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Analysis and identification of lipolytic bacterial species for the degradation of wastewater lipids

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An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

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Under the mentorship of Dr. Tiehang Wu

ABSTRACT

The aim of this study was to isolate native bacterial strains from the wastewater treatment facilities of Statesboro, GA to identify their lipolytic activities. Obtained bacterial strains were further assessed via morphological and biochemical methods to determine their enzymatic capabilities. Use of the detergent Tween-20 in growth mediums was the first criteria to assess lipase activity, and these isolates were further investigated to quantitatively measure lipase presence and activity. Lipase protein was precipitated and dialyzed to perform a lipase activity assay, followed by Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) to confirm the presence of the lipolytic enzymes. Sanger sequencing was then employed to determine the identity of the isolated bacterial strains.

Results indicated lipolytic activity of industrial agents is lower than the lipase specific activity of the isolated bacteria. After two days of incubation, isolates and industrial agents did not have a significant difference in specific activities, but after allowing eight days of incubation, isolates showed significantly higher specific activity than industrial agents. Isolates with the highest levels of enzymatic activity were identified as *Bacillus velezensis* and *Bacillus subtilits*. This analysis showed that the bacteria specific to a wastewater plant can be successfully employed to degrade wastewater lipids without chemical additives.

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1. Introduction

As long as people have lived in large groups together, human waste disposal has been a prominent issue when planning living conditions. Though wastewater management has become more advanced, the fact still remains that wastewater treatment has lasting impacts on human health and the environment. Chemical treatment is the standard method, which poses a risk to human health, as these chemicals have been linked to adverse effects on human health, including links to cardiovascular disease and diabetes (Prasse et al., 2018). As vital as water is to survival, the possibility of chronic illness from it should not be a concern. Instead, bacteria isolated from the wastewater can be used to replace these chemicals. *Bacillus* is one of the most commonly reported bacterial species with lipolytic properties and has the capability to naturally break down lipids found in its environment (Bell et al, 2002). Though this is only one of the macromolecules found in wastewater, lipids are an important piece of the puzzle to completely break down waste products using bacteria. At the end of processing, water can be sterilized via UVC light, a practice that has already been effectively used to disinfect water (Juarez-Leon et al., 2020). Using this method, treatment facilities can improve their processes and protect the health of communities. Though bioremediation is not a novel concept, by isolating bacteria from local wastewater, it could be ascertained that the bacteria would not need to be replaced on a regular basis because it is found in the source of contamination naturally. This makes bioremediations of wastewater more sustainable long term.

It is hypothesized that microbial lipases in bacterial strains isolated from local wastewater would exhibit greater specific activity than those in industrial agents, and that culturing the bacteria in lipid rich environments would improve their lipid degrading

ability. In this study, lipolytic bacterial strains will be isolated and assessed via morphological and biochemical methods to determine their enzymatic capabilities, and molecular methods will be employed to identify the species. It is predicted that isolated bacterial strains can be practically applied in situations where it is not appropriate to use industrial agents for waste degradation. On top of being a more consumer friendly waste treatment method, isolating bacteria native to an environment can be a potential way to treat environmental disasters without lasting effects on that environment.

2. Materials and Methods

2.1 Isolation of lipolytic bacterial strains

Using samples isolated from Statesboro wastewater treatment plants as well as samples from industrial enzymatic agents, cultures of bacteria were grown for the following methods. These samples were isolated via the streak plate method outlined by "Benson's Microbiological Applications" (Brown & Smith, 2015) in either Tryptic Soy Agar(TSA) (3% Tryptic Soy Broth (TSB), 1.5% agar), Tween 20 (1% peptone, 0.5% NaCl, 0.01% $CaCl_2 \cdot 2H_2O$, 1.5% agar, 1% Tween 20), or olive oil (1% peptone, 0.5% NaCl, 0.01% $CaCl_2 \cdot 2H_2O$, 1.5% agar, 1% olive oil) inoculated plates. Once it was determined these were pure cultures of each bacterial sample, the isolates were stored at room temperature in both TSB (1% TSB) and Lysogeny Broth (LB) (1% LB) broth for future use. Isolates were gram stained and kept for morphological and molecular identification purposes and biochemical studies in the future.

2.2 Morphological assessment

Testing for lipase activity began by inoculating plates with Tween 20 detergent and streaking isolates over the plate (El-Bestawy et al., 2005). Over a 48-hour testing

period, results were collected detailing the reaction of microbes in the presence of the detergent. These results included checking for growth every 24 hours for a “clear halo” formation within 48 hours (Figure 1). A positive result was indicated by the formation of a halo around the bacterial growth. This occurs because free fatty acids from lipase activity react with the Tween 20 detergent and precipitate calcium onto the plate. Using these methods to compare the isolate’s lipid degrading abilities, isolates were chosen for further molecular and biochemical analysis.

2.3 Enzymatic and biochemical analysis

The size, intensity, and speed at which the halo formed was compared between isolates. Samples of isolates 8b and 17 were grown in lipid enriched broths (5% lipid, 2% LB) of either olive oil or duck fat inside of a shaking incubator at 30°C for 48 hours. An industrial agent, IA 2, was also brewed in both duck fat and olive oil for comparison. The replicates of each sample then underwent protein precipitation using ammonium sulfate to extract lipase from the bacterial cells to be tested for their purity and concentration in bacterial cells via SDS-PAGE. The broth was refrigerated to separate free floating lipids from the proteins, as the presence of lipids in the precipitated proteins made them difficult to extract. A 10mL sample of each broth was saturated with 40% ammonium sulfate then incubated at 22°C for 1 hour in a shaking incubator. Samples were centrifuged at 4250xg for 15 minutes. The saturation of ammonium sulfate was then increased to 60% and the samples were treated with the same incubation and centrifugation methods (Wingfield, 2001). Precipitated protein was isolated in pellet form and dialyzed at 3-, 6-, and 15-hour intervals using a Biotech Cellulose Ester 20 kDa

dialysis membrane in deionized water. Once the samples were desalted, they were stored for later use at 4°C.

A lipase activity assay was used to determine enzymatic activity of the brewed isolates. The assay was performed by incubating 30 µL of each brewed sample at 30°C for 30 minutes in 160 µL of 0.1M sodium phosphate buffer, pH7, and 30 µL of 0.01M p-nitrophenylphosphate (p-NPP) solution. The reaction between p-NPP and lipase was stopped using 50µL of 0.1M solution of sodium carbonate. Absorbance of each sample was measured at 410nm before and after the reaction. Absorbance of the breakdown product p-nitrophenol (p-NP) at 410nm and protein concentration at 280nm were measured using the Nanodrop 2000 spectrophotometer. One lipase unit was defined as the amount of enzyme required to liberate one nanomole of p-NP per minute from p-NPP (Phukon et al., 2020). Enzymatic units per mg were calculated to determine each sample's specific activity, and an ANOVA test was run to determine if there was a significant difference in activity between the bacterial samples and the industrial agent. Activity was calculated by first using Beer's law to calculate the change in concentration of p-NP, then by using the equation (Pham et al., 2021):

$$Enzyme\ activity\ \left(\frac{U}{\mu L}\right) = \frac{nmol\ p-NP}{\mu L} * \frac{1}{incubation\ time}$$

Using dialyzed samples, SDS-PAGE was performed on replicates of the industrial agent and isolates 8b ad 17 to determine the purity of the enzyme extracted and confirm its presence. Wells were loaded with samples of the industrial agent, isolate 8b, and isolate 17 that were grown in either duck fat or olive oil to compare the amounts of lipase protein present in each type of lipid broth. Running buffer (15.0 g of Tris base, 72.0 g of glycine, and 5.0 g of SDS in 500 ml of H_2O) and loading buffer (100 mM Tris-Cl, 4%

(w/v) sodium dodecyl sulfate (SDS), 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM β-mercaptoethanol) was prepared and a mix of 20µL of 2x loading buffer and 20µL dialyzed protein was heated at 95°C for 5 minutes. Running buffer was diluted from 10x to 1x, and then samples were loaded and run at 150V for 55 minutes. The Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard (Bio-Rad, CA, USA) was used as a molecular weight marker for the run. Following electrophoresis, the lane containing the marker was separated from the rest of the gel to prevent it from becoming unreadable during staining. The gel was placed in fixing solution (50% v/v methanol, 10% v/v acetic acid) for two hours, then allowed to stain overnight in Coomassie staining solution: 0.1% (w/v) Coomassie Blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid. The gel was placed in destaining solution (50% (v/v) methanol,10% (v/v) acetic acid), which was changed out several times over the course of 2 hours until the bands of protein could be seen clearly over the color of the background of the gel. The stained gel was allowed to sit in storage solution (5% (v/v) acetic acid) for one hour to allow it to expand back to its original size then compared to the molecular weight marker to determine the size of the protein bands present in each well (Figure 4) (Simpson, 2004). The gel was kept in storage solution at 4°C for future analysis.

2.4 Molecular analysis

To identify the bacteria isolated from industrial agents and wastewater, DNA was extracted from the pure cultures of isolated bacteria that indicated lipase activity. PCR was then performed using primers confirmed to amplify the 16 rRNA gene in *Bacillus* species. Isolated DNA was amplified at 55°C for annealing over 35 cycles. The 16S rRNA gene sequencing results were determined by sequencing using PCR amplicons of

27F/1392R primer pairs. A gel was run to determine the size of the PCR products (Figure 5). Sequencing was conducted by Eton Biosciences (NC, USA), and the sequencing data was then blasted using the National Center for Biotechnology Information Nucleotide BLAST. Samples were identified based on matched genetic sequences to determine the species of each isolate (Table 1).

2.5 Statistical analysis

The treatment effects of bacterial isolates on enzymatic activities were statistically analyzed using analysis of variance (ANOVA). The homogeneity of variances and normality of distribution were tested with the Levene and Kolmogorov–Smirnov tests, respectively. All data were analyzed by one-way ANOVA with means and a post-hoc method (Tukey HSD) for multiple comparisons at a 5% significance level. All the statistical analyses were performed with JMP® Pro 16.0.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

Overall, a total of 12 isolates were obtained displaying lipase activity as noted by clear halo formation (Table 1). It was determined that isolates 8b and 17 had the strongest lipid degrading abilities, although 10 other isolates were also found to have a halo (Table 1). This was based off whether a “clear halo” formed in 48 hours, but the strains with the highest lipolytic activity formed a halo within 36 hours (Figure 1). Figure 1 shows the halos of isolates 8b and 17 after 36 hours. The remaining isolates did not exhibit as large a halo, and if they did, it took several days for them to do so.

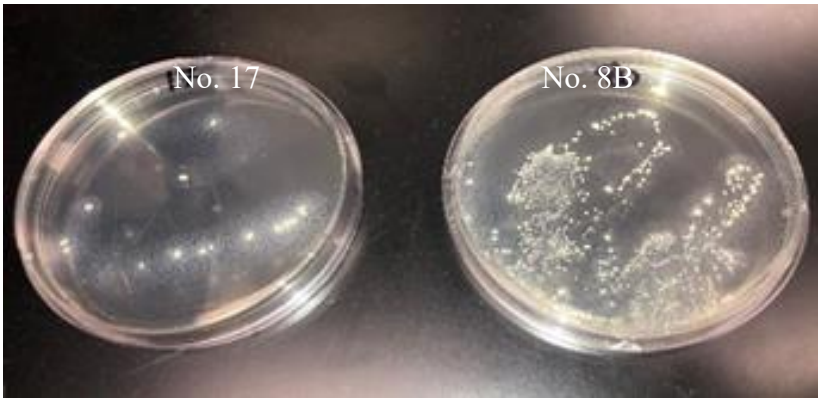

Figure 1. Clear halo formed by isolate 8b and 17 indicating lipolytic activity following 36 hours of incubation.

The lipase activity was not significantly different among duck fat treatment samples (IA2, isolate 8b, and 17) after two-day treatment (Figure 2), the broths were allowed to grow for another six days. Once allowed to fully take over their broth after eight days, bacterial isolates were able to out compete industrial agents in duck fat enriched broth (Figure 2). The olive oil enriched broth was given the same treatment, even though there was a significant difference between the industrial agent and isolates after two days (Figure 3). This meant that the difference between the isolates given the eight days to grow in olive oil was more dramatic than the results after two days.

The PCR products formed in preparation for Sanger sequencing were found to be roughly 1300 base pairs in size (Figure 4). Following analysis of Sanger sequencing, all lipolytic strains isolated during these methods were determined to be various species of *Bacillus* (Table 1), with six isolates identified at *B. subtilits*, three identified as *B. velezensis*, two identified as *B. licheniformis*, and one as *B. halotolerens*. The strains with the highest levels of enzyme activity were found to be *B. subtilits* (isolate 17) and *B. velezensis* (isolate 8b). The next most lipolytic was *B. halotolerens* based on the speed at which its halo formed.

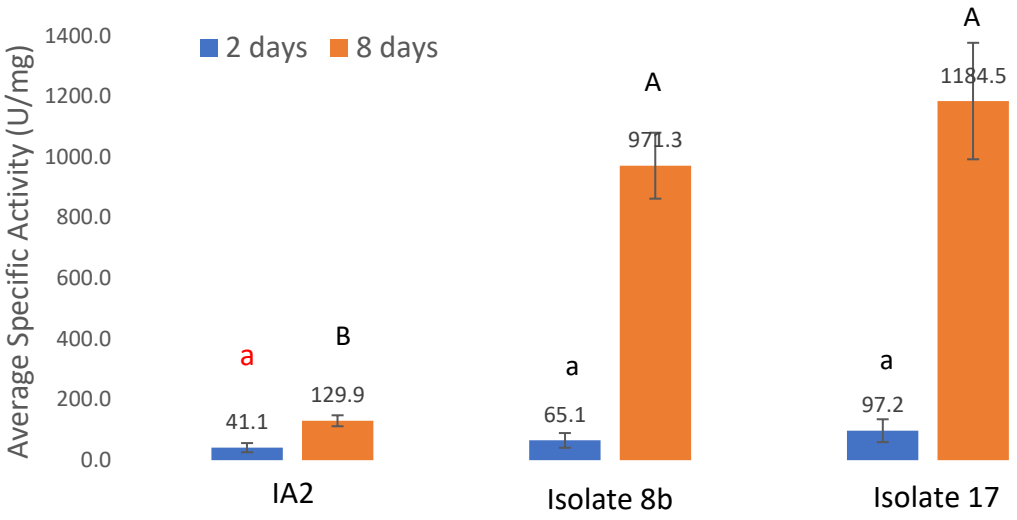

Figure 2: Means (± 1 SE) of specific lipase activity from two isolated bacteria and industrial agent (IA) in duck fat after 2 and 8 days of incubation. Means followed by the same letter in 2 or 8 days are not significantly different according to Tukey HSD test

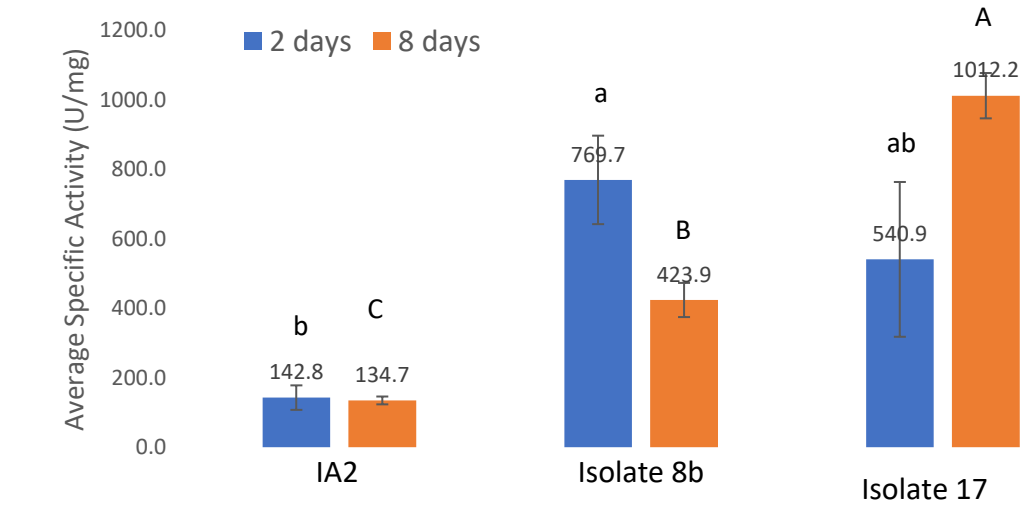

Figure 3: Means (±1 SE) of specific lipase activity from two isolated bacteria and industrial agent (IA) in olive oil after 2 and 8 days. Means followed by the same letter in 2 or 8 days are not significantly different according to the Tukey HSD (P≤0.05)

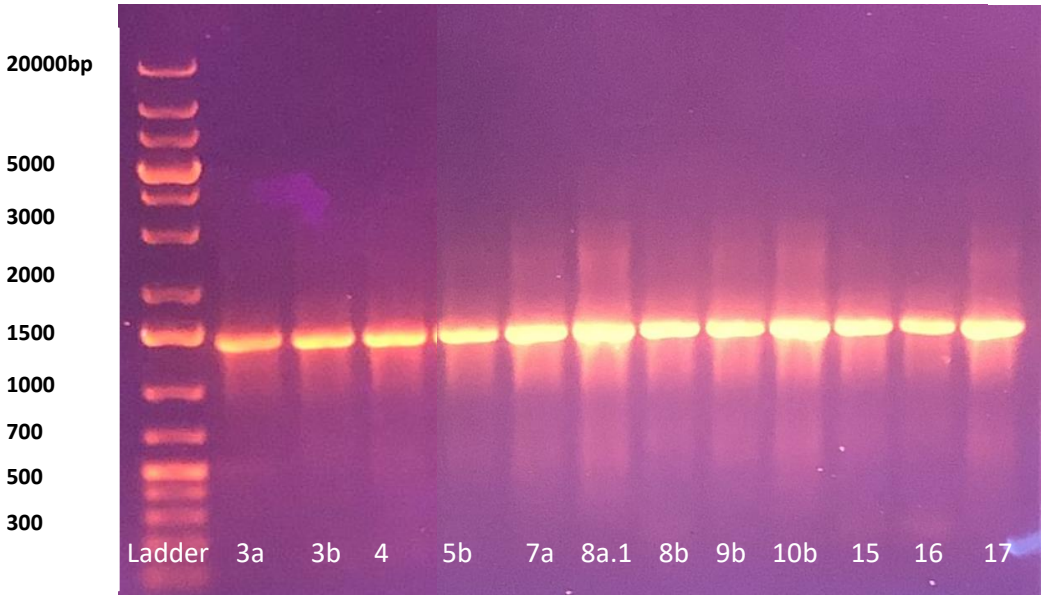

Figure 4: Agarose gel displaying the amplified 16S rRNA gene from the 12 isolated lipolytic strains. The amplified PCR products were used for Sanger sequencing.

Table 1. Source, culture method, and species identification of lipolytic isolates.

Isolate Number	Source	Culture Method	BLAST ID
3a	Wastewater	Tween 20	*Bacillus subtilis*
3b	Wastewater	Tween 20	*Bacillus subtilis*
4	IA-1	Olive Oil	*Bacillus velezensis*
5b	Wastewater	TSA	*Bacillus halotolerens*
7a	Wastewater	TSA	*Bacillus subtilis*
8a.1	Wastewater	TSA	*Bacillus licheniformis*
8b	Wastewater	TSA	*Bacillus velezensis*
9b	IA-1	Olive Oil	*Bacillus subtilis*
10b	IA-2	Olive Oil	*Bacillus licheniformis*
15	Wastewater	Olive Oil	*Bacillus velezensis*
16	Wastewater	Olive Oil	*Bacillus subtilis*
17	Wastewater	Olive Oil	*Bacillus subtilis*

SDS-PAGE showed *B. velezenis* and *B. subtilis* had proteins present between 37 and 75 kDa (Figure 5). The bands in the 37-75 kDa ranges of isolates 8b and 17 stained darker than those in the same range as the industrial agent's showing there was a larger concentration of proteins in that range for the isolates. Isolate 17 had significantly darker staining in the 37-75 range than that of isolate 8b. The banding patterns between lipid types were comparable for isolate 17, but there were larger amounts of protein in the duck fat broth of the industrial agent, and the olive oil broth of isolate 8b yielded larger amounts of protein based on the intensity of bands after staining.

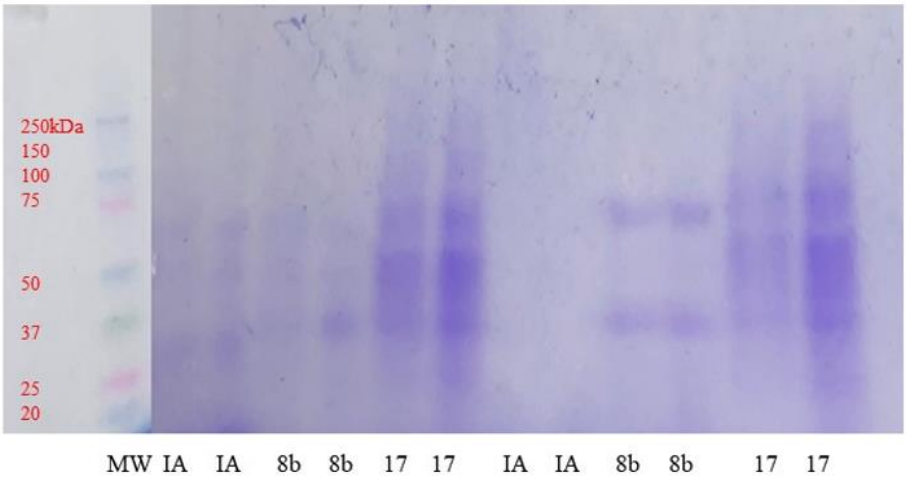

Figure 5. SDS-PAGE result for lipases isolated from *B. velezenis* (8b) and *B. subtilis* (17) compared with industrial agent (IA).

4. Discussion

The results of this experiment led to several findings. First, the choice of culture medium affected the potential of an individual's lipase to break down different lipids, based on the majority of isolated lipolytic bacterial species being grown on olive oil enriched plates. Lipases first isolated from olive oil inoculated plates had higher enzymatic capability and typically developed "clear halos" faster than other isolates that

eventually developed halos. Of the 33 bacterial strains isolated throughout using the streak plate method to find pure cultures of bacteria, only 12 were found to have lipase activity. Of those 12, six were isolated from olive oil inoculated plates. This indicates lipases react well when an organism is grown in an environment suitable for its activity. There was a statistically significant difference between the lipase activity in isolate 17, which was originally isolated from olive oil enriched plates, and isolate 8b, which was initially grown in TSA plates. Isolate 17 had a consistently higher specific activity than isolate 8b and the industrial agent's lipase.

Though the difference between lipase activity was not always statistically significant at first, once the strains took over the broth, there were higher levels of lipase in the isolate mediums. Through this, it can be inferred that if a bacterial strain is given enough time, it will break down more than the industrial agents it is competing against. As has been found in other studies, *Bacillus* spp. are highly lipolytic. The identified strains were often times the same species of *Bacillus*, meaning that the strains can be selected for based off of their abilities, and their lipid degrading abilities can be improved over generations.

The bands present in the SDS gel allow comparison of the various amounts of lipase in each sample. Since lipase tends to be in the range of 19-60 kDa (Chandra et al., 2020), the stronger banding patterns, especially in isolate 17, indicate a higher concentration of proteins with the same size as lipase. Because the proteins were precipitated specifically to isolate lipase, it leads to the conclusion that the high levels of protein in the size range of lipase were in fact lipase. The stronger banding in isolate 17 is most likely due to higher levels of the enzyme, which may be the explanation for the

higher specific activity. The higher intensity of the SDS-PAGE bands in the olive oil replicate of isolate 8b is also likely tied to the lower level of calculated specific activity. Isolate 8b had higher protein concentrations after being allowed to grow for the additional six days, and because of the method of calculation, this meant the specific activity was higher, even when the concentrations of p-NP increased compared to the assay after two days. The lack of banding in industrial agent wells in both lipid treatments also aligns with their lower specific activity.

5. Conclusion

Overwhelming evidence throughout this study points to *Bacillus* species as the optimal bacteria for lipid degradation. This is clear based on the only identified strains being *Bacillus*. Most suitable strains can be isolated by growing in lipid enriched plates to ensure they are fit for their environment. By isolating the strains from the source of contamination, using these strains makes bioremediation more practical, as the bacteria do not need to be reintroduced to the environment regularly. They can grow in the environment without outside stimulation, so growing large batches of the bacteria and allowing the species to overtake others inside wastewater may make them more likely to succeed in breaking down lipids without outside assistance.

Bacillus subtilits was the best suited for lipid degradation, and more work may be done with the individual strain of isolate 17, as it displayed higher enzymatic activity than its other *B. subtilis* counterparts. This could be promising for its future use, as its lipase could be improved over generations by growth in lipid rich environments to select for lipid degrading ability. Through it could be applied to wastewater as originally

intended, the strain could also be introduced to environmental disasters like oils spills to aid in clean up. Otherwise, the same methods of isolation to find a native lipolytic strain can be employed to make cleaning the environment easier.

Future research can investigate whether *Bacillus* is as effective with other lipids than those used in this study, and if bioremediation using bacteria isolated from the source of contamination is a practical choice for wastewater treatment. The specific strain of *B. subtilis* still needs to be reintroduced to wastewater since its isolate to allow this project to come to completion to ensure the strain would thrive in wastewater conditions. If the isolate does not thrive in the environment it is given, the gene specific for isolate 17's lipase may be located and introduced into the genome of other bacteria more suited for the environment. Additionally, to watch the increase in protein concentration over the course of the bacteria's brewing, it would be important to precipitate and dialyze each sample of bacterial lipase after two and eight days to watch the increase of proteins over time.

Works Cited:

1. Bell, P. J., Sunna, A., Gibbs, M. D., Curach, N. C., Nevalainen, H., & Bergquist, P. L. (2002). Prospecting for novel lipase genes using PCR. *Microbiology*, 148(8), 2283-2291. DOI:10.1099/00221287-148-8-2283

2. Brown, A. E., & Smith, H. (2017). Benson's microbiological applications: Laboratory manual in general microbiology (15th ed.). McGraw-Hill Higher Education.

3. El-Bestawy, E., El-Masry, M. H., & El-Adl, N. E. (2005). The potentiality of free gram-negative bacteria for removing oil and grease from containing industrial effluents. *World Journal of Microbiology and Biotechnology*, 21(6): 815-822. DOI:10.1007/s11274-004-2239-8

4. Chandra, P., Enespa, R. S., & Arora, P. K. (2020). Microbial lipases and their industrial applications: a comprehensive review. *Microbial Cell Factories*, 19(1). https://doi.org/10.1186/s12934-020-01428-8

5. Juarez-Leon, F. A., Soriano-Sanchez, A. G., Rodriguez-Licea, M. A., & Perez-Pinal, F. J. (2020). Design and implementation of a germicidal UVC-LED lamp. *IEEE Access*, 8: 196951-196962. doi:10.1109/access.2020.3034436

6. Pham, V. H., Kim, J., Chang, S., & Chung, W. (2021). Investigation of lipolytic-secreting bacteria from an artificially polluted soil using a modified culture method and optimization of their lipase production. *Microorganisms*, 9(12), 2590. https://doi.org/10.3390/microorganisms9122590

7. Phukon, L. C., Chourasia, R., Kumari, M., Godan, T. K., Sahoo, D., Parameswaran, B., & Rai, A. K. (2020). Production and characterization of lipase for

application in detergent industry from a novel *Pseudomonas helmanticensis* HS6. *Bioresource Technology*, 309, 123352. https://doi.org/10.1016/j.biortech.2020.123352

8. Prasse, C., Ford, B., Nomura, D. K., & Sedlak, D. L. (2018). Unexpected transformation of dissolved phenols to toxic dicarbonyls by hydroxyl radicals and UV light. *Proceedings of the National Academy of Sciences*, 115(10), 2311-2316. doi:10.1073/pnas.1715821115

9. Shahinyan, G., Margaryan, A., Panosyan, H., & Trchounian, A. (2017). Identification and sequence analyses of novel lipase encoding novel thermophillic *Bacilli* isolated from Armenian geothermal springs. *BMC Microbiology*, 17(1). doi:10.1186/s12866-017-1016-4

10. Wingfield, P. (2001). Protein precipitation using ammonium sulfate. *Current Protocols in Protein Science*, 13: Appendix 3F.

11. Simpson, R. J. (2004). *Purifying proteins for proteomics: A laboratory manual*. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press.