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Research Article

Regulation of thiamine synthesis in *Saccharomyces cerevisiae* for improved pyruvate production

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Abstract

Metabolic engineering of *Saccharomyces cerevisiae* for high-yield production of carboxylic acid requires a cytosolic pyruvate pool as precursor. In this study, a novel strategy to improve pyruvate production and reduce metabolic by-products via regulating thiamine synthesis was explored. Two of the thiamine biosynthesis regulatory genes, *THI2* and *THI3*, were disrupted in the *S. cerevisiae* parent strain FMME-002. The mutants FMME-002Δ*THI2* and FMME-002Δ*THI3* both exhibited an enhanced pyruvate yield. Moreover, FMME-002Δ*THI2* achieved a relatively higher pyruvate production, and the highest concentration of pyruvate was achieved when 0.04 μM thiamine was added. Enzyme assays and fermentation profiles of the *THI2*-complemented strain indicated that the observed metabolic changes represented intrinsic effects of *THI2* deletion on the physiology of *S. cerevisiae*. Under optimal C:N ratio conditions, FMME-002Δ*THI2* produced pyruvate up to 8.21 ± 0.30 g/l, whereas the ethanol titre decreased to 2.21 ± 0.24 g/l after 96 h of cultivation. These results demonstrate the possibility of improving pyruvate production by regulating thiamine synthesis in *S. cerevisiae*. **Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: **pyruvate; *Saccharomyces cerevisiae*; thiamine synthesis; *THI2***

Introduction

The yeast *Saccharomyces cerevisiae* is a robust, well-established industrial production organism; as a metabolic engineering platform, it has been used to synthesize such biotechnologically interesting carboxylic acids as lactate (Tokuhiro *et al*., 2009), malate (Zelle *et al*., 2008, 2010) and succinate (Raab *et al*., 2010, 2011) from pyruvate by expression of heterologous enzymes and transporters or disruption of homologous enzymes. Moreover, if the aim of the *S. cerevisiae* metabolic process is to produce maximum amounts of other carboxylic acids, a high pyruvate titre (cytosolic pyruvate pool) is required. However, *S. cerevisiae* can accumulate only very small amounts of pyruvate naturally. In yeasts, pyruvate is located at the branch-point between respiratory dissimilation of sugars and alcoholic fermentation (Pronk *et al*., 1996), and ethanol formation is regarded as one of the major directions of carbon flow under fully aerobic conditions when sugars are present in excess. In this respect, substantial research effort has been directed to developing a method to improve pyruvate production and reduce metabolic by-products.

Historically, studies on improving pyruvate production and reducing ethanol formation in *S. cerevisiae* have mainly focused on interrupting pyruvate decarboxylase, but this method inhibited the ability of the strain to grow on glucose as the sole carbon source and generated hypersensitivity to high glucose concentrations in synthetic media (Flikweert *et al*., 1996, 1997; van Maris *et al*., 2004).

Co-factor levels are known to play an essential role in a large number of biochemical reactions and regulate flux through various metabolic pathways (San *et al*., 2002). For example, the first

committed step towards ethanol production in alcoholic fermentation requires the ThDP-dependent enzyme PDC to catalyse the irreversible conversion of pyruvate to acetaldehyde and carbon dioxide (Figure 1A). Thiamine was confirmed to be the most important factor affecting pyruvate production in *Torulopsis glabrata* (Li *et al*., 2001). *S. cerevisiae* utilizes external thiamine for the production of thiamine diphosphate (ThDP), but can also synthesize the co-factor *de novo* (Figure 1B). Three genes have now been identified as positive regulatory factors in the thiamine biosynthetic pathway. *THI2* is allelic with *PHO6*, and *THI2* mutants have been found to be defective in thiamine biosynthesis but fully capable of normal thiamine transport (Kawasaki *et al*., 1990; Nishimura *et al*., 1992a). The *THI3* mutation prevents expression of all thiamine-regulated genes, which indicates that the THI3 protein, or its isoforms, may act as a global regulator (Nishimura *et al*., 1992b). *PDC2* is the third positive activator, which is necessary for the expression of not only *THI* genes but also PDC structural genes (*PDC1* and *PDC5*) (Nosaka, 2006). Deletion of either *THI2* or *THI3* inhibits the strain's ability to grow in the absence of thiamine, but only on glucose medium (Mojzita and Hohmann, 2006). Deletion of *PDC2*, on the other hand, inhibits growth in the presence of glucose, even with supplemented thiamine (Hohmann, 1993). Many studies have since concentrated on elucidating the molecular mechanisms of this novel type of regulation (Figure 1C) (Nosaka, 2006), but the mode of action of these regulatory proteins and the nature of their interactions have remained elusive. Surprisingly, to the best of our knowledge, no reports in the literature have yet described attempts to decrease the activity of PDC via regulating thiamine synthesis with the aim of improving pyruvate production and reduce metabolic by-products.

To investigate the feasibility of such an approach, we derived *THI2*-deleted and *THI3*-deleted mutants from the reference strain FMME-002. The ability of manipulation of either of these genes to effectively improve pyruvate production was evaluated. Comparative analysis was then carried out to identify the more effective of the two genetic modifications to improve pyruvate production under pyruvate-producing conditions with suboptimal thiamine.

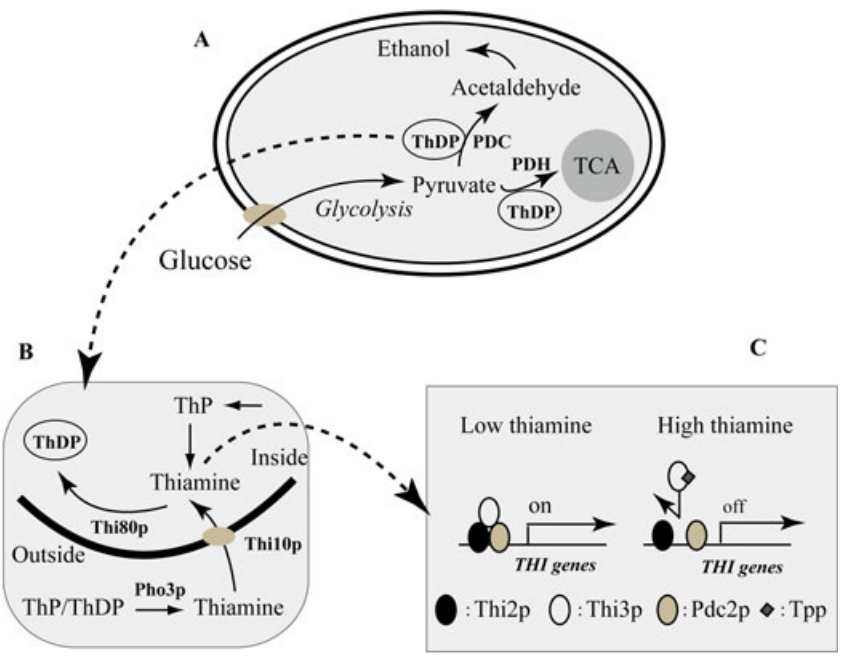

Figure 1. The relationships among the ethanol pathway, thiamine metabolism and *THI* regulatory system in *S. cerevisiae*: (A) thiamine diphosphate (ThDP) in yeast pyruvate metabolism; (B) schematic outline of thiamine metabolism in yeast; (C) hypothetical model of the yeast *THI* regulatory system

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Materials and methods

Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* CEN.PK2-1 C (*MAT***a** *ura3-52 leu2-3,112 trp1-289 his3Δ MAL2-8c SUC2*) was obtained from EUROSCARF (Frankfurt, Germany). All yeast strains used in this study were derived from strain *S. cerevisiae* CEN.PK2-1 C. *Escherichia coli* strain JM109 was used for plasmid construction and plasmid maintenance.

Plasmid pUG27 was constructed as previously described (Gueldener *et al*., 2002). Briefly, the *kanMX* marker in pUG6 was replaced with the his5$^+$ marker from pFA6a–HIS3MX6 (which complements the *S. cerevisiae his3* mutation), using the *Bgl*II and *Sac*I restriction sites. The Cre-expressing plasmid pSH47 was used for marker rescue.

Deletion of *THI2* and *THI3*

The vector pUG27 was used to generate deletions of *THI2* (1353 bp) and *THI3* (1707 bp) in FMME-002. All primers used in this study are listed in Table 1. After plasmid preparation, a fragment of pUG27 was amplified by PCR to obtain a cassette consisting of *loxP–kanMX–loxP*. The resulting PCR product was composed of the *kanMX* gene, loxP sites and homologous regions for the target gene in FMME-002. The PCR products were transformed into yeast by the LiAc/SS Carrier DNA/PEG method. Homologous recombination in yeast led to the deletion of the target genes. Positive clones were selected by incubation in synthetic complete (SC) selection medium (Difco Yeast Nitrogen Base supplemented with a mixture of amino acids, including uracil but without histidine). The HIS marker was then removed from the positive clones via transformation with pSH47, as previously described (Guldener *et al*., 1996). Subsequently, the Cre plasmid pSH47 was removed from this yeast strain.

Media and cell cultivation

Medium for slant and seed cultures contained 10 g/l yeast extract, 20 g/l tryptone and 20 g/l glucose. The fermentation medium consisted of 60 g/l glucose, 7 g/l NH_4Cl, 5 g/l KH_2PO_4 and 0.8 g/l $MgSO_4{\cdot}7H_2O$. The initial pH was adjusted to 5.0, and different concentrations of filter-sterilized thiamine HCl were added to the fermentation medium. The pH buffer $CaCO_3$ was sterilized by dry heat at 160 °C for 30 min before being

Table 1. Lists of strains, plasmids and primers used in this study

Strain/plasmid/primer	Description/genotype/sequence (5′ to 3′)	Reference or source
Strains		
FMME-002	*MAT***a** reference strain	EUROSCARF
FMME-002*ΔTHI2*	*MAT***a**; *THI2*::*loxP*	This study
FMME-003*ΔTHI3*	*MAT***a**; *THI3*::*loxP*	This study
Plasmids		
pFA6a-HIS3MX6	HIS3	Lab collection
pUG27	Amp, *Sz. pombe* HIS5	This study
pSH47	Amp, GAL1–cre, URA3	(Guldener *et al*., 1996)
*Primers**		
THI2-F	ACCACGTATATATATAGCCTATATATATATCCGCA CTAGAACCAACAGCTGAAGCTTCGTACGC	This study
THI2-R	TGGCTTTTTTTTTCTTGAAATGAGTGAAGGGAAG GCTCAATAAGCGCATAGGCCACTAGTGGATCTG	This study
THI3-F	CAGCTGAACATACATACCATATTTGGACTCTCCG GAGAATTTAGCCAGCTGAAGCTTCGTACGC	This study
THI3-R	AGCGGTAATCATGAGGGTCCCTGGTAGTAGGGC GGAGAGATCAGAGCATAGGCCACTAGTGGATCTG	This study
*Spe*I-F(THI2)	GGACTAGTATGGTCAATAGTAAGAGGCAGCAG	This study
*Sal*I-R(THI2)	GCGTCGACCTAGTCCTGCATGGCATATACATC	This study

Underlining indicates 19–22 nucleotides homologous to sequences flanking the disruption marker on a plasmid or restriction sit.
*F, forward; R, reverse.

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added into the medium. *Escherichia coli* cells were grown in Luria–Bertani medium containing 0.5% yeast extract, 1% tryptone and 1% NaCl, which was heat-sterilized for 15 min at 121 °C. For culturing cells carrying the plasmid, the sterile medium was supplemented with ampicillin (100 mg/l).

A seed culture was inoculated with a single colony from a fresh YPD plate and incubated for 24 h in a 250 ml flask containing 20 ml seed medium. The cells were harvested, washed twice in sterile water and then inoculated into a 250 ml flask containing 50 ml fermentation medium. The inoculum size was 5% v/v. Cultures were incubated in a shaker set at 200 rpm for 96 h at 30 °C. All experiments were carried out in triplicate.

Analytical methods

To determine cell growth, each culture broth was diluted to the appropriate fold with 0.1 M HCl and optical density (OD) measured at 600 nm on a spectrophotometer. Extracellular concentrations of pyruvate, ethanol and glucose were determined by HPLC, using an Aminex HPX-87 H column (Bio-Rad). Fractions were eluted with 0.0275% v/v H_2SO_4 at a flow rate of 0.6 ml/min at 35 °C. Pyruvate was detected using an Agilent 1100 series VWD detector at 210 nm. Ethanol and glucose were detected using a 1100 series Agilent refractive index detector.

Measurement of enzyme activity

Cells harvested at different stages were obtained by centrifuging fermentation broth at 8000 rpm for 5 min. Cell pellets were washed with ice-cold 40 mM imidazole–HCl buffer, pH 6.5. The cell suspension was ultrasonicated for 10 min at 0 °C and the cell debris was recovered by centrifugation at 8000 rpm for 5 min and washed with the same buffer. Pyruvate decarboxylase activity was assayed at 30 °C immediately after preparation of the extracts, using a Hitachi model 100–60 spectrophotometer set at 340 nm. Reaction rates were linearly proportional to the amount of cell-free extract added. The assay mixture consisted of 40 mM imidazole–HCl buffer, pH 6.5, 0.2 mM thiamine pyrophosphate, 0.15 mM NADH, 88 U/ml alcohol dehydrogenase (Boehringer), 5 mM $MgCl_2$ and cell-free extract. Enzyme assays were performed at 30 °C with freshly prepared extracts. Total protein concentration was measured by the Lowry *et al*. (1951) method.

Construction of a *THI2*-complement strain

The *S. cerevisiae THI2* gene was amplified by PCR from chromosomal DNA of FMME-002, using the primers *Spe*I-F(*THI2*) and *Sal*I-R(*THI2*) (Table 1). The resulting fragment and pY14TEF1 plasmid were digested with *Spe*I and *Sal*I and then ligated to create pY14TEF1–*THI2*. The final plasmid was then used to transform strain FMME-002*ΔTHI2*.

The plasmid was introduced into yeast cells using a Frozen-EZ Yeast Transformation II kit (Zymo Research), following the manufacturer's protocol. The transformants were selected on SC selection medium agar plates lacking various specific amino acids, used as auxotrophic markers (prototrophy). The transformant that showed trptophan prototrophy was selected, and restoration of its thiamine synthesis was confirmed by the fermentation profiles investigation.

Results

Effect of *THI2* or *THI3* deletion on pyruvate production

To investigate the effect of thiamine synthesis defect on pyruvate accumulation and ethanol formation, two approaches were used to disturb the synthesis of thiamine: deleting either the *THI2* gene or the *THI3* gene. Wild-type FMME-002 can produce 0.82 ± 0.08 g/l pyruvate and 5.27 ± 0.23 g/l ethanol after 96 h cultivation, while these two deletions resulted, respectively, in 3.12- and 2.18-fold increases in the final pyruvate titre (Figure 2a) and 89.9% and 81.0% decreases in final ethanol titre (Figure 2b). Moreover, in comparison with the FMME-002*ΔTHI3* mutant, this mutant FMME-002*ΔTHI2* exhibited more pyruvate production, high growth rate and specific glucose consumption rate (Figure 2c, d). Based upon these findings, the FMME-002*ΔTHI2* mutant was chosen for subsequent detailed study.

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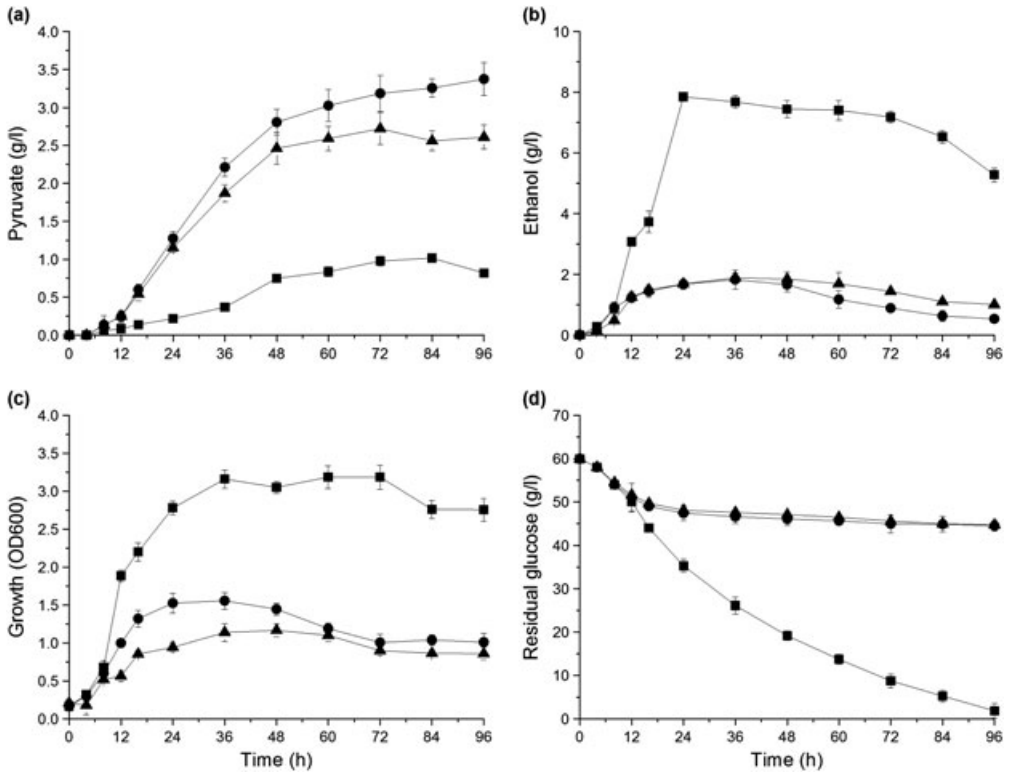

Figure 2. Profiles of pyruvate metabolism by FMME-002, FMME-002Δ*THI2* and FMME-002Δ*THI3*: (a) pyruvate; (b) ethanol; (c) growth; (d) residual glucose. Square, FMME-002; circle, FMME-002Δ*THI2*; triangle, FMME-002Δ*THI3*. Values are presented as means of three independent experiments; bars represent SD

Effect of thiamine concentration on pyruvate production

In order to gain further insights into how pyruvate production by *S. cerevisiae* may be maximized, the effect of thiamine concentration on the pyruvate production and ethanol formation was also studied. For the FMME-002Δ*THI2* mutant, the highest concentration of pyruvate (3.82 ± 0.14 g/l) and a relatively low ethanol titre (4.57 ± 0.27 g/l) were achieved at 36 h when 0.04 μM thiamine was added to the medium broth. The results also demonstrated

Table 2. Comparison of growth and compound yields of FMME-002, FMME–002Δ*THI2* during aerobic batch growth on 6% glucose under different concentrations of thiamine

Parameter	FMME-002[a]	FMME-002Δ*THI2*[a]			
	VB1 = 0 μM	VB1 = 0 μM	VB1 = 0.04 μM	VB1 = 0.08 μM	VB1 = 0.3 μM
Fermentation time (h)	36	36	36	36	36
Biomass (OD_{600})	3.16 ± 0.12	1.56 ± 0.11	2.24 ± 0.08	2.78 ± 0.09	2.95 ± 0.11
Growth rate (h^{-1})	0.291	0.258	0.267	0.275	0.282
Pyruvate (g/l)	0.37 ± 0.04	3.08 ± 0.10	3.82 ± 0.14	2.95 ± 0.11	0.81 ± 0.08
Pyruvate yield (mol/mol)	0.022	0.401	0.335	0.229	0.053
Ethanol (g/l)	7.68 ± 0.29	1.87 ± 0.11	4.57 ± 0.27	5.76 ± 0.16	6.78 ± 0.22
Ethanol yield (mol/mol)	0.914	0.268	0.766	0.809	0.846
Residual glucose (g/l)	25.58 ± 1.76	42.68 ± 1.14	35.07 ± 0.64	32.13 ± 1.44	28.64 ± 1.26

[a]Triplicate fermentations.
Data ± SD.

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that further increase of thiamine concentration led to great improvement of the growth rate and the specific glucose consumption rate, but decreased pyruvate yield and increased ethanol yield (Table 2).

Pyruvate decarboxylase activity measurements

To elucidate the metabolic alterations associated with *THI2* deletion and thiamine addition, the specific activity of PDC, which is the first key enzyme involved in the pathway between pyruvate and ethanol, was measured at various times during the fermentation process. Figure 3 shows PDC activity in FMME-002 and in FMME-002*ΔTHI2* cells. In the early-stationary phase, no apparent decrease was observed in the specific activity of PDC when *THI2* was deleted. After 48 h of incubation without supplemented thiamine, PDC reached its maximum specific activity in FMME-002, while it had a dramatical decrease after *THI2* deletion (67.0%; $p < 0.05$, $n = 3$). When 0.04 μM thiamine was added, the PDC activity of FMME-002*ΔTHI2* was increased by 10.5%. These results suggested that relative decreased PDC activity was sufficient to improve pyruvate production.

Fermentation profiles of *THI2*-complemented strain

To confirm that the observed metabolic alterations were actually caused by *THI2* deletion, the profiles of fermentation by the parent strain, *THI2*-deleted mutant and *THI2*-complemented strain were investigated. As shown in Figure 4a, b, the parent strain and *THI2*-complemented strain showed similar pyruvate production and ethanol formation. Similarly, the *THI2*-complemented strain showed even higher growth rate during the logarithmic growth phase, while the *THI2*-deleted mutant exhibited significantly decreased growth rate as compared to the parent strain (Figure 4c). Moreover, no apparent difference was observed in the glucose consumption rate between the parent strain and *THI2*-complemented strain (Figure 4d). Thus, the *THI2*-complemented strain showed similar fermentation profiles to the parent strain, which indicated that the *THI2* deletion was responsible for the observed metabolic changes.

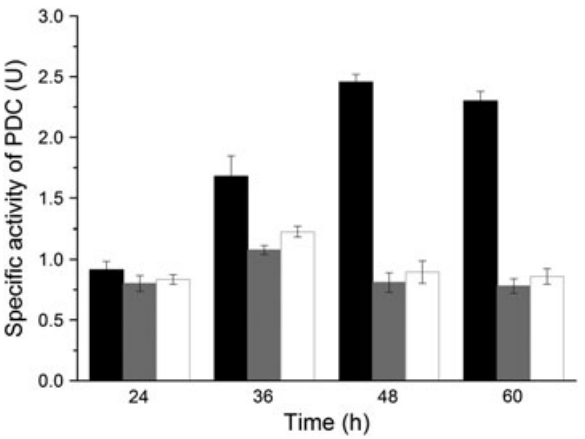

Figure 3. Activity of pyruvate decarboxylase in the fermentation process: dark grey bar, FMME-002; grey, FMME-002*ΔTHI2*; white, FMME-002*ΔTHI2* supplemented with 0.04 μM thiamine

Batch culture process of *THI2*-deleted mutant

To further increase the biomass and pyruvate titre, we explored the effect of nitrogen and C:N ratio on pyruvate production. In the batch culture of FMME-002*ΔTHI2*, a high pyruvate titre (8.21 ± 0.30 g/l) and biomass ($OD = 3.61 \pm 0.16$) were obtained, while a slightly high ethanol titre was achieved when 2.00 g/l urea was used and the C:N ratio was set at 35:2 after 96 h cultivation (Figure 5). This overall performance represents a 9.0-fold improvement in pyruvate production and a 58.06% reduction in ethanol formation compared to the parent strain. These results confirm that pyruvate production can be improved effectively by regulating thiamine synthesis and optimizing medium composition in *S. cerevisiae*.

Discussion

In this study, a novel genetic-based approach was designed to improve pyruvate production and reduce ethanol formation in *S. cerevisiae* by regulating thiamine synthesis. We investigated the primary effects of *THI2* and *THI3* knockout on pyruvate production and ethanol formation, and found that both of these single mutants were able to produce an enhanced pyruvate production (Figure 2a) and a decreased ethanol formation (Figure 2b). Furthermore, the *THI2*-deleted mutant produced a higher pyruvate production during the middle-stationary growth phase, leading us to focus

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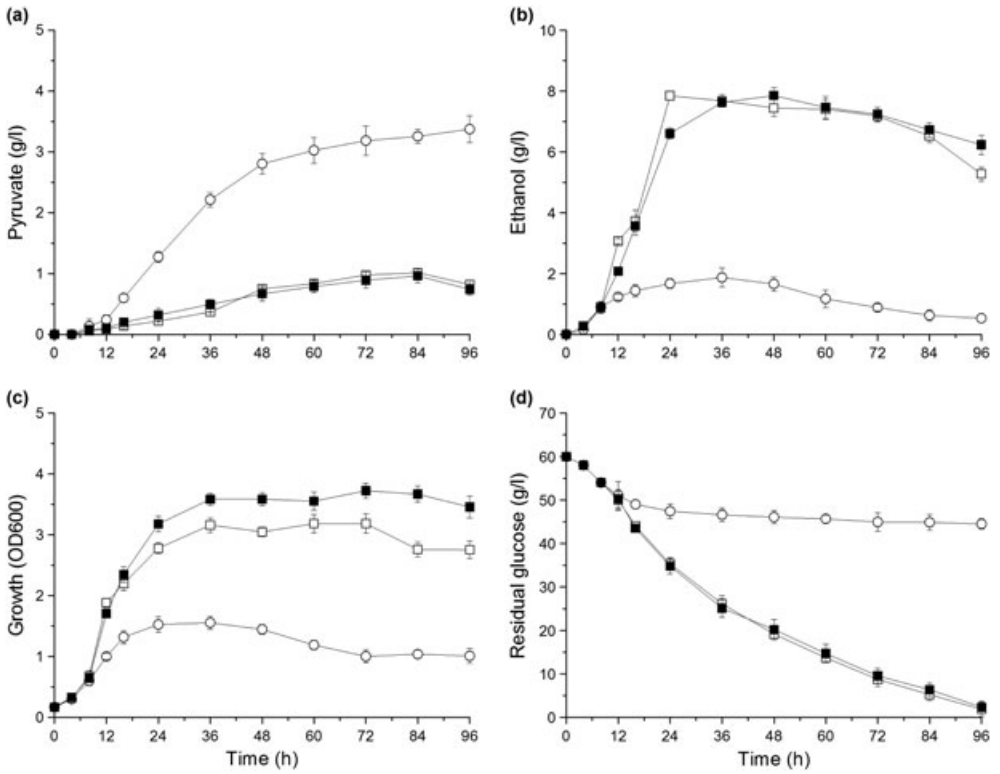

Figure 4. Profiles of pyruvate metabolism by the parent strain, *THI2*-deleted mutant and *THI2*-complemented strain: (a) pyruvate; (b) ethanol; (c) growth; and (d) residual glucose. Open square, parent strain; open circle, *THI2*-deleted mutant; closed square, *THI2*-complemented strain. Values are presented as means of three independent experiments; bars represent SD

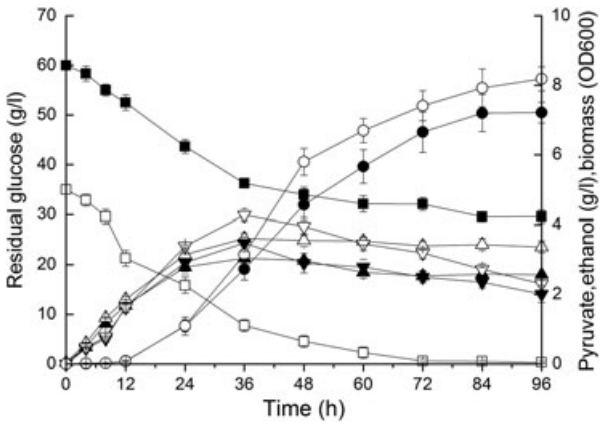

Figure 5. Fermentation profiles for cell growth, glucose utilization and product accumulation during batch cultures of FMME-002Δ*THI2*. Square, glucose; circle, pyruvate; triangle, growth; inverted triangle, ethanol; closed symbols, C:N = 60:2; open symbols, C:N = 35:2. Values are presented as means of three independent experiments; bars represent SD

subsequent studies on FMME-002Δ*THI2*. Then the effect of thiamine concentration on pyruvate production was studied. Since these fermentation characteristics were similar between *THI2*-complemented strain and the parent strain (Figure 4), specific studies were carried out to confirm that these metabolic changes were due to the *THI2* deletion. Under the optimal C:N ratio condition, FMME-002Δ*THI2* showed a 9.0-fold improvement in pyruvate production and a 58.06% reduction in ethanol formation. These results have confirmed the utility of this alternative genetic-based approach to improving pyruvate production in *S. cerevisiae*.

The reasons for pyruvate accumulation in this study was that the genetic manipulation and sub-optimal thiamine concentration supplementation led to a relatively low activity of both the pyruvate dehydrogenase complex and pyruvate decarboxylase, which are responsible for the breakdown to acetyl-CoA and acetaldehyde, respectively. After

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the deletion of *THI2*, the pyruvate yield on glucose increased to 0.335 mol/mol from 0.022 mol/mol, while the ethanol yield on glucose decreased to 0.766 mol/mol from 0.914 mol/mol between 0 and 36 h in the fermentation medium supplemeted with 0.04 μM thiamine (Table 2). From these data, it was calculated that about 16.2% carbon flow redirected from ethanol to pyruvate or other metabolites can be seen; in other words, about 47.3% carbon flow of pyruvate could be derived from the reduced carbon flow of ethanol. In addition, a relatively low growth rate was also observed, which could be caused by a relatively activity of pyruvate dehydrogenase.

The decreased biomass production observed in both FMME-002*ΔTHI2* and FMME-002*ΔTHI3* under pyruvate-producing conditions without thiamine addition (Figure 2c) was unexpected, since deletion of *THI2* and *THI3* had previously been reported to inhibit growth in the absence of thiamine on glucose medium (Mojzita and Hohmann, 2006). On the one hand, in the first 24 h, the mutants both showed ethanol formation, cell growth, glucose consumption and pyruvate accumulation in the absence of thiamine supplementation. These unexpected phenomena could be explained by the existence of a small quantity of thiamine in the cell after it was inoculated from the complex medium of the preculture to the defined medium of the fermentation. On the other hand, the roles of *THI2* and *THI3* in regulating thiamine synthesis need to be discussed. Mutation in the *THI2* gene has been demonstrated to cause defects in thiamine synthesis (Hohmann and Meacock, 1998), suggesting that *THI2* is required for proper thiamine synthesis. However, deletion of the *THI2* gene has also been shown to disturb the expression of other *THI* genes, with the notable exception of *THI10*. Specifically, a *THI2* mutant exposed to thiamine starvation conditions was shown to have significantly reduced expression of *THI4*, *THI5*, *PHO3*, *THI6* and *THI80* genes (Nosaka, 2006). This finding may indicate that *THI2* is not uniquely required for thiamine synthesis in *S. cerevisiae*, but may act either in conjunction with or upstream of other *THI*s. Similarly, it has been proposed that the *THI3* gene is required for the induction of all *THI* genes in response to thiamine deprivation (Nosaka, 2006); this notion is inconsistent with our study's findings. Furthermore, *thi3p* has no similarity to any other known transcriptional regulator and must act via interaction with other protein(s). Thus, the roles of *THI2* and *THI3* in regulating thiamine synthesis and their interaction remain unknown and need to be further investigated.

In this study, FMME-002*ΔTHI2* exhibited a relatively low specific rate of glucose consumption under a suboptimal thiamine condition, especially after the logarithmic growth phase. On the one hand, the pentose phosphate pathway might be affected, due to transketolase catalysing the reversible, ThDP-dependent transfer reactions, and the impaired pentose phosphate pathway decreased the rate of NADPH synthesis, which led to a slow glucose consumption rate because the decrease in NADPH inhibited the synthesis of cell materials (Hua *et al*., 1999). On the other hand, aerobic ethanol formation in *S. cerevisiae* has been demonstrated to be due to a limited capacity of the respiratory system involved in oxidation of mitochondrial NADH (Vemuri *et al*., 2007). Moreover, the NADH/NAD^+ co-factor pair is generally considered to play a central role in glucose catabolism, and studies have demonstrated that glycolytic flux can be regulated by cytosolic NADH/NAD^+ (Luttik *et al*., 1998), while a lack of NAD^+ results in termination of the glycolysis process. Thus, manipulating the redox balance may be an effective approach to improve the glucose consumption rate and further improve pyruvate production.

In summary, the present study has provided an alternative approach to increasing pyruvate yield, and reduce ethanol production, by regulating thiamine synthesis, which is an important step for achieving high yields of carboxylic acid from engineered yeast strains.

Conclusions

The use of *S. cerevisiae* for interesting carboxylic acid production from renewable feedstock has evoked great interest, due to dwindling petroleum resources and concerns about climate change. Moreover, significant progress has been made in improving carboxylic acid yield using metabolic engineering. To further enhance desired product production, any metabolic engineering strategy for carboxylic acid production requires a cytosolic pyruvate pool as precursor. In this study, the

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strategies focused upon exploring the effect of *THI2* deletion on pyruvate accumulation and investigating the effect of thiamine concentration on pyruvate production. Modification of regulatory protein in the thiamine biosynthetic pathway to improve pyruvate production, shown in this study, would provide the precursor for desired carboxylic acid production.

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