Design Brief

Road salt alternative using antifreeze proteins*

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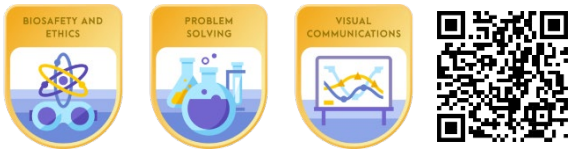
Every winter, drivers are threatened by hazardous icy roads. Among the methods used to prevent snow-related traffic accidents, the most common measure is road salt. However, applying road salt causes many problems, such as corrosion of pavement and cars and harmful environmental impacts. The adverse effects of road salt necessitate an alternative solution to treating winter roads. Our plan involves inserting three antifreeze protein (AFP) gene sequences from mealworms, perennial ryegrass, and arctic yeast into plasmids. Then we will transform the plasmids into the expression system in the yeast Pichia pastoris before purifying three AFPs for subsequent experiments. These AFPs adhere to the surface of newly formed ice crystals, inhibiting ice crystal formation in sub-zero temperatures. We can apply the product as a cost-effective road salt alternative by expressing and purifying the AFPs from our three organisms. This innovative solution addresses environmental concerns and uses a more eco-friendly approach. Testing this design's efficacy and safety will prove its feasibility as a replacement for road salt.

Keywords: Road salt, antifreeze proteins, sustainability, genetic engineering of *Pichia pastoris*



Winter roads pose a threat to drivers worldwide. Every year, more than 1,300 people are killed, and over 116,800 are injured in vehicle crashes in the U.S. due to snowy and icy roads (American Highway Users Alliance, n.d.). To combat these high casualties, the U.S. applies 15 to 32 million tons of road salt on icy pavement yearly (Cary Institute of Ecosystem Studies, n.d.). Road salt, or sodium chloride, works to clear icy surfaces by lowering the freezing point of water, causing ice to melt when temperatures are below the freezing point. However, there have been numerous efforts to find alternative solutions due to the severe issues that road salt causes, including contamination of water, infiltration into ecosystems, and corrosion of vehicles (Pieper et al., 2018).

Management of icy roads

Managing icy roads occurs in two defined steps: anti-icing and de-icing (Massachusetts Department of Transportation, n.d.). Before the onset of snowfall, a liquid solution is applied to the pavement to prevent snow and ice from binding to roads; this step is referred to as anti-icing. Although anti-icing helps keep roads clear, heavy snowfall can still accumulate ice and snow on roads, reducing its effectiveness against persistent precipitation as the treatment may be washed or diluted (Mainroad, n.d.). To combat these dangerous driving conditions, de-icing is put into effect.

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The products used in anti-icing and de-icing may reduce dangers on winter roads. However, many negative consequences occur with their application. Anti-icing products include liquid magnesium chloride or liquid brine to prevent water molecules from rebonding, delaying the freezing process, and generating heat to melt ice (Loeffler, 2022; Massachusetts Department of Transportation, n.d.). However, liquid magnesium chloride is expensive and only works when temperatures exceed -15 degrees Celsius. Its counterpart, liquid brine, is a 23% crystal salt and 77% water mixture that is easy to apply, less costly, and it functions in temperatures as low as -29 degrees Celsius (Lilek, 2017; Mainroad, n.d.). Unfortunately, liquid brine is more corrosive to materials and the surrounding environment, leading to significant infrastructure damage. The anti-icing step is a preventative measure that yields results. However, if products such as liquid magnesium chloride or liquid brine encounter excessive snowfall, a reactionary measure – the de-icing step – is needed.

Road salt

The most common products in the de-icing step include road salt and sand. In 2013, a study shows that a 10% improvement in surface friction could lead to a 20% reduction in crashes (American Highway Users Alliance, 2018). Road salt is the most affordable and efficient deicer to ensure vehicle safety during winter. American roads and highways use road salt, which has been proven to reduce collisions by up to 88% and injuries by 85% (American Highway Users Alliance, 2018). On four-lane roads, this estimate is raised to 93%. The economic impact of road closures due to inclement weather, including costs associated with snow plowing and removal, can escalate to 300 to 700 million dollars in cost. In this context, the application of salt represents a relatively modest investment. As of today, road salt is the most cost-effective option for ensuring road safety, but improvements still need to be made.

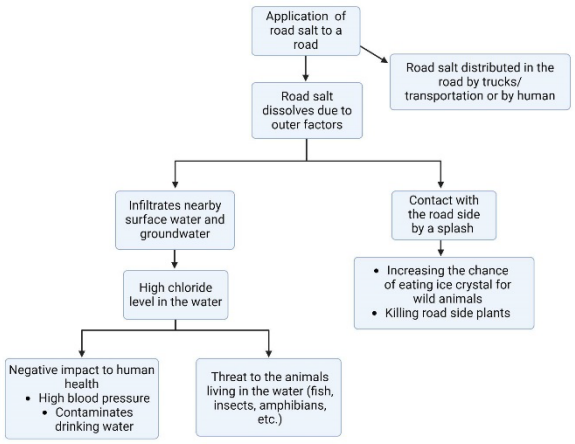

Figure 1: Flowcharts of the environmental and human impacts of road salt after it dissolves into melted snow (Sentryair, 2023). Created with BioRender.com.

Despite being the most commonly used method for keeping roads safe during winter, road salt has many flaws. After trucks disperse road salt in initial discharge, it becomes environmental runoff through winds and roadway drainage, resulting in many side effects (Figure 1). Road salt significantly impacts vehicles, vegetation, and the ecosystem. Due to corrosion from road salt, approximately 3.5 to 7 billion dollars are spent annually on repairs of cars, trucks, and infrastructures in the U.S. (Bridgestone, 2021). When road salt infiltrates nearby water sources and contaminates water reservoirs, high salt pollution harms people with high blood pressure, fish, insects, and amphibians (United States Environmental Protection Agency, 2020). In response to this issue, roadside areas accumulate excess road salt, which can kill vegetation and harm wildlife that ingest the salt crystals. Although road salt has been proven to be the most effective deicer, sand is also commonly used in the de-icing step.

Sand

Sand is regarded as an acceptable alternative for road salt to improve road safety (Forestell, 2020). According to the American Geosciences Institute, about nine million tons of sand were used on U.S. roadways during the winter months of 2015 (Lilek, 2017). Sand is usually used as an alternative because it carries less negative environmental impacts on water and infrastructure than road salts do. It is non-corrosive, meaning that metals, underbellies of vehicles, and infrastructure

are not eroded (Forestell, 2020; Salt Vs. Sand, n.d.). However, there are many drawbacks to using sand in the de-icing step. Specifically, sand's sole purpose is to increase traction, resulting in the potential for ice to form over sand without melting ice or snow (Salt Vs. Sand, n.d.). Also, using sand as a road salt alternative results in a significant expense in cleaning the sand-clogged drains and pipelines after winter in the northern hemisphere. Sand cannot be reused for the next year, because when it is first used, the vehicles crush the particles. The sand particles get finer and finer, making it ineffective after a season (*Good Question*, 2012). Although sand does not affect water quality and infrastructure to the same extent as road salt, it lowers air quality by producing Particulate Matter (PM; California Air Resources Board, n.d.; Salt Vs. Sand, n.d.).

The solution

Tenebrio molitor (*T. molitor*), a strain of yellow mealworm beetle, generates thermal hysteresis protein (THPs), including Cys-rich and Thr-rich (Graham et al., 2000). Thermal hysteresis refers to the phenomenon where the freezing point of a substance is lowered without interfering with its melting temperature. Thermal hysteresis proteins are types of antifreeze proteins (AFPs) that lower *T. molitor*'s hemolymph freezing point, enabling their survival in subzero temperatures. Organisms, including insects, fish, and plants, can synthesize THPs when they live in environments where the temperature is consistently below 0°C (32°F). Previous research on THPs involved the isolation of one functional THP, xylomannan, from the freeze-tolerant beetle *Upis ceramides* through an ice affinity protocol (Walters et al., 2009), which marks the first instance of an antifreeze substance isolated from a freeze-tolerant animal. This groundbreaking finding paves the way for our design as we explore the real-world application of freeze tolerance – the ability of organisms to survive in conditions where the body fluids freeze – in the context of mass protein production for environmental use.

AFPs function by adhering to the surface of newly formed ice crystals. As shown in Figure 2, when AFPs attach to the ice, they hinder water molecules from reaching the ice nucleus at the specific attachment site. AFPs modify how these ice crystals grow. Typically, ice crystals expand symmetrically and uniformly as they freeze more water molecules. AFPs disrupt this process, altering the shape and growth pattern of the ice crystals by creating a convex ice surface on the side facing the liquid in the areas where AFPs are attached (Hakim et al., 2013). This convex created by the AFPs leads to an alteration in the ice crystal's growth. According to the Gibbs-Thompson-Herring effect, as depicted in Figure 3, these convex surfaces are less stable and energetically less favorable for ice growth. Consequently, AFPs inhibit the additional attachment of more water molecules (Gharib et al., 2022).

Based on AFPs' types determined by their structure, they attach to specific ice crystal planes, thereby preventing further ice formation. AFPs have a specialized area on their surface called the ice-binding site. Researchers believe an ice-binding site arranges water molecules into a formation similar to how they would be arranged in an ice crystal, nonetheless, not turning water into ice (Kondo, 2012). This arrangement

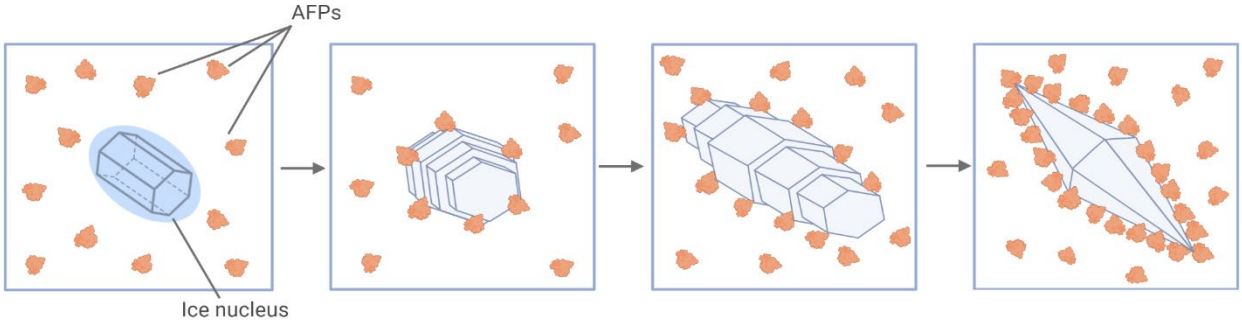

Figure 2: Schematic representation of the effects of Antifreeze Proteins (AFPs) on the icing process. Created with BioRender.com.

resembles ice planes, enabling AFPs to prepare their binding target, as they create a pattern like ice on its surface before attaching to the ice (Eskandari et al., 2020). When the AFP comes into contact with ice, the ice-binding site (IBS) "matches" with a particular plane of the ice crystal, because the pattern of water molecules at the IBS is complementary to the pattern on that specific ice plane. This precise matching enables the AFP to bind effectively to the ice, preventing further ice formation by attaching to specific ice planes in a targeted manner (Eskandari et al., 2020). For example, Type 1 AFPs from the winter flounder are known to adhere to the pyramidal and secondary prism planes of ice, and hyperactive AFPs from the *Flavobacterium frigoris PS1*, a bacterium found in Antarctica known as FfIBP binds specifically to the basal plane of ice crystals (Kim et al., 2017).

Considering the environmental concerns, health risks, and high costs associated with these deicing products, an environmentally friendly and cost-effective alternative is needed in the long run. This project aims to prevent corrosion caused by road salt and improve road longevity with a bioengineered yeast strain that combines antifreeze proteins from multiple species. Here, we propose bioengineering the yeast *Pichia pastoris* with the genes encoding AFPs from *T. molitor, Leucosporidium sp*. strain 30, and *Lolium perenne* to enable the sustainable production of biodegradable AFPs as a road salt alternative. Compared to traditional chemical road salt, which is attributed to infrastructure corrosion, high cost, and water infiltration, the innovative and comprehensive approach of biosynthesizing three AFPs from three sources holds promise for addressing environmental concerns and economic burdens while meeting the demand for effective road salt alternatives.

Systems level

The yeast species *P. pastoris* has been selected as the chassis for bioengineering for its well-documented efficiency in large-scale protein production. Specifically, X-33 is a *P. pastoris* strain often utilized in synthetic biology due to its robust growth and high levels of recombinant protein expression, and it will be utilized as the host for plasmid transformations. To successfully integrate AFP genes into X-33, we employed a selection method based on three antibiotic-resistance genes inserted in the three constructed plasmids. After transforming the three plasmids carrying genes from *Tenebrio molitor, Leucosporidium sp. strain 30*, and *L. perenne*, X-33 will be cultured in a buffered complex glycerol medium containing the antibiotics hygromycin B, bleomycin, and kanamycin. As Figure 4 illustrates, only yeast cells that have successfully integrated all three plasmids confer resistance to these antibiotics, thus providing a measure of yeast

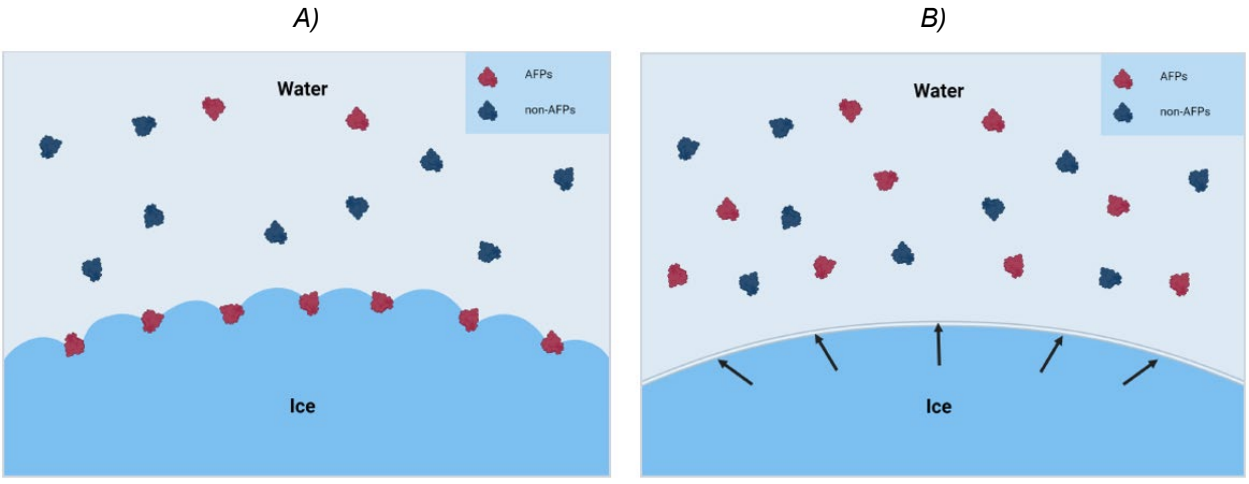

Figure 3: (A) The image illustrates the mix of non-AFP and AFP in front of the ice front. (B) AFPs bind to the ice while non-AFPs do not. AFP binding into the ice front will increase the curvature of the ice (Kuiper et al., 2015). Created with BioRender.com.

selection.

A successfully transformed colony on the antibiotics-supplemented medium will then be expanded in a chemostat to maintain steady-state conditions, allowing for consistent growth and AFP expression. This stable and controlled culturing environment enables X-33 to reproduce and produce AFPs sustainably. Purifying the expressed AFPs starts with filtering, concentrating the proteins, and removing cellular debris. After purification, AFPs will be stored in a buffer solution at low temperatures to maintain their structural integrity and biological activity, which ensures their effectivity until their future experimentations or applications.

Device level

A plasmid vector is a circular DNA molecule separate from chromosomal DNA that can replicate independently and is often used to introduce foreign DNA into a host cell for cloning or genetic modification purposes. The pGHYB vector serves as the cloning vehicle for the antifreeze gene from *T. molitor* (Yang et al., 2014). The *T. molitor* gene will be synthesized and inserted into the vector via a restriction enzyme located downstream of the GAP promoter and upstream of the AOX1 terminator and hygromycin-resistant gene, ensuring the proper expression of the AFP as shown in Figure 5A. This configuration verifies that the introduced gene is expressed constitutively while the hygromycin-resistant gene provides resistance to the antibiotic hygromycin, facilitating the selection of successfully transformed yeast cells. By leveraging the constitutive expression capabilities of the GAP promoter, we ensure that the *T. molitor* gene is actively transcribed within the yeast host, enabling the expression of the product AFP.

In a similar fashion to the *T.molitor* gene in the pGHYB plasmid, gene AY30 from *Leucosporidium sp*. strain 30 will be integrated into a yeast expression system. The BB3aZ_14* plasmid's robust TEF promoter can facilitate strong constitutive protein expression (Prielhofer et al., 2017). Thus, gene AY30 will be synthesized and positioned downstream of this promoter and upstream from the bleomycin-resistant marker (Figure 5B). This precise placement at the initiation site of the multiple cloning area right after the TEF promoter preceding the terminator sequence ensures the accurate transcription of the AY30 AFP (Prielhofer et

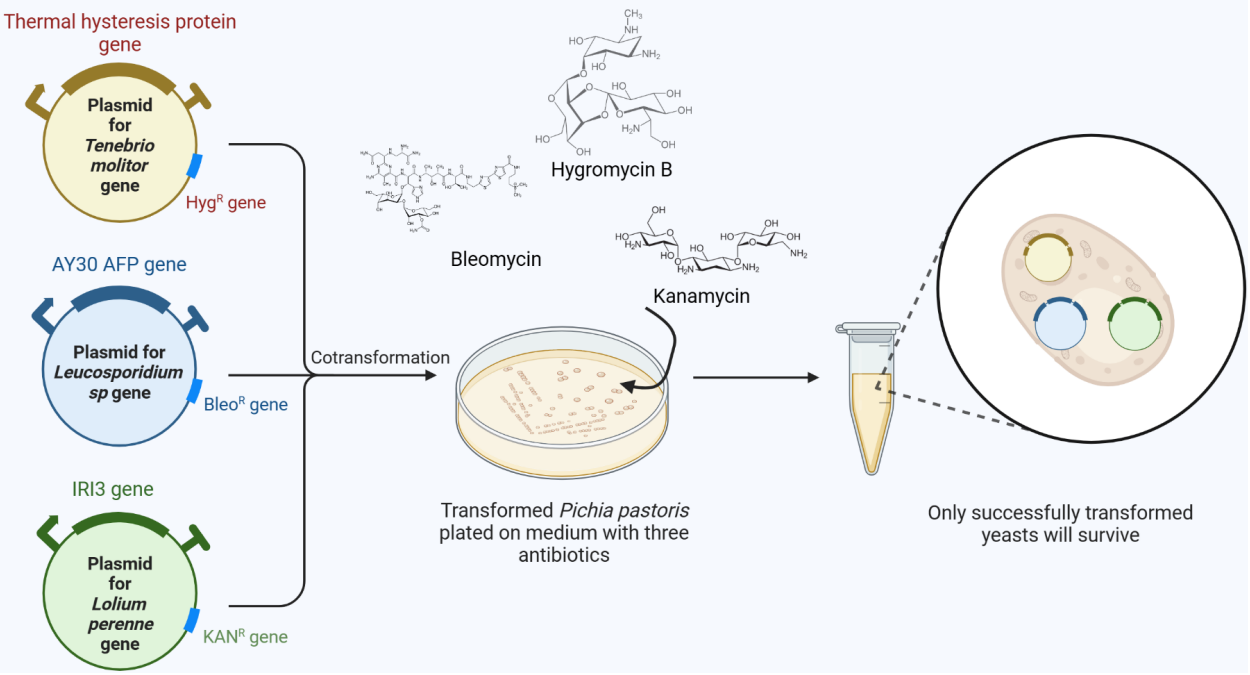

Figure 4. Co-transformation of Three Plasmids for AFP Production in P. pastoris, followed by selection using three antibiotics. Adapted from "Plasmid Transfection Workflow (Layout)", by BioRender.com (2024).

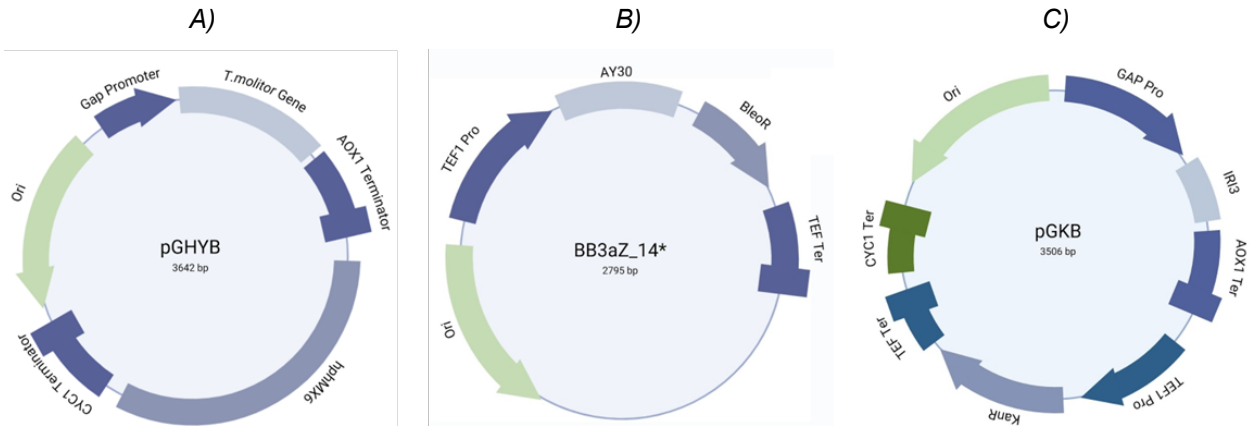

Figure 5. Plasmid maps of the three plasmid designs incorporated with AFP genes. (A) The thermal hysteresis protein gene from T. Molitor is inserted in the pGHYB plasmid backbone with a hygromycin-resistant selectable marker. (B) Gene AY30 from Leucosporidium sp. strain 30 in the BB3aZ_14 plasmid backbone with a bleomycin-resistant selectable marker. (C) Gene IRI3 from L. perenne in the pGKB plasmid backbone with a kanamycin-resistant selectable marker. Created with BioRender.com.*

al., 2017). The bleomycin-resistant gene downstream grants a selectable trait, simplifying the selection for successfully transformed X-33.

To express the IRI3 gene that encodes an AFP protein inhibiting ice recrystallization in *L. perenne*, we have chosen the pGKB vector as our plasmid framework (Yang et al., 2014). pGKB's plasmid's constitutive GAP promoter can offer a potent and consistent expression of target genes, making it a desirable candidate for harvesting AFP. The IRI3 gene will be synthesized and inserted downstream of the GAP promoter and upstream of the AOX1 terminator, as illustrated in Figure 5C. The kanamycin resistance marker in the plasmid will be used as the selection marker to identify successfully transformed X-33.

Parts level

In this antifreeze protein production system's design, we aimed to offer an eco-friendly alternative to road salt. Three genes encoding AFPs from various organisms will be inserted into three separate plasmids. These genes first include the thermal hysteresis protein gene from the mealworm *T. molitor*, which produces AFPs to help it survive in cold temperatures. The AY30 AFP gene from *Leucosporidium sp.* strain 30, a yeast known for its glycosylated ice-binding protein (LeIBP), will also be synthesized in a compatible plasmid vector. Lastly, we aim to introduce Gene IRI3, which encodes an ice recrystallization inhibition protein-like protein from *L. perenne* utilizing AFPs for cold protection. These proteins work by selectively binding to the surface of microscopic ice crystals to prevent them from growing larger. AFPs essentially 'coat' the small ice crystals, creating a barrier that hinders further ice crystal fusion and growth. This protects the organism's cells from the potentially lethal expansion that occurs when water freezes and expands. This design aims to utilize the ice-inhibiting mechanism of AFP to prevent road ice formation.

Safety

P. pastoris, the chassis, is categorized as a biosafety level 1 (BSL-1) organism, which is known to have little to no threat of infection in healthy adults. Furthermore, there are minimal hazards for those working directly with it in the laboratory and those interacting with it in the environment (ATCC, n.d.). Our system, which consists of three AFP genes from *T. molitor, L. perenne, and Leucosporidium sp.*, is also safe to handle. Approved for consumption, *T. molitor* has no known safety risks (*EFSA* Panel on Nutrition, Novel Foods and Food Allergens et al., 2021). The only potential risk of using *L.*

perenne is its pollen-related allergies. Wearing medical masks can protect against pollen during the experiment process and reduce allergy symptoms (Hycor News Archive, 2022). Using appropriate personal protective equipment (PPE), such as lab coats, gloves, and eye protection, is essential when handling microbes and chemicals to minimize risk. Good laboratory ventilation, including fume hoods and biosafety cabinets, should be implemented to prevent the accumulation of potentially harmful aerosols. *Leucosporidium sp.* is also categorized as BSL-1 (ATCC, n.d.). The organisms used in this study are generally safe to work with, and the AFPs we are extracting provide no existing evidence of hazardous risks. AFPs have many applications within the food industry, such as food preservation (Crevel et al., 2002). According to current knowledge, their characteristics do not exhibit any toxic effects.

During gene extraction and transformation, preventing contamination is crucial to ensure the genetic material's integrity and the modified organisms' safety. The gene extraction and transformation process will all be carried out in laminar flow cabinets and clean rooms to minimize the risk of contamination (Sentryair, 2023). All tools and equipment, from pipettes to glassware, will be sterilized through autoclaving to eliminate biological contaminants. Gel electrophoresis will be conducted on each constructed plasmid to verify that only the genes of interest have been successfully inserted (Swanson, 2014). Transformed *P. pastoris* will only be worked with in a biosafety cabinet, and waste will be disposed of according to regulatory guidelines to prevent accidental release of genetically modified organisms.

Storing AFPs at four degrees Celsius in autoclaved glassware is standard practice to inhibit enzymatic activity and prevent protein degradation, ensuring protein stability for research purposes (Thermo Scientific, 2009). As for chemical hazards, there are no hazardous components in the buffer solutions. As for biological risks, the AFPs are all derived from non-pathogenic sources. While the direct bioactivity of these proteins on human health has yet to be extensively researched, our research team will conduct a thorough safety assessment. We will do this by evaluating potential allergenicity and any other bioactive effects of the AFPs produced by the bioengineered *P. pastoris* to ensure the products are safe for applications and lab research (Fernandez et al., 2021).

The stability and longevity of AFPs in the environment, particularly regarding their functional structure, have not been extensively detailed in the available literature for the specific sources–*T. molitor, L. perenne, and Leucosporidium sp* – in our research. However, AFP's application in cryopreservation, which protects cells and tissues at low temperatures, underscores their stability and functional retention under cold conditions (Kim et al., 2017). This stability suggests a baseline resilience that could extend to environmental exposures. Our research group will conduct specific longevity and stability studies to test the AFPs product's functionality when exposed to environmental conditions outside the laboratory in real-world icy road testing sites (The Protein Man, 2018). Specific experimental approaches include field testing, temperature fluctuation studies, and structural analysis using X-ray crystallography to analyze the structural integrity of the AFPs after exposure to environmental conditions (Smyth & Martin, 2000).

Concerns about the potential impact of AFPs on human health also center on their persistence in the environment, as long-lived proteins that maintain their structural integrity could interact with local ecosystems and thus indirectly affect human health through environmental pathways. Our safety assessment will evaluate the potential impact of AFPs on local water and soil sources, aquatic life, the soil microbial community, and overall environmental quality. Ecotoxicological assessments will also be employed to determine if the AFPs or their degradation products have unknown toxic effects on local flora, which includes aquatic toxicity testing exposing *Daphnia magna* to different concentrations of AFPs to observe any acute and chronic toxic effects (United States Environmental Protection Agency, 2023).

Furthermore, our safety assessment will consider the potential direct human exposure

to AFPs, evaluating scenarios such as direct skin contact or accidental ingestion. While AFPs are not inherently toxic, their novel use in environmental applications requires thorough *in vitro* and *in vivo* investigations to prevent unforeseen health risks caused by the potential allergenicity of these proteins: *in vitro* screening, such as protein sequence analysis that compares AFPs' sequences with known allergens; immune reactivity assays which detect antibodies against the AFPs in serum samples from individuals with known allergies; and cell-based assays that assess the triggering of immune responses by AFP. *In vivo* testing subject to ethical approval, including skin prick and oral tests, will also assess allergic reactions (Albert-Vega et al., 2018; Gromiha, 2010; Mayo Clinic Staff, n.d.).

Discussions

As the design for the alternative road salt progresses into a usable product, it will be necessary to seek government approval to use our product on public roads. To achieve this approval, environmental testing will take place. We will test the longevity of AFPs to assess their impact on the surrounding ecosystems and their effect on human health, since AFPs may infiltrate water systems. Through ecotoxicological assessments, like *Daphnia magna* aquatic toxicity tests mentioned in the safety section, we can see if AFPs or their degradation products have any unknown toxic effects on the environment. However, since our chassis is categorized as a Biosafety Level 1 organism alongsidethe source organisms of our inserted genes, we will most likely have success with environmental testing. Since AFPs are frequently used within the food industry, it is unlikely that they would threaten human health (Crevel et al., 2002). However, in case AFPs infiltrate water systems, monitoring water protein levels and effective filtration methods for macromolecule removal will be needed to ensure no contamination has occurred (Ezugbe & Rathilal, 2020). If we receive undesirable results in our environmental testing, we will revise our design to make our product road-ready.

To further improve our design, we will lower production costs by enhancing the batch culturing protocol of our bioengineered yeast product and optimizing the protein extraction procedures. With careful budgeting in production and storage, our product will offer a cost-effective road salt alternative to icy roads worldwide. Another improvement that will strengthen the quality of our design will be finding a filtration method, like ultrafiltration, to keep AFPs out of water systems. Although digestible, monitoring protein levels in the water will be vital to ensure no water contamination occurs (Ezugbe & Rathilal, 2020).

A successful design will protect the environment from corrosion and salt-infiltrated water systems. Our completed design will allow for an easy application and a cost-effective solution to combat icy roads with advantages over current road salt alternatives. This AFP road salt alternative will be safe for drivers in winter conditions and will reduce collisions, casualties, and other safety concerns.

Next steps

In our forthcoming research, we will delve into codon optimization strategies to align the codon preferences of plant and mealworm genes with those of the *P. pastoris* chassis. This optimization can modulate translation elongation rates and enhance heterologous protein expression, thus improving the synthesis of our antifreeze proteins. We also plan to research the transformation processes available for the three plasmids to improve efficiency. Electroporation will be the most feasible method. However, there could be difficulty in transforming three plasmids into one organism. We will consider transforming each plasmid into separate batches of chassis so that we can express different AFPs separately and combine them in the application process. We will focus on implementing and developing a transformation protocol to effectively introduce the three plasmids into our *P. pastoris* chassis. This method involves preparing a competent chassis exposed to an electric field to uptake plasmid DNA, optimizing electroporation parameters and selecting successfully transformed yeast

using antibiotics. This step is pivotal in establishing a robust antifreeze protein expression, ensuring an efficient system for advancing our batch culturing and AFP harvesting. Lastly, we will test the functionality of AFPs on simulative road-testing sites for prototypes before applications on icy roads.

Author contributions

M.Z. came up with the original idea and began introductory research. P.D., H.P., T.S., and L.W. conducted the early research process and contributed to the writing and proofreading of the paper. P.D., H.P., T.S., and L.W. designed this project's images, graphics, and videos.

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