Increased Riboflavin Production from Activated Bleaching Earth by a Mutant Strain of *Ashbya gossypii*

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RUNNING TITLE: RIBOFLAVIN PRODUCTION BY A MUTANT STRAIN OF *ASHBYA GOSSYPII*

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Abstract

The production of riboflavin from vegetable oil was increased using a mutant strain of *Ashbya gossypii* (ATCC 10895). This mutant was generated by treating the wild-type strain with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG). Riboflavin production was 10-fold higher in the mutant compared to the wild-type strain. The specific intracellular catalase activity after 3 d of culture was 6-fold higher in the mutant than in the wild-type strain. For the mutant, riboflavin production in the presence of 40 mM hydrogen peroxide was 16% less than that in the absence of hydrogen peroxide, whereas it was 56% less for the wild-type strain. The isocitrate lyase (ICL) activity of the mutant was 0.26 mU/mg of protein during the active riboflavin production phase, which was 2.6-fold higher than the wild-type strain. These data indicate that the mutant utilizes the carbon flux from the TCA cycle to the glyoxylate cycle more efficiently than the wild-type strain, resulting in enhanced riboflavin production. This novel mutant has the potential to be of use for industrial-scale riboflavin production from waste-activated bleaching earth (ABE), thereby transforming a useless material into a valuable bioproduct.

[**Keywords:** *Ashbya gossypii*, riboflavin, waste activated bleaching earth, strain improvement, biorefinery]

INTRODUCTION

Activated bleaching earth (ABE) is a commonly used adsorbent for the removal of carotene, chlorophyll, and other components formed during the refinement of crude vegetable oil (*e.g.* phosphatides and soaps). ABE is generated when montmorillonite clay is treated with mineral acids (generally sulfuric acid) to elute basic components such as aluminum, iron, and magnesium. The ABE crystal lattice is a three-layered structure of silica-alumina-silica, and its activated porous structure has a large surface area and adsorption capacity (1). During crude oil refinement, ABE adsorbs approximately 40% of vegetable oil by weight (2) and is disposed of as waste material. Annually, Japan discharges more than 80,000 metric tons of waste ABE.

Traditionally, waste ABE has been transferred from the oil-refining industry to the cement industry for incineration; however, cement manufacturers have difficulty treating waste ABE because of its high oil content. In the near future, it is likely that incineration or landfill disposal will become impossible due to environmental restrictions, spoilage of released waste oil, lack of suitable new sites, and need to reduce greenhouse gases. We previously attempted to convert the vegetable oil in waste ABE into useful bioproducts, such as riboflavin, using an oil-utilizing microorganism (3, 4). Riboflavin, a yellow water-soluble vitamin, is an essential component of basic cellular metabolism since it is a precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) coenzymes (5). Many microorganisms, plants, and fungi can

biosynthesize riboflavin from carbon sources. However, vertebrates, including humans, lack this ability and must obtain this vitamin from their diet (6). Currently, industrial riboflavin production by microbial fermentation generates approximately 6000 tons per year. Several microorganisms produce high levels of riboflavin, including the fungi *Eremothecium ashbyii* and *Ashbya gossypii*, yeast *Candida flaveri*, and bacteria *Bacillus subtilis* (7).

We investigated the capacity of several microorganisms to produce riboflavin from the vegetable oil present in waste ABE and determined that *A. gossypii* was the most efficient (2, 8, 9). However, this wild *A. gossypii* strain was still unsuitable for economic large-scale production of riboflavin from waste ABE because of low riboflavin productivity.

In this study, we isolated a mutant strain that overproduced riboflavin using the vegetable oil adsorbed in waste ABE as a sole carbon source for the process. The mutant was characterized in a 3-L jar fermentor using ABE containing crude vegetable oil as an artificial form of waste ABE. Proteomic analysis was performed on the isolated mutant strain, and the results indicated that the levels of several enzymes involved in the riboflavin synthesis pathway were altered in the mutant strain.

MATERIALS AND METHODS

Strain, media, and culture conditions The *A. gossypii* (ATCC 10895) strain was used in this study. Cells were grown at pH 6.0 and 28°C on a solid medium containing (per liter) 10 g yeast extract (Oriental Yeast Co. Ltd., Tokyo, Japan), 10 g glucose (Wako Pure Chem. Ind. Ltd., Osaka, Japan), and 20 g agar (Shimizu Shokuhin Kaisha, Ltd., Shizuoka, Japan) (pH 6.0). After 3 d of culture, the agar-plates were stored at 4°C until further analysis. The seed medium described by Szczesniak et al. (10) was modified and consisted of (per liter): 30 g corn steep liquor (CSL; Sigma Chem. Co., St. Louis, MO, USA), 10 g yeast extract (Wako), and 15 g rapeseed oil (pH 6.8). The rapeseed oil was provided by Mizusawa Chem. Ind. Ltd. (Niigata, Japan). The production medium consisted of (per liter): 30 g gelatin (Wako), 60 g CSL, 1.5 g glycine (Wako), 1.5 g KH_2PO_4, 4.4 mg $CoCl_2$, 17.9 mg $MnCl_2 \cdot 4H_2O$, 44.2 mg $ZnSO_4 \cdot 7H_2O$, 10.3 mg $MgSO_4 \cdot 7H_2O$, and 50 g rapeseed oil. These ingredients were dissolved in 1 liter of distilled water, and the pH was adjusted to 6.8.

The YR medium consisted of (per liter) 10 g yeast extract and 10 g rapeseed oil (pH 6.0), which was used to select for rapeseed oil-utilizing *A. gossypii*. To screen for riboflavin-overproducing mutants, 100 mM itaconate was added to the YR medium (itaYR) as an antimetabolite. Solid YR and itaYR media were prepared by adding 20 g/l agar to each.

A seed culture was grown for 40 h at 28°C on a rotary shaker (Bio Shaker; Takasaki Sci. Instr. Co., Saitama, Japan) at 220 rpm. For riboflavin production in flask cultures, 2

ml of seed culture was inoculated into a 500-ml Erlenmeyer flask containing 50 ml of production medium, and these cultures were grown for 6 d at 28°C on a rotary shaker at 200 rpm. Artificial waste ABE (Mizusawa Chem. Ind. Co., Niigata, Japan) that adsorbs approximately 40% (w/w) crude vegetable oil was used as the supplemental carbon source.

Riboflavin production by the wild-type and isolated mutants was compared in a 3-L bioreactor (Bioneer-300, Marubishi, Co., Ltd., Tokyo) containing 1.5 L production medium. The inoculum size was 10% (v/v), and the culture was grown with agitation at 600 rpm and an aeration rate of 1 vvm at a constant temperature of 28°C.

Spore isolation, mutagenesis, and mutant screening After 1 week of growth on solid seed medium, the mycelia were isolated by adding 0.5 ml of steam-sterilized distilled water. Zymolyase 20T (0.25 ml) from *Arthrobacter luteus* (Seikagaku Co., Tokyo, Japan; 15 mg/ml), and the mycelial suspension (0.5 ml) were mixed and incubated for 30 min at 37°C with gentle shaking. The solution was centrifuged at 2700 *g* for 5 min, and the resulting pellet was resuspended in 1 ml of distilled water containing 0.03% Triton X-100. This centrifugation process was repeated twice. The hydrophobic spores of *A. gossypii* were resuspended in 500 µl Triton X-100, and 100 µl of glycerol was added. The spore suspension was stored at −80°C until further analysis.

The spore suspension (500 µl; 1.0×10^7 spores/ml) from the wild-type strain was treated with 1 mg/l *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in TM buffer (200 mM 2-amino-2 hydroxymethyl-1,3-propanediol, 170 mM maleic acid, and 0.03% Triton

X-100, pH 8.0) for 2 h at 28°C. The spores were washed twice with 10 ml TM buffer, and the resulting pellet was resuspended in 5 ml of sterilized saline solution. The MNNG-treated spores were placed on the itaYR medium and incubated at 28°C for 6–14 d. Yellow-colored colonies growing in the itaYR medium were selected, and each colony was cultured for an additional 7 d in the itaYR medium. This screening process was repeated three times.

Proteome analysis The wild-type and mutant strains were grown in the YR medium for 72 h at 28°C. To isolate the soluble proteins, the cells were washed in distilled water and resuspended in 50 mM potassium phosphate buffer (pH 7.5). The cells were then sonicated four times for 30 s using an ultrasonicator (Vibracell; Sonics & Materials Inc., Newtown, CT, USA). After centrifugation at 20,000 *g* for 5 min at 4°C, the supernatant was collected. This supernatant, which contained the soluble protein fraction, was subjected to two-dimensional electrophoresis proteome analysis at Shimadzu Biotech (Tsukuba, Japan).

Analytical method Cell-free extracts to assess enzyme activity were prepared in buffer containing 50 mM imidazole/HCl (pH 7.5), 10 mM KCl, 1 mM EDTA (pH 8.0), 5 mM $MgSO_4$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 30% glycerin. The harvested cells were washed in buffer and sonicated four times for 30 s using an ultrasonicator. The suspension was centrifuged for 30 min at 20,000 *g* and 4°C. The supernatant was collected for assessment of enzyme activity

and protein concentration. The latter was measured by Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Isocitrate lyase (ICL) activity was assayed according to a previously reported method (11). The reaction buffer contained 25 mM imidazole/HCl, 4 mM phenylhydrazine/HCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, and 4 mM threo-*DL*-isocitrate. The reaction was initiated by the addition of glyoxylate phenylhydrazone ($\varepsilon = 14630\ M^{-1}\ cm^{-1}$), and the absorbance was measured at 340 nm at 30°C. One unit of enzyme activity was defined as the amount of the enzyme required to produce one micromole of glyoxylate phenylhydrazone per minute. Catalase activity was measured using the catalase assay kit (Cayman Chem. Co., Ann Arbor, MI, USA).

The residual vegetable oil concentration was measured by the solvent extraction method. Prior to sampling, the culture broth was thoroughly mixed, and a 10-ml sample was withdrawn. *n*-Hexane (10 ml) was added to the sampled culture broth, and the two were vigorously shaken for 1 min in a screw-capped Falcon tube, followed by centrifugation at 2000 *g* for 15 min. The upper hexane layer was evaporated by blowing with nitrogen gas on a heat block, and the residue was dried at 75°C for 3 h. The weight of the extracted oil was measured.

To measure the riboflavin concentration, 0.5 ml of culture broth was mixed with 4.5 ml of distilled water and centrifuged at 1000 *g* for 10 min. The supernatant (1.6 ml) was removed and thoroughly mixed with 0.8 ml of 1 N NaOH and 5 ml of 50 mM phosphate buffer (pH 7.0), and then the samples was centrifuged at 9000 *g* for 5 min. The

supernatant (0.8 ml) was filtered, and the optical density at 444 nm was measured. The obtained riboflavin concentration was converted using a conversion factor of 127.297 mg (unit of optical density per liter) (12). In the case of the culture broth containing ABE, the sample was thoroughly mixed with 0.8 ml of 1 N NaOH and centrifuged at 2000 *g* for 15 min. The supernatant was used to measure the riboflavin concentration.

RESULTS AND DISCUSSION

Isolation and screening of riboflavin-overproducing variants created by MNNG-induced mutation The survival rates of wild-type *A. gossypii* cultured in medium containing various concentrations of MNNG relative to that in medium without MNNG was as follows: 15% in medium containing 30 mg/l MNNG; 10% in medium containing 60 mg/l MNNG; 5% in medium containing 90 mg/l MNNG; and 1% in medium containing 100 mg/l MNNG (data not shown). Based on these data, 100 mg/l was selected as the optimal concentration of MNNG for inducing mutation.

The MNNG-induced mutant colonies were grown on agar-itaYR medium containing 100 mM itaconate as the antimetabolite. The total number of colonies that grew in the screening medium was 186,025. There were 571 intensely yellow-colored colonies (data not shown), and these were selected by visual identification for follow-up analysis. These yellow colonies were cultured in YR medium in a test tube. Among the 571 variants, there were 18 mutants that produced more than 250 mg/l of riboflavin, and these were selected for follow-up studies. In contrast, the wild-type strain produced only

50 mg/l of riboflavin. The riboflavin production levels of eight mutants that were identified as high-level producers were evaluated in 500-ml flask cultures using the production medium. The Mutant 13a strain produced 3.8 g/l of riboflavin, which was 3-fold higher than that produced by the wild-type strain (Fig. 1A). On YR agar plates, the mycelia of the wild type spread out to a greater extent than those of mutant 13a; however, the intensity of the yellow color of the mutant 13a strain was much stronger than the wild-type (Fig. 1B). The morphology of mutant 13a in the liquid medium showed that the organism tended to aggregate and form pellets, unlike the wild-type strain that spread its mycelia throughout the medium (data not shown).

Phenotypic changes in the mutant strain To investigate the difference in riboflavin production between the wild-type and mutant 13a strains, the itaconate resistance of the two strains was compared using YR medium containing 0–100 mM itaconate. In the presence of 100 mM itaconate, the dry cell weight of the wild-type strain was one-third of that in the presence of 50 mM itaconate (Fig. 2A), while the dry cell weight of mutant 13a was not affected by the presence of itaconate. Riboflavin production by mutant 13a in the presence of 100 mM itaconate was 0.38 g/l, which was 50% lower than the amount produced in the absence of itaconate (Fig. 2B). In the wild-type strain, riboflavin production in the presence of 100 mM itaconate was reduced to 70% of that in the absence of itaconate. The specific riboflavin concentration of the mutant 13a strain was 5-fold higher than that of the wild-type strain at every itaconate concentration range (Fig. 2C). These results indicated that the mutant 13a strain is

resistant to itaconate, and it can maintain high riboflavin productivity even in the presence of high itaconate concentrations.

Proteomic analysis was performed to investigate whether there were changes in the expression of proteins involved in the riboflavin metabolic pathway in the mutant strain. During the riboflavin production phase in the mutant 13a strain, there was increased expression of five proteins: thioredoxin peroxidase, cytoplasmic thioredoxin isoenzyme, 2-methylbutyraldehyde reductase, NADPH oxidoreductase, and phosphoglycerate kinase. However, the expression of mitochondrial cytochrome *b2* decreased in this mutant strain. Oxidoreductases catalyze the transfer of electrons from a fatty acid to another hydrogen or electron acceptor. Thioredoxin peroxidase is a mitochondrial antioxidant protein that regulates basal intracellular levels of hydrogen peroxide, and it protects against reactive oxygen species-induced damage by catalyzing the reduction of hydrogen peroxide into water (13-15). In addition, Kowaltowski et al. (16) demonstrated that thioredoxin peroxidase and catalase function together to maintain cell viability and protect the mitochondria from reactive oxygen species. After 3 d of culture, the mutant 13a strain exhibited specific intracellular catalase activity that was 6-fold higher than that in the wild-type strain (Fig. 3A). To confirm the resistance of the mutant 13a strain to reactive oxygen species, its resistance to hydrogen peroxide was investigated. When the mutant 13a and wild-type *A. gossypii* strains were cultured in YR medium containing 40 mM hydrogen peroxide, riboflavin production levels were maintained at 84% and 44%, respectively, in comparison to the levels in medium

without hydrogen peroxide (Fig. 3B). In comparison to the wild-type strain, the mutant 13a strain had developed hydrogen peroxide resistance. Cytochrome *b*, an electron-transfer protein, mediates a wide range of functions during a multitude of different redox processes. The reduced levels of mitochondrial cytochrome *b2* suggested that catalytic activity decreased during mitochondrial oxidative phosphorylation in the mutant 13a strain.

When vegetable oil is used as the sole carbon source, the glyoxylate cycle assumes an essential role in mediating cell growth and riboflavin production. ICL, the key enzyme in this metabolic pathway, converts isocitrate into glyoxylate, which contributes to cell growth and riboflavin synthesis (17, 18). The ICL activity of the two strains was compared (Fig. 4). The specific ICL activity of the wild-type strain was 0.32 mU/mg of protein on the first day of culture, suggesting that the ICL activity contributed to the increased mycelial growth of the wild-type strain initially. However, the ICL activity of the mutant 13a strain gradually increased to 0.26 mU mg/protein on the third day of culture, which was 2.6-fold higher than that of the wild-type strain at this time point during the active riboflavin production phase. These data indicate that the mutant strain utilizes carbon flux from the TCA cycle to the glyoxylate cycle strain more efficiently than the wild-type strain.

Riboflavin production using the mutant 13a strain Riboflavin production by the two strains was compared in a 3-L jar fermentor. The oxygen consumption of the mutant 13a strain was lower than that of the wild-type strain because of the decreased

expression of cytochrome *b2*. The decrease in mitochondrial cytochrome *b2* might lower the catalytic activity during mitochondrial oxidative phosphorylation, resulting in decreased oxygen consumption and less mycelial growth (Fig. 5A). Higher ICL activity of mutant than that of wild-type strain (Fig. 4) accelerates carbon flux from TCA cycle to glyoxylate cycle, which may be another reason of decreased mitochondrial oxidative phosphorylation, resulting in decreased oxygen consumption. When nonfermentable carbon source was used in the culture of succinate dehydrogenase-deleted *Kluyveromyces lactis*, oxygen consumption was one-eighth that of wild-type strain (19), because of using alternative pathway, glyoxylate cycle.

The maximum riboflavin concentrations of the mutant 13a and wild-type strains were 3.7 and 0.8 g/l, respectively (Fig. 5B). In the case of the wild-type strain, the residual oil concentration after 6 d of culture was 0.8 g/l in contrast to 2.8 g/l for the mutant 13a strain (Fig. 5B). Therefore, the oil consumption of the mutant 13a strain was lower than the wild-type strain. However, the riboflavin yield based on the consumed substrate was 0.08 g riboflavin/g consumed oil, which was 4-fold higher for the mutant as compared to the wild-type strain.

When 75 g/l of artificial ABE waste containing 50 g/l of crude rapeseed oil was used, the riboflavin concentration increased by 1.6 fold to 6.0 g/l (Fig. 6). The effect of ABE on riboflavin levels produced by *A. gossypii* cultures using vegetable oil as the carbon source has been previously reported (8). After 48 h of culture, when a nitrogen mixture containing 7.5 g of yeast extract and 3.75 g of glycine was added to the culture

with the same concentration of carbon source, the riboflavin concentration significantly increased and reached 5.5 g/l on the third day of culture. The increased riboflavin production rate (Fig. 6) was maintained, and the maximum riboflavin concentration reached 8.7 g/l after 6 d of culture. The riboflavin production yield based on the consumed substrate was 0.17 g riboflavin/g of substrate, which was 8-fold higher than the wild type.

At the end of the culture, ABE was separated from the culture broth, and the precipitate (ABE cake) was dried at 105°C for 2 h. More than 80% (w/w) of the produced riboflavin was adsorbed into ABE, causing formation of a thick yellow powder. The amount adsorbed onto ABE was 20 mg of riboflavin/g ABE (data not shown).

The results of this study demonstrated that in comparison to the wild-type strain, the MNNG-induced mutant 13a strain, which used ABE as the oil absorbent, improved riboflavin production by 10-fold. The mutant 13a strain exhibited higher specific intracellular catalase activity after 3 d of culture, and this level was 6-fold higher than the wild-type strain. In the presence of 40 mM hydrogen peroxide, the mutant 13a strain maintained 84% of the original level of riboflavin production in the absence of hydrogen peroxide, whereas the wild-type strain only retained 44%. These data suggested that the mutant 13a strain was resistant to hydrogen peroxide. The ICL activity of the mutant 13a strain was 0.26 mU/mg of protein during the active riboflavin production phase, which was 2.6-fold higher than the wild-type strain. This indicates

that the mutant 13a strain utilizes the carbon flux from the TCA cycle to the glyoxylate cycle more efficiently than the wild-type strain, resulting in enhanced riboflavin production.

In summary, this study identified a novel mutant of *A. gossypii* that has the potential to be used in industrial-scale riboflavin production from waste ABE, and showed that a useless material like waste ABE can be transformed into a value-added bioproduct.

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Figure legends

FIG. 1. Riboflavin concentration (A) and colony formation in YR medium (B). Mutants were cultured for 6 d in 500-ml flasks containing 50 ml production medium. W.T. and 13a denote the wild type and isolated mutant strains, respectively.

FIG. 2. Effect of itaconic acid concentration on mycelial growth (A), riboflavin concentration (B), and specific riboflavin concentration (C) in the mutant 13a and wild-type strains. Both the mutant 13a and wild-type strains were cultured in 500-ml flasks containing 50 ml YR medium and 0–100 mM itaconic acid for 4 d.

FIG. 3. Specific catalase activity of mutant 13a and wild-type strains during culture in 500-ml flasks containing 50 ml YR medium (A). Effect of hydrogen peroxide on riboflavin production (B). The wild type (white bars) and mutant 13a (black bars) strains were cultured for 6 d in 500-ml flasks containing 50 ml YR medium with various hydrogen peroxide concentrations.

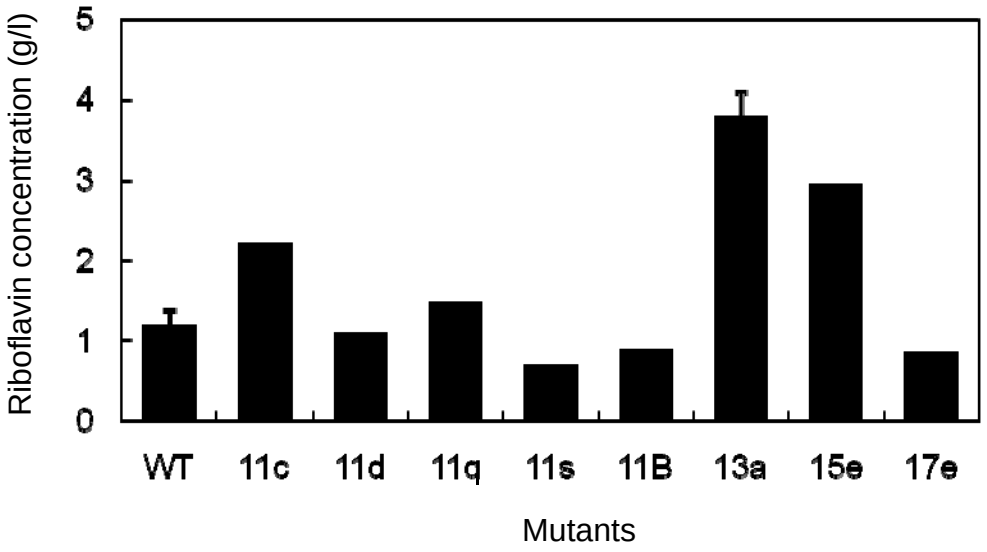
FIG. 4. Comparison of specific ICL activities of the wild type (white bars) and mutant 13a (black bars) strains. Both strains were cultured for 4 d in 500-ml flasks containing 50 ml YR medium.

FIG. 5. Comparison of the dissolved oxygen concentration (A) and concentrations of riboflavin and residual oil (B) in the 3-L jar fermentor of working volume 1.5 L.

Wild-type (dotted lines in A and open squares in B) and mutant 13a (straight lines in A and closed circles in B) strains were cultured in the production medium.

FIG. 6. Riboflavin production by the mutant 13a strain under various culture conditions. The strain was cultured in a 3-L jar fermentor of working volume 1.5 L (production medium). The carbon source in the production medium was 50 g/l rapeseed oil (opened squares), 75 g/l ABE containing 50 g/l rapeseed oil (closed triangles), and 75 g/l ABE containing 50 g/l rapeseed oil with the addition of 5 g/l yeast extract and 2.5 g/l of glycine after 48 h of culture (closed squares).

A


Riboflavin concentration (g/l)
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3
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1
0
WT
11c
11d
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11B
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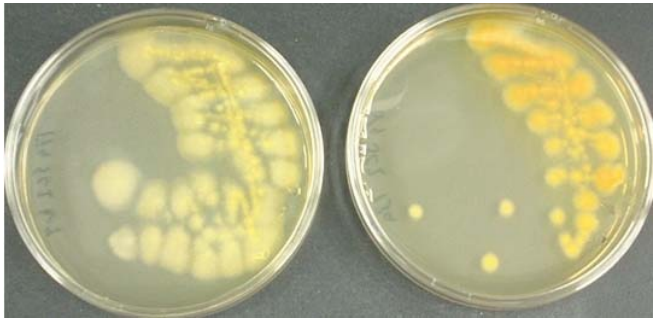
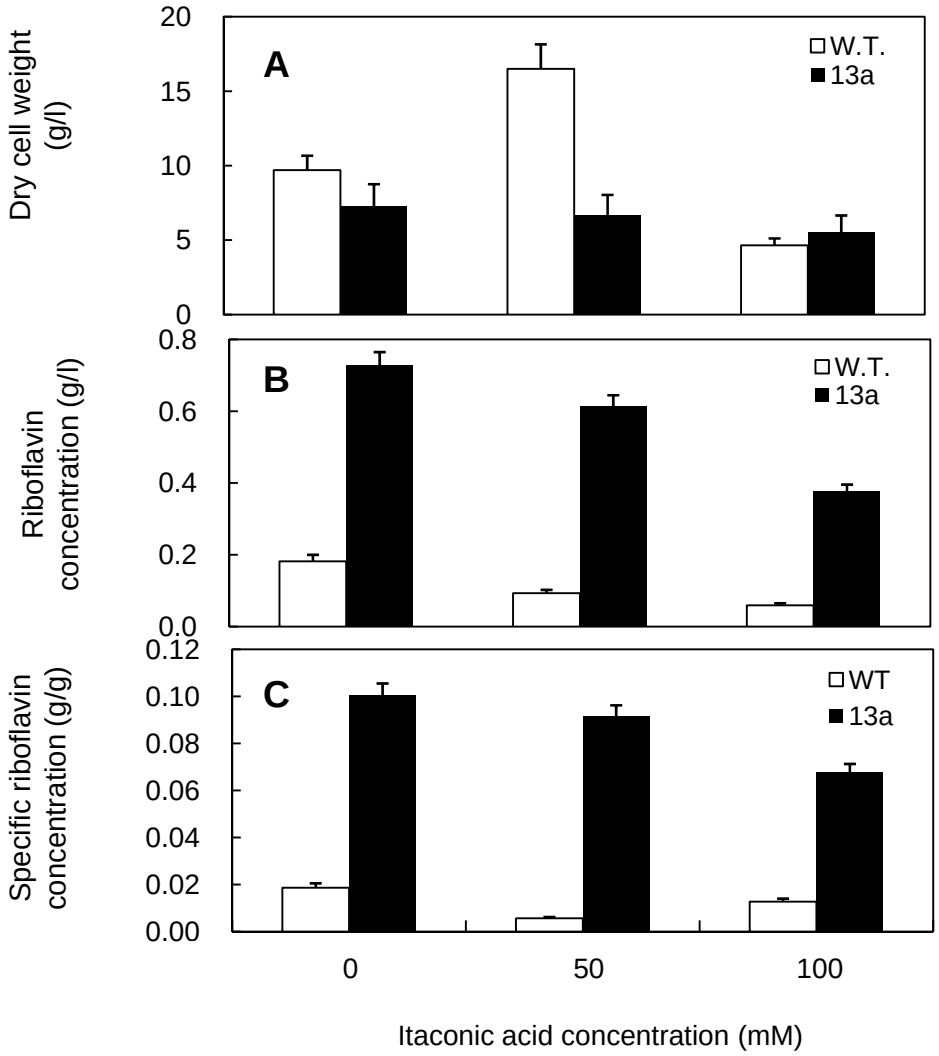
15e
17e
Mutants

B


W.T.
13a


A
W.T.
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Dry cell weight (g/l)
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B
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Riboflavin concentration (g/l)
0.8
0.6
0.4
0.2
0.0
C
WT
13a
Specific riboflavin concentration (g/g)
0.12
0.10
0.08
0.06
0.04
0.02
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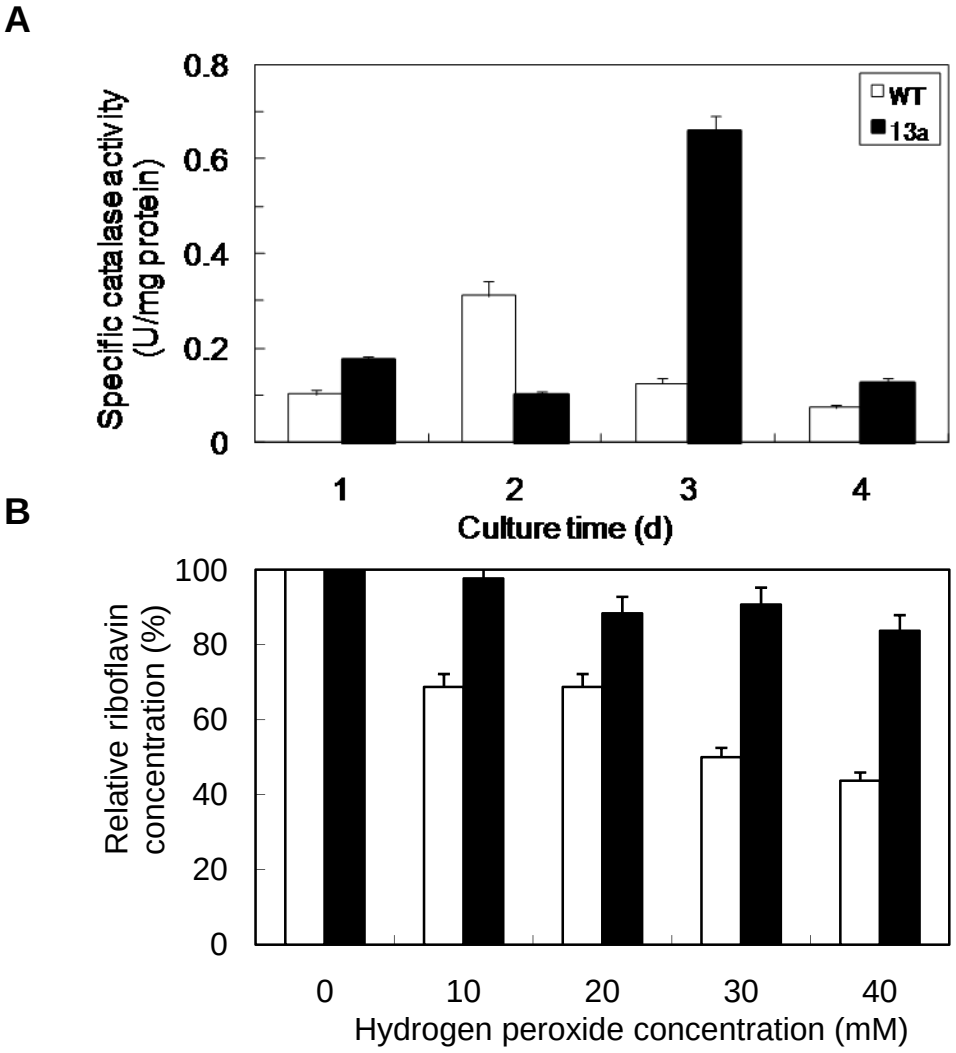
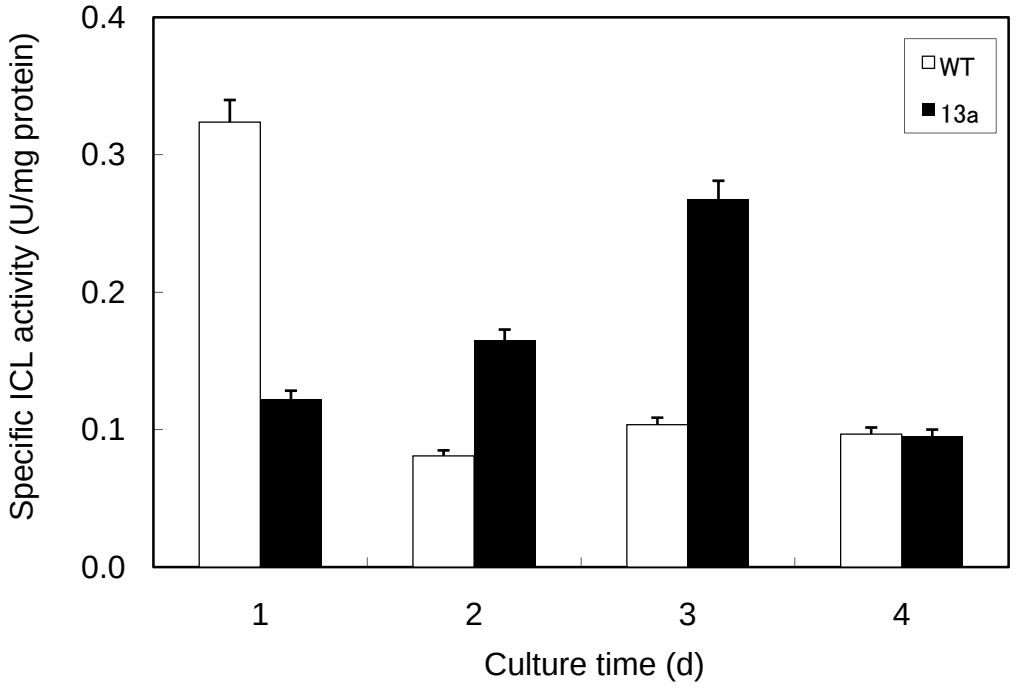
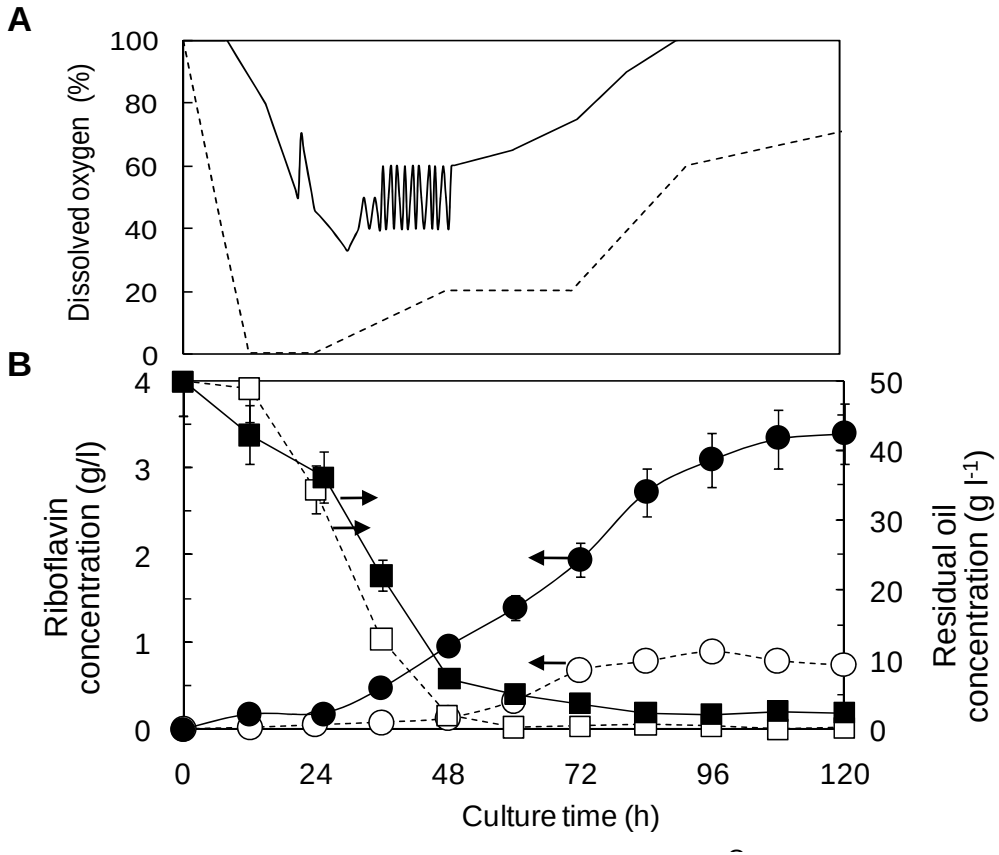
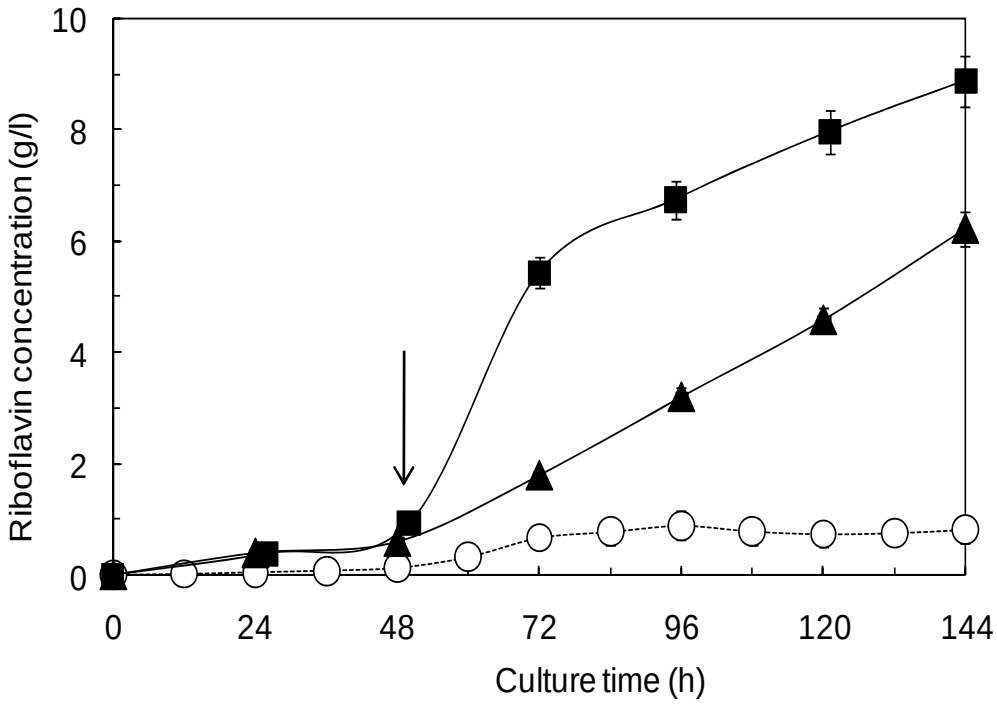
100
Itaconic acid concentration (mM)


A
Specific catalase activity (U/mg protein)
0.8
0.6
0.4
0.2
0
WT
13a
1
2
3
4
Culture time (d)
B
Relative riboflavin concentration (%)
100
80
60
40
20
0
0
10
20
30
40
Hydrogen peroxide concentration (mM)


Specific ICL activity (U/mg protein)
0.4
0.3
0.2
0.1
0.0
WT
13a
1
2
3
4
Culture time (d)


A
Dissolved oxygen (%)
100
80
60
40
20
0
B
Riboflavin concentration (g/l)
Residual oil concentration (g l-1)
4
3
2
1
0
50
40
30
20
10
0
0
24
48
72
96
120
Culture time (h)


10
8
6
4
2
0
Riboflavin concentration (g/l)
0
24
48
72
96
120
144
Culture time (h)