BIOREMEDIATION COUPLED CELLULAR IMMOBILIZATION PLATFORM

ABSTRACT

Water filtration is a demand that has required increasing innovation to keep up with the demands of a progressively urbanizing society. This problem has been compounded by the results of phenomena such as climate change and global pollution. Water filtration techniques and the separation of solids from water, however, have been unable to match the pace of this demand, largely due to inefficient techniques based on principles of mechanical water remediation. Bioremediation, however, is a new evolving method, with immense potential. The 2018 iGEM Toronto team has developed a platform, based on the optimization of bacterial gene expression to produce gas vesicles in *E. coli*, that demonstrates the viability of bioremediation as a water filtration technique.

INTRODUCTION

The finite supply of fresh, clean water is declining throughout the world. This is a result of global warming, polluted runoff, poorly managed wastewater practices, and overdevelopment. Industries, such as mining operations and wastewater treatment plants, have put a great demand on the world's water. Existing techniques used in various industries for cleaning water are costly, have high energy expenditure, or potentially dangerous to the environment. Biological water treatment methods, on the other hand, show great promise in combating these hurdles. The 2018 iGEM Toronto team is optimizing a bacterial gene cluster to act as a bioremediation platform to combat the current inefficiencies faced by major industries and bring a more reliable form of biological water treatment. By removing unwanted pollutants or highly valuable materials, wastewater effluent from mining processes and wastewater treatment plants can return to its state of purity. Furthermore, the safe extraction method used by this platform will preserve the relevant materials providing a financial incentive.

SECTION 1: CURRENT AFFLUENT SEPARATION TECHNIQUES IN INDUSTRY

Currently, industrial facilities use chemical, mechanical, and biological methods to separate high-value materials and pollutants from water. Many of these methods, while still widely used, remain inefficient on a large scale. This section will look at how well these methods perform remediation in four major industries: microalgae-based biomass production, wastewater treatment, and the mining and fermentation industries.

1.1. Biofuel and Biomass Separation From Algae:

Microscopic algae, or microalgae, are limited to nanometers in size. Despite their small stature, they are immensely important for both the global climate and the biofuel industry; they produce a significant portion of the world's oxygen, and their rapid growth time and highly efficient resource uptake systems make them, even more than conventional plants, a cornerstone of biofuel production. The application of microalgae for the production of valuable products and biofuels have created many filtration techniques on the industrial scale. The need to generate low-cost methods for biomass production is a priority; thus, harvesting microalgae biomass more efficiently from aqueous solutions is a challenge within this industry. Currently, microalgae biomass is harvested through mechanical, electrical, chemical, and biological methods.

1.1.1 Mechanical Methods within the Microalgae Biomass Industry:

Companies such as Algae to Energy (A2E) and Algae venture Systems Inc., use different mechanical methods to obtain suspended algae from water waste. In the algae industry, centrifugation is a rapid and reliable method for the recovery of suspended algae, resulting in 12-22% solids concentration after harvesting yielding >90% recovery.[1] However, while, rapid and reliable, centrifugation is a high investment and consumes energy at a high rate. To avoid such high expenses, companies may turn to filtration[1]. Tangential filtration is argued to be more feasible than dead-end filtration, as it is reliable, results in a high solids concentration and has a recovery rate of approximately 70-90 percent. However, tangential filtration can result in "membrane fouling and replacement", which require high investment and power use.[1] Sedimentation, another mechanical technique, is another low-cost method that can result in concentrations of 1.5 solids for algae harvesting[1] However, sedimentation has low reliability due to the fluctuating density of algae cells and is a slow process overall. In addition to being slow, sedimentation may cause biomasses to break down during this process.

Another process, referred to as dissolved air flotation (DAF), is used in wastewater treatment sludge removal. This process is very successful on the industrial scale, however, the use of flocculants -- compounds that cause "clumping" in solution, often necessary during mechanical microalgal biomass harvesting -- can interfere when the microalgae are later processed and converted into viable biofuel[1]. Lastly, techniques where artificially-constructed algae biofilms mechanically harvest microalgae and are vacuumed or scraped after coagulation. A2E and Algaeventure Systems Inc. use a continuous algae biofilm belt to extract suspended algae.

1.1.2 Electrical Methods within the Microalgae Biomass Industry:

The microalgae harvesting industry also employs electrical methods. The negative charge of microalgae, which are single-celled organisms, allows them to be concentrated by movement in

an electric field[2]. However, this method requires high power and electrodes, which are implausible on a large scale application.

1.1.3 Chemical Methods within the Microalgae Biomass Industry:

Companies that aim to harvest microalgae may use chemical methods, such as the use of electrolytes and synthetic polymers, to coagulate and flocculate algae cells for harvest.[2] Many companies treat microalgae with aluminum to increase the viability of starch-based coagulants and ease harvesting. The benefit to using aluminum is the reliability or high recovery of algae.[1] However, there are a number of adverse effects when using aluminum treatment, and similar chemical methods. Firstly, aluminum and sulfate coagulants have been reported to hinder methanogenic activity in bacteria-fed wastewater sludge, while land application of aluminum-treated microalgae biomass can increase heavy metal uptake, and may lead to phosphorus uptake problems in plants.[1] Furthermore, the use of aluminum and sulfate as coagulants may lead to excessive residual aluminum in the microalgae culture, which leads to the potential contamination and limitation in the reuse of culture medium, and the potential to inhibit subsequent microalgae growth.[1] Ultimately, harvested algae biomass would be contaminated with heavy metals, which results in unusable for animal feed and human consumption. Natural polymers, on the other hand, such as chitosan and rice starch, are less harmful than aluminum and sulfate.[2]

1.1.4 Biological Methods within the Microalgae Biomass Industry:

Biological methods are both more financially viable, and less harmful to the environment, than mechanical, electrical, and chemical methods. However, current biological methods for the harvesting of algae are not impeccable, and more optimization methods need to be researched. Currently, the most economically efficient technology is flocculation of algal biomass.[3] Bio-flocculation is a cheap and eco-friendly flocculation method which uses microorganisms or polymer substances.[3] Plant-based bio flocculation methods yield microalgae biomass with high biodegradability and low toxicity levels.[3] However, the needed addition of cationic quaternary amine group compounds into some plant-based polymer flocculants may produce a cost issue.[3] "Microbial flocculants associated bio flocculation involves the cultivation of microbes and the purification of bio flocculants. A drawback of this kind of flocculants are very less productivity and high dosage of flocculants are required that further leads to the high production cost of flocculants that consequently increase the high operation cost of bio flocculation driven cell harvesting."[3] Furthermore, bio flocculants need to be species-specific to be efficient in algae recovery, limiting their potential application.[3] Another biological method used for microalgae flocculation is flocculation induced by fungus, which is highly efficient, and does not require the addition of any known toxic compounds, nor does it use significant energy on an industrial scale.[3] Additionally, unlike many current bacterial flocculation methods, the fungal medium can be reused without further treatment.[3] The algal-fungal interactions that lead to the flocculation process are largely unexplored; however, it has been hypothesized that fungal matter is shown to

be positively charged, while algal matter contains a negative surface charge, resulting in algal attachment to the fungal cell wall. Limitations that arise with this technique include the demand of organic substrates for the generation of fungus, the pH required for microalgae and its effect on the fungus, and fungal contamination in harvested biomass. Another biological method for microalgae flocculation is auto flocculation, which can occur naturally within certain microalgae species; auto flocculation results in cell aggregation and adhesion of microalgae cells to each other. Auto flocculation does not occur in all microalga species, however, and is slow and unreliable. The use of planktivorous fish such as tilapia is a final biological method for microalgae flocculation. This entails microalgae being batch-fed to caged fish; any remaining sedimentation of algae within fish droppings is brought to the surface on a conveyor belt and is fed to an anaerobic digester.[4]

1.2. Water Waste Treatment:

Wastewater treatment is one of the most important needs of any developing society. The increasing pressures of urbanization have placed a large impetus on wastewater plants around the world to develop more efficient ways of removing pollutants that become ever more present as cities grow, such as heavy metals. While mechanical methods comprise the bulk of the remediation performed at most wastewater treatment plants, biological methods, currently in burgeoning use in many plants, have much-unexplored potential in this area.

1.2.1 Preliminary Treatment of Water (Mechanical and Biological methods) within the Wastewater Treatment Industry:

Preliminary water treatment at water waste plants involves screening and grit removal. Wastewater simply enters the Headworks (using the example of the City of Toronto's water treatment plant) to remove large pieces of debris. Preliminary treatment also removes sand, gravel, and similar heavy inorganic material by gravity separation. The next process is the primary treatment, which occurs in the Primary Clarification Tanks. In these tanks, the flow velocity of the wastewater is reduced, to allow heavier solids settle at the bottom and primary sludge and scum to float to the top. Sludge collectors gather settled sludge in the tanks and push it into "sludge hoppers" at the bottom of the tanks. Primary sludges are also drained periodically from the top. The non-sludge wastewater, or "primary effluent," is then transported to secondary treatment. Part of the secondary treatment process involves mixing primary effluent and return activated sludge (RAS), which is composed of part of the already-filtered sludge mixed with compounds such as alcohols and ammonia. RAS is removed from the Final Clarification Tanks, from the end of the process (see below), and contains microorganisms that naturally occur in wastewater, which facilitate its degradation. The microorganisms break down organic material in the wastewater in the presence of oxygen, which is supplied to the Aeration Tanks. This process results in the production of phosphorus in the remaining wastewater. To help eliminate this production of phosphorus, ferrous chloride is added to the RAS, as well as other compounds,

such as ammonia and alcohols; these compounds also help to remove any nutrients present in the wastewater. The effluent is then moved to the Final Clarification Tanks as the Final Effluent, where the activated sludge is able to settle. A regulated amount of sludge is returned to the Aeration Tanks as RAS to maintain a sufficient biomass concentration, and any excess is removed as waste activated sludge (WAS), then put into the Primary Clarification Tank to settle with the raw sludge. Treatment of the Final Effluent involves chemical methods. The Final Effluent is treated with sodium hypochlorite, to disinfect and kill pathogens. Sodium bisulfite (SBS) is then added to remove excess chlorine from the wastewater. This prevents any toxic elements from entering major water sources, into which the treated water is deposited -- in Toronto's case, the Don River.

1.2.2 Chemical Methods within the Wastewater Treatment Industry:

During the treatment process at water waste treatment plants, numerous chemical treatments are employed. For example, ferrous chloride, sodium hypochlorite, and sodium bisulfite are used to treat wastewater. Ferrous chloride is used in the removal of phosphorus. In 2017, Toronto's water treatment plant used 8.98 tonnes of ferrous chloride, at a unit cost of $800/tonne.[5] Sodium hypochlorite (12%) is used as a disinfectant in the Final Effluent stage. Toronto's water treatment plant 's usage of sodium hypochlorite in 2017 was 21.72 m^3, at a cost of $132/$m^3$.[5] To remove sodium hypochlorite from, or dechlorinate, the water, 5.94 m^3 of sodium bisulfite (38%) was used in 2017, at a cost of $361/$m^3$.[5]

1.3. The Mining Industry:

Mining industries produce millions of tonnes of pollutants, often present in wastewater tailings, per year. As such, have an important duty to eliminate pollutants from mining tailings, and any other wastewater produced.Current methods are efficient but have high financial and energetic demands. Experts continue to develop methods that are more efficient, more cost-effective and have shorter execution times.

1.3.1. Chemical & Physical Methods for Filtration of Mining Tailings

One process that is sometimes used for the remediation of mining tailings is electrocoagulation. Electrocoagulation, a physical/chemical process, is also known as "plate technology". It removes "total suspended solids (TSS), heavy metals, emulsified oils, bacteria and other contaminants from water".[6] It requires the electrification of water and uses anodes and cathodes to provide metal hydroxides to the tailings, which causes the particles in wastewater to coagulate.[7] This method is commonly used in the laboratory-scale and rarely on real-world wastewater, which contains more complex components -- specifically, much higher chloride and sulfate salt contents.[8] Recent studies have aimed to use this technology on real-world wastewater, and studied parameters including power supply, current density, aeration intensity, flow rate, and

anions to determine optimal conditions.[82] Conclusively, high current density increased removal of toxic metals, but effectively increased energy consumption. The highest removal efficiency for each of Zn2+, Cd2+, and Mn2+ reached 99.93%, 97.15%, and 85.46% respectively.[8] It used an electrical energy consumption of 14.76 kWh/m3 (42 kWh/kg), electrode consumption of 2.09 kg/m3 (5.88 kg/kg) and an operational cost of 2.2 US$/m3 (6.21 US$/kg).[8] In summary, electrocoagulation is highly efficient and minimizes chemical use and toxicity in post-treatment products. However, potential downfalls include the fact that the required plates can be heavy and may occupy a large space, as well as the high energy levels that the process requires, which increases costs and health risks.[9]

1.3.2. Mechanical Methods for Filtration of Mining Tailings:

Soil washing is a physical separation process of heavy metals from soil, based on size separation, gravity concentration, froth flotation, attrition scrubbing, and magnetic separation.[10] This technology uses "a mixer for soil extraction, screen for soil/gravel separation, filter chamber presses for soil/liquid and recycled EDTA separation and soil rinsing, continuous centrifuge separator for removal of precipitated metals and electrolytic cells for process water cleansing".[11] It may also be combined with chemical leaching. "Physical separation [soil washing] is primarily applicable when metal contaminants are under particulate forms (ideally liberated particle), whereas chemical extraction is primarily suitable for ionic forms (free ions or adsorbed on soil, salts)."[10] Soil washing costs and efficiency are dependent on soil properties (such as particle size distribution, particulate shape, clay content, moisture content, humid content, heterogeneity of soil matrix, density between soil matrix and metal contaminants, magnetic properties, and hydrophobic properties of particle surface)[10], as well as concentrations and types of potentially toxic metal (PTM) contaminating the soil.[11] Soil washing removes up to 97% of Pb and 96% of Zn concentrations, and sample costs amounted to 50.5€ per ton [3].[11] The company ENVIT claims that their soil washing process removes up to 90% of toxic metals, generates no liquid waste and produces only 1.1% weight of solid wastes.[11] Advantages include soil remediation, after which soil can be returned to the site for reuse, for a short- to medium-treatment duration.[10] However, this process is limited when dealing with soils that contain "high clay content, high humic content, and organic contaminants with high viscosity".[10]

1.3.3. Biological Methods for Filtration of Mining Tailings:

Industries are increasingly exploring bioremediation and metal sequestration in hopes of increasing metal uptake, which conventional methods are not capable of processing. Some bacteria are resistant and capable of binding metals to enhance bioprecipitation/intracellular accumulation, which would enable a more efficient bioremediation process. Biomining is an increasingly common biological procedure used in the mining industry. Biomining is the use of microorganisms to extract metals from ores or mine waste. Bioleaching extracts these metals in a

solubilization process. Advantages of bioleaching include: its inexpensive cost, its relative environmentally friendliness (with no use of noxious gaseous emissions, no use of high energy and consumption, as in smelting), the enhancement of low grade gold ores (which are usually not economically feasible for extraction via conventional methods), and non-chemical and non-biologically active tailings.[12] However, it has long duration time of 6 to 24 months or longer, low and inconsistent yield of minerals, high risk of contamination, and requires a large, open area.[12]

One biological method is used for mining in areas such as Chile is acid mine drainage (AMD), causing large Arsenic (As), and other metallic, contamination. Sulfate Reducing Bacteria (SRB) reduces sulfate, the generation of alkalinity, and removes "dissolved heavy metals and metalloids by precipitation as insoluble metal sulfides."[13] SRB can tolerate high metal concentrations of As and Iron (Fe) and can "develop metal resistance mechanisms to protect their cellular components".[13] However, this process is dependent on pH levels and metalloid (As) concentrations, since these control the rate of SRB growth, and effectively the amount of sulfate reduced.[13] One study looked at the acclimatization of SRB by observing AMD samples in reactors every 3 days for 30 days.[13] The reactors produce N2 gas to reduce oxygen and were stored in a dark room at 24℃ .[13] Regarding efficiency, SRB removed up to 73% As and 78% Fe.[13] To optimize treatment, more information is needed on microbial speciation and sensitivity to different environments.

1.4. The Fermentation Industry:

The adhesion properties of yeast cells are of remarkable use in the industry, because of their ability to ease the process of cell separation from fermentation products. The following paragraphs will discuss the biological, chemical, and mechanical aspects of yeast cell separation techniques.

1.4.1. Biological Methods of Yeast Cell Separation:

One method to harvest cells biologically is dissolved air flotation techniques, which use buoyancy force over sedimentation to improve the rate of separation.[14] Although dissolved air flotation techniques are industry's most commonly used bubble based techniques, they are not yet used to separate yeast cells due to high energy requirements and their invasive nature. Furthermore, when saturated fluid is released from the dissolved air systems, the high energy causes the flocculated yeasts to break-up and disintegrate.[14] To compensate for this, a similar technique called microflotation was introduced. In short, this is a recycled version of dissolved air flotation techniques that use low power with desirable bubble size under laminar (constant streamlines, not turbulent[4]) flow conditions.[15] Microflotation produced microbubble clouds using fluidic oscillation which is also used in microalgae harvesting. This not only significantly

reduces the energy requirements to run the system, but does so without losing yeast biomass quality.[15]

1.4.2. Mechanical Methods of Yeast Cell Separation:

Saccharomyces cerevisiae is the most commonly used species of yeast in the production of bread, alcohol, and bio-ethanol.[16] The enzymes within these cells catalyze specific reactions that yield desirable products. After they have performed their function, they must be removed from the products via various cell separation techniques. However, this is not as easy as it may seem, due to the yeast cells' small size and density. Traditional separation techniques include sedimentation, filtration, and centrifugation, which are often time and energy consuming. For instance, sedimentation ("the action of the process of depositing") requires very long durations to allow for the cells to settle, whereas filtration requires expensive equipment.[17] Furthermore, centrifugation can negatively impact yeast cells by disturbing the flocculation process.[16]

1.4.3 Chemical Methods of Yeast Cell Separation:

Yeast cells can also be precipitated by adding natural or synthetic polymers such as chitosan, which is derived from chitin, an essential compound of the fungal cell wall. Added polymeric particles and polyelectrolytes disrupt cell-surface charge which promotes the cells to settle to the bottom. However, these substances cannot be used in the food industry, because they may cause toxicity.[13]

1.5. Conclusion:

Currently, water processing is done by 3 overarching methods, chemical, mechanical and biological. All with their own costs and benefits illustrated in the 4 industries above. The two main facets that challenge future water processing methods is cost and environmental impact. Mechanical processes require large industrial machines that have high energy costs, maintenance costs and purchasing costs. Chemical processes have negative environmental impacts through contamination and biological techniques have much-foreseen promise in challenging those two facets. However, biological techniques have yet to prove to be consistent and reliable in the industrial scale. The next two sections outline the processes in which iGEM Toronto hopes to solve those challenges for biological remediation platforms.

SECTION 2: iGEM TORONTO'S BIOREMEDIATION-COUPLED CELLULAR IMMOBILIZATION PLATFORM

The 2018 iGEM Toronto project revolves around optimizing the Arg1 operon in bacteria to induce consistent gas vesicle formation. Gas vesicle production is mainly employed for floatation in aqueous environments in organisms such as *Aphanizomenon flos-aquae* and Bacillus megaterium. These vesicles are a construct of multiple proteins that have been conserved across different species, a concept which will be discussed below. The ability of

bacteria to float in water using vesicle generation has a wide range of applications, the main one being in processing and removing target compounds from bodies of water. Current water processing techniques are not sustainable for a growing, urbanized world population, and current biological methods are relatively rudimentary. As illustrated above in the previous section, there are significant financial and environmental inefficiencies present in current water treatment methods. Our floatation platform has numerous pragmatic applications within these industries.

2.1. Genes/Proteins and Properties of Gas Vesicle Formation

Gas vesicle production in bacteria is largely performed by a few conserved genes. The parameters of these vesicles are modulated by the transcription levels of these genes and their association enhancers/inhibitors. The precise method of this modulation is of great importance to our platform and is a large reason behind the versatility and utility of our platform itself.

2.2.1. Structure and Function of GvpA and GvpC

Gas vesicle production in bacteria has been mainly attributed to two primary proteins: GvpA and GvpC. The genes encoding these two proteins are highly conserved across vesicle-producing bacteria; they also exist in bacteria that don't produce vesicles. GvpA and GvpC are the primary proteins that control gas vesicle width and length; in principle, a smaller gas vesicle is more robust, but can also hold fewer gas particles. Balancing the expression of these two proteins (as well as other secondary proteins, for which more information is provided below) is the primary modulator of the efficacy of the gas vesicles.[18]

Of the two primary proteins, GvpA, a hydrophobic protein, is the primary structural protein in gas vesicles. Gas vesicles can be modeled as a number of GvpA protein subunits. The assembly of these subunits depends on a number of forces, including Van Der Waals forces and forces exerted by salt bridges. The GvpA subunits form "ribs" that are positioned perpendicularly to the main anterior-posterior axis of the cylindrical vesicle.[19]

GvpA structure is the most highly conserved out of the proteins that form gas vesicles; it is formed of roughly equal parts alpha-helices and beta sheets (the latter of which forms the interior lining of the vesicle) and is highly aggregative. In the cyanobacterium *A. flos-aquae* and the archaeon *H. salinarum*, which are the two most well-known microbiological systems to use gas vesicles, there is no post-translational modification of the GvpA protein. It has been postulated that high beta-sheet activity is primarily responsible for the vesicle's structure, with the vesicles of *A. flos-aquae* having significant beta-sheet scaffolding present, spaced equally, roughly 1.15nm apart throughout the cylindrical portion of the vesicle. The GvpA protein has several other amino acid residues that may perform specific functions on their own; deletion of up to 7 amino acids (A70-A76) from the peptide does not alter the formation of the gas vesicles. However, a variant of GvpA with 11 amino acids deleted upstream of A70 proved unable to

form gas vesicles. In addition, deletion of I66-A76 destabilized helix-helix interactions in the vesicles that formed. This and several other experiments were able to localize a point of importance in the GvpA protein to R15; R15 mutants (with point mutations comprising either deletions or replacements of R15 with A15 or K15) could not form gas vesicles. Lastly, K60 and E56 have been identified as responsible for creating bridging forces between helices that allow the gas vesicle to hold itself together; elimination of K60 created increasingly small, infirm vesicles.[18]

In contrast to GvpA, the primary structural protein, GvpC is a hydrophilic external scaffolding protein which strengthens the vesicles and increases stability. While much less is known about the structure of GvpC, we do know that is aggregates on the outside of the gas vesicles, with the cytoplasm facing it on one side and the GvpA alpha-helices facing it on the other. Washing with urea to remove GvpC in *A. flos-aquae* drastically reduces the mean critical pressure for gas vesicle collapse, meaning GvpC maintains the integrity of the structure formed by the GvpA subunits. In addition, GvpC structure is far more varied than GvpA's, providing a source of variance for gas vesicle shape and structure.[20]

2.1.2. Structure and Function of Secondary Proteins:

There is immense variation in just the GvpA and GvpC expression patterns among the organisms that produce gas vesicles; across these known species, the mean critical pressure for vesicle collapse varies from 0.3MPa to 1MPa.[19] However, there are many other proteins that are involved in the formation of gas vesicles in different bacteria/archaea.

The first variety of these is unknown proteins, or proteins unrelated to the Gvp family, that perform a species-specific function. For example, in *H. salinarum*, other proteins must form the biconical structures on each end of the cylindrical vesicle.[19] In *A. flos-aquae*, gas vesicle formation is initiated in response to light; this light-sensitive protein must, presumably, be related to the function of the primary operational proteins for the formation of the vesicles. However, the more important class of secondary proteins for gas vesicle formation is comprised of other proteins in the Gvp family. The function of many of these are not yet known; however, our lab is optimizing these secondary proteins in the Arg1 operon, a synthetically produced operon which induces flotation in *Escherichia coli*, provided to us by the Shapiro Lab at Caltech. This operon contains multiple repeats of the GvpA and GvpC genes derived from *A. flos-aquae*, as well as secondary Gvp genes from *B. megaterium*.

Specifically, formation of a gas vesicles requires secondary Gvp genes in 8-14 gvp gene cluster, with their fundamental roles in production is not fully yet understood (although many of them serve different functions in different species of archaea and bacteria -- for example, in some archaea, elimination of up to 5 Gvp genes doesn't affect vesicle formation at all). Screens of the specific Gvp proteins in gene clusters yielded gvp genes A, O, F, G, J, L, and M as essential for

vesicle formation, and genes C, H, I, V, and N as non-essential.[17,18] We also know that more fundamental regulatory proteins are essential: for example, GvpE is partly responsible for the recruitment of RNA polymerase in *Hfx. mediterranei*, and GvpD is involved in general repression of expression.

The roles of these secondary Gvp family proteins have largely been defined through knockout studies and other similar experiments. For example, the absence of GvpN and GvpV in *H. salinarum* produces smaller gas vesicle that contain only the bicone-shaped end pieces, with smaller or absent trace of the mature cylindrical bodily structure, suggesting that GvpN and GvpV are responsible, in part, for the coordination of GvpA in the formation of the vesicle's body. GvpJ and GvpM appear to be similar in structure and function to GvpA.[17]

These secondary proteins, in addition to other non-Gvp proteins (such as the transcription factor, TbpD), can be used to regulate the multidimensionality and expression of the gas vesicles. In *H. salinarum*, selective expression of many of the secondary Gvp proteins, in addition to insertions of an enhancer cassette into the GvpC coding sequence, were able to maximize vesicle formation, as measured through light scattering techniques.[19] The secondary Gvp proteins have also been the target of manipulation for modulation of the formation and function of gas vesicles in the past, and this area of research is showing promise for the future; expression of the Gvp complex partnered with other proteins has successfully been used as both a reporter system[5] and a novel way to deliver both pathogens[6] and immunogenic particles,[17] even in deep mammalian tissue.

There is much that is not yet known about the structure and function of the secondary Gvp family proteins. However, their level of interaction with GvpA and GvpC, as well as other important transcription factors, is an important avenue for potential future research into optimizing gas vesicle formation.

2.2. iGEM Toronto's Experiments:

Our lab's aim is to optimize this gene cluster and reduce the size of the Arg 1 operon, greater than 7kb is size, and rely on the primary Gvp A and C proteins. This will be observed through gene knockouts of specific secondary proteins to test how they affect floatation of E. coli cells. Laboratory experimentation will test and characterize the Arg1 operon through buoyant forces imparted by gas vesicles and modeling growth dynamics of our cultures. All of this data is compiled and fed into mathematical models, coupled with metal surface binding protein dynamics, to compare the performance of our platform versus conventional industrial methods.

2.3. Theoretical Integration with Existing Cell Separation Technologies:

Our platform could yield a cellular bioremediation platform that is superior, in both monetary and energetic efficiencies, to current approaches. The overarching theoretical mechanism to achieve this will be the coupling of cell surface engineering with the expression of the Arg1 operon, as discussed above. Target compounds can be received via cell-surface receptors, which bind metals or organic molecules; they can also be uptaken by the bacteria through membrane channels. Both methods are meant to induce a signaling cascade within the bacteria, which will prompt the overexpression of the Arg1 operon. Experiments on gas vesicles in the past have evaluated that gas vesicle formation is known to be stimulated in conditions of reduced oxygen, and is strictly controlled by the quorum sensing molecule N-acyl homoserine lactone. Quorum sensing will lead to overexpression of floatation, and will significantly reduce the amount of our target molecule within the water, bioaccumulating it within our cells. Existing cellular separation techniques already in use in the fermentation industry discussed in section 1 can be used to separate the bacteria from the water once they have floated to the surface. Later on, the bacteria may be lysed or induced to excrete the target compound which was pulled from the water, and the target compound may be used or disposed of appropriately. Examples of the potential uses of this construct can be seen in the mining industry, where we may couple our platform with metal-binding peptides on the bacteria's surface, which can be theoretically used as a bioreactor, with effluent containing a particular metal of interest. Analogous mechanisms can be used for the extraction of organic molecules as well, with applications within the wastewater processing and biofuel industries. Further investigations on certain organic molecule binding receptors or antibodies or intake channels must be done for application into specific industrial interests. In retrospect, this platform can be shaped to eliminate multiple target compounds and or one target compound with an overarching coupling of our experimentally designed Arg1 construct and the accumulation mechanisms discussed above.

SECTION 3: OUR PLATFORM VERSUS PLATFORMS CURRENTLY IN USE

The 2018 iGEM Toronto project has multiple advantages over current methods of cell separation. As detailed above, there are numerous inefficiencies in mechanical and chemical methods used in the biofuel, wastewater, mining and fermentation industries. Our platform is not just a unique solution to many of these specific issues, but also an innovative approach to solving the broader, urgent problems that face developing society as a whole, such as the need for more efficient heavy metal extraction and antibody extraction from our drinking water.

3.1. Inefficiencies in current industries:

There are a number of disadvantages to current microalgal separation techniques. As mentioned in section one of this paper, Algae to Energy and Algaeventure Systems Inc. use various mechanical methods such as centrifugation, sedimentation, and dissolved air flotation. The advantages of centrifugation include its rapid processing time, as well as a reliability level which yields 90% recovery.[24] However, it also has its drawbacks. It has high energy requirements, and

requires high investment. Tangential filtration, on the other hand, can result in “membrane fouling” which is when a solution or particle is deposited on a membrane surface or in membrane pores.[7] This means that needs high investment and power use. Sedimentation, another approach to microalgal separation, is a process whereby the particles are left to settle to the bottom in accordance with their densities. Although this comes with a low cost, this process is impractical due to very long waiting times. Dissolved air flotation uses “flocculants that may cause issues in the downstream processing of algae”.[24]

In addition to mechanical methods, chemical methods are also used for algae separation. Synthetic polymers and electrolytes that aid in the flocculation of these cells, which eases the separation process.[12] However, when using electrolytes, certain metals can interfere with cellular processes and results in contamination. For instance, aluminum and sulfate hinder methanogenic activity in bacteria-fed wastewater sludge. This would make it unusable for animal feed and human consumption.[12] In terms of biological processes, the most common method is flocculation, which is simply the clumping of algae cells to ease cell separation. This is cheap, as well as eco-friendly.[8] Disadvantages to this cell separation method include less productivity because these cells need to be species-specific in order for the process to work. Another method is flocculation induced by fungus.[15] This is effective because it is not known to be toxic thus far, and the fungus can be recycled.[15]

The process of wastewater treatment by which standard municipalities around the world function is largely mechanical; more details can be read in section 1. This intricate and in-depth process, that slightly integrates chemical methods as well, ensures that as many toxicities are removed from the sludge.[13]It succeeds in removing a high amount of contaminants from the water, but it also has noticeable gaps, and requires a large amount of energetic, financial, and spatial investment, due to the mechanical processes involved.

In the mining industry, there are mechanical, chemical and biological mining techniques that may also be used. Chemical processes include electrocoagulation, which removes suspended solids and heavy contaminants. However, the plates used in electrocoagulation are heavy, occupy large spaces and require high voltages.[11] Mechanical processes include soil washing, where costs and energy requirements depend on soil properties.[12] Lastly, under biological methods, bioremediation is a technique that is commonly used to break down pollutants. In contrast to most other processes, bioremediation is natural, which means that bacteria can have mutations in their genomes that may allow them to enhance intracellular accumulation of metals.[14] Biomining and bioleaching are both inexpensive, and environmentally friendly; they do not emit any greenhouse gasses on their own. Disadvantages long waiting times, because these processes can take from up to 6 months to one year. Furthermore, they require a large open area; this spatial

investment often presents a critical problem

Lastly, yeast cells may also be used (in addition to algae and mining techniques) for cell separation. Since both algae and yeast are living cells, the methods used to separate these cells are also very similar to each other. The nature of dissolved air flotation techniques are invasive and energy consuming.[15] Microflotation, on the other hand, is not as invasive (due to its use of laminar flow), has low energy requirements and prevent the loss of biomass quality. As mentioned before, centrifugation, sedimentation, and filtration are some mechanical methods that may be used in algae cell separation. However, each method comes with its drawbacks. Filtration equipment is expensive, sedimentation is time-consuming and centrifugation is energy-consuming[12]. There are a number of chemical methods, that used synthetic polymers that aid in the coagulation of yeast cells. Although they ease the process of yeast separation, they cannot be used in the food industry due to their toxic nature.[12]

3.2. The Issue We Hope to Challenge:

The relevance of our gas-vesicle coupled bioremediation platform is to provide a framework to build a much more reliable and cost-effective methods of cleaning wastewater effluent. Global water security dialogue has been at the center of the United Nations sustainable development goals. 70% of industrial waste in developing countries is dumped untreated into usable water supplies. Only 2.5% of the total volume of water on earth is comprised of freshwater resources, and of that 2.5%, less than 1% is usable freshwater for humans. Global water security is in the underpinning of global food security. 70% of our useable freshwater resources are allocated to irrigation, and rainfall-dependent agriculture practices are predicted to fall up to 50% by 2020, based on the findings of the Intergovernmental Panel on Climate Change.[28]

One of the hurdles to combatting this challenge is to improve our current water and wastewater management.[28] As previously illustrated, the removal of certain heavy metals and organic compounds have many problems with existing conventional methods. Bioremediation is a much more cost-friendly and efficient platform.[26] The liability of our gas vesicle-mediated platform means an immense amount of compounds may be extracted from wastewater effluent through the coupling of binding peptides and gas vesicle expression. We will explore the theoretical applications of our platform in two potential future settings: heavy metal extraction in the mining industry and antibiotic extraction from municipal wastewater.

3.2.1. Heavy Metal Extraction:

Mining industry wastewater effluent is commonly plagued by heavy metals. Conventional methods of water treatment, such as chemical processes, are impractical in this situation, because of the intrinsic environmental impacts; mechanical processes are not feasible because the cost of building and operating treatment motifs on-site are too large. Transporting wastewater to appropriate treatment facilities also poses another cost barrier, and is environmentally unfriendly,

due to more greenhouse gas emissions by large transportation vehicles. Bioremediation is much more efficient and cost-effective is this respect, because it empowers the mining industry to treat their water on site.[31] Heavy metals are toxic to most organisms and are biologically managed in multiple ways. One of these approaches is to bioaccumulate or sequester a metal in physiologically inaccessible form.[30] Metal-binding proteins (rich in histidine and cysteine residues) are commonly used by bacteria, in the form of metallothioneins (MTs).[30] The synthesis of MTs may be induced by a host of cytotoxic metals, including cadmium (Cd), zinc (Zn), mercury (Hg), copper (Cu), gold (Au), silver (Ag), cobalt (Co), nickel (Ni) and bismuth (Bi).[30] Intracellular expression of MTs, however, runs into various complications within the cell. A more viable option to couple with our platform would be to introduce an MT that would be expressed on the cell surface. The sequence of this MT would be placed directly upstream of our gas vesicle gene cluster to ensure that flotation is induced by the induction of MT, due to the presence of a heavy metal of interest in our effluent. MT expression has shown great promise with Cd and Hg accumulation in the previous experiments.[31] The expression of a certain cysteine-rich metal-binding peptide motif fused with a maltose-binding protein in *E.coli* enhanced cadmium and mercury binding tenfold.[31] Further expressing these cell surface MTs as fusions to LamB, a protein which spans the outer membrane which is anchored to the bacterial peptidoglycan layer, increased metal-binding capacity between 15-20 times over.[32] Thus, cell-surface MTs show great promise in applying our bioremediation platform in the mining industry where the ability to extract valuable and toxic metals from wastewater is vital.

3.2.2. Antibiotic Extraction:

A recent UN report concluded that 70% of industrial waste in developing countries is dumped untreated into useable water supplies.[28] Included in these pollutants that make it into wastewater are pharmaceuticals, pesticides, and petrochemicals, which later bioaccumulate in the environment or persist as other toxic metabolites. Remediation of these compounds either too costly, or inefficient overall; many current chemical methods simply convert one toxic pollutant to another one.[27] An estimated 26 metric tonnes of pharmaceutical waste, in particular, is disposed of down the drain in municipal waste in North America[9]. These antibiotics, painkillers, hormones, tranquilizers, and other drug classes are of significant regulatory concern to municipal water officials. Pharmaceutical companies do not manufacture most drugs with biodegradation in mind and are more concerned with producing products with longer persistence within the human body.[27] These drugs tend to persist the most in the environment because of these particular pharmacokinetic and chemical modifications made to them.

Bioremediation is a very promising tool to combat this problem. The gas-vesicle mediated platform constructed by our team investigates a potential application into relieving penicillin, a common antibiotic, from municipal water systems. One of the possible theoretical applications of our platform in this realm consists of using the expression of highly variable penicillin-binding

proteins (PBPs). The expression PBPs, are a class of proteins with very high binding-affinity to penicillin. These binding proteins are known to have similar binding sites to that of beta-lactamase.[26] Beta-lactamase is a widely known enzyme that hydrolyzes a common beta-lactam ring found on penicillin and its derivative which renders the drug inactive and harmless to bacteria.[29] Expression of PBPs are already found in clinical strains of bacteria expressing beta-lactamase.

The coupling of this very well studied process with the expression of our Arg1 gas-vesicle gene construct may lead to a simple and effective route of sequestering penicillin within our bacteria. The bacteria can then float to the surface of the water, our target antibiotic compound, and removing it from wastewater.

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