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Biodegradation of 2-methylisoborneol (2-MIB) and geosmin by bacteria isolated from aquaculture pond water and sediment

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Abstract. The biodegradation of taste and odour compounds, 2-methylisoborneol (2-MIB) and geosmin, was investigated using bacteria isolated from an earthen fish pond. A simple enrichment technique was used with 2-MIB and geosmin serving as the sole carbon source. Two isolates, *Pseudomonas sp.* and *Bacillus cereus* from pond water and sediment, were identified as possible 2-MIB and geosmin degraders, respectively. Significant reduction of MIB was observed from day 0 to day 1 of experiment from an initial concentration of 5µg L^{-1} to 0.01µg L^{-1}. On the other hand, significant reduction of geosmin was observed from day 0 to day 3 of the experiment from an initial concentration of 5µg L^{-1} to 0.41µg L^{-1}.
Key Words: biodegradation, geosmin, 2-methylisoborneol, *Bacillus cereus*, *Pseudomonas sp*.

Rezumat. Biodegradarea compuşilor de gust şi miros, 2-metillisoborneol (2-MIB) şi geosmin, a fost studiată folosind bacterii izolate dintr-un eleşteu de pământ. S-a folosit o metodă simplă de îmbogăţire cu 2-MIB şi geosmin, servind ca singură sursă de carbon. Două izolate, *Pseudomonas* sp. şi *Bacillus cereus* din apă şi sediment din heleşteu, au fost identificate ca posibili degradatori ai 2-MIB şi respectiv geosminului. Reducerea semnificativă a MIB a fost observată din ziua 0 în ziua 1 a experimentului de la o concentraţie iniţială de 5µg L^{-1} la 0.01µg L^{-1}. Pe de altă parte, reducerea semnificativă a geosminului a fost observată din ziua 0 până în ziua 3 a experimentului de la o concentraţie iniţială de 5µg L^{-1} la 0.41µg L^{-1}.
Cuvinte cheie: biodegradare, geosmin, 2-metilisoborneol, *Bacillus cereus*, *Pseudomonas* sp.

Introduction. Milkfish (*Chanos chanos*), an important component of the fisheries sector and economy in the Philippines, and other freshwater aquaculture species reduce their quality and marketability when characterized with an earthy-muddy taint and a musty taste and odor (Martinez et al 2006; Guttman & van Rijn 2008; Smith et al 2008; Pahila & Yap 2013). This is caused by 2-methylisoborneol (2-MIB) and geosmin, which are produced as secondary metabolites of *Actinomycetes* and *Cyanobacteria* especially during eutrophic conditions (Tucker 2000; Watson et al 2008). A lot of studies seek on water treatment technologies since it is also a continued concern to the water industry (McGuire 1995; Srinivasan & Sorial 2011). Physical and chemical means, such as activated carbon adsorption and advanced oxidation process (AOPs), are not completely effective for the removal of 2-MIB and geosmin. Natural organic material in the water negatively affects carbon adsorption. Advanced oxidation process poses risks in producing harmful disinfection byproducts such as aldehydes and ketones from ozonation (Ho et al 2002; White 2010; Yuan et al 2013; Kim et al 2014). However, the biological method offers a promising means of removal (Izaguirre et al 1988; Saito et al 1999; McDowall et al 2009). Drikas et al (2009) and Kim et al (2014) consider the role of bacteria in 2-MIB and geosmin removal using granular activated carbon and membrane system combined with powdered activated carbon, respectively. Several gram-negative and gram-positive species of bacteria are associated with 2-MIB and geosmin

degradation (Tables 1 and 2). Different species or strains of bacteria are known to work cooperatively on the degradation, for example *Pseudomonas* species on 2-MIB degradation and *Sphingopyxis sp.*, *Novosphingobium sp.*, and *Pseudomonas sp.* on geosmin degradation (Izaguirre et al 1988; Hoefel et al 2006).

Table 1

*Bacteria responsible for 2-MIB degradation

Bacteria	*References*
Bacillus spp.	Ishida & Miyaji (1992)
	Lauderdale et al (2004)
Bacillus subtilis	Yagi et al (1988)
Candida spp.	Sumitomo (1988)
Enterobacter spp.	Tanaka et al (1996)
Flavobacterium spp.	Egashira et al (1992)
Flavorbacterium multivorum	Egashira et al (1992)
Pseudomonas spp.	Izaguirre et al (1988)
	Egashira et al (1992)
	Tanaka et al (1996)
Pseudomonas aeruginosa	Egashira et al (1992)
Pseudomonas putida	Oikawa et al (1995)

* - The list is the same with Ho et al 2007

Table 2

Bacteria responsible for geosmin degradation

Bacteria	*References*
Arthrobacter atrocyaneus	Saadoun & El-Migdadi (1998)
Arthrobacter globiformes	Saadoun & El-Migdadi (1998)
Bacillus cereus	Silvey et al (1970)
	Narayan & Nunez (1974)
Bacillus subtilis	Narayan & Nunez (1974)
	Yagi et al (1988)
Chlorophenolicus strain N 1053.	Saadoun & El-Migdadi (1998)
Chrysobacterium sp.	Zhou et al (2011)
Novosphingobium sp.	Hoefel et al (2006)
Pseudomonas sp.	Hoefel et al (2006)
Rhodococcus moris	Saadoun & El-Migdadi (1998)
Sinorhizobium sp.	Zhou et al (2011)
Spingopyxis sp.	Hoefel et al (2009)

The need to substantiate and optimize microbial degradation and to create a feasible biodegradation technology has always been emphasized (Saadoun & El-Migdadi 1998; McDowall et al 2009; Srinivasan & Sorial 2011). This study aims to investigate the biodegradation of 2-MIB and geosmin by bacteria isolated from freshwater aquaculture pond water and sediment.

Materials and Methods

Chemicals. Analytical grade standard solution of 2-Methylisoborneol and (+/-) Geosmin (*Supelco® Analytical*) initially containing 100µg L^{-1} in methanol was diluted with triple distilled water to prepare 5µg L^{-1} of each compound for the enrichment and degradation procedures. Prepared solutions were kept in air tight sealed scintillation vials.

Enrichment procedures. Water and sediment samples were collected in sterile bottles from tilapia ponds in Freshwater Aquaculture Station, University of the Philippines Visayas. Portions of the samples were placed in sterile 250-mL screw-cap flasks and spiked with 2-MIB or geosmin. This was done separately to screen for the presence of 2-MIB and geosmin degraders. Distilled water was added to the sediment sample and was shaken prior to spiking. The control was the autoclaved spiked sample. All flasks were aerated and securely capped to prevent volatilization of the compounds until cloudy in appearance.

Enrichment of biodegraders was conducted with a mineral salts medium (MSM, NH_4NO_3 0.1%, K_2HPO_4 0.1%, $MgSO_4 \bullet 7H_20$ 0.05%, KCl 0.02% (W/V), trace elements). Portions of 100mL from the previous culture were used to inoculate 150mL of MSM spiked with 2-MIB or geosmin. All flasks were again aerated and securely capped.

Isolation of 2-MIB and geosmin-degrading bacteria. Enrichment cultures at 10^{-3} dilution were spread plated onto MSM agar supplemented with either 2-MIB or geosmin. Agar at 1.5% was added to MSM. It was autoclaved and supplemented with either 2-MIB or geosmin upon cooling to about 40^0C. The plates were secured with sealing films, and were incubated at room temperature for 48 hours. Distinct bacterial colonies were streaked onto MSM agar for purification.

Identification of bacteria. Bacterial colonies from the enrichment culture were purified and identified using BD BBL Crystal™ Identification Systems. It is a miniaturized identification method employing modified conventional, fluorogenic and chromogenic substrates. A bacterial suspension in the inoculum fluid was used for rehydration of biochemical and enzymatic substrates. The tests used in the system were based on microbial utilization and degradation of specific substrates detected by various indicator systems. The cultures were also streaked onto several selective agar media.

2-MIB and geosmin degradation. Bacterial isolates that showed possible degradation activity from the enrichment culture at 10^{-3} dilution were inoculated to 250mL flasks containing sterile distilled water with an initial concentration of 5µg L^{-1} 2-MIB or 5µg L^{-1} geosmin. These were then tightly covered without aeration. Sampling was done at day 0, day 1, day 3, and day 7 of the experiment for degradation analysis.

Analytical method. Gas chromatography analysis by Gas Chromatography/Mass Spectroscopy (GCMS) (*Perkin Elmer Clarus 600*) via SPME method was used to measure 2-MIB and geosmin content of the samples. Samples of 15mL were stored in sealed vials.

Results and Discussion

Isolation of 2-MIB and geosmin-degrading bacteria. Samples from pond water and sediment except for the control start to appear cloudy during the first two weeks of the enrichment procedure. This assumes the degradation of 2-MIB or geosmin. Izaguirre et al (1988) have also observed the presumptive disappearance of 2-MIB within 11 to 16 days of the enrichment period with no detection of 2-MIB at day 13. Geosmin and 2-MIB are not encountered as the sole carbon source at levels sufficient to support bacterial growth in nature, however, it can be metabolized by degrading-bacteria (Zhou et al 2011).

Identification of 2-MIB and geosmin-degrading bacteria. Bacterial colonies used in biodegradation experiment are repeatedly streaked onto supplemented MSM agar for purification and identification. Two kinds of bacteria (A and B) grow distinctly on MSM agar supplemented with geosmin and with 2-MIB. Isolate A is a nonmotile gram-positive bacteria which appears to be semitransparent and creamy white. On the other hand, isolate B is a motile gram-negative bacteria which appears to be light orange or peach in color. Both isolates are negative for hydrogen sulfide test (H_2S), thus are not able to reduce sulfides during metabolism (Table 3).

Table 3

Morphology and characteristics of bacterial isolates

Isolate	*Morphology*	*Gram stain*	*Motility*	*H_2S*
A	Cream white, semitransparent, lobate margin, smooth edge	+	-	-
B	Light orange or peach, entire margin, smooth edge	-	+	-

"+" - positive; "-" – negative.

The resulting profile number in BBL Crystal Gram Positive ID of isolate A is 2637541563. Using the BBL Crystal Autoreader the organism is identified as *Bacillus cereus* at 86.06% confidence. Isolate B from pond water grows on cetrimide base agar (CBA). Cetrimide base agar is used for the selective isolation *Pseudomonas aeroginosa*. The identification results agree with the morphology and characteristics of bacterial isolates (Table 3) and confirm several studies such as with Narayan & Nunez (1974), Tanaka et al (1996), Lauderdale et al (2004) and Hoefel et al (2006).

2-MIB and geosmin degradation. The degradation of 2-MIB and geosmin is observed using the isolates *Pseudomonas sp.* and *Bacillus cereus*, respectively. The graphs below shows the degradation activity of *Pseudomonas sp.* on 2-MIB and of *Bacillus cereus* on geosmin in a week.

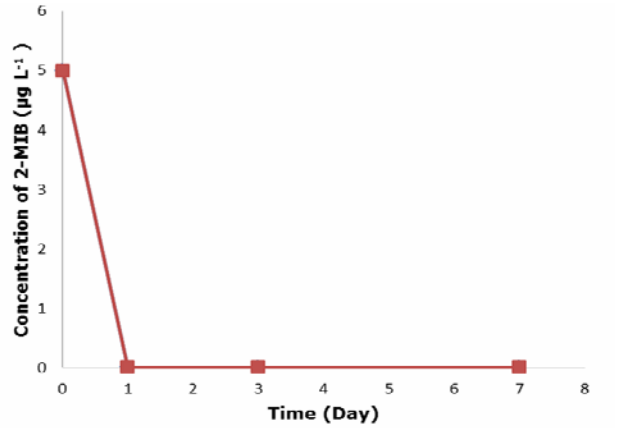

Figure 1. Degradation of 2-MIB by *Pseudomonas sp.* isolated from pond water.

In order to investigate the biodegradation of 2-MIB as the sole carbon source, 2-methylisoborneol in MSM, with an initial dose of 5ppb, was employed. Figure 1 shows the change of 2-MIB concentration with time. Based on Friendman's test, there was no statistically significant difference on the concentration of 2-MIB through time, X2 (2) = 9.240, p = 0.026. Post hoc analysis with Wilcoxon signed-rank tests is conducted with a Bonferroni correction applied, resulting in a significance level set at p < 0.017. Statistically significant reduction in 2-MIB concentration is found to be from Day 0 to Day 1(Z = -2.023, p = 0.043) only. The results indicate that the 2-MIB concentration is significantly reduced from the initial time that the bacteria are introduced until the first day only. The degradation activity on 2-MIB by *Pseudomonas* bacteria isolated from pond water resulted to a concentration of 0.013µg L^{-1} at day 7.

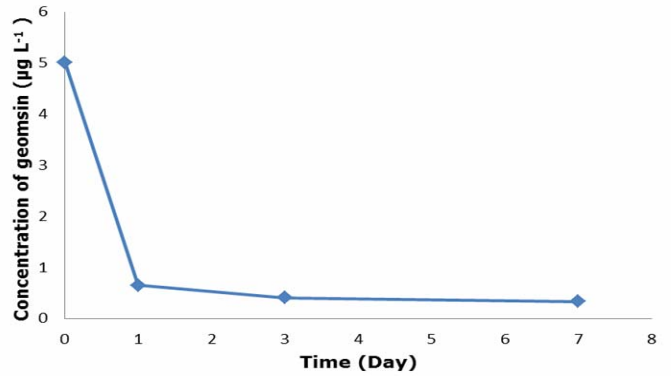

Figure 2. Degradation of geosmin by *Bacillus cereus* isolated from pond sediment.

Geosmin in MSM, with an initial dose of 5ppb, is also employed to investigate the biodegradation of geosmin as the sole carbon source. Figure 2 shows the change of geosmin concentration with time. The results indicate that the geosmin concentration is significantly reduced from the initial time that the bacteria at concentration of 10^{-3} are introduced until the third day. Based on Friendman's test, there is a statistically significant difference on the concentration of geosmin through time, $X^2 (2) = 13.560$, $p = 0.004$. Post hoc analysis with Wilcoxon signed-rank tests is conducted with a Bonferroni correction applied, resulting in a significance level set at $p < 0.017$. Statistically significant reduction in geosmin concentration is found to be from Day 0 to Day 1($Z = -2.023$, $p = 0.043$) and Day 1 to Day 3 ($Z = -2.023$, $p = 0.043$); but there is no significant reduction from Day 3 to Day 7 ($Z = -0.674$, $p = 0.500$). *Bacillus cereus* isolated from the pond sediment resulted to the degradation of geosmin from 5µg L^{-1} to 0.33µg L^{-1} at day 7.

Conclusions. This study showed that 2-MIB and geosmin can be degraded by bacteria isolated from freshwater aquaculture pond. The biodegraders are identified as *Bacillus cereus* and *Pseudomonas sp.* Elucidation of 2-MIB and geosmin biodegradation with the isolates are currently underway.

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