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Purification and Activity Analysis of a Highly Active Recombinant Catalase from Psychrophilic Bacterium Psychrobacter cryohaloentis

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ABSTRACT

To date, the genus Psychrobacter includes 37 recognized bacterial species isolated from different sources ranging from Siberian permafrost (*P. cryohalolentis*) to seal feces (*P. lutiphocae*). Due to its diverse ecological niches, several species belonging to the genus have different enzymatic activities with potential applications in bioremediation and the food industry. In this study, a bioprospecting approach was used to perform a in depth analysis of a Psychrobacter-like bacterial strain isolated from goose feces (designated as *Psychrobacter unknown*). The phylogenetic analysis of 16S rRNA gene and homologous sequence studies suggest that the strain may represent a novel species of the Psychrobacter genus. Further genomic analysis on this novel species suggests to a non-canonical central carbon metabolism and potential catalase enzymatic activity. Upon expression and purification of catalse from *P.* cryohaloentis, a cobalt based catalase assay was optimized. *P. cryohaloentis* was shown to have an elevated catalase activity exceeding that of commercial bovine liver catalase.

INTRODUCTION

Since the 1960s, researchers at the National Aeronautics and Space Administration (NASA) have been puzzling over one question: can humans adapt to live on Mars? Although we are still rather far from travelling to Mars, we are closer to answering that question than ever before. Rather than scouring the surface of Mars looking for life sources, NASA researchers have turned their focus on the biochemical exploration of microbial extremophiles here on Earth—particularly *Psychrobacter sp.* A study released in 2009 suggests that *Psychrobacter cryohalolentis*—a psychrotolerant microorganism obtained from Siberian permafrost, is capable of surviving Martian surface conditions such as high ultraviolet irradiation, low temperature, and low atmospheric pressure (Smith et. al., 2009). Although we as humans are several taxonomic branches away from *Psychrobacter sp.*, understanding how this organism can survive on Mars, is the first step towards our future lives on the red planet. This practice of analyzing an organism to understand its survivability extends far past the realm of space exploration. By employing various basic science and bioprospecting techniques, novel species research is being pushed further than ever, leading to the advent and revolution of several industries.

BIOPROSPECTING

Defined as the investigation of a subject to increase knowledge and understanding about it, basic science research is the driving force behind all scientific advancements. For every vaccine, pesticides, antibiotic, and space flight there is a litany of fundamental research supporting it. Over the past century, the advent of basic science research has branched out into several smaller disciplines. At the forefront of the basic science trend, however, is the idea of biodiversity prospecting—or bioprospecting. Bioprospecting is the exploration of natural sources for biochemical and genetic information that could be harnessed to advance a wide array of industries including pharmaceuticals, cosmetics, agriculture, and aquaculture. Over the past 40 years, 30% of all medications approved by the Food and Drug Administration (FDA) were derived from natural products using bioprospecting techniques (Cicka & Quave, 2019). Despite this success, however, due to the nature of bioprospecting revolving around the disruption of an ecosystem, there is some controversy surrounding the topic. Environmentalist and human rights groups argue that an increase in bioprospecting—particularly with regards to the pharmaceutical industry— puts the wellbeing of indigenous groups at risk for the benefit to the western world. This notion is

supported by the fact that the majority of current bioprospecting programs focus on terrestrial plants, fungi, and Acinetobacter species all found in rainforest areas (Beattie et. al., 2010). Although bioprospecting studies continue to produce novel research, its current trajectory may present some environmental issues. Fortunately, however, by shifting bioprospecting efforts away from plants and other sustainable resources to bacteria, a compromise can be reached. Due to inherent difficulties with isolation and *in vitro* cultivation, many species of bacteria—especially that of microbial extremophiles—are often overlooked as a potential bioprospecting source. These unique organisms, however, have a genome rich with interesting proteins (extremozymes) that allow them to survive and thrive in such inhabitable (extreme) environments. Common types of extremophiles include thermophiles, psychrophiles, alkaliphiles, acidophiles, halophiles, and piezophiles (Rampelotto, 2013). A notable example of an enzyme isolated from an extremophile is taq polymerase from *Thermus aquaticus*. Due to the thermophilic nature of *T. aquaticus*, Taq polymerase is thermostable at high temperatures. This allows for the enzyme to be readily used in polymerase chain reaction (PCR) DNA amplification. **The following study analyzes a novel species of Psychrobacter to determine whether proteins encoded in the species warrant further bioprospecting and industrial application.**

PSYCHROBACTER SPECIES

The genus Psychrobacter was originally proposed by Juni and Heym (1986) to accommodate a group of non-motile (sometimes twitching), oxidase-positive, non-pigmented, psychrotolerant (cold-tolerant), aerobic, catalase-positive, and Gram-negative coccobacilli (elongated spheres) considered to be a subset of the family *Moraxellaceae (Garcia-Lopez et. al., 2014)*.

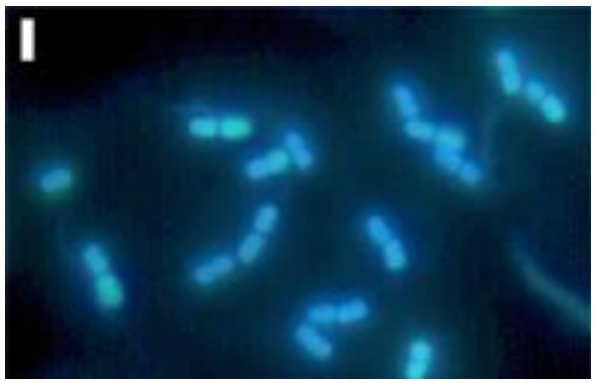

Figure 1. *Psychrobacter cryohalolentis* K5. *P. cryohalolentis* cells were stained with DAPI and imaged on a confocal fluorescent microscope. Scale Bar = 1 micron. Image by Corien Bakermans (Genome Portal).

The *Moraxellaceae* family currently encompasses the genera *Acinetobacter*, *Moraxella*, and *Psychrobacter*. Several of the microorganisms included in this family have a history of debate and changes in their names over the past few decades leading to difficulties in the interpretation of early literature (Teixeira, 2014). The family is a heterogeneous group of bacteria distributed over a wide variety of natural habitats and having diverse ecological and clinical significances. Although sharing many of the same properties as Moraxella and Acinetobacter, several authors have argued that there is a significant number of biological differences between them including optimal growth temperatures, salt tolerance, and glucose utilization (Yang, 2014). Unlike Moraxella and Acinetobacter, the genus *Psychrobacter* is typically found and isolated in natural low-temperature saline environments. Additionally, some species are associated with terrestrial and marine animals, and with foodstuff. Psychrobacters are rarely isolated from human sources; as such, little is known about their clinical significance (Le Guern, 2014).

Upon discovery, Psychrobacter were initially isolated from fish, chicken, and various fermented foods. Now, Psychrobacter is considered to be a ubiquitous bacterium with virtually a global distribution. As mentioned previously, many members of the genus have been isolated from several cold environments including Antarctic glacier mud, Antartic ornithogenic soils, Siberian permafrost, and Artic seawater (Amato et. al., 2009; Ayala et. al., 2010; Bakermans et. al., 2006). Aside from being found in harsh frigid environments, some species of Psychrobacter have been isolated from warmer environments such as cheese, human blood, and seal feces (Yang, 2014). This broad distribution suggests that Psychrobacter species are able to withstand temperatures ranging from -10° to 42°C and a pH from 5.5 to 9.0. This wide range of growth conditions coupled with its diverse global distribution, alludes to a unique pattern of survivability that could be indicative of biological activities useful worthy of bioprospecting efforts. In particular, Psychrobacter may be a source of cold-adapted proteins and enzymes which have broad applicability in industrial processes applications, such as bioremediation of waste waters and effluents, degradation of petrochemicals, production of biopolymers and biosurfactants, biomass production, and production of immune adjuvants and probiotics (Teixeira, 2014). Current literature surrounding these applications of Psychrobacter is sparse. Based on preliminary findings, Psychrobacter species warrants further bioprospecting efforts and should be closely studied.

PSYCHROBACTER UNKNOWN

As of September 2020, the genus Psychrobacter consists of 37 named species. In Summer 2019, bacteria isolated from fresh goose feces in Cold Spring Harbor, NY, were cultured on LB agar at 30 and 37˚C (Lu et. al., 2009). The slow growing, convex, off-white colonies were observed by light microscopy to be a monoculture of ~2 µm coccobacilli with twitching motility. A genomic DNA library was prepared, sequenced to 800-fold coverage, and assembled into 24 contigs. The contigs were annotated using the DDBJ Annotation and Submission Tool (DFAST; Tanizawa & Nakamura, 2018). The genome of *P. unknown* is 3.333 Mbp with 43.7% GC content. DFAST annotated 2,754 protein coding regions, three rRNAs, 41 tRNAs, and one CRISPR. The 3.333 Mbp sequence of the 16S rRNA gene of the isolate was determined and its similarities with other Psychrobacter spp. were estimated using BLASTN. The isolate showed the highest similarity with many subspecies of *P. piscatorii* (1509/1510 nt), though the first 22 nt of the 16S sequence of our species was not included in these alignments. The DNA G + C content was 44.6%. On the basis of the above results, in silico DNA-DNA hybridization calculations between the isolate and *P. piscatorii* or *P. pacificensis* were calculated. The highest score for any calculation was 70%. Taking all the aforementioned findings together, coupled with the slow growing nature of the bacterial strain, the goose feces isolate was identified a novel Psychrobacter species and designated as *Psychrobacter unknown* (**Figure 2**).

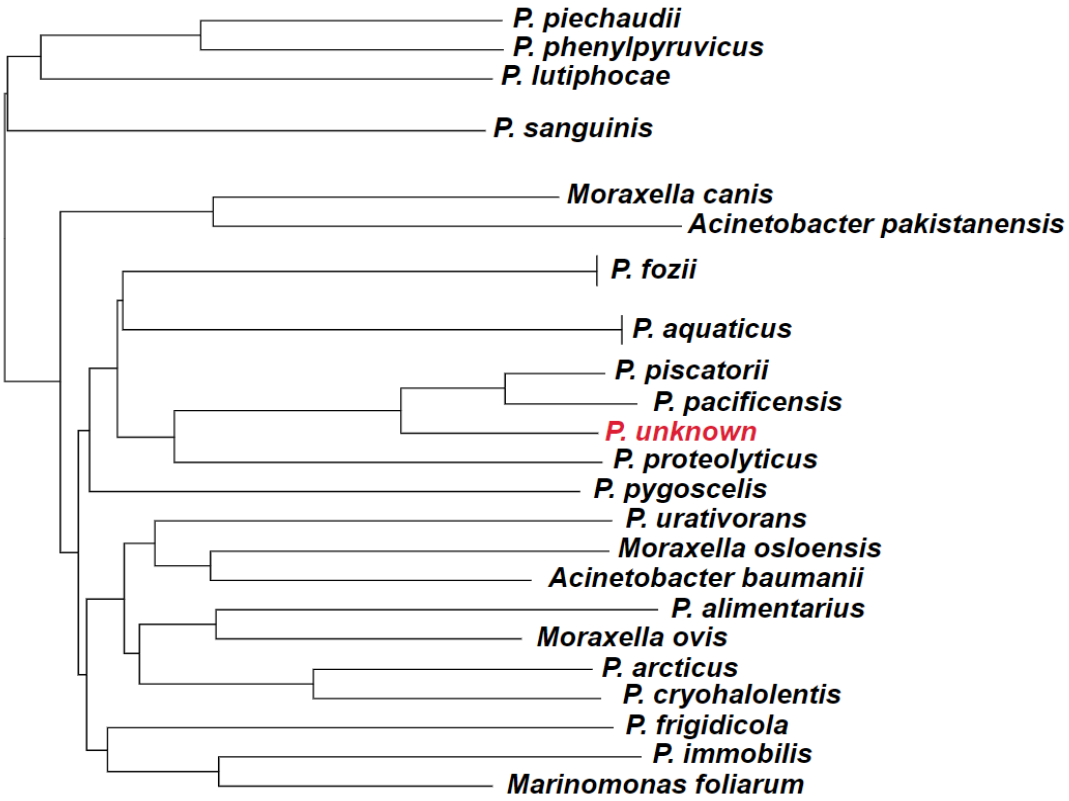

Figure 2. *Psychrobacter unknown* is a novel species. A 16S RNA phylogenetic analysis was performed using TYGS (Meier-Kolthoff & Goker, 2019). The resulting phylogenic tree suggests that *P. unknown* is a novel organism most closely related to *P. piscatorii* and *P. pacificensis*.

Preliminary *in silico* genomic analyses suggest that *Psychrobacter unknown* is catalase, oxidase, and urease positive. These traits—found in a variety of *Psychrobacter spp.*— are indicative of an opportunistic pathogen. Furthermore, some of these species show penicillin resistance—making it difficult to kill in a clinical setting (Le Guern, 2014; Caspar, 2013). These possible human health and safety risk factors make *P. unknown* unsafe to test in an undergraduate facility. In modern biochemistry, it is not strictly necessary to study a live pathogen to learn more about its function and bioprospecting potential.

In accordance with the aforementioned phylogenetic analysis, bacteria species closely related *P. unknown* based on whole genome DNA-DNA hybridization calculations, the gold standard in establishing a novel species, include *P. piscatorii* (70%), *P. pacificensis* (67%), and *P. proteolyticus* (56%) (Meier-Kolthoff & Goker, 2019). As such, a homologous study approach to the bioprospecting of *P. unknown* is wise. A homologous study is largely based on the fact that different parts of the genome evolve at different rates, and that many organisms in the same genus share homologous proteins and biochemical activities. Traditional homologous studies begin with sequence similarity searching. Sequence similarity searches can identify homologous proteins or genes by detecting a statistically significant level of sequence conservation, often indicative of

common ancestry. This can be largely useful when exploring a novel organism. In addition to any human health and safety risks that may be present (such as in the case of *P. unknown*), protein identification and analysis is expensive and time consuming yet may not yield useful results. This is of the largest downsides of bioprospecting. A major cause of these detrimental rabbit holes is the fact that many of the protein structure identification and imaging processes such as x-ray crystallography and cryo-electron microscopy are antiquated and yield low resolution images. Despite mass improvements in this technology and an increase in overall accessibility over the past 50 years, there is still much work to be done. To a large extent, the biochemical relevance of a protein lies within its structural dynamics and arrangement of distinct conformations yielding a unique shape and charge. For a protein to fulfill its biological niche, it must be able to move and conform to its various substrates. Conceptually, for the majority of proteins at least two conformers are needed. The current accessible imaging techniques only render images in a single fixed state. There are ways around this by averaging several images together, but when a protein image is averaged, associated structural details are often lost. Before a protein can be adequately detailed, it requires several rounds of this expensive and time-consuming imaging. As such, before investing in an analysis of a novel organisms, it is beneficial to compare proteins of interest with more well-studied organisms via *in silico* and *in-vitro* analyses to determine if further bioprospecting research on novel organisms is warranted.

Even with the inherent benefits in homologous studies, homologous sequences do not always share significant sequence similarity (Pearson, 2014). There are thousands of proteins that are considered to be homologous, yet do not have a statistically significant primary sequence. Consider a hypothetical protein X and a hypothetical protein Y. Despite being isolated from different species, protein X and Y both share the same function and convert molecule W to molecule Z. They take the same biochemical route and utilize the same cofactors. The difference between protein X and Y, however, lies in the sequence similarity. Assume that the sequence of the active site of protein X is **aaabbbccc** and the active site sequence of protein Y is **eeebbbggg**. In this case, even though the proteins are homologous in function, they only have a 33% sequence similarity—this is statistically insignificant. Furthermore, the existence of a gene in a genome does not guarantee gene expression. Depending on how the specific gene is processed and manipulated can change the resulting protein entirely. As such, in addition to a comparative analysis using sequence similarity search tools such as NCBI BLAST (Basic Local Alignment Search Tool, it is of paramount importance to consider statistically significant structural and enzymatic similarities to determine if proteins are a significant match and perform *in vitro* analyses before any complete conclusions can be drawn (Pearson, 2014).

CENTRAL CARBON METABOLISM

In order to fully understand how an organism lives in and interacts with its environmental niche, it is vital to study its central carbon metabolism. In biochemistry, metabolism refers to the sum of the chemical reactions that take place within each cell of a living organism and that provide energy for vital processes and for synthesizing new organic material. In a simplified sense, metabolism any biochemical reaction that drives a process. With regards to central carbon metabolism, this is the metabolism of an organism revolving around the anabolic and catabolic conversion of carbon compounds for energy or as a carbon source. By analyzing the central carbon pathway, a better understanding of how an organism utilizes its resources can be determined. As such, a more in-depth understanding is achieved, allowing for a more accurate classification of an organism. Furthermore, understanding how an organism obtains energy and other vital

biochemical cofactors from naturally occurring carbon sources can lead to the identification of enzymes that may prove to be useful in various bioprospecting efforts.

One of the more distinguishing features of the Psychrobacter genus is the lack of essential enzymes crucial for traditional carbohydrate metabolism via glycolysis. Nearly all organisms convert and harness energy from carbon sources via glycolysis. Glycolysis is a ten step catabolic conversion of glucose to pyruvic acid. Bacteria belonging to the genus of Psychrobacter, however, lack many of the essential enzymes— such as hexokinase—that are crucial for carbohydrate metabolism via glycolysis. As such, bacteria belonging to this genus are unable to grow on simple carbohydrates and require pathways other than glycolysis to generate essential intermediates for metabolic reactions. Glucose can, however, be used as an energy source—rather than a carbon source— in the case of *P. arcticus* 273-4 (Bakermans et. al., 2006) and *Psychrobacter sp.* DAB_AL43B (Lasek et. al., 2017) due to the presence of a putative glucose dehydrogenase. Additionally, it has been noted that some Psychrobacter species produce glucose from acid. This utilization of glucose further supports the possible presence of putative glycolysis enzymes in Psychrobacter (Lasek et. al., 2017). A detailed *in silico* analysis was performed to analyze the annotated proteome of *P. unknown*, however, revealed that this is not the case for *P. unknown*. As a result, it is hypothesized that *P. unknown* cannot metabolize glucose, and instead relies on acetate compounds for both a carbon and energy source. **Figure 3**, presents a reconstruction of the hypothesized central carbon metabolism of *P. unknown*, including the tricarboxylic acid (TCA) cycle and its variants. The list of enzymes catalyzing particular reactions is given in **Table 1**.

Table 1. Enzymes represnetned in *P. unknown* central carbon metabolism. The numbers correlate with **Figure 3**.

Number	Enzyme	Gene
1	Pyruvate dehydrogenase	aceE
2	Citrate synthase	gltA
3	Acetyl-CoA acetyltransferase	thlA
4	Aconitate hydratase	aco2
5	Isocitrae dehydrogenase (NADP/NADP-dependent)	icd/ icd2
6 & 7	Isocitrate lyase	aceA
8	Malate synthase	glcB
9	Oxoglutarate dehydrogenase	sucB
10	Succinate-CoA ligase	sucC/D
11	Succinate dehydrogenase	sdh
12	Fumarate hydratase	fumC
13 & 14	Malate dehydrogenase	mdh
15	Phosphoenolpyruvate carboxylase	ppc
16	L-lactae dehydrogenase	lldD2
17	D-lactate dehydrogenase	N/A
18	Hydroxyacylglithione	gloB
19	Lactoylglutathione lyase	gloA
20	Phosphoenolpyruvate synthase	ppsA
21	Phosphoenolpyruvate enolase	eno
22	Phosphoglycerate mutase	N/A
23	Glycerate 3-kinase	glxK
24	Glycerate dehydrogenase	N/A
25	Phosphoglycertae kinase	pgk
26	Glyceraldehyde-3-phosphate dehydrogenase	gap
27	Triose-phosphate isomerase	tpiA
28	Fructose-bisphosphate aldolase	fba
29	Fructose-bisphosphotase	fbp
30 & 31 &32	Glucose-6-phosphate isomerase	pgi

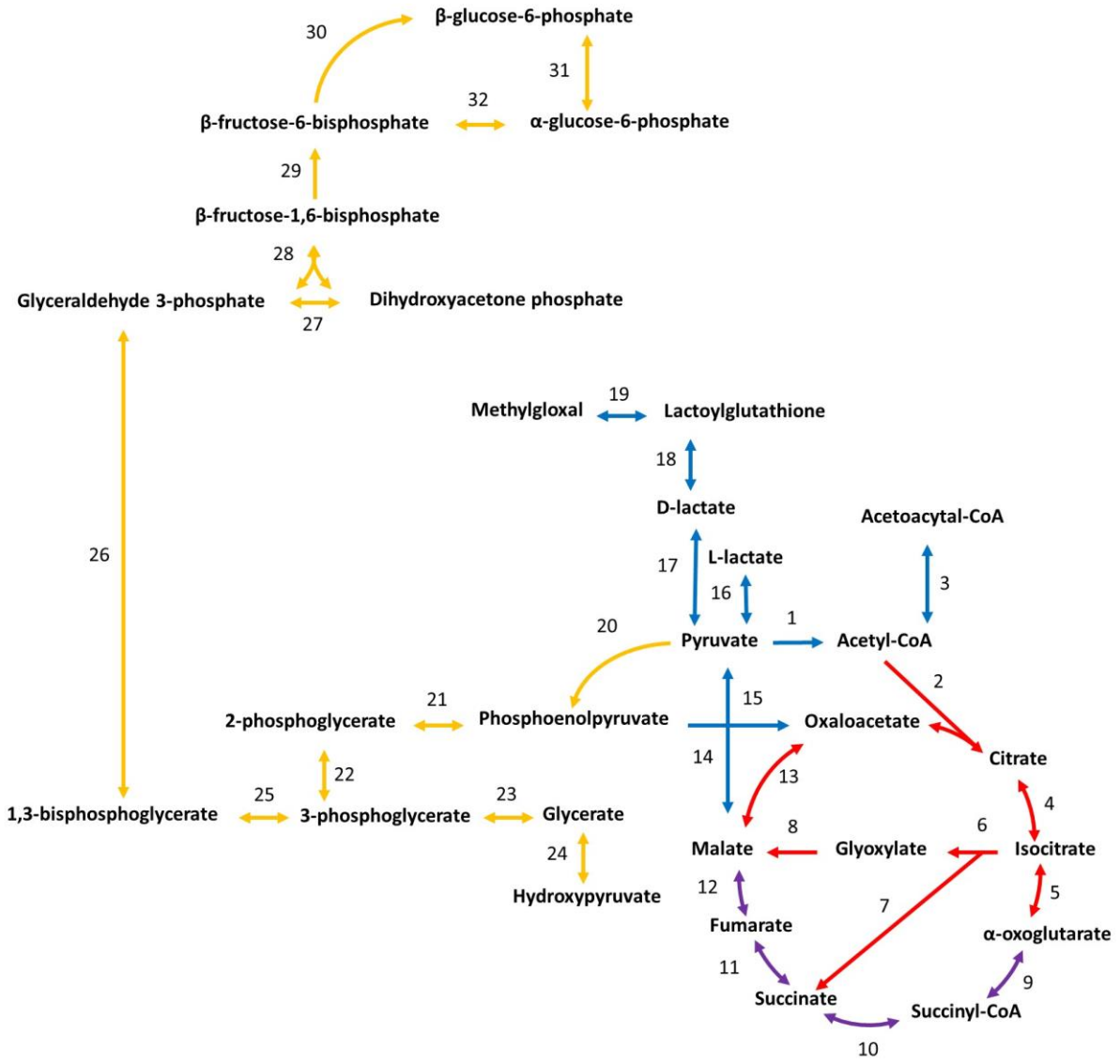

Figure 3. Hypothesized central carbon metabolism of *Psychrobacter unknown*. An in depth *in silico* analysis of the proteome of *P. unknown* was done to reconstruct its central carbon metabolism. The numbers correlate to the enzymes in **Table 1**. The colors group various metabolic processes.

Although the canonical glycolytic pathway is not fully present in *P. unknown*, some enzymes involved in the glycolysis are present. One enzyme of particular interest is Glyceraldehyde 3-Phosphate (G3P). The presence of G3P suggests that *P. unknown* can acquire basic carbohydrates via gluconeogenesis and the pentose phosphate pathway. This affirmation is further supported by the lack of sugar transport enzymes. Without these enzymes, an organism must contain an internal pathway for producing carbohydrates. The various intermediates used in this anabolic pathway is adequately replenished by a series of anaplerotic reactions. As predicted

by an *in silico* analysis of the proteome these reactions include the bidirectional conversion of malate and oxaloacetate into pyruvate and the conversion of isocitrate to malate via the glyoxylate shunt— an augmentation of the standard TCA cycle (Lasek et. al., 2017).

The glyoxylate shunt (GS) is an anabolic variation of the TCA that occurs in a wide range of plants, bacteria, and fungi. Much like the TCA, the GS centers around the conversion of acetyl-CoA to succinate and malate for the synthesis of carbohydrates. The difference, however, is that the GS consists of a series of reactions that serves as a bypass step of the oxidative decarboxylation stages of the TCA, allowing for the conservation of carbon groups. This allows for the organism to utilize low carbon substrates—such as acetate—when simple sugars are not available, or in the case of *P. unknown*, there genome lacks the proper metabolic genes for said sugar. The key enzymes involved in these bypass steps are malate synthase and isocitrate lyase. As noted in **Figure 3**, both of these enzymes are present.

Previous studies on the role of the GS in bacterial organisms indicate that its function is largely upregulated by oxidative stress (Xie et. al., 2019; Ahn et. al., 2016). Oxidative stress is a phenomenon resulting from an imbalance between the synthesis oxygen reactive species (ROS) in cells and their ability to detoxify these reactive products. This buildup of ROS can have a detrimental effect on a variety of biochemical processes. Despite their inherent detriment, exposure to several of environmental factors such as salinity, drought, toxicity, air pollutants and ultraviolent radiation may lead to the upregulation and production of ROS, furthering the oxidative stress in many microbial species (Den et. al., 2009). Exposure to a variety as such, any rapid change in environment—such as exiting the digestive tract of a goose—may result in elevated levels of oxidative stress and generation of ROS. Such an observation suggests that a constitutively active GS, such as the one found in the *in silico* analysis of *P. unknown*, serves as an indicator of chronic oxidative stress. Outside of a rapid change in environmental conditions, some literature on Psychrobacter species points to an elevated internal production of ROS as a biproduct of their own metabolism. Although chronic exposure to high internal levels of ROS is a well-studied and accepted contributing factor to oxidative stress, ultimately, further *in vivo* analyses would need to be performed to determine the source of said oxidative stress—if any—within *p. unknown*.

CATALASE

As previously discussed, much like every aerobic microorganism, Psychrobacter encounter intracellular reactive oxygen species (ROS)—such as hydroxyl radicals and superoxide—as byproducts of their own oxygen metabolism. One side effect of these ROS bioproducts, is the activation of a positive feedback loop leading to the synthesis of hydrogen peroxide (H_2O_2). Thusly the subsequent break down of H_2O_2 may lead to the generation of more harmful ROS (Hertel et. al., 1998). As such, the elimination of intracellular H_2O_2 is crucial for microorganisms to prevent ROS reacting with their cell and biochemical functionality. Catalase is an enzyme found in nearly all living organisms exposed to oxygen. Catalase is a multiunit protein containing four heme groups that allow for it to react with various ROS. The main function of catalase is to promote the

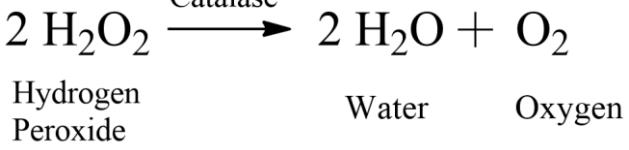

Figure 4. Decomposition of hydrogen peroxide via catalase. Catalase decomposes hydrogen peroxide into water and oxygen.

decomposition of hydrogen peroxide to water and oxygen (**Figure 4**), proving to be a vital enzyme in the metabolism of ROS, thus largely protecting the cell from oxidative damage. As such, in addition to the upregulation of the GS, an *in silico* analysis of *P. unknown*, suggests an increased activity of the enzyme catalase. Catalase is one of three regulatory proteins responsible for the disproportionation reaction of hydrogen peroxide (H_2O_2) in microorganisms. In most cases, catalase production is a defense mechanism to protect the organisms against harsh ROS. Therefore, an elevated level of catalase activity would suggest oxidative stress within the organism. This is consistent with the aforementioned findings, furthering the suggestion of exposure to an oxidative environment, or internal metabolic pathways generating elevated ROS.

In general, few bacteria have been reported to be able to actively survive in highly oxidative environments. Those that have been discovered to be capable to survive have been initially extracted from oxidative environments. In a 2012 study done by Kimoto et al., a strain (T-3) of Psychrotolerant *Psychrobacter piscatorii*, was isolated from a fish processing plant that uses H_2O_2 as a bleaching agent. The cell extract from the T-3 strain exhibited a higher catalase activity (19,700 U*mg/ protein) than that of *Micrococcus luteus* (5,000—10,900 U*mg/ protein) commonly used for industrial catalase production. Utilizing a BLASTp analysis and ClustalOmega alignment, the T-3 catalase primary protein sequence was aligned and compared with that of the catalase sequence in *P. unknown* (**Figure 5**; Kimoto et. al., 2012). In this alignment, both catalase active sites—H^{83} and N^{156}—remain conserved and the overall protein sequence had a 40.08% similarity (BLASTp). This conservation suggests that the activity of *P. unknown* catalase, may be similar to that of *P. piscatorii* T-3. In order to appropriately test this theory, however, a further *in vitro* analysis of the *P. unknown* catalase must be completed to determine its specific activity and other kinetic parameters. Due to rising concerns over its pathogenicity and opportunistic nature, we do not find it safe to culture in our lab settings at this time. Despite this, however, a phylogenetic analysis of the *P. unknown* catalase, shows a strong relatedness (87.91% identical) to *Psychrobacter cryohalolentis*, suggesting that *P. cryohalolentis* can be used as an accurate model system for studying homologous catalase activity in *P. unknown.*

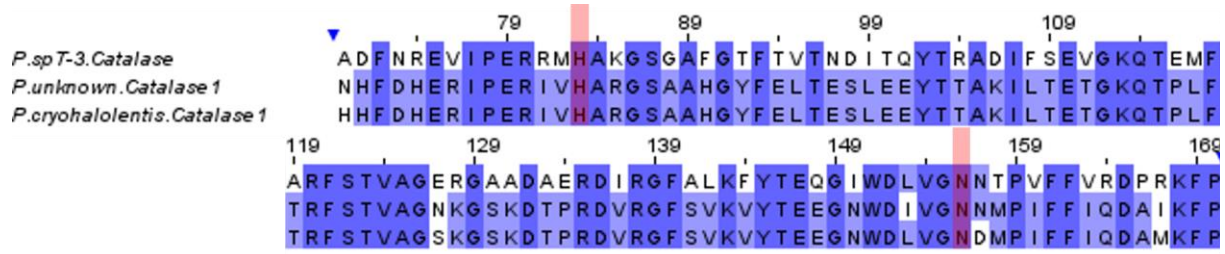

Figure 5. Alignment of homologous catalase enzyme primary structure from Psychrobacter species reveals highly conserved active site residues. Using CLUSTALW software, catalase from *P. unknown*, *P. piscatorii* T-3, and *P-cryohalolentis* were aligned. The alignment indicates one highly conserved active site (residues shown in red). The shade of blue correlates to the conservation across species. The darker the hue, the more conserved the residue. 100/ 702 residues shown.

With the expansion of bioprospecting interests and the enzyme industry, catalase has been exploited in many industrial applications. In the field of bioremediation, catalase assists in the removal of hydrogen peroxide from bleach effluents created by the textile industry and serves as

an indicator of hydrocarbon degradation (Kaushal et. al., 2018). With regards to the food industry, catalase also plays an important part in several different processes. Catalase is often incorporated in food wrappers to help create *active packaging* that extends the shelf life of foodstuffs. Catalase is also used in the measurement of milk quality and an alternative to pasteurization in the production of certain cheeses such as swiss. Due to the highly applicable nature of this enzyme and overall abundance, further natural sources should be investigated. By determining the catalase enzyme activity in novel organism *Psychrobacter unknown*, a potential source of easy catalase production may be discovered. As such, *P. unknown* may serve as an effective microbial bioremediation agent and catalyst in cheese production.

Given the conservation of catalase primary sequence of *P. cryohalolentis* and *P. unknown* and the inherent safety risks associated with the growth and opportunistic nature of *P. unknown*, going forward, an *in vitro* homologous study of *P. cryohalolentis* catalase activity will be completed. By expressing, isolating, and purifying the catalase enzyme, we can quantitatively assess its enzymatic activity via a simple astrophotometric *in vitro* assay (Hadwan, 2018). Colleting such data will allow for a further analysis on the use of use of Psychrobacter catalase in industry.

RESULTS AND DISCUSSION

This project began with the isolation and characterization of catalase from gram-negative, psychrophilic organism *Psychrobacter cryohalolentis* K5 (Bakermans et. al., 2006). To date, there have been several reports of unique catalases from extremophiles (Gomez & Steiner, 2004), however, there are limited reports of psychrophilic catalases isolated from psychrophilic microorganisms (Yumoto et. al., 2000). *P. cryohalolentis* catalase was selected after extensive homology searching in the NCBI database using BLASTP (**Figure 5**). *P. cryohalolentis* bears extensive (85.29% identity) primary sequence to the catalase discovered in the novel organism *Psychrobacter unknown*. *P. unknown* was isolated from goose feces in Summer 2019, and sequence analysis suggests that it is a novel species and potential human pathogen warranting further study (Riley; personal communications). As an experimental starting place, *P. cryohalolentis* is employed here as a better established and safer model organism.

Most bacterial catalases are characterized as one of two types: mammal type (typical) catalases and bifunctional catalase-peroxidases. The typical catalases are isolated from animals, plants, fungi, and bacteria. Despite being sourced from divergent species, these typical catalases share similar molecular features: they are composed of four subunits of equal size and have an overall molecular mass range of 225 to 270 kDa. Furthermore, they exhibit a broad optimum pH range of 5 to 10, are resistant to treatment with organic solvents, and are inhibited by some triazole groups (Brown & Salin, 1995; Pool et. al., 1986; Klotz & Hutcheson, 1992). The catalase-peroxidases, which are most typically expressed in bacteria and fungi, have several properties distinguishing them from mammal type catalases: they are sensitive to organic solvents, insensitive to triazole groups, and express a pH-dependent activity. In both *P. cryohaloentis* and *P. unknown*, both types of catalase can be found. As such, it is important to identify the appropriate catalase type in order to avoid unwanted assay results due to the variation in hydrogen peroxide metabolism. Preliminary classification and *in silico* analysis of *Psychrobacter cryohalolentis* suggests that the catalase of interest (NCBI:txid330922) is classified as a monofunctional mammal

type (typical) catalase. Due to the limited surrounding literature of *P. cryohaloentis* catalase, there is no published crystal structure. Through a PDB sequence search, however, the most similar crystallized catalase is KatE from *E. coli* (PDB ID: 6JQQ). Considering *P. cryohaloentis* to be rather divergent from *E. coli*, it is interesting that the tertiary structure of their typical catalase is semi-convergent. Furthermore, when comparing the primary amino acid sequence to *E. coli* monofunctional catalase, the histidine and arginine active site residues are conserved indicating a single binding site/ function (**Figure 6**).

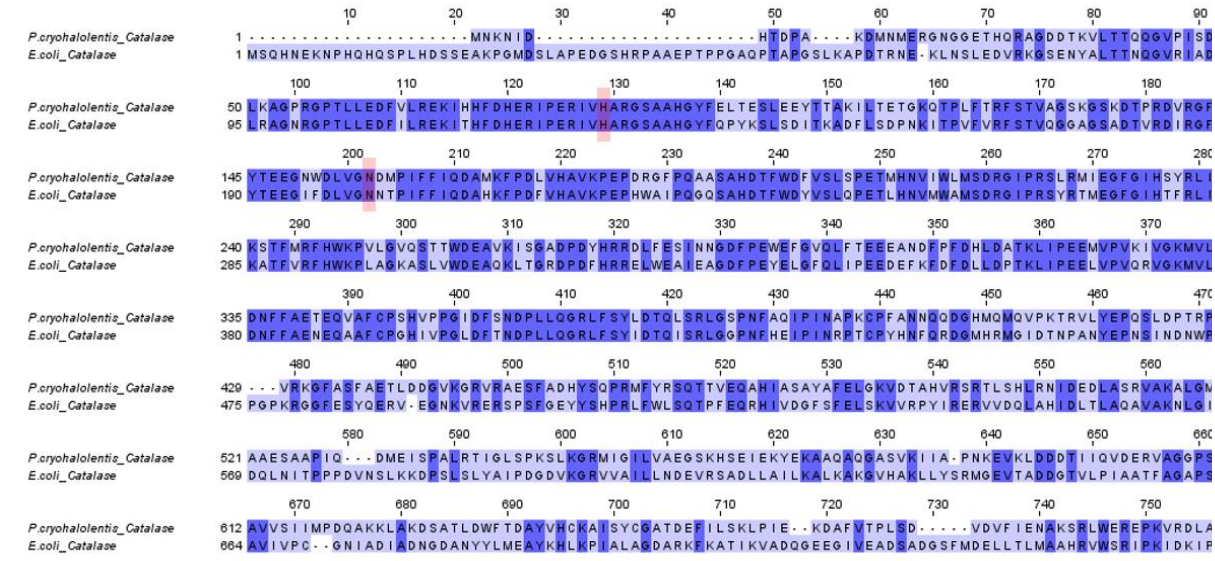

Figure 6. Conserved active site residues of monofunctional catalase from *E. coli* and *P. cryohaloentis*. An alignment of two catalase amino acid sequences were rendered using Clustal Omega and visualized with Jalview. The darker the blue, the more conserved the residues. The active site residues are highlighted in red.

More often than not, cold-adapted metabolic enzymes—such as citrate synthase, from psychrophilic organisms exhibit a shift in optimum activity toward low temperatures. Cold-adapted enzymes have a combination of specific adaptations in their structural features that give them more flexible structures than mesophilic enzymes. This trait allows for high catalytic activity at low temperatures (Sarmiento et. al., 2015). These structural features correspond to specific genetic changes, which are a consequence of long-term selection. In recent reviews on psychrophilic enzymes, the following features were mentioned as important adaptations: (a) decreased core hydrophobicity, (b) lower arginine/lysine ratio (c) decreased secondary structures and oligomerization, and (d) an increase in conformational entropy of the unfolded protein state (Feller, 2010; Cavicchioli et. al., 2011). In the *in silico* analysis of *P. cryohaloentis* catalase, the only determinable feature discovered was a lower arginine/ lysine ratio as compared to the *E. coli* catalase (0.97 and 1.17, respectively). Further imaging and crystallography research must be carried out to clearly determine the presence of these features.

Regardless of the presence of traditional psychrophilic enzyme structural features, psychrophilic enzymes are generally characterized by having a higher and more stable specific activity over a broad temperature range often covering 0 to 30°C (Gerday et. al., 1997). In an early analysis of novel Antarctic bacterial isolate DS2-3R, a cold-active citrate synthase—a metabolic enzyme utilized in the glyoxylate shunt of many Psychrobacter species—was overexpressed in *E. coli* and found to have an optimum activity at 31°C and a comparable activity (~95%) at room temperature (Gerike et. al., 1997). Based on these results, enzyme activity at a low temperature is not always a good indicator of psychrophilic enzymes. The optimum temperature of an enzyme may change depending on how much activity is necessary to sustain its function and ensure survival within an organism. As such, the following expression, purification, and analysis of *P. cryohaloentis* catalase, was performed in room temperature environement.

CLONING AND PROTEIN EXPRESSION

In the present study, we purified and characterized a catalase that **exhibited relative elevated activity** from other catalase positive organisms. As a starting place for analysis, the purification and assay conditions were carried out at room temperature (~22°C). Expression of *P. cryohaloentis* catalase started with the coding region synthesized as a dsDNA GeneBlock (IDT), cut with NheI/XhoI and inserted into pET28a in frame with its N-terminal hexahistidine tag.

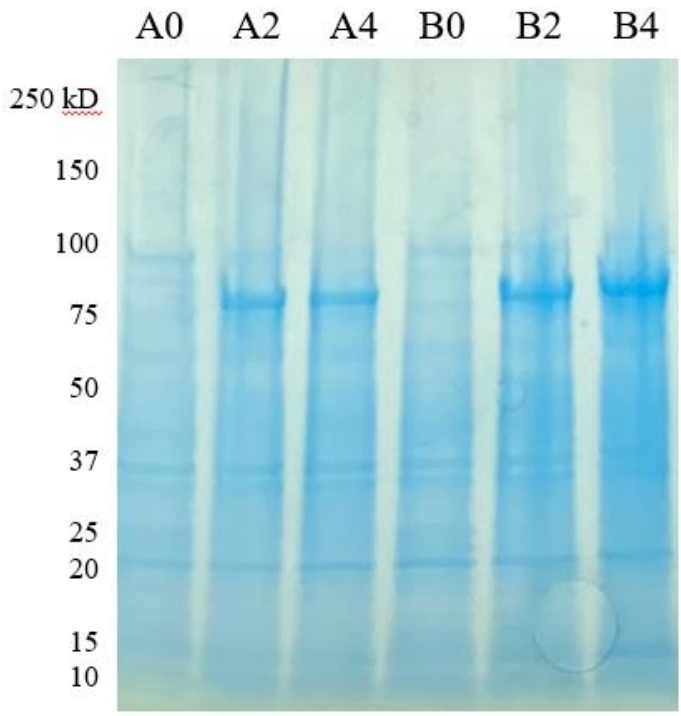

Figure 7. Optimal *P. cryohaloentis* catalse protein expression occurs 4 hours post induction. Saturated cultures A and B were diluted 1:50 and grown to mid-log phase (OD600= 0.584; t=4 hr; A/B 0). The expression of the recombinant catalase with the N-terminal hexahistidine tag was induced with 1 mM IPTG. Aliquots (25 mL) were incubated at 37°C with vigorous shaking (250 rpm) for various time points post-induction (t= 2, 4; A/B2,4) at which a small sample of the culture was pelleted by centrifugation (16,000 rpm for 2 minutes). Collected pellets were analyzed by SDS-PAGE using 4-20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad).

Due to the novel nature of this enzyme purification, a hexahistidine tag was selected due to its relatively small size, hydrophilic nature, and versatility in the presence of detergents and many other additives. Additionally, the ability to detect and purify proteins without the need for an additional probe or protein-specific antibody makes for the His tag to be an attractive choice. The subsequent plasmid DNA was transformed into competent BL21(DE3)pLysS *Escherichia coli* cells, which were used for expression within one week of transformation. BL21(DE3)pLysS cells allow for high efficiency protein expression of virtually any gene that is cloned into a plasmid with a T7 promoter region (Davanloo et. al., 1984). In the case of this synthesis, the pET28a plasmid contains the T7 promoter region and required ribosome binding sites necessary for proper synthesis within BL21(DE3)pLysS cells. Furthermore, BL21(DE3)pLysS also contains the plasmid pLysS which carries the gene encoding for T7 lysozyme. When expressed, T7 lysozyme lowers the background expression of level target genes under the control of the T7 promoter but is suppressed upon induction with growth hormone IPTG (Davanloo et. al., 1984). This shut off suppresses leaky expression of possibly toxic proteins and allows for the upregulation of the gene in the pET28a plasmid.

In the case of *P. cryohaloentis* catalase K5. The bacteria were induced with IPTG (1 mM) following a four hour incubation period (**Figure 7**). The newly induced BL21(DE3)pLysS *E. coli* cells were harvested four hours later in preparation for protein purification and extraction.

CATALASE PURIFICATION

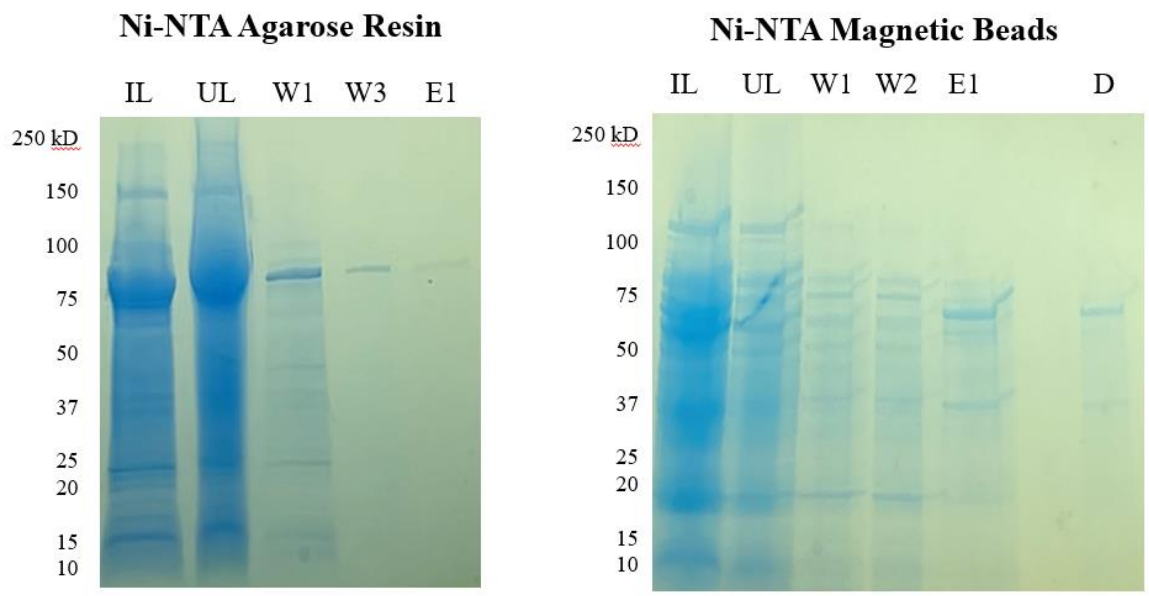

Figure 8. Catalase protein purification utilizing Ni-NTA agarose resin results in significant loss in protein. Proteins were extracted from cell lsytae by incubation with lysozyme (4 mM) and DNAseI (4 mM) for 15 minutes at RT while nutating and cell debris was removed after centrifugation at 15,000 rpm for five minutes at 4°C; sample of the supernatant (IL) were saved. The enzyme was purified in batch mode by affinity chromatography with HisPur Ni-NTA Agarose Resin or Ni-NTA Magentic Beads (1 M; ThermoFisher); samples of unbound lystate (UL) were saved. The column was washed with wash buffer (W1,2,3); finally, the enzyme was eluted with elution buffer (E1). The protein eluent from the magnetic bead system was dialyzed against 50 mM Tris-HCl buffer pH 7.0 for 16 hours. Collected fractions were analyzed by SDS-PAGE using a 4-20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad).

Following successful induction, the pellets were subject to varying rounds of purification. Using a ThermoFisher HisPur Ni-NTA agarose resin purification system, catalase was successfully purified from all contaminating proteins visible by Coomassie staining. Despite its overall success, however, a large portion of the protein was lost during the third wash step (**Figure 8**).

When using the ThermoFisher HisPur Ni-NTA magnetic bead purification system, the purified catalase was present in a larger concentration. However, with reduced wash steps, the protein was less pure immediately following elution (**Figure 8**). In both purification systems, the final target catalase was eluted with an imidazole solution (20 mM). In preliminary optimization tests of the catalase assay (see *catalase assay* section), imidazole buffers were shown to inhibit the formation of the desired carbanato-colbaltate (III) complex necessary for the activity assay. Thus, the eluted protein was dialyzed against a non-reactive buffer. The eluted catalase from the Ni-NTA magnetic bead purification was dialyzed against 50 nM Tris-HCl solution (pH 7.0) for 16 hours total. The resulting catalase was stabilized to a 50% concentration with glycerol. The final reported concentration via Bradford assay was 59.5 µg/ mL.

CATALASE ASSAY OPTIMIZATION

Catalase enzyme is characterized by its ability to decompose hydrogen peroxide into water and oxygen. To date, several methods have been described for measuring catalase activity in bacteria. One of the simplest qualitative methods involves determination of the enzyme's presence in bacterial isolate through the observation of bubbles (oxygen gas) in the presence of hydrogen peroxide (Iwase et. al., 2013). This simple method, however, is often overlooked in favor of more quantitative approaches focusing on careful measurements through colorimetric and spectrophotometric assays (Iwase et. al., 2013). Despite the inherent speed and low cost of this method, there are several limitations, including cumbersome procedures and high cost. The standard catalase kit from Sigma Aldrich, for instance, costs over $700. In the confines of an undergraduate facility, this is not a practical or cost-effective method for measuring catalase activity. As such, the catalase assay utilized in this research was modified from a published simple spectrophotometric assay for measuring catalase activity in biological systems (Hadwan, 2018).

Catalase activity is directly proportional to the rate of dissociation of hydrogen peroxide. Due to this linear relationship, it can be assumed that an increase in activity is associated with a decrease in hydrogen peroxide (Jones and Sugget, 1968). For reactions regulated by the catalase enzyme, the addition of cobalt bicarbonate can act as a stop bath. When added to a previously incubated solution of catalase sample and hydrogen peroxide (10 mM), any unreacted hydrogen peroxide will oxidize cobalt (II) to cobalt (III) and then react with the carbonate to produce a carbonato-cobaltate (III) complex ($[Co(CO_3)_3]Co$), which has an intense olive green color (Hadwan, 2018). When scanned on a spectrophotometer from 200 to 700 nm, the formed complex shows bands at 440 and 640 nm (Hadwan, 2018). Using this knowledge, an inverse relationship between relative catalase activity and the presence of a green color (absorbance at 440 nm), can be postulated. By then comparing the absorbance of the catalase sample to the standard, the relative activity can be calculated (**Equation 1**).

$$\frac{2.303}{t} * \log \frac{S°}{S}$$

Equation 1. Relative activity of catalase based on measured absorbance. Equation as described by Hadwan (2018) for calculation of catalse activity based on standard absorbance where t= time (s), S°= absorbance of standard, and S= absorbance of sample test. Activity defined in kU.

In preparation and optimization of this assay, a few key factors not discussed in Hadwan (2018) were discovered. First, the prepared solutions as described by Hadwan (2018) are not shelf stable. Further investigation of the cobalt "stop bath" solution revealed the formation of a pinkish precipitate. Based on the concentrations of the reagents in the "stop bath" (cobaltous nitrate hexahydrate, 3.485E-3 M; sodium hexametaphosphate, 8.15E-4; sodium bicarbonate, 0,96417 M) and the reported literature value of Cobalt (II) carbonate (1.0 E-10; Guo et. Al., 2019), it is reasonable to assume that the pinkish precipitate is this salt. Due to the formation of a precipitate, the working "stop bath" solution must be made fresh and thoroughly mixed at the start of the assay.

Additionally, when stored at room temperature, the prepared hydrogen peroxide—given its low 10 mM concentration—is not stable after long due to oxidative effects of the surrounding air (Pędziwiatr et. al., 2018). This means that all reagents used must be made fresh at the start of the assay.

Another discovery was the optimization of a purification protocol to isolate bacterial catalase. According to Hadwan (2018), the proposed assay can work with bacterial catalase and was even tested with *S. aureus* and *E. coli* catalase proteins. The purification method used, the potential tags on the proteins, nor the buffer conditions for protein storage were not discussed in the paper. As discussed previously, the purification method used eluted the final catalase protein in an imidazole (20 mM) based elution buffer (pH 7.0). When running preliminary tests with bovine catalase (Sigma Aldrich), the formation of the green carbonato-cobaltate (III) complex end product was not observed. Instead, the solution maintained its initial pink color before fading to a gray-purple. Qualitative analysis showed that the addition of the imidazole elution buffer to hydrogen peroxide and "stop bath" did not result in the formation of the desired green solution, whereas hydrogen peroxide plus the "stop bath" did (**Figure 9**).

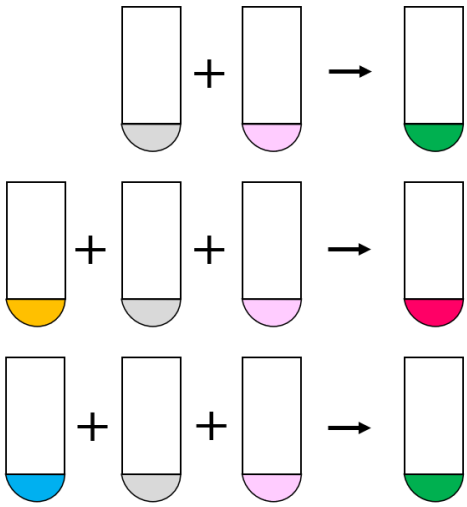

Figure 9. Addition of imidazole inhibits formation of green carbonato-cobaltate (III) complex. Graphical representation of addition of imidazole (yellow) solution to hydrogen peroxide/ stop bath solution (grey and pink respectively). Tris-HCl pH 7.0 (blue) does not inhibit the reaction.

Although there is no published literature on the effect of imidazole with this specific cobalt complex, a paper published in 1959 suggests the interaction of the imidazole with a cobalt nitrate solution to form a Co-imidazole complex (Mather & Lal, 1959). Due to the role cobalt nitrate plays in the formation of the desired carbonato-cobaltate (III) complex, it is likely that the addition of imidazole shifted the equilibrium of the reaction reversing the synthesis of the carbonato-cobaltate (III) complex. As such, in order to proceed with the assay as designed, the catalase buffer needed to be dialyzed against a non-reactive buffer. In this case, Tris-HCl 50 mM pH 7.0 was selected.

Another factor considered in optimizing the Hadwan (2018) assay is the stability of the catalase. The initial protocol describes two vortexing steps between the incubation periods. As with most *in vitro* enzyme, vortexing has the potential to unfold them resulting in a loss in activity. Previous literature surrounding the preparation of catalase notes that vortexing the enzyme was less stable than other mixing methods such as sonication (Qi et. al., 2011). Although no clear loss in activity resulted from the inclusion of the vortex steps, the assay performed more consistently when vortexing was omitted and replaced with a simple inversion.

P. CRYOHALOENTIS CATALASE QUANTIFICATION

Using the optimized Hadwan (2018) protocol and provided equation (**Equation 1**), the relatively activity of *P. cryohaloentis* catalase was determined at various concentrations. At the maximum *P. cryohaloentis* catalase concentration tested (59.5 µg/ mL), the relative activity was calculated to be 0.0131 kU (**Figure 10**).

When performing the same assay using a commercial bovine catalase (Sigma Aldrich) at the same catalase, the relative activity was 0.0127 kU. Although displaying similar activity at higher concentrations, an interesting observation is the divergence in activity as the concentration decreases. At the lowest concentration tested (9.92 µg/ mL), the *P. cryohaloentis* catalase relative activity was 1.45 times that of the commercial bovine catalase. This data suggests that *P. cryohaloentis* may exhibit a higher catalase activity than both catalase positive organisms. An alternative postulate to this result, however, is that the optimized catalase does not remain a linear function at lower concentrations. In order to determine the efficacy and accuracy of the assay, a kinetics assay must be performed.

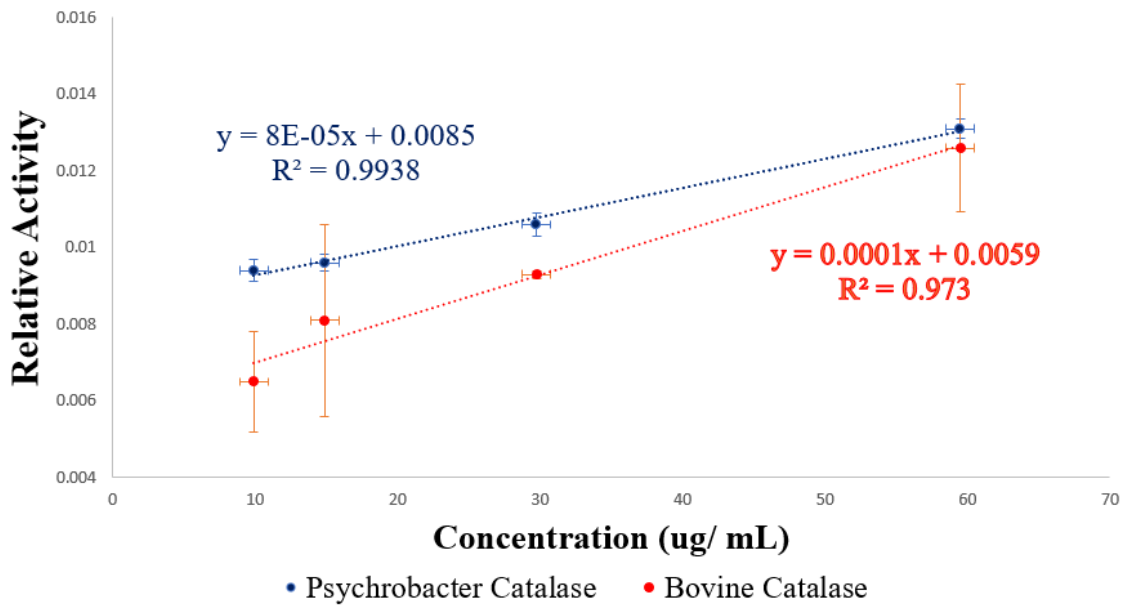

Figure 10. *P. cryohaloentis* has a relatively higher catalase activity than commercial catalase from bovine liver. A simple spectrophotometric assay utilizing a carbonato-cobalt (III) complex was optimized for purified *P. cryohaloentis* catalase from an existing protocol (Hadwan, 2018). The catalase source sample (varying concentrations) was mixed with 10 mM hydrogen peroxide in a phosphate buffer solution (pH 7.0, 50 mM) and incubated at 37°C for two minutes. The sample was thoroughly mixed by inversion and then treated with a cobalt-bicarbonate stop bath. The solution incubated at RT in the dark for 10 minutes. The resulting solution was analyzed using a Nanodrop spectrophotometer at 440 nM. The absorbances were compared to the standard (catalse source sample and working solution) using Equation 1.

To a large extent, however, this project demonstrates the first published isolation and purification of *P. cryohaloentis* catalase and measurement of relative activity using a modified

simple spectrophotometric assay. Through further kinetic analyses and organism specific optimizations such as pH and temperature, this method can be successfully employed to quantify the activity of *P. cryohaloentis*, and in turn, *P. unknown*.

MATERIALS & METHODS

CLONING AND BACTERIAL TRANSFORMATION

Psychrobacter cryohaloentis catalase-k5 coding region was synthesized as dsDNA (GeneBlock by IDT), cut with NheI/XhoI and inserted into pET28a in frame with its N-terminal hexahistidine tag— later verified by Sanger sequencing (Eurofins Genomics). pET-HisCat DNA was added to calcium competent BL21(DE3)PLyS *E. coli* and placed on ice for 30 minutes. The mixture was then heat shocked at 42°C for 30 seconds and then transferred to ice for five minutes. The transformed bacteria were plated on Lb/Kan/Cm agar plates (Kan= 50 µg/ mL; Cm= 34 µg/ mL) and incubated overnight at 37°C. Colonies were harvested and grown overnight in Lb/Kan media (Kan= 50 µg/ mL) at 37°C with vigorous shaking (250 rpm).

PROTEIN EXPRESSION OPTIMIZATION AND SDS-PAGE

Saturated cultures were diluted 1:50 and grown to mid-log phase (OD600= 0.584; t=4 hr). The expression of the recombinant catalase with the N-terminal hexahistidine tag was induced with 1 mM IPTG. Aliquots (25 mL) were incubated at 37°C with vigorous shaking (250 rpm) for various time points post-induction (t= 2, 4) at which a small sample of the culture was pelleted by centrifugation (16,000 rpm for 2 minutes). Collected pellets were analyzed by SDS-PAGE using 4-20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad).

PROTEIN EXTRACTION AND PURIFICATION UTILIZING Ni-NTA AGAROSE RESIN

Pellets of induced *E. coli* cells containing His-catalase were suspended in B-PER native protein purification buffer (Pierce; RT) with protease inhibitors (Roche, mini-EDTA-free;1 mL/g pellet mass). Proteins were extracted by incubation with lysozyme and DNAseI (4 mM each) for 15 minutes at RT while nutating and cell debris was removed after centrifugation at 15,000 rpm for five minutes at 4°C. The enzyme was purified in batch mode by affinity chromatography with HisPur Ni-NTA Agarose Resin (1 M; ThermoFisher). The column was washed 3 times 10 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 20 mM Imidazole; finally, the enzyme was eluted with 10 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 200 mM Imidazole. Pellets, crude extracts, and collected fractions were analyzed by SDS-PAGE using a 4-20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad). Final protein concentration was insignificant when read on a Nanodrop.

PROTEIN EXTRACTION AND PURIFICATION UTILIZING Ni-NTA MAGNETIC BEADS

Following the lysate prep steps from *Protein Extraction and Purification Utilizing Ni-NTA Agarose Resin*, the enzyme was purified in batch mode by affinity chromatography with HisPur Ni-NTA Magnetic Beads (1 M; ThermoFisher). The column was washed 2 times 10 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 20 mM Imidazole; finally, the enzyme was eluted with 10 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 200 mM Imidazole. The final eluted

fraction showing the presence of a band the expected size (~70) was dialyzed against 50 mM Tris-HCl buffer pH 7.0, diluted 50 % with glycerol and stored at -20°C. The concentration was measured via Bradford assay to be 59.5 µg/ mL (Sigma-Aldrich). Pellets, crude extracts, and collected fractions were analyzed by SDS-PAGE using a 4-20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad). Final protein concentration was determined via Bradford assay to be 222.83 ug/ mL.

SPECTROPHOTOMETRIC CATALASE ASSAY

A simple spectrophotometric assay utilizing a carbonato-cobalt (III) complex was optimized for purified *P. cryohaloentis* catalase from an existing protocol (Hadwan, 2018). The catalase source sample (varying concentrations) was mixed with 10 mM hydrogen peroxide in a phosphate buffer solution (pH 7.0, 50 mM) and incubated at 37°C for two minutes. The sample was thoroughly mixed by inversion and then treated with a cobalt-bicarbonate stop bath (cobaltous nitrate hexahydrate, 3.485E-3 M; sodium hexametaphosphate, 8.15E-4; sodium bicarbonate, 0,96417 M). The solution incubated at RT in the dark for 10 minutes. The solution was analyzed using a Nanodrop spectrophotometer at 440 nM. The resulting absorbances were compared to the standard (catalase source sample and working solution) using **Equation 1**. A sample prep table is provided below.

Table #. Sample catalase assay preparation table.

Reagents	1X dil.	Standard	Blank
Catalase Source Sample	50 uL	--	--
50 mM Tris-HCl	--	50 uL	150 uL
Hydrogen Peroxide Solution	100 uL	100 uL	--
Invert and incubate at 37°C for 2 minutes then ADD:			
Working Solution	600 uL	600 uL	600 uL
Invert and incubate at RT for 10 minutes in the dark.			

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