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The Study and Identification of Bacterial Spoilage Species Isolated from Catfish during Refrigerated Storage

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Abstract

Catfish farming is relatively new in the state of Delaware although it has been a long standing profitable business in the southern United States. Catfish fillets used in this study were stored at 4°C for 1-2 weeks, after which, bacterial growth was expected on the surface of the fish. Bacteria were isolated and cultured on selective and differential media. Bacterial growth curves formulated from results in this study can be used to assess the shelf-life of catfish fillets under refrigerated storage. A specific spoilage organism was targeted to further understand and prevent microbial degradation of catfish fillets. The need for target gene 16S to be used for the discrimination of *Pseudomonas* spp. was confirmed rather than target gene *rpo*D; as it does not discriminate sufficiently to permit resolution of *Pseudomonas* spp. intrageneric relationships. *Pseudomonas* spp., *Shewanella* spp., *Bacillus* spp., *Myroides* spp., *Aeromonas* spp., and *Enterobacter* spp. were found to be contributors to the spoilage of catfish in the state of Delaware through method of 16S rDNA sequencing. The comparison of spoilage rates from both retail purchased and pond obtained catfish fillets illustrated need for increased catfish farming and production for sustainability in the State of Delaware.

Keywords: Catfish; Bacterial spoilage; Identification; Geographical origin

Introduction

Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States accounting for more than 60% of its aquaculture production [1]. Fish is one of the most vulnerable of the world's resources as fillets deteriorate rapidly due to microbial degradation [2]. Fish quality is a complex concept that incorporates many factors for consumers such as safety, nutritional quality, availability, freshness, eating quality, and physical attributes of the species [3]. Bacterial spoilage of iced and refrigerated fishery products increases during storage time, however, the shelf life of freshly harvested fish is dependent upon factors such as bacterial flora, processing, storage temperature, and handling [3]. Commercial processing of channel catfish spread skin and gut microflora on work surface and processing equipment, which ultimately increase contamination of final retailed product [4].

Microbial spoilage can manifest itself as visible growth with slime, colonies, textural changes as polymers degrade, gapping, off-odors, or off-flavors [5]. Worldwide, microbial spoilage of food leads to considerable economic losses. It has been estimated that as much as 25% of all food produced is lost post-harvest due to microbial activity [6]. Minimizing the financial impact of food spoilage by industry requires strategies that control organisms responsible for product degradation [7]. The establishment of the "specific spoilage organism" (SSO) concept has helped fundamentally shape the understanding of seafood spoilage. Studies of many seafood products have shown that the amount of SSOs present can be used to predict the remaining shelf life of products making identification of these bacteria a top priority [6].

This study was conducted to identify bacterial spoilage species present on catfish fillets. Through the selection of unique bacterial colonies from spoiled catfish fillets, this research aimed to classify the diversified bacterial communities that aid in the spoilage of retail and pond catfish within the state of Delaware. As specific bacterial species contributing to catfish spoilage were determined, this research provides the industry and consumers with significant information towards understanding, delaying, and possibly preventing microbial degradation on fishery products. Stakeholders in the seafood business would be highly interested in keeping their investment of fish safe through processing in order to see their anticipated profit. This investigation also offers insight into the shelf-life of fish during refrigerated storage and could be useful for manufacturers looking to optimize processing and shipping techniques without compromising the overall quality of their product. This study distinguishes itself because it is the first known attempt to isolate spoilage bacteria from catfish in the state of Delaware. The accurate characterization of bacteria attributed to spoilage in this area is extremely beneficial to the state and surrounding catfish farmers. All data was cross referenced with results from tests performed on selective and differential agar, Polymerase Chain Reaction (PCR) screening, 16S rDNA sequencing, and phylogenetic tree construction. Results can be compared with other states or regions and effective precautions to inhibit bacterial growth could be implemented. The accurate identification of spoilage bacteria affecting catfish in the mid-Atlantic region increases awareness and encourages further testing of microbial degradation in this industry. This research served as a foundation for more detailed studies into fish spoilage mechanisms. In determining which species of bacteria contribute to fish spoilage, a better chance of reversing their adverse effects is possible. Issues such as food safety, seafood contamination, and post-harvest preservation remain a concern in the food industry.

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Identifying specific spoilage organisms indicative of this region allow for the comparison of other identified species that have been unknown contributors to seafood spoilage.

Materials and Methods

Fish sample

The fish used in this research was North Atlantic channel catfish. Catfish samples from a local retail source in Dover, Delaware were acquired from an official U.S. farm-raised catfish processor in North Carolina. The Aquaculture Research and Demonstration Facility Earthen Aquaculture Ponds at Delaware State University also provided channel catfish to this study. All catfish fillets were purchased and used for sampling from early spring to summer. Sampling of retail source fillets began on March 16, 2011 and ended in May 16, 2011. Sampling of pond source fillets began on May 26, 2011 and ended in July 22, 2011. The fish were filleted by experienced staff and samples were packed in bags, kept on ice, and immediately transported to the food microbiology laboratory. Samples were stored at 4°C for 2-3 weeks depending on fillet spoilage. Half of each fillet was used for a sensory evaluation; the other half was divided into 10 g portions and put into stomacher bags (Stomacher 3500, Seward Inc., Bohemia, New York, USA).

Bacterial isolation

Bacteria were isolated every two days using 10 g catfish sample was homogenized with 50 ml of saline solution. Tenfold dilution were performed using 100 µl of pure sample into 900 µl of Tryptic Soy Broth (TSB) (Carolina, Burlington, North Carolina, USA) plated onto Tryptic Soy Agar (TSA) (Carolina, Burlington, North Carolina, USA) and Pseudomonas Isolation Agar (Difco™, Sparks, Maryland, USA). Isolated cultures were incubated at 25°C for 3 days and bacteria colonies (CFU) were enumerated. Catfish fillets reached a spoiled state after 12 to 18 days and the cycle was repeated using fresh catfish fillets. Candidate colonies of unknown spp. were collected at each sampling. Isolates were plated on Pseudomonas Putida Agar and Pseudomonas Fluorescens Agar (Difco, Sparks, Maryland, USA) for further identification.

DNA extraction

DNA extraction was performed using 100 µl of stored glycerol stock sample into 10 ml of TSB. Once turbid, 1 ml of inoculated TSB was transferred to a 2 ml microcentrifuge tube, centrifuged at 14,000 xg for 4 minutes at 14°C and supernatant was discarded. Pellet was re-suspended in 200 µl of molecular grade water and put into a 100˚C water bath for 10 min. Tube was centrifuged at 14,000 xg for 4 min at 14°C. Fifty µl of DNA template containing supernatant was transferred to a new micro centrifuge tube and used for PCR assay.

PCR for detection of *Pseudomonas* spp.

Each PCR tube contained: 2 µl of extracted DNA template, 10 µl of PCR water, 1 µl of forward primer, 1 µl of reverse primer, and 15 µl of 2x Taq (New England BioLabs Inc. OneTaq, Ipswich, Massachusetts, USA). PCR was performed using the following parameters: initial DNA denaturation at 95°C for 4 min, 34 cycles of DNA denaturation at 95°C for 35 sec, annealing at 54°C for 30 sec, extension at 72°C for 50 sec, and final extension of DNA at 72°C for 4 min. Primers used to identify *Pseudomonas* spp. were 1 µmol universal Pseudomonas primers PSEG30F and PSEG79R consisting of sequences 5'-ATY-GAA-ATC-GCC-AAR-CG-3' and 5'-CGG-TTG-ATK-TCC-TTG-A-3'(Sigma Genosys, Woodlands, Texas, USA) that targeted the *rpo* D gene of DNA.

Gel electrophoresis

PCR amplicons positive for *Pseudomonas* spp. were run on 82 V agarose gel electrophoresis for 30 min and stained with ethidium bromide (Amresco 0.625mg/mL, Solon, Ohio, United States). A 1% agarose (Amresco Agarose I, Solon, Ohio, United States) gel in 0.5X Tris-Acetate-EDTA (TAE) buffer was used for separating PCR products. Bands were photographed (Syngene G: Box, Frederick, Maryland, United States) for visual analysis, sample comparison, and separation.

DNA sequencing

Twenty candidate bacterial isolates were DNA sequenced based on unique colony morphologies. One-hundred µl of each isolate was inoculated into 10 ml of TSB for overnight incubation. Each candidate was streaked onto TSA and incubated at 28°C to obtain pure culture for GENEWIZ analysis. 16S rDNA sequencing was performed by GENEWIZ, Inc (South Plainfield, NJ, USA).

Phylogenetic tree reconstruction

Isolates 16S rDNA sequencing data was used to identify bacterial species using Nucleotide BLAST in Genbank database (www.ncbi.nlm.nih.gov/genbank/). Identity was chosen based on 99% or greater matches. Strain sequences were acquired through access to List of Prokaryotic Names with Standing in Nomenclature (LPSN) (www.bacterio.cict.fr/). Multiple gene alignments were performed using the software program Clauster W [8]. Phylogenetic tree was constructed by the neighbor-joining method using the software MEGA4 [8]. This data was compared to a known strain and bootstrap values were computed.

Results

Bacterial enumeration

Plating on Pseudomonas Isolation agar showed a log reduction of 2 when the initial spoilage of retail fillets were compared to initial spoilage of pond fillets. Plating on TSA, showed a 1.6 log reduction of initial spoilage of retail fillets compared to initial spoilage of pond fillets. Final spoilage rates calculated during the stationary phase of both retail and pond fillets showed that pond fillets have less bacteria present with 1 log reduction shown on selective media and 1.2 log reduction shown on rich media. Therefore, it was confirmed that fillets obtained from the local retail source would have higher spoilage rates than those fillets obtained from the pond source. Change in bacterial growth was likely due to the significant spoilage that took place prior to retail fillets reaching their final destination for sale. This data proves the importance of understanding a products dependency on variations in the storage conditions [9].

Bacterial isolation

Total of 237 bacterial colonies were isolated after six isolation trials. Based on colony morphology, the majority of isolated colonies maintained a circular shape. Colonies frequently appeared yellow, orange, white, or translucent in color with slight elevation. As indicated by Gram and Huss [10] in a related study, *Pseudomonas* spp. and *Shewanella* spp. presence were expected on fresh catfish fillets. Fillets obtained from retail sources reached a spoilage state much faster than fillets obtained from pond source (Figure 1).

DNA extraction, PCR, and gel electrophoresis

Agar plate, PCR screening, and gel electrophoresis results showed that bacterial colonies isolated from retail catfish had higher percentages

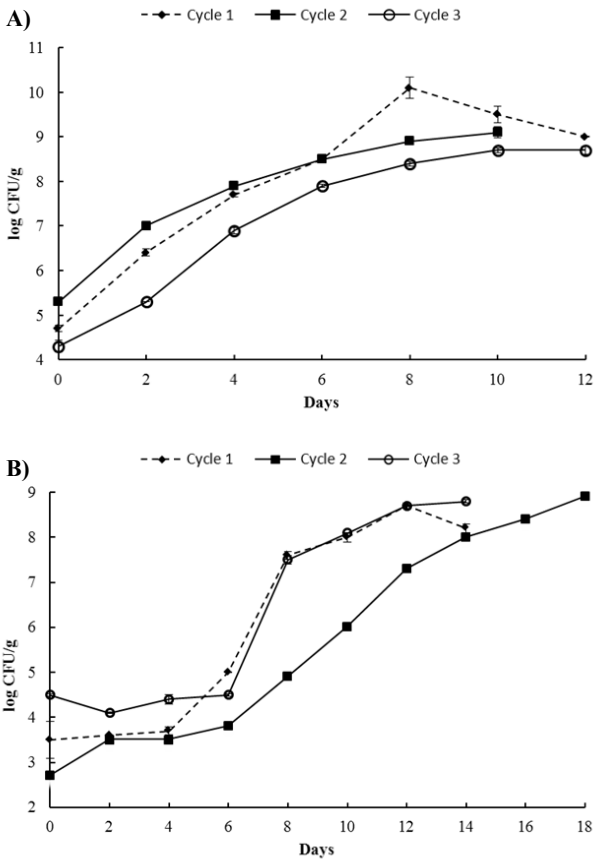

Figure 1: Increase of log CFU/g on a catfish fillets stored at 4°C. (A) Spoilage rates of catfish fillets purchased from a retail source on TSA for cycles 1, 2, and 3. (B) Spoilage rates of catfish fillets from the Aquaculture Research and Demonstration Facility Earthen Aquaculture Ponds at Delaware State University on TSA for cycles 1, 2, and 3.

Pseudomonas spp. than those of the pond sources. Thirty percent of retail fillets tested positive for *Pseudomonas fluorescens* using agar plating method while 24% of pond fillets tested positive. Fifty-three percent of samples from retail fillets screened positive for *Pseudomonas* spp. through PCR assay and gel electrophoresis while 42% of pond fillet samples showed positive test results. No positive results for agar plate tests on *Pseudomonas putida* were obtained which remained consistent in both retail and pond samples.

16S rDNA gene analysis

Samples 1, 4, 5, 9, 10, 11, 13, 14 and 15 were all identified as *Pseudomonas* spp. (Table 1). Sample 1 showed 100% match to *Pseudomonas reactans, Pseudomonas grimontii,* and *Pseudomonas fluorescens*. These results compared to other samples with just one match at 100% accuracy raised concern. Results obtained by Mulet et al. [11] showed the 16S rDNA gene sequence is a good tool for phylogenetic studies, however, in many cases it does not discriminate sufficiently to permit resolution of *Pseudomonas* spp. intrageneric relationships because of its slow rate of evolution. The matching of *Pseudomonas* spp. resulted in a single sample obtained from the 16S rDNA sequencing results found in table 1 support these findings (Figure 2) [11].

Consistency between data received from gene alignment and phylogenetic tree analysis was maintained in most samples. Only 2 of 20 isolates showed variation; likely due to the contrast in protocol between gene alignment findings and phylogenetic tree data. Sequences were matched using 700–1000 bp sequences. In phylogenetic tree reconstruction, a larger range of base pairs usually greater than 1400 are recommended. This explains why sample 14 and 20 were not directly joined to a standard strain as shown in figure 3. Twenty unique isolates from both retail and pond sources were combined into 1 phylogenetic tree. *Pseudomonas* spp., *Shewanella* spp., *Bacillus* spp., *Myroides* spp., *Aeromonas* spp., and *Enterobacter* spp. were all represented (Figure 3).

Discussion

Data confirmed that catfish fillets acquired directly from pond source have significantly lower bacterial growth rates despite being obtained during the summer months with higher anticipated bacterial activity than those obtained from the retail sources. Bacterial spoilage on iced and refrigerated fishery products is dependent upon many factors including processing, storage, and handling [3]. The process of moving catfish fillets to allotted retail establishments causes increased bacteria detection. Less handling, decreased transportation, and reduced storage time significantly decrease the risk of contamination.

Both *Pseudomonas* spp. and *Shewanella* spp. identification were expected during sequencing and phylogenetic analysis. *Shewanella putrefaciens* is the primary spoilage bacteria of marine temperate-water

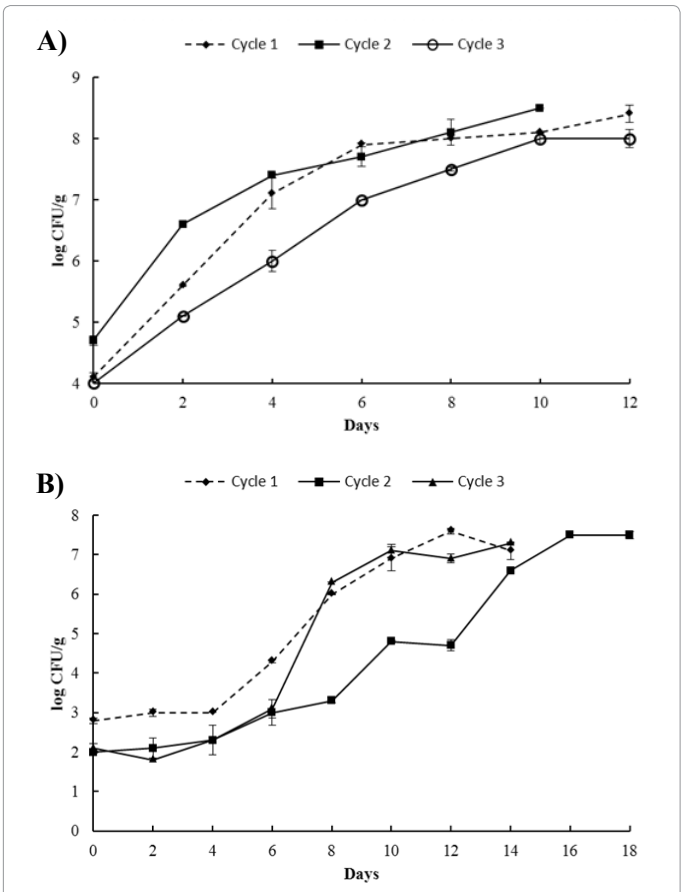

Figure 2: Increase of log CFU/g on a catfish fillets stored at 4°C. (A) Spoilage rates of catfish fillets from a retail source when plated on *Pseudomonas* Isolation Agar for cycles 1, 2, and 3. (B) Spoilage rates of catfish fillets from the Aquaculture Research and Demonstration Facility Earthen Aquaculture Ponds at Delaware State University on *Pseudomonas* Isolation Agar.

Catfish Isolate	Bacterial Identification 16S rDNA Sequencing Result	Similarity (%)[a]	Length (bp)[b]	GenBank Accession	Marine Type Origin
1	*Pseudomonas reactans*	100	1374	JN411452	Fresh
	Pseudomonas grimontii	100	1382	JQ282836	Fresh
	Pseudomonas fluorescens	100	1319	JN411357	Fresh
2	*Shewanella profunda*	99	1530	FR733713	Fresh
	Shewanella putrefaciens	99	1468	AB681550	Fresh/Brackish
3	*Shewanella baltica*	99	1481	JF327458	Fresh/Brackish
4	*Pseudomonas fragi*	99	1462	AB680221	Fresh
5	*Pseudomonas reactans*	99	1374	JN411452	Fresh
	Pseudomonas grimontii	99	1382	JQ282836	Fresh
	Pseudomonas fluorescens	99	1319	JN411357	Fresh
6	*Shewanella profunda*	99	1530	FR733713	Fresh
	Shewanella putrefaciens	100	1468	AB681550	Fresh/Brackish
7	*Pseudomonas fragi*	100	1459	AB685609	Fresh
8	*Myroides marinus*	99	1388	GQ857652	Fresh/Brackish
9	*Pseudomonas auricularis*	100	1463	AB681727	Fresh
	Pseudomonas poae	100	1326	HQ898911	Fresh
10	*Pseudomonas gessardii*	100	1516	AF074384	Fresh
	Pseudomonas fluorescens	100	1457	JQ236807	Fresh
11	*Pseudomonas gessardii*	100	1516	AF074384	Fresh
	Pseudomonas fluorescens	100	1457	JQ236807	Fresh
12	*Bacillus aryabhattai*	100	1475	JN700141	Fresh/Brackish
13	*Pseudomonas veronii*	99	1459	AB494445	Fresh
14	*Pseudomonas oryzihabitans*	100	1463	AB681726	Fresh
15	*Pseudomonas marginalis*	100	1489	HE586396	Fresh
16	*Rahnella aquatilis*	99	1432	JN712163	Fresh
17	*Aeromonas veronii*	99	1458	JQ301791	Fresh/Brackish
18	*Bacillus thuringiensis*	100	904	HE648112	Fresh
	Bacillus cereus	100	909	HQ873674	Fresh/Brackish
19	*Bacillus thuringiensis*	100	1396	JQ342872	Fresh
	Bacillus cereus	100	1450	JQ248587	Fresh/Brackish
20	*Enterobacter asburiae*	99	1465	HQ242717	Fresh/Brackish
	Enterobacter cloacae	99	1437	HQ220157	Fresh

[a]: Similarity of 16S rDNA region between catfish isolated samples and closest relative found in GenBank database
[b]: base pair used for gene alignment.

Table 1: Gene Alignment of 20 Bacteria Isolated after DNA Sequencing.

fish stored aerobically in ice. *Pseudomonas* spp. is the specific spoiler of ice stored tropical freshwater fish stored at psychotropic temperature. *Shewanella putrefaciens*, is another known spoiler of marine tropical fish stored on ice [9].

Bacillus spp. is ubiquitous and diverse in terrestrial and marine ecosystems and can be introduced in food during processing [12]. Bacillus includes species such as *Bacillus cereus, Bacillus licheniformis*, or *Bacillus subtilis* that may be present in fresh and pasteurized food products due to their ability to generate heat-resistant spores under adverse environmental conditions [12]. *Bacillus cereus* has been recognized as a causative agent of food poisoning for more than 40 years and has been linked to foodborne emetic and diarrheal syndromes [12]. Moreover, this species is known to cause spoilage in bread. Ropy spoilage of bread, for example is usually caused by Bacillus spp., especially Bacillus subtilis and Bacillus licheniformis. Other endospore formers like *Bacillus pumilus* and *Bacillus cereus* have also been isolated from spoiled bread [13]. The natural vulnerability of catfish makes such a resistant bacterial species a serious threat.

The genus *Myroides* was formed in 1996 in which two species are derived from a single phylogenetic branch; *Myroides odoratus,* formerly *Flavobacteria odoratum,* and *Myroides odoratimimus* [14]. *Myroides* spp. are widely distributed in water and soil particularly. *Flavobacteria,* previously classified under the genus *Myroides*, was known for its presence on fish and shellfish but their role in the spoilage of chilled fish was thought to be minor compared with that of *Pseudomonas* spp. [15].

Aeromonas spp. are inhabitants of a wide range of aquatic ecosystems such as freshwater, estuarine, coastal waters, and in water with levels of chlorine [16]. Foods of animal origin, including seafood, have been considered important sources of *Aeromonas spp.* infection [16]. *Aeromonas* spp., principally *Aeromonas hydrophila*, currently have the status of foodborne pathogen of emerging importance. It has attracted attention primarily because of its ability to grow at psychotropic temperature. *Aeromonas hydrophila* has been isolated from a wide range of animal and plant food products including raw meat, poultry, fin fish, seafood, dairy products, vegetables, and miscellaneous foods [17].

Food may be contaminated with *Enterobacter sakazakii* under conditions of hygiene mismanagement by contaminated insects and rats. *Enterobacter sakazakii* has been detected in food production as well as in domestic environments [18].

Based on these findings, it is important to broaden the scope of future research studies in this area. It is also essential to conduct sampling of both pond and retail fillets during the same season to decrease spoilage variables. Broadening the scope of this investigation by incorporating more retail and pond sources to sample in the area would increase the accuracy of bacterial identification in the state and Mid-Atlantic region. It would also encourage more cross-discipline collaboration and partnerships between neighboring universities.

The identification of six specific bacterial species contributing to catfish spoilage in this study now offer the industry and seafood consumers valuable data as it relates to microbial degradation on fishery products. Investors in the state of Delaware also benefit from these results as this study gives deeper insight on bacterial species that jeopardize aquaculture profits. It is the hope of the authors to highlight the necessity of more catfish farming in the state of Delaware. Benefits such as decreased spoilage and less points of contamination make implementing locally produced and harvested fish products a clear

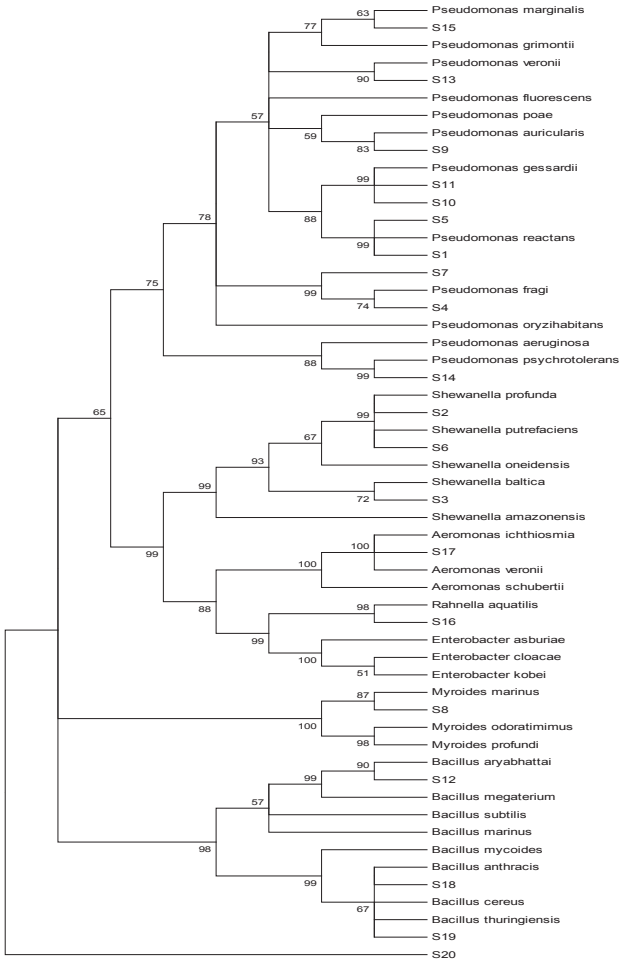

Figure 3: Phylogenetic Tree Reconstruction. Diagram shows the species identified from the 20 samples selected. *Pseudomonas* spp., *Shewanella* spp., *Bacillus* spp., *Myroides* spp., *Aeromonas* spp., and *Enterobacter* spp. were represented.

solution. In addition to more sustainable products, locally produced catfish would boost the economy and keep resources allotted for shipping in the state of Delaware.

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