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Research paper

Metabolic engineering of *Aspergillus oryzae* for efficient production of L-malate directly from corn starch

Jingjing Liu[a,b], Jianghua Li[a,b], Hyun-dong Shin[c], Guocheng Du[a,b], Jian Chen[d], Long Liu[a,b,*]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[b] Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[c] School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA
[d] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China

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ABSTRACT

L-Malate, an important chemical building block, has been widely applied in the food, pharmaceutical, and bio-based materials industries. In previous work, we engineered *Aspergillus oryzae* by rewiring the reductive tricarboxylic acid pathway to produce L-malate from glucose. To decrease the production cost, here, we further engineered *A. oryzae* to efficiently produce L-malate directly from corn starch with simultaneous liquefaction-saccharification and fermentation. First, a promoter PN5 was constructed by quintuple tandem of the 97-bp fragment containing the cis-element of the glucoamylase gene promoter (P*glaA*), and with the promoter PN5, the transcriptional level of *glaA* gene increased by 25–45%. Then, by co-overexpression of *glaA,* a-amylase (*amyB*) and a-glucosidase (*agdA*) genes with the promoter PN5, the L-malate titer increased from 55.5 g/L to 72.0 g/L with 100 g/L corn starch in shake flask. In addition, to reduce the concentration of byproducts succinate and fumarate, a fumarase from *Saccharomyces cerevisiae*, which facilitated the transformation of fumarate to L-malate, was overexpressed. As a result, the concentration of succinate and fumarate decreased from 12.6 and 1.29 g/L to 7.8 and 0.59 g/L, and the L-malate titer increased from 72.0 g/L to 78.5 g/L. Finally, we found that the addition of glucose at the initial fermentation stage facilitated the cell growth and L-malate synthesis, and the L-malate titer further increased to 82.3 g/L by co-fermentation of 30 g/L glucose and 70 g/L corn starch, with a productivity of 1.18 g/L/h and a yield of 0.82 g/g total carbon sources.

1. Introduction

As an essential intermediate of cell metabolism and an important platform chemical (Werpy and Petersen, 2004), malate is widely used in the food and beverage industries as an acidulant and flavor enhancer, and bulk use is carried out for alkyl and unsaturated polyester resins and coatings (Thakker et al., 2015; Liu et al., 2017a). Malate can also be used to treat hyperammonemia and liver dysfunction since calcium citrate malate is a widely used source of calcium that does not increase the risk of kidney stones while improving bone strength (Battat et al., 1991).

Currently, L-malate is typically produced by genetically engineered or adaptive evolution strains, including *Aspergillus flavus* (Battat et al., 1991), *A. oryzae* (Brown et al., 2013; Liu et al., 2017b), *Rhizopus delemar* (Li et al., 2014), *Saccharomyces cerevisiae* (Zelle et al., 2008; Zelle et al., 2010; Nakayama et al., 2012), *Zygosaccharomyces rouxii* (Taing and Taing, 2006), *Torulopsis glabrata* (Chen et al., 2013), *Escherichia coli* (Moon et al., 2008; Zhang et al., 2011; Dong et al., 2017), and *Ustilago trichophora* (Zambanini et al., 2016a, 2016b). Although great advancement has been made in the microbial production of L-malate, to the best of our knowledge, the industrial production of L-malate by microbial fermentation has not been achieved due to the higher production cost compared with petrochemical synthesis or biotransformation approaches. Glucose and glycerol are the most commonly used carbon sources, and production costs can be significantly decreased if L-malate can be efficiently produced directly from starch.

Starch, is a major storage, natural and digestible carbohydrate polymers, large-exiting in nature, and it's the main form of energy store in cells. Renewable starch sources, such as corn (maize), wheat, oats, rice, potato and tapioca, make it have more economic attractiveness (Roy Goswami et al., 2016). However, most of starch must be hydrolyzed to glucose before fermentation. Conventionally, for the use of starch as a carbon source, a two-step process, namely, liquefaction/saccharification and fermentation, is necessary in most cases (Huang et al., 2014). Liquefaction of starch is processed by amylase at high temperatures of 90–130 °C for about 15 min, and then saccharification

* Corresponding author at: Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China.
E-mail address: longliu@jiangnan.edu.cn (L. Liu).

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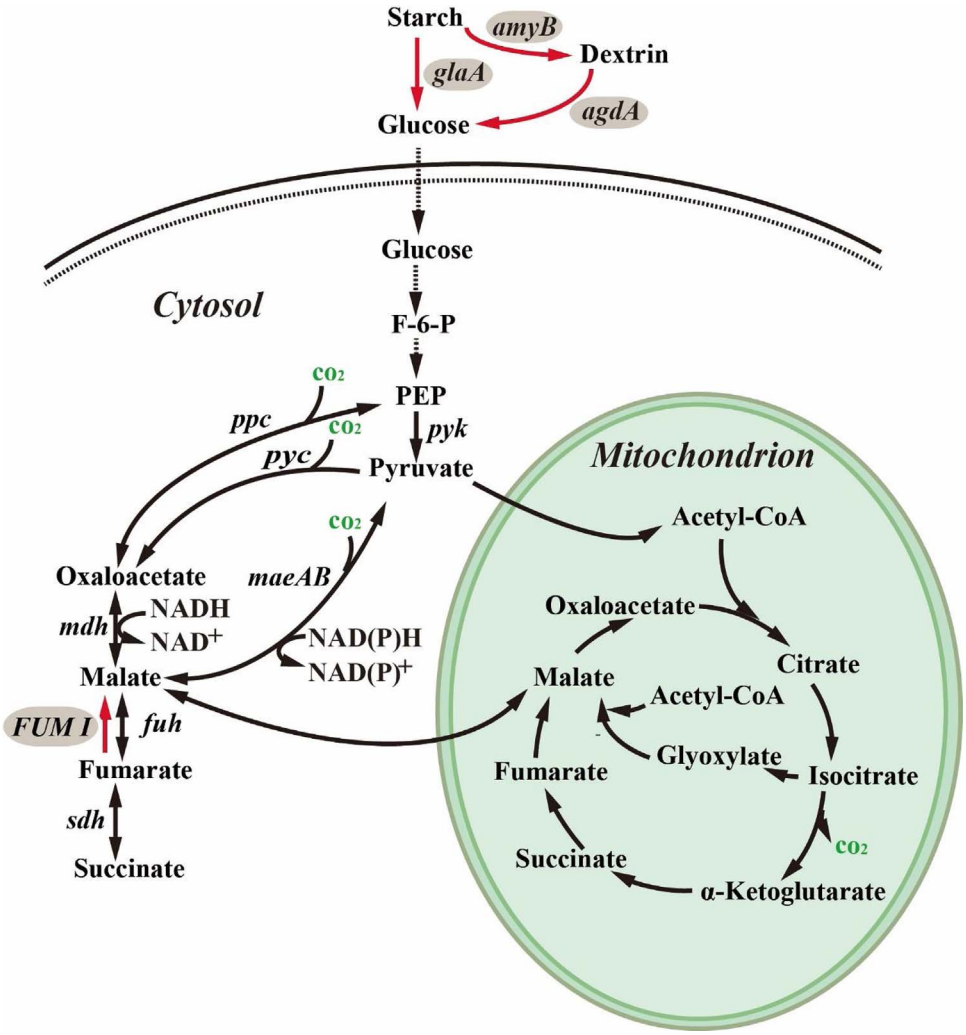

Fig. 1. Metabolic pathways of L-malate from starch in *Aspergillus oryzae*.

is carried out by glucoamylase at 60 °C, liquefied starch was further hydrolyzed into glucose (Huang et al., 2014; Tabah et al., 2015). However, this two-step process is time-consuming and has a high energy requirement. For cost-effectively, the consecutive enzymatic hydrolysis and fungal fermentation can be implemented simultaneously (Haq et al., 2003). For example, direct production of citric acid from starchy material by *Aspergillus niger* (Wang et al., 2015).

In a previous work, we engineered *A. oryzae* by rewiring the reductive tricarboxylic acid pathway, and 93.2 g/L L-malate was produced from glucose by the strain *A. oryzae* WS-M-P-PP-C4-MA-PFK in shake flask culture (Liu et al., 2017b). Here, we engineered *A. oryzae* to efficiently produce L-malate directly from corn starch with simultaneous liquefaction/saccharification and fermentation. In *A. oryzae*, there are three native amylolytic enzymes, namely, α-amylase, α-glucosidase, and glucoamylase (Sugimoto and Shoji, 2012) (Fig. 1). α-Amylases are enzymes that hydrolyze α-1,4-glycosidic bonds to release dextrin, oligosaccharides, and monosaccharide, whereas α-glucosidases can hydrolyze one-molecule α-1,4-glycosidic bonds and release a molecule of glucose. Glucoamylase, which hydrolyzes both α-1, 4- and α-1, 6-glycosidic bonds, plays important roles in the production of glucose directly from starch. In this context, L-malate production from corn starch with metabolically engineered *A. oryzae* be studied, by integrating metabolic engineering and medium optimization strategies. First, a-amylase, a-glucosidase and glucoamylase were overexpressed and analyzed, respectively. In order to further increase the transcriptional level of glucoamylase-encoding gene (*glaA*), five copies of the 97-bp fragment containing the cis-element of the *glaA* promoter (P*glaA*) were tandemly fused and inserted to the *glaA* promoter, according to deletion and site-specific mutation analysis (Hata et al., 1992). Then, *A. oryzae* GAA with simultaneous overexpression of the three amylolytic enzyme genes can efficiently utilize starch for L-malate production. Next, through heterologous expression of the *Saccharomyces cerevisiae* fumarase gene (*FUMI*), the concentration of main byproducts succinate and fumarate was significantly reduced. Finally, the mixed carbon sources were used and tested for fermentation. L-Malate titer was significantly enhanced by an *A. oryzae* GAAF41 with the high enzyme activities for amylohydrolysis from mixed carbon of glucose and starch.

2. Materials and methods

2.1. Plasmid construction and transformation

The strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 was used as the host strain for DNA manipulation. The genes *glaA*, *amyB*, and *agdA* were amplified from the cDNA of *A. oryzae* NRRL3488, and the *FUMI* gene was amplified from the genome of *S. cerevisiae* by PCR. Genes were amplified by PCR with the primer pairs are listed in Table 2. The resulting plasmids were constructed using the pMD19-T vector (TaKaRa Bio Inc., Otsu, Shiga, Japan), and all plasmids were constructed as vectors designed to integrate randomly into the *A. oryzae* genome. The procedures for protoplast transformation and isolation of transformants were carried out as described previously (Liu et al., 2017b). *A. oryzae* WS-M-P-PP-C4-MA-PFK was used as the initial host for engineering to produce L-malate.

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