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Biodegradation of Geosmin by a Newly Isolated *Xanthobacter flavus*-YW

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Geosmin is a typical odorant in potable water sources, but this substance is hardly removed by conventional water treatment. In this study, a promising facultative anaerobic bacterial strain was successfully isolated from a bioreactor and exhibited a high biodegradation rate of geosmin. 16S rRNA sequencing and physiological analysis were performed. Results showed that this strain belongs to Gram-negative *Xanthobacter flavus*. To date, this is the only study to report the isolation and characterization of a facultative anaerobic bacterium from a bioreactor capable of biodegrading geosmin as the sole carbon source. As additional carbon source of *X. flavus*-YW, beef extract (0.5 g/L) may enhance the biodegradation of geosmin. At an initial concentration of 50 μg/L, 60% of geosmin was biodegraded after 2 d, and 71.3% of geosmin was biodegraded after 10 d. This study is important in basic research and application of the efficient removal of geosmin from potable water sources.

Key words: Geosmin; Biodegradation; Isolation; *Xanthobacter* spp.

The occurrence of taste and odor problems in drinking water is widespread and has been reported in Asia, Australia, North America, and Europe[1-3]. One of the typical odorants is geosmin (Tran-1,10-dimethyl-trans-9-decalol) that produces an earthy-musty odor in drinking water[4]. This odorant is released by *Cyanobacteria* (blue-green algae) at high concentrations reported reaching 100 ng/L[5]. At a low threshold of approximately 2 ng/L, this substance can cause malodorous drinking water[6]. Although compounds that cause these problems do not pose health threats, offensive odor and taste may lead to psychosomatic effects, such as headaches and eye, nose, and skin irritations. Water utilities typically rely on conventional treatment methods, such as pre- and post-chlorination, coagulation, and sedimentation, to remove taste- and odor-causing compounds. However, these technologies fail to remove geosmin. Advanced treatment options, such as oxidation by chlorine or ozone, are also not entirely effective[7, 8]. Activated carbon can also be used to treat geosmin, but this technique is often limited by the presence of other far more abundant natural organic matter (NOM) in water sources[9]. Recent studies have begun to examine biological treatment as an alternative method for

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geosmin removal because it has a high potential in environmental technology.

In 1970, Silvey et al. first reported that microorganism could be used to biodegrade geosmin, and the strain was identified as *Bacillus cereus*[10]. Narayan and Nunez further enriched and cultured geosmin-biodegrading bacteria previously reported and other strains from soil. They found that *B. cereus* and *B. subtilis* isolated from the soil were the most effective in oxidation of geosmin[11]. However, MacDonald and other researchers failed to replicate the same experimental results with the same strains[12]. Saadoun and El-Migdadi reported that biodegradation of geosmin produced by *Streptomyces halstedii* (A-1 strain) was investigated using seven Gram-positive bacteria; among these bacteria, *Arthrobacter atrocyaneus*, *Arthrobacter globiformis*, *Chlorophenolicus* N-1053, and *Rhodococcus maris* had ability to biodegrade geosmin[13].

These geosmin-biodegrading strains above all belonged to Gram-positive bacteria. In 2009, Hoefel enriched and isolated a Gram-negative bacterium labeled as Geo48 from the biofilm in a biologically active sand filter, where the isolate effectively biodegraded geosmin individually. Geo48 belonged to *Sphingomonadaceae* of the sub-class *Alphaproteobacteria* and biodegraded geosmin in a planktonic state by a pseudo-first-order mechanism[14].

In general, geosmin-biodegrading strains are classified as heterotrophic aerobic species; however, there are knowledge gaps regarding facultative-anaerobic and anaerobic bacteria that can biodegrade geosmin. Moreover in isolating geosmin degraders, some culture media containing other carbons except geosmin reduce abilities to obtain highly efficient geosmin degraders, such as Luria-Bertani (LB) agar medium (1% peptone, 0.5% yeast extract powder, 0.5% NaCl, 1.5% agar (W/V), pH 7) used by Tanaka[15] and Beihai Zhou[16]. Furthermore, geosmin in aqueous solutions is highly volatile. MacDonald reported, in their preliminary experiments the substantial loss of geosmin occurred if its solutions were incubated in flasks equipped with cotton plugs or permeable membrane[12].

This study aimed to isolate a facultative anaerobic geosmin-biodegrading bacterium by collecting biofilm from a bioreactor. This study also aimed to isolate stains on a culture plant with geosmin as the sole carbon source. To prevent geosmin loss, flasks equipped with glass restriction stoppers were used in the experiments. The effect of culture conditions on the removal rates of geosmin was also investigated. This study is important in basic research and application of the efficient removal of geosmin from potable water sources.

MATERIALS AND METHODS

Source of Bacteria

The target bacteria were from a BAF (biological aerated filter) bioreactor by collecting filler surface biofilm. This bioreactor is a laboratory-scale BAF (Fig. 1). It could remove geosmin efficiently and stably at an average removal rate of 94.1% for 100 d and at an initial concentration of 100 ng/L[17]. Influent water consisted of 1 g of NH_4NO_3, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.168 g of $NaHCO_3$, 30 mg of sodium humate, and 100 ng of geosmin (100 mg/L in methanol solution, chemical purity ≥ 99%; Supelco, USA) in 1000 mL of tap water flowing downward the column with hydraulic retention time (HRT) of 2 h.

Culture Medium and Conditions

A basal mineral salt medium (MSM) containing 1 mg of ferrous sulfate, 1 mg of $CaCl_2$, 20 mg of $MgSO_4$, 50 mg of KH_2PO_4, 10 mg of NH_4Cl, and 100 mg of $Na_2HPO_4 \cdot 12H_2O$ in 1000 mL of deionized water was used to isolate and culture bacterial strains. As the sole carbon source, geosmin (solid standard substance; Supelco, USA) was added at an initial concentration of 50 µg/L. Both media except geosmin and all experimental utensils were sterilized at 121 °C for 20 min. Geosmin was dissolved in ultrapure water and filtered using a 0.22 µm membrane for degerming. The bacterial strains inoculated in 100 mL grinding mouth triangular flasks equipped with glass restriction stoppers were cultured at 30 °C and shake at a rate of 200 r/min.

Isolation and Identification of Microorganisms

One of the biological ceramsites with well-domesticated biofilm from a bioreactor was obtained, added to 10 mL of sterile water, and mixed. Afterward, 0.1 mL of supernatant was inoculated

in 10 mL of MSM containing geosmin of 50 µg/L and incubated at 30 °C for 5 d. Then 0.1 mL of culture supernatant was transferred to a new MSM containing geosmin and incubated in the same condition, repeatedly. Repeating several times like this, the culture obtained was diluted and spread onto solid plates in the presence of 50 µg/L geosmin. Single colonies grown on the plates were picked and inoculated into liquid media (MSM containing geosmin) to examine their abilities to biodegrade geosmin. This procedure was repeated several times until a pure bacterial strain with the highest geosmin-biodegrading rate was isolated. A promising isolated bacterial strain was identified based on its morphological, physiological, and biochemical characteristics as well as the results of 16S rRNA analysis. 16S rRNA was selectively amplified with PCR (Eppendorf-5331) for a total of 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. Upstream and downstream primers were 8F (52 -AGAGTTTGATCCTGGCTCAG-32) and 1492R (52 -GGTTACCTTGTTACGACTT-32), respectively. The amplified product was then sent to Beijing Sunbiotech Co. (China) for sequencing.

Analysis of Geosmin

A headspace solid-phase micro-extraction (HS-SPME) coupling with gas chromatography-mass spectrometry (GC-MS; Shimadzu QP2010E, Japan) was performed to analyze geosmin qualitatively and quantitatively. A sample (1 mL) with a magnetic stir bar and 30% (w/w) NaCl was placed in a vial, which was sealed with a septum type cap. SPME needle (50/30 µm DVB/CAR/PDMS; Supelco, USA) was used to pierce the septum. Fiber was then exposed to the vapor phase above the liquid phase of the sample. Analytes were adsorbed on fiber coating by crossing the air barrier present between extraction phases and sample surface. The sample was warmed at 70 °C and stirred at a rate of 850 r/min for 31 min. The needle, along with the analytes on fiber coating, was immediately injected into the GC-MS injection port at 230 °C for 4 min. An Rxi-5Sil-MS capillary column (30 m × 0.25 mm, i.d., × 0.25 µm) was applied in the separation system, and 99.999% helium gas was used as carrier gas at a flow rate of 15 mL/min in a splitless mode. The gradient temperature program of the column oven was operated from 40 °C to 105 °C at 20 °C/min. Temperature was then increased to 110 °C at 1 °C/min and to 125 °C at 10 °C/min; this temperature was maintained for 2 min. The column box was warmed to 250 °C at 30 °C/min and maintained for 3 min finally. The overall time was 19 min. SIM (selected ion mode) mode at the ions of 112, 149, and 125 (*m*/*z*) was used at an electronic ionization temperature of 220 °C and joint temperature of 250 °C. Thus, the retention time of geosmin was 13.34 min.

RESULTS

Isolation of Geosmin-biodegrading Bacteria

To examine their abilities in the biodegradation of geosmin, single colonies grown on the MSM plates with geosmin as the sole carbon source after 5 d were picked and inoculated in liquid media containing geosmin of 50 µg/L in grinding mouth triangular flasks with glass restriction stoppers. Four monoclonal colonies with different sizes and colors were found (Fig. 2). Table 1 showed the apparent characteristics of colonies.

Fig. 3 shows the change in geosmin concentration with time and the comparison of removal rates among different species from monoclonal colonies. At an initial concentration of 50 µg/L, 40% of geosmin was removed by strain C after 2 d. This result indicated that strain C could strongly biodegrade geosmin. By contrast, a small amount of geosmin was removed by strains D, A, and B after 2 d at the same initial concentration. Strain C exhibited 99% homology with

Table 1. Apparent characteristics of colonies

Number of bacteria	A	B	C	D
Colony size	middle	big	small	very small
Colony color	yellow	yellow	yellow	pink
Single cell shape at 1000× magnification	ellipsoid	small ellipsoid	rod shaped, sometimes twisted	ellipsoid
Geosmin degradation ability	middle	middle	high	low

Xanthobacter flavus identified by 16S rRNA and physiological analysis. The biochemical characteristics of this strain were as follows. The results of oxidase and catalase tests were positive and weakly positive, respectively. Hence, *X. flavus*-YW was a heterotrophic facultative anaerobic strain. Fig. 4 shows the phylogenetic tree of *X. flavus*-YW. Thus far, studies have not yet described the biodegradation of geosmin by *X. flavus*-YW. Hence, the result of this study provided the basis of research and application to remove geosmin efficiently from drinking water.

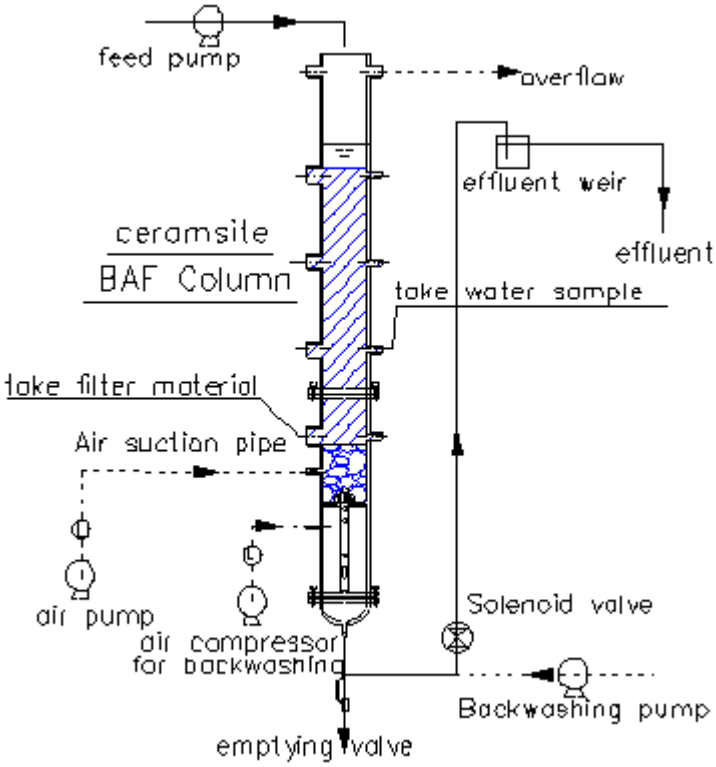

Fig. 1. Test equipment of biological aerated filter (flow rate: 0.8 L/h, size of ceramic filter layer: (100 mm × 600 mm)

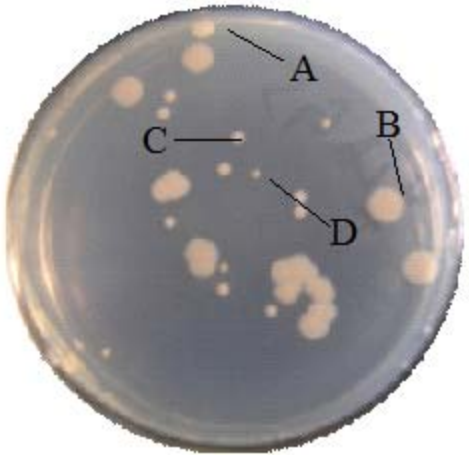

Fig. 2. Plate for isolation

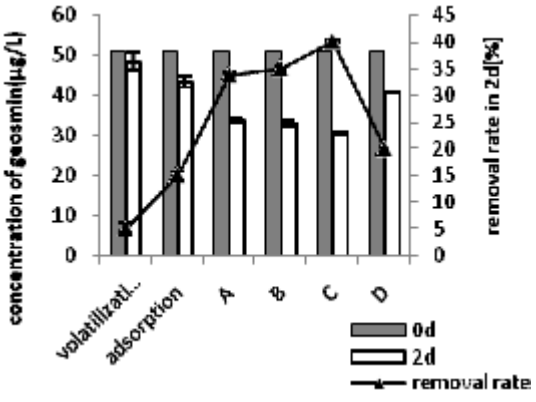

Fig. 3. Geosmin biodegradation by different isolated strains

Optimization of the Nutritional Conditions of *X. flavus*-YW

Fig. 5 shows the *X. flavus*-YW can biodegrade geosmin under different nutritional conditions. Under restrictive nutritional conditions in which geosmin was used as the sole carbon source, *X. flavus*-YW could biodegrade this substance from an initial concentration of 50 µg/L

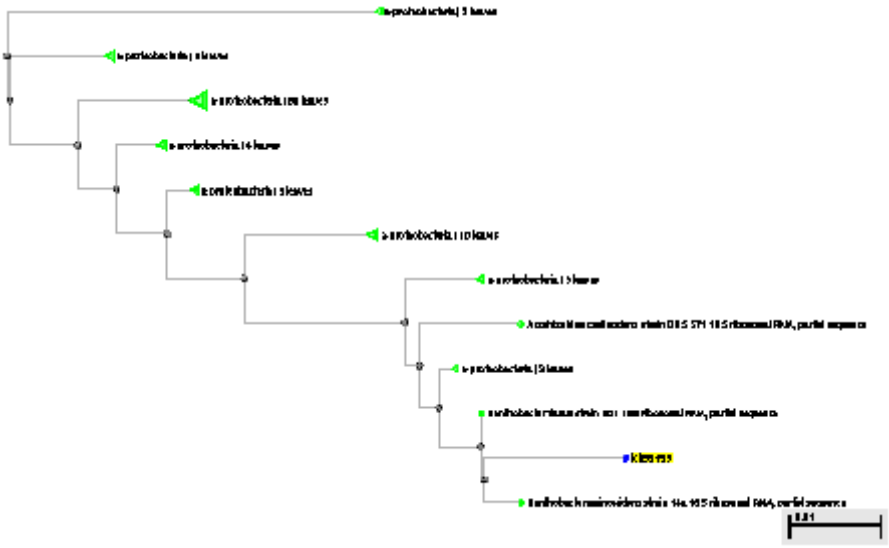

Fig. 4. Phylogenetic tree of *Xanthobacter flavus*-YW for the efficient biodegradation of geosmin (GenBank Accession Number: KC192788)

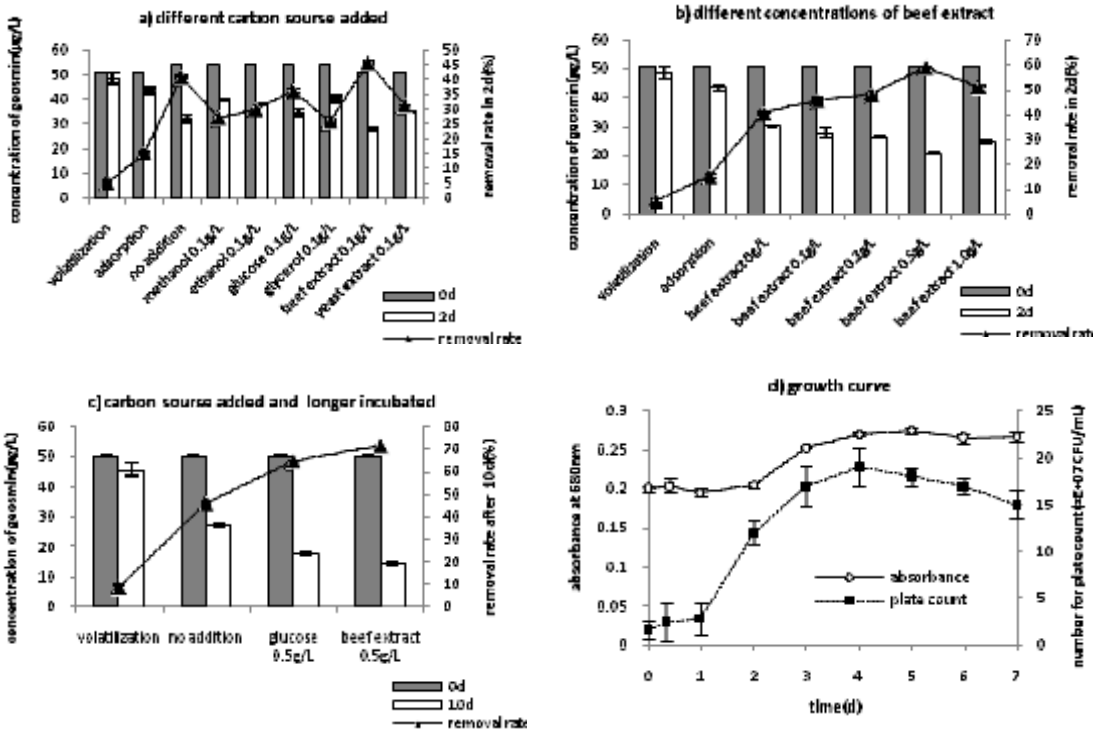

Fig. 5. Effects of different culture conditions on the biodegradation of geosmin by *Xanthobacter flavus*-YW (basal mineral salt medium with 50 µg/L geosmin cultured at 30 °C and 200 r/min and sealed with a glass stopper under anaerobic conditions)

to a final concentration of 32 µg/L by 40% after 2 d [Fig. 5 (a)]. At 0.1 g/L of other carbon sources, such as methanol, ethanol, glucose, glycerol, and yeast extracts added in the medium, geosmin removal was reduced. Only at 0.1 g/L of beef extract, geosmin biodegradation was greatly enhanced and the removal rate was 45% after 2 d [Fig. 5 (a)]. At 0.5 g/L beef extract, geosmin biodegradation was accelerated [Fig. 5 (b)]. Approximately 59% of geosmin was removed after 2 d; this result was higher by 14% than that at 0.1 g/L beef extract. Fig. 5 (c) shows that longer incubation time in the presence of additional carbon source resulted in an improved rate at which *X. flavus*-YW biodegraded geosmin. At 0.5 g/L beef extract, the rate of geosmin biodegradation for 10 d was 71.3%, which was higher by 11.3% than the biodegradation rate for 2 d. At 0.5 g/L beef extract, the rate of geosmin biodegradation for 10 d was also higher by 23.4% compared with that in the absence of additional beef extract for 10 d. At 0.5 g/L glucose, geosmin removal was also increased by 18.7% compared with that in the absence of additional glucose for 10 d. Fig. 5 (d) shows the exponential phase of *X. flavus*-YW was at 3 d.

Biodegradation of Geosmin by *X. flavus*-YW

Fig. 6 shows GC-MS chromatograms of a sample containing 50 µg/L geosmin biodegraded by *X. flavus*-YW at different reaction times in the presence of 0.5 g/L beef extract. The peak of geosmin was evidently decreased for 2 d. This result suggested that *X. flavus*-YW strain could highly biodegrade geosmin.

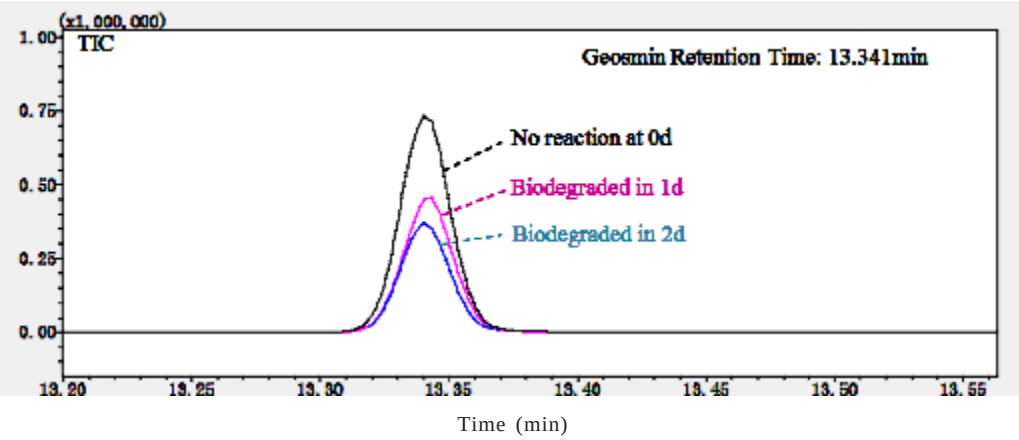

Fig. 6. GC-MS Chromatograms for the biodegradation of geosmin by *Xanthobacter flavus*-YW for different reaction periods (SIM mode, ions selected were 112, 125, and 149)

DISCUSSION

Source of bacteria

In this study, geosmin-biodegrading microorganisms were isolated from domesticated biofilm in a bioreactor. This bioreactor comprised abundant microbial species; as such, target strains can be highly obtained from this bioreactor compared with lake water, soil, and other natural environments. Narayan and Nunez reported that *B. cereus* and *B. subtilis* isolated from soil can biodegrade geosmin only at some mg/L[11]. Hoefel and Ho of the Australian Water Quality Centre found a member of *Alphaproteobacteria* isolated from biofilm in a water treatment plant sand filter; this microorganism biodegraded geosmin individually only at some mg/L after 42 d[14]. However, high pertinence and low operational difficulty in isolating strains from mature biofilm were observed in this study.

Biodegradation mechanism

The biodegradation of trace geosmin in water may undergo secondary utilization[18]. The biodegradation of specific compounds (such as xenobiotics or trace compounds) is feasible if sufficient microbial growth occurs by using natural organic materials and if the secondary utilization kinetics of the micropollutants is sufficiently rapid. In 1992, Manem and Rittmann reported that an easily degraded organic substrate fed in a

bioreactor increased the removal rate of xenobiotics at a relatively small amount[19]. Therefore, this study added beef extract as an easily biodegraded organic substrate to the medium for sufficient microbial growth; as a result, the removal rate of trace geosmin could be increased. Therefore, beef extract was the optimal choice among other carbon sources, including methanol, ethanol, glucose, glycerin, and yeast extracts. Microorganism biodegradation was improved by extending incubation time in the presence of easily degraded organic substrates. The biodegradation of odorous pollutants was slow, even in the presence of easily degraded organic substrates. This finding may be attributed to degradation that could occur only if bacterial growth has reached a certain level or when geosmin may be toxic for cell growth[20].

Intermediates and products

Intermediates and products have yet to be identified. The biodegradation pathway of geosmin may be similar to that of cyclohexanol[21]. Saito et al. reported four suspected biodegradation products of geosmin (+), an isomer of geosmin (-) which cause odor in water. Among these products, a dehydration product and an oxidation product were identified. Two products, namely, 1,4a-dimethyl-2,3,4,4a,5, 6,7,8-octahydronaphthalene and enone, unambiguously synthesized authentic samples [(±) geosmin][22-24]. However, all of these four suspected products were not found in this study. This result suggested that metabolites and biodegradation pathways may vary because of different strains and target materials. The bacterial metabolites of geosmin biodegraded by *X. flavus*-YW are subjected to identification tests now.

In conclusion, *X. flavus*-YW isolated is a promising strain that could efficiently biodegrade geosmin. This strain is the only facultative anaerobic bacterium reported capable of biodegrading geosmin as the sole carbon source. As additional carbon source of *X. flavus*-YW, beef extract is an optimal choice among other carbon sources, including methanol, ethanol, glucose, glycerin, and yeast extracts. Beef extract (0.5 g/L) may enhance the biodegradation of geosmin. The result of this study could be used as a basis of research and application in efficiently removing geosmin from micropolluted water sources.

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