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Metabolic engineering of *Rhizopus oryzae*: Effects of overexpressing *fumR* gene on cell growth and fumaric acid biosynthesis from glucose

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ABSTRACT

Fumaric acid is a dicarboxylic acid used extensively in synthetic resins, food acidulants, and other applications, including oil field fluids and esters. The filamentous fungus *Rhizopus oryzae* is known for its ability to produce and accumulate high levels of fumaric acid under aerobic conditions. In this work, the overexpression of native fumarase encoded by *fumR* and its effect on fumaric acid production in *R. oryzae* were investigated. Three plasmids containing the endogenous *fumR* gene were constructed and used to transform *R. oryzae*, and all transformants showed significantly increased fumarase activity during both the seed culture (growth) and fermentation (fumaric acid production) stages. However, fumarase overexpression in *R. oryzae* yielded more malic acid, instead of fumaric acid, in the fermentation because the overexpressed fumarase also catalyzed the hydration of fumaric acid to malic acid. The results suggested that the overexpressed fumarase, encoded by *fumR*, by itself was not responsible for the over-production of fumaric acid in *R. oryzae*.

1. Introduction

Fumaric acid, a four-carbon dicarboxylic acid with a C=C double bond, is widely used in food and chemical industries with applications ranging from the manufacturing of synthetic resins, biodegradable polymers, and plasticizers to food and beverage additives [1]. Currently, fumaric acid is primarily produced through chemical synthesis using hydrocarbons (e.g., benzene, n-butane or n-butane/n-butene mixture) as the precursors [2], with an estimated industrial production capacity of 90,000 ton/a [3]. Although the current petroleum-based chemical synthesis can reach a high production yield, recent increases in oil prices and accompanying chemical pollution problems have generated renewed interest in the production of biobased fumaric acid by fermentation using filamentous fungi, mainly *Rhizopus* species that have been identified as the best microorganisms for fumaric acid production [1,3,4].

Fumaric acid is an important intermediate in the tricarboxylic acid (TCA) cycle present in most aerobic organisms. While in fumaric acid producing strains of *Rhizopus oryzae*, fumaric acid is mainly accumulated in the cytosol via the C3 plus C1 mechanism with CO_2 fixation as the pathway for fumaric acid biosynthesis [5–7]. Fig. 1 shows the metabolic pathways in *R. oryzae*. Three reactions, starting from pyruvate, catalyzed by pyruvate carboxylase, malate dehydrogenase, and fumarase are involved in the biosynthesis of fumaric acid in the cytosol [8]. The maximal theoretical molar yield of fumaric acid from glucose is 200%. The actual fumaric acid yield in the fermentation is usually much lower than the theoretical yield since a significant amount of pyruvate is entering the TCA cycle and there are other competing cytosolic pathways leading to the production of byproducts such as malic acid and ethanol.

For economical production of biobased fumaric acid, past 30 years have seen extensive research efforts focusing on screening for hyper-producing *Rhizopus* species and fermentation process optimization, including use of cheaper biomass feedstocks, optimizing fermentation conditions and medium formulation [1,4,9,10], and developing novel bioreactors and separation methods [10]. Strain development through metabolic engineering offers a promising approach to increase fumaric acid production in filamentous fungi, but has rarely been tried due to the limited availability of molecular techniques and lack of knowledge of the regulatory network in these organisms [11]. Skory [12] overexpressed native *ldhA* in *R. oryzae* NRRL395 and the transformants with higher levels of lactic acid were obtained. The expression of *ldhA* gene in fumaric acid producing strain *R. oryzae* 99880 resulted in lactic acid production with a concurrent decrease in fumaric acid, ethanol and glycerol [13]. However, to date metabolic engineering of *R. oryzae* to overproduce fumaric acid has not been reported yet.

The goals of this study were to overexpress fumarase catalyzing the final step of fumaric acid biosynthesis in *R. oryzae* and evaluate its possible effects on cell growth and fumaric acid production. Fumarase, encoded by a single gene *fumR*, catalyzes the reversible

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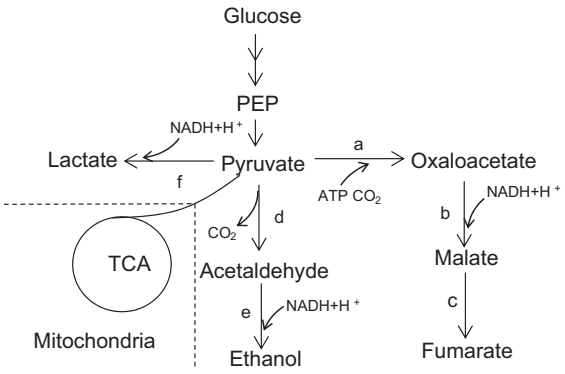

Fig. 1. Metabolic pathways for fumaric acid, lactic acid, and ethanol biosynthesis from glucose in *R. oryzae*. (a) Pyruvate carboxylase; (b) malate dehydrogenase; (c) fumarase; (d) pyruvate decarboxylase; (e) alcohol dehydrogenase; (f) lactate dehydrogenase and (g) phosphoenolpyruvate carboxylase.

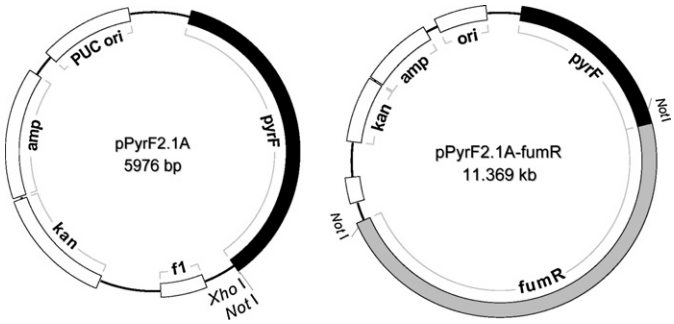

Fig. 2. Plasmid maps of expression vectors pPyrF2.1A and *pPyrF2.1A-fumR*.

dehydration of L-malic acid to fumaric acid. Fumarase in fumarate-producing *R. oryzae* has been studied extensively [14–18], but *fumR* gene has never been directly overexpressed in *R. oryzae* to elucidate its role in fumaric acid biosynthesis. In this paper, we report the first metabolic engineering study of *R. oryzae* attempting to overexpress fumarase for its potential effects on cell growth and fumaric acid biosynthesis.

2. Materials and methods

2.1. Strains and culture media

R. oryzae M16, a uracil auxotrophic mutant strain of *R. oryzae* 99880, was used as the parental strain of various transformant strains (see Table 1) developed in this work. The uracil auxotroph has a mutation in *pyrF* gene encoding OMP pyrophosphorylase [13]. The DNA isolated from *R. oryzae* NRRL 6400 was used as template to obtain the *fumR* gene, while *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) was used for the preparation of all recombinant plasmids. Unless otherwise noted, *R. oryzae* was cultured at 35 °C in a medium containing 50 g/L glucose and 2.5 g/L yeast extract. The *Rhizopus* (RZ) minimal medium containing 100 g/L glucose, 2 g/L $(NH_4)_2SO_4$, 0.5 g/L KH_2PO_4, 0.25 g/L $MgSO_4$, 2.2 mg/L $ZnSO_4{\cdot}7H_2O$, 0.5 mg/L $MnCl_2{\cdot}4H_2O$, and 0.5% Trypticase peptone was used in the selection of *R. oryzae* transformants [19]. *E. coli* was grown at 37 °C in LB with ampicillin (50 μg/mL) or on solid LB plates (15 g/L agar, 100 μg/mL ampicillin). All strains were in the same class of fumarate-producing *R. oryzae*. Table 1 lists all strains and plasmids with their relevant characteristics used in this study. Fig. 2 shows detailed maps of the expression plasmids used in this work.

2.2. Construction of plasmids expressing fumR gene

The DNA of *R. oryzae* NRRL 6400 was extracted by using Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA) and used as template to amplify *fumR* gene. The coding region for the native fumarase gene (GenBank: X78576) [14] has 1.9 kb nucleotides, including nine introns, which was amplified along with its promoter sequence in three different total DNA lengths (3.0 kb, 5.4 kb, and 4.9 kb) using PCR primers listed in Table 2.

The PCR reaction mixture (50 μl) was prepared from 5 μl 10× High Fidelity PCR buffer, 1 μl 10 mM dNTP mixture, 2 μl 50 mM $MgSO_4$, 1 μl primer mix (10 μM each), 1 μl template DNA and 0.2 μl Platinum *Taq* high fidelity. The PCR was performed for 30 thermal cycles under the following conditions: initial denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s; extension at 68 °C for 5 min. The PCR amplification products containing *fumR* genes were purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) and cloned into the pGEMT vector (Promega, Madison, WI). The resulting plasmids were digested with *XhoI* and ligated with *XhoI* linearized plasmid pPyrF2.1A [13], which contained the *pyrF* gene as the selection marker. The resulting expression vectors containing the *fumR* gene were designated as *pPyrF2.1A-fumR1*, *pPyrF2.1A-fumR2*, *pPyrF2.1A-fumR3*, respectively.

2.3. Transformation

The expression plasmids for *fumR* gene were transformed into *R. oryzae* M16 spores using microprojectile particle bombardment (PDS-1000/He system, BioRad Laboratories, Hercules, CA) following the procedures described by Skory [20]. Ungerminated spores were transformed directly on RZ medium plates. Approximately 5–7 days after bombardment, spores were collected from the plates, diluted in sterile water, and replated to obtain single-spore isolates, which were tested for their genetic stability, enzyme activities, and fermentation kinetics.

2.4. Southern hybridization analysis

Spores of *R. oryzae* 99880 and transformant isolates were inoculated at 1×10^5 spores/ml in growth media containing 50 g/L glucose and 2.5 g/L yeast extract. After 16 h cultivation at 30 °C, 200 rpm, the mycelia were collected, washed with distilled water, and filtered. The genomic DNA was extracted using OmniPrep kit (G-Biosciences, St. Louis, MO) and digested with *Hpa*I. Southern analyses were performed using DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany). DIG labeled Lambda DNA, cleaved with *Hind*III was used as the molecular weight marker on the gel. The *pyrF* probe used for hybridization was an internal 582-bp PCR fragment obtained with the primers: P1 5′-TGCACTTGCCAATGATGTCTTA-3′ and P2 5′-CAAAGCCAATTCAGCCTCAAATG-3′.

Table 1
Strains and plasmids used in this study.

	Relevant characteristics or applications	Reference/Source
Strains		
R. oryzae 99880, *R. oryzae* M16	Wild type strain *R. oryzae* 99880 uracil auxotrophic mutant	Skory and Ibrahim, 2007 [13]
R. oryzae NRRL 6400	Genomic DNA for *fumR* and *pyc* genes	NRRL
R. oryzae fumR1, R2, R3	*R. oryzae* M16 transformed with plasmids *pPyrF2.1A-fumR1*, *pPyrF2.1A-fumR2*, *pPyrF2.1A-fumR3*	This work
E. coli DH5α	Host cells for plasmids preparation	Invitrogen
Plasmids		
pPyrF2.1A	*R. oryzae* cloning vector. *pyrF*, orotate phosphoribosyl transferase gene for uracil complementation	Skory and Ibrahim, 2007 [13]
pGEMT	*f1 ori*, *lacZ*, *amp*, *ori*	Promega
pPyrF2.1A-fumR1	*fumR1* overexpressing plasmid	This work
pPyrF2.1A-fumR2	*fumR2* overexpressing plasmid	This work
pPyrF2.1A-fumR3	*fumR3* overexpressing plasmid	This work