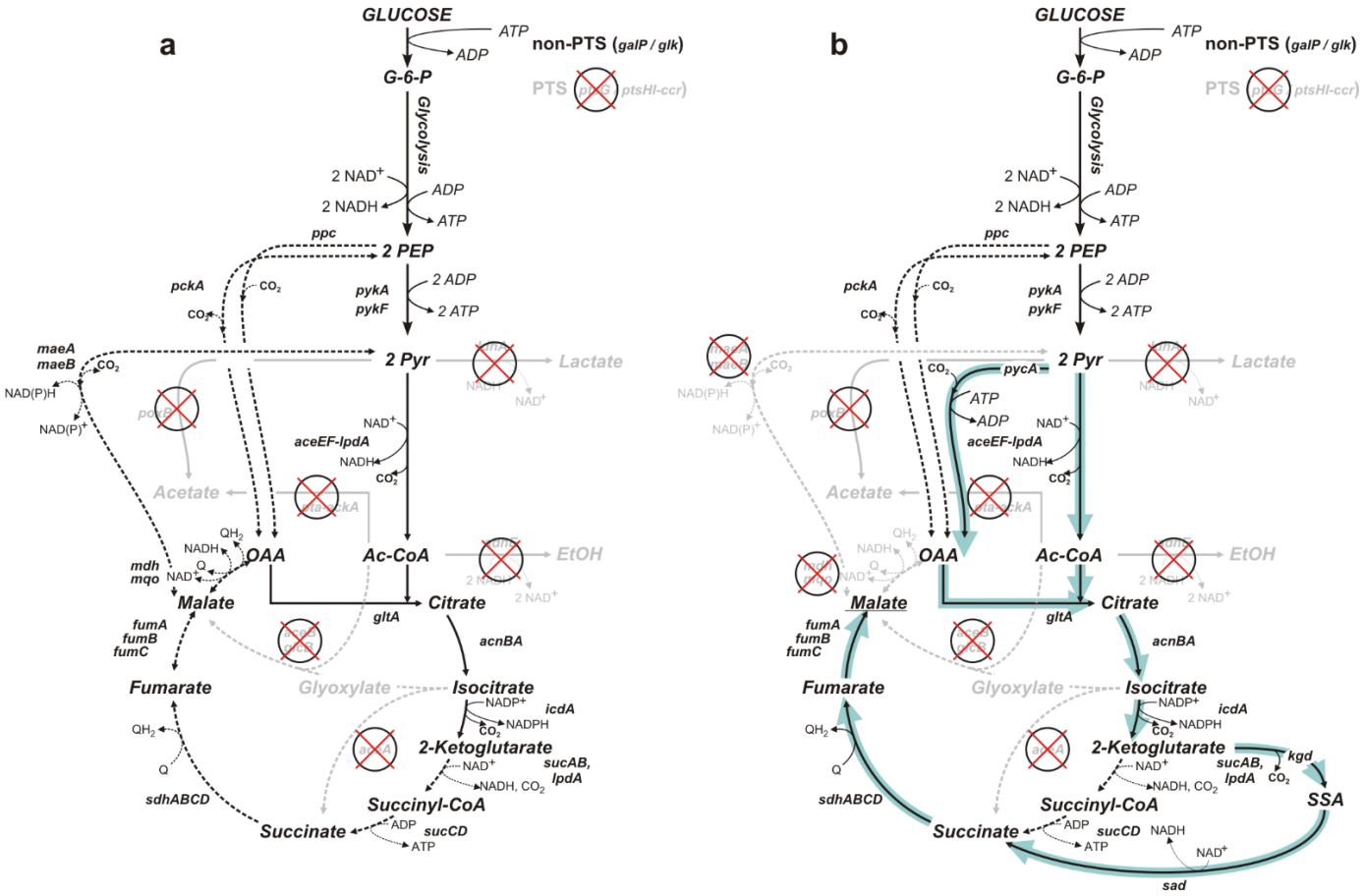

Figure 1. Central carbon metabolism in engineered *E. coli* strains under aerobic conditions with glucose as a carbon source. (**a**) The core strain MAL 0; (**b**) The best-performing malic acid-producing strain MAL 4MPA [pMW-*kgd*]. The enzymes are indicated by their gene names. Heterologous genes and enzymes are as follows: *kgd*, *M. tuberculosis* 2-ketoglutarate decarboxylase; *pycA*, *B. subtilis* pyruvate carboxylase. Abbreviations of key metabolites in the pathways are *Ac-CoA*, acetyl-CoA; G-6-P, glucose-6-phosphate; *OAA*, oxaloacetate; *PEP*, phosphoenolpyruvate; *Pyr*, pyruvate; *SSA*, succinate semialdehyde. The deleted genes are marked with crossed circles. Dotted arrows indicate reactions with decreased intensity. The reactions, as well as the genes of the respective enzymes, substrates, products, and cofactors that are absent or inactive in the constructed strains, are shown in grey. The reactions of the artificial pathway of malic acid biosynthesis, starting from pyruvate and including shunting of 2-ketoglutarate to succinic acid via intermediate SSA formation, which was engineered in the current study, are highlighted by wide blue arrows.

The design of the target malic acid-producing strain implied the formation of the end product through the pathway resembling the variant TCA cycle of myco- and cyanobacteria [30,31]. These bacteria lack 2-ketoglutarate dehydrogenase activity and are unable to convert 2-ketoglutarate to succinic acid via intermediate succinyl-CoA formation. The respective TCA cycle variant bypasses the 2-ketoglutarate dehydrogenase- and the succinyl-

CoA synthetase-mediated reactions and ensures the closing of the cycle resulting from the sequential action of 2-ketoglutarate decarboxylase, EC 4.1.1.71, catalysing the decarboxylation of 2-ketoglutarate with SSA formation, and succinate-semialdehyde dehydrogenase oxidising SSA to succinic acid. *E. coli* naturally possess two succinate-semialdehyde dehydrogenases, represented by both NAD^+-dependent Sad and $NADP^+$-dependent GabD. The expression of the NAD^+-dependent succinate-semialdehyde dehydrogenase gene, *sad*, is known to be markedly increased in *E. coli* in the presence of the appropriate substrate, assuming the participation of the enzyme in the cellular defence from the toxic effect of this reactive aldehyde [36]. Thus, the intracellular formation of SSA resulting from 2-ketoglutarate decarboxylation could serve as a signal for an adaptive response and ensure a rather high level of native cellular SSA-neutralising succinate-semialdehyde dehydrogenase activity. However, *E. coli* lack 2-ketoglutarate decarboxylase classified as EC 4.1.1.71 and cannot convert 2-ketoglutarate to SSA. Therefore, to allow the channelling of 2-ketoglutarate to succinic acid via intermediate SSA formation, the *Mycobacterium tuberculosis kgd* gene, encoding 2-ketoglutarate decarboxylase, was expressed in strain MAL 0 and its engineered derivatives (Figure 1b).

Upon the aerobic utilisation of glucose during growth in a complex nutrient medium, the strain MAL 0 [pMW-*kgd*] did not synthesise detectable amounts of malic acid, irrespective of the induction of *kgd* gene expression, secreting pyruvic, acetic, and succinic acids as the main products (Table 2).

Table 2. Molar yields of metabolites produced by the MAL 0 [pMW-*kgd*], MAL M [pMW-*kgd*], MAL 3M [pMW-*kgd*], MAL 4M [pMW-*kgd*], and MAL 4MP [pMW-*kgd*] strains during 8 h of aerobic glucose utilisation.

Strain	IPTG	Pyruvate	Acetate	Succinate	Fumarate	Malate	% C-Recovery [a]	DCW (g/L)
MAL 0	–	71.9 ± 1.6	15.7 ± 0.4	7.0 ± 0.2	n.d.	n.d.	46	4.18 ± 0.12
[pMW-*kgd*]	+	12.5 ± 0.3	24.8 ± 1.1	13.6 ± 0.6	n.d.	n.d.	24	4.90 ± 0.15
MAL M	–	98.0 ± 1.7	13.7 ± 0.5	5.2 ± 0.4	n.d.	n.d.	59	4.16 ± 0.10
[pMW-*kgd*]	+	5.8 ± 0.3	24.2 ± 0.9	4.0 ± 0.3	n.d.	n.d.	14	4.82 ± 0.16
MAL 3M	–	98.7 ± 1.5	12.0 ± 0.4	4.9 ± 0.4	n.d.	n.d.	58	4.20 ± 0.12
[pMW-*kgd*]	+	1.5 ± 0.1	20.7 ± 1.0	7.1 ± 0.6	n.d.	n.d.	13	4.96 ± 0.15
MAL 4M	–	159.6 ± 2.1	11.2 ± 0.5	2.9 ± 0.2	3.6 ± 0.2	12.5 ± 0.4	96	2.75 ± 0.07
[pMW-*kgd*]	+	135.9 ± 1.8	29.0 ± 1.2	6.6 ± 0.5	5.2 ± 0.2	14.9 ± 0.5	95	2.27 ± 0.06
MAL 4MP	–	62.3 ± 1.0	12.1 ± 0.6	6.5 ± 0.4	8.8 ± 0.3	35.1 ± 0.6	69	2.72 ± 0.08
[pMW-*kgd*]	+	3.8 ± 0.2	23.5 ± 1.2	16.9 ± 0.7	20.7 ± 0.8	55.9 ± 0.9	72	1.74 ± 0.04

Data are the means ± standard deviations of three replicates. Molar yields of the secreted metabolites are given in % per mole of consumed glucose (mol/mol, %). n.d.—not detected. [a]—Carbon recovery was calculated as the ratio of total moles of carbon in the end-products per moles of carbon in total glucose consumed and expressed on a percentage basis.

The carbon recovery, which was calculated based on the amounts of detected carboxylic acids secreted by the strain, reached the value of 46% in the absence of IPTG, the specific inducer of the expression of the *kgd* gene, in the medium. This indicated that without expression of the 2-ketoglutarate decarboxylase gene, the activity of the native oxidative TCA cycle in the strain was rather low, resulting in glucose overflow metabolism [37,38]. The corresponding effect was not so pronounced in the case of the strain MAL 0 [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase. The strain secreted markedly less pyruvic acid, apparently channelling most of it through an operational TCA cycle. The drop in the value of carbon recovery demonstrated by the strain to 24% supported the latter assumption since the multiple turnovers of the entire oxidative TCA cycle lead to considerable carbon loss via CO_2-dissimilating reactions. It should be noted that the observed secretion of acetic acid by the strain lacking phosphotransacetylase, acetate kinase, and pyruvate oxidase could have resulted, in this case, from the background activity of *E. coli* acyl-CoA thioesterases YciA and TesB. This suggested non-optimal action in the cells of the TCA cycle or its initial stages that led to the accumulation of excessive intracellular acetyl-CoA levels. Nevertheless, to ensure the formation of malic acid as the terminal product of aerobic utilisation of glucose

by the strain MAL 0 [pMW-*kgd*], the TCA cycle should be interrupted at the stage of the conversion of the respective intermediate to the gluconeogenic derivatives.

E. coli possesses several enzymes that can participate in the gluconeogenic conversion of malic acid to OAA and pyruvic acid. These are malate dehydrogenase, Mdh, and malate:quinone oxidoreductase, Mqo, which oxidise malate to generate OAA, using NAD^+ or quinone as an electron acceptor, as well as NAD^+- and $NADP^+$-dependent malic enzymes, MaeA and MaeB, correspondingly, which oxidatively decarboxylate malic acid to pyruvate [39]. Malate dehydrogenase serves in *E. coli* as the main enzyme catalysing the terminal step of the TCA cycle, while the contribution of malate:quinone oxidoreductase to the oxidation of malic acid to OAA in this bacterium appears to be insignificant [40]. Moreover, the expression of the *mdh* gene is activated by CRP-cAMP [41,42] and, therefore, could increase in PTS-negative strains with a constitutively active adenylate cyclase. On the other hand, malic enzymes are known to be subjected to allosteric inhibition by OAA, and their activity could be rather low in the strains with intact malate dehydrogenase [43]. Therefore, to prevent the unwanted drainage of the target product formed in the TCA cycle via malate-consuming reactions, the *mdh* gene encoding malate dehydrogenase was initially deleted in the strain MAL 0 [pMW-*kgd*].

The metabolite profiles formed by the resulting strain MAL M [pMW-*kgd*] during aerobic utilisation of glucose were similar to those of the parent strain MAL 0 [pMW-*kgd*], and malic acid was absent among the secreted products (Table 2 and Figure 2a,b). Nevertheless, the yield of pyruvic acid secreted by the strain MAL M [pMW-*kgd*] without induction of *kgd* gene expression slightly increased compared to the strain MAL 0 [pMW-*kgd*] (Table 2). This observation, along with the increased carbon recovery, could indicate the further retardation of the depressed native TCA cycle in the strain MAL 0 [pMW-*kgd*] due to the inactivation of malate dehydrogenase. On the contrary, in the case of the induced expression of the heterologous 2-ketoglutarate decarboxylase, the yield of pyruvic acid and the value of carbon recovery demonstrated by the Mdh-deficient strain were lower than those of the parent strain. This could suggest the relative increase in the carbon flux through the modified TCA cycle due to the derepression of malic enzymes resulting from the decrease in the intracellular free OAA level. Therefore, the genes *maeA* and *maeB*, encoding malic enzymes, were then deleted in the strain MAL M [pMW-*kgd*].

During aerobic utilisation of glucose, the strain MAL 3M [pMW-*kgd*], devoid of malate dehydrogenase and both malic enzymes, formed metabolite profiles nearly identical to those of the parent strain MAL M [pMW-*kgd*] (Figure 2a,b), and the yields of the respective products were almost unchanged (Table 2). Moreover, the strain still did not secrete malic acid either with or without induction of the expression of the *kgd* gene. Since the metabolite production by MAL 3M [pMW-*kgd*] was not affected by the absence of *maeA* and *maeB* genes, it could be supposed that the malic enzymes were not responsible for the intracellular malate leakage in the parental strain MAL 1M [pMW-*kgd*] and that malate:quinone oxidoreductase was the primary enzyme oxidising malic acid in the engineered Mdh-deficient strains. However, it has previously been shown that the concomitant inactivation in *E. coli* of only malate dehydrogenase and malate:quinone oxidoreductase is insufficient to ensure the efficient TCA cycle-dependent conversion of glucose to malic acid, and the activity of the malic enzymes prevents the accumulation of the target product [25]. Therefore, to eliminate potential pathways of gluconeogenic malate consumption completely, the *mqo* gene was further deleted in the strain MAL 3M [pMW-*kgd*] with the inactivated malic enzymes rather than in MAL 1M [pMW-*kgd*].

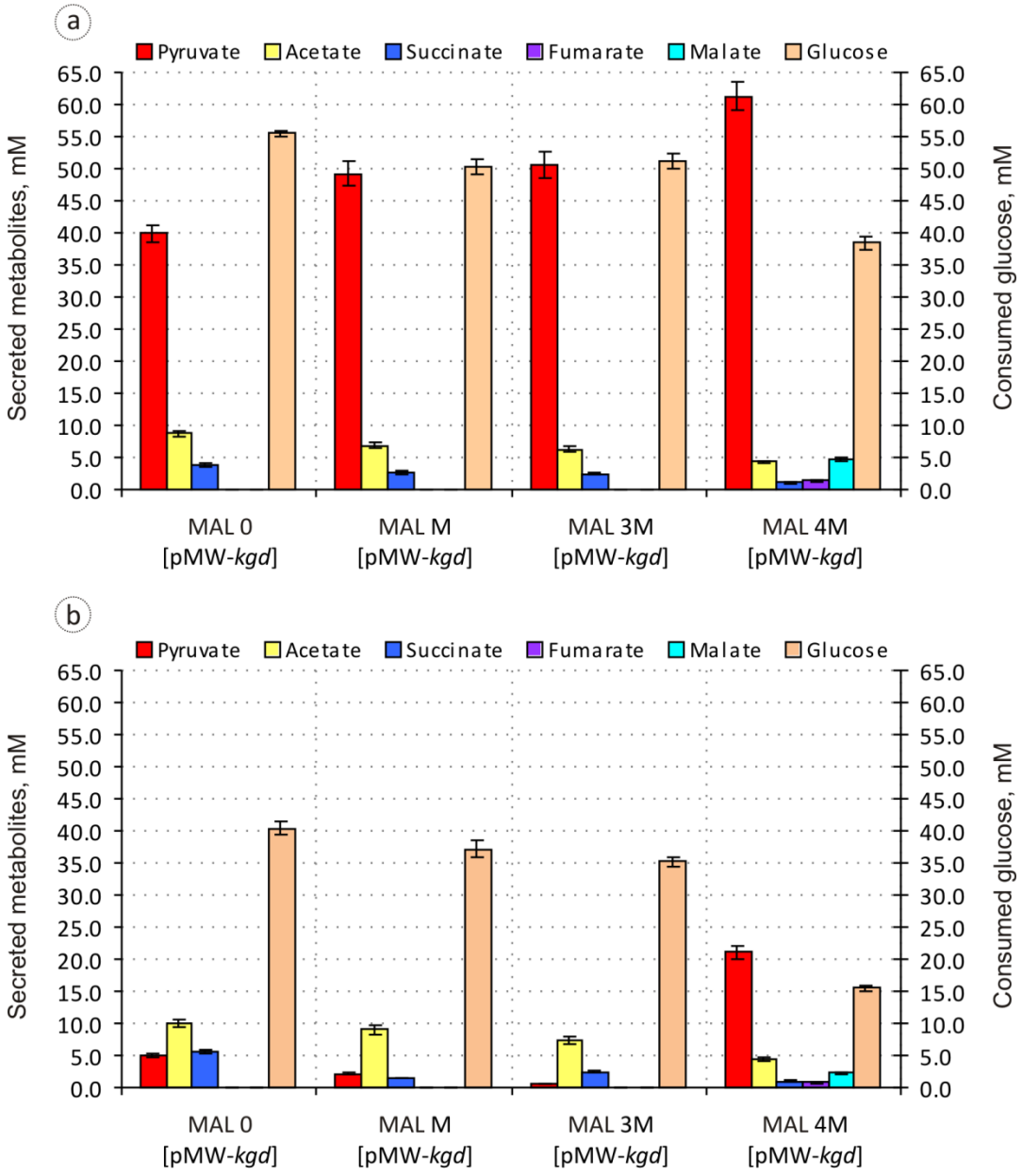

Figure 2. Metabolite production and substrate consumption by the strains MAL 0 [pMW-*kgd*], MAL M [pMW-*kgd*], MAL 3M [pMW-*kgd*], and MAL 4M [pMW-*kgd*] during 8 h of aerobic glucose utilisation in the absence (**a**) or presence (**b**) of IPTG.

The resulting strain MAL 4M [pMW-*kgd*] synthesised malic acid from glucose aerobically both in the absence and presence of IPTG in the medium, converting most of the consumed glucose to pyruvic acid (Table 2 and Figure 2a,b). At the same time, the carbon recovery values increased to 96 and 95%, indicating that the turnover of the TCA cycle was almost completely terminated in the strain. As a result, the induced expression of the 2-ketoglutarate decarboxylase gene led to only a minor increase in the yield of the target product from 12.5 to 14.9%. The decline in the replenishment of OAA via the TCA cycle limited the availability of the corresponding intermediate not only for the initiation of new rounds of the cycle and the biosynthesis of malic acid but also for the biosynthesis of aspartate-family amino acids. The latter led to a drastic decrease in biomass formation by the strain since, during the growth in rich or complex LB-like media, *E. coli* completely consumes available amino acids at the early stages of growth [44,45] and then needs to synthesise corresponding building blocks intracellularly to maintain further proliferation.

This effect was more pronounced in the case of the induced *kgd* gene expression. It was most likely caused by an additional decline in the intracellular availability of succinyl-CoA for the biosynthesis of the methionine, lysine, and the cell wall components due to the diversion of 2-ketoglutarate towards succinate via the intermediate SSA formation instead of succinyl-CoA. Similar bypassing of the succinyl-CoA synthetase-catalysed reaction during 2-ketoglutarate-dependent hydroxylation of proline coupled with the formation of succinic acid has recently been shown to inhibit the growth of the engineered *E. coli* strains [46,47]. The retardation in biomass formation by the strain MAL 4M [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase led, in turn, to a decrease in glucose consumption (Figure 2b).

Nevertheless, despite the negative effect of the introduced genetic modifications on the biomass formation and substrate consumption by MAL 4M [pMW-*kgd*], the strain was able to aerobically convert glucose to malic acid both through the native and modified TCA cycle, demonstrating a potential for the further optimisation of its biosynthetic characteristics.

3.2. Improvement of the Engineered Malic Acid-Producing Strain and Evaluation of Its Biosynthetic Potential

The observed pronounced secretion of pyruvic acid by MAL 4M [pMW-*kgd*] and the low activity in the strain of the truncated TCA cycle was most likely caused by the insufficient intracellular generation of OAA from the glycolytic precursors. *E. coli* cells possess two enzymes responsible for the anaplerotic formation of OAA. These are PEP-carboxylating phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase [39]. The activity of anaplerotic pyruvate carboxylase is absent in *E. coli*. Phosphoenolpyruvate carboxylase irreversibly carboxylates the corresponding substrate to form OAA, while the reaction catalysed by phosphoenolpyruvate carboxykinase is reversible. Consequently, during the utilisation of glycolytic substrates, phosphoenolpyruvate carboxylase serves in *E. coli* as the main anaplerotic enzyme [39]. However, phosphoenolpyruvate carboxylase competes for the common precursor metabolite, PEP, with glycolytic pyruvate kinases synthesising pyruvic acid, which is required for further acetyl-CoA formation. Thus, the presence of heterologous pyruvate carboxylase in the engineered *E. coli* strains could favour the production of malic acid via the oxidative TCA cycle, providing more flexibility to anaplerotic processes and redirecting excess pyruvate towards the OAA formation. It should be noted that in PTS-negative strains, namely pyruvate kinases are primarily responsible for the formation of pyruvic acid from PEP during utilisation of glucose instead of a PEP-dependent system of glucose transport and phosphorylation. The overexpression of phosphoenolpyruvate carboxylase in the PTS-negative strain MAL 4M [pMW-*kgd*] could therefore cause limitation in the relative intracellular acetyl-CoA availability preventing the proper functioning of the TCA cycle. Taking into account these considerations, we decided to introduce heterologous pyruvate carboxylase in the engineered strain to ensure the efficient formation of OAA instead of the overexpression of the malate-insensitive variant of the native phosphoenolpyruvate carboxylase that was performed previously [25]. Accordingly, the *Bacillus subtilis pycA* gene, encoding pyruvate carboxylase, was integrated into the chromosome of the strain MAL 4M [pMW-*kgd*] under the control of the lambda phage P_L promoter.

Without induction of *kgd* gene expression, the resulting strain MAL 4MP [pMW-*kgd*] converted glucose to malic acid with a yield of 35.1% (Table 2). The yield of pyruvic acid dropped more than 2 times to 62.3% (Table 2). The consumption of the carbohydrate substrate by the strain slightly improved (Figures 2a and 3a), while the carbon recovery decreased to 69% (Table 2).

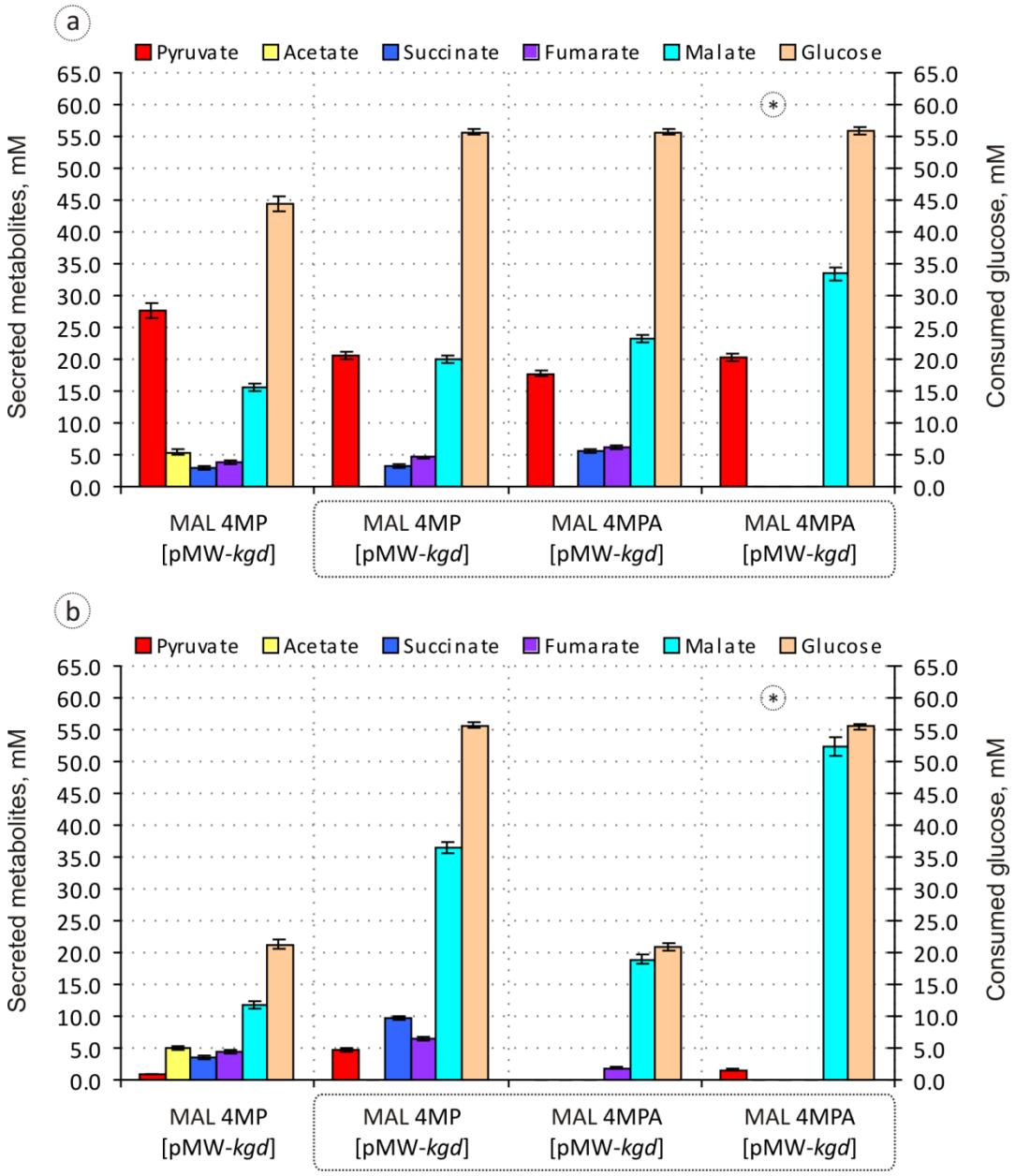

Figure 3. Metabolite production and substrate consumption by the strains MAL 4MP [pMW-*kgd*] and MAL 4MPA [pMW-*kgd*] strains during 8 h and 19 h (marked with dotted square) of aerobic glucose utilisation in the absence (**a**) or presence (**b**) of IPTG. The parameters for the strain MAL 4MPA [pMW-*kgd*] functioning in "biocatalyst" mode are marked with asterisks.

These results indicated that the truncated native TCA cycle was activated in the strain resulting from the action of pyruvate carboxylase, which supplied it with an extra OAA. However, this activation was incomplete. Indeed, when accounting for the amounts of CO_2 generated during the synthesis of succinic, fumaric, and malic acids through the reactions of the oxidative TCA cycle in the total sum of the products formed by the strain, the value of carbon recovery reached only 86%, indicating a partial carbon loss during the glucose catabolism. The corresponding loss of carbon was apparently caused by the emergence in the strain of the glutamate overflow [44,48] resulting from the inefficient operation of the TCA cycle due to the insufficient activity of 2-ketoglutarate dehydrogenase. Glutamate overflow is a metabolic effect that occurs when the carbon flux through the oxidative TCA cycle exceeds the catalytic capacity of 2-ketoglutarate dehydrogenase, leading to

the formation of glutamic acid from excess 2-ketoglutarate instead of its conversion to succinyl-CoA. As mentioned above, the expression of 2-ketoglutarate dehydrogenase in *E. coli* during aerobic growth in glucose-containing media is rather low [26].

Upon the expression of the 2-ketoglutarate decarboxylase gene, the strain MAL 4MP [pMW-*kgd*] synthesised malic acid as the main product of aerobic utilisation of glucose (Figure 3b) with a yield that increased to ~56% (Table 2). The secretion of pyruvic acid by the strain almost ceased, while succinic and fumaric acids, along with acetic acid, accumulated as the main by-products (Figure 3b and Table 2). The profile of metabolites secreted by the strain was therefore shifted towards the preferred formation of four-carbon intermediates of the TCA cycle instead of three- and two-carbon acetic and pyruvic acids originating from glycolysis (Figure 3b). Consequently, the proportion of four-carbon dicarboxylates among the products formed by the strain MAL 4MP [pMW-*kgd*] increased from 40% to 77% resulting from the *kgd* gene expression. This indicated the markedly intensified carbon flux through the truncated TCA cycle in the strain MAL 4MP [pMW-*kgd*] expressing both pyruvate carboxylase and 2-ketoglutarate decarboxylase and the diminished contribution of glucose overflow metabolism to the formation of the end-products of carbohydrate substrate utilisation. Although the carbon recovery, which was calculated based on the amounts of products secreted by the strain in the culture medium, constituted only 72% (Table 2), the value, calculated by taking into account the amounts of CO_2 generated during the synthesis of intermediates of the oxidative TCA cycle in the total sum of products formed by the strain, was almost 100%. The above observation suggested that glutamate overflow was also successfully overcome in the strain MAL 4MP [pMW-*kgd*] with the active 2-ketoglutarate decarboxylase due to the efficient channelling of 2-ketoglutarate towards the synthesis of succinic acid via the intermediate generation of SSA followed by the further formation of malic acid through the downstream reactions of the modified TCA cycle.

Nevertheless, despite the strain being able to convert glucose to malic acid via the modified TCA cycle with relatively high efficiency, succinic acid synthesised in the strain resulting from the action of the artificial 2-ketoglutarate decarboxylase-mediated bypass was incompletely converted to the target product. The yields of succinic and fumaric acids formed by the strain MAL 4MP [pMW-*kgd*] upon the induction of the expression of the 2-ketoglutarate decarboxylase gene were even higher than those in the case of the absence of the induction. Moreover, the strain did not consume all the glucose available. In this regard, it should be noted that *E. coli* can utilise both succinate and fumarate as carbon sources under aerobic conditions. Thus, the problem of incomplete substrate consumption and the by-production of succinic and fumaric acids by the engineered malic acid-producing strain can be overcome, in particular, by prolonging the cultivation process. In this case, succinic and fumaric acids that accumulate in the medium will be gradually re-consumed by the strain and converted to the target product via the succinate dehydrogenase- and fumarase-catalysed reactions of the truncated oxidative TCA cycle.

The effect of prolonged cultivation on aerobic fumaric acid production from glucose by the strain MAL 4MP [pMW-*kgd*] was assessed for both induced and uninduced cultures. Upon prolongation of the cultivation period to 19 h, the strain MAL 4MP [pMW-*kgd*] consumed glucose completely irrespective of the induction of *kgd* gene expression (Figure 3a,b). Without induction, the strain synthesised succinic, fumaric, and malic acids with yield values similar to those demonstrated in 8 h of cultivation (Tables 2 and 3), while the yield of pyruvic acid decreased 1.7-fold, and acetic acid was absent among the end-products formed by the strain (Table 3 and Figure 3a). This observation implied that part of the pyruvic acid and all of the acetic acid were re-assimilated from the medium by the strain after glucose exhaustion in accordance with the well-known effect of the acetate switch [49]. While acetic acid was apparently utilised by acetyl-CoA synthetase Asc to form acetyl-CoA and pyruvic acid was converted to OAA by pyruvate carboxylase, the almost unchanged yields of four-carbon dicarboxylates synthesised by the strain, along with the decreased carbon recovery, suggested that both of these metabolites, replenishing the pool of key TCA cycle precursors, primarily contributed to glutamate overflow.

Table 3. Molar yields of metabolites produced by the MAL 4MP [pMW-*kgd*] and MAL 4MPA [pMW-*kgd*] strains during 19 h of aerobic glucose utilisation.

Strain	IPTG	Pyruvate	Acetate	Succinate	Fumarate	Malate	% C-Recovery [a]	DCW (g/L)
MAL 4MP	–	36.9 ± 0.7	n.d.	5.9 ± 0.4	8.3 ± 0.3	35.7 ± 0.7	52	2.74 ± 0.08
[pMW-*kgd*]	+	8.6 ± 0.4	n.d.	17.2 ± 0.7	11.5 ± 0.5	65.5 ± 1.2	67	2.76 ± 0.09
MAL 4MPA	–	31.8 ± 0.6	n.d.	10.1 ± 0.5	11.1 ± 0.6	41.8 ± 0.7	58	1.32 ± 0.03
[pMW-*kgd*]	+	n.d.	n.d.	n.d.	9.1 ± 0.2	90.4 ± 1.5	67	0.58 ± 0.02

Data are the means ± standard deviations of three replicates. Molar yields of the secreted metabolites are given in % per mole of consumed glucose (mol/mol, %). n.d.—not detected. [a]—Carbon recovery was calculated as the ratio of total moles of carbon in the end-products per moles of carbon in total glucose consumed and expressed on a percentage basis.

On the other hand, during 19 h of cultivation, the strain MAL 4MP [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase synthesised malic acid with a yield that increased from 55.9% to 65.5 % along with the concomitant and nearly proportional decrease in the yield of fumaric acid from 20.7% to 11.5% (Tables 2 and 3). This could indicate that the strain partially re-assimilated secreted fumaric acid and successfully converted it to the target product. The strain did not accumulate acetic acid but secreted more pyruvic acid compared to 8 h of incubation (Table 3). Contrary to the uninduced culture, acetyl-CoA derived from acetic acid was presumably utilised by the strain to support cellular growth. The relatively decreased carbon recovery and improved biomass formation demonstrated by the strain (Table 3) supported this assumption. Increased secretion of pyruvic acid, in this case, was most likely caused by an elevated carbon flux through glycolysis due to the intensification of substrate consumption resulting in a relative increase in the residual glucose overflow metabolism. At the same time, the yield of succinic acid synthesised by the strain MAL 4MP [pMW-*kgd*] with the active 2-ketoglutarate decarboxylase was not affected by the prolongation of the cultivation period. Thus, the strain was unable to utilise succinic acid secreted into the medium and channel it to malic acid even after the depletion of glucose, suggesting the limitation in the carbon flux through the terminal reactions of the truncated TCA cycle. Taking into account the observed re-assimilation by the strain of synthesised fumaric acid, this limitation could primarily be due to the insufficiency of the activity of succinate dehydrogenase caused by the overload of the respiratory electron transfer chain.

It is known that *E. coli*, like the mitochondria of eukaryotic organisms, is subjected to respiratory control [50] when ATP is not produced through oxidative phosphorylation until enough ADP is available. The ADP level increases when ATP is consumed, and the demand of non- or poorly growing cultures for ATP is decreased, thus limiting the relative intracellular ADP availability. The strain MAL 4MP [pMW-*kgd*] demonstrated notable retardation in biomass formation (Tables 2 and 3) that could lead to the accumulation of excessive intracellular levels of ATP formed both glycolytically and via oxidative phosphorylation. The consequent decline in ATP generation by (F_1F_0) H^+-ATP synthase could cause an increase in the membrane proton gradient, inhibiting cytochromes *bo* and *bd* and resulting in the limitation in the relative availability of quinones required for the action of succinate dehydrogenase. Therefore, the biosynthetic characteristics of the strain could benefit from the enforcement of ATP hydrolysis.

The intracellular ATP supply can be decreased in *E. coli* using several approaches that are primarily based on the manipulation of components of the (F_1F_0) H^+-ATP synthase complex in order to prevent oxidative phosphorylation and/or enhance ATP hydrolysis. The overexpression of the genes encoding the components of the cytoplasmic F_1 subunit comprising the catalytic site will promote ATP hydrolysis. Conversely, the inactivation of the genes that code for the components of the membrane-bound F_0 subunit, which forms the proton channel, will block ATP production via oxidative phosphorylation with a concomitant increase in ATPase activity. A similar effect will be achieved due to the uncoupling of the subunits resulting from the deletion of the genes encoding components that are responsible for the assembly of the complex. Although these approaches have

previously been applied to promote glycolysis in the strains engineered for the production of pyruvic and acetic acids [51–53], they could also be used for the derepression of the electron transfer chain and associated succinate dehydrogenase. Thus, to stimulate ATP hydrolysis by the (F_1)-ATPase while uncoupling the proton channelling through the F0 component from the subsequent ATP generation, the genes *atpF* and *atpH*, which code for the delta and b subunits involved in the binding of the respective parts of (F_1F_0) H^+-ATP synthase complex, were deleted in the strain MAL 4MP [pMW-*kgd*].

Without induction of *kgd* gene expression, the resulting strain MAL 4MPA [pMW-*kgd*] synthesised malic acid from glucose with a yield of 41.8%, which was only slightly higher than that demonstrated by the parent strain (Table 3). The overall yield of four-carbon dicarboxylates produced by the strain was also increased insignificantly and reached 60% versus 49.9% in the case of MAL 4MP [pMW-*kgd*]. Thus, the enforced ATP hydrolysis had little impact on the biosynthetic performance of the engineered strain synthesising malic acid through the suboptimally functioning native TCA cycle. However, the decline in the intracellular availability of ATP led to a two-fold decrease in biomass accumulation by the strain (Table 3).

The activation of the modified TCA cycle in the ATP synthase deficient MAL 4MPA [pMW-*kgd*] due to the expression of the 2-ketoglutarate decarboxylase gene led to dramatic changes in the metabolite production, substrate consumption, and biomass formation by the strain. The yield of malic acid produced by the strain rose almost 1.4 times compared to the parent strain and more than two times compared to the uninduced culture, reaching 90.4% (Table 3). The strain did not secrete pyruvic, acetic, and succinic acids, accumulating minute amounts of fumaric acid as the only by-product formed. This indicated that the electron transfer chain was successfully derepressed in the strain as a result of the enforcement of ATP hydrolysis, enabling efficient operation of the entire modified TCA cycle. Nevertheless, although the strain converted the substrate to the target product almost quantitatively, the glucose consumption by the strain dropped nearly 2.7 times concomitantly with the drastic decrease in biomass formation (Figure 3b, Table 3). It has previously been shown that the deletion of *atpFH* genes in pyruvate-producing *E. coli* strains decreases the growth rate slightly but promotes the glycolytic flux [51,52]. Thus, the decrease in glucose consumption by the strain MAL 4MPA [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase was apparently caused by the growth retardation due to the 2-ketoglutarate decarboxylase-mediated bypassing of the reaction catalysed by succinyl-CoA synthetase. During the conversion of succinyl-CoA to succinic acid, succinyl-CoA synthetase generates ATP via substrate-level phosphorylation. Therefore, the avoiding of the corresponding reaction in the ATP synthase deficient strain resulted in an additional limitation in ATP availability for the cellular anabolic pathways participating in proliferation.

Since the namely impaired biomass formation by the MAL 4MPA [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase led to a decrease in substrate consumption, limiting the accumulation of the target product, the biosynthetic potential of the engineered malic acid-producing strain was also evaluated during a two-stage fermentation. This process comprised a growth phase for biomass accumulation and a subsequent production phase. An increased amount of biomass of the strain MAL 4MPA [pMW-*kgd*] was obtained at the first stage, and then, during the second stage, which was uncoupled from growth, the producing strain performed as a whole-cell biocatalyst, efficiently converting substrate to the target product.

The yield of malic acid synthesised from glucose by the uninduced culture of the strain MAL 4MPA [pMW-*kgd*] functioning in biocatalyst mode increased compared to that achieved upon prolonged growth and reached ~60% (Tables 3 and 4). It was caused by the cessation of accumulation by the strain of succinic and fumaric acids, which were absent among the detected products of glucose utilisation and were apparently completely re-assimilated from the medium, after the exhaustion of carbohydrate substrate, by an increased cellular mass. Nevertheless, the secretion of pyruvic acid by the strain continued

and carbon recovery remained incomplete, reaffirming the suboptimal functioning of the native TCA cycle.

Table 4. Molar yields of metabolites produced by the strain MAL 4MPA [pMW-*kgd*] during functioning in biocatalyst mode.

Strain	IPTG	Pyruvate	Acetate	Succinate	Fumarate	Malate	% C-Recovery [a]	DCW (g/L)
MAL 4MPA [pMW-*kgd*]	–	36.2 ± 0.8	n.d.	n.d.	n.d.	59.9 ± 1.3	58	2.56 ± 0.07
	+	2.8 ± 0.1	n.d.	n.d.	n.d.	93.7 ± 1.8	64	2.58 ± 0.05

Data are the means ± standard deviations of three replicates. Molar yields of the secreted metabolites are given in % per mole of consumed glucose (mol/mol, %). n.d.—not detected. [a]—Carbon recovery was calculated as the ratio of total moles of carbon in the end-products per moles of carbon in total glucose consumed and expressed on a percentage basis.

Activation of the modified TCA cycle by enabling shunting of 2-ketoglutarate to succinic acid due to the induction of *kgd* gene expression led to an elevation in the yield of malic acid synthesised from glucose by MAL 4MPA [pMW-*kgd*] performing as a biocatalyst up to the value of 93.7%, which was comparable to that demonstrated by the strain expressing 2-ketoglutarate decarboxylase in the case of growth prolongation. However, the respective yield value was achieved along with the complete consumption of available glucose by the engineered strain. The strain accumulated no other intermediates of the TCA cycle besides malic acid, secreting trace amounts of pyruvic acid as the only by-product detected. The carbon recovery, which was calculated using the amounts of secreted products, was almost unchanged (Tables 3 and 4), and the value calculated accounting for the CO_2 emission retained as high as nearly 100%. These results indicated that the engineered malic acid-producing strain MAL 4MPA [pMW-*kgd*] could serve as an efficient whole-cell biocatalyst converting glucose to the target product almost quantitatively. Moreover, its biosynthetic performance may be further improved by both the optimisation of the cultivation conditions and the additional metabolic engineering interventions.

Indeed, the observed residual secretion of pyruvic acid by the strain could be caused by either the insufficiency of pyruvate carboxylase activity or the limitations in the availability of dissolved bicarbonate required for OAA formation. The insufficiency of pyruvate carboxylase may be overcome by the integration of the additional copies of the *pycA* gene into the chromosome of the strain, whereas the cellular demand for bicarbonate may be satisfied by increasing the concentration of HCO_3^- ions in the medium. Alternatively, pyruvate carboxylase with a low K_m for HCO_3^- may be used along with the overexpression of carbonic anhydrase.

It should be noted that the specific productivity of malic acid and specific rate of glucose consumption demonstrated by the strain MAL 4MPA [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase and functioning in biocatalyst mode constituted 0.143 g/g DCW/h and 0.204 g/g DCW/h, which were lower than those achieved during growth-coupled production (0.230 g/g DCW/h and 0.342 g/g DCW/h). Thus, the consumption of glucose and the accumulation of the target product by the biocatalytically performing strain appeared to be restricted by amounts of the substrate (55 mM) added to the medium and may improve upon its excess.

4. Conclusions

We metabolically engineered *E. coli* to efficiently convert glucose to malic acid aerobically through the modified oxidative TCA cycle, implying artificial channelling of 2-ketoglutarate to SSA and then to succinic acid in a manner analogous to that of the variant TCA cycle of myco- and cyanobacteria. The yield of the target product reached 0.65 mol/mol and was further improved to 0.94 mol/mol by the derepression in the strain of the electron transfer chain and succinate dehydrogenase resulting from the enforcement of ATP hydrolysis. The respective value amounted to 94% of the theoretical maximum and exceeded the best value, 0.85 mol/mol, reported previously for an *E. coli* strain engineered for aerobic production of malic acid. While the current work represents a proof-of-concept

study, the implemented strategy enabling almost quantitative conversion of substrate to target product by the directly engineered *E. coli* strain provides a framework for the further development of highly efficient strains and processes for the bio-based production of malic acid from renewable raw materials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8120738/s1, Table S1: Primers used in the study.

Author Contributions: Conceptualisation, A.Y.G. and A.Y.S.; methodology, A.Y.G., A.A.S., and A.Y.S.; validation, N.V.K., A.A.S., and A.Y.S.; formal analysis, A.Y.G. and A.Y.S.; investigation, A.A.S., N.V.K., and A.Y.S.; data curation, A.Y.G. and A.Y.S.; writing—original draft preparation, A.Y.G. and A.Y.S.; writing—review and editing, A.Y.G., A.Y.S., and V.G.D.; supervision, V.G.D.; project administration, A.Y.G.; funding acquisition, A.Y.G. All authors have read and agreed to the published version of the manuscript.

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