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Original Research Article

Screening and identification of acetic acid bacteria (AAB) producing coenzyme Q_{10} from plant materials

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ABSTRACT

The major sources of coenzyme Q_{10} (CoQ_{10}) used in pharmaceutical and cosmetic industries are generally synthesized from bacteria such as *Agrobacterium tumefaciens*, *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum*, but the yields of CoQ_{10} obtained from these bacteria have not been enough for its demand. In order to satisfy the need of CoQ_{10}, the new potential CoQ_{10} producing bacteria have to be obtained. In this study, various parts (i.e. flower, fruit, and root) of plants such as grape, pineapple, orchid, sleep plant, apple, krachai, hibiscus, cogon grass and nut grass, etc., were used for isolation and screening of CoQ_{10} producing bacteria. From the screening results obtained by using high performance liquid chromatography, 29 isolates from 65 isolates can produce CoQ_{10} in the range of 144 to 1,536 µg/g of dried sample. Interestingly, the 16S rRNA gene sequencing analysis indicated that all isolates were acetic acid bacteria including *Asaia bogorensis* (9), *Gluconobacter japonicus* (13), *Gluconobacter oxydans* (3), *Gluconobacter roseus* (2), *Gluconobacter liquefaciens* (1), and *Gluconobacter thailandicus* (1). The top 4 strains which can produce CoQ_{10} more than 1,500 µg/g of dried sample (*G. japonicus* BK86-2, *G. japonicus* BK81-1, *A. bogorensis* BD-1, and *A. bogorensis* BK51-2) were selected as the candidates for CoQ_{10} production. The growth rate and yield of CoQ_{10} of each strain were monitored every 24 h for 120 h in order to obtain the optimum condition for CoQ_{10} production. The highest yield of CoQ_{10} about 2,169±68 µg/g of dried sample was obtained from *G. japonicus* BK81-1 after culturing at 30°C in Glucose-yeast-peptone medium (GYP) for 96 h.

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INTRODUCTION

Coenzyme Q_{10} or ubiquinone-10 (CoQ_{10}) is a naturally fat-soluble substance occurred from the conjugation of a 2, 3-dimethoxy-5-methyl-benzoquinone ring and ten units of isoprenoid side-chain. CoQ_{10} plays a crucial role in the generation of cellular energy and the scavenging of free radicals so that it has been extensively used in pharmaceuticals, food supplements, and cosmetics (Littarru and Tiano, 2007). CoQ_{10} is generally found in animals, plants, and microorganisms; however, the production of CoQ_{10} is only relied on certain microbes such as *Agrobacterium* (Ha *et al*., 2007), *Paracoccus* (Yoshida *et al*., 1998, Choi *et al*., 2005), *Rhodobacter* (Urakami and Yoshida, 1993), and *Sporobolomyces* (Yurkov *et al*., 2008) due to the high levels of biological CoQ_{10} produced inside their cells. However, the production of CoQ_{10} is still not enough to satisfy increasing customer demand, resulting in high cost of CoQ_{10}. Screening for a new microbial source of CoQ_{10} is essential and for solving its commercial production.

Acetic acid bacteria (AAB) are a group of Gram-negative bacteria which can oxidize sugars, sugar alcohols, and ethanol with the production of acetic acid as the major end product. AAB not only play a positive role in the production of selected foods and beverages (Raspor and Goranovic, 2008), but they also has been reported as a potential source of CoQ_{10} (Yamada *et al*., 1968, Park *et al*., 2005). Although the habitats of AAB are diverse, they are normally found and isolated from plant materials (Gillis *et al*., 1989, Fuentes-Ramírez *et al.*, 2001, Seearunruangchai *et al*., 2004). Therefore, the aim of this study is to screen and isolate AAB from plants for new potential CoQ_{10} producing bacteria.

MATERIALS AND METHODS

Screening of AAB from plant materials

Various parts (i.e. flower, fruit, and root) of plants such as grape (*Psidium guajava* L.), pineapple (*Ananas comosus*), orchid (*Vanda lilacina*), sleepy plant (*Mimosa pudica),* apple (*Malus domestica*), spike flower (*Ixora coccinea* L.), krachai flower (*Curcuma sessilis),* hibiscus flower (*Hibicus syriacus*), grass root (*Brachiaria mutica*), were collected from Ratchaburi province and used for bacterial isolation in this study. A gram of each plant sample was ground and soaked in 9 ml of acidified Glucose Yeast Peptone (GYP) broth (pH 3.5) at 30°C for 72 h. Then, 100 µl of mixtures from its various 10 fold serial dilutions were spread on GYP agar supplemented with $CaCO_3$ (pH 5.2). After incubation at 30°C for 72 h, a single colony was gathered in which clear zones were observed and purified. The colony was transferred, re-dissolved in 1 ml of GYP broth (pH 5.2) and stored in 15% glycerol at -80°C for further study.

Bacterial identification by 16*S* rRNA gene sequencing analysis

Selected strains were thawed on ice and grown on GYP agar (pH 5.2) at 30°C. After 72 h, the cells were collected by washing twice with 0.85% sterile sodium chloride and centrifuged at 8000 rpm (Mikro 200R Centrifuge, Hettich, UK), respectively. Genomic DNA was extracted by a DNeasy Tissue kit (Qiagen, Hilden, Germany). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') as described by Yukphan *et al.,* 2004. The amplification was carried out in a TProfessional® thermocycler (Biometra, Göttingen, Germany) with the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 60 s, annealing at 50°C for 60 s, and extension at 72°C for 2 min, and a final extension step at 72°C for 3 min. The PCR product was sent and sequenced at Macrogen, Seoul, South Korea. The 16S rRNA gene sequence was multiple-aligned with the related sequences obtained from the EzbioCloud database by using the CLUSTAL_X program (Thompson *et al.,* 1997). Gaps and ambiguous bases were removed before reconstructing the phylogenetic tree by the neighbor-joining method in the MEGA 6 program. Confidence values of the branches of phylogenetic tree were evaluated by using the bootstrap resampling method with 1000 replications.

Screening for CoQ_{10} producing bacteria

Cell cultivation

Twenty nine isolates of AAB were evaluated for their ability to produce CoQ_{10}. A loop full with each AAB cells was collected from 72 h cell culture and inoculated into a test tube containing 5 ml GYP broth (pH 5.2). The culture was incubated at 30°C for 24 h on a rotary shaker at 100 rpm. The cell concentration of the culture was adjusted with 0.85% sterile sodium chloride until it was equivalent with optical density 0.8 at wave length 660 nm. One percent of diluted culture was transferred to a 250-ml flask containing 100 ml of GYP broth (pH 5.2) and incubated at 30°C for 72 h on rotary shaker at 200 rpm.

Cell extraction and measurement of CoQ_{10}

The CoQ_{10} extraction from cells was adapted from method of Yoshida *et al*. (1998). Briefly, the cell culture obtained from previous section was harvested by centrifugation at 8,000 rpm (Suprema 25, Tomy, Japan) for 10 min at 4°C and washed with sterile distilled water. The cells were frozen at -80°C and dried by vacuum freeze dryer (Gamma 2-16 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Ten mg of dried cells were added into 10 ml of the mixture between chloroform and methanol (2:1), mixed vigorously for 18 h, and filtered through Whatman filter paper No.1. The filtrate was evaporated until dryness. The residue was resuspended in ethanol and CoQ_{10} concentration was analyzed by HPLC (LC-10AD, Shimadzu, Japan) according to the method of Kien *et al.* (2010) with certain modifications. The analysis was performed on an InertSustain® C18 column (250 mm X 4.6 mm, 5 µm) with the mixture of methanol and isopropanol (1:1) as the mobile phase at a flow rate of 1.2 ml/min. The CoQ_{10} was detected at 275 nm. All samples were done in triplicate.

Cells and CoQ_{10} production during growth of AAB

The highest top 4 of CoQ_{10}-producing AAB in this study were chosen to study their growth and CoQ_{10} production. The cell inoculum was prepared as mentioned previously, and 1 l of Erlenmeyer flask containing 200 ml GYP broth (pH 5.2) was used instead of a 250-ml flask containing 100 ml of GYP broth (pH 5.2). The cells were incubated at 30°C harvested every 24 up to 120 h for measuring cell dried weight and amount of CoQ_{10} production.

RESULTS AND DISCUSSION

Isolation of AAB from plant materials

Sixty five isolates obtained from plant materials, particularly fruits and flowers (Figure 1) showing clear zone on GYP agar with 0.3% $CaCO_3$. Gram's strain and morphological analysis of colony selection showed that 54 isolates were Gram negative, creamy color, circular, convex, and entire with slimy which was a major characteristic of AAB for further investigation, while the rest as Gram positive bacteria were omitted from this study. According to lactate and acetate oxidation, these Gram negative bacteria can be simply divided into 3 groups; 1) Genus *Acetobacter* which have ability to oxidize both lactate and acetate (25 isolates); 2) Genus *Gluconobacter* which have no ability to oxidize both lactate and acetate (20 isolates); 3) Unknown genus which have ability to oxidize only lactate or acetate (9 isolates). As previous study from Cleenwerck *et al*. (2002) and Li *et al*. (2014) indicated that bacterial isolates in group 1 cannot produce CoQ_{10} due to a lack of ubiquinone-10. Therefore, only bacterial isolates in group 2 and 3 were selected for 16S rRNA gene sequencing analysis.

Identification of AAB by 16S rRNA gene sequencing analysis

Based on 16S rRNA gene sequences and Neighbour-joining tree, the sequences of 29 isolates from plant materials belong to 2 genera which are *Gluconobacter* and *Asaia* as shown in figure 2. These genera are divided into 6 species including *G. japonicus* (13 isolates), *G. oxydans* (3 isolates), *G. roseus* (2 isolates), *G. liquefaciens* (1 isolate), *G. thailandicus* (1 isolate), and *A. bogorensis* (9 isolates). Interestingly, all isolates obtained from various parts of plant materials in this study are AAB.

CoQ10 production of AAB

The amounts of CoQ_{10} extracted from 29 isolates were listed in Table 1. The CoQ_{10} production from each strain was range between 144 to 1,536 µg/g of dried sample. The greatest amount of CoQ_{10} (approximately 1,536 µg/g of dried sample) was obtained from *G. japonicus* BK86-2. Although the amount of CoQ_{10}-derived from *G. japonicus* was lower than CoQ_{10} which produced by famous plant-pathogen strains, *A. tumefaciens*, (Rho *et al*., 2001, Subramoni *et al*., 2014), but it was nonpathogenic and safe use for human (Weenk *et al*., 1984, Singh and Kumar, 2007, García-García *et al*., 2017). Therefore, the top 4 strain which can produce CoQ_{10} more than 1,500 µg/g of dried sample (*G. japonicus* BK86-2, *G. japonicus* BK81-1, *A. bogorensis* BD-1, and *A. bogorensis* BK51-2) were selected for studying their growth and CoQ_{10} production during batch fermentation in order to obtain the maximum CoQ_{10}.

Cells and CoQ_{10} production during growth of AAB

The highest amount of dried cells-obtained from each strain was in the range of 2.0 to 2.3 g/l. They were not much different in terms of growth during 120 h fermentation. The growths of every stain were followed the typical sigmoidal curve obtained for most bacteria. The lag phases of cells were observed during 24 to 48 h. The cell contents increased from 0.5 to 2.0 g/l within approximately 96 h, after which the slope became stable as shown in figure 3A. In terms of CoQ_{10} production, the highest amount of CoQ_{10} of each stain was observed at 96 and 120 h. The CoQ_{10} productions from *G. japonicus* BK86-2, *G. japonicus* BK81-1, *A. bogorensis* BD-1, and *A. bogorensis* BK51-2 were 2,177±119 (120 h.), 2,169±68 (96 h.), 1,796±127 (96 h.) and 1,816±118 (120 h.) µg/g dried cell, respectively. (Figure 3B). The greatest amount of CoQ_{10} production among 4 strains studied was obtained from *G. japonicus* BK81-1 (2,169±68 µg/g dry weight) at 96 h. which this amount of CoQ_{10} production was not different from *G. japonicus* BK86-2 at 120 h fermentation. This was different from previous results which showed that *G. japonicus* BK86-2 (1,536±43.3 µg/g dry weight) was the best CoQ_{10} producing isolate. This might be due to lower number of cells produced during the growth of *G. japonicus* BK81-1 and the time use for culturing.

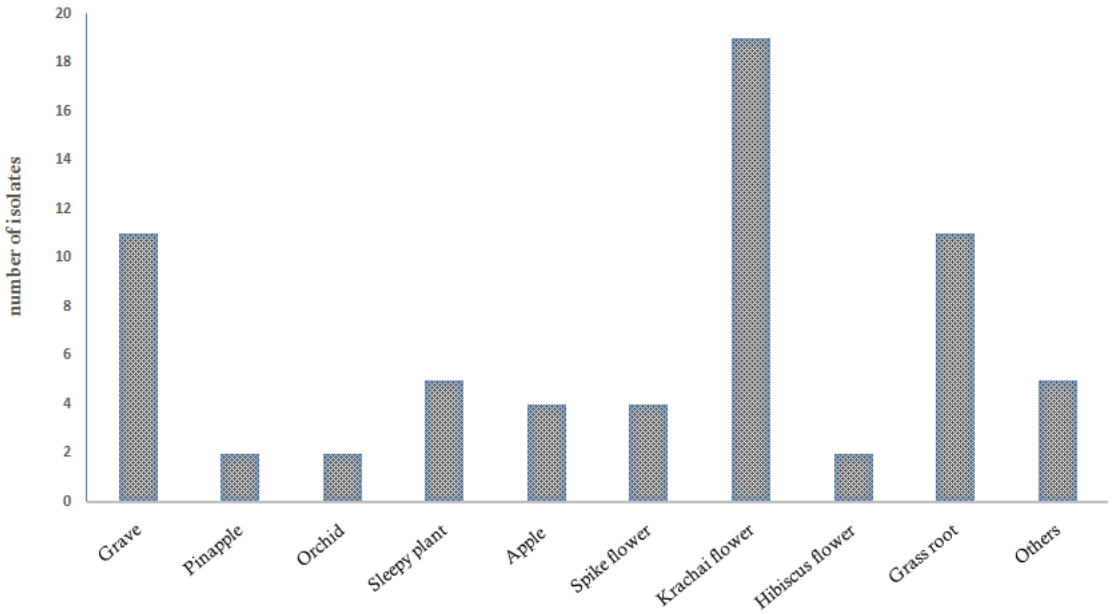

Figure 1. Number of isolates and sources of AAB

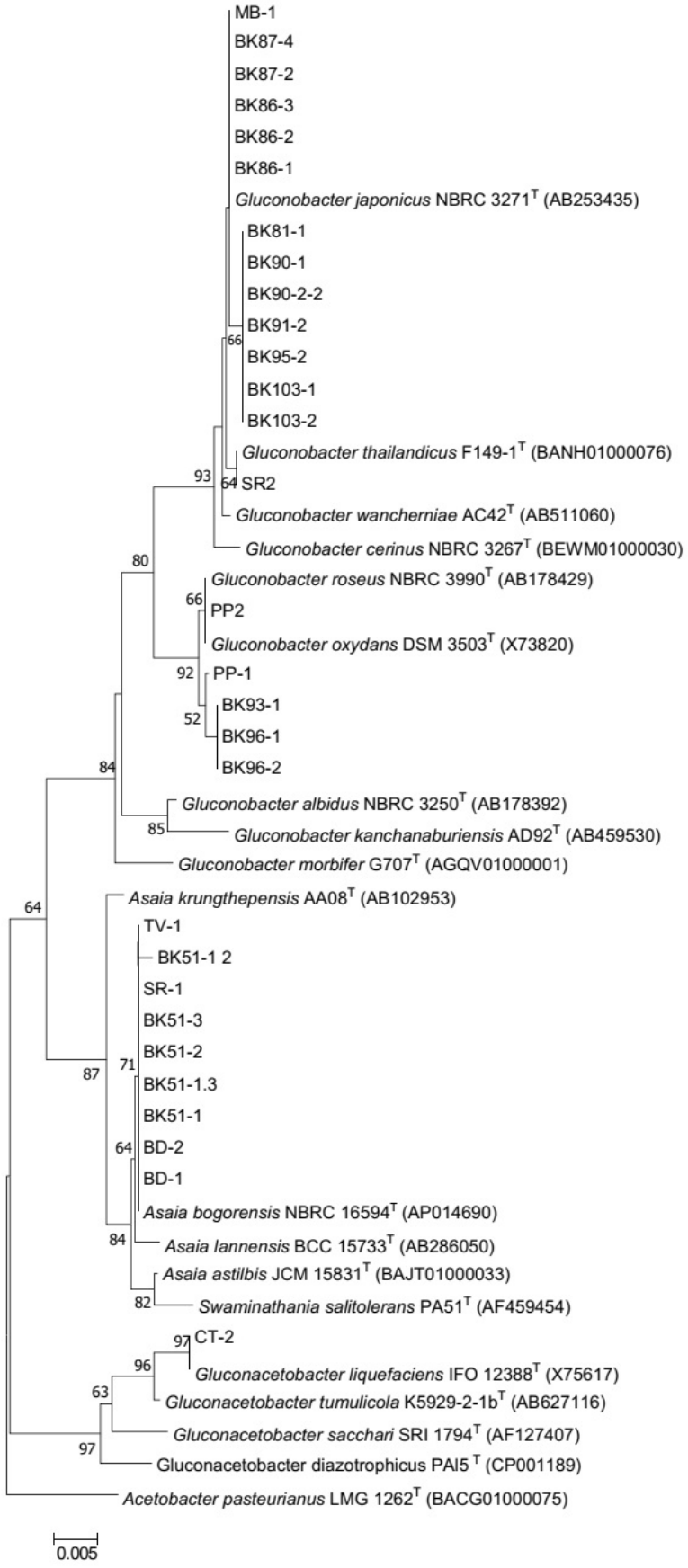

Figure 2. Neighbor-joining tree based on 16S rDNA sequences showing the relationship among 29 bacterial strains and related species. Bootstrap percentages >50%, based on 1000 replications, are given at nodes. Bar 0.005 substitutions per nucleotide position

Table 1. CoQ_{10} production of AAB isolated from plant materials in GYP broth (pH 5.2, 30°C for 72 h)

No.	Isolates	Species	CoQ_{10} (µg/g dry weight)
1	BK86-2	*Gluconobacter japonicus*	1536±43.3
2	BK81-1	*Gluconobacter japonicus*	1523±31.7
3	BD-1	*Asaia bogorensis*	1512±37.5
4	BK51-2	*Asaia bogorensis*	1510±41.2
5	BK86-1	*Gluconobacter japonicus*	1413±27.4
6	BK90-1	*Gluconobacter japonicus*	1360±39.3
7	BK103-2	*Gluconobacter japonicus*	1262±48.3
8	BK95-2	*Gluconobacter japonicus*	1251±53.1
9	BK93-1	*Gluconobacter oxydans*	1219±51.8
10	BK87-4	*Gluconobacter japonicus*	1206±29.8
11	BK103.1.1	*Gluconobacter japonicus*	1203±43.1
12	MB-1	*Gluconobacter japonicus*	1201±22.0
13	BK86-3	*Gluconobacter japonicus*	1200±15.3
14	BK96-1	*Gluconobacter roseus*	1175±10.2
15	BK91-2	*Gluconobacter japonicus*	1173±43.7
16	BK87-2	*Gluconobacter japonicus*	1155±25.4
17	SR-2	*Gluconobacter thailandicus*	1153±27.0
18	PP-2	*Gluconobacter roseus*	1150±25.2
19	PP-1	*Gluconobacter oxydans*	1067±34.2
20	SR-1	*Asaia bogorensis*	1030±26.2
21	BD-2	*Asaia bogorensis*	977±35.3
22	BK51-3	*Asaia bogorensis*	950±14.5
23	BK51-1.3	*Asaia bogorensis*	945±32.0
24	BK90-2.2	*Gluconobacter japonicus*	902±28.4
25	TV-1	*Asaia bogorensis*	839±24.0
26	BK51-1	*Asaia bogorensis*	818±10.4
27	BK51-1.2	*Asaia bogorensis*	792±21.3
28	BK96-2	*Gluconobacter oxydans*	773±22.3
29	CT-2	*Gluconacetobacter liquefaciens*	144±16.2

CONCLUSIONS

The screening method used in this study is very suitable and specific for AAB. Three main genera of AAB i.e. *Acetobacter*, *Gluconobacter*, and *Asaia* were observed in plant materials, particularly fruits and flowers. The highest amount of CoQ_{10} about 2,169±68 µg/g of dried sample was obtained from *G. japonicus* BK81-1 after culturing at 30°C in GYP for 96 h. The results clearly show that *G. japonicus* BK81-1 is potent for use as a novel source of CoQ_{10}; however, its CoQ_{10} need to be evaluated for anti-oxidant, anti-wrinkle and anti-melanogenesis activity before using in cosmetic and pharmaceutical products.

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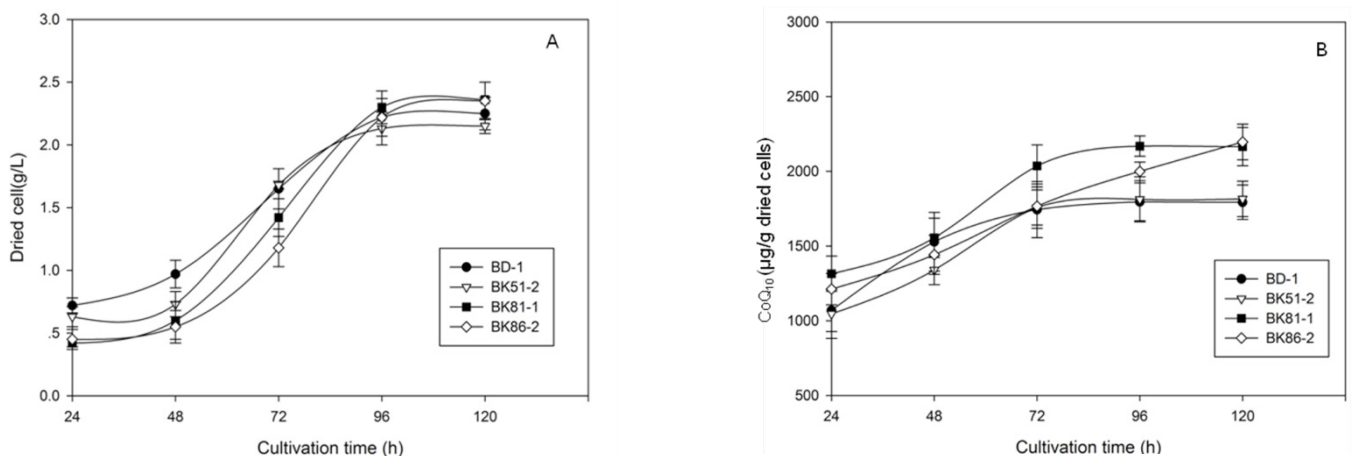

Figure 3. The total amount of dried cells (A) and CoQ_{10} (B) obtained from each bacterial strain (*G. japonicus* BK86-2, *G. japonicus* BK81-1, *A. bogorensis* BD-1, and *A. bogorensis* BK51-2) cultured in GYP broth (pH 5.2, 30°C for 120 h).

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