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Proteomic Evaluation of Cellular Responses of *Saccharomyces cerevisiae* to Formic Acid Stress

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Formic acid is a representative carboxylic acid that inhibits bacterial cell growth, and thus it is generally considered to constitute an obstacle to the reuse of renewable biomass. In this study, *Saccharomyces cerevisiae* was used to elucidate changes in protein levels in response to formic acid. Fifty-seven differentially expressed proteins in response to formic acid toxicity in *S. cerevisiae* were identified by 1D-PAGE and nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analyses. Among the 28 proteins increased in expression, four were involved in the MAP kinase signal transduction pathway and one in the oxidative stress-induced pathway. A dramatic increase was observed in the number of ion transporters related to maintenance of acid-base balance. Regarding the 29 proteins decreased in expression, they were found to participate in transcription during cell division. Heat shock protein 70, glutathione reductase, and cytochrome c oxidase were measured by LC-MS/MS analysis. Taken together, the inhibitory action of formic acid on *S. cerevisiae* cells might disrupt the acid-base balance across the cell membrane and generate oxidative stress, leading to repressed cell division and death. *S. cerevisiae* also induced expression of ion transporters, which may be required to maintain the acid-base balance when yeast cells are exposed to high concentrations of formic acid in growth medium.

KEYWORDS : Formic acid, MAPK signal transduction, Nano-LC-MS/MS, Proteomics, *Saccharomyces cerevisiae*

The production of non-wood biomass-based transportable ethanol (NWBBTE) has been accompanied with increasing oil prices and environmental pressures. Possible feedstocks for ethanol production of NWBBTE are sugarcane bagasse [1], rice straw [2], palm kernel press cake [3], corn stover [4], miscanthus [5], and switchgrass [6]. These non-wood biomasses are composed of cellulose, hemicellulose, and lignin and therefore need to be converted to fermentable sugars and compounds, which further can be used by microorganisms to produce various products. Pretreatment for the enzymatic saccharification of polysaccharides was performed by mineral acid-catalyzed hydrolysis [7]. Depending on the severity of the hydrolytic conditions (acid concentration, temperature and time), the polysaccharides are then cleaved by different degrees into soluble mono- and oligo-sugars [8, 9]. Inhibitory byproducts are concomitantly generated by this pretreatment process [10]. In particular, short-chain organic acids such as formic, acetic, and levulinic acids are formed as the major degradation products from hemicellulosic biomass [11]. These hydrolytic byproducts are often non-fermentable, inhibitory, or toxic to microorganisms, including yeast and *Escherichia coli* [12, 13]. Therefore, microorganisms that use biomass hydrolysates for bioethanol production should be able to survive under the stressful environment created by the byproducts.

Proteomic techniques are often employed for the profiling of whole proteins in target cells as well as differently expressed proteins in a stressful environment, along with the detection of protein interactions and modifications [14]. Analyses using 1D-PAGE and nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) have been recognized as powerful and fast compared to 2D-PAGE and matrix-assisted laser desorption/ionization-time of flight mass spectrometry analyses [15]. Herein, we used proteomic techniques to investigate the mode of inhibition of formic acid on the growth and survival of *Saccaromyces cerevisiae* fermenting biomass hydrolysates. In this regard, to measure formic acid toxicity, the differential expression of proteins in yeast cells with or without formic acid was profiled by 1D-PAGE and nano-LC-MS/MS. We identified the presumable target site of formic acid inhibition as well as the defense mechanisms responsible for formic acid-generated toxicity.

Materials and Methods

Strain and cultivation. *S. cerevisiae* (ATCC26603) was used in this study. Standard yeast media, culture conditions, and bioassays for pheromone response were prepared as previously described [16]. The flask cultures were shaken at 200 rpm and 30°C for 48 hr. In the first

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24 hr, the cells were grown on glucose (2 g/L) to a dry cell mass concentration of about 1 g/L. The external pH was controlled at 6.9, and the pH increased to 7.4 after the cultivation. Solutions (pH 6.5~7) of formic acid were aseptically added to the cultures to a concentration of 5 g/L. Glucose was also added to one flask for comparison purposes. The flask cultures were shaken under the same conditions for 24 hr. The cells were harvested by centrifugation at 5,000 g for 20 min and then freeze-dried for later use.

1D SDS-PAGE. 1D SDS-PAGE was performed as described by Laemmli [17]. Samples of 20 μg were mixed with SDS-PAGE sample buffer and heated at 100°C for 5 min. The denatured proteins were separated on 10~20% gradient polyacrylamide SDS gels and then stained by Coomassie dye G-250 (Bio-Rad, Hercules, CA, USA). For determination of molecular weight, 10 μL of precision plus protein standards (Bio-Rad) was applied to the gels. All protein bands were sliced from the gel, destained with 50% (v/v) acetonitrile in 50 mM NH_4HCO_3, and then completely dried in a speed-vacuum centrifuge. Then, 20 μL of sequencing-grade modified porcine trypsin (20 μg/μL in 50 mM NH_4HCO_3) was added to the dried gel slices treated previously with dithiothrietol and iodoacetamide. The unabsorbed solution was removed before 20 μL of NH_4HCO_3 was added to the rehydrated slices. These samples were then incubated at 37°C overnight. Tryptic digestion was stopped by addition of 5 μL of 2% trifluoroacetic acid (TFA). The digested peptides were extracted from each gel slice by sonication of 0.1% TFA and 50% acetonitrile/0.1% TFA for 45 min. Both supernatants were combined for LC-MS/MS analysis.

Nano-electrospray LC-MS/MS analysis. LC-MS/MS analyses were carried out using the Ultimate™ system interfaced to a quadruple ion trap mass spectrometer (Bruker Dlatonics, Billerica, MA, USA). The gradient consisted of (A, 0.1% formic acid; B, 0.1% formic acid in acetonitrile) 5% B for 5 min, 60% B for 88 min, 95% B for 10 min, 5% B for 15 min, and 5% B for 20 min. Peptide spectra were recorded over a mass range of m/z 300~2500, and MS/MS spectra were recorded using information-dependent data acquisition over a mass range of m/z 50~1600. One peptide spectrum was recorded, followed by two MS/MS spectra; the accumulation time was 1 sec for the peptide spectra and 2 sec for the MS/MS spectra. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Doubly or triply charged ions were selected for product ion spectra. MS/MS spectra were interpreted by Mascot (Matrix Science Ltd., London, UK) using Biotools ver. 2.2 software (Bruker Daltonics).

Analysis of peptide sequences. Peptide mass fingerprint (PMF) searches based on the measured peptide masses were performed using the SWISSPROT database or MSDB database with the Mascot program. PMF searches were based on the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. Mass tolerance of 1.0 Da was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparing the search results against the estimated random match populations and were reported as: $10 \times \log_{10}(p)$, where p is the absolute probability. Scores in Mascot greater than that at $p = 0.05$ were considered significant. Therefore, for scores higher than that at $p = 0.05$, the probability of that match being a random event is lower than 0.05.

The algorithm used to determine the probability of a false-positive match with a given mass spectrum is described elsewhere [18].

Western immunoblot analysis. Western blots were performed with a semidry Fasblot apparatus (Bio-Rad, Richmond, CA, USA) as follows. Anti-serum against heat shock protein (HSP) 70 of *S. cerevisiae* (Darwinbio, Seoul, Korea) was used. This anti-serum was applied to a nitrocellulose

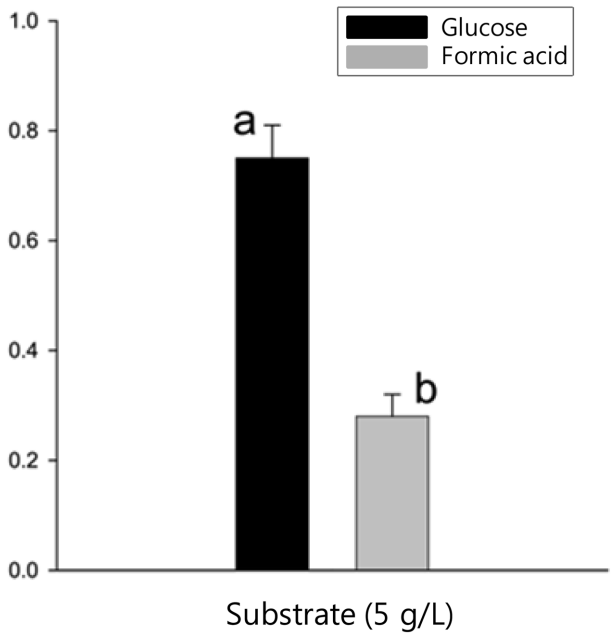

Fig. 1. Cell mass concentrations of *Saccharomyces cerevisiae* cultivated in mineral solution (pH 6.8~7.5) at 30°C. Cells were grown on glucose (2 g/L) for 24 hr and then exposed to 5 g/L of glucose and formic acid for 24 hr. Different letter indicates a significant difference between the two groups ($p = 0.05$).

membrane, and horseradish peroxidase-antibody conjugate (Darwinbio) was also applied to the membrane. Bound antibodies were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA) containing luminol.

Determination of cytochrome c oxidase (COX) and glutathione reductase activities. Mitochondria were prepared essentially as described by Faye *et al*. [19], except that zymolyase 20,000 instead of glusulase was used to digest the cell wall. The post-mitochondrial supernatant was kept for the detection of CytC. Mitochondria were washed twice with 0.5 M sorbitol at 12,000 rpm for 15 min and suspended in 0.5 M sorbitol. Protein concentration was measured by the method published by Lowry *et al*. [20]. COX activity was measured at room temperature in

Table 1. Identification of upregulated proteins in *Saccharomyces cerevisiae* after exposure to formic acid

No.	Protein name	No. of matched peptides	Mascot score (value $p = 0.05$)	Accession No.	Species	DB search engine
			Signal transduction proteins			
1	MAP kinase kniase kinase wis4	4	32 (27)	O14299	*Saccharomyces cerevisiae*	Mascot
2	MAP kinase kinase kinase mkh1	6	30 (30)	Q10407	*S. cerevisiae*	Mascot
3	Nuclear protein SON1	4	32 (31)	SON1_Yeast	*S. cerevisiae*	OWL31.4
			Regulatory proteins			
4	Regulatory protein Sir3 (silent information regulator 3)	6	39 (30)	P06701	*S. cerevisiae*	Mascot
5	Osmolarity two-component system protein SLN1	3	41 (31)	SLN1_Yeast	*S. cerevisiae*	OWL31.4
6	Peroxide stress-activated histidine kinase	9	35 (27)	O14002	*S. cerevisiae*	Mascot
7	Pyrimidine pathway regulatory protein 1	4	33 (29)	PPR1_Yeast	*S. cerevisiae*	OWL31.4
			Transporters			
8	Mitochondrial carnitine carrier	3	30 (30)	Q12289	*S. cerevisiae*	Mascot
9	Myo-inositol transporter	4	30 (27)	Q10286	*S. cerevisiae*	Mascot
10	Electron transporter	5	36 (27)	Q10361	*S. cerevisiae*	Mascot
11	Probable cation-transporting ATPase	4	32 (27)	O74431	*S. cerevisiae*	Mascot
12	Calcium-transporting ATPase 2 (vacuolar $Ca^{(2+)}$-ATPase)	3	31 (28)	P38929	*S.cerevisiae*	Mascot
13	Potassium transporter protein	4	28 (28)	P47946	*S. cerevisiae*	Mascot
14	Protein transporter protein	7	38 (27)	O13817	*S. cerevisiae*	Mascot
15	Vacuolar membrane ATPase sub unit a	3	40 (30)	Q874G9_Yeast	*S. cerevisiae*	Mascot
16	Sodium transport ATPase 5	5	31 (29)	Q12691	*S. cerevisiae*	Mascot
17	Nitrogen permease protein	2	30 (29)	P39923	*S. cerevisiae*	Mascot
18	Vitamin H transporter	3	31 (29)	P53241	*S. cerevisiae*	Mascot
			Other proteins			
19	Activator of HSP70 and HSP90 chaperons	6	34 (30)	T41531	*S. cerevisiae*	Mascot
20	Halotolerance protein	4	34 (27)	O94505	*S. cerevisiae*	Mascot
21	2-Isopropylmalate synthase	4	32 (31)	LEU1_Yeast	*S. cerevisiae*	OWL31.4
22	Cytochrome c oxidase polypeptide VI	3	32 (26)	Q9UTF6	*S. cerevisiae*	OWL31.4
23	Fatty acid synthase beta subunit	5	28 (27)	Q9UUG0	*S. cerevisiae*	OWL31.4
24	Glucose repressible protein	3	31 (28)	Q02197	*S. cerevisiae*	Mascot
25	Glucose-induced degradation protein 8	2	34 (29)	P40208	*S. cerevisiae*	Mascot
26	Long-chain fatty acid-CoA ligase	5	42 (26)	O60135	*S. cerevisiae*	Mascot
27	Cullin-4 homolog	3	26 (25)	O14122	*S. cerevisiae*	Mascot
28	Urease	4	36 (27)	O00084	*S. cerevisiae*	Mascot

Results are LC-MS/MS data processed with Mascot search engine and the homology alignments. Uniprot and TIGR classification were used to search cellular roles of the identified proteins.

LC-MS/MS, liquid chromatography-tandem mass spectrometry; HSP, heat shock protein.

20 mM K_2HPO_4, pH 7.5, containing 65 μM reduced CytC. COX activity was assayed in mitochondria (10 μg of protein) permeabilized with potassium deoxycholate by measuring oxidation of ferrocytochrome c at 550 nm in order to maximize substrate accessibility to the enzyme active site.

Table 2. Identification of downregulated proteins in *Saccharomyces cerevisiae* after exposure to formic acid

No.	Protein name	No. of matched peptides	Mascot score (value $p = 0.05$)	Accession No.	Species	DB search engine
	Signal transduction proteins					
1	1-Phosphatidylinositol-3-phosphate 5-kinase FAB1	8	46 (29)	P34756	*Saccharomyces cerevisiae*	Mascot
	Regulatory proteins					
2	Transcription regulator CRZ1	3	30 (29)	P53968	*S. cerevisiae*	Mascot
3	Transcription regulatory protein SWI3	2	32 (30)	P32591	*S. cerevisiae*	Mascot
4	Histone transcription regulator 2	3	34 (29)	P32480	*S. cerevisiae*	Mascot
5	Cell division control protein 37	3	40 (30)	CC37_Yeast	*S. cerevisiae*	OWL31.4
6	Cell division control protein 66 Myosin-2	4	31 (29)	P19524	*S. cerevisiae*	Mascot
7	Transcription regulatory protein SNF 12	3	31 (29)	P53628	*S. cerevisiae*	Mascot
8	Transcription factor SPT8	2	48 (29)	P38915	*S. cerevisiae*	Mascot
	Other proteins					
9	Origin recognition complex subunit 1	5	33 (28)	P54784	*S. cerevisiae*	Mascot
10	Myosin tail region-interacting protein MTI1 (BBC1 protein)	3	32 (28)	P47068	*S. cerevisiae*	Mascot
11	Imidazole glycerol phosphate synthase hisHF (IGP synthase)	5	37 (29)	P33734	*S. cerevisiae*	Mascot
12	SIT4-associating protein SAP55	5	38 (30)	P43612	*S. cerevisiae*	Mascot
13	Acetyl-CoA synthetase 1	3	33 (30)	Q01574	*S. cerevisiae*	Mascot
14	GTPase-activating protein (Bud emergence protein)	6	35 (30)	P39960	*S. cerevisiae*	Mascot
15	5-Aminolevulinate synthase, mitochondrial precursor	3	33 (29)	P09950	*S. cerevisiae*	Mascot
16	Ribosome biogenesis protein MAK21	3	33 (29)	Q12176	*S. cerevisiae*	Mascot
17	Histone deacetylase complex 1 subunit 2	5	31 (29)	Q06629	*S. cerevisiae*	Mascot
18	Glutathione reductase	3	32 (29)	P41921	*S. cerevisiae*	Mascot
19	Neutral trehalase	5	30 (29)	P32356	*S. cerevisiae*	Mascot
20	PAB1-binding protein	5	48 (29)	P53297	*S. cerevisiae*	Mascot
21	Calcium-binding mitochondrial carrier SAL1 (suppressor of AAC2 lethality)	5	29 (29)	P48233	*S. cerevisiae*	
22	Succinyl-CoA ligase	2	31 (29)	P53312	*S. cerevisiae*	Mascot
23	Bud site selection protein BUD3	4	37 (29)	P25558	*S. cerevisiae*	Mascot
24	D-lactate dehydrogenase	7	33 (29)	P39976	*S. cerevisiae*	Mascot
25	Nuclear control of ATPase messenger RNA expression protein	4	37 (29)	P32493	*S. cerevisiae*	Mascot
26	Ubiquitin conjugation factor	4	33 (29)	P54860	*S. cerevisiae*	Mascot
27	Sterol O-acyltransferase 2	2	30 (29)	P53629	*S. cerevisiae*	Mascot
28	Alpha-1,3-mannosyltransferase MNN1	2	38 (29)	P39106	*S. cerevisiae*	Mascot
29	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	2	35 (29)	P19262	*S. cerevisiae*	Mascot

Results are LC-MS/MS data processed with Mascot search engine and the homology alignments. Uniprot and TIGR classification were used to search cellular roles of the identified proteins.

Glutathione reductase activity was measured according to the method described previously by Smith *et al*. [21] by monitoring the reduction of 5,5-dithiobis (2-nitrobenzoic acid) by glutathione, which is produced by glutathione reductase.

Results

Fig. 1 shows the cell concentrations of *S. cerevisiae* grown on media with or without 5% formic acid. The low cell mass concentration obtained with formic acid suggests that the acid inhibited cell growth. For comparison, the cells grew very well on media without formic acid.

Electrospray tandem LC-MS/MS measurements of the samples prepared by 1D SDS-PAGE showed mass differences between *S. cerevisiae* cells with or without formic acid. Approximately 300 proteins out of more than 1,000 hits were identified by Mascot search, and 57 of them were chosen to measure the formic acid toxicity in *S. cerevisiae*. The cells contained 29 upregulated proteins (Table 1) and 28 downregulated ones (Table 2) after exposure to formic acid for 24 hr. Overexpression of COX and downregulation of glutathione reductase were reevaluated by determining the activities of the enzymes (Table 3). Two enzymes showed significantly different activities according to their expression in response to formic acid stress.

Among the proteins increased in expression, 11 were transporters for the efflux of toxic products or the maintenence of acid-base balance in the cells (Table 1). These proteins were identified as mitochondrial carnitine carrier, myo-inositol transporter, electron transporter, probable cation-transporting ATPase, calcium-transporting ATPase, potassium transporter protein, vacuolar membrane ATPase, sodium transporter ATPase, nitrogen permease protein, and vitamin H transporter. One chaperone protein, HSP70, was increased (Table 1, Fig. 2). Among the upregulated proteins, the homologies of mitogen-activated protein kinase (MAPK) kinase kinases wis4 and mkh1 were investigated in the formic acid-treated yeast cells (Table 1). Sir3 and SLN1 proteins and a peroxide stress-activated histidine kinase were also enhanced in response to surplus formate in the environment. In addition, halotolerance protein, fatty acid synthase, glucose-induced degradation protein, long-chain fatty acid-CoA ligase, and cullin-4 homolog were also upregulated after the addition of formic acid and might have been involved in the reduction of oxidative stress generated by formic acid (Table 1).

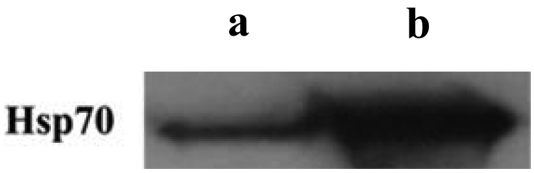

Fig. 2. Effect of formic acid on expression of heat shock proteins (HSPs) in *Saccharomyces cerevisiae* as analyzed by Western blotting. a, *S. cerevisiae* grown in a media containing glucose; b, *S. cerevisiae* grown in media containing formic acid at a concentration of 5 g/L.

Formic acid causes the downregulation of transcription factors such as CRZ1, SWI3, CDC37, CDC66, SNF12, and SPT8 (Table 2). Among the decreased proteins, GTPase-activating protein and bud site selection protein BUD3 played roles in the replication mechanisms (Table 2). Proteins such as ribosome biogenesis protein MAK21, nuclear control of ATPase messenger RNA expression protein, and succinyl-CoA synthetase participated in the biogenesis of biomolecules. Lactate dehydrogenase, which plays an important role in gluconeogenesis, was also downregulated (Table 2).

Discussion

Yeast cells are enclosed by a semi-permeable membrane and maintain ionic balance for normal cell function. Thus, cells exposed to osmotic stress can protect themselves by osmoregulation, including regulation of cell volume, increase of osmolyte concentration, change of ion transport systems, and protection of DNA or proteins from oxidative attack. In relation to osmoregulation, MAPKs play an important role in the response to stress by multi-relay phosphorylation in cells.

Mode of inhibitory action of formic acid in *S. cerevisiae* and induction of HSP. Formic acid is a well-known toxicant on microbial growth, and its mode of inhibition is based on breakdown of the acid-base balance in living bacterial cells [22]. Penetration of free acid molecules causes acidification of the cell cytoplasmic membrane by dissociation. As a result, the proton gradi-

Table 3. Cytochrome c oxidase and glutathione reductase activities in *Saccharomyces cerevisiae* in response to formic acid

Classification	Cytochrome c oxidase[a]	Fold	Glutathione reductase[b]	Fold
Control	2.93 ± 0.21*a*[c]	1	6.26 ± 0.77*a*	1
Formic acid-treated yeast	3.56 ± 0.15*b*	1.22	4.53 ± 0.38*b*	0.72

[a]Cytochrome c oxidase activity was expressed as micromoles of cytochrome c oxidized per minute per milligram of mitochondrial protein.
[b]One milliunit is defined as the activity that produces 1 nmol of 5-thio(2-nitrobenzoic acid) per min.
[c]The means in the column followed by the different italic letters are significantly different from the control ($p < 0.05$) using t-test.

ent through the membrane cannot be maintained, which decouples the energy generation process. Compared to acetic acid (pKa 4.75), formic acid can acidify the cytoplasm very fast due to its 10-fold higher dissociation constant (pKa 3.75). Formic acid, which differs from other short chain aliphatic acids, is an aldehyde that may inhibit the central enzymes of glycolysis and the citric acid cycle [13].

Several proteins involved in acid-base balance were increased in the formic acid-treated *S. cerevisiae* cells (Table 1), especially sodium transport ATPase, potassium transport protein, and calcium-transporting ATPase. Most of these proteins were transporters that require energy to pump protons outwards across the membrane. In bacterial cells, electron transfer produces energy through a consecutive respiratory chain, which is essential for ATP synthesis downstream of most catabolic pathways, including carbohydrates. Therefore, COX may be increased after formic acid treatment in *S. cerevisiae* cells in order to promote energy production (Table 1).

Among the 29 proteins increased in expression, one heat shock protein was increased after formic acid treatment (Table 1, Fig. 1). Heat shock proteins are highly conserved and have been demonstrated as molecular chaperones that bind non-covalently to exposed hydrophobic surfaces of non-native proteins. Little information on this finding is available. Therefore, further studies may explain the roles of heat shock proteins in *S. cerevisiae* in response to formic acid stress.

Upregulation of MAPK signal transduction pathways. Yeast cells contain five MAPK cascades that respond to various environmental and physiological stimuli [22]. In this study, three different MAPKs, wis4, mkh1, and ssk2 were upregulated in response to formic acid in yeast cells.

Interestingly, wis4 protein was active in a signal transduction pathway activated under conditions of heat shock, oxidative stress, or limited nutrition. Unusually, it was not activated by changes in osmorality of the extracellular environment. In addition to wis4 protein, peroxide stress-activated histidine kinase was also upregulated in response to formic acid. This protein is involved in the control of the SAPK-dependent transcriptional response to peroxide stress found in *S. pombe* [23]. Thus, the upregulation of wis4 and homology of peroxide stress-activated histidine kinase may indicate that formic acid addition induces oxidative stress.

MAPK cascade activiation may lead to Sir3p hyperphosphorylation and strengthen transcriptional silencing [24]. Therefore, the upregulation of Sir3 in this study (Table 1) may have resulted from the activation of MAPK signal transduction pathways, which led to various transcriptional silences in response to formic acid toxicity. However, it may be worthwhile to investigate the hyperphosphorylation of Sir3 since an increase in Sir3 protein in cells did not contribute to gene silencing.

Induction of fatty acid biosynthesis in *S. cerevisiae*. Fatty acid synthetase catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH. The beta subunit of this protein contains domains for acetyltransferase and malonyltransferase, S-acyl fatty acid synthase thioesterase, enoyl-[acyl-carrier-protein] reductase, and 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase. Malonyl-CoA, which is a precursor of fatty acid biosynthesis, is produced from acetyl-CoA by enzyme acetyl-CoA carboxylase. In yeast cells, acetyl-CoA carboxylase was expressed similarly in the media with or without formic acid (data not shown). Therefore, the two substrates, acetyl-CoA and malonyl-CoA, might have been formed by acetyl-CoA carboxylase during fatty acid biosynthesis. Moreover, a long-chain fatty acid-CoA ligase was also upregulated in the yeast cells grown in media containing formic acid, whereupon it catalyzed the esterification of exogenous long-chain fatty acids into metabolically active CoA thioesters for subsequent degradation or incorporation into phospholipids. Therefore, induction of these two proteins appeared to play a role in maintaining fatty acid biosynthesis and degradation during exposure to formic acid.

Toxic effects of formic acid in *S. cerevisiae* via downregulation of factors. The growth of yeast cells was inhibited by exposure to formic acid under aerobic conditions in our study (Fig. 1). The distinct inhibitory effect of formic acid on the growth of *S. cerevisiae* seems to have resulted from three different routes. Firstly, transcription factors were silenced after formic acid treatment (Table 2). Downregulation was observed for cell division control proteins 37 and 66, which plays a role in Ste11 function and pheromone-induced cell cycle arrest and acts as a target site of calmodulin, respectively [25, 26]. Transcription factor CRZ1 and histone transcription regulator 2 were also downregulated, which may have affected cell progression and development by altering the deacetylation of lysine residues in the N-termini of core histones [27, 28]. In response to these reductions, histone deacetylase 1 was also reduced. Other transcription factors such as SWI3/SNF12, which is involved in an ATP-dependent chromatin remodeling complex, were decreased in response to formic acid toxicity [29]. Downregulation of other transcription factors in response to formic acid may have resulted in the upregulation of Sir3 protein in yeast cells. Secondly, three proteins that participate in the biosynthesis and transformation of succinyl-CoA in cells, succinyl-CoA ligase, 5-aminolevulinate synthase, and succinyltransferase component, were downregulated. However, we could not determine why formic acid affected these biochemi-

cal routes. Finally, formic acid inhibited protein biosynthesis in yeast cells by downregulating ribosome biogenesis and nuclear control of ATPase mRNA expression (Table 2). Therefore, formic acid has potent toxicities to a variety of proteins involved in the cell cycle and biosynthetic pathways in *S. cerevisiae*.

Conclusively, proteomic analysis showed that *S. cerevisiae* differentially expressed proteins in yeast cells exposed to formic acid. According to The Institute for Genomic Research protein classification, most of the overexpressed proteins were MAPK cascade proteins involved in the responses to formic acid breakdown of ion homeostasis. *S. cerevisiae* also had increased numbers of ion transporters to control the extracellular changes in ion milieu. Several transcription factors were downregulated possibly be due to Sir3, which was upregulated in response to formic acid toxicity in yeast cells. Further studies that examine the genetic and metabolomic changes in *S. cerevisiae* will be necessary to understand formic acid stress and address the overall effects of short-chain organic acids on biomass hydrolysates during yeast fermentation.

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References

1. Cardona CA, Quintero JA, Paz IC. Production of bioethanol from sugarcane bagasse: status and perspectives. Bioresour Technol 2010;101:4754-66.
2. Binod P, Sindhu R, Singhania RR, Vikram S, Devi L, Nagalakshmi S, Kurien N, Sukumaran RK, Pandey A. Bioethanol production from rice straw: an overview. Bioresour Technol 2010;101:4767-74.
3. Jøjensen H, Sanadi AR, Felby C, Lange NE, Fischer M, Ernst S. Production of ethanol and feed by high dry matter hydrolysis and fermentation of palm kernel press cake. Appl Biochem Biotechnol 2010;161:318-32.
4. Li X, Kim TH, Nghiem NP. Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF). Bioresour Technol 2010;101:5910-6.
5. Sørensen A, Teller PJ, Hilstrøm T, Ahring BK. Hydrolysis of Miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment. Bioresour Technol 2008;99:6602-7.
6. Keshwani DR, Cheng JJ. Switchgrass for bioethanol and other value-added applications: a review. Bioresour Technol 2009;100:1515-23.
7. Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 2000;25:1503-55.
8. Grohmann K, Bothast RJ. Saccharification of corn fibre by combined treatment with dilute sulphuric acid and enzymes. Proc Biochem 1997;32:405-15.
9. Olsson L, Hahn-Hagerdal B. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. Proc Biochem 1993;28:249-57.
10. Fan LT, Lee YH, Gharpuray MM. The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis. Adv Biochem Eng Biotechnol 1982;23:157-87.
11. Torget R, Walter P, Himmel M, Grohmann K. Dilute-acid pretreatment of corn residues and short-rotation woody crops. Appl Biochem Biotechnol 1991;28/29:75-86.
12. Larsson S, Palmqvist E, Hahn-Hagerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant NO. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Technol 1999;24:151-9.
13. Yu J, Wang JP. Metabolic flux modeling of detoxification of acetic acid by *Ralstonia eutropha* at slightly alkaline pH levels. Biotechnol Bioeng 2001;73:458-64.
14. Aebersold R. Constellations in a cellular universe. Nature 2003;422:115-6.
15. Lee SE, Li QX, Yu J. Proteomic examination of *Ralstonia eutropha* in cellular responses to formic acid. Proteomics 2006;6:4259-68.
16. Bardwell L, Cook JG, Chang EC, Cairns BR, Thorner J. Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. Mol Cell Biol 1996;16:3637-50.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
18. Berndt P, Hobohm U, Langen H. Reliable automatic protein identification from matrix-assisted laser desorption/ionization mass spectrometric peptide fingerprints. Eletrophoresis 1999; 20:3521-6.
19. Faye G, Kujawa C, Fukuhara H. Physical and genetic organization of petite and grande yeast mitochondrial DNA. IV. *In vivo* transcription products of mitochondrial DNA and localization of 23 S ribosomal RNA in petite mutants of *Saccharomyces cerevisiae*. J Mol Biol 1974;88:185-203.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193:265-75.
21. Smith IK, Vierheller TL, Thorne CA. Assay of glutathione reductase in crude tissue homogenates using 5,5′-dithiobis(2-nitrobenzoic acid). Anal Biochem 1988;175:408-13.
22. Maeda T, Wurgler-Murphy SM, Saito H. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 1994;369:242-5.
23. Buck V, Quinn J, Soto Pino T, Martin H, Saldanha J, Makino K, Morgan BA, Millar JB. Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. Mol Biol Cell 2001;12:407-19.
24. Stone EM, Pillus L. Activation of an MAP kinase cascade leads to Sir3p hyperphosphorylation and strengthens transcriptional silencing. J Cell Biol 1996;135:571-83.
25. Abbas-Terki T, Donzé O, Picard D. The molecular chaperone Cdc37 is required for Ste11 function and pheromone-induced cell cycle arrest. FEBS Lett 2000;467:1111-6.
26. Brockerhoff SE, Stevens RC, Davis TN. The unconventional myosin, Myo2p, is a calmodulin target at sites of cell growth is *Saccharomyces cerevisiae*. J Cell Biol 1994;124:315-23.

27. Stahopoulos-Gerontides A, Guo JJ, Cyert MS. Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev 1999;13: 798-803.
28. Spector MS, Raff A, DeSilva H, Lee K, Osley MA. Hir1p and Hir2p function as transcriptional corepressors to regulate histone gene transcription in the *Saccharomyces cerevisiae* cell cycle. Mol Cell Biol 1997;17:545-52.
29. Peterson CL, Herskowitz I. Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription. Cell 1992;68:573-83.