Bioresource Technology 148 (2013) 91–96

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Fumaric acid production in *Saccharomyces cerevisiae* by simultaneous use of oxidative and reductive routes

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## HIGHLIGHTS

- The simultaneous use of reductive and oxidative routes was investigated.
- The introduction of *RoFUM1* gene led to the decreased of fumaric acid titer.
- Pyruvate carboxylase plays as a valve in fumaric acid synthetic pathway.
- The level of biotin affects the distribution of carbon flow.
- Fumaric acid production is regulated by carbon–nitrogen ratio.

## ARTICLE INFO

*Article history:*
Received 19 June 2013
Received in revised form 16 August 2013
Accepted 19 August 2013
Available online 26 August 2013

*Keywords:*
*Saccharomyces cerevisiae*
Fumaric acid
Oxidative and reductive routes
Pyruvate carboxylase
Carbon/nitrogen ratio

## ABSTRACT

In this study, the simultaneous use of reductive and oxidative routes to produce fumaric acid was explored. The strain FMME003 (*Saccharomyces cerevisiae* CEN.PK2-1C*ΔTHI2*) exhibited capability to accumulate pyruvate and was used for fumaric acid production. The *fum1* mutant FMME004 could produce fumaric acid via oxidative route, but the introduction of reductive route derived from *Rhizopus oryzae* NRRL 1526 led to lower fumaric acid production. Analysis of the key factors associated with fumaric acid production revealed that pyruvate carboxylase had a low degree of control over the carbon flow to malic acid. The fumaric acid titer was improved dramatically when the heterologous gene *RoPYC* was overexpressed and 32 μg/L of biotin was added. Furthermore, under the optimal carbon/nitrogen ratio, the engineered strain FMME004-6 could produce up to 5.64 ± 0.16 g/L of fumaric acid. These results demonstrated that the proposed fermentative method is efficient for fumaric acid production.



## 1. Introduction

Recently, bio-based fumaric acid production has received considerable attention because it could be a cost-effective and ecologically sustainable alternative to the current petrochemical process, thus promising a significantly higher market potential. Fungi such as *Rhizopus oryzae* and *Rhizopus arrhizus* can naturally accumulate and excrete large amounts of fumaric acid via cytosolic reductive TCA pathways under aerobic conditions with limited amount of nitrogen (Ferreira et al., 2013; Gu et al., 2013; Kenealy et al., 1986; Wang et al., 2013); however, their industrial-scale use is limited because their morphology can strongly affect production characteristics. Furthermore, product safety is questionable because these fungi have potential pathogenic properties. The yeast *Saccharomyces cerevisiae* is a well-established and robust industrial production host, exhibiting extraordinarily high acid- and osmotolerance, and is generally regarded as safe. These features, in conjunction with the sophisticated toolbox for genetic engineering, make *S. cerevisiae* particularly suitable for the production of carboxylic acids such as lactic acid (Pacheco et al., 2012), malic acid (Zelle et al., 2008, 2010), and succinic acid (Otero et al., 2013; Raab et al., 2010).

It was proposed that the challenge in metabolic engineering of *S. cerevisiae* for the production of carboxylic acids involves at least four levels, one of which is engineering fast and efficient metabolic pathways that link the high-capacity glycolytic pathway in *S. cerevisiae* to the target compound, taking into account the redox and free-energy constraints (Abbott et al., 2009). *S. cerevisiae*, in its natural state, cannot produce fumaric acid, and thus, an efficient synthetic pathway of this chemical needs to be constructed. Two metabolic pathways can be used for fumaric acid production. In the first pathway, fumaric acid production via reductive TCA cycle

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http://dx.doi.org/10.1016/j.biortech.2013.08.115

**Table 1**
*S. cerevisiae* strains used in this study.

| Strain | Relevant genotype |
|---|---|
| FMME002 | CEN.PK2-1C |
| FMME003 | CEN.PK2-1C *ΔTHI2* |
| FMME004 | CEN.PK2-1C *ΔTHI2*, *ΔFUM1* |
| FMME004-1 | CEN.PK2-1C *ΔTHI2*, *ΔFUM1*, *pY26TEF/GPD* |
| FMME004-2 | CEN.PK2-1C *ΔTHI2*, *ΔFUM1*, *↑RoMDH*, |
| FMME004-3 | CEN.PK2-1C *ΔTHI2*, *ΔFUM1*, *↑RoMDH*, *↑RoFUM1* |
| FMME004-4 | CEN.PK2-1C *ΔTHI2*, *ΔFUM1*, *pY15TEF*, *↑RoMDH*, *↑RoFUM1* |
| FMME004-6 | CEN.PK2-1C *ΔTHI2*, *ΔFUM1*, *↑RoPYC*, *↑RoMDH*, *↑RoFUM1* |

(reductive route) provides a maximum theoretical yield of 2 mol/mol glucose. Moreover, this process involves $CO_2$ fixation, instead of release, which is of great interest because of increasing concerns about climate change. In our previous study, an exogenous fumaric acid biosynthetic pathway involving reductive reactions of the TCA cycle was successfully introduced in *S. cerevisiae* via a series of simple genetic modifications (Xu et al., 2012a). However, the energy balance for fumaric acid synthesis via reductive TCA cycle is barely even and does not provide any ATP for maintenance and active transport processes, and the redox balance is uneven. In the second pathway, fumaric acid production is carried out via oxidative TCA cycle (oxidative route) and the engineered strain is stable in the fermentation process. However, this process leads to carbon loss, thus limiting the maximum theoretical fumaric acid yield to 1 mol/mol glucose. Our previous study showed that fumaric acid can be produced via an oxidative TCA cycle with the aid of *in silico* metabolic engineering (Xu et al., 2012c).

A perfect metabolic model for fumaric acid production should simultaneously take into account redox and free-energy constraints as well as a relatively high maximum theoretical fumaric acid yield. Stoichiometrically, when compared with purely oxidative synthesis (1 mol/mol glucose), the simultaneous use of reductive and oxidative TCA cycles (oxido/reductive route) leads to a higher theoretical fumaric acid yield from glucose (1.71 mol/mol glucose), because the released $CO_2$ is again fixed. Moreover, this model can provide a positive energy balance, and even redox balance.

In the present study, the simultaneous use of reductive and oxidative routes to produce fumaric acid was explored, and the key factors for fumaric acid production were analyzed. Furthermore, the key enzyme that restricts the carbon flow redirected towards fumaric acid synthesis pathway was overexpressed. In addition, to further improve fumaric acid production, the effect of carbon/nitrogen (C/N) ratio on fumaric acid production was also investigated.

## 2. Methods

### 2.1. Microorganisms and cell maintenance

All yeast strains used in this study (Table 1) were derived from the strain *S. cerevisiae* CEN.PK2-1C (MATa ura3-52 leu2-3,112 trp1-289 his3Δ MAL2-8c SUC2). The yeasts were kept frozen at −80 °C in stock culture of 25% glycerol and 75% yeast culture (volume fraction). *Escherichia coli* JM109 was used as a host for recombinant DNA manipulation and plasmid maintenance, and was routinely cultured using Luria–Bertani (LB) medium supplemented with 100 mg/L of ampicillin.

### 2.2. Strain construction

The yeast strain FMME003 was constructed as described in our previous study, which could accumulate up to 8.21 ± 0.30 g/L of pyruvate under thiamine-limited condition (Xu et al., 2012b). The yeast strain FMME004 was obtained by deleting the *FUM1* gene of FMME003 using the primers 45-F(FUM1) and 45-R(FUM1).

The genes *RoMDH* and *RoFUM1* were amplified by PCR using the cDNA of *R. oryzae* NRRL1526 as template, and the pY26TEF-*RoFUM1*-GPD-*RoMDH* plasmid was obtained as described in our previous study (Xu et al., 2012a). The gene *RoPYC* was also amplified by PCR using the cDNA of *R. oryzae* NRRL1526 as template, and the resulting PCR fragment of *RoPYC* and expression vector pY15-TEF1 were digested with SpeI and SalI and ligated together to create the pY15TEF1 plasmid. All primers used in this study are listed in Table 2.

### 2.3. Yeast transformation

The plasmids were introduced into the yeast cells using a Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. Transformation of disruption cassette was performed by using lithium-acetate method (Gietz and Woods, 2006). The transformants were selected on synthetic complete (SC) selection medium lacking specific amino acid or pyrimidine for the auxotrophic markers.

### 2.4. Medium formulation

LB medium (5 g/L of yeast extract, 10 g/L of tryptone, and 10 g/L of NaCl) was used for plasmid purification from *E. coli* JM109. The fermentation medium contained the following (per L): 50 g of glucose, 2 g of $CO(NH_2)_2$, 5 g of $KH_2PO_4$, and 0.8 g of $MgSO_4{\cdot}7H_2O$, and 10 mL of trace metal solution (0.2 g/L of $MnCl_2{\cdot}H_2O$, 2 g/L of $FeSO_4{\cdot}7H_2O$, 2 g/L of $CaCl_2{\cdot}2H_2O$, 0.05 g/L of $CuSO_4{\cdot}5H_2O$, and 0.5 g/L of $ZnCl_2$). In a flask, 40 g/L of $CaCO_3$ (dry-heat-sterilized at 160 °C for 30 min) were used as buffer for the fermentation medium. Precultures were grown in seed medium containing the following: 20 g/L of glucose, 1.7 g/L of Yeast Nitrogen Base (without amino acid or ammonium sulfate), and 5 g/L of ammonium sulfate. The pHs of the seed and fermentation media were adjusted to 5.5 with 2 M NaOH, and both the media were heat-sterilized for 20 min at 115 °C. After cooling, the corresponding filter-sterilized amino acid mix and uracil were added. For nitrogen-limited cultivations, urea (to a final concentration of 0.1, 0.2, 0.4, 1.0, and 2.0 g/L) was added.

### 2.5. Shake flask cultivations

The seed culture was inoculated with well-grown yeast on an agar slant and incubated for 24 h in a 250-mL flask containing 20 mL of seed medium. Then, the broth was centrifuged, the pellet

**Table 2**
Primers used in this study.

| Primers | Sequences (5′–3′) |
|---|---|
| 45-F(FUM1) | GAAATTCCATAAAGTCTAACTATTAAACGGATAAGAGATACAATCCAGCTGAAGCTTCGTACGC |
| 45-R(FUM1) | TTATTTAGGACCTAGCATGTGTTCAGGAACAACCCATTCATCAAAGCATAGGCCACTAGTGGATCTG |
| 5′RoPYC | ATGCCTGCTGCACCAGTAC |
| 3′RoPYC | TTAGGCTTCCTCTTTGACAACC |
| SpeI-5′RoPYC | GGACTAGTATGCCTGCTGCACCAGTAC |
| 3′RoPYC-SalI | ACGCGTCGACTTAGGCTTCCTCTTTGACAACC |