Bioconversion of AHX to AOH by resting cells of Burkholderia contaminans CH-1

Bioconversion of AHX to AOH by resting cells of *Burkholderia contaminans* CH-1

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Abstract

Fairy rings are zones of stimulated grass growth owing to the interaction between a fungus and a plant. We previously reported the discovery of two novel plant-growth regulating compounds that related to forming fairy rings, 2-azahypoxanthine (AHX) and 2-aza-8-oxohypoxanthine (AOH). In this study, a bacterial strain CH-1 was isolated from an airborne-contaminated nutrient medium containing AHX. The strain converted AHX to AOH and identified as *Burkholderia contaminans* based on the partial gene sequence of its 16S rDNA. The quantitative production of AOH by resting cells of the strain was achieved. Among seven *Burkholderia* species, two bacteria and two yeasts tested, *B. contaminans* CH-1 showed the highest rate of conversion of AHX to AOH. By batch system, up to 10.6 mmol AHX was converted to AOH using the resting cells. The yield of this process reached at 91%.

Key words: bioconversion; *Burkholderia contaminans*; fairy chemicals; fairy ring; plant growth regulator

Introduction

Fairy rings are zones of stimulated grass growth due to the interaction between a fungus and a plant. Since the first scientific article about "fairy rings" in 1675 and subsequent studies reviewed in Nature in 1884,[1] this phenomenon had been attributed to "fairy" before our study in 2010.[2-5] In that year, we reported that isolation and identification of the fairy-ring causing principle produced by a fairy-ring forming fungus *Lepista sordida*, 2-azahypoxanthine (AHX) (Fig. 1).[6] After that, it was found out that AHX was absorbed into plants from their roots and then was converted into a common metabolite, 2-aza-8-oxohypoxanthine (AOH) (Fig. 1).[7] On the other hand, this fungus occasionally causes growth-suppressed rings and we purified the suppressor from the fungus and identified it as imidazole-4-carboxamide (ICA) (Fig. 1A).[8] Furthermore, we proved the existence of endogenous AHX and AOH in plants and found a novel purine pathway producing them in plants (Fig. 1B). We named the three compounds "fairy chemicals" after the title of the article in Nature that covered our research about them (Fig. 1A).[9] Fairy chemicals regulated growth of all the plant tested and increased the yields of rice, wheat and various other crops in pot and/or field experiments, suggesting that they might find practical application in agriculture.[10,11] For practical use of the compounds, field experiments are necessary and large amounts of them are required for them. AHX and ICA are chemically synthesized readily. On the other hand, only method for synthesis of AOH is enzymatic conversion of AHX to it by xanthine oxidase (XOD) at present (Fig. 2).[12] However, commercially available XOD is inappropriate for large-scale preparation of AOH because the enzyme is very expensive and the enzymatic reaction should be carried out with low concentration of AHX (Fig. 2A).

During searching for better methods to synthesize AOH, we happened to notice that aqueous solution of AHX was accidentally contaminated by microorganisms and HPLC analysis of the solution indicated that AHX in the solution was converted to AOH. Therefore, we tried to isolate the airborne microorganism that produced AOH.

Biotransformation to produce value-added substances from natural resources have been given attention as an alternative tool to replace conventional chemical synthesis of the substrate.[13,14] The use of microorganisms as catalysts in the manufacture of economically important substances is a rapidly developing field of biotechnology.

On the other hand, resting cells are non-growing but metabolically active. The use of resting cells in a bioconversion process would be more advantageous than that of growing cells.[15] Production with resting cells often allows easier and more flexible operation of reaction systems, which results in higher yield.[16,17]

In the present study, we succeeded in isolation of a strain of a bacterium that converted high concentrations of AHX to AOH with high yield, and resting cells of the strain were applied to production of AOH from AHX.

Materials and methods

Materials. Strains (*Burkholderia caryophilli* NBRC 13591, *B. capaci* NBRC 14595, *B. caledonica* NBRC 102488, *B. fungorum* NBRC 102489, *B. ferrariae* NBRC 106233, *B. ginsengisoli* NBRC 100965, *B. mimosarum* NBRC 106338, *Bacillus subtilis* NBRC 13719, *Saccharomyces cerevisiae* NBRC 2377 and *Candida albicans* NBRC 1060) were purchased from the National Institute of Technology and Evaluation (Chiba, Japan). Xanthine oxidase from butter milk (XOD; 0.35 U/mg powder) was obtained

from Oriental Yeast Co. (Tokyo, Japan). All solvents used throughout the experiments were obtained from Kanto Chemical Co. (Tokyo, Japan).

General. ^{13}C NMR spectra were recorded on a JEOL lambda spectrometer at 67.5 MHz. HPLC separations were performed with a Jasco Gulliver system using reverse-phase HPLC columns (Develosil C30-UG-5, Develosil ODS-UG-5, Nomura chemical Co., Ltd, Japan).

Isolation of bacterial strain. A nutrient solution (for plant culture; 1 mM NH_4NO_3, 0.6 mM Na_2HPO_4, 0.3 mM K_2SO_4, 0.4 mM $MgCl_2$, 0.2 mM $CaC1_2$, 45 μM Fe-ethylenediaminetetraacetic acid, 50 μM H_3BO_3, 9 μM $MnSO_4$, 0.3 μM $CuSO_4$, 0.7 μM $ZnSO_4$ and 0.1 μM Na_2MoO_4) was used to isolate airborne bacterial strains. Uncovered petri dish (90 mm) containing a twenty milliliter of AHX solution (0.1 mM in the nutrient solution) was incubated at room temperature for 3 days. The culture solution was spread on nutrient agar plates. One colony capable of converting AHX to AOH was isolated.

Culture conditions. Strain CH-1 was grown in TSB medium containing polypeptone, 17 g, soy peptone, 3 g, NaCl, 5 g, K_2HPO_4, 2.5 g, and glucose, 2.5 g per litter. pH of the medium was adjusted to 7.3 with 2 N NaOH. Cultures were inoculated at an OD_{600} of 3.0 and incubated at 30 ºC at 180 rpm. Bacterial growth was measured at 600 nm using a Hitachi U-2001 spectrophotometer.

Measurement of AOH conversion activity by xanthine oxidase. Xanthine oxidase (XOD, 0.08 unit or 0.5 unit) was dissolved in 10 mM phosphate buffered saline (PBS, pH 7.4, 900 μL). One hundred μL of 0.36 mM (or 11.5 mM) AHX was added to the buffer containing 0.08 unit (or 0.5 unit) of XOD and reacted in a test tube. After the reaction, the reaction mixture was analyzed by HPLC (column, C30-UG-5; mobile phase, 0.02% TFA in H_2O; flow rate, 0.8 mL/min; wavelength, 254 nm).

Measurement of AOH conversion activity by resting cells. The strain was grown in TSB medium 5 mL (test tube φ18 mm, 30ºC, 180 rpm). Cells were harvested by centrifugation at 11,228×*g* for 5 min. The cell pellet was rinsed and resuspended in 10 mM PBS (900 μL). Various concentrations of AHX (100 μL) were added to the suspension and reacted in a test tube. After the reaction, the reaction mixture was heated (98ºC, 5 min) and centrifuged at 11,228×*g* for 5 min. Concentration of AHX and AOH in the supernatant was determined by HPLC (column, C30-UG-5; mobile phase, 0.02% TFA in H_2O; flow rate, 0.8 mL/min; wavelength, 254 nm).

For a conversion of AHX at a large scale, the strain was precultured in TSB medium (5 mL, test tube φ18 mm, 30ºC, 180 rpm, 16 h) and then the precultured cells were grown in 100 mL of TSB medium in 500 mL-Erlenmeyer flask (30ºC, 180 rpm, 6 h). The cells were harvested by centrifugation at 15,652×*g* for 5 min. The obtained cell pellet was rinsed and resuspended in 10 mM PBS (20 mL). Various concentration of AHX (180 mL) were added and reacted in the flask (40ºC, 80 rpm). Concentrations of AHX and AOH were determined by HPLC.

In order to examine whether further metabolism of AOH occurs by resting cells of CH-1, the strain was grown in 5 mL of TSB medium (test tube φ18 mm, 30ºC, 180 rpm).

Cells are harvested by centrifugation at 11,228×*g* for 5 min. The obtained cell pellet was rinsed and resuspended in 10 mM PBS (900 μL). One hundred μL of 0.46 mM AOH was added to the suspension and reacted in a test tube. After the reaction, a concentration of AOH was determined by HPLC.

Purification of AOH. AOH produced by the resting cell method was purified as follows. After the reaction at the 500 mL scale, the reaction mixture was heated at 98ºC for 5 min, and the cells were removed by centrifugation (39,120×*g*, 10 min). Then, the solution was concentrated 7-fold by an evaporator and AOH was crystallized at 4ºC for 24 h and the obtained crystals were collected by filtration and dried in a vacuum desiccator.

Measurement of xanthine oxidase activity of resting cells of strain CH-1. The strain was grown in 5 mL of TSB medium (test tube φ18 mm, 30ºC, 180 rpm). Cells are harvested by centrifugation at 11,228×*g* for 5 min. The obtained cell pellet was washed and resuspended in 50 mM Tris-HCl buffer (pH 7.5, 1.45 mL). Fifty μL of 10 mM xanthine was added to the suspension and the reaction mixture was incubated in a test tube. After incubation, the reaction mixture was heated at 98ºC for 5 min and was centrifuged at 11,228×*g* for 5 min. Concentration of xanthine and uric acid in the supernatant was determined by HPLC (column, ODS-UG-5; mobile phase, 10 mM potassium phosphate buffer, pH 5.5; flow rate, 1 mL/min; wavelength, 260 nm).

Bacterial identification by sequence analysis of 16S rDNA. To extract the genomic DNA, bacterial pellet was suspended in 0.2 mL of Insta Gene Matrix (Bio-Rad,

USA), incubated at 56°C for 30 min and then heated at 100°C for 8 min. After heating, the supernatant was used for the PCR reaction. Bacterial 16S rDNAs were amplified by using two universal primers, 9 F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541 R (5'- AAGGAGGTGATCCAGCC-3'). Polymerase chain reaction was performed using a kit with Hot Star tag DNA polymerase (Qiagen). The PCR program used for amplification was as follows: 95 °C for 15 min, followed by 25 cycles consisting of 94°C for 30 sec, 50°C for 1 min, and 72°C for 1 min, and a single final extension step consisting of 72°C for 10 min. The sequencing analysis of amplified products was performed with four universal primers, 9 F (5'-GAGTTTGATCCTGGCTCAG-3'), 785 F (5'-GGATTAGATACCCTGGTAGTC-3'), 926 R (5'-CCGTCAATTCCTTTGAGTTT-3') and 1541 R (5'-AAGGAGGTGATCCAGCC-3') by Fasmac Co., Ltd.

Results

Isolation and identification of AOH-forming bacterium

Since AHX in the nutrition medium contaminated by airborne microorganisms was converted to AOH, we were triggered to investigate airborne microorganisms for AOH production from AHX. An uncovered petri dish containing AHX solution was left alone at room temperature for 3 days. Conversion of AHX to AOH in the solution was analyzed by HPLC and part of AHX was converted to AOH (Fig. 3). Therefore, we tried to isolate the microorganism that converted AHX to AOH. The culture solution was spread on nutrient agar plates. As a result, one colony capable of converting AHX to AOH was isolated and we named the strain CH-1.

The partial sequence (1423 bp) of its 16S rDNA showed that the strain CH-1 is *Burkholderia contaminans* with similarity of 99.9% to the type strain (Table 1).

Bioconversion of AOH by resting cells of Burkholderia contaminans *CH-1*

After cultivation, the isolated *B. contaminans* CH-1 cells were harvested via centrifugation, washed, and then resuspended in the buffer containing AHX. AOH yield was greatly improved with the increase in reaction time with the resting cells (Fig. 4). Fig. 4A shows that AHX (initial concentration of 0.5 mM) was completely biotransformed to AOH by the cells at 24 h. The produced AOH was stable with the cells without further degradation even after 98 h of reaction (Fig. 4C).

Bioconversion of AOH by resting cells of various microorganisms

We compared the ability of the conversion of the obtained strain, CH-1, with other *Burkholderia* species and other microorganisms. For the comparison, reaction time was set to be 24 h, because the conversion with CH-1 almost reached maximum at 24 h. Seven *Burkholderia* species (*B. caryophilli* NBRC 13591, *B. capaci* NBRC 14595, *B. caledonica* NBRC 102488, *B. fungorum* NBRC 102489, *B. ferrariae* NBRC 106233, *B. ginsengisoli* NBRC 100965, and *B. mimosarum* NBRC 106338), other two bacteria (*Escherichia coli* JM 109 and *Bacillus subtilis* NBRC 13719), and two yeasts (*Saccharomyces cerevisiae* NBRC 2377 and *Candida albicans* NBRC 1060) were tested (Fig. 5A). Among them, only two species were able to produce AOH from AHX quantitatively like CH-1 (Fig. 5A). As shown in Fig. 5B-D, when the reaction time was shortened to 6 h from 24 h, AOH ratio between the contents of AHX and AOH produced by CH-1, *B. cepacia*, and *B. fungorum* were 89%, 70%, and 16%, respectively.

As a consequence, *B. contaminans* CH-1 exhibited the highest converting ability of AHX to AOH among all the strains tested.

Production of AOH by the resting cells of B. contaminans *CH-1*

The results mentioned above indicated that AOH could be produced from AHX by the resting cell method and that 0.5 mM AHX was completely converted to AOH. To confirm the result at higher concentrations of AHX, the resting cell method was carried out at a larger scale. 14.6 mM AHX was completely converted to AOH within 24 h of incubation (Fig. 4B). AOH was then purifyed by centrifugation and crystallization from reaction mixture of 11.6 mmol AHX, and we obtained 1.63 g (10.6 mmol) of purified AOH in 91% yield. The purified AOH showed the same retention time in HPLC and the same signals in ^{13}C NMR as authentic AOH (Fig. 6).

Discussion

AHX and AOH are useful chemicals with potential applications in a wide range of fields.[10,11] A production of AOH from AHX has been reported by xanthine oxidase.[7] However, this production method is insufficient for industrial use owing to several disadvantages such as low yields and slow reaction rates.[12]

In the current study, *B. contaminans* CH-1 with high AOH-producing ability was screened and identified (Fig. 3). *B. contaminans* is a member of the *Burkholderia cepacia* complex, a group of closely related gram-negative.[18-20] Characterization of this strain showed that it inhibited the growth of a broad range of fungal pathogens.[21-23] It has been reported about a degradation of 3-nitrotyrosine, 2-naphthoate and lactose by *Burkholderia* sp.[24-26]

Biotransformation of resting cells has some advantages such as short reaction time, non-sterile reaction conditions and convenient product separation. In the enzymatic reaction of AOH from AHX by XOD, high concentration of AHX strongly inhibited the activity of XOD (Fig. 2B). On the other hand, the resting cells almost completely converted AHX to AOH. These results indicated that the cells produced more active and/or more stable enzyme(s) or larger amounts of the enzyme(s) than the commercially available XOD. Considering the diverse environment of biotransformation between the resting cells and XOD, the resting cell method for AOH production is attractive in aspects of bioconversion time, substrate concentration and purification. To investigate whether the enzyme that produced AOH from AHX in the resting cells of CH-1 is XOD or not, we used xanthine as the substrate and xanthine was completely converted into uric acid within 4 h of incubation (Fig 7). This result suggested that AOH might be produced by XOD in the resting cells. We are now trying to purify the enzyme that catalyzes the reaction from AHX to AOH, because the enzyme would be useful for further improvement of the present biochemical method of AOH production and for investigation of the biosynthetic route from AHX to AOH in microorganisms and/or plants. A production level of AOH (10.6 mmol) was achieved using the bioconversion that successively used resting cells, which is a novel procedure for large scale production of AOH. Highly purified AOH is easily obtained through a simple purification procedure of centrifugation and crystallization. This suggests that AOH may be easily produced by our method to practical use in agriculture.

Author contributions

J.-H.C., S.T., and H.K. designed the experiments. J-H.C. and A.K. performed the experiments. H.H., S.T. and H.K. assisted with experiments and contributed to discussions. J-H.C. and H.K. wrote the manuscript.

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Figure captions

Fig. 1. Structures of fairy chemicals (A) and purine metabolic pathway in animals, plants and microorganisms (B).

Note: **1** : 2-azahypoxanthine (AHX); **2** : 2-aza-8-oxohypoxanthine (AOH); **3** : imidazole-4-carboxamide (ICA); **4** : 5-aminoimidazole-4-carboxamide (AICA); **5** : AICA ribotide; **6**: 5-formyl AICAR; XOD: xanthine oxidase. The novel pathway in plants is in the framed rectangle.

Fig. 2. Time course of production of AOH from AHX by xanthine oxidase.

Note: Symbols, ● and ○, indicate AHX and AOH, respectively. Initial concentrations of AHX were 0.36 mM (A) and 11.5 mM (B), respectively.

Fig. 3. HPLC profile of authentic AHX and AOH, and culture broth of CH-1

Notes: A: authentic AHX; B: culture broth of CH-1; C: authentic AOH. Column: C30-UG-5; mobile phase: 0.02% TFA in H_2O; flow rate: 0.8 mL/min; wavelength: 254 nm.

Fig. 4. Time course of AOH production (A, B) and AOH degradation (C) by resting cells of CH-1.

Note: Symbols, ● and ○, indicate AHX and AOH, respectively. Initial concentrations of AHX were 0.5 mM (A) and 14.6 mM (B), respectively.

Fig. 5. Concentration ratio of AHX and AOH in the reaction by resting cells of various microorganisms.

Notes: A: AHX (0.5 mM) was incubated for 24 h by resting cells of various microorganisms; conversion rate at each reaction time by resting cells of CH-1 (B), *B. cepacia* NBRC 14595 (C) and *B. fungorum* NBRC 102489 (D). Gray and black columns indicate AHX and AOH contents, respectively.

Fig. 6. Identification of the purified product.

Notes: HPLC chromatograms of AOH (A). Authentic AOH (above), purified AOH in this study (below). Column: C30-UG-5; mobile phase: 0.02% TFA in H_2O; flow rate: 0.8 mL/min; wavelength: 254 nm. ^{13}C NMR spectra of AOH in DMSO-d_6 (B). Authentic AOH (above), purified AOH in this study (below).

Fig. 7. Time course of production of uric acid from xanthine by resting cells of CH-1

Note: Symbols, ● and ○, indicate xanthine and uric acid, respectively.

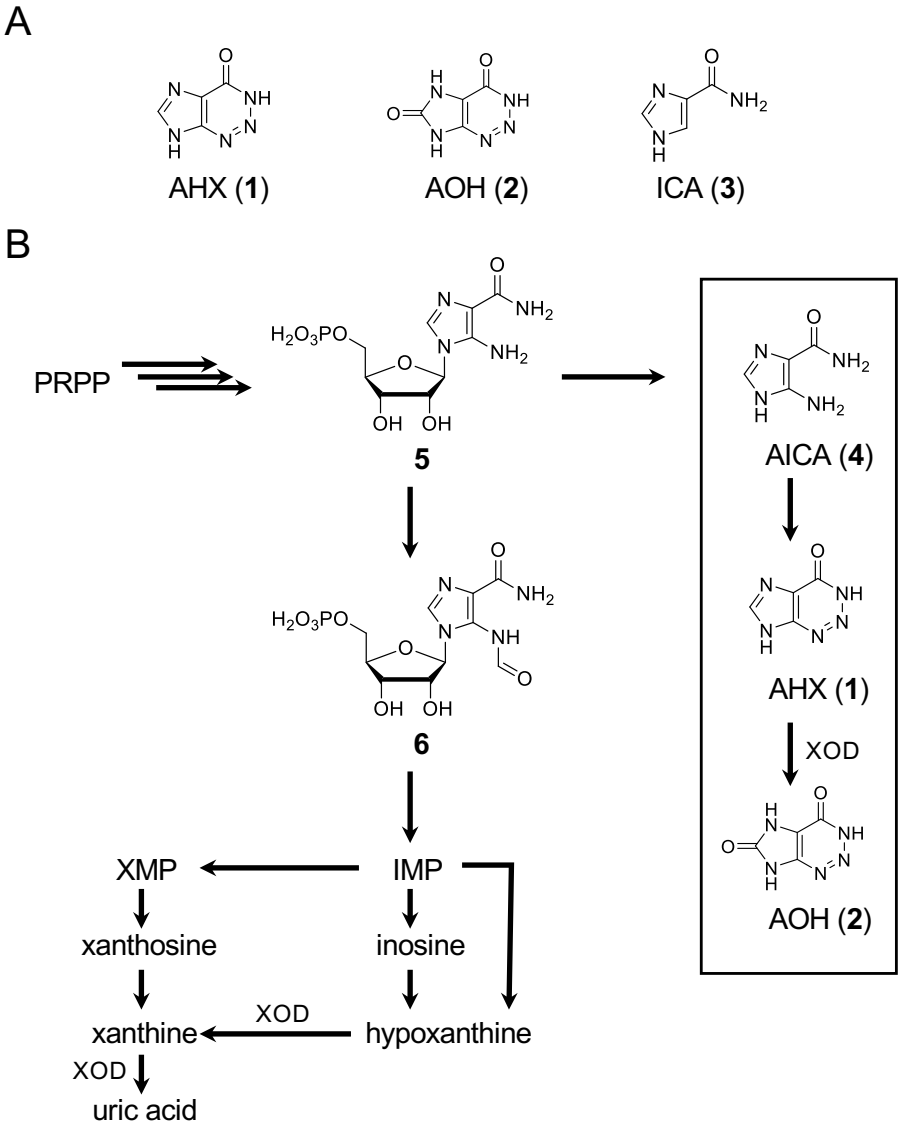
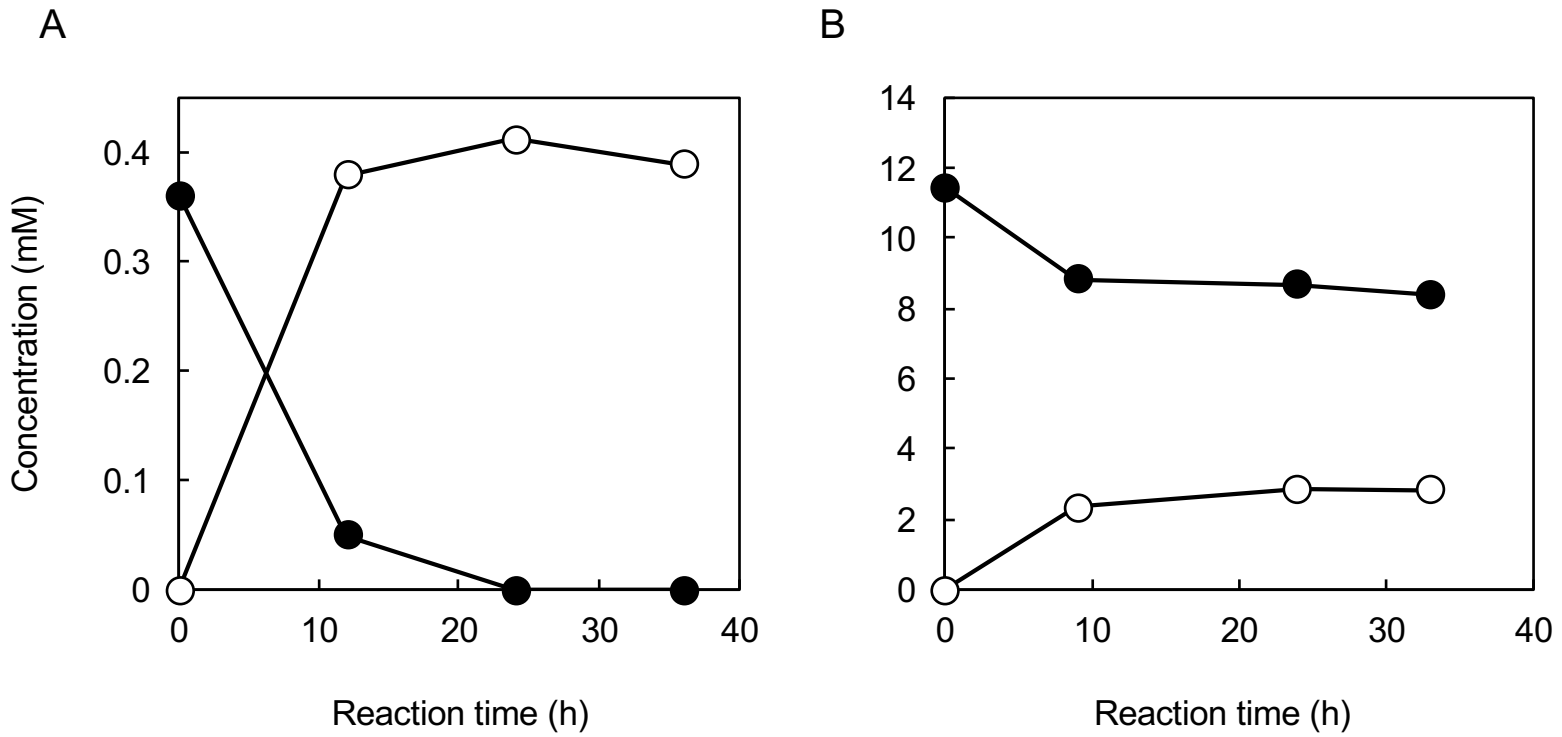

A
AHX (1)
AOH (2)
ICA (3)
B
PRPP
5
6
AICA (4)
AHX (1)
XOD
AOH (2)
XMP
IMP
xanthosine
inosine
XOD
xanthine
hypoxanthine
XOD
uric acid

Fig. 1


A
B
Concentration (mM)
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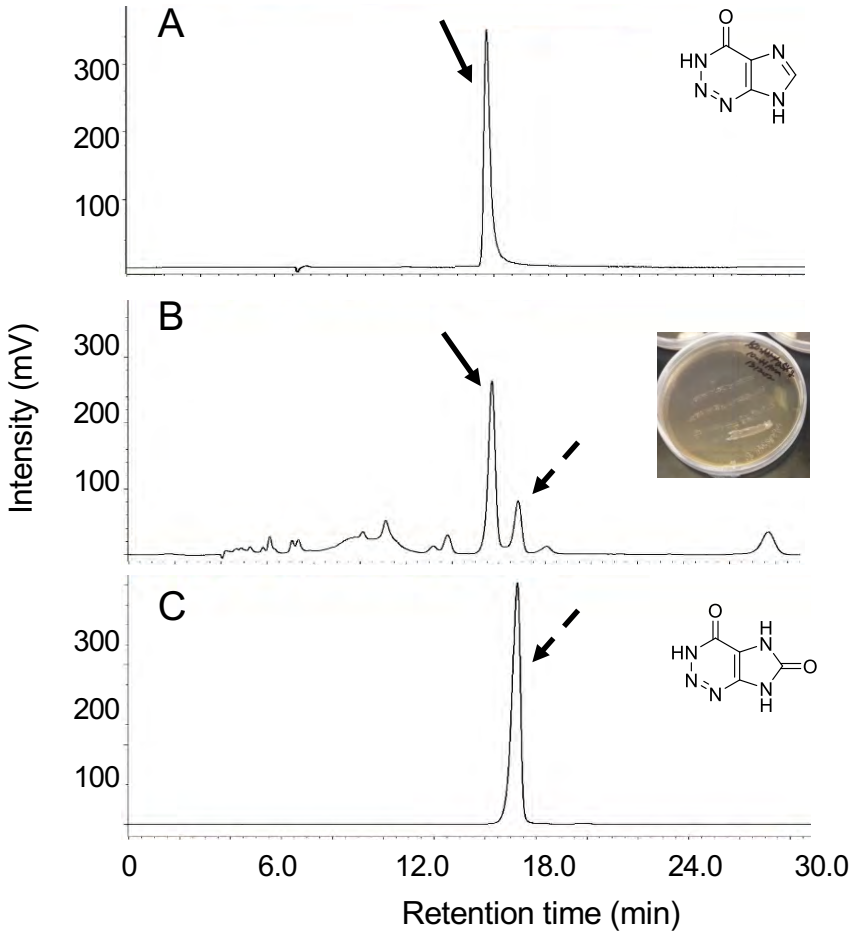
0.3
0.4
0
2
4
6
8
10
12
14
0
10
20
30
40
Reaction time (h)
Reaction time (h)

Fig. 2


A
B
C
300
200
100
Intensity (mV)
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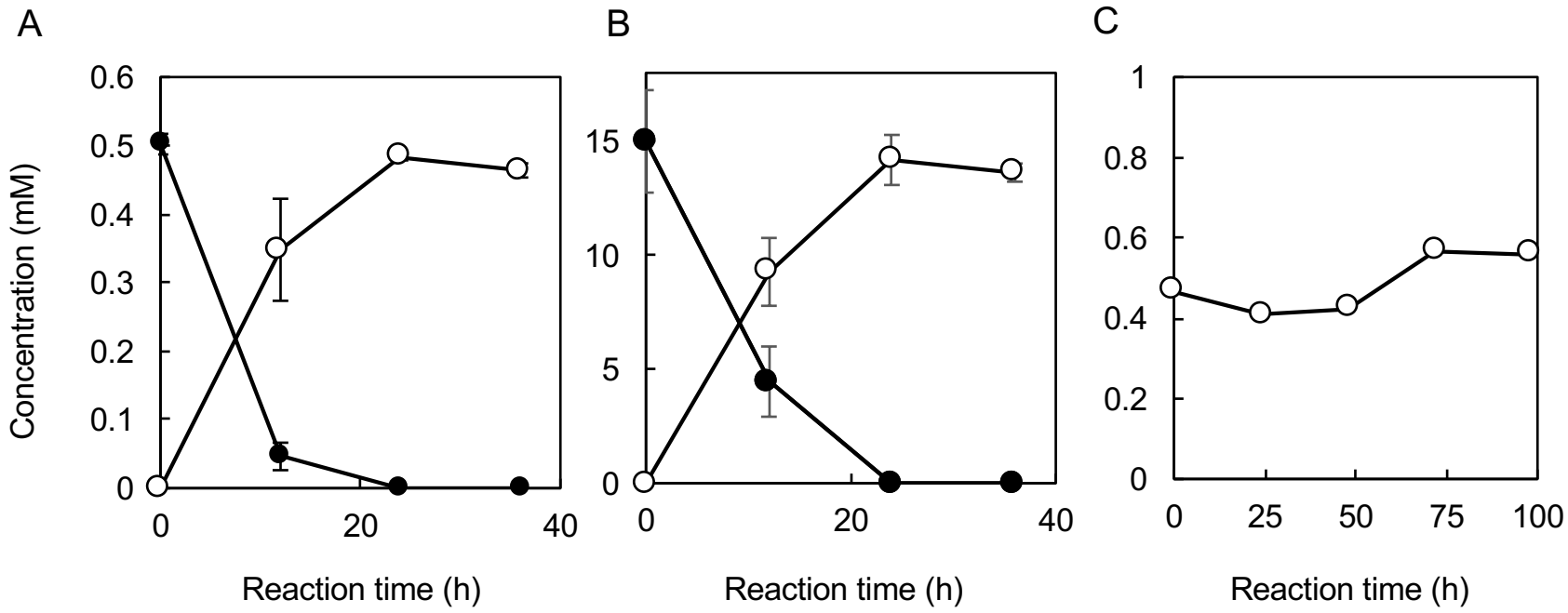
18.0
24.0
30.0
Retention time (min)
HN
N
N
O
H

Fig. 3


A
B
C
Concentration (mM)
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0.5
0.4
0.3
0.2
0.1
0
15
10
5
1
0.8
0.6
0.4
0.2
0
0
20
40
25
50
75
100
Reaction time (h)
Reaction time (h)
Reaction time (h)

Fig. 4

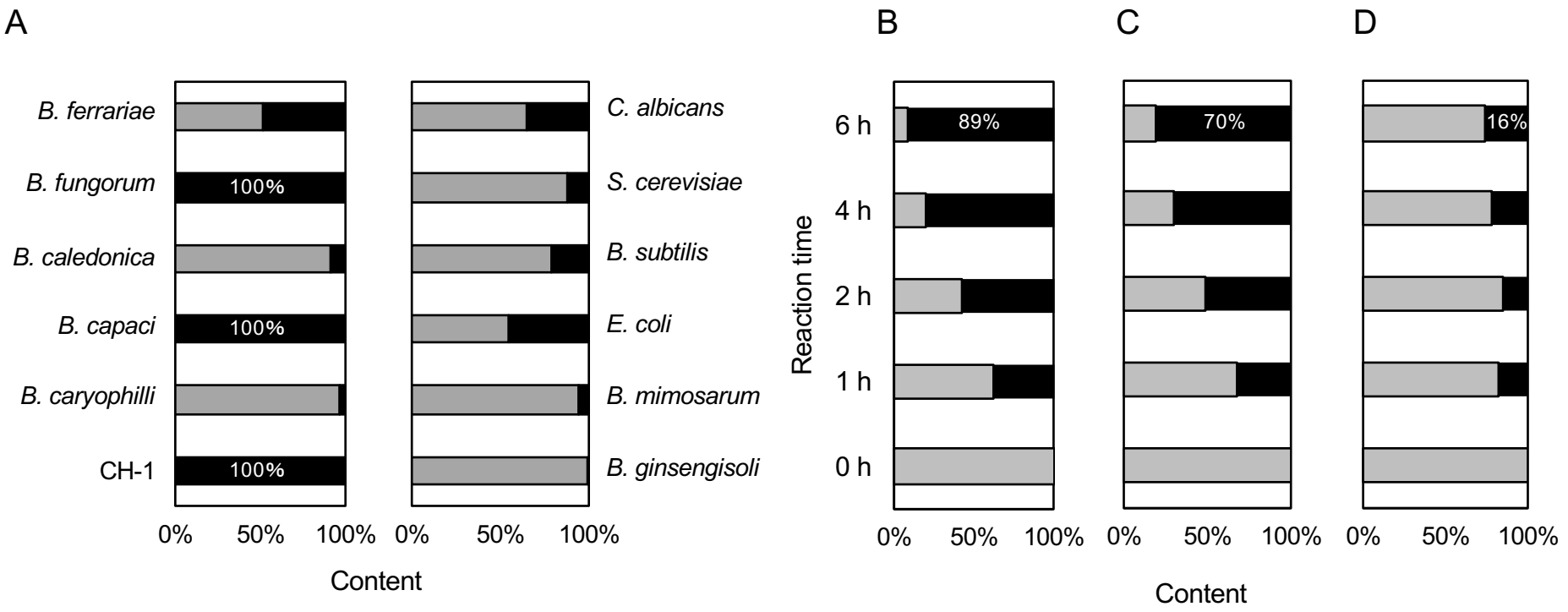
A
B
C
D
B. ferrariae
B. fungorum
B. caledonica
B. capaci
B. caryophilli
CH-1
C. albicans
S. cerevisiae
B. subtilis
E. coli
B. mimosarum
B. ginsengisoli
100%
100%
100%
0%
50%
100%
Content
Reaction time
6 h
4 h
2 h
1 h
0 h
89%
70%
16%

Fig. 5

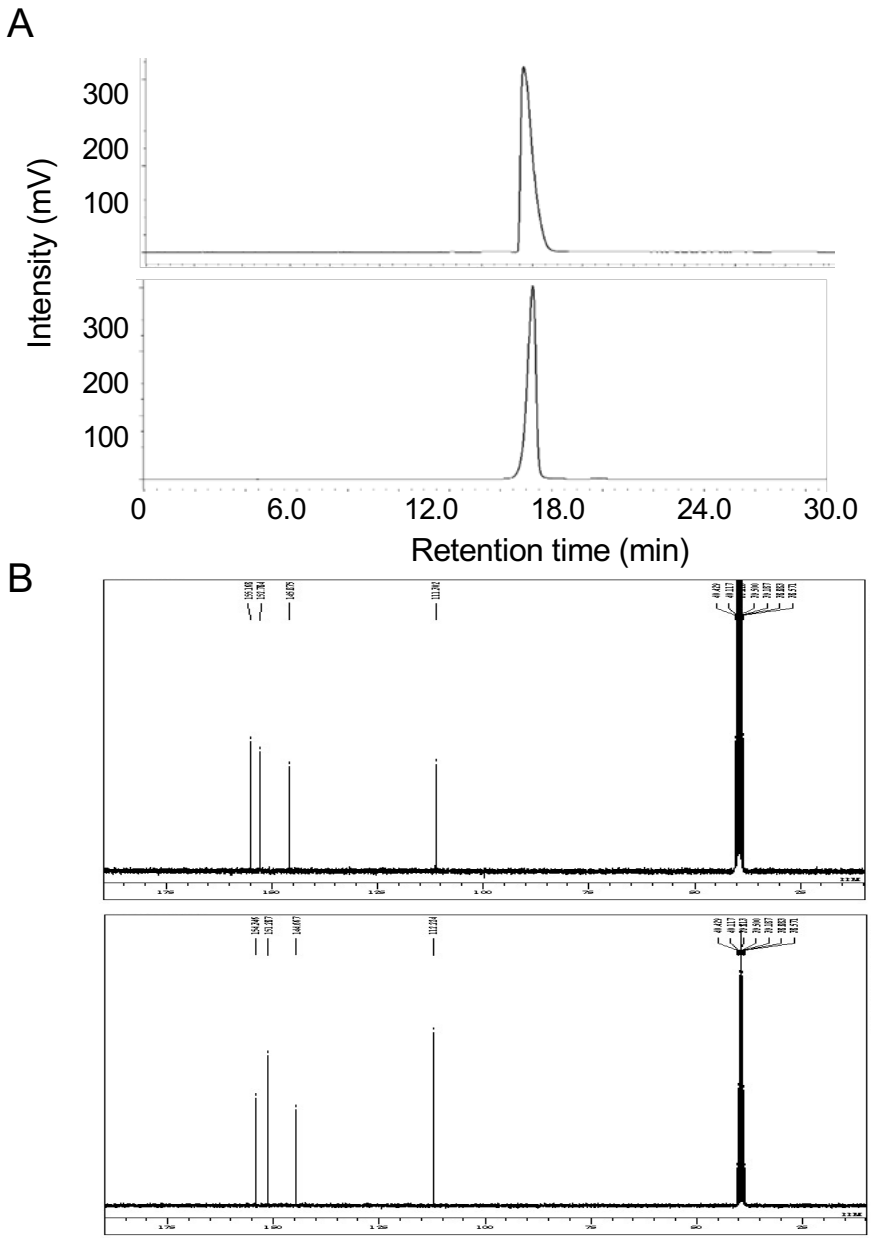
A
300
200
100
Intensity (mV)
300
200
100
0
6.0
12.0
18.0
24.0
30.0
Retention time (min)
B

Fig. 6

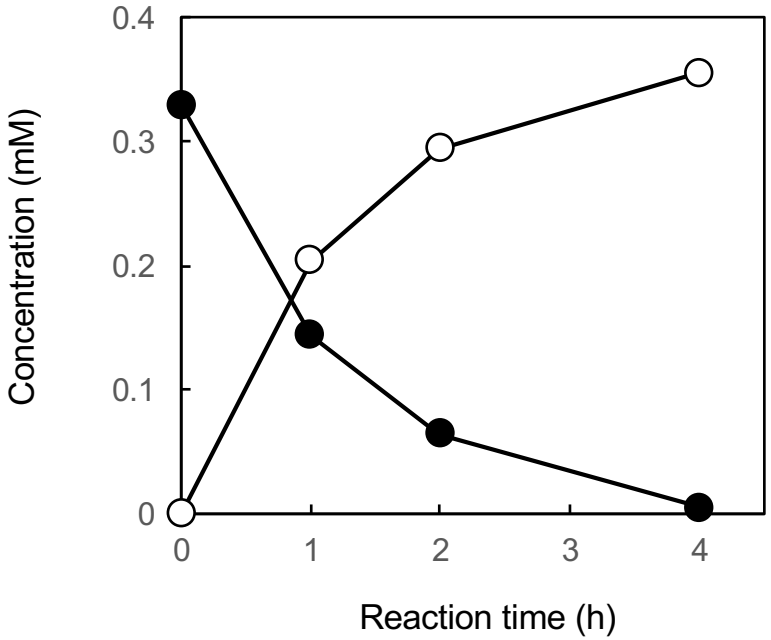

Fig. 7

Table 1. The 16S rDNA similarity between strain CH-1 and other *Burkholderia* genus

Number	Strains	Similarity (%) (Length)
1	*Burkholderia contaminans* LMG 23361^{T}	99.9 (1421/1423)
2	*Burkholderia lata* 383^{T}	99.7 (1419/1423)
3	*Burkholderia arboris* R-24201^{T}	99.6 (1418/1423)
4	*Burkholderia cepacia* NBRC14074^{T}	99.4 (1413/1421)
5	*Burkholderia cenocepacia* LMG 16656^{T}	99.2 (1410/1421)