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Limitation of nutrients stimulates musty odor production by *Streptomyces* sp. isolated from a tropical environment

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Musty odor production by actinomycetes is usually related to the presence of geosmin and 2-methylisoborneol (2-MIB), which are synthesized by enzymes encoded by the *geoA* and *tpc* genes, respectively. *Streptomyces* spp. strain S10, which was isolated from a water reservoir in Malaysia, has the ability to produce geosmin when cultivated in a basal salt (BS) solid medium, but no 2-MIB production occurred during growth in BS medium. Strain S10 could produce higher levels of geosmin when the phosphate concentration was limited to 0.05 mg/L, with a yield of $17.53 \pm 3.12 \times 10^5$ ng/L, compared with growth in BS medium. Interestingly, 2-MIB production was suddenly detected when the nitrate concentration was limited to 1.0 mg/L, with a yield of $1.4 \pm 0.11 \times 10^5$ ng/L. Therefore, it was concluded that phosphate- and nitrate-limiting conditions could induce the initial production of geosmin and 2-MIB by strain S10. Furthermore, a positive amplicon of *geoA* was detected in strain S10, but no *tpc* amplicon was detected by PCR analysis. Draft genome sequence analysis showed that one open reading frame (ORF) contained a conserved motif of geosmin synthase with 95% identity with *geoA* in *Streptomyces coelicolor* A3 (2). In the case of the *tpc* genes, it was found that one ORF showed 23% identity to the known *tpc* gene in *S. coelicolor* A3(2), but strain S10 lacked one motif in the N-terminus.

Key Words: geosmin; 2-methylisoborneol; nutrient limitation; *Streptomyces*

Introduction

Musty odor in water environments is associated with geosmin and 2-methylisoborneol (2-MIB). According to the World Health Organization and the United States Environmental Protection Agency, these two compounds do not have any toxic effects on human or aquatic life (Young et al., 1996). However, the presence of these compounds in reservoirs and aquaculture ponds can be troublesome. Actinomycetes are one of the major producers of taste and odor compounds in terrestrial soil environments (Zaitlin and Watson, 2006). Geosmin and 2-MIB were first isolated from actinomycetes (Gerber, 1969; Gerber and Lechevalier, 1965) and later isolated from cyanobacteria (Izaguirre et al., 1982; Medsker et al., 1968; Negoro et al., 1988; Rashash et al., 1995; Safferman et al., 1967). However, compared with cyanobacteria, limited information is known about the contribution of actinomycetes to the musty odor in drinking water (Park et al., 2016).

Musty odor compound production mostly occurs during the warmer season in temperate countries (Klausen et

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al., 2004). Therefore, it is believed that temperature is an important environmental factor that affects the metabolic activity of *Streptomyces* (Anuar et al., 2017). Moreover, the production of geosmin and 2-MIB is affected by several environmental factors, such as eutrophication, especially when nutrients exceed critical levels (nutrient enrichment) in lakes (Saadoun et al., 2001). This situation results in oxygen depletion, an increase in algae production and the creation of imbalanced ecosystems within species in aquatic environments. The presence of algae in lake water will decrease the water quality index. The Department of Environment (DOE) in Malaysia uses the Water Quality Index (WQI) to evaluate the status of water quality (Nurul-Ruhayu et al., 2015). The most commonly known pollutants in water are phosphate and nitrate (Fulazzaky et al., 2010). In addition, phosphate in detergents, livestock wastewater and municipal sewage contributes to phosphate in well water and runoff water. Moreover, exposure to 10 ppm nitrate in drinking water can cause methemoglobinemia (inability to use oxygen) in infants (Smith et al., 2008). The availability of phosphates and nitrates in water sources can now be considered a factor responsible for musty odor in various countries. The effect of nutrients on the production of geosmin and 2-MIB by *Streptomyces*, especially in tropical areas, has received little attention.

The detection and quantification of genes involved in the production of geosmin and 2-MIB in microorganisms are relevant indicators for the production of taste and odor compounds (Wang et al., 2011). Geosmin is formed from the cyclization of farnesyl diphosphate (FPP) by geosmin synthase (Jiang et al., 2006). 2-MIB is synthesized from the methylation of geranyl diphosphate (GPP) to 2-methyl GPP and further cyclization to 2-MIB catalyzed by 2-MIB synthase (Giglio et al., 2011; Wang et al., 2011). The geosmin synthase gene is *geoA* responsible for producing geosmin, and the 2-MIB synthase gene is *tpc* responsible for producing 2-MIB. These genes are used as indicators to investigate the production of geosmin and 2-MIB. The primer pairs 248F/1832R for *geoA* and AmMIBF/AmMIBR for *tpc* are generally used for the detection of these genes in musty odor-producing bacteria (Auffret et al., 2011; Du et al., 2013). In a previous study, Auffret et al. (2011) worked with the same primers AmMIBF and AmMIBR in 11 *Streptomyces* strains and successfully amplified the putative *tpc* gene in four strains. Therefore, if the *tpc* gene is present, the isolates are also expected to produce 2-MIB. However, only 2 strains (strains AMU11 and AMU14) produced 2-MIB when cultured in the AIA medium supplemented with glycerol, but 2-MIB was not detected in strains iafB and iafH when cultured under the same conditions. These findings proved that environmental or chemical factors can affect the production of 2-MIB, and the detection of this *tpc* gene is helpful for the rapid monitoring of potential musty odor compound-producing *Streptomyces* sp. (Blevins et al., 1995).

Currently, there are limited studies regarding the production of geosmin and 2-MIB by *Streptomyces* in Malaysia, as well as the occurrence of these bacteria. There are no published reports regarding the production of geosmin and 2-MIB by *Streptomyces* in tropical areas under nutrient-limited conditions. In this study, the influence of environmental stress conditions on geosmin and 2-MIB production by isolated *Streptomyces* sp. from tropical environments was studied, and the genes involved in geosmin and 2-MIB production were identified.

Materials

Sampling and isolation of musty odor-producing Streptomyces sp. The water samples and sediments were collected from Batu Dam, Malaysia (3°16′23.7″ N, 101°41′17.0″ E), for the isolation of *Streptomyces* spp. Basal salt (BS) medium was used for the growth of isolates. The medium contained the following ingredients: KNO_3, 2.0 g/L; $FeSO_4{\cdot}7H_2O$, 0.1 g/L; $MgSO_4{\cdot}7H_2O$, 0.1 g/L; K_2HPO_4, 0.5 g/L; starch, 10.0 g/L; and agar, 20.0 g/L; pH 8.0 (Hikida et al., 2012). Plates were incubated at 28°C for 7 days until the colonies were visible. After 7 days, each colony was purified via several inoculations to obtain pure culture bacteria for measuring the production of geosmin and 2-MIB.

GC-MS analysis for geosmin and 2-MIB detection. Purified isolates were inoculated into 100 mL of Yeast Meat Peptone Dextrose (YMPD) medium (yeast extract, 2.0 g/L; meat extract, 2.2 g/L; bacto extract, 4.0 g/L; NaCl, 2.0 g/L; $MgSO_4{\cdot}7H_2O$, 1.0 g/L; glucose, 1.0 g/L; pH 7.2) (Hikida et al., 2012). The culture was incubated by continuous shaking at 120 rpm and 28°C. After 3 days, cells were harvested by centrifugation at 5,000 rpm for 5 min at 25°C and washed with 1 mL of wash BS medium ($[NH_4]_2SO_4$, 2.0 g/L; NaCl, 2.0 g/L; K_2HPO_4, 0.5 g/L; $MgSO_4{\cdot}7H_2O$, 1.0 g/L; $FeSO_4{\cdot}7H_2O$, 0.05 g/L; pH 8.0).

For studies on the effects of phosphate, modified BS medium was prepared using different concentrations of dipotassium phosphate (K_2HPO_4). To study the effects of nitrate, different concentrations of potassium nitrate (KNO_3) were used in the BS medium. An aliquot of 5 mL of HPLC grade methanol (Fisher Chemical, USA) was pipetted uniformly onto the plates and left to stand at room temperature for 30 min. Then, 1 mL of methanol from the plate was collected and mixed with 2 mL of *n*-hexane. The mixture was shaken vigorously for 30 min before centrifuging at 800 × *g* for 10 min. The upper layer was then carefully collected and passed through Na_2SO_4 to remove any humidity (Jensen et al., 1994). The extracted solution was stored at –20°C prior to being used for gas chromatography-mass spectrometry (GC-MS) analysis with a model 7890A device (Agilent Technologies USA) to measure the geosmin and 2-MIB concentrations.

Geosmin and 2-MIB analytical standard solutions with a concentration of 100 μg/mL in methanol from Supelco (USA) were obtained from Sigma-Aldrich. The calibration standards for diluted concentrations of 10^7, 5×10^6, 10^6, 5×10^5, and 10^5 were prepared in *n*-hexane (R&M Chemicals, USA) and analyzed using GC-MS. The *n*-hexane blank negative control was used to check for any contamination. Column separation was performed using a model 19091S-433 (5%-phenyl) methylpolysiloxane (HP-5ms) Ultra Inert GC Column (Agilent, USA) with a length of 381–1,524 mm and a diameter of 0.18–0.32 mm. The

carrier gas, helium, was applied at a flow rate of 1.0 mL/min. The oven was set at an initial temperature of 50°C and raised to 200°C at a rate of 10°C/min. The temperature was further raised to a final temperature of 260°C at a rate of 30°C/min and held for 1 min. The total run time was 18 min per sample.

Geosmin and 2-MIB were monitored using the single ion monitoring mode. The geosmin parent ion was *m/z* 112, whereas *m/z* 95 was monitored for 2-MIB. To identify both compounds from the isolated strain, the expected retention time ranges were 11.3 min ± 0.3 and 8.9 min ± 0.2 for geosmin and 2-MIB, respectively. A calibration curve was plotted after the completion of the standard geosmin and 2-MIB analysis. Based on the calibration curve, the R^2 values obtained for geosmin and 2-MIB were 0.9838 and 0.9725, respectively.

DNA extraction, 16S rDNA sequencing and detection of the geoA and tpc genes by PCR. A pure isolated strain was selected from the agar medium using an inoculation loop and dispersed in 100 μL of autoclaved Milli-Q water. To disrupt the strong cell wall of the actinomycetes, glass beads (150–200-μm diameter) were added to the tube. The cells were then disrupted using a Fast Prep FP120 cell disruptor (Thermo Savant, Japan) operating at 5 m/s for 10 s. DNA was extracted by adding 100 μL of a 25:24:1 solution of phenol:chloroform:isoamyl alcohol to the tube, and the solution was mixed by flicking the tube. The mixture was centrifuged at 15,000 rpm at 25°C for 3 min. The uppermost layer containing the DNA was collected and stored at 4°C for further analysis. PCR was performed using a Veriti® 96-well Thermal Cycler (Applied Biosystems, USA). 16S rRNA regions were amplified with universal primer pairs 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) for forward and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) for reverse (Park et al., 2016; Sneath 1994). Specific target primers were used to investigate the prevalence of the *geoA* and *tpc* genes, which encode the enzymes responsible for the production of geosmin and 2-MIB, respectively. Primer pairs from Du et al. (2013) were used for the detection of *geoA*, including the forward primer 248F (5′-TCTTCTTCGACGACCACTTCCT-3) and the reverse primer 1832R (5-CCCTCGTACTCGATCTCCTTCT-3). The primer pairs for the detection of *tpc* from Auffret et al. (2011) were used, viz, AmMIBF (5′-TGGACGACTGCTACTGCGAG-3) as the forward primer and AmMIBR (5-AAGGCGTGCTGTAGTTCGTTGTG-3) as the reverse primer.

The master mix was prepared in a volume of 20 μL with 0.3 μM each primer, 200 μM dNTPs, 0.5 U of PrimeSTAR HS DNA polymerase enzyme (Takara Biotechnology (Dalian) Co. Ltd), 5× PrimeSTAR Buffer (Mg^{2+}) (Takara Biotechnology (Dalian) Co., Ltd.), and 1 μL of DNA. PCR was carried out with the following protocol: 95°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s (16S rRNA and *geoA*) and 95°C for 30 s (*tpc*), with annealing for 30 s at 54°C for 16S rRNA, 55.5°C for *geoA*, and 58°C for *tpc*. Extension was carried out at 72°C for 1.3 min and terminated with a final extension at 72°C for 7 min. After PCR was completed, the size of the PCR product was verified by 0.8% agarose gel electrophoresis and staining with 0.4 μL of Red safe (New England Biolabs). The gel was electrophoresed for 35 min at 60 V.

Bioinformatics analysis of the 16S rRNA gene and draft genome sequence. The sequencing of the 16S rRNA gene PCR product was performed using the ABI 3500xl Genetic Analyzer sequencer. After assembling the sequence reads, a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to search for any similarities between the 16S rRNA nucleotide sequences obtained in this study. The nucleotide sequences of 16S rRNA genes identified in this study were deposited in the NCBI database under the reference number $MK_2$40497. The BLAST algorithm was used to search for sequences homologous with GeoA and Tpc in the bacterial genome database of NCBI (www.blast.ncbi.nlm.nih.gov/Blast.cgi). All genes homologous with GeoA and Tpc were selected and analyzed. The identification of the open reading frames (ORFs) in the amplified sequences was completed using the NCBI ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession RXXG00000000. The version described in this paper is version RXXG01000000.

Real-Time PCR (RT-PCR). Total RNA was extracted using the Macherey-Nagel Kit according to the manufacturer's instructions. Briefly, 0.05 μg of total RNA was reverse transcribed to cDNA in a volume of 10 μL with 0.2 μM for each primer, 5× RT Buffer, and RT enzyme mix. The mixture was incubated at 37°C for 25 min, followed by heating at 98°C for 5 min. Real-time PCR (RT-PCR) was performed in a MyiQ mini real-time system (Bio-Rad, USA). In this study, the primer pairs from Auffret et al. (2011), viz, 245F (5′-TCTTCTTCGACGACCACTTCCT-3′) and 551R (5′-CGGCGCATCTCGATGTACTC-3′), were used to amplify the *geoA* gene. The primer pairs for 16S rRNA from Rintala et al. (2001), viz, 139F (5′-ACAAGCCCTGGAAACGGGGT-3′) and 237R (5′-GATAGGCCGCGGGCTCAT-3′), were used as the control to normalize the gene expression level of the gene of interest, because the expression levels of these control genes remain relatively stable in response to any treatment (Bas et al., 2004; Livak and Schmittgen 2001; Morse et al., 2005). Amplification of *geoA* and the 16S rRNA gene was performed in a volume of 20 μL according to the manufacturer's instructions. PCR was carried out as follows: preheating for 30 s at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 64°C for 16 s (*geoA*) and at 66°C for 32 s (16S rRNA). Extension was carried out at 72°C for 32 s. The data obtained were analyzed for fold changes according to the MyiQ handbook. In this study, relative quantification techniques were applied to investigate the expression level of a gene of interest, *geoA*. C_t values were obtained for expression of the gene of interest from both a test and calibrator sample, and the difference between them was ΔC_t. The fold difference was then simply 2 to the power of ΔC_t. The resulting ΔC_t value was incorporated to determine the fold difference in the expression level for the targeted gene of interest (Bustin et al., 2009).

Table 1. The expression of the *geoA* gene in geosmin production and production of 2-MIB in different phosphate concentrations.

Phosphate concentration (mg/L)	Geosmin		2-MIB (10^5 ng/L)
	(10^5 ng/L)	Fold change	
0.05	17.53 ± 3.12*	4.61 ± 0.80	ND
0.08	5.84 ± 1.67	0.43 ± 0.07	ND
0.11	4.58 ± 2.51	0.50 ± 0.95	ND
272.0 (Normal BS)	1.02 ± 0.07	0.96 ± 0.46	ND

$n = 3$, $*p < 0.05$, each concentration against normal BS (control), respectively, ND = not detected.

Table 2. Production of geosmin and 2-MIB in different nitrate concentrations.

Nitrate concentration (mg/L)	Geosmin (10^5 ng/L)	2-MIB (10^5 ng/L)
0.1	1.8 ± 0.09*	1.5 ± 0.15
0.3	1.4 ± 0.09*	4.5 ± 0.48
1.0	0.6 ± 0.06	1.4 ± 0.11
10.0	4.0 ± 0.44*	ND
100.0	7.0 ± 0.26*	ND
1226.0 (Normal BS)	1.02 ± 0.07	ND

$n = 3$, $*p < 0.05$, each concentration against normal BS (control), respectively, ND = not detected.

Results

Isolation of musty odor-producing Streptomyces sp. isolated from a tropical environment

Basal salt (BS) solid medium was used for the isolation of musty odor-producing *Streptomyces* sp. isolated from a water reservoir in Malaysia. After several screenings, a total of 19 actinomycete isolates, which have the ability to produce a musty odor, were obtained; all isolates were chosen based on the actinomycete morphology showing clear white spores with a leathery appearance, which is similar to spore-forming *Streptomyces* sp. (Wildermuth, 1970; Wildermuth et al., 1971). After cultivation of all isolates in BS solid medium, GC-MS analysis was performed to investigate the ability of the isolates to produce either geosmin or 2-MIB or both compounds. After several screenings by GC-MS analysis, strain S10 was selected for further study based on the ability of the strain to consistently produce musty odor compounds. The 16S rRNA gene of strain S10 was amplified and analyzed using BLAST in the NCBI database. The BLAST results revealed that S10 shared a 99% sequence similarity with *Streptomyces chilikensis* (accession No. NR_118246.1). Therefore, strain S10, which was isolated as a geosmin producer, was confirmed to be under the genus *Streptomyces* sp.

To evaluate the presence of genes responsible for the production of musty odor compounds, PCR analysis was performed to identify the *geoA* and *tpc* genes in strain S10. A PCR amplicon of *geoA* using primer sets of 248F/1832R was observed with the correct size of 1,360 bp, but no amplicon was observed using the primer sets of AmMIBF/AmMIBR for the detection of the *tpc* gene.

Effect of nitrate and phosphate concentrations on geosmin and 2-MIB production

One of the primary objectives of this study was to evaluate the effect of nutrient limitation on the production of musty odor compounds. It was confirmed that strain S10 has the ability to produce geosmin when cultivated on solid BS medium; therefore, strain S10 was grown on phosphate-limited and nitrate-limited BS solid medium to evaluate the production of musty odor compounds. Table 1 shows the effect of a limited phosphate concentration on the production of geosmin and 2-MIB. Geosmin was consistently detected in the presence of all different phosphate concentrations tested, whereas no 2-MIB production was detected. The concentration of 0.05 mg/L phosphate was chosen based on the total phosphate in the National Water Quality Index (NWQI). At the lowest phosphate concentration (0.05 mg/L) tested in this study, S10 showed the highest production of geosmin, with a yield of $17.53 \pm 3.12 \times 10^5$ ng/L, and this yield was significantly different ($P < 0.05$) from the yield produced in the presence of phosphate at the other concentrations tested. Meanwhile, approximately 0.08 mg/L phosphate in water can cause eutrophication (Huang et al., 2015). There was a slight difference in geosmin production at 0.08 mg/L and 0.11 mg/L phosphate, with yields of $5.84 \pm 1.67 \times 10^5$ ng/L and $4.58 \pm 2.51 \times 10^5$ ng/L, respectively. However, there was no significant difference ($P > 0.05$) between the 0.08 mg/L and 0.11 mg/L groups. When the phosphate concentration was increased to 272.0 mg/L (normal BS medium), geosmin synthesis was decreased to $1.02 \pm 0.07 \times 10^5$ ng/L compared with the limited nitrate concentration.

The effect of limited nitrate concentrations on geosmin and 2-MIB production in strain S10 is shown in Table 2. The production of geosmin was highest at a nitrate concentration of 100 mg/L, with a yield of $7.0 \pm 0.26 \times 10^5$

ng/L, which was significantly different ($P < 0.05$) from the yield in the presence of nitrate at the other concentrations tested in this study. At a nitrate concentration of 10 mg/L, geosmin was produced with a yield of $4.0 \pm 0.44 \times 10^5$ ng/L. A slight difference in geosmin production was observed at nitrate concentrations of 0.1 mg/L and 0.3 mg/L, with yields of $1.8 \pm 0.09 \times 10^5$ ng/L and $1.4 \pm 0.09 \times 10^5$ ng/L, respectively. At a nitrate concentration of 1.0 mg/L, the lowest geosmin production was observed, with a yield of $0.6 \pm 0.06 \times 10^5$ ng/L, and this yield was not significantly different ($P < 0.05$) from the yield in the presence of the nitrate at the other concentrations tested in this study. Geosmin production was decreased in normal BS medium (1226 mg/L of nitrate) with a yield of $1.02 \pm 0.07 \times 10^5$ ng/L compared with the lower nitrate concentration in this study. Interestingly, 2-MIB was detected under conditions of limited nitrate concentration, except at 10 mg/L and 100 mg/L. The highest production of 2-MIB from strain S10 was observed at a nitrate concentration of 0.3 mg/L, yielding $4.5 \pm 0.48 \times 10^5$ ng/L. There was a slight difference in 2-MIB production at 0.1 mg/L and 1.0 mg/L nitrate, yielding $1.5 \pm 0.15 \times 10^5$ ng/L and $1.4 \pm 0.11 \times 10^5$ ng/L 2-MIB, respectively. However, there was no significant difference ($P > 0.05$) in all nitrate concentrations tested for 2-MIB.

Draft genome sequence analysis of strain S10

To identify the genes responsible for the production of geosmin and 2-MIB, a draft genome sequence analysis was conducted. The draft genome of *Streptomyces* sp. S10 was 8,611,899 bp in length, with an overall GC content of 71.67%. Additionally, annotation results indicated the presence of 1,480 contigs and gave functional predictions for 6,837 genes. According to the literature, *cyc2* in *Streptomyces coelicoler* A3 (2) is coding geosmin synthase, and *geoA* in *Streptomyces avermilitis* is also coding geosmin synthase. These two are showing the same EC number (4.1.99.16) with a high similarity (Cane et al., 2006; Gust et al., 2003). Therefore, "*cyc2*" and "*geoA*" are names of the gene which encodes the same functional enzyme as goemin synthase. Based on the homology search by the BLAST program, one ORF in contig No. 345 of S10 showed 95% identity (E-value 0.0) with the *geoA* gene encoding geosmin synthase in *Streptomyces coelicolor* A3(2) (accession No. Q9X839.3). The *tpc* in S10 shares approximately 23% identity with *tpc* in *Streptomyces coelicolor* A3(2) (accession No. Q9F1Y6.1).

Evaluation of geoA expression under phosphate-limiting concentrations

The transcription of the *geoA* gene in S10 growing under phosphate-limiting conditions was analyzed to confirm its involvement in the production of geosmin. To normalize the amount of DNA added for each real-time PCR, the 16S rRNA gene was used as the housekeeping gene. The expression of the *geoA* gene was 4.61 ± 0.80-fold higher at 0.05 mg/L phosphate compared with the expression of *geoA* in normal BS medium. When strain S10 was grown in 0.08 mg/L and 0.11 mg/L phosphate, the fold change decreased to 0.43 ± 0.07 and 0.50 ± 0.95 compared with normal BS medium, respectively.

Discussion

In tropical countries, the production of geosmin and 2-MIB compounds by actinomycetes has received only minimal attention. In this study, we successfully isolated strain S10 as a producer of geosmin and 2-MIB from a tropical water environment in Malaysia. S10 was found to be similar to *Streptomyces chilikensis*, which has not been reported as a geosmin or 2-MIB producer. The geosmin and 2-MIB compounds produced by S10 under nitrate and phosphate limitation conditions were analyzed by GC-MS. The results showed that the isolate has the ability to produce geosmin under both phosphate and nitrate limitation conditions, while 2-MIB was produced only under nitrate limitation conditions. Actinomycetes have been associated with earthy or musty odor events in water reservoirs (Francisco and Silvey, 1971). Several species, such as *Streptomyces coelicolor* A3 (2), *Streptomyces avermitilis* and *Streptomyces griseu*s, have been proven to produce geosmin and 2-MIB (Cane et al., 2006; Hikida et al., 2012; Schrader and Blevins, 2001). Therefore, the findings from this study revealed that the water reservoir in Malaysia might face the problems of taste and odor since our isolate produced geosmin and 2-MIB.

Globally, many aquatic ecosystems have been negatively affected by the phosphate concentration (Xu et al., 2010). In Malaysia, phosphate issues in water reservoirs have become a major consideration in the past 40 years (Ghani and Mahmood, 2011). The results of this study demonstrated that geosmin production by *Streptomyces* sp. in tropical areas was affected by the concentration of phosphate. In actinomycetes, phosphate can directly regulate secondary metabolism. According to Martin and Demain (1976), secondary metabolite biosynthesis by *Streptomyces griseu*s is controlled by the phosphate concentration, with phosphate concentrations at 5 ± 10 mM greatly inhibiting biosynthesis of the antibiotic candicidin. As in this study, the biosynthesis of geosmin by S10 showed a negative regulation at higher phosphate concentrations (>0.05 mg/L) (Xu et al., 2010). The concentration of 0.05 mg/L phosphate in our experiments was chosen based on the total phosphate in the National Water Quality Index (NWQI) (Ghani and Mahmood, 2011). The present results indicated that the maximum geosmin concentration, $17.53 \pm 3.12 \times 10^5$ ng/L, was found at a phosphate concentration of 0.05 mg/L, and the previous reports mentioned that a threshold value of geosmin at 12.8 ng/L can elicit customer complaints (Martin and Demain, 1976; Ömür-Özbek et al., 2011). These results agree with the findings of Schrader et al. (2001), in which the highest production of geosmin, 1.35×10^{10} ng/L, was achieved in *Streptomyces halstedii* when the phosphorous concentration was limited to 1.12 mg/L. Data from this study revealed that maximal geosmin production was favored under phosphate-limiting conditions. Many studies have indicated that phosphate concentrations in the sea, rivers and lakes in Malaysia exceed the capacity of receiving waters (Radojevic and Bashkin, 2007). Based on the Status of Eutrophication of Lakes in Malaysia by National Hydraulic Research Institute of Malaysia (NAHRIM), 56 lakes or 62% of the 90 lakes and reservoirs evaluated were eutrophic (Huang et

al., 2015). Additionally, excessive input of phosphate into lakes and reservoirs, approximately 0.0844 mg/L (Schrader and Blevins, 2001), can lead to eutrophication. In the Pearl River Delta, China, phosphate has been responsible for algal blooms since the 1980s, although the concentration of phosphate is generally less than 0.04 mg/L (Huang et al., 2003). Therefore, phosphate is an important factor affecting the quality of freshwater, and the presence of phosphate in water reservoirs can lead to water quality problems, such as eutrophication (Radojevic and Bashkin, 2007). Basal Salt (BS) media was normally used for the isolation and characterization of musty odor-producing Actinomycetes, and normal BS media contains 272.0 mg/L of phosphate and 1226.0 mg/L of nitrate. These phosphate and nitrate concentrations in BS media is considered as a "eutrophic" condition, because the concentration of these are higher than the acceptable range as drinking water. Interestingly, a limited amount of phosphate in our study enhanced the production of geosmin, reportedly averaging $17.53 \pm 3.12 \times 10^5$ ng/L at 0.05 mg/L phosphate and this yield was significantly different ($P < 0.05$) compared with 0.08 mg/L and 0.11 mg/L of phosphate. Thus, the present data suggest that actinomycetes from tropical areas tend to produce a higher amount of geosmin at the limited amount of nutrients. This study showed that a limited amount of phosphate is the key for the production of geosmin. The main sources of phosphate are domestic and municipal wastewater, agricultural effluents, and industrial wastewaters. Thus, it is essential to control and monitor the level of phosphate in water reservoirs.

Apart from affecting the concentration of phosphate, the concentration of nitrate also appears to affect geosmin and 2-MIB in S10. The pattern for geosmin production by S10 at different nitrate concentrations increased up to $7.0 \pm 0.26 \times 10^5$ ng/L at 100 mg/L nitrate, but decreased to $1.02 \pm 0.07 \times 10^5$ ng/L in normal BS medium (1,226 mg/L). This result was supported by a study performed by Saadoun et al. (2001), in which geosmin produced from an *Anabaena* sp. isolate increased with the tested nitrate concentrations and reached a maximum, but decreased as the nitrate level continued to increase. The nitrate concentrations used in this study were consistent with the recommended raw water quality and the drinking water quality standards of the Ministry of Health, Malaysia (http://kmam.moh.gov.my). In this study, 10 mg/L nitrate was the maximum acceptable value in drinking and raw water, and the effect of 10 mg/L nitrate on the yields of geosmin and 2-MIB was further investigated. Table 2 shows that S10 was still producing geosmin, $4.0 \pm 0.44 \times 10^5$ ng/L, in the presence of 10 mg/L nitrate. These nitrate levels create problems in drinking water and give water an unpleasant taste. This approach possibly improves the management of drinking water sources and can be applied to other uses, such as aquaculture systems and industries.

Interestingly, 2-MIB production by S10 was detected in the presence of 0.1 mg/L, 0.3 mg/L, and 1.0 mg/L nitrate, but not when S10 was cultivated in normal BS medium. The amount of 2-MIB produced was affected under nitrate-limiting conditions, implying that 2-MIB production is more dependent on nitrate than phosphate. When nitrate was increased from 0.3 mg/L to 1.0 mg/L, the 2-MIB produced started to decrease dramatically from $4.5 \pm 0.48 \times 10^5$ ng/L to $1.4 \pm 0.11 \times 10^5$ ng/L. However, 2-MIB was not produced at the highest nitrate concentrations tested (>1.0 mg/L), similar to normal BS medium. The results pattern for the reduced nitrate concentration demonstrated that S10 produces significantly more geosmin and 2-MIB when the nitrate concentration is reduced than when it is abundant (1,226 mg/L) as in normal BS medium. These results indicate that with reduced nitrate, metabolism was directed towards the production of both geosmin and 2-MIB. However, with excess nitrate (1,226 mg/L), the production tends to be suppressed. This research provides data and scientific evidence relating to the presence of musty odor, which will be of use in taking action about this problem.

The gene encoding geosmin synthase, *geoA*, has been identified in actinomycetes such as *S. coelicolor* A3 (2), *S. avermitilis*, and *S. peucetius* (Cane and Watt, 2003; Cane et al., 2006; Ghimire et al., 2008). When we compared the alignment of *geoA* sequences in S10 with full-length amino acid sequences of geosmin synthase in *S. coelicolor* A3(2), *Streptomyces avermitilis* (accession No. BAC69874.1), and *Streptomyces peucetius* subsp. *caesius* (accession No. ABY50951.1), as shown in Fig S1, the conserved N-terminal region sequences DDHGLE and NDLFSYQRE were also conserved in S10. According to Wang et al. (2014), the conserved N-terminal motifs DDHFLE and NDLFSYQRE are necessary for the production of geosmin rather than the C-terminal regions, namely, DDYYP and NDVFSYQKE. The gene expression of the identified *geoA* gene in S10 was also investigated, and it was found that limited phosphate concentration did induced the expression of *geoA* in S10. However, at the higher concentration of phosphate, the expression of *geoA* started to decrease. The expression pattern of *geoA* and the production of geosmin under phosphate limitation conditions paralleled; therefore, it was suggested that *geoA* in S10 encodes geosmin synthase. Based on a previous study, the geosmin synthase gene (*geo*) and its flanking regions were identified in *A. ucrainica* (Wang et al., 2014). The *geo* gene was found to be located in a transcription unit with two cyclic nucleotide-binding protein genes (*cnb*) and was predicted to be a member of the cyclic adenosine monophosphate (cAMP) receptor protein/fumarate nitrate reductase regulator (Crp-Fnr) family. The evolution of the geosmin synthesis gene probably involves HGT. However, this presumption needs further investigation.

In this study, the detection of *tpc* gene in S10 was performed using a set of AmMIB primer, as reported by Auffret et al. (2011), Park et al. (2016) and Anuar et al. (2017). However, no *tpc* gene was observed in S10 based on PCR analysis. Performing the alignment of the possible *tpc* gene in S10 with *S. coelicolor* A3(2) showed only one conserved motif in the C-terminus (NDLYSYTKE) in S10, but a conserved motif (DDCYCED) in the N-terminus was not observed in the *tpc* gene (Fig. S2). This may be one of the reasons why PCR detection of the *tpc* gene, by using the AmMIBF/AmMIBR primer set based on the two motifs in the N- and C-terminus conserved motif of the *tpc* gene in S10, failed. These two motifs are required for the transformation of geranyl pyrophosphate (GPP) to

2-MIB. From draft genome analysis, it was found that contig no 77_4 of S10 contained one conserved motif, DDCYCED, in the N-terminus, as shown in Fig S3. Based on the results from NCBI BLASTP analysis, this contig, no 77_4, belongs to the terpene synthase protein family. Since 2-MIB also belongs to the terpene group, it is possible that the *tpc* gene in S10 could work together with contig no 77_4 to produce 2-MIB in S10 under nitrate-limiting conditions. Therefore, further research should be carried out on the transcriptional induction of the possible *tpc* gene and the gene no 77_4 to verify the function of the genes responsible for the production of 2-MIB-producing *Streptomyces* obtained from tropical freshwater sources.

Besides, although no 2-MIB was detected by S10 under a phosphate-limited condition, some production was observed when S10 is cultivated under a nitrate-limited condition. Furthermore, S10 was found to produce significantly more 2-MIB when the concentration of nitrogen was reduced, indicating that 2-MIB production may be induced under nitrate-limiting conditions. Based on findings from Auffret et al. (2011) two out of four *Streptomyces* strains can produce 2-MIB, but not the other two strains when cultured under the same conditions. Therefore, based on this result, the presence of specific nutrients can notably impact the secondary metabolite production by actinomycetes. Nutrients, such as glycerol, nitrogen, and phosphorus, and other micronutrients, affected the production of the musty odor in most *Streptomyces* sp. which was confirmed by Komatsu et al. (2008) where *S. coelicolor* A3 (2) start to produce 2-MIB when grown on soy flour-mannitol (SFM) medium. However, no 2-MIB was detected under inorganic salts-starch-yeast extract (M4YE) medium. Thus, we can conclude that environmental, or chemical, factors can affect the production of 2-MIB, and the detection of this *tpc* gene is helpful for the rapid monitoring of potential musty odor compound-producing *Streptomyces* sp. (Blevins et al., 1995).

To the best of our knowledge, this study is the first to investigate the effects of nutrient factors on the production of musty odors in bodies of water in tropical countries. The identified *geoA* gene in S10 is involved in the production of geosmin when S10 is cultured under both phosphate- and nitrate-limiting conditions. S10 was found to produce significantly more 2-MIB when the concentration of nitrogen was limited, indicating that 2-MIB production may be induced under nitrate-limiting conditions. The genes responsible for the production of 2-MIB in S10 are still unknown, but *tpc* and other ORFs may contribute to the production of 2-MIB under environmental stress conditions. This study found that S10 produced more geosmin and 2-MIB when the phosphate and nitrate were limited. It can be concluded that the amount of phosphate and nitrate in the water surface should be monitored regularly to prevent the production of these odor-producing strains in tropical areas.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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