Comprehensive metabolic profiling of Geotrichum candidum and comparison with Saccharomyces cerevisiae

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(Citation)
Journal of Bioscience and Bioengineering,137(1):9-15

(Issue Date)
2024-01

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/0100491712

Title

Comprehensive metabolic profiling of *Geotrichum candidum* and comparison with *Saccharomyces cerevisiae*

Short title

Comprehensive metabolic profiling of *G. candidum*

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Abstract

Geotrichum candidum is a dimorphic yeast used in cheese processing. However, to our knowledge, no major metabolites have been identified to date in *G. candidum* except for some amino acid and fatty acid metabolites. This has limited research on the commercial use of *G. candidum*. In this study, we aimed to analyze temporal changes in the intra- and extra-cellular metabolites of *G. candidum* and *Saccharomyces cerevisiae* as reference, cultured in YM medium. As a result of metabolite analysis, *G. candidum* proclivity to accumulate the pentose phosphate pathway compounds, which is involved in nucleic acid synthesis, after 48 h of cultivation in comparison to *S. cerevisiae*. In addition, *G. candidum* accumulated higher amounts of the antioxidant glutathione in the medium than did *S. cerevisiae*. In addition, *G. candidum* accumulated large amounts of B vitamins such as pantothenic acid and nicotinic acid in the medium. Finally, we examined the potential of *G. candidum* as a host for the production of useful compounds, such as pantothenic acid. When cultured in medium supplemented with the pantothenic acid precursor *β*-alanine, *G. candidum* produced 12-fold higher amounts of pantothenic acid (30 μM) than that by *S. cerevisiae*. This study indicates that *G. candidum* accumulates various useful compounds that are dissimilar to those produced by *S. cerevisiae*. Furthermore, *G. candidum* has the potential to produce useful chemicals under appropriate culture conditions.

Keywords

Saccharomyces cerevisiae, Geotrichum candidum, Ultrastructure, Metabolome analysis, Pantothenic acid

Introduction

Yeasts have been widely used to produce various foods and metabolites; for example, *Saccharomyces cerevisiae* is used in the production of beer, bread, and wine (1), and yeasts such as *Geotrichum candidum* are important for the surface ripening of cheese (2). *G. candidum* is a dimorphic yeast existing in, both, the yeast and hyphal forms (3). This morphological switch may be associated with gene expression and/or metabolomic changes. Certain strains of *S. cerevisiae* are known to undergo a morphological change to pseudohyphal due to changes in gene expression upon nitrogen starvation (4). However, the determinants of *G. candidum* dimorphism and the effects of cell morphology on cheese production remain unknown.

The key to the ideal utilization of yeast is to understand the distinct metabolomic features of each yeast strain. This understanding has further developed by recent advances in the mass spectrometry (MS)-based identification and quantification of metabolites, leading to the data-driven engineering of yeasts to optimize fermentation or the production of useful metabolites. This is particularly true for the model yeast *S. cerevisiae*, in which metabolomic data can be readily validated by genetic manipulation and multi-omics information can be integrated (5).

In a previous study, we developed an ion pair-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantitative metabolite profiling, which permitted the high-precision quantification of more than 100 metabolites (6). In the present study, we used this method and aimed to characterize metabolites from *G. candidum* using *S. cerevisiae* as a reference strain. Majority of the metabolic pathways in *S. cerevisiae* have been extensively characterized and a well-developed metabolic and genetic database. (Saccharomyces Genome Database; https://www.yeastgenome.org). On the other hand, although the absence of a dedicated database for *G. candidum*, genomic information is

readily accessible (Genbank accession number; GCA_001402995.1) (7). A limited number of research have been conducted regarding the metabolic processes of *G. candidum*. In a previous study, the metabolism of sulphur amino acids, branched-chain amino acids, and fatty acids, which are involved in the production of volatile compounds that affect the flavor of cheese, was investigated in *G. candidum* (8). On the other hand, in this study, we conducted a comprehensive analysis of an expanded array of metabolites encompassing central metabolic pathways in yeast, in order to explore novel applications of *G. candidum*, including the production of valuable chemical compounds. This study also may provide some insights regarding the dimorphism of yeast at the molecular level and its applications in cheese ripening and other industrial applications.

Materials and methods

Strains and media

G. candidum ATCC 204307 (ATCC, Manassas, VA, USA) and *S. cerevisiae* BY4741 (Life Technologies, Carlsbad, CA, USA) were used in this study. Precultivation was performed in a test tube with 10 mL yeast and mold (YM) medium [3.0 g/L yeast extract (Difco Laboratories, Detroit, MI, USA), 3.0 g/L malt extract, 5.0 g/L peptone, and 10 g/L glucose] in a shaker incubator at 180 rpm for 48 h at 25 °C. For metabolome analysis, the precultured cells were then inoculated into a new shake flask with 200 mL YM medium to an initial optical density at 600 nm (OD_{600}) of 0.5, and cultivated in a shaker incubator at 180 rpm and 25 °C. For pantothenic acid production, the glucose concentration in the YM medium was varied to 10, 20, 30, 40, 50, or 100 g/L, under the same conditions.

Transmission electron microscopy

Yeast cells were collected from 5 mL of culture medium after main cultivation by

centrifugation at 3,000 × g for 5 min at 4 °C. Collected cells were washed thrice with distilled water. Cells were prefixed with 2 % glutaraldehyde in 40 mM phosphate buffer pH 7.2 (9). The samples were washed thrice with 100 mM phosphate buffer. Post-fixation was performed with 1.2 % potassium permanganate overnight at 4 °C and embedded in Epon812. Ultrathin sections were stained with 4 % uranyl acetate and 0.4 % lead citrate, and observed using a transmission electron microscope (TEM; JEM 1011; JEOL, Tokyo, Japan) operating at 80 kV.

Sample preparation for metabolome analysis

Intracellular metabolites were prepared using leakage-free quenching and cold methanol extraction (10). Briefly, 5 mL of broth was withdrawn and injected (<1 s) into a tube containing 7 mL of pure methanol cooled to -40 °C. The mixture was quickly vortexed and placed back in the cryostat (-40 °C). The extracellular medium was obtained using centrifugation at 5000 ×*g* and -20 °C for 5 min. After decantation, 10 µL of 17 µM (+)-10-camphorsulfonic acid was added to the samples as an internal standard. For determining dry cell weight, yeast cells were collected from 5 mL of broth by centrifugation at 5,000 × g for 5 min. Collected cells were washed twice with distilled water, and then freeze-dried using the FreeZone 2.5 Plus system (Labconco, Kansas City, MO, USA).

Metabolite analysis using LC-MS/MS

Quantitative analysis of more than 100 metabolites, including organic acids, amino acids, nucleosides, nucleotides, coenzymes, and other small molecules, was performed using LC-MS/MS as described previously (6). The LC-MS/MS system consisted of a Nexera X2 high-performance liquid chromatograph and an LCMS-8060 triple-quadrupole mass

spectrometer (Shimadzu Corporation, Kyoto, Japan). LC-MS/MS analysis was performed using the following conditions: column, Discovery HS F5-3 (2.1 mm × 150 mm, particle size: 3 μm, pentafluorophenylpropyl as a stationary phase, Sigma-Aldrich); mobile phase, 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in acetonitrile (B); The flow rate was 0.25 mL/min with a gradient of 0-2 min - 0 % B, 2-5 min - linear gradient from 0 % B to 25 %, 5-11 min - linear gradient from 25 % B to 35 %, 11-15 min - linear gradient from 35 % B to 95 %, 15-20 min - kept at 95 % B; 20–20.1 min - linear gradient from 95 % B to 0 %, 20.1-35 min - kept at 0 % B (re-equilibrate); injection volume: 3 μL; column temperature, 40 °C; nebulizer flow, 3.0 L/min, drying gas flow, 10.0 L/min; heating gas flow, 10 L/min; desolvation line temperature, 250 °C; interface temperature, 300 °C; block heater temperature, 400 °C. The other MS parameters were determined via auto-tuning. The acquired data were analyzed for peak selection using Traverse MS (version 1.2; Reifycs Inc., Tokyo, Japan). The multiple reaction monitoring conditions, including the collision energy and fragmentation voltage of each metabolite, were set automatically with the aid of LabSolution (Shimadzu), using a standard solution (1 μmol/L).

High-performance liquid chromatographic analysis

Glucose and ethanol concentrations in the fermentation medium were analyzed using a high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (7.8 mm × 300 mm, particle size 9 μm; Bio-Rad, Hercules, CA, USA) and a RID-10A refractive-index detector (Shimadzu). The system was operated at 65 °C, with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min.

Ammonia analysis of culture medium

Ammonia concentration in the culture supernatant was assessed using LabAssay ™ Ammonia (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's protocol.

Glycogen analysis

Yeast cells were collected from 1.5 mL of culture medium after main cultivation by centrifugation at 3,000 × g for 5 min at 4 °C. Collected cells were washed twice with distilled water and then freeze-dried using the FreeZone 2.5 Plus system (Labconco). Extraction and analysis of intracellular glycogen was determined as described previously (11).

Results and discussion

Growth profiles of *S. cerevisiae* BY4741 and *G. candidum* ATCC 204307 in YM medium

We cultured *G. candidum* and *S. cerevisiae* as reference organisms in YM medium. The glucose-consumption rate of *S. cerevisiae* (0.61 g/L/h) was approximately 1.8 times faster than that of *G. candidum* (0.34 g/L/h) (Fig. 1A). *S. cerevisiae* produced glycerol (1.1 g/L) and ethanol (5.4 g/L) at 48 h of culture, whereas *G. candidum* did not produce these metabolites (Fig. 1B,C). The maximum cell amount of *S. cerevisiae* reached 2.5 g-dry cell weight/L, whereas that of *G. candidum* reached 8.3 g-dry cell weight/L at 48 h and 72 h of cultivation, respectively (Fig. 1D). *G. candidum* showed a high cell density without ethanol production, suggesting that it is a Crabtree-negative yeast strain. The initial pH of the medium was approximately 6.2; with *S. cerevisiae*, the pH decreased continuously during culture and reached a value of 4.4 after 168 h of cultivation (Fig. 1E), whereas with *G. candidum*, the pH decreased to 4.3 after 24 h of

cultivation and then slowly increased to 6.1 after 168 h of cultivation.

Metabolome analyses of *S. cerevisiae* BY4741 and *G. candidum* ATCC 204307

To characterize the metabolism of *G. candidum*, intracellular and extracellular metabolites from *S. cerevisiae* BY4741 and *G. candidum* ATCC 204307 (at 4, 8, 24, 48, 72, 96, 120, 144, and 168 h of cultivation) were quantitatively analyzed using LC-MS/MS.

The intracellular amounts of pentose phosphate (PP) pathway compounds, such as fructose 6-phosphate, xylulose 5-phosphate, sedoheptulose 7-phosphate, and ribulose 5-phosphate, increased in *G. candidum* compared to those in *S. cerevisiae* after 48 h of culture (Fig. S1), when glucose was depleted. The major tricarboxylic acid (TCA) cycle compounds detected in the culture media differed between *S. cerevisiae* and *G. candidum* (Fig. S2). *S. cerevisiae* showed higher extracellular accumulation of 2-oxoglutarate, succinate, fumarate, and malate than those in *G. candidum* after 48 h of cultivation. In contrast, the accumulations of citrate, aconitate, and isocitrate were higher in *G. candidum* than those in *S. cerevisiae* after 48 h of culture.

There was no difference in nucleic acid accumulation between *S. cerevisiae* and *G. candidum* (data not shown). However, accumulations of the nucleobases, uracil and thymine, and ribonucleosides, guanosine, uridine, and cytidine, were higher in the culture medium of *G. candidum* than those in the culture medium of *S. cerevisiae* (Fig. S3).

The intracellular accumulation of major amino acids (except methionine) was lower in *G. candidum* than that in *S. cerevisiae* (Fig. 2). In *S. cerevisiae*, methionine and asparagine were completely consumed, whereas most of the other amino acids remained in the medium (Fig. 3). In contrast, in *G. candidum*, almost all amino acids, except glutamate and aspartate, were depleted from the medium after 72 h of cultivation. As an amino acid-related compound with characteristic differences, the accumulation of 4-

hydroxyproline, a proline derivative, in the culture medium was higher in *G. candidum* than that in *S. cerevisiae* (Fig. S4). The tripeptides, glutathione and ophthalmic acid, are known antioxidants. The intracellular accumulation of these compounds was higher in *S. cerevisiae* than that in *G. candidum* (Fig. S5A). In contrast, the extracellular accumulation of these compounds was higher in *G. candidum* (Fig. S5B).

Regarding water-soluble B vitamins, *G. candidum* accumulated more pantothenic acid (vitamin B5) in the medium than that by *S. cerevisiae* (Fig. S6). Both the strains accumulated similar amounts of niacinamide (vitamin B3) in the culture medium, whereas nicotinic acid accumulation was slightly higher in *G. candidum* than that in *S. cerevisiae*. In contrast *S. cerevisiae* accumulated more intracellular niacinamide and nicotinic acid than did *G. candidum* (Fig. S7). Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide hydrogen (NADH), which are derivatives of niacinamide, accumulated to a greater extent in *S. cerevisiae* than those in *G. candidum*. NAD and NADH are involved in aging and longevity, and have recently attracted industrial attention.

Among the vitamin-like compounds, dimethylglycine and the choline derivatives acetylcholine, carnitine, and acetylcarnitine were more abundant in *G. candidum* than those in *S. cerevisiae* (Fig. S6).

Metabolomic analysis showed that *G. candidum* intracellularly accumulated PP-pathway compounds and extracellularly accumulated nucleic acids. As *G. candidum* is superior to *S. cerevisiae* in growth, it is reasonable that *G. candidum* synthesizes higher amounts of these growth-essential compounds than does *S. cerevisiae*. The Crabtree-negative yeasts, *Pichia pastoris* and *Candida utilis,* are known to have stronger metabolic fluxes in the PP pathway than those in *S. cerevisiae* (12). One reason for the intra- and extracellular accumulation of glutathione and ophthalmic acid in both *S. cerevisiae* and

G. candidum may be a reduction in oxidative stress. Glutathione is a cellular antioxidant currently being investigated. Many antioxidants used to block oxidative stress are chemically converted into oxidation products that react with glutathione to form glutathione adducts, which protect against free radicals (13,14). Ophthalmic acid is an analogue of glutathione, wherein the cysteine group of glutathione is replaced by L-2-aminobutyrate; it has recently attracted attention as a biomarker of oxidative stress and is presumed to have an antioxidant effect similar to that of glutathione (15, 16).

In *S. cerevisiae*, the pH in the medium gradually decreased during cultivation. In contrast, in *G. candidum*, the pH of the medium initially decreased but gradually increased after glucose depletion (Fig. 1E). A similar trend in pH was also reported in a previous study of *G. candidum* (17). *G. candidum* can utilize sugars, such as glucose, and amino acids as carbon sources; the consumption of amino acids contributes to the formation of ammonium ions, thus increasing pH (17). In this study, *G. candidum* consumed most of the amino acids that were originally present in the medium or released after 72 h of culture. In contrast, some amino acids, such as cysteine and aspartic acid, were not consumed by *G. candidum*. In addition, some organic acids such as succinate, fumaric acid, and 2-oxoglutaric acid, released into the medium by *G. candidum*, were consumed after 24 h of culture, which might have contributed to the increase in pH. *G. candidum* is used in cheese production. Amino acid metabolism is a major process in the formation of aromatic compounds in cheese. Volatile sulfur-containing compounds produced by the metabolism of methionine, as well as organic acids and aldehydes produced by the metabolism of leucine, isoleucine, valine, and tyrosine, are known components contributing to the aroma of cheese. Although leucine was not detected in the present culture, *G. candidum* consumed these amino acids to a greater degree than that by *S. cerevisiae*.

Changes in the intracellular structure with morphological changes over time

G. candidum is a dimorphic yeast species, displaying, both, hyphal and yeast-like morphologies. As the first step in the morphological change, fully grown hyphae initially form septa and then desorb to produce columnar conidia called arthrospores, which appear to have a yeast-like morphology (18). In this study, changes in the morphology and intracellular structure of *G. candidum* during culture in YM medium were observed using TEM, to elucidate the relationship between morphological changes and changes in the intra- and extracellular metabolites of *G. candidum* (Fig. 4). At 4 h of culture, the cells of *G. candidum* grew from 10 to 20 μm; after 8 h of culture, they further grew to 20–30 μm and adopted a hypha-like structure with connected cell walls. After 48 h of culture, the spores were transformed into articular asexual spores. This was consistent with amino acid consumption. In *G. candidum*, the formation of yeast-like mitotic cells is enhanced on the depletion of nitrogen sources rather than carbon sources (19). Here, mitotic cells were observed after 96 h of culture when the amino acids, which served as nitrogen sources, were depleted. On the other hand, accumulation of ammonia was observed in the culture medium (Fig. S8). This result suggested that among nitrogen sources, depletion of amino acids affected morphological changes of *G. candidum*. Previous study has also suggested a relationship between amino acid consumption and morphological changes in G. candidum (20). We focused on changes in the number of mitochondria, nuclei, and vacuoles as characteristic changes in the cells. The number of mitochondria was approximately 6–20 at the start of culture but increased to 23–38 at 4 h and to approximately 35–60 at 8 h of cell growth. In the state where conidia were present (96 h of culture), the number returned to approximately 10–34. In eukaryotes, the metabolism of the TCA cycle occurs in the mitochondria; therefore, an increase in the number of

mitochondria suggests enhancement of the TCA cycle. In *G. candidum*, the extracellular accumulation of citric, aconitic, and isocitric acid increased up to 48 h during cell proliferation (Fig. S2). The number of nuclei increased from 2–5 at 4 h of culture to 3–7 at 8 h of culture due to the elongated cells; however, at other culture times, this number was 1–4 per section. There was a tendency for the number of vacuoles to be low (0–3) at 48 h of preculture and high (3–12) at 96 h. There was large variation within the same culture time, and no distinct differences were observed at different culture times. In this study, we did not observe any significant morphological changes or changes in intracellular structure after 96 h of culture. In addition, a very high electron density structure (black appearance) was observed in the cells of *G. candidum*. Remarkable aggregation was observed in cell sections at 96 h (Fig. 4). The details of the electron-dense structures are unknown; however, similar structures observed in *S. cerevisiae* contain glycogen (21). In fact, after 96 h of cultivation, glycogen accumulation was observed in both *G. candidum* (4.0 μg glucose equivalents /mg-dry cell weight) and *S. cerevisiae* (2.2 μg glucose equivalents/mg-dry cell weight) (Fig. S9). After 96 h, we observed autophagy-like cytoplasmic incorporations, wherein the structures were fused (Fig. S10).

Evaluation of pantothenic acid production by *G. candidum*

Metabolomic analysis showed that pantothenic acid (also known as vitamin B5) is a characteristic product of *G. candidum*. As a constituent of coenzyme A (CoA), pantothenic acid is involved in important reactions in the metabolic pathways of glucose and fatty acids (22).

In this study, we investigated the effects of culture conditions and addition of the precursor *β*-alanine on pantothenic acid production, to verify the potential of *G. candidum*

as a natural host for its production.

First, we investigated the effect of altering the amount of glucose used as the substrate on the amount of pantothenic acid produced. In this experiment, cells were cultured in YM medium containing 10, 20, 30, 40, 50, or 100 g/L glucose, and glucose consumption, growth, and pantothenic acid production were examined. *G. candidum* consumed up to 40 g/L of glucose. In 14-day cultures with initial glucose concentrations of 50 and 100 g/L, residual glucose levels of 4.5 and 65.4 g/L, respectively, were observed. The OD value was the highest when cultured with 40 g/L glucose, reaching 27.8 after 14 days of culture. The production of pantothenic acid was highest when the yeast was cultured at 40 g/L or 100 g/L and it reached 30 μM after 14 days of culture (Fig. 5).

Pantothenic acid is produced by the condensation of pantoate with *β*-alanine through the action of pantoate-*β*-alanine ligase. We therefore added 2.5 g/L of *β*-alanine to a medium containing 40 g/L glucose to evaluate its effect on pantothenic acid production. As a result, 40 g/L of glucose was completely consumed after 9 days and the amount of pantothenic acid reached 40 μM after 14 days of culture; therefore, a 30 % increase in pantothenic acid production was observed on addition of *β*-alanine, compared to that where *β*-alanine was not added (Fig. 6). Furthermore, concerning the amount of pantothenic acid produced per dry cell weight, *G. candidum* consistently exhibited higher values than *S. cerevisiae* (Fig. S11).

The increase in pantothenic acid production in *G. candidum* was only 10 μM upon addition of 28 mM (2.5 g/L) *β*-alanine. On the other hand, *S. cerevisiae* produced almost no pantothenic acid even with the addition of *β*-alanine. Therefore, although the wild type strain of *G. candidum* does not have a high conversion efficiency of *β*-alanine to pantothenic acid, it is considered to be more suitable host for pantothenic acid production than *S. cerevisiae*. Additionally, since *G. candidum* does not produce ethanol as a by-

product, it may be able to produce higher amounts of pantothenic acid than *S. cerevisiae*. There have been several studies on the production of pantothenic acid using *Escherichia coli* (23). Another advantage of using yeast as a host is that it is resistant to phage contamination, which is a problem in industrial production of *E. coli* (24).

There have been very few basic studies on *G. candidum*, and until now, the major intra- and extra-cellular changes in metabolites during culture have been unknown. In this study, we found that *G. candidum* proliferates vigorously and accumulates compounds of the pentose phosphate pathway, maintains high metabolic activity even after nutrient depletion, and exhibits a characteristic metabolic profile of stress-resistant substances including antioxidants (such as glutathione), vitamins (such as pantothenic acid and nicotinic acid), and various amino acids (such as hydroxyproline) (Table S1). Many compounds accumulated in *G. candidum* are commercially useful, indicating that *G. candidum* is a potential host for the production of several useful compounds.

Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

This work was supported by Chieko Soh and Kazumi Toyama, employees of Procter and Gamble Innovation GK, and Tomohiro Hakozaki, an employee of Procter and Gamble Company. These associations do not alter the authors' adherence to the journal policy on sharing experimental data.

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Figure legends

Fig. 1 Cultivation of *Saccharomyces cerevisiae* BY4741 (diamonds) and *Geotrichum candidum* ATCC204307 (squares) in YM medium. Time course of (A) glucose, (B) glycerol, and (C) ethanol concentrations during cultivation. (D) Dry cell weight and (E) pH is also monitoring during cultivation. Error bars indicate the standard deviations of three independent experiments.

Fig. 2 Intracellular amino acid concentrations of *S. cerevisiae* B4741 (diamonds) and *G. candidum* ATCC204307 (squares) cultured in YM medium. The x-axis shows time (h). The y-axis shows the relative intensity of metabolites. Relative intensity of each metabolite was normalized by the internal standard and dry cell weight. Error bars indicate the standard deviations of three independent experiments.

Fig. 3 Extracellular amino acid concentrations of *S. cerevisiae* BY4741 (diamonds) and *G. candidum* ATCC204307 (squares) cultured in YM medium. The x-axis shows time (h). The y-axis shows the relative intensity of metabolites. Relative intensity of each metabolite was normalized by the internal standard. Error bars indicate the standard deviations of three independent experiments.

Fig. 4 Morphological and ultrastructure analysis of *G. candidum* ATCC204307.

Fig. 5 Evaluation of pantothenic acid production by *G. candidum* ATCC204307 cultured in YM medium with various glucose concentrations. Time course of (A) glucose concentration, (B) dry cell weight, and (C) pantothenic acid estimated concentration during cultivation. Pantothenic acid was analyzed by LC-MS/MS, yet the influence of

matrix effects was disregarded. Initial glucose concentration is shown by the following symbols: open circles, 10 g/L; open triangles, 20 g/L; open diamonds, 30 g/L; open squares, 40 g/L; crosses, 50 g/L; closed circles, 100 g/L. Error bars indicate the standard deviations of three independent experiments.

Fig. 6 Evaluation of pantothenic acid production by *S. cerevisiae* BY4741 (diamonds) and *G. candidum* ATCC204307 (squares) cultured in YM medium with 2.5 g/L *β*-alanine. Time course of (A) glucose concentration, (B) dry cell weight, and (C) pantothenic acid estimated concentration during cultivation. Pantothenic acid was analyzed by LC-MS/MS, yet the influence of matrix effects was disregarded. Error bars indicate the standard deviations of three independent experiments.

Fig. 1

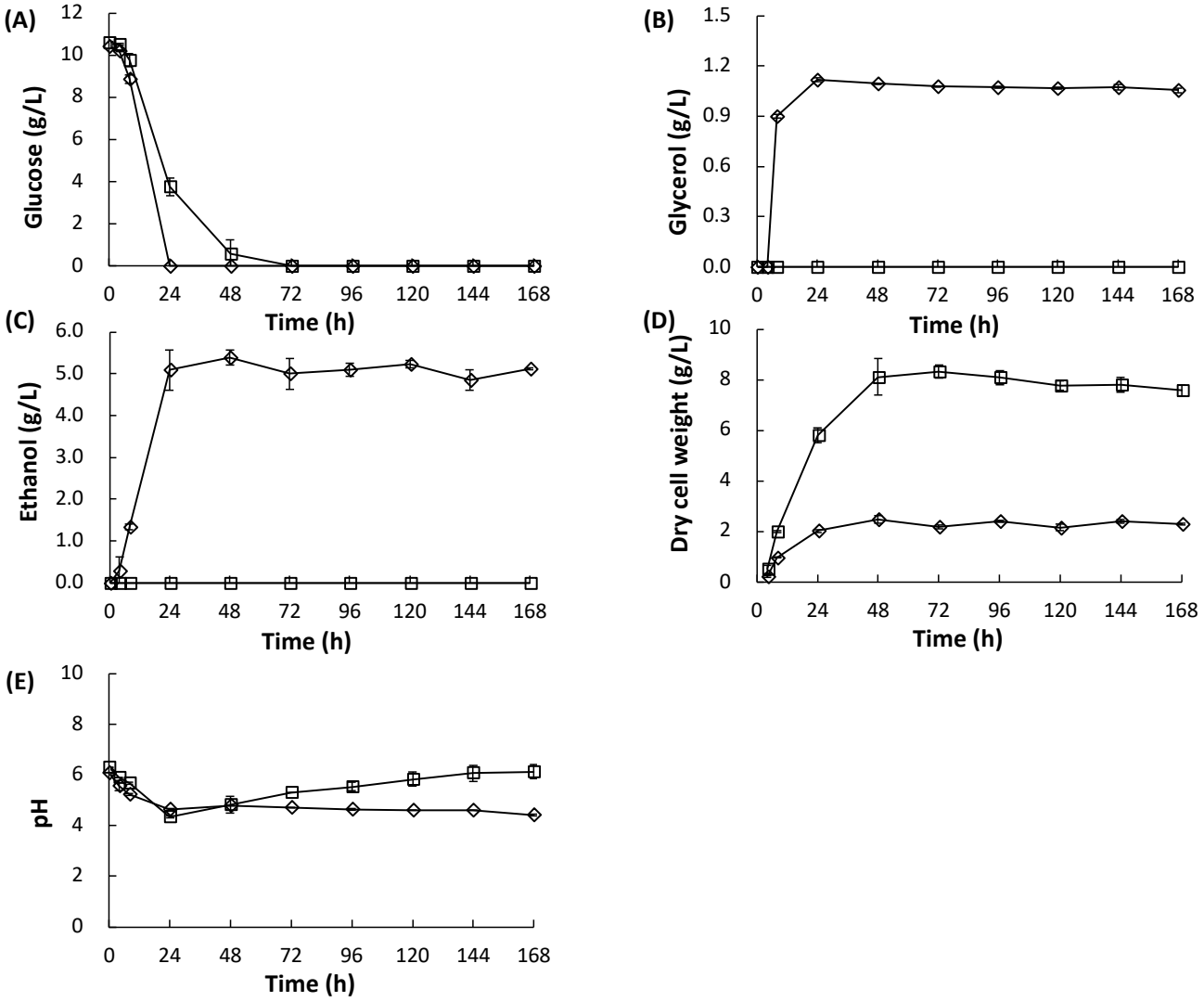
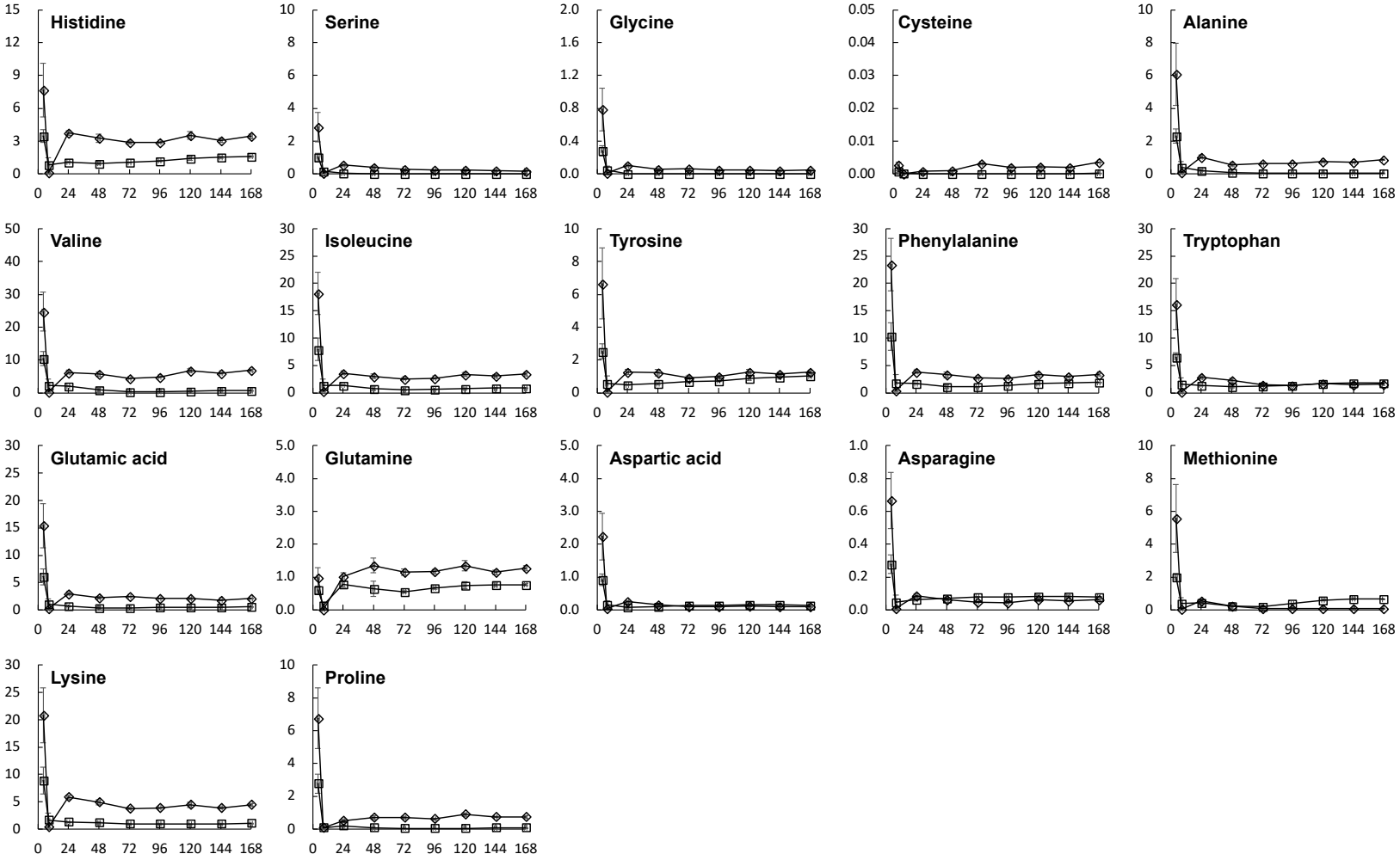

(A)
Glucose (g/L)
Time (h)
(B)
Glycerol (g/L)
Time (h)
(C)
Ethanol (g/L)
Time (h)
(D)
Dry cell weight (g/L)
Time (h)
(E)
pH
Time (h)

Fig. 2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Serine
Glycine
Cysteine
Alanine
Valine
Isoleucine
Tyrosine
Phenylalanine
Tryptophan
Glutamic acid
Glutamine
Aspartic acid
Asparagine
Methionine
Lysine
Proline
0 24 48 72 96 120 144 168

Fig. 3

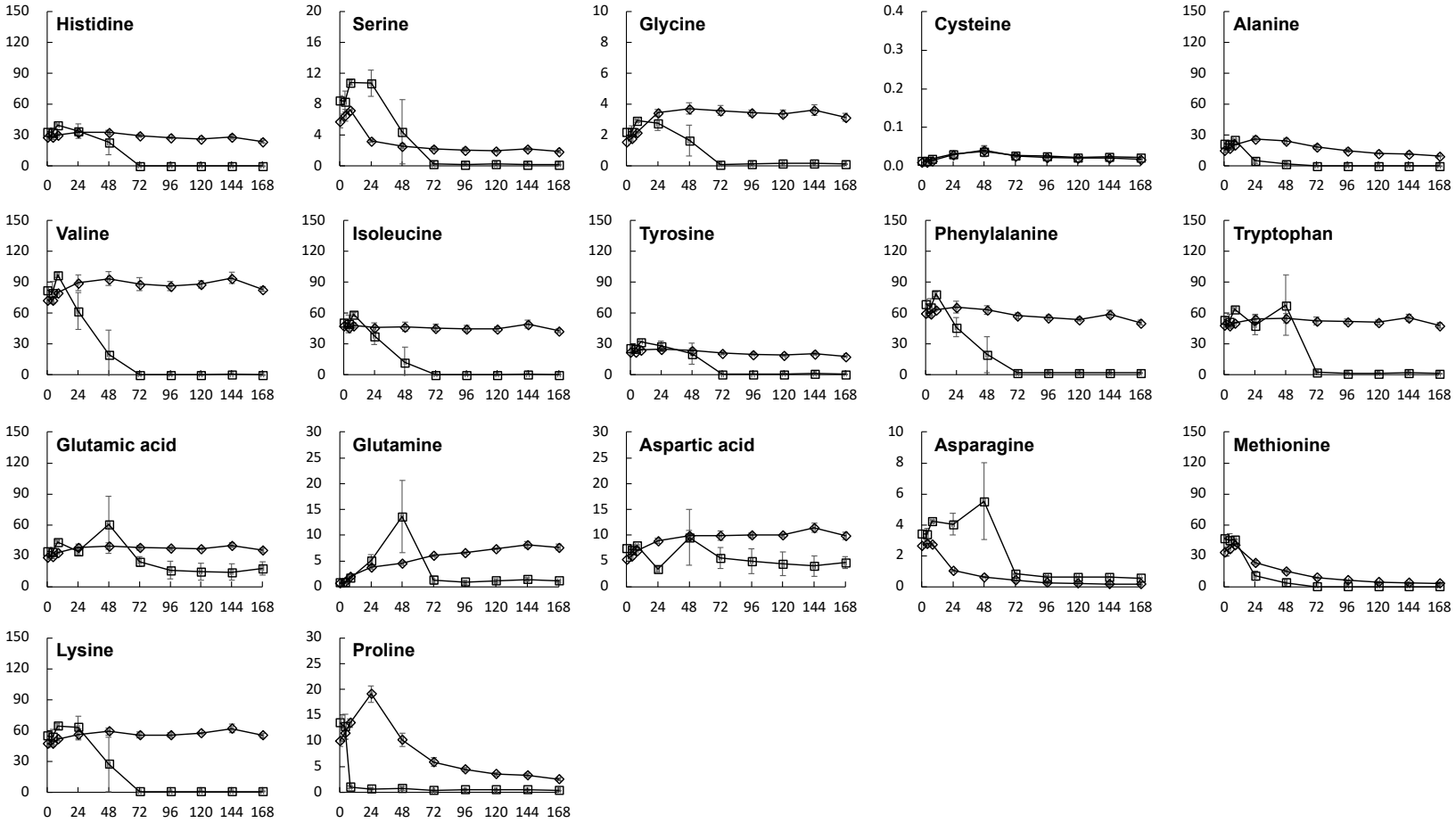

Histidine
Serine
Glycine
Cysteine
Alanine
Valine
Isoleucine
Tyrosine
Phenylalanine
Tryptophan
Glutamic acid
Glutamine
Aspartic acid
Asparagine
Methionine
Lysine
Proline
0 24 48 72 96 120 144 168

Fig. 4

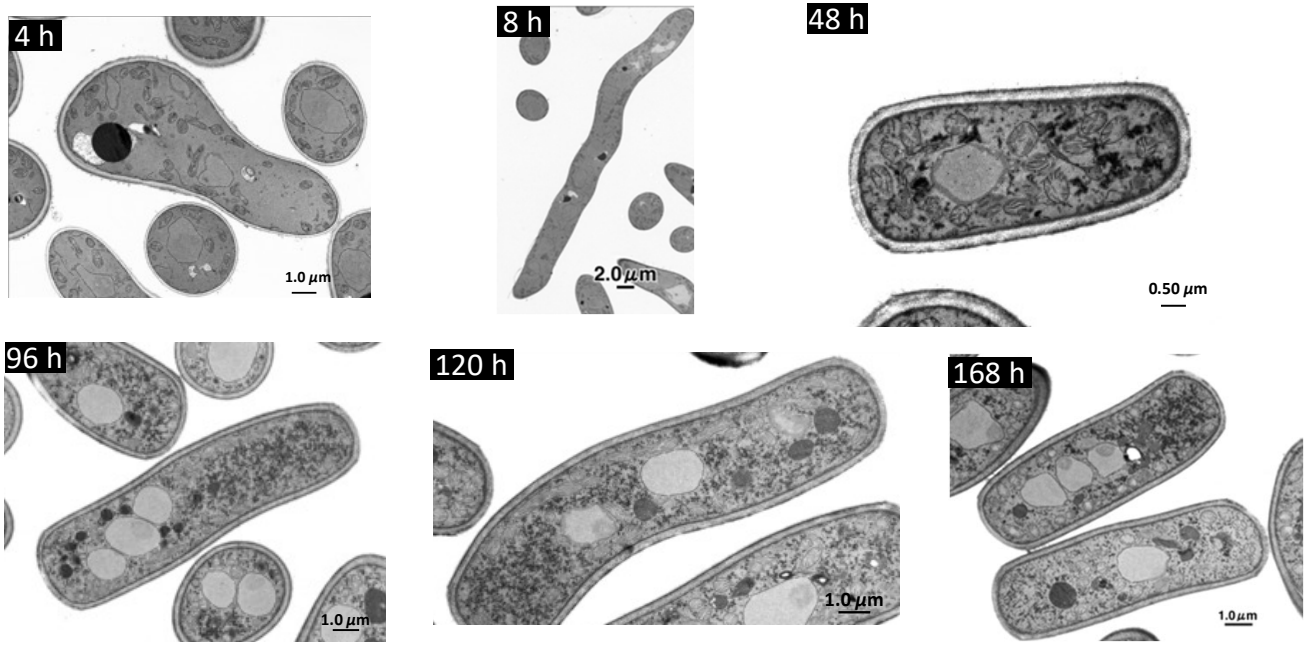


Fig. 5

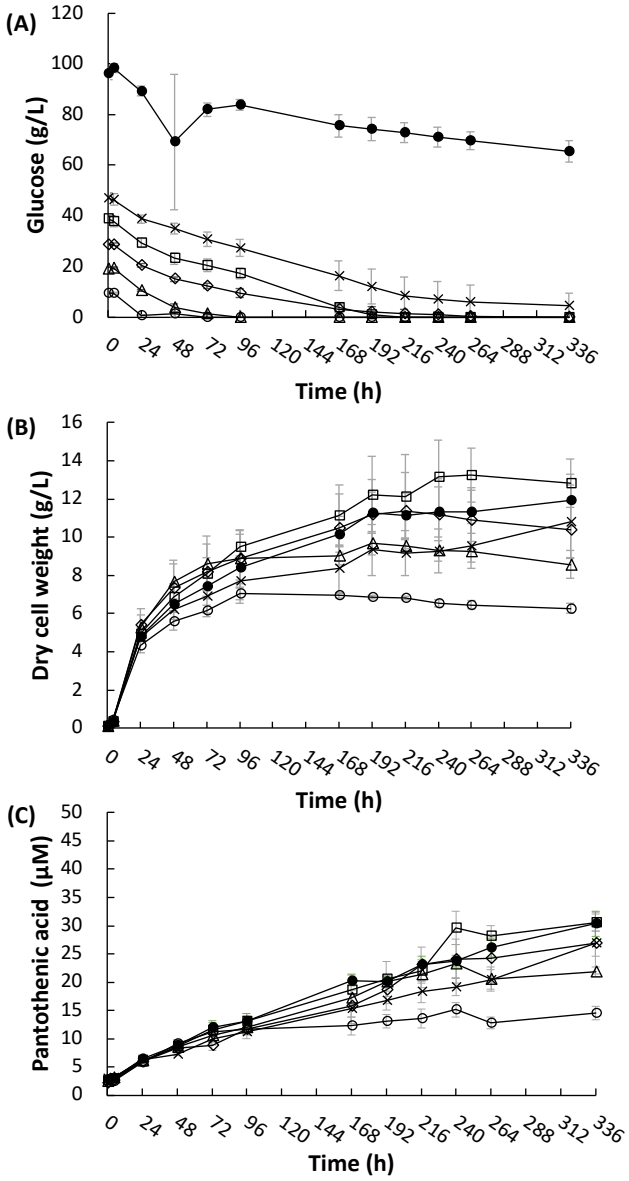
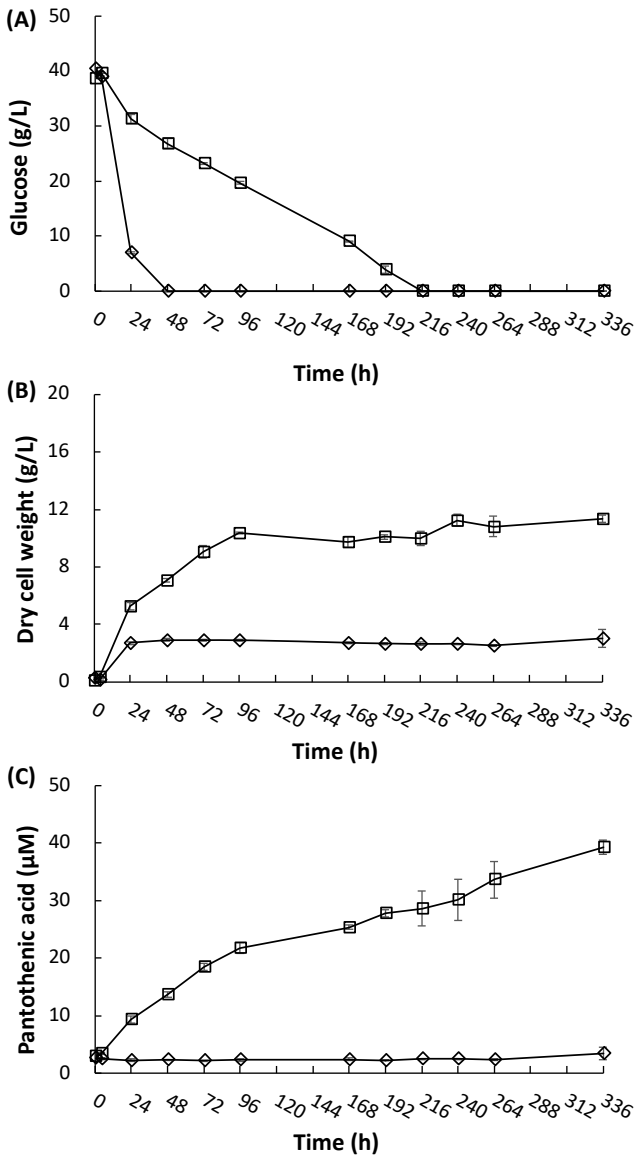

(A)
Glucose (g/L)
120
100
80
60
40
20
0
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
Time (h)
(B)
Dry cell weight (g/L)
16
14
12
10
8
6
4
2
0
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
Time (h)
(C)
Pantothenic acid (μM)
50
45
40
35
30
25
20
15
10
5
0
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
Time (h)

Fig. 6


(A)
Glucose (g/L)
50
40
30
20
10
0
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
Time (h)
(B)
Dry cell weight (g/L)
20
16
12
8
4
0
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
Time (h)
(C)
Pantothenic acid (μM)
50
40
30
20
10
0
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
Time (h)