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REVIEW ARTICLE

Microbial biosynthesis and secretion of l-malic acid and its applications

Zhe Chi, Zhi-Peng Wang, Guang-Yuan Wang, Ibrar Khan, and Zhen-Ming Chi

UNESCO Chinese Center of Marine Biotechnology, Ocean University of China, Qingdao, China

Abstract

L-Malic acid has many uses in food, beverage, pharmaceutical, chemical and medical industries. It can be produced by one-step fermentation, enzymatic transformation of fumaric acid to L-malate and acid hydrolysis of polymalic acid. However, the process for one-step fermentation is preferred as it has many advantages over any other process. The pathways of L-malic acid biosynthesis in microorganisms are partially clear and three metabolic pathways including non-oxidative pathway, oxidative pathway and glyoxylate cycle for the production of L-malic acid from glucose have been identified. Usually, high levels of L-malate are produced under the nitrogen starvation conditions, L-malate, as a calcium salt, is secreted from microbial cells and $CaCO_3$ can play an important role in calcium malate biosynthesis and regulation. However, it is still unclear how it is secreted into the medium. To enhance L-malate biosynthesis and secretion by microbial cells, it is very important to study the mechanisms of L-malic acid biosynthesis and secretion at enzymatic and molecular levels.

Keywords

Application, biosynthesis, fumurase, L-malic acid, one-step fermentation secretion

History

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Introduction

Malic acid is a four-carbon dicarboxylic acid and an intermediate in the tricarboxylic acid (TCA) cycle. It has many applications in the beverage and food industry as an acidulant and flavor enhancer, and in metal cleaning, textile finishing, pharmaceuticals and agriculture. In the chemical industries, it is also used as a feedstock for chemical synthesis of polymalic acid (PMA). It can be synthesized by chemical means through the hydration of fumaric acid under high temperature and pressure, yielding a racemic mixture of D- and L-malate (Goldberg et al., 2006) or by enzymatic ways that transform fumaric acid to L-malate using the purified fumarase and the microbial cells containing fumarase (Presecki & Vasic-Racki, 2005). However, these processes have many disadvantages. In recent years, L-malic acid production from glucose by one-step fermentation has received increasing attention and many microorganisms have been found to be able to produce L-malate as it can be produced from sustainable and eco-friendly sugars, not from petroleum-based feedstocks where the source is being depleted and price is increasing. Because L-malic acid is an intermediate in the TCA cycle, it is partially clear how it is synthesized in microbial cells. At present, it is significant to screen microbial strains which can actively transform glucose to high levels of L-malic acid and can secrete it into medium. In the last 2 years, L-malic acid also has been produced via acid hydrolysis of PMA which is secreted by PMA-producing fungi (Zhang et al., 2011; Zou et al., 2013).

In this review article, our main interests are focused on L-malate-producing microorganisms, pathways of L-malate biosynthesis, secretion of L-malate and industrial applications of L-malate.

L-malate-producing microorganisms

Although malate and calcium malate can be extracted from fruit and eggshells, these processes are not economical because fruit juices comprise less than 1.0% L-malate (Tsao et al., 1999) and extraction from eggshells requires high energy-consumption, low extraction rates, a complicated operation, high cost and heavy pollution (Lin et al., 2012). In fact, L-malic acid can be produced from pyruvic acid via oxaloacetic acid by a one-step fermentation or is produced via the conversion of fumaric acid under catalysis of fumarase or is produced through acid hydrolysis of PMA.

There are three microbial groups which can synthesize L-malic acid, one of them synthesizes L-malic acid from glucose by a one-step fermentation, the other transforms fumaric acid to L-malic acid using pure fumarase or fumarase in its cells and the third group synthesizes PMA which can be hydrolyzed into L-malic acid.

L-malic acid production by one-step fermentation

Several native strains and metabolically engineered strains were found to be able to produce L-malic acid. For example, the accumulation and excretion of L-malic acid, and to a lesser extent succinic and fumaric acids, by Aspergillus flavus occur under aerobic conditions in a medium containing a high

Address for correspondence: Dr Professor Zhen-Ming Chi, UNESCO Chinese Center of Marine Biotechnology, Ocean University of China, Yushan Road, No. 5, Qingdao, China. Tel/Fax: 0086-532-82032266. E-mail: zhenming@sdu.edu.cn

glucose concentration, a limiting amount of nitrogen and a neutralizing agent ($CaCO_3$) and malate dehydrogenase is essential for its L-malic acid production and accumulation. However, this one-step process is not applied in actual production due to the strain potential aflatoxin production capacity (Battat et al., 1991). A maximal amount of 113 g of L-malic acid per liter and a productivity of 0.59/l/h were achieved after incubation of *A. flavus* for 6–8 days (Battat et al., 1991). In addition to *A. flavus*, many other species of *Aspergillus*, including *Aspergillus niger* ATCC 9029, *A. niger* ATCC 9142, *A. niger* ATCC 10577 were able to produce malic acid from glucose as a carbon source. For example, the highest malic acid of 17 g/l was produced from thin stillage by *A. niger* ATCC 9142 and the highest malic acid yield was 0.8 g/g of consumed glucose. Thus, thin stillage has the potential to act as a substrate for the commercial production of food-grade malic acid by the *A. niger* strains (West, 2011). *A. oryzae* NRRL3488 cultivated in laboratory-scale fermentors resulted in 30.27 ± 1.05 g/l of malic acid (Knuf et al., 2013). *A. oryzae* NRRL 3488 overexpressing the native cytosolic alleles of pyruvate carboxylase, malate dehydrogenase and a native C4-dicarboxylate transporter achieved a malate titer of 154 g/l in 164 h, a productivity of 0.94 g/l/h and a yield of 1.38 mol/mol of glucose (69% of the theoretical maximum) (Brown et al., 2013). However, succinic acid (13 g/l), fumaric acid (0.6 g/l) and citric acid (6 g/l) were simultaneously produced by the genetically engineered *A. oryzae* NRRL 3488. At the same time, *A. oryzae* NRRL 3488 overexpressing the native cytosolic alleles of pyruvate carboxylase, malate dehydrogenase and a native C4-dicarboxylate transporter also was able to transform xylose and mixture of glucose and xylose into malate. However, the molar yield of malate on xylose fell below the value of the wild type on glucose (Knuf et al., 2014). It was also found that at 35 °C, up to 42 g/l malic acid was produced by *A. oryzae* DSM 1863 in a 168-h batch process with fumaric acid as a by-product. When this mold was grown in the mixture of xylose or glycerol with a carbon-to-nitrogen (C/N) ratio of 200:1, both organic acids were produced but the formation of malic acid was decreased to 39.4 g/l (Ochsenreither et al., 2014). All the results above demonstrate that in addition to malic acid, the by-products such as fumaric acid, citric acid and others are also produced by different strains of *A. oryzae*. So far, both *A. niger* and *A. oryzae* have achieved a ''Generally Recognized As Safe'' (GRAB) status.

It was found that a glucose concentration of 30%, initial pH 5.0 and 25 °C incubation temperature were the optimum conditions for *Zygosaccharomyces rouxii* V19 to ferment glucose to produce L-malic acid. The presence of glutamic, malic and succinic acid precursors in the medium increased the production of malic acid. Finally, the maximum amount of malic acid produced was 74.9 g/l (32.8% yield, based on glucose consumption) in the medium with 0.5% glutamic acid supplement, and titer of succinic acid was 7.7 g/l of the medium (8.1% yield) when 0.3% malic acid was added in the medium (Taing & Taing, 2007). Recently, it has been found that many strains of *Penicillium* spp. isolated from marine environments can also synthesize and secrete high levels of calcium malate into the medium. For example, *Penicillium rubens* 67, isolated from the surface of marine algae, can produce over 110.0 g of calcium malate per liter of the medium within 3 days and *Penicillium clerotiorum* K304 can yield 92.0 g of calcium malate per liter of the medium within 3 days (Wang et al., 2013). In another study (Khan et al., 2013), corn steep liquor (CSL) was found to be able to stimulate calcium malate production by *Penicillium viticola* 152 isolated from marine algae and 0.5% (v/v) CSL was the most suitable for calcium malate production. Under the optimal conditions, a titer of calcium malate in the supernatant was 132 g/l at flask level. During a 10 -l fermentation, a titer of 168 g/l, a yield of 1.28 g/g of glucose and a productivity of 1.75 g/l/h were reached within 96 h of the fermentation, and 93.4 % of the sugar was used for calcium malate production by *P. viticola* 152 and its cell growth. This demonstrates that the titer, yield and productivity of calcium malate by this fungal strain are very high and the fermentation period is very short. Furthermore, only calcium malate was detected in the supernatant. Therefore, *P. viticola* 152 has highly potential applications in L-malic acid production.

It has been well regarded that *Saccharomyces cerevisiae* is one of the most safe and accepted eukaryotic microorganisms. However, *S. cerevisiae* strains produce only low levels of L-malic acid. To achieve efficient malate production with this yeast, the simultaneous introduction of all three genetic modifications were carried out: (i) overexpression of the *PYC2* gene encoding the native pyruvate carboxylase, (ii) high-level expression of the *MDH3* gene encoding malate dehydrogenase and (iii) functional expression of the *Schizosaccharomyces pombe* malate transporter gene *SpMAE1*. The resulting engineered strain produced 59 g of L-malic acid per liter of medium at a malate yield of 0.42 mol (per mol glucose). However, the engineered strains still produced substantial amounts of pyruvate, indicating that the pathway's efficiency can be further improved (Zelle et al., 2008). Nakayama et al. (Nakayama et al., 2012) discovered that malic acid production by the respiratory-deficient strain of *S. cerevisiae* sake yeast strain No. 28 was 2.5-fold higher than that by its wild-type K1001 and wild-type No. 28, demonstrating that the high malic acid production by a respiratory-deficient mutant is attributed to the suppression of mitochondrial activity.

The vitamin-auxotroph of *Torulopsis glabrata* is a well-established microorganism used for the industrial production of pyruvate. Under the optimal vitamin concentrations, a high pyruvate concentration (94.3 g/l) and yield (0.635 g/g) can be achieved by *T. glabrata* after 82 h of cultivation. Furthermore, *T. glabrata* has many advantages such as high glucose and acid tolerance and these advantages make *T. glabrata* a promising alternative host for the use of metabolic engineering strategies to redirect the carbon flux from pyruvate to malate. Therefore, the genes *RoPYC* encoding pyruvate carboxylase, *RoMDH* encoding malate dehydrogenase and *SpMAE1* encoding malate transporter simultaneously were overexpressed in *T. glabrata.* However, using these strategies, only 8.5 g/l malate was accumulated in the engineered strain T. G-PMS (Chen et al., 2013).

The mushroom *Schizophylium commune* IFO-4928 was also used to produce L-malic acid. When the optimum temperature was 27 °C and the optimal concentrations of both calcium carbonate and glucose were 50 g per liter,

around 21 g of L-malic acid per liter of the medium was produced and the productivity was 0.18 g per liter per hour (Kawagoe et al., 1997).

As *Bacillus subtilis* has been widely used in food and pharmaceutical industries, it may be a possible malate producer. After both the *Escherichia coli* phosphoenolpyruvate carboxylase gene and the *S. cerevisiae* malate dehydrogenase gene were introduced into *B. subtilis* in which the gene encoding lactate dehydrogenase had been deleted, the L-malate production by the engineered *B. subtilis* was increased 1.5-fold to 9.2 ± 0.22 mM. Under the two-stage fermentation conditions, the engineered *B. subtilis* produced up to 15.65 ± 0.13 mM L-malate. However, the L-malate production by the recombinant *B. subtilis* was still low so that the engineered *B. subtilis* could not be used for L-malate production in industries (Mu & Wen, 2013).

Escherichia coli was also used as the producer of malate. It was found that expression of the gene encoding fumarate reductase in *E. coli* strains (KJ060 and KJ073) could effectively redirect carbon flow into malate even in the presence of fumarase. The engineered strain (XZ658) produced 163 mM malate, with a yield of 1.0 mol of malate per mol of the consumed glucose. Using a two-stage process (aerobic cell growth and anaerobic malate production), this engineered strain produced 253 mM malate (34 g per liter of the medium) within 72 h, with a higher yield (1.42 mol of malate per mol of the consumed glucose) and productivity (0.47 g/l/h) (Zhang et al., 2011).

After the gene encoding a malic enzyme derived from *Thermococcus kodakarensis* was introduced into *E. coli* cells with a thermodynamically favorable non-ATP-forming Embden–Meyerhof pathway, at high concentration of HCO_3^-? >, direct conversion of glucose to malate by the engineered *E. coli* could be enhanced. As a result, approximately 2.6 mM malate and 0.6 mM lactate were produced from 1.8 mM glucose within 3 h and the malate yield was calculated to be 72% (Ye et al., 2013).

As the PEP carboxylase in *E. coli* converts PEP to oxaloacetate without generating ATP, thus losing the high-energy phosphate bond of PEP, overexpression of this gene may not positively affect malic acid production. Thus, the *pckA* gene encoding PEP carboxykinase in *Mannheimia succiniciproducens* was introduced into the *pta* mutant *E. coli* strain WGS-10 and the final malic acid concentration of 9.25 g/l was obtained after 12 h of aerobic cultivation of the engineered *E. coli* (Moon et al., 2008).

All the strains used for production of malate from glucose by one-step fermentation are summarized in Table 1.

In all the processes for malate production from glucose by a one-step fermentation, $CaCO_3$ is required in the fermentation medium because $CaCO_3$ plays an important role in malate biosynthesis by keeping pH constant of around 6.5 and providing CO_2 as a substrate for efficient production of malate. The results have also shown that calcium is involved in cellular signaling pathways and influences pyruvate carboxylase activity and different concentrations of $CaCO_3$ have an effect on malate formation (Zelle et al., 2010). All the acid end products as calcium salts can be easily precipitated after addition of methanol (Goldberg et al., 2006). This may be a promising approach to greenhouse gas CO_2 fixation and the production of useful biocommodities (Ye et al., 2013). Furthermore, in the one-step fermentation, renewable feedstocks or wastes can be used for malic acid production. Therefore, in recent years, the one-step fermentation has received more and more attention. However, so far, no fermentation process has been developed for industrial production of malic acid. Recently, the results from our research have shown that one-step fermentation using some strains of *Penicillium* spp. has the potential for the commercial production of malic acid (Table 1) as titer of L-malic acid (over 92 g per liter) in the culture is high, fermentation period (3 days) is short and no byproducts in the culture are detected (Khan et al., 2013; Wang et al., 2013).

As stated above, it has been well known that high L-malic acid production can be achieved only in the medium with a high carbon-to-nitrogen ratio. In response to the nitrogen starvation, it was found that expression of most of the glycolytic genes and all the genes related to the cytosolic malic acid production pathway from pyruvate via oxaloacetate to malate in *A. oryzae* was highly upregulated and the pyruvate carboxylase reaction was identified as a target for metabolic engineering as its encoding gene was transcriptionally regulated through the correlation between intracellular fluxes and transcriptional changes and the pyruvate carboxylation seems to be a rate-limiting step in malic acid biosynthesis (Knuf et al., 2013). In contrast, the expression of the genes involved in the TCA cycle was downregulated. After analysis of the upstream sequences of the upregulated genes including the gene encoding pyruvate carboxylase, it was found that they had the conserved 6-oligonucleotide sequence (CCCCTC) which was the binding site for the *S. cerevisiae* yeast transcription factor Msn2/4, which is the transcriptional activator of the multistress response including nitrogen starvation in *S. cerevisiae*. The transcription activators Msn2/4 are key players in the general stress response pathway (Knuf et al., 2013). During mitotic growth, Msn2/4 s reside in the cytoplasm where they are inactive. Upon exposure to stress or nutrient depletion, these transcription factors rapidly accumulate in the nucleus and stimulate expression of more than 150 genes (Santhanam et al., 2004). In addition, in response to the nitrogen starvation, the degradation of proteins and other nitrogen containing cellular compounds and reduction of protein synthesis occurred in *A. oryzae* cells (Knuf et al., 2013).

Acid hydrolysis of PMA

In recent years, many researchers have tried to produce malic acid from PMA through acid hydrolysis (Zhang et al., 2011; Zou et al., 2013). About 47.3 g of malic acid per liter was obtained from the batch fermentation medium and 87.6 g of malic acid per liter was yielded from a fed-batch fermentation medium. However, the fermentation pH decreased slightly from the initial pH 6.8 to 6.0 due to the production of 0.6 and 1.2 g of succinic and acetic acids per liter (Zou et al., 2013). Zhang et al. (Zhang et al., 2011) found that 15 g of malic acid per liter was the yield from PMA after acid hydrolysis at 90 °C for 10 h. Like the one-step fermentation mentioned above, PMA also comes from glucose and other glucose-containing materials and $CaCO_3$ through fungal fermentation.

Table 1. L-Malate production by one-step fermentation by the native and metabolically engineered microorganisms.

Microorganism	Medium/conditions	Titer (g per liter)	Yield (mol per mol)	Productivity (g per l per h)	References
Native L-malate producers					
A. flavus	Glucose (120 g/l) in mineral salts medium, 90 g/l $CaCO_3$, microaerobic, 25 °C, pH 7.5	113	1.26	0.59	(Battat et al., 1991)
A.niger	Thin stillage (3.4 g/l glucose; 17.1 g/l glycerol; 15.8 g/l lactic acid)	17	0.8	0.09	(West, 2011)
A. oryzae NRRL 3488	Glucose (100 g/l), $CaCO_3$ (40 g/l), NH_4SO_4 (0.46 g/l), KH_2PO_4 (0.15 g/l), K_2HPO_4 (0.15 g/l), $MgSO_4{\cdot}7H_2O$ (0.10 g/l) $CaCl_2{\cdot}2H_2O$ (0.1 g/l), $FeSO_4{\cdot}7H_2O$ (0.005 g/l), NaCl (0.005 g/l),	30.3	–	–	(Knuf et al., 2013)
A. oryzae DSM1863	120 g/l glucose monohydrate, 1.2 g/l $(NH_4)_2SO_4$, 0.1 g/l KH_2PO_4, 0.17 g/l $K_2HPO_4{\cdot}3H_2O$, 0.1 g/l $MgSO_4{\cdot}7H_2O$, 0.1 g/l $CaCl_2{\cdot}2H_2O$, 5 mg/l NaCl, and 60 mg/l $FeSO_4{\cdot}7H_2O$, 90 g/l $CaCO_3$	42	–	0.25	(Ochsenreither et al., 2014)
Z. rouxii	Glucose (300 g/l) with 5 g/l yeast extract, 10 g peptone, 5 g/l glutamate; microaerobic, 25 °C, pH 5	75	0.52	0.54	(Taing & Taing, 2007)
S. commune	Glucose 50 g/l, $CaCO_3$ 50 g/l, NH_4NO_3 1.0 g/l, KH_2PO_4 1.0 g/l, $MgSO_4$. $7H_20$ 0.5 g/l, KC1 0.5 g/l, Thiamine 0.005 g/l, MnCl, 0.005 g/l	21	–	0.18	(Kawagoe et al., 1997)
Penicillium sp. K034	Glucose 150.0 g/l, NH_4NO_3 2.0 g/l, KH_2PO_4 0.1 g/l, $MgSO_4{\cdot}7H_2O$ 0.1 g/l, KCl 0.5 g/l, $CaCO_3$ 50.0 g/l, 30 °C,180 rpm 3 days	92.0	0.88	1.23	(Wang et al., 2013)
P. rubens 67	Glucose 150.0 g/l, NH_4NO_3 2.0 g/l, KH_2PO_4 0.1 g/l, $MgSO_4{\cdot}7H_2O$ 0.1 g/l, KCl 0.5 g/l, $CaCO_3$ 50.0 g/l, 30 °C,180 rpm 3 days	112	0.92	1.53	(Wang et al., 2013)
A. pullulans ZD-3 d	120 g/l $NaNO_3$, 2 g/l KH_2PO_4, 0.1 g/l $MgSO_4{\cdot}7H_2O$, 0.2 g/l KCl, 30 g/l $CaCO_3$, 25 °C, 200 rpm, 7 days	57.2	–	0.35	(Zhang et al., 2011)
A. pullulans strain ZX-10	Glucose (100 g/l), NH_4NO_3 (2 g/l), KH_2PO_4 (0.1 g/l), $MgSO_4{\cdot}7H_2O$ (0.1 g/l), KCl (0.5 g/l), $ZnSO_4$ (0.1 g/l) and $CaCO_3$ (30 g/l)	41.2	–	0.49	(Zou et al., 2013)
P.viticola 152	Glucose (140 g/l), CSL (0.5 % v/v), KH_2PO_4 (0.1 g/l), $MgSO_4{\cdot}7H_2O$ (0.1 g/l), KCl (0.5 g/l), $CaCO_3$ (40.0 g/l), pH 6.7, 28 °C	168	1.28	1.75	(Khan et al., 2013)
Aureobasidium sp. P6	120.0 g/l of glucose, 2.0 g/l of NH_4NO_3, 0.1 g/l of KH_2PO_4, 0.1 g/l of $MgSO_4{\cdot}7H_2O$, 0.5 g/l of KCl and 65.0 g/l of $CaCO_3$.	98.7	0.85	0.63	(Ma et al., 2013)
Aureobasidium sp. P6	140.0 g/l of glucose, 2.0 g/l NH_4NO_3, 0.1 g/l KH_2PO_4, 0.1 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l KCl and 65.0 g/l $CaCO_3$.	118.3	0.87	0.67	(Ma et al., 2013)
Engineered strains					
E. coli WGS-10	Glucose (20 g/l) in mineral salts medium, aerobic batch, 37 °C, pH 6.7	9.25	0.56	0.74	(Zhang et al., 2011)
S. cerevisiae	Glucose (188 g/l) in mineral salts medium with 150 g/l $CaCO_3$, aerobic flask, 30 °C, pH 6	59	0.42	0.19	(Zelle et al., 2008)
Engineered *A. oryzae* NRRL 3488	Glucose (160 g/l), $CaCO_3$ (120 g/l), Bacto Peptone (9 g/l), KH_2PO_4 (0.15 g/l), K_2HPO_4 (0.15 g/l), $MgSO_4{\cdot}7H_2O$ (0.10 g/l) $CaCl_2{\cdot}2H_2O$ (0.1 g/l), $FeSO_4{\cdot}7H_2O$ (0.005 g/l), NaCl (0.005 g/l), 0.5 ml/l Pluronic L61	154	0.94	1.38	(Brown et al. 2013; Knuf et al. 2014)
The engineered *T. glabrata*	Fresh medium B	8.5	0.19	0.18	(Zhang et al., 2011)
E. coli XZ658	Glucose (50 g/l) in mineral salts medium with 100 mM $KHCO_3$, anaerobic batch, 37 °C, pH 7	22	1.0	0.15	(Zhang et al., 2011)
E. coli XZ658	Glucose (50 g/l) in mineral, salts medium with 100 mM $KHCO_3$, two-stage process, 37 °C, pH 7	34	1.42	0.47	(Zhang et al., 2011)
The *pta* mutant *E. coli* strain WGS-10 harboring *pckA* gene	R/2 medium supplemented with 20 g glucose per liter	9.25	–	–	(Moon et al., 2008)
B. subtilis 168	100 mM glucose, 50 mM sodium pyruvate, 13.6 mM glutamate and 24.5 mM tryptophan two-stage, process fermentations	15.7 mM	–	with 52.4 ± 1.7 mM acetate	(Mu & Wen, 2013)

Recently, it has been found that a novel *Aureobasidium* sp. P6 strain isolated from mangrove system can produce 98.7 g/l of Ca^{2+}-PMA from 12.0% glucose and 118.3 g/l of Ca^{2+}-PMA from 14.0% glucose during 10-l fermentation and most of the produced PMA can be hydrolyzed into calcium malate (Ma et al., 2013).

Biotransformation using fumarase

As many microbial cells contain fumarase which catalyzes the conversion of fumaric acid to malic acid, they can be used for the conversion of fumaric acid to malic acid. After the gene encoding fumarase was over-expressed in *S. cerevisiae* and their cells over-producing fumarase were immobilized, it was found that the immobilized cells could transform 80–90% of fumaric acid into malic acid with a productivity of about 2 g per liter per hour (Neufeld et al., 1991). However, in order to reduce the costs, the unconsumed fumaric acid must be recycled. After the permeabilized *S. cerevisiae* cells were immobilized, they could be continuously used for production of L-malic acid, resulting in the biocatalyst productivity of 11.8 mmol of L-malic acid per gram cell dry weight per day (Stojkovic & Znidarsic-Plazl, 2012). In addition to *S. cerevisiae*, the yeast *Dipodascus magnusii* was found to be able to produce high level of fumarase (18.8 U per mg of cell dry weight) while *S. cerevisiae* only yielded 1.9 U per mg of cell dry weight (Rosenberg et al., 1999). When the intact cells of *D. magnusii* were used to transform sodium fumarate (1 M) into malic acid, the maximum specific productivity of malic acid obtained was 1.72 g per g of cell dry weight per hour while in the case of ammonium fumarate, it was 2.25 g per g of cell dry weight per hour. Furthermore, it was noticed that there were no by-products from the yeast (Mikova et al., 1999).

As *Saccharomyces bayanus* had the highest activity of fumarase, the permeabilized cells of it were used to convert fumaric acid to malic acid. The average conversion of fumaric acid was up to 82% and gave 21, 40, 83 and 175 mM L-malic acid from 25, 50, 100 and 210 mM fumaric acid, respectively. Especially, by-product, such as succinic acid, was not detected and the conversion was up to 82% (Presecki & Vasic-Racki, 2005).

The fumarase immobilized in a membrane reactor also could catalyze conversion of fumaric acid into L-malic acid and showed no activity decay during more than 2 weeks of continuous operation. Finally, a steady-state conversion degree of 80% was achieved (Giorno et al., 2001).

Malic enzyme catalyzes the decarboxylation of L-malic acid to pyruvic acid and releases CO_2 from L-malic acid by using the oxidized form of the coenzyme, $NAD(P)^+$, as electron acceptor. In contrast, in the reverse reaction, pyruvic acid is transformed into L-malic acid with the fixation of CO_2 while the reduced form of the coenzyme, NAD(P)H, is oxidized to $NAD(P)^+$. Therefore, the malic enzyme was prepared and purified from *Brevundimonas diminuta* IFO13182 and the L-malic acid production for HCO_3^- fixation system with layer-by-layer immobilization of NAD+/malic enzyme multilayer film on the electrode was done. Finally, an L-malic acid production of nearly 11.9 mmol and an HCO_3^- fixation rate of nearly 47.4% were achieved (Zheng et al., 2009).

The resting cells of *Brevibacterium* sp. B2D have a high rate of L-malic acid production with high yield without coproduction of unwanted byproducts. When fumarate concentration was 60 g per liter, 89.8% of it was converted into L-malic acid by the resting cells and average specific productivity was 0.41 g of L-malic acid per gram of glucose per hour (Gong et al., 1996).

When the immobilized cells of *Brevibacterium ammoniagenes* MA-2 and *Brevibacterium flavum* MA-3 were used to catalyze ammonia fumarate to L-malic acid, the product concentrations were 210 g of L-malic acid per liter and 218 g of L-malic acid per liter, respectively. At the same time, to reduce the residual fumaric acid, a conversion system was established to produce L-malate coupling with immobilized *E. coli* No. 1 cells for converting the remnant fumarate to L-aspartic acid (Hu & Ouyang, 2010).

The *S. cerevisiae* cells with fumarase were immobilized on the microchannel surface via covalent immobilization and permeabilized to improve mass transfer across the membrane. Then, the whole-cell microreactors were used to continuously biotransform fumaric to L-malic acid (Stojkovic & Znidarsic-Plazl, 2012).

However, as the enzymatic conversion processes mentioned above require rather expensive chiral resolution agent, complex reaction processes or expensive enzyme and recycled substrate, these disadvantages make them difficult to be used practically (Moon et al., 2008). In addition, these processes require petroleum-derived maleic anhydride or fumaric acid as feedstocks for the synthesis of L-malic acid.

Pathways of L-malate biosynthesis

Three metabolic pathways for the production of L-malic acid from glucose have been identified (Figure 1). These include non-oxidative pathway, oxidative pathway and L-malic acid from the glyoxylate cycle.

Non-oxidative pathway

The first of these pathways is carboxylation of pyruvate (*S. cerevisiae* lacks phosphoenolpyruvate carboxylase) to oxaloacetate, followed by reduction of oxaloacetate to malate. If pyruvate is produced during glycolysis, this non-oxidative pathway is ATP neutral and involves a net fixation of CO_2, resulting in a maximum theoretical malate yield of 2 mol (per mol of the consumed glucose). In this case, if glucose concentration in the medium plus $CaCO_3$ is 180 g per liter, theoretically, 344 g of calcium malate per liter will be produced and the yield will be 1.9 g of L-malic acid per gram of the consumed glucose. In fact, no such titer and yield were obtained in the one-step fermentation (Table 1), because some of the added glucose is transformed into both CO_2 by respiration and cell mass by growth so that it is impossible for the yield to be 1.9 g of L-malic acid per gram of the consumed glucose. Even some of the added glucose is transformed into other organic acids, such as citric acid, succinic acids and fumaric acid. Many researchers think that pyruvate carboxylase, phosphoenolpyruvate (PEP) carboxylase and malate dehydrogenase are involved in malic acid biosynthesis in the non-oxidative pathway. However, as mentioned above, when the PEP carboxylase converts PEP to oxaloacetate, no

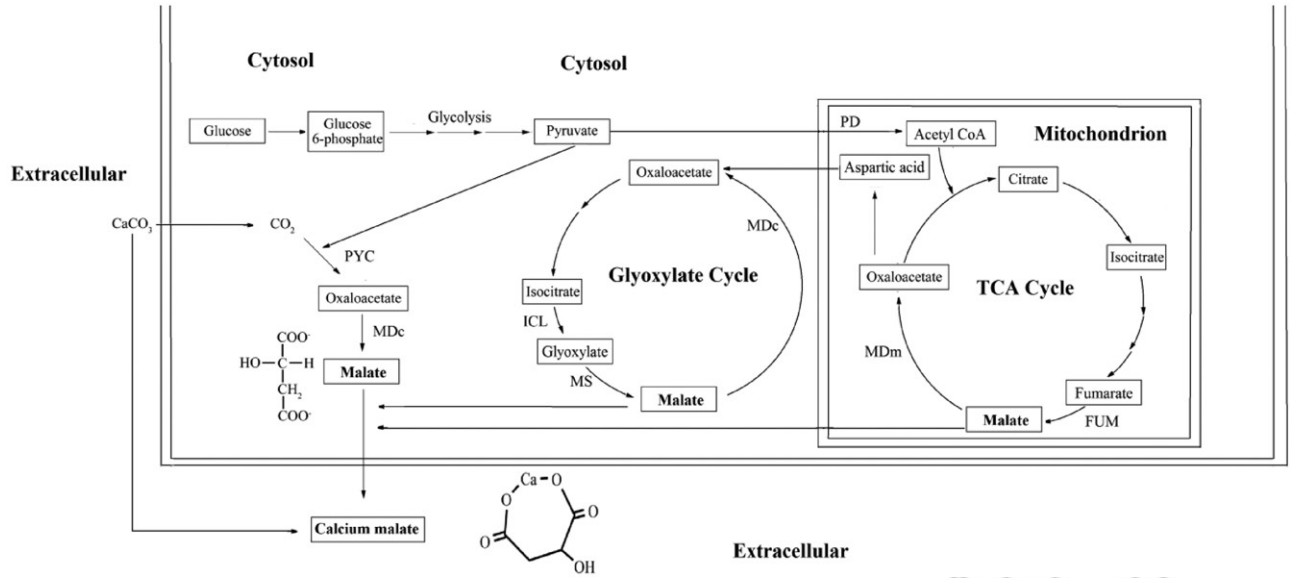

Figure 1. The proposed pathways of L-malic acid biosynthesis in microorganisms. PYC, Pyruvate carboxylase; MDc, Cytosolic malate dehydrogenase; MDm, mitochondrial malate; dehydrogenase; MS, Malate synthetase; ICL, Iso-citrate lyase; PD, Pyruvate decarboxylase; FUM, fumarase.

ATP is produced, leading to losing of the high-energy phosphate bond of PEP. Therefore, the PEP carboxylase may not be used for production of oxaloacetate by the microorganisms mentioned above. Pyruvate carboxylase is a biotin-dependent tetrameric enzyme that catalyzes the carboxylation of pyruvic acid while malate dehydrogenase catalyzes the NAD(H)-dependent reversible conversion of L-malic acid to oxaloacetic acid, suggesting that biotin and NAD(H) are required during biosynthesis of malic acid. This pathway is regarded to occur in the cells of *S. cerevisiae*, *A. flavus*, *A. oryzae, E. coli*, *B. subtilis* and *Penicillium* spp. as a near-neutral pH, which is achieved by adding $CaCO_3$, is required for efficient malic acid production and increased calcium concentrations has positive effect on malate formation (Zelle et al., 2008). Overexpression of either fumarase, which is involved in the oxidative tricarboxylic acid (TCA) cycle, or malate dehydrogenase in the reductive TCA cycle of mitochondria increases malic acid production (Peleg et al., 1990; Pines et al., 1996; Pines et al., 1997; Wang et al., 1998).

Oxidative pathway

The second pathway is condensation of oxaloacetate and acetyl-coenzyme A (acetyl-CoA) to citric acid, followed by its oxidation to malate via the tricarboxylic acid (TCA) cycle (Figure 1). If acetyl-CoA is generated by pyruvate dehydrogenase, the conversion of glucose to malate via this oxidative pathway will result in the release of two CO_2, thus limiting the maximum theoretical malate yield to 1 mol (per mol glucose). In this case, fumarase which catalyzes the reversible hydration of fumaric acid to L-malic acid may play an important role in malate formation. As stated above, many microbial cells carrying fumarase have been widely used for transformation of fumaric acid to L-malic acid. In *S. cerevisiae*, overexpression of either fumarase, which is involved in the oxidative tricarboxylic acid (TCA) cycle, or malate dehydrogenase in the reductive TCA cycle of mitochondria increases malic acid production (Koganemaru et al., 2001; Pines et al., 1996). Nakayama et al. (Nakayama et al., 2012) found that the respiratory-deficient strain of *S. cerevisiae* can produce more L-malic acid than its parent strain. However, overexpression of the malic enzyme, which catalyzes the conversion of malic acid to pyruvic acid in mitochondria, leads to a decrease in malic acid production (Redzepovic et al., 2003).

L-malic acid from the glyoxylate cycle

Formation of malate from two molecules of acetyl-CoA via the glyoxylate cycle is the third pathway (Figure 1). In this alternative oxidative pathway for malate production, the maximum malate yield on glucose is limited to 1 mol per mol due to the oxidative decarboxylation reaction required for acetyl-CoA production from pyruvate. In the case, iso-citrate lyase and malate synthetase are involved in malate biosynthesis. The iso-citrate lyase catalyzes lysis of iso-citrate into glyoxylate and succinic acid. Then, the malate synthetase in *S. cerevisiae* catalyzes the reaction: Acetyl-S-CoA + $Glyoxylate^{-}$ + $H_2O \rightarrow Malate^{--}$ + HS-CoA + H^{+}. The malate synthetase in *S. cerevisiae* requires Mg^{2+} for activity and is specific for glyxoxylate. For example, the glyoxylate pathway enzyme malate synthase and the reductive pathway enzymes pyruvate carboxylase and cytosolic malate dehydrogenase in *S. cerevisiae* can enhance malic acid production (Koganemaru et al., 2001; Zelle et al., 2008). However, some researchers think that the glyoxylate cycle cannot be involved in L-malate biosynthesis as the cycle will be repressed by high concentration of glucose in the medium (Straathof & van Gulik, 2012). Usually, L-malic acid is produced at high concentration of glucose (Khan et al., 2013; Wang et al., 2013).

From the three pathways of L-malic acid biosynthesis, it can be clearly seen that the first pathway is preferred by industries as 1 mol of glucose is transformed into 2 mol of L-malic acid. However, it is still a problem how to further enhance yield of L-malic acid by metabolic engineering.

Secretion of L-malate

To date, the exact mechanism of L-malate secretion of the cell has not been definitively understood. As L-malic acid accumulation in the cells is toxic to the producers, it is also important to enhance diffusion of the synthesized L-malic acid across the plasma membrane into the medium, Especially, *S. cerevisiae* cells do not have a membrane transporter for L-malate and the diffusion rate of the L-malate across the its plasma membrane is very slow. The carboxylate transporter (was shared by malate, succinate, fumarate and α-ketoglutarate) encoded by the gene *KlJEN2* was found in *K. lactis* (Queiros et al., 1998).

Many years ago, the malate transporter gene *SpMAE1* was cloned from *Schizosaccharomyces pombe* and the malate transporter encoded by this gene was found to be involved in import of malate. In fact, this transporter corresponds to a 49-kDa protein with 10 transmembrane predicted segments. Furthermore, the *SpMAE1* gene encodes a permease for malate and other C4 dicarboxylic acids and its protein behaves as a proton symporter not subjected to glucose repression (Sousa et al., 1992). The heterologous expression of the gene *KlJEN2* cloned from *K. lactis* enables *S. cerevisiae* to uptake succinic and malic acids (Queiros et al., 1998). At the same time, as stated above, functional expression of a heterologous malate transporter expression of the *SpMAE1* gene indeed enabled increased malate production (Chen et al., 2013; Knuf et al., 2013; Zelle et al., 2008). However, it is still unknown if the transporter gene can function in other malate-producing fungi.

As mentioned above, *A. flavus* is a good producer of malic acid. Peleg et al. (Peleg et al., 1990) thought that it was possible that in *A. flavus*, malic, succinic and fumaric acids were excreted to the broth from the hyphae. Immediately upon secretion, these acids reacted with $CaCO_3$ present in the medium to form insoluble calcium salts of the acids which crystallized on the hyphae. During fermentation, the crystals grew until they reached a critical size and were detached to the broth in an insoluble form (Peleg et al., 1988). According to our results (Khan et al., 2013; Wang et al., 2013), during the fermentation of the early 3 days, the calcium malate formed is soluble. However, as the concentration of calcium malate is increased, the calcium malate is saturated in the broth, leading to crystal formation of calcium malate.

After analyzing the genomic DNA and proteins of *A. flavus*, it was found that there are six putative proteins (the accession numbers are XP_002376605.1, XP_002372510.1, XP-002386672.1, XP_002381607.1, XP_06580654.1 and XP_002378692.1, respectively) which may be involved in L-malic acid C4-dicarboxylate transport (NCBI). It is being under investigation whether they take part in L-malic acid and other C4-dicarboxylate transport in this laboratory.

Applications of L-malate

Currently, the annual worldwide production of malic acid is 40 000 tones whereas the projected market volume is 200 000 tones (Kajiyama et al., 2003). The classical chemical process for production of malic acid occurs at high temperature and high pressure yielding a racemic mixture of D- and L-malic acid and these current production costs are too high to allow a more wide spread use of L-malic acid.

As it possesses a greater acid taste and better taste retention than citric acid, malic acid as an acidulant and flavor enhancer is mainly used in beverages, candy and food. For example, organic acids, such as malic, succinic and lactic acids, are crucial factors in determining the taste of Japanese saké. The succinic and malic acids produced by the yeast *S. cerevisiae* during saké fermentation confer an umami and a refreshing taste to the saké (Nakayama et al., 2012). Malic acid is also used as one component of antimicrobial agents. For example, the combination of Panax ginseng (2% v/v), malic acid (0.5 % v/v) and potassium sorbate (0.05% v/v) show the highest antimicrobial effectiveness against *Salmonella enteric* ser. Saintpaul and *E. coli* O157:H7 in sterile and fresh mango and orange juices, in addition to a higher microbiological inhibition during storage (21 days) (Raybaudi-Massilia et al., 2012). L-Malic acid is also used in amino acid infusions to treat hyperammonemia and liver dysfunction. Nonfood applications for malic acid include metal cleaning and finishing, textile finishing, electroless plating and paints (Goldberg et al., 2006). As the malic acid has been proposed as potential bulk chemical precursors, it can be used as a specialty chemical intermediate and a feedstock for chemical synthesis of biodegradable polymers, such as PMA as stated above (Sauer et al., 2008). L-malic acid was also used for synthesis of optically pure 4-bromo-2-hydroxybutanoic acid esters which could be applied to synthesize peptide secondary structure mimetics (Kim et al., 2007). L-Malic acid can be metabolized to provide ATP in the mammalian tricarboxylic acid cycle.

Calcium malate, ferric malate and zinc malate, formed during the fermentation, can be used as medicines to provide human and animals with calcium, iron and zinc. Calcium malate is a source of bio-available calcium in which calcium is bound to the naturally occurring malic acid. Also known as fruit acid, malic acid is a naturally occurring organic acid in the plants and animals, including humans. The absorption potential of calcium is enhanced in calcium malate as compared to inorganic forms of calcium. Calcium malate also finds application in ''functional beverages''. Awareness of the necessity of calcium for long-term bone health has grown substantially and calcium fortification has exploded across the food chain. This has lead to increased demand for bioavailable calcium delivery forms such as calcium malate.

Phosphate utilization efficiency in soils is very low because applied phosphorus is mostly fixed to aluminum ($AlPO_4$) and iron ($FePO_4$) in acidic soils and to calcium [$Ca_3(PO_4)_2$] in alkaline soils. Malic acid produced by *Penicillium oxalicum* may be mediated in aluminum phosphate solubilization in soil. For example, *P. oxalicum* demonstrated higher levels of $Ca_3(PO_4)_2$ and $AlPO_4$ solubilization than that of $FePO_4$ solubilization (Gadagi et al., 2007). This means that malic acid may also be applied to agricultural industries.

Concluding remarks

L-Malic acid has many uses in industry. Although the biosynthetic pathways of L-malic acid in microorganisms

are partially clear, no suitable native microorganisms and engineered microorganisms have been suitable for industrial production of L-malic acid. Recently, it has been found that some strains of *Penicillium* spp. may be suitable for industrial production of L-malic acid in the future because titer of L-malic acid is high, fermentation period is short and no byproducts in the culture are detected. However, it is still unknown how it is secreted into the medium. To enhance L-malate biosynthesis and secretion by microbial cells, it is very important to study the biosynthesis and secretion mechanisms of L-malic acid at enzymatic and molecular levels.

Declaration of interest

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