Effects of pH on Biomass, Geosmin, and 2-Methylisoborneol Production and Cellular Activity by *Streptomyces luridiscabiei* Isolated from a Rainbow Trout Recirculating Aquaculture System

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**Abstract**

Rainbow trout (*Oncorhynchus mykiss*) grown in recirculating aquaculture systems (RAS) can acquire “earthy” and “musty” taints due to bioaccumulation of geosmin and 2-methylisoborneol (MIB), respectively, in the fish flesh and will result in an unmarketable product. Certain species of actinomycetes produce these compounds, and *S. luridiscabiei* was previously determined to be a contributor to geosmin and MIB presence in the RAS. In this study, the effects of pH on growth (dry-weight biomass), geosmin and MIB production (solid phase microextraction and gas chromatograph-mass spectrometer), and cellular activity (adenosine 5'-triphosphate via luminometer) by *S. luridiscabiei* were determined from cultures of *S. luridiscabiei* grown at different pH (0.5 increments from pH 6.0 to 9.0) over a 7-day period. There was not a dramatic difference in growth by *S. luridiscabiei* at different pH, with significantly (*P* < 0.05) highest biomass production on day 7 at pH 7.5. Cellular activity was significantly (*P* < 0.05) higher at pH 7.0 on day 7. Significantly (*P* < 0.05) higher amounts of geosmin and MIB were produced at pH 7.5 and 8.0. Results indicate that a large difference in the growth of *S. luridiscabiei* in the RAS is unlikely to occur from pH 7.0 to 9.0. However, pH 7.5 and 8.0 in the RAS are likely to promote greater geosmin and MIB production by *S. luridiscabiei*. Maintaining water pH in the RAS from 6.5 to 7.0 may decrease geosmin and MIB production by *S. luridiscabiei* and subsequently reduce the intensities of off-flavor problems while maintaining adequate nitrification.

**ABBREVIATIONS**

RAS: Recirculating Aquaculture Systems; MIB: 2-Methylisoborneol; ATP: Adenosine 5'-Triphosphate

**INTRODUCTION**

The growth of certain segments of the United States of America aquaculture industry, including fish raised in recirculating aquaculture systems (RAS), continues to be hampered by preharvest “off-flavor” problems. The most common environmentally-derived, preharvest off-flavors are caused by the bioaccumulation of geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol or MIB ([1-*R*-exo]-1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol) in the flesh of the cultured fish which causes “earthy” and “musty” taints, respectively, and result in unpalatable and unmarketable products [1]. The tainted fish must be held by producers in water free of off-flavor compounds until the earthy and/or musty off-flavors are purged. Furthermore, the process of transferring the tainted fish from the RAS culture tank to clean water for depuration is time-consuming and requires an adequate depuration system. A depuration system increases the following: 1) capital investment; 2) operating costs (due to energy required to pump more water); and 3) the water-use requirement because large volumes of water must be exchanged in the depuration system for optimal purging of the off-flavors from the fish flesh. Additional adverse impacts of these common preharvest off-flavors in fish cultured in RAS include the reduction of consumer market demand due to inconsistent product quality, inhibition of growth into new markets, and economic losses associated with delays in stocking a new crop while holding the off-flavor fish until flavor quality improves [1].

The sources of geosmin and MIB in RAS have been attributed...
to their production by certain species of actinomycetes such as *Nocardi*a spp. and *Streptomycyes* spp. [2-4]. Specifically, in a study by Schrader and Summerfelt [4], four species of actinomycetes (*Nocardi*a *cummi*dela*n*, *Nocardi*a *fluminea*, *Streptomycyes* *albidoflavus*, and *Streptomycyes* *luridiscabiei*) were isolated from biosolids contained within a series of RAS used to produce rainbow trout (*Oncorhynchus mykiss*) and confirmed to be producers of geosmin and MIB production by certain species of actinomycetes [6,7]. Analysis. A subsequent study [5] to determine the effects of temperature on growth and off-flavor compound production by these four isolates discovered that MIB was also produced by each isolate.

The effects of particular environmental factors such as temperature have been demonstrated to directly impact biomass and geosmin production by certain species of actinomycetes [6,7]. More recently, cellular activity and biomass, geosmin, and MIB production by *N. cummi*dela*n*, *N. fluminea*, *S. albidoflavus*, and *S. luridiscabiei* were found to be significantly impacted by various temperatures [5]. In fact, *S. luridiscabiei* was determined to be the likely contributor to the geosmin and MIB present in the RAS because it produced significantly higher biomass, geosmin, and MIB than the other three isolates at 15°C, the water temperature maintained in the RAS [5]. Another study using the geosmin-producing *Streptomycyes halstedii* determined that different pH will also impact biomass and geosmin production [6]. Therefore, the determination of the effects of pH on biomass, geosmin, and MIB production by *S. luridiscabiei* would further identify certain environmental conditions that could enhance the intensities of off-flavor episodes in the RAS and provide insight into potential management strategies.

In the current study, the impacts of pH on cellular activity and biomass, geosmin, and MIB production by *S. luridiscabiei* were evaluated. A pH range from 6 to 9 was studied because this is the pH range usually maintained within RAS; most aquatic animals grow best within this range and nitrifying biofilters in RAS operate better in this range [8].

MATERIALS AND METHODS

Bacterial species, growth media, and preparation of inocula

An isolate of *Streptomycyes luridiscabiei* originally isolated from biosolids contained within a RAS [4] was cultured on actinomycete isolation agar (AIA) (Difco®; Becton, Dickinson and Company, Sparks, MD, USA) [5] and stored in a sterile solution of 10% (w/v) glycerol and 5% (w/v) peptone at -80°C until physiology studies were performed at which time the isolate was aseptically streaked for confluent growth onto yeast-dextrose (YD) agar plates. The plates were incubated at room temperature (25°C) for 5-12 d to obtain confluent growth before preparation of a propague suspension. A propague suspension of *S. luridiscabiei* was obtained by “harvesting” aseptically from YD plates containing confluent growth and transferred to sterile 0.85% saline solution to obtain a final cfu/mL of 9.1 x 10⁷.

Media and culture conditions for physiology studies

For pH studies, 100 µL aliquots of *S. luridiscabiei* propague suspension (maintained at 4°C) were aseptically pipetted into separate sterilized 1-L Erlenmeyer flasks with foam enclosures and containing 250 mL of sterile YD broth in triplicate. The pH studies were conducted in 0.5 increments from pH 6.0 to 9.0, and each test pH was obtained by adding either 1.0 N HCl or 1.0 N NaOH to YD broth before filter-sterilization using sterile 0.22 µm bottle-top filters (Corning Inc., Corning, NY, USA). Triplicate flasks of each test pH were incubated at 15°C (the water temperature maintained in the RAS and the optimal temperature for growth[5]) for 7 d on a rotary shaker (Model Excella E24R; New Brunswick Scientific, Enfield, CT, USA) at 200 rpm. Cell culture material (35 mL/flask per each sampling time) was aseptically removed from each flask at 3, 5, and 7 d (determined to be adequate from previous studies [5]) for biomass determination, cell viability assay, and analysis of geosmin and MIB concentrations.

Cell viability assay

Cell viability was determined by measuring adenosine 5’-triphosphate (ATP) production. An ATP (Sigma-Aldrich; St. Louis, MO, USA) standard curve was created according to the Bac-Titer-Glu™ Microbial Cell Viability Assay (Promega, Madison, WI, USA) protocol used previously [9]. Specifically, 100 µL of ATP standard was serially diluted (1:10) using sterile YD broth (90 µL per well) in flat-bottom wells of a 96-well, white-bottom polystyrene plate (Thermo Scientific™ Nunc™; Waltham, MA, USA) to obtain final concentrations of 1 µM to 10 pM (triplicate wells per concentration). Additionally, on the same microplate, culture material from each flask was aseptically transferred to wells (100 µL per well) and to the ATP standard wells. The microplate was then placed in a luminometer (Model Synergy™ 2, Biotek®; Winooski, VT, USA), mixed with moderate rotary shaking for 7 sec at room temperature (25°C), and kept in the dark for an additional 5 min before measuring luminescence for 1 sec per well. Control wells containing sterile YD broth (200 µL per well) were used to obtain background luminescence values which were subtracted from the other luminescence readings.

Biomass determination

Glass-fiber filters (dia. 42.5 mm, grade G4; Fisher Scientific, Pittsburgh, PA, USA) were dried at 121°C for 24 h, and the initial weight was obtained after cooling. For each flask, 10 mL of culture material (in triplicate) were vacuumed filtered. Filtrate was rinsed with 10 mL of 0.85% saline, dried for 24 h in a drying oven (121°C), and the final filter weight recorded. The initial weight of the filter was subtracted from the final weight to determine dried biomass (mg/mL).

Analysis of geosmin and MIB concentrations in cultures

For each sample, 0.6-mL aliquots of culture material were micropipetted into individual 2-mL glass, crimp-top vials containing 0.3 g sodium chloride per vial. The method used to quantify levels of geosmin and MIB was similar to the solid phase microextraction (SPME) GC-MS procedure used previously [10]. Specifically, vials were heated at 40°C for 20 min before the volatile compounds were absorbed onto a 100 µm polydimethyl siloxane solid-phase microextraction fiber (Supelco, Bellfonte, PA, USA).
PA, USA). The fiber assembly was then shaken for 10 min during the absorption period and then desorbed for 2 min at 250°C in the injection port of a HP 6890 gas chromatograph-mass spectrometer (Agilent, Palo Alto, CA, USA) with a 5973 mass selective detector operated in selected-ion-monitoring mode. The conditions of the gas chromatograph were as follows: (1) initial oven temperature was 60°C for 0.5 min; (2) the first ramp rate was 30°C/min to 100°C; (3) the second ramp rate was 20°C/min to 300°C with an isotherm time of 2 min; and (4) the maintenance of flow pressure was at 124 kPa (18 lb/in²), with helium used as a carrier gas. The molecular ion base peaks were monitored at the ratio of molecular mass to ionic charge (m/z) of 168, 95, and 135 for MIB and at m/z of 182, 112, and 126 for geosmin. A DB-5 capillary column ([5%-phenyl]-methylpolysiloxane, 30 m, 0.25 mm inside diameter, 0.25-µm film thickness; J&W Scientific, Folsom, CA, USA) was used. The retention time for geosmin was 6.8 min and for MIB was 5.2 min. Standards for MIB and geosmin were prepared at 0.1, 0.5, 1.0, and 2.5 µg/L in deionized water. The original standards were obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA), and were included at the beginning, middle, and end of each group of samples analyzed using a CombiPal autosampler (LEAP Technologies, Inc., Carrboro, NC, USA). Each sample was run in triplicate and detection threshold limits for geosmin and MIB were 1 ng/L [11].

Data analysis

Means and standard deviations (±SD) were calculated from determinations of geosmin and MIB concentrations. Means and standard errors (±SE) were calculated from biomass determinations and cell viability assays. The calculated means and respective standard deviations and standard errors were graphed against time. Analysis of variance was performed for mean comparisons of each sampled variable (e.g., biomass) among the different pH on the same sampling day using SigmaPlot software, Version 11.0 (Systat Software Inc., San Jose, CA, USA).

RESULTS AND DISCUSSION

Biomass production by S. luridiscabiei increased for each test pH throughout the study period, with highest biomass production of 8.1 ± 0.2 mg/mL occurring on day 7 at pH 7.5 (Figure 1A). Among the various test pH, only pH 7.0 differed significantly (P < 0.05) compared to the other test pH for biomass production at day 7, with a yield of 4.3 ± 0.2 mg/mL. Overall, there was not a dramatic difference in biomass production between the different test pH, though there was a drop at pH 7.0. A repeat of test pH 7.0 yielded similar results at day 7. A relatively broad pH optimum for growth, usually between pH 6 and 8, exists for most streptomycetes, with growth rate rapidly declining outside of this range [12]. In our study, S. luridiscabiei did not show a significant change in biomass production when collectively comparing the different test pH within the test range of 6 to 9. Therefore, biomass production by S. luridiscabiei is not expected to be dramatically affected by minor changes in water pH between 6 and 9 in the RAS.

Cellular activity by S. luridiscabiei was highest for most of the test pH, except pH 7.0, after 5 days of incubation (Figure 1B). At day 5 of incubation, pH 6.0 had the highest cellular activity with an ATP level of 147,612 ± 16,298 pg/mL while pH 7.5 had the lowest activity with an ATP level of 20,059 ± 3,880 pg/mL. There was not a significant difference (P > 0.05) between the ATP levels of any test pH at 5 days. At day 7, pH 7.0 had a significantly (P < 0.05) higher ATP level of 506,690 ± 49,597 pg/mL compared to the other test pH which were dramatically lower. Similar results were obtained after repeating test pH 7.0. The lowest cellular activity levels among the different test pH after 7 days incubation of S. luridiscabiei were pH 7.5 and 8.0, with ATP levels of 377 ± 2 and 574 ± 108 pg/mL, respectively. It is possible that at day 7 the pH 7.0 cultures were at an earlier stage of growth (e.g., logarithmic) while cultures grown at the other pH were at a later stage of growth (e.g., stationary) and had greater biomass accumulation and lower cell viability.

Geosmin production by S. luridiscabiei was highest at 7 days incubation at pH 8.0, with a yield of 115,707 ± 25,879 ng/L (Figure 2A). Test pH 7.5 and 6.0 were next highest for geosmin production at day 7, with yields of 74,906 ± 2,885 and 64,908 ± 38,065 ng/L, respectively. The lowest geosmin production at 7 days incubation was at pH 7.0, with a yield of 4,125 ± 826 ng/L. Test pH 6.5 also yielded low geosmin production at day 7 of 19,209 ± 1,275 ng/L. At 5 days incubation, pH 7.5 yielded significantly (P < 0.05) the highest geosmin production of 3,098 ± 1,353 ng/L among the different test pH. Greatest geosmin production by S. luridiscabiei for all test pH occurred at 7 days of incubation.

Geosmin/biomass production by S. luridiscabiei was highest after 7 days incubation at pH 8.0, with a yield of 16.6 ± 3.6 ng/mg (Figure 2B). Test pH 7.5 and 6.0 were next highest for geosmin/biomass production after 7 days, with yields of 9.2 ± 0.1 and 10.0 ± 5/5
MIB production by *S. luridiscabiei* were the same as for geosmin production in terms of highest production at test pH 7.5 and 8.0. This narrower pH range for the optimum production of the secondary metabolites geosmin and MIB compared to growth by *S. luridiscabiei* is characteristic for actinomycetes as the pH range for obtaining the greatest yields of secondary products is usually more restricted than the pH range supporting luxurious growth [12].

The optimum pH range for nitrification is 7.0 to 9.0 [13]. The process of nitrification, which occurs mainly via biological filtration in RAS, is essential for the effective removal of ammonia that is produced as an end product of fish metabolism and excreted by fish as waste. Nitrification involves the conversion of ammonia to nitrite by ammonia oxidizing bacteria (e.g., *Nitrosomonas*) and then conversion of nitrite to nitrate by nitrite oxidizing bacteria (e.g., *Nitrobacter*). The optimum pH for *Nitrosomonas* growth is 7.2 to 7.8 and for *Nitrobacter* growth is 7.2 to 8.2 [8]. The effective removal of ammonia and nitrite from RAS is essential due to their toxicities to fish. Ammonia toxicity in fish can cause disruptions of the central nervous system, osmoregulatory disturbances, blood acidosis, and reduced respiratory efficiency [14]. Nitrite toxicity in fish causes brown-blood disease which results in the reduced oxygen carrying capacity of the blood hemoglobin [8].

Results from our study indicate that within the pH range of 7.0 to 9.0 a large difference in the growth of *S. luridiscabiei* in the RAS is unlikely to occur. However, pH values of 7.5 and 8.0 in the RAS are likely to promote greater geosmin and MIB production by *S. luridiscabiei* (Figures 2A and 3A, respectively). Unfortunately, these pH values are within the ranges that

![Figure 2](image1.png)

**Figure 2.** Effects of pH on geosmin production (A) and geosmin/biomass production (B) by *Streptomyces luridiscabiei*. Standard deviation bars of the mean (n = 3) are included. For each bar graph, means on the same day with the same letter are not significantly different (P > 0.05).

5.2 ng/mg, respectively. The lowest geosmin/biomass production at 7 days incubation was at pH 7.0 and 6.5, with yields of 1.0 ± 0.2 and 3.2 ± 0.3 ng/mg, respectively. There was no significant difference (P > 0.05) in geosmin/biomass production between the different test pH at 3 and 5 days of incubation. Greatest geosmin/biomass production by *S. luridiscabiei* for all test pH was after 7 days of incubation. The results of higher geosmin and geosmin/biomass production by *S. luridiscabiei* at pH 8.0 are somewhat similar to results from a previous study [6] in which *S. halstedii*, isolated from the sediments of an Alabama catfish pond, was found to produce highest geosmin and geosmin/biomass at a more alkaline pH (e.g., pH 9) within the test pH range of 6 to 12.

Production of MIB by *S. luridiscabiei* was highest at 7 days incubation at pH 8.0, with a yield of 288,725 ± 54,781 ng/L (Figure 3A) and this yield was significantly (P < 0.05) higher than the other test pH. The next highest MIB production after 7 days was 184,252 ± 48,223 ng/L at pH 7.5 which was also significantly different (P < 0.05) from the other test pH. Test pH 6.5 had the lowest MIB yield of 8,182 ± 10,152 ng/L after 7 days of incubation. At 5 days of incubation, test pH 7.5 yielded significantly (P < 0.05) higher MIB production by *S. luridiscabiei* of 65 ± 6 ng/L. Highest MIB production was obtained after 7 days of incubation for each test pH.

MIB/biomass production was highest for all test pH after 7 days of incubation, and pH 8.0 had a significantly (P < 0.05) higher MIB/biomass production yielding of 41.6 ± 8.6 ng/mg (Figure 3B). Test pH 7.5 was second highest, with a MIB/biomass yield of 22.9 ± 6.8 ng/mg. The lowest MIB/biomass production at 7 days was at pH 6.5, with a yield of 1.3 ± 1.5 ng/mg. Results for

![Figure 3](image2.png)

**Figure 3.** Effects of pH on 2-methylisoborneol production (A) and 2-methylisoborneol/biomass production (B) by *Streptomyces luridiscabiei*. Standard deviation bars of the mean (n = 3) are included. For each bar graph, means on the same day with the same letter are not significantly different (P > 0.05).
are optimal for the growth of *Nitrosomonas* and *Nitrobacter*. Because nitrifying bacteria in the filter can adapt to the operating conditions within the RAS, maintenance of pH towards the lower end of the optimum pH range for the nitrifying bacteria has been recommended in order to minimize ammonia stress on the cultured fish [8]. Therefore, maintaining water pH in the RAS in the range of 6.5 to 7.0 may provide adequate nitrification and also decrease geosmin and MIB production by *S. luridiscabiei* with subsequent reduction of the intensities of these off-flavor problems.

The water pH in RAS is largely controlled via management of concentrations of dissolved carbon dioxide which originates mainly from the cultured fish and the biofilter [8,15] (Summerfelt and Sharrer 2004). Carbon dioxide stripping columns, the addition of chemicals to adjust the pH, or utilization of both methods can be used to manage carbon dioxide concentrations in RAS [8]. In addition, maintenance of alkalinity at 70 mg/L as CaCO$_3$ using chemical dosing pumps to supply sodium bicarbonate (NaHCO$_3$) to the water will increase pH stability within certain RAS [16].

Efficacy studies should be conducted within RAS to verify that maintaining water pH at 6.5 to 7.0 will reduce the intensities of common off-flavors caused by geosmin and MIB. Additional studies to identify other environmental factors that directly impact geosmin and MIB production by *S. luridiscabiei* and other off-flavor compound-producing actinomycetes may provide additional management approaches for reducing the adverse impacts of common off-flavor problems in RAS.

**CONCLUSION**

A significant difference in the biomass production of *S. luridiscabiei* is unlikely to occur from pH 7.0 to 9.0. However, pH values of 7.5 and 8.0 in the RAS promote greater geosmin and MIB production by *S. luridiscabiei*. These differences in off-flavor compound production at different pH by *S. luridiscabiei* may be useful in developing management strategies for mitigating geosmin and MIB-related off-flavor problems in RAS.

**ACKNOWLEDGEMENT**

Mention of trade names of commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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