# Isolation and Identification of a Potential Sulfolane Degrading Fungus

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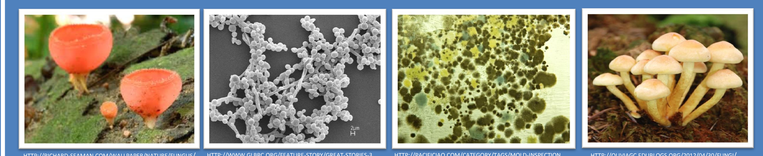
## INTRODUCTION

### • WHAT ARE FUNGI?

FUNGI ARE EUKARYOTIC, HETEROTROPHIC ORGANISMS THAT REPRODUCE VIA SPORES. THERE ARE CURRENTLY 100,000 FUNGI DESCRIBED, BUT MAY BE AS MANY AS 1.5 MILLION SPECIES. THESE SPECIES INCLUDE MUSHROOMS, YEASTS, AND MOLDS. FUNGI ARE INCREDIBLY DIVERSE, AND CAN BE FOUND IN THE SOIL, WATER, AND AIR.



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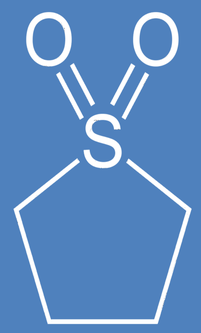
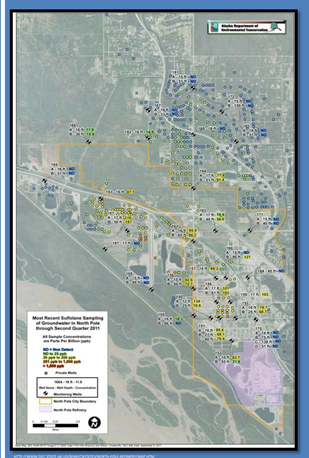
*ABOVE: SEVERAL EXAMPLES OF FUNGI, INCLUDING TWO MUSHROOM SPECIES, YEAST, AND VARIOUS MOLDS.*

### • WHAT IS SULFOLANE?

SULFOLANE IS AN ARTIFICIAL CHEMICAL SOLVENT, COMMONLY USED IN OIL REFINING AND GASOLINE PRODUCTION, ALTHOUGH IT IS ALSO COMMONLY USED IN THE MANUFACTURING OF TEXTILES, PLASTICS, AND ELECTRONICS. AMONG ITS PHYSICAL FEATURES IS ITS LOW VISCOSITY, MEANING IT FLOWS EASILY, ITS LOW VOLATILITY, MEANING IT DOES NOT EASILY EVAPORATE, AND ITS TENDENCY TO DISSOLVE IN WATER. SO, ONCE SULFOLANE ENTERS A WATER SOURCE, IT WILL DISSIPATE AND FLOW WITH THE WATER, RATHER THAN ATTACHING TO SOIL PARTICLES OR EVAPORATING. THIS MAKES IT DIFFICULT TO CLEAN IF THERE IS A SPILL.

*RIGHT: THE CHEMICAL STRUCTURE OF SULFOLANE.*




### • SULFOLANE IN NORTH POLE

SULFOLANE HAS BEEN FOUND IN THE GROUNDWATER OF MUCH OF THE NORTH POLE AREA, INCLUDING MANY PRIVATE DRINKING WATER WELLS. THIS CONTAMINATION ORIGINATES FROM THE FLINT HILLS REFINERY. LOOKING AT THE MAP BELOW, IT IS EASY TO SEE CONTAMINATION IS WIDESPREAD (APPROXIMATELY 3 MILES LONG BY 2 MILES WIDE). FLINT HILLS IS PROVIDING CLEAN DRINKING WATER TO AFFECTED RESIDENTS; HOWEVER, THIS IS NOT A PERMANENT SOLUTION TO THE GROUNDWATER CONTAMINATION ISSUE. WELL WATER IS STILL USED FOR BATHING, ALONG WITH COOKING AND GARDENING. THE FATE OF SULFOLANE IN THE AQUIFER AND ITS POTENTIAL BIODEGRADATION BY MICROBES ARE NOT WELL UNDERSTOOD.

*LEFT: A MAP SHOWING CONCENTRATIONS OF SULFOLANE IN NORTH POLE, FROM A STUDY CONDUCTED BY THE ALASKA DEPARTMENT OF ENVIRONMENTAL CONSERVATION.*

### • BIOREMEDIATION

GROUNDWATER CONTAMINATION HAS SPARKED INTEREST IN HOW TO BEST REMEDIATE (GET RID OF) THE SULFOLANE. ONE PROMISING TECHNIQUE IS BIOREMEDIATION. THIS REFERS TO THE USE OF ORGANISMS TO BREAK DOWN A HARMFUL COMPOUND INTO HARMLESS COMPONENTS. FOR EXAMPLE, SOME SPECIES OF BACTERIA ARE KNOWN TO CLEAN UP OIL SPILLS. IN THEORY, THE SAME PRINCIPAL COULD BE APPLIED TO SULFOLANE IN NORTH POLE. SEVERAL STUDIES WERE CONDUCTED BY THE LAB OF DR. MARY BETH LEIGH AT UAF, IN WHICH SEVERAL SPECIES OF BACTERIA FROM NORTH POLE GROUNDWATER WERE ISOLATED AND IDENTIFIED, A FEW OF WHICH SHOWED THE ABILITY TO DEGRADE SULFOLANE. SEVERAL OF THESE BACTERIA WERE NOT KNOWN TO DEGRADE SULFOLANE PRIOR TO EXPERIMENTATION.

### • DISCOVERY OF A POTENTIAL SULFOLANE DEGRADING FUNGUS

WHILE THE BACTERIA WERE BEING TESTED FOR SULFOLANE DEGRADING QUALITIES, A FUNGUS WAS FOUND GROWING IN A TUBE CONTAINING DISCARDED SULFOLANE. THIS WAS A SURPRISING DISCOVERY, AS SULFOLANE WAS THE ONLY USABLE CARBON SOURCE WITHIN THE TUBE, AND THERE ARE NO KNOWN REPORTS OF FUNGI WITH THE ABILITY TO DEGRADE SULFOLANE. HOWEVER, SOME FUNGI ARE KNOWN TO BIODEGRADE OTHER CONTAMINANTS LIKE PETROLEUM. IF THE FUNGUS TRULY COULD DEGRADE SULFOLANE, IT WOULD BE ANOTHER PLAUSIBLE OPTION FOR BIOREMEDIATION IN NORTH POLE

*RIGHT: THE ORIGINAL FUNGUS IN TUBE OF DISCARDED SULFOLANE SOLUTION.*


## ABSTRACT

SULFOLANE IS AN INDUSTRIAL CHEMICAL THAT IS CURRENTLY CONTAMINATING THE GROUNDWATER OF NORTH POLE, ALASKA. SEVERAL KINDS OF REMEDIATION ARE BEING INVESTIGATED, AND ONE METHOD IS BIOREMEDIATION BY MICROBES, ESPECIALLY BACTERIA. IN THE COURSE OF A RECENT EXPERIMENT IN THE LABORATORY, ANOTHER KIND OF MICROBE, A FUNGUS, WAS FOUND GROWING IN A TUBE CONTAINING SULFOLANE. SULFOLANE WAS THE ONLY KNOWN SUBSTANCE IN THE TUBE THAT THE FUNGUS COULD USE TO GROW AND METABOLIZE, WE HYPOTHESIZED THAT THIS FUNGUS MIGHT BE A POTENTIAL BIODEGRADER OF SULFOLANE. IN THIS STUDY WE GREW THE FUNGUS ON VARIOUS NUTRIENT MEDIA, INCLUDING SOME WITH SULFOLANE AS ONLY CARBON SOURCE. ALL AGAR PLATES WERE SUCCESSFUL IN GROWING FUNGUS, WHILE LIQUID MEDIA ON CULTURES RESULTED IN NO VISIBLE GROWTH DURING THE COURSE OF OUR STUDY. WE ALSO USED PCR TO AMPLIFY THE INTERNALLY TRANSCRIBED SPACER (ITS) REGION OF THE FUNGUS, AND SANGER SEQUENCING WAS USED TO IDENTIFY THE FUNGUS AS BELONGING TO THE GENUS *CLADOSPORIUM*, A DIVERSE AND WIDESPREAD GROUP OF ASCOMYCETE FUNGI.

## METHODS

### PART 1. FUNGAL ISOLATES

FUNGAL SAMPLES WERE GROWN ON VARIOUS AGAR MEDIA PLATES TO 1) CREATE MORE FUNGAL BIOMASS FOR DNA EXTRACTION 2) TEST THE TOLERANCE OF THE FUNGUS TO SULFOLANE. TWO SPECIALIZED SULFOLANE MEDIA WERE CREATED FOR THIS PURPOSE. THE SECOND (SOCS) WAS ALSO PREPARED AS LIQUID ONLY, WITHOUT ADDITION OF AGAR. A FEW MILLIGRAMS FROM THE ORIGINAL FUNGUS WAS USED TO INOCULATE ALL MEDIA, AND ALLOW TO GROW AT ROOM TEMPERATURE FOR THE DURATION OF THE STUDY.

PAT AGAR

| Reagent | 500 mL – Mass (g) |
|---|---|
| Bacto PCA | 1.15 |
| Bacto Agar | 8.5 |
| Sulfolane | 0.5 |

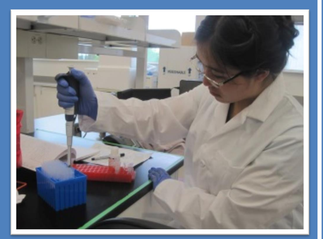
SOCS LIQUID MEDIA (ADDITION OF 8.5 G OF AGAR CREATES SOCS AGAR)

| Reagent | 500 mL - Mass (g) |
|---|---|
| K2HPO4 | 0.25 |
| NaCl | 1 |
| MgCl2*6H2O | 0.22 |
| KNO3 | 1 |
| NH4Cl | 0.5 |
| Sulfolane | variable |
| Trace Metal Solutions | 0.5 mL |

### PART 2. DNA IDENTIFICATION

### • DNA EXTRACTION

DNA WAS EXTRACTED FROM TISSUES OF BOTH THE ORIGINAL FUNGAL COLONIES AND FUNGUS GROWN ON AGAR. WE USED TWO METHODS TO ACHIEVE OPTIMAL RESULTS, THE POWERWATER DNA EXTRACTION KIT (MOBIO) AND THE FASTDNA SPIN KIT FOR SOIL (MP BIOPREP). EACH METHOD CONSISTED OF FOUR MAIN STEPS: 1) LYSING (BREAKING APART CELLS BY PHYSICAL AND CHEMICAL MEANS), 2) SEPARATING THE DNA BY PRECIPITATION (CAUSING NON-DNA CELL MATERIAL TO BECOME SOLID AND FALL TO THE BOTTOM OF THE TUBE), 3) BINDING AND WASHING THE DNA THROUGH SPECIALIZED MEMBRANE FILTERS, 4) ELUTING THE PURE DNA IN WATER. ALL DNA WAS ANALYZED ON A NANODROP 1000 SPECTROPHOTOMETER TO DETERMINE QUALITY AND QUANTITY



*RIGHT: CONNOR ITO PREPARING SAMPLES OF FUNGI.*

### • PCR

A POLYMERASE CHAIN REACTION WAS USED TO MULTIPLY THE "INTERNALLY TRANSCRIBED SPACER" REGION OF THE FUNGAL GENOME. THIS WOULD AID IN IDENTIFYING THE SPECIES. THE PCR PROCESS CONSISTS OF A FEW MAIN STEPS. THE PCR CHEMISTRY IS MIXED, AND THEN HEATED IN CERTAIN PATTERNS TO DUPLICATE THE DNA. THESE PATTERNS BEGIN WITH THE HEATING OF THE DNA UNTIL THE BONDS HOLDING IT TOGETHER MELT, CAUSING THE DNA TO UNRAVEL LIKE A ZIPPER. THEN, THE TEMPERATURE COOLS, AND SPECIALIZED PROTEINS CALLED POLYMERASE ATTACH TO THE STRANDS. THEN THE TEMPERATURE RISES, AND THE POLYMERASE DUPLICATES THE DNA IT IS ATTACHED TO. THIS PROCESS IS REPEATED 32 TIMES, DOUBLING THE AMOUNT OF DNA EACH CYCLE.

### • SEQUENCING

THIS IS A TWO STEP PROCESS, THE FIRST STEP BEING VERY SIMILAR TO PCR, AND IS CALLED CYCLE SEQUENCING. THE CYCLE SEQUENCING PRODUCT IS THEN PURIFIED, ENSURES THAT NO CONTAMINANTS ARE IN THE SEQUENCING PRODUCT, ACHIEVED BY FILTERING THE PRODUCT THROUGH A SEPHADEX GEL. THIS WAS THEN VACCUFUGED, AND RESUSPENDED WITH HIDI FORMAMIDE. THE SECOND MAJOR STEP OF SEQUENCING WAS RUNNING THE FINAL PRODUCT THROUGH A 3130X1 GENETIC ANALYZER TO SEQUENCE THE DNA. SEQUENCE DATA WERE ANALYZED WITH SOFTWARE PROGRAMS (SEQUENCING ANALYSIS 5.1, SEQUENCHER 4.9, GENBANK BLAST SEARCHES, AND MEGAN 4.7).

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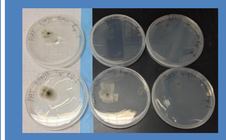
## RESULTS

### PART 1. FUNGAL CULTURES

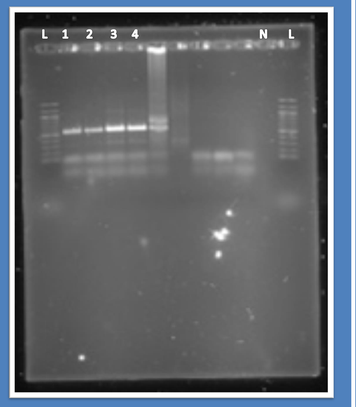
ALL AGAR PLATES WERE SUCCESSFUL IN GROWING FUNGUS, ALTHOUGH THE PAT PLATES AND SOCS MEDIA GREW IN DIFFERENT PATTERNS THAN THE FUNGUS GROWN ON NUTRIENT AGAR, WITH PAT FUNGI BEING LESS DENSE AND SLOWER TO GROW, AND SOCS FUNGI BEING COMPLETELY FLAT AND COLORLESS, AS WELL AS SLOWER GROWING. THE LIQUID MEDIA DID NOT PRODUCE VISIBLE GROWTH OF FUNGUS DURING THE STUDY PERIOD.

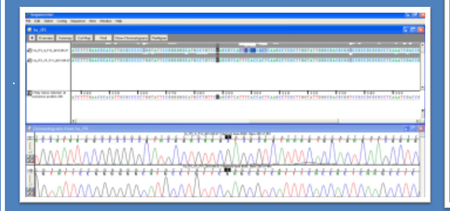


### PART 2. DNA ANALYSES AND IDENTIFICATION

THE RESULTS FROM THE POWERWATER EXTRACTION SHOWED HIGHER QUANTITY AND QUALITY OF DNA RESULTS THAN THE FASTDNA KIT, BUT DNA FROM BOTH KITS WERE SUCCESSFULLY AMPLIFIED BY PCR.

*RIGHT: AGAROSE GEL OF PCR PRODUCTS. L= 100 BP LADDER. 1-4 AMPLIFIED DNA FROM UPPER AND LOWER FUNGAL COLONIES, EXTRACTED WITH THE TWO DNA KITS. N=NEGATIVE CONTROL PCR. (OTHER SAMPLES ON GEL ARE SEPARATE STUDY.)*



THE RESULTS FROM THE SEQUENCING OF PCR PRODUCTS WERE GOOD READS FOR 3 OF THE 4 SAMPLES. ALL READS PROVED TO BE IDENTICAL SEQUENCES. RESULTS OF GENBANK BLAST HITS AND MEGAN ANALYSES SHOWED OUR FUNGUS TO BE 100% IDENTICAL TO MULTIPLE SPECIES WITHIN THE GENUS ***CLADOSPORIUM***.

*ABOVE LEFT: SCREEN CAPTURE OF THE RAW DATA (CHROMATOGRAM) OF THE FUNGAL SEQUENCE FROM THE ABI 3130XL DNA SEQUENCER.*

## CONCLUSIONS

IN THIS STUDY, WE SUCCESSFULLY ISOLATED AND IDENTIFIED A SULFOLANE TOLERANT FUNGUS. STILL, IT IS NOT KNOWN IF THIS FUNGUS DEGRADES SULFOLANE OR JUST TOLERATES IT. THE SOCS LIQUID CULTURES FROM THIS STUDY WILL CONTINUE TO BE MONITORED FOR FUNGAL GROWTH. IF FUNGI DO GROW (AND MEASURED SULFOLANE CONCENTRATION DECREASES), THIS WILL INDICATE THE FUNGUS IN FACT DEGRADES SULFOLANE. ALSO, IN THE FUTURE THE FUNGUS COULD BE CULTURED ON DIFFERENT MEDIA TO SEE IF IT CAN DEGRADE SULFOLANE IF ADDITIONAL NUTRIENTS ARE PROVIDED. WE DISCOVERED THIS FUNGUS TO BE A SPECIES WITHIN THE GENUS CLADOSPORIUM WHICH IS A WIDESPREAD AND DIVERSE GENUS. ADDITIONAL SECTIONS OF THE THE GENOME CAN BE SEQUENCED TO ACHIEVE A MORE FINELY RESOLVED TAXONOMIC IDENTIFICATION TO DETERMINE IF THIS IS AN ALREADY KNOWN STRAIN OF THIS GENUS. EVEN IF THE FUNGUS IS NOT FOUND TO HAVE SULFOLANE DEGRADING ABILITIES, PERHAPS OTHER CLOSELY RELATED SPECIES OF FUNGI OF COULD BE TESTED FOR SULFOLANE DEGRADATION ABILITIES, AND OUR STUDY DEMONSTRATES AT A MINIMUM THAT FUNGI CANNOT BE IGNORED AS POTENTIALLY USEFUL AGENTS OF BIODEGRADATION.

## ACKNOWLEDGEMENTS

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