Chapter 25

Physiological Strategies To Control Geosmin Synthesis in Channel Catfish Aquaculture Systems

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Several microbial taxa produce 1α,10β-dimethyl-9α-decalol (geosmin) from farnesyl precursors resulting in an intense musty "off-flavor" that negatively impacts the flavor quality of channel catfish (*Ictalurus punctatus*) and other food and water resources. Methods to selectively control geosmin production are not available, and the sporadic occurrence of geosmin hinders identification of potential microbial sources of geosmin. Physiological investigations were conducted to provide information necessary to develop molecular controls of geosmin production. Growth of the geosmin-producing filamentous bacterium *Streptomyces tendae* was inhibited by exogenous farnesol, whereas geosmin did not inhibit growth. Media were identified that both promote and repress geosmin production, and cultures of *S. tendae* grown on geosmin-promoting media were more resistant to farnesol than cultures grown on media that repress geosmin production, suggesting that geosmin production may provide a mechanism to remove farnesyl moieties. This information is being used to search for non-geosmin producing isolates to competitively exclude problematic strains and to provide sequence information required for gene probes to identify potential sources of geosmin.

Impact of Off-flavors

Geosmin (1α,10β-dimethyl-9α-decalol) and 2-methylisoborneol ((1-R-exo)-1,2,7,7-tetramethyl-bicyclo-[2,2,1]-heptan-2-ol) (MIB) are isoterpenoid (**Figure 1**) derivatives that can impart intense earthy/muddy "off-flavors" to a wide variety of food and water resources (*1 - 4*). As little as 0.01 μg/L of geosmin in water can be detected by olfaction (*4*), yet surface waters have been reported to contain 2 μg/L

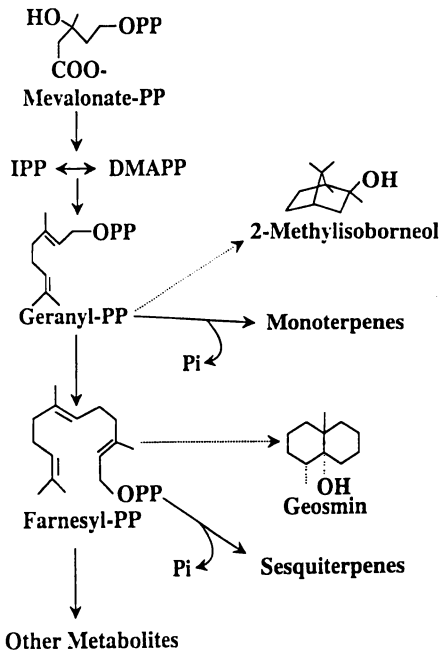

Figure 1. A schematic representation of the terpene pathway showing the possible derivation of the musty/earthy "off-flavor" microbial metabolites 2-methylisoborneol (M*B) and geosmin. Abbreviations: PP, pyrophosphate; IPP, isopentyl pyrophosphate; DMAPP, dimethylally pyrophosphate; Pi, inorganic phosphate (Adapted from ref. *42*).

of geosmin (*5*). Geosmin may also round-off the flavor of whiskey, red beetroot, and perfumes by adding earthy notes (*6*). Several types of phototrophic bacteria (algae), heterotrophic bacteria, fungi and other organisms have been reported to produce geosmin in culture (**Table I**). In addition to geosmin and MIB, the dehydration products of MIB, 2-methyleneborane and 2-methyl-2-bornene, have been implicated as potential off-flavor metabolites (*46, 47*). However, recent reports indicate that 2-methyleneborane and 2-methy-2-bornene obtained from channel catfish (*Ictalurus punctatus*) filets did not exhibit an earthy or musty odor after purification (*48*).

Economic impacts associated with "off-flavors" have encouraged research into the bioregulation of geosmin and MIB synthesis. Although this chapter focuses primarily upon controlling geosmin production in the context of the channel catfish aquaculture industry, the approaches discussed may have relevance in the control of undesirable metabolites in other systems.

Despite the widespread perception that channel catfish production is a small-scale "cottage" industry, commercial catfish production in the U.S. is a large-scale, technologically sophisticated, capital intensive, and expanding industry. To be economically competitive, producers must maintain highly efficient, relatively large scale operations. Catfish impoundments contain dense populations of fish and receive large amounts of feed (75 kg/ha/day; *49*). Channel catfish are fed by broadcasting a pelleted mixture of feed grains, oils, fish meal, vitamins, minerals, and other components onto the water. Most of the commercial channel catfish production in the United States is located in the Southeastern States. This area often experiences warm temperatures and abundant sunlight. These climatic factors coupled with nutrients from feed contribute to the establishment of dense algal and bacterial populations (blooms) on the surface and near the surface of the water (*50*).

Unfortunately, a wide variety of soil and water microorganisms produce off-flavor metabolites (**Table I**) that negatively impact fish production, processing, marketing, and consumption. Fish can rapidly absorb off-flavor metabolites through their gills and other tissues (*51*). To avoid consumer rejection and market share losses, producers and processors screen channel catfish for off-flavors and do not harvest and process fish found to be "off-flavor." This rejection, in turn, delays harvesting, restocking, and pond management, and disrupts cash flow and processing schedules. Fish that are harvested but not accepted for processing must be returned to the pond which requires additional handling and transportation.

Fish mortality may be extensive during handling. Fish not harvested continue to require feed, aeration, and other inputs while continuing to grow. This results in fish that are larger than the optimal size for marketing and a further lowering of profits. Additionally, it is not feasible to check every fish for flavor quality prior to processing. Because only a small proportion (one or two fish) of a consignment of fish can be evaluated for flavor quality prior to processing, some off-flavor fish may escape detection. Processors that allow off-flavor catfish to be sold risk the alienation of customers. Conversely, producers may have an entire consignment of catfish rejected because a single or small number of off-flavor fish. In 1987, channel catfish producers in Mississippi reported that about 64 million kg of their fish were off-flavor (*52*).

Table I. Organisms that have been reported to contain geosmin in culture. Data presented are an update of those presented in reference (*7*). Organism (*Ref.*)

BLUE-GREEN ALGAE:
Anabaena macrospora (8, 9, 10)
A. cirinalis (11, 12, 13)
A. schieremetiev (7)
A. viguiera (14)

Aphanizomenon sp. (15)

Fischerella muscicola (16)

Lygbya aestuarii (17)

Oscillatoria sp. (18, 19)
O. agardii (17, 20)
O. amphibia (20)
O. amoena (15, 19, 21)
O. brevis (20, 22, 29)
O. cortiana (17)
O. flos-aquae (19, 21)
O. limosa (20)
O. macrospora (19, 29)
O. prolifica (17)
O. simplicissma (9)
O. splendida (15, 19, 20, 21)
O. tenuis (22, 23, 24)
O. variablis (10)

Phomidium autumnale (20, 25)

Pseudoanabaena catenata (20)

Scchizothrix muelleri (26)

Symploca muscorum (23, 27, 28)

FUNGI:
Basidiobolus ranarum (30)
Chaetomium globosum (26, 31)
Pennicilium expasum (32)
P. citrium (33)
P. farenosum (33)

MYXOBACTERIA:
Nannocystis exedens (34)

AMOEBA:
Vannella sp. (35)

ACTINOMYCETES:
Actinomyces biwako (10)

Microbispora resea (28)

Nocardia sp. (28)

Streptomyces spp. (36)
S. albidoflavus (17)
S. alboniger (28)
S. antibioticus (28)
S. chibaensis (19)
S. fradiae (28)
S. fargilis (19)
S. griseoflavus (19)
S. griseoluteus (37, 38)
S. griseus (38, 39, 40)
S. lavendulae (28)
S. odorifer (38, 41)
S. neyegawaensis (19)
S. phaeofaciens (19)
S. prunicolr (19)
S. tendae (42, 43, 44)
S. versipellis (19)
S. virdochromogenes (29)
S. werraensis (19)

LIVERWORT:
Symphogyna brongniartii (45)

Dissipation and Degradation of Off-flavor Metabolites

It has been suggested that microbial catabolism of off-flavor metabolites may be enhanced to provide an effective method to control off-flavors. Indeed, microbial cultures have been reported to degrade relatively high levels of off-flavor metabolites *(53, 54, 55)*, but whether microbial activity can be used practically to improve the flavor quality of aquaculture systems remains to be determined.

In addition to biological processes, off-flavor metabolites may be adsorbed onto substrates and lost by volatilization *(56)*. These physical factors must be considered in the evaluation of the effectiveness of control strategies, and may account for some of the reductions in metabolite concentration attributed to bacterial action *(56)*.

Fish can rapidly accumulate off-flavors from water containing even very low concentrations of off-flavor metabolites *(46)*. Therefore, to be totally effective, removal systems must continually biodegrade or dissipate even trace amounts of metabolites from very large volumes of water which reduces the potential effectiveness of this approach. An increased understanding of the flux rate of metabolites within a system will enhance channel catfish production, but research efforts must also focus upon preventing the occurrence of off-flavor metabolite synthesis to be fully effective.

Nonselective Control Agents

If microbes cause off-flavors, it might seem logical that the destruction of microbial populations with chemical control agents (biocides) would prevent the occurrence of off-flavor metabolites. Unfortunately, nonselective biocides can initiate a series of events that may be ultimately more problematic than off-flavors *(49, 58, 59)*.

Although nonselective biocides may reduce off-flavor-producing populations along with other populations *(49)*, widespread destruction of microbial and/or macrophyte biomass can render large amounts of nutrients available for decomposition and a complete disruption of the biological community *(59)*. Increased biological oxygen demand associated with biomass decomposition coupled with a loss of phototrophic populations could rapidly deplete oxygen reserves, resulting in extensive fish mortality. Algal populations are beneficial in that they reduce the penetration of light into the water and thereby reduce macrophyte weed growth. Macrophyte weeds hinder pond maintenance, harvesting, and may harbor geosmin-producing bacteria (*54*). Microbial populations are also critical to nutrient cycling and contribute to fish vigor by removing nitrogenous wastes products from the water.

Even if fish populations could be maintained by aeration, biocides may not be effective in controlling off-flavors. Most of the geosmin present (ca. 90-99%) is associated with cells rather than the media (*16, 43, 60, 12*). Biocide induced cell lysis may release off-flavor metabolites from cells (*61*) and render them available for adsorption by fish (*8, 9, 13*). Off-flavor metabolites require long periods of time to clear from fish (*51*). Off-flavor-producing populations may become reestablished before off-flavor metabolite clearance is complete. In addition, the biocides used may be carried off-site or taken up by fish.

Although chemical agents may control certain macrophyte weed populations, their effectiveness for the control of off-flavors is limited. For example, application of a saponified castor oil derivative (Solricin-135), reported to inhibit algal growth, did not reduce off-flavor incidence (*62*). Application of dyes to the water to reduce algal populations by shading may actually foster other problematic algal taxa (*63*). Copper sulfate and other copper containing compounds are widely used algicides (*64*). However, copper sulfate is not completely effective (*59*). Additionally, the use of copper compounds has resulted in the occurrence of copper-tolerant populations of problematic algae (*25*), water quality problems (*59*), and adverse affects on fish quality and health (*65*). While peroxide evolving compounds have been reported to affect off-flavor metabolite-producing populations in channel catfish ponds, their practical effectiveness may be limited (*66*).

Although many terrestrial herbicides may also control phototrophic bacteria (algae; *67 - 70*), several species of non-phototrophic bacteria, such as *Streptomyces*, are prolific producers of off-flavor metabolites (**Table I**). The growth of non-phototrophic populations may be fostered by nutrients released from phototrophic populations exposed to herbicides (*71*), thereby limiting the potential effectiveness of adapting terrestrial herbicides for the control of off-flavor metabolite synthesis.

Selective Chemical Control Agents

The agricultural chemical research community has developed and marketed many agents to selectively control aquatic and terrestrial weeds. However, there are no agents currently available to selectively inhibit off-flavor-producing populations. Typically, control agents are discovered and developed through large scale screening programs preformed on crops and pests that represent major markets. Agents that exhibit phytotoxic properties of interest receive further research attention. The selective properties of an agent are often identified secondarily rather than by a direct search for a specific set of properties.

The process of registration and marketing is extremely costly and can only be justified for markets that represent a significant profit potential. The current aquaculture market is not sufficient to justify an effort to synthesize and develop specific agents to selectively inhibit off-flavor metabolite production.

Recovery of Flavor Quality

One approach to the problem of off-flavor fish may be to allow fish to purge these compounds from their tissue prior to processing (*72*). Purging fish offers producers and processors the advantages of being able to harvest and process fish on schedule while avoiding the marketing of off-flavor fish. However, several technical concerns must be addressed before purging systems can be made fully effective and practical (*72*).

Purging requires either a continuous flow of water through the system or the recirculation of water through filters. The large amounts of "clean" water required by flow-through systems is expensive and may not be widely available (*73*). Recirculating systems use much less water than flow-through systems. However, the filters used to purify the water may become a source of off-flavors (*74*).

Purging systems offer a more limited and manageable environment to attempt the selective control of off-flavors than an open impoundment (*72*). Methods similar to those used to treat off-flavors in potable water sources may be applied to recirculating systems (*39*), and might include comparatively uncomplicated approaches, such as limiting the influx of light to reduce phototrophic populations and flushing the system with fresh water. However, to be fully effective, off-flavor metabolite synthesis must be controlled in purging facilities (*72*).

Geosmin Biosynthesis

The episodic and species specific occurrence of MIB and geosmin suggest that they are secondary metabolites, and that their biosynthesis may be induced by environmental stimuli (for reviews see *75-88*). Unlike the primary metabolites, secondary metabolites are not essential for growth and tend to be strain specific (*89*). Secondary metabolites may exhibit a wide range of chemical structures and biological activities, and are derived by unique biosynthetic pathways. These pathways are often long and complex, and are under the control of specific regulatory genes that control the synthesis of these metabolites so that they are well integrated into the physiology of the organism (*89*). For example, in strains genetically capable of producing geosmin, expression of geosmin synthesis may be associated with specific developmental stages and environmental stimuli (*83, 85*).

The biosynthesis of geosmin is of interest because an enhanced understanding of the pathways and enzymes involved may support the development of effective off-flavor control strategies. Increased biochemical information could also enhance the use of biochemical systems to produce large amounts of geosmin for the flavor and fragrance industries, which may be interested in this compound to provide a desirable earthy note to certain products (*88*). Currently, difficulties associated with the synthesis of the three chiral carbons renders the chemical synthesis of geosmin extremely difficult (*88*).

Although the many details of the biosynthetic pathway of geosmin synthesis are not known (*88*), research with blue-green algae (*90*) and *Streptomyces spp.* (*91*) indicate that geosmin and MIB are derived from the terpene pathway which produces a wide variety of secondary metabolites (**Figure 1**). Research with *Streptomyces spp.* indicates that geosmin may be derived from a sesquiterpene precursor, such as farnesyl pyrophosphate (*91*), and appears to be coregulated with aerial mycelium synthesis genes carried on giant linear plasmids (*92*). Mixed-function oxidase activity may account for hydroxylation (*44*), L-methionine, and folic acid may be involved in the methylation of geosmin precursors (*93*).

The adaptive advantage of geosmin production (if any) is not known. However, secondary metabolite; i.e. geosmin, production requires carbon and energy indicating that geosmin synthesis may confer an adaptive advantage to the organism or geosmin synthesis would not be widely expressed in natural populations (*90*). For example, geosmin synthesis has been associated with nitrogen fixation in blue-green algae (*72*), dissipation of excess carbon associated with photosynthetic pigment production (*22*), and with the coordination of cytodifferentiation among

populations (i.e. aerial mycelium and spore formation) (*86*). Cells separated from the media contained much greater concentrations (ca.>99%) of geosmin than the medium (*43*) which agrees with reports indicating that less than 1% of the total geosmin produced by the blue-green algae *Fischerella muscicola* (*16*) and *Oscillatoria tenuis* (*22*) was released from the cell prior to culture senescence. The large proportion of geosmin retained within these cells suggests that geosmin may have a role within the organism in addition to any possible exogenous roles.

Detection of Geosmin-Producing Organisms

Off-flavor episodes may correspond to changes in species composition within a particular aquatic system and/or the induction of metabolite synthesis within existing populations (*93, 94*). Culturability is a significant problem in investigation of the microbial component of environmental samples (*95*), since estimates indicate that only 0.01 -12.5% of the viable bacterial population from marine samples can be cultured (*96*). Thus it is very difficult to determine effectively which taxa or isolates are actually responsible for a particular off-flavor episode. Even if an organism can be successfully cultured, an individual isolate may not produce off-flavors, but other isolates, at other times or under different culture conditions may exhibit off-flavors. Therefore, the taxon the culture represents cannot be definitively ruled out as a potential producer. If the culture is found to contain off-flavors, all that can be determined definitively is that the individual culture has the ability to produce off-flavors in the laboratory. However, the taxon the culture represents may or may not be a significant producer of off-flavors in the environment of interest. An additional problem lies in the length of time required before a particular culture can be grown to a sufficient density to allow an accurate quantification of geosmin or MIB. Because fish take up lipophilic off-flavor metabolites very rapidly, the identification of problematic taxa after a long incubation period would not be timely enough to provide useful information to catfish producers.

This lack of information concerning which organisms are actually responsible for off-flavor episodes, and the need to culture organisms greatly hinders efforts to monitor problematic populations. However, if specific genes and/or gene products can be identified that are co-regulated or directly involved with MIB or geosmin production, samples could be screened rapidly for the presence of the "genetic machinery" associated with off-flavor metabolite synthesis. This would provide a rapid indication of the potential for off-flavors without culturing.

The use of molecular probes to track specific microbes in the environment, specifically those not easily cultured, has been recently reviewed (*95, 97, 98*). The sensitivity of these probes may be further enhanced by using amplification strategies (e.g., polymerase chain reaction or PCR), to amplify segments of DNA from samples obtained from production systems (*95, 99*). However, gene probes for geosmin or MIB synthesis are not currently available.

Induction of Geosmin Biosynthesis

The search for probes for off-flavor metabolite-producing taxa is facilitated by the development of model systems to induce metabolite synthesis. *Streptomyces spp.* have been reported to produce odorous compounds under several environmental conditions (*57*). *Streptomyces tendae* and other actinomycetes are a widely occurring filamentous bacteria that are useful as sources of antibiotics (*30, 100*). The relatively simple culture requirements and high concentrations of geosmin produced by some of these bacteria make them useful model systems for investigations concerning induction off-flavor metabolite synthesis. Sivonen (*101*) qualitatively examined odor production and sugar utilization by several taxa of filamentous bacteria and Weete et al. (*102*) and Yagi et al. (*36*) investigated the effects of chemical and physical parameters on geosmin synthesis by cultures of *Streptomyces* grown in broth. Dionigi et al. (*43*) reported that cultures of *S. tendae* [ATCC 31160] cultures could be grown either Actinomyces (ACT; *103*) or Hickey-Tresner (HT; *104*) media covered with a polycarbonate membrane (0.05 μm pore size). Growing cultures on a membrane allowed quantitative separation of the cells from the medium. Cells and media samples were subjected to gas chromatography with flame ion detection (see *42, 105*) and factorial analysis according to McIntosh (*106*). Although cultures produced biomass on both the HT and ACT media (**Figure 2**), the point of onset of geosmin biosynthesis differed (*43*). Geosmin was not detected by olfaction or gas chromatography in ACT medium-grown cultures before 55 hr after inoculation. However after 55 hours, geosmin was detected in these cultures (**Figure 2**). In contrast, geosmin was detected in HT medium-grown cultures throughout the time course indicating that growth on HT media promoted geosmin synthesis to a greater extent than did growth on ACT medium (**Figure 2**).

The observation that cultures from the same inoculum grown on differing media exhibit a very different level of geosmin synthesis provides an opportunity to search for gene products; e.g., proteins, that are co-regulated with geosmin synthesis or regulate geosmin synthesis. Preliminary results indicate that at least one protein band appeared to be associated with geosmin production (*Dionigi, C. P.; Spanier, A. M. personal communication, 1992*). Further research is required to characterize this protein. Purified protein can be used to develop immunological markers to localize geosmin production at a subcellular level. Sequence information obtained from the protein could be used to construct probes to identify potential geosmin producers without culturing.

Selection of Geosmin Synthesis Mutants

Mutant strains of bacteria that have lost the ability to produce geosmin could be used to exclude problematic populations from environments, such as purging systems, by competition. Additionally, genetic analysis of strains in which the insertion of a transposon (*107*) prevented geosmin synthesis could provide DNA sequence information associated with geosmin synthesis. This information can be used to construct probes to rapidly identify sources of geosmin production from environmental samples without culturing (see *95, 97, 98*). However, a large number

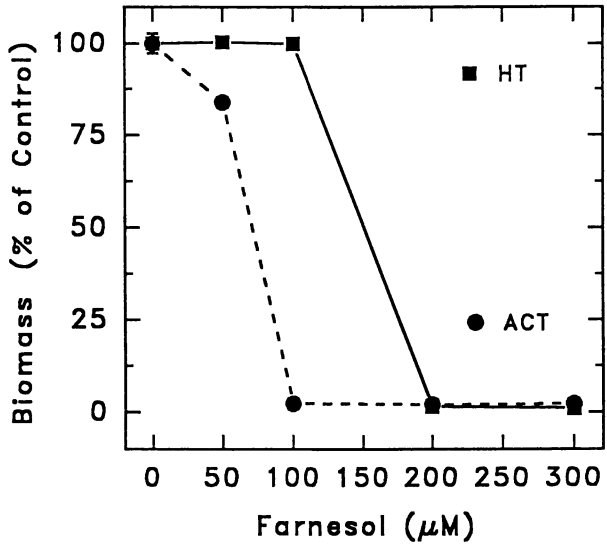

Figure 2. Geosmin production and fresh biomass accumulation by *Streptomyces tendae* cultures on Hickey Tresner and Actinomyces Media. Data are expressed ± 2 SE of a mean (Adapted from ref. *43*).

of transposon mutants would have to be generated (*107*). Gas chromatographic analysis would have to be preformed on each mutant rendering this approach prohibitively time consuming. A prescreening strategy is required to reduce the number of mutants requiring gas chromatographic analysis.

Terpenes, such as geraniol and farnesol, can inhibit the growth of microorganisms (*108-110*), and Dionigi et al. (*42*) hypothesized that geosmin synthesis may provide a mechanism to convert potentially inhibitory farnesyl precursors into a less inhibitory compound, geosmin. Indeed, cultures of *S. tendae* grown on HT medium containing 300 μM farnesol produced 97% less ($Pr \geq 0.0001$) biomass than untreated controls (*42*). In contrast, biomass accumulation in cultures exposed to 300 μM geosmin was not affected ($Pr \geq 0.85$) compared to untreated controls, indicating that, geosmin was less inhibitory than farnesol (*42*). If geosmin production provides a detoxification mechanism that allows cultures to avoid the accumulation of farnesyl moieties, then cultures that produce relatively greater amounts of geosmin should be more tolerant of farnesol than cultures that produce less geosmin. Cultures grown on ACT medium (a non-geosmin promoting medium) exhibit an increased ($Pr \geq 0.0001$) sensitivity to farnesol compared to those grown on HT medium (a geosmin-promoting medium) (**Figure 3**). For example, cultures

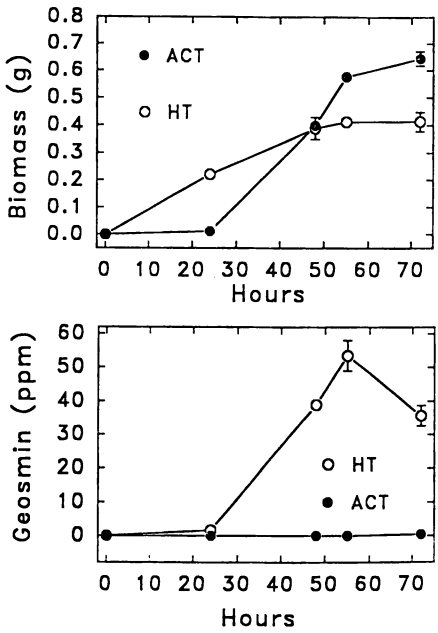

Figure 3. Effects of farnesol on fresh matter accumulation by *Streptomyces tendae* cultures grown on either Hickey-Tresner (HT) or Actinomyces (ACT) medium. Data are expressed ± 1 SE of the mean (Adapted from ref. *42*).

grown on HT medium containing 100 μM farnesol accumulated as much biomass as untreated controls, whereas 100 μM farnesol coincided with a nearly complete inhibition of biomass accumulation on AB medium. However, 200 and 300 μM farnesol produced a nearly complete inhibition of biomass production on both AB and HT medium.

Prescreening a mutant library to indicate which mutants exhibit an increased sensitivity to sublethal doses of farnesol, would greatly reduce the number of samples requiring gas chromatographic analysis. A proportion of the farnesol sensitive mutants identified by prescreening may exhibit increased farnesol sensitivity because they have lost the ability to produce geosmin. Non-geosmin producing strains (e.g., deletion mutatants) inoculated into environments, such as those used to purge off-flavors from fish, may competitively exclude populations that produce off-flavors. If the loss of geosmin production in selected strains is non-adaptive relative to "wild types" in these environments, the establishment of selected strains may be facilitated by a selective substrate readily utilizable by the desired strain (*111*). Genes that preclude off-flavor metabolite production may be contained on plasmids and transferred to other populations, thereby further inhibiting off-flavor metabolite production. More far reaching approaches may include specific inhibition of gene expression (*112*) which may provide the selective inhibition of geosmin or MIB synthesis needed for the continued expansion of the aquaculture industry.

Conclusions

Although the earthy/muddy off-flavors caused by the microbial metabolites geosmin and MIB hinder the production, processing, and marketing of channel catfish, other microbial metabolic activities are critical to the overall stability and productivity of the aquatic ecosystem. Therefore, nonselective control agents that cause a massive destruction of aquatic biomass in an effort to control off-flavors do not represent a viable control option. Until effective and safe agents are available to selectively control off-flavors, the channel catfish industry will have to continue to avoid the production and marketing off-flavor fish. One approach may include the use of systems that allow fish to recover flavor quality prior to processing. However, these systems will have to be kept free of off-flavors. This will require a sensitive means to detect the occurrence of problematic populations, and effective management strategies to deal with these populations once they occur. Genetic probes that are specific for geosmin and/or MIB producers would provide a rapid detection of problematic taxa and could be used to direct the efforts of production and processing facility managers. An increased understanding of the physiology and genetics of geosmin and MIB production will contribute to the development of genetic probes for geosmin and MIB production and strategies to effectively control off-flavor metabolite production.

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