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Immobilization of Enzymes from Rhodococcus Rhodochrous DAP 96253 with Targeted Uses for the Bioconversion of Acrylonitrile to Bioacrylamide and for the Novel Therapeutic Treatment of Acute Lymphoblastic Leukemia

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IMMOBILIZATION OF ENZYMES FROM *RHODOCOCCUS RHODOCHROUS* DAP 96253 WITH TARGETED USES FOR THE BIOCONVERSION OF ACRYLONITRILE TO BIOACRYLAMIDE AND FOR THE NOVEL THERAPEUTIC TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

by

BRIANNA CHILTON

Under the Direction of George Pierce, PhD

ABSTRACT

Acrylamide (AMD) is an important industrial chemical used in coagulators, water treatment, soil conditioners, mineral refining, paper treatment, adhesives, paints, and petroleum recovering agents. It is typically shipped in an aqueous solution of 30-50% w/w acrylamide, with 40% being preferred where transportation distances may be significant. 20% w/w is employed where the AMD is used to make PAM at the point of manufacture. To bypass the cost of shipping a solution that is 80-50% w/w water, one can locally produce AMD or poly-AMD using microorganisms to convert acrylonitrile (AN) to AMD. Induced cells of *Rhodococcus rhodochrous* DAP 96253 produce high levels of the enzyme nitrile hydratase, which is capable of converting acrylonitrile to acrylamide. In this study, whole cells, immobilized whole cells,

lysate, free purified enzyme, and immobilized purified enzyme derived from induced cells of *R. rhodochrous* DAP 96253 were used to compare the production of 20% to 40% AMD from AN.

Acute lymphoblastic leukemia (ALL) is a rare blood cancer of the bone marrow. While ALL is less than 1% of adulthood cancers, it comprises 25% of childhood cancers and 80% of leukemia in children. The regression of acute lymphoblastic leukemia has been linked to the enzyme L-asparaginase. This enzyme catalyzes the degradation of L-asparagine into aspartic acid and ammonia. While this enzyme is readily found in bacteria, plants, and animals, it is not readily found in humans. The cancerous cells of ALL rely on asparagine from human cells to supplement their metabolism in order to grow and divide. Adding L-asparaginase to a patient's plasma and depleting L-asparagine leads to cell apoptosis, killing the cancerous cells. The survival rate for patients 14 and younger has increased to >90%. However, 50% of patients showing recurrence within 10 years with the majority of those patients showing relapse within the first 2 years. Induced cells of *Rhodococcus rhodochrous* DAP 96253 exhibit commercially comparable asparaginase activity with decreased levels of endotoxins. Further research on the expression, purification, and pegylation of nitrile hydratase with asparaginase activity was conducted with the immediate goal of preserving enzymatic activity in pegylated, i.e. immobilized, cells.

INDEX WORDS: Bioacrylamide, Bioconversion, Biotechnology, Cell Immobilization, Enzyme Immobilization, Enzyme Utilization, Industrial Microbiology, Medical Microbiology, Pharmaceuticals, Therapeutics

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Office of Graduate Services

College of Arts and Sciences

Georgia State University

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DEDICATION

I would like to dedicate this dissertation to everyone in my life. The accumulation of this final stage of my educational science career has not come easy.

My husband, James Chilton. I remember early on in my career sitting around with fellow lab mates and them talking about how their significant others were bored or lost as they talked about the intricacies of microbial metabolism. My husband is not that person. Since I started on this microbiological journey, my husband has been reading journal articles behind my back just so he can bring up my favorite subject at the dinner table . . . or at a dinner party. We're real fun at parties but we have fun together. I love you.

From day one, my parents have been a constant source of support in my life. From being a veterinarian to a park ranger to a shepherdess to a microbiologist, Jimmy and Jheri Galbreath told me "You've got this!". I didn't always believe them but here we are. I love y'all.

To my brother, you may in the medical field but I'm glad we can talk about science together. I love you.

To my in-laws, friends (looking at you Emily Windham), extended family, acquaintances, network connections, bosses, coworkers, colleagues, lab members, mentors, and more: thank you. I truly appreciate the support and guidance over the past 5 years and beyond.

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I would like to thank both the biology department and Georgia State University for allowing me a coveted spot into their graduate program.

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LIST OF ABBREVIATIONS

1-Hexene Monooxygenase	(1-HMO)
Acrylic Acid	(AA)
Acrylonitrile	(AN)
Acrylamide	(AMD)
Acute Lymphoblastic Leukemia	(ALL)
Amidase	(AMDase)
Asparaginase	(ASNase)
Bioacrylamide	(BioAMD)
Calcium Alginate	(Ca-Alg)
Cell Free Lysate	(CFL)
Cobalt	(Co)
Distilled Water	(DI)
Glutaminase	(GLNase)
Hardened Calcium Alginate	(H-CaAlg)
High-Molecular-Mass Nitrile Hydratase	(H-NHase)
Low-Molecular-Mass Nitrile Hydratase	(L-NHase)
Monomethoxypolyethylene glycol	(PEG)
Nickel	(Ni)
Nitrile hydratase	(NHase)
Non-hardened Calcium Alginate	(NH-CaAlg)
Polyacrylamide	(PAM)
Polyethyleneimine	(PEI)
Polyethyleneimine-Glutaraldehyde	(PEI-Glu)
Purified Enzyme	(PU)
Urea	(U)

1 INTRODUCTION

1.1 Historical Use of Microbes

Humans have been using microbes for the production of goods and services starting as early as 7000 BC. In ancient times, microbes were used to produce fermented breads and beverages, as was the case in China with rice, honey, and fruit [1, 2]. While some people had budding ideas about the unseen science happening in these products, it wasn't until Antonie van Leeuwenhoek developed a lens powerful enough to see these microscopic organisms in 1675 that the science of microbiology was enabled [3]. Decades later, the science behind fermentation, specific human diseases, and spoilage was burgeoning [4]. These and other advancements in microbiology, like the discovery of enzymes produced by microorganisms [5], helped lead to microbes being used in industrial settings and being manipulated genetically and situationally, i.e biotechnology, for better use in industrial settings. This process, even before being named, was used for the scaled-up fermentation of beer in ancient times [6, 7].

Besides fermentation, another microbial process used in industry is enzymatic conversion. In contrast to the biological nature of fermentation, enzymatic conversion is a biologically mediated chemical process in which one substance (the substrate) is converted into another substance (the product) using an enzyme(s). This chemical conversion is carried out at a faster rate by the use of a catalyst in the form of a microorganism's enzyme [8]. These enzymes may either be within the living cell in which they are produced, free or immobilized, or in free form, which can also be immobilized. This process is also often referred to as biocatalysis [9]. Leading up to 1916, the use of microbes to produce industrially important chemicals began when Chaim Weizmann converted starch into butanol and acetone using *Clostridium acetobutylicum*. The acetone was later used in explosives during World War 1. The use of microbes in the production of items,

such as paper, chemicals, medicine, and more has been successful in decreasing environmental pollution, increasing the quality of one's final product, and decreasing overall cost in comparison to the more conventional chemical processes. To be widely used, decreasing cost along with a competitive product must be achieved. These accomplishments have led to the increase in using microbes in the commercial production industry [10]. One of the most successful industrial applications of enzymatic hydrolysis is the conversion of acrylonitrile to acrylamide [11]. While chemical production is still the common approach for acrylonitrile used in most production facilities, the enzyme nitrile hydratase has been used to produce 30,000 tons/year of acrylamide in smaller boutique-sized processes while creating lower energy consumption, safer bioprocesses, and higher purity. This bioconversion system involves numerous non-genetic components that can be manipulated in order to change both the cost and efficiency. For some industrial products, these manipulations have been tested [8]. For certain modern medicines, such as the enzyme L-asparaginase, certain structural changes have been found to benefit the efficacy of the product. When new microorganisms are being used to develop an enzyme, these structural changes must be tested as well as the original structural form [12]. By changing some of these conditions and testing them, scientists can provide the best, known way in which these industrial or pharmaceutical processes should be carried out.

1.1.1 *Rhocococcus rhodochrous*

The genus *Rhodococcus* was first used to describe red-pigmented bacteria in 1891 by Zopf [13]. Eighty-six years later, the genus had been expanded to include other species of bacteria within the rhodochrous complex. Today, this complex is used to define a new genus of varying species that appeared to be closely related to but did not belong in the genera *Nocardia*,

Corynebacterium, or *Mycobacterium* based on varying characteristics [14]. These four genera are often grouped together (Mycolata) due to that fact that they all possess characteristic mycolic acids in the cell walls [15]. *Rhodococcus* species are aerobic, Gram-positive, non-motile, mycolate-containing, non-spore forming, no aerial mycelium formation, nocardioform actinomycetes [16]. In the decades since then, multiple species have been combined, created, moved to other established genera, or moved to newly established genera [17]. Currently, there are 12 species assigned to the *Rhodococcus* generus, including ten non-pathogenic species: *R. coprophilus, R. erythropolis, R. globerulus, R. marinonascens, R. opacus, R. percolatus, R. rhodnii, R. rhodochrous, R. ruber, and R. zopfii*, and two pathogenic species: *R. equi* and *R. fascians* [18]. While opportunistic infections in humans have been reported in a few of the non-pathogenic species (*R. erythropolis, R. globerulus, R. ruber,* and *R. fascians*), the virulence factors of *R. equi* and *R. fascians* in horses/humans and plants, respectively, lead to their inability to be used in industrial settings [19]. However, many of the environmental species of *Rhodococcus* are used for industrial and environmental applications due to their ability to degrade a large number of organic compounds, despite toxicity, to catalyze steroid conversion, and to bioremediate chlorinated hydrocarbons and phenolics [20, 21].

The heightened industrial use of *Rhodococcus* species for numerous clinical, industrial, and environmental applications is largely accredited to the genetic and physiological diversity of this genus. This diversity is responsible for giving the microorganism a broad range of metabolic functions, which is ultimately an effect of the large numbers of enzymes genetically possessed by the species. The biodegradation and biotransformation of many organic compound by members of the *Rhodococcus* genus is carried out by enzymes involved in the metabolism of the microorganism. While the complete genome for the known strains of *R. rhodococcus* is not

commonly found in literature, genomic sequencing completed on *Rhodococcus rhodochrous* ATCC 17895 was found to consist of 6,869,887 base pairs on average with 6,662 predicted genes and 6,609 protein-coding genes. This does not include plasmid DNA [22]. However, one of the largest bacterial genomes analyzed to date belongs to the *Rhodococcus* genus. *Rhodococcus jostii* strain RHA1 was found to consist of 9,702,737 base pairs [23]. This number is larger than the much used and highly documented bacteria *Escherichia coli,* which consists of 4,600,000 base pairs and 4,000 genes [24]. Besides size, the genome of *Rhodococcus* species is unique in its possession of genes that encode for multiple catabolic enzymes and pathways, small circular plasmids, large linear plasmids that contribute to substrate diversity and act as "mass storage", and the redundancy of many genes [21].

Rhodococcus rhodochrous has been of industrial significance due to its large number of mono- and dioxygenases and hydratases, specifically nitrile hydratase (NHase). Outside of just possessing nitrile hydratase, it is found that this microorganism possesses a "full nitrile metabolizing operon [], consisting of nitrile hydratases, regulators, amidase, and aldoxime dehydratase" with many proteins coding for genes with overlapping function (p. 180) [22]. This system is the main reason *Rhodococcus rhodochrous* was first looked at for biocatalytic hydration, specifically that of bioconverting acrylonitrile to acrylamide [22]. *Rhodococcus rhodochrous* DAP 96253 (pictured below in Figure 1) has been studied for its potential use in chemical, agriculture, wildlife, and pharmaceutical industries given its documented production of nitrile hydratase (NHase), asparaginase (ASNase), glutaminase (GLNase), 1-hexene monooxygenase (1-HMO), and amidase (AMDase) [25-28].

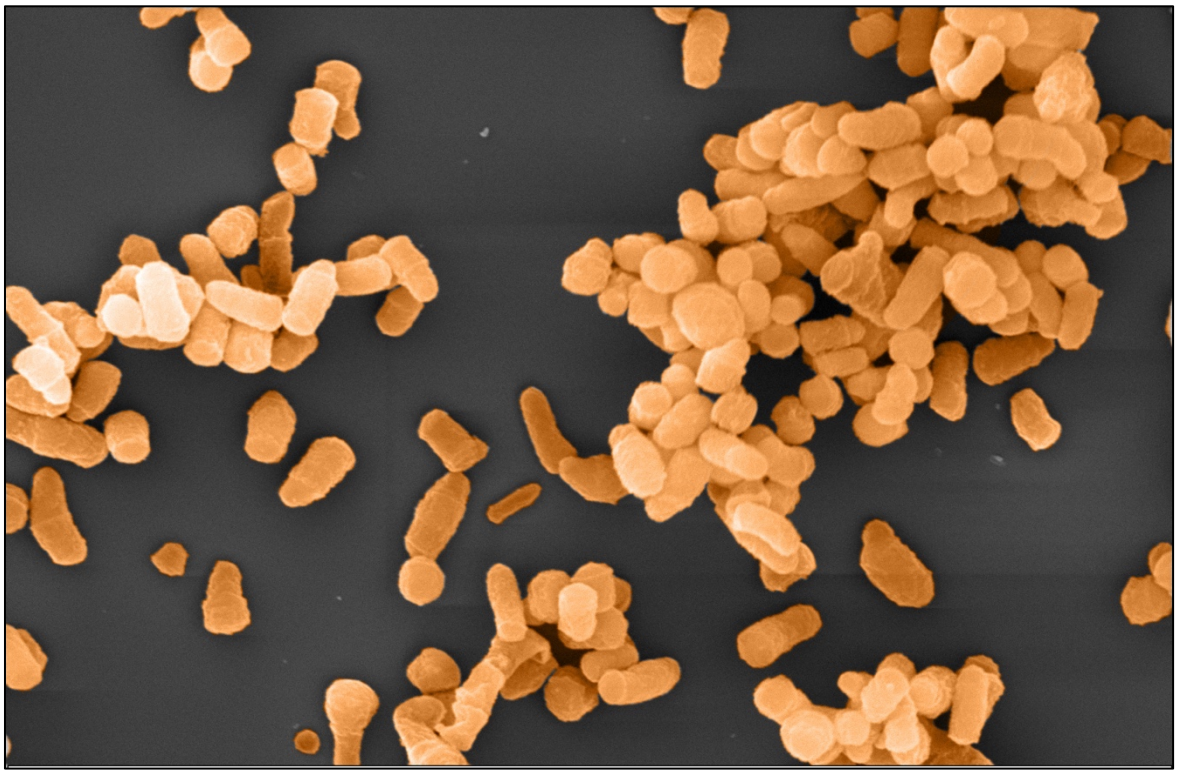


Figure 1: A pseudo-colored scanning electron microscope image of Rhodococcus rhodochrous DAP 96253 fermentation cells. The pseudon-color used here was matched to the actual pigment produced by the cells. (©John Neville, Georgia State University) [28]

Current research using the metagenomic data generated from IonTorrent and adapting work from Kobayashi and Zhou being conducted by Fortino Pineda Veloz in Dr. George Pierce's lab proposes the schematic in Figure 2 for the nitrile operon in *Rhodococcus rhodochrous* DAP 96253 by using the metagenomic data generated from IonTorrent and adapting work from Kobayashi and Zhou [29-31]. In this study, *Rhodococcus rhodochrous* DAP 96253 will be utilized to produce highly-active immobilized enzymes, such as nitrile hydratase and nitrile hydratase with asparaginase-like activity, for the enhancement of bioconversion, bioremediation and other therapeutic treatments.

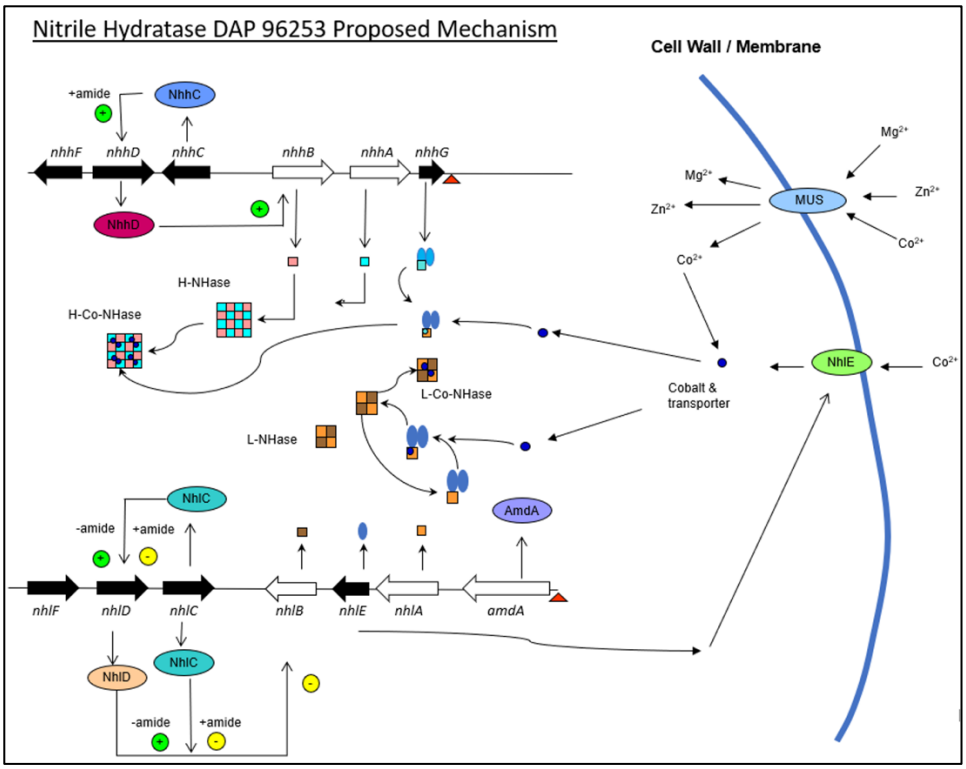


Figure 2: Schematic for the proposed nitrile operon in Rhodococcus rhodochrous DAP 96253. (F. Pineda modification of the Pierce model based upon the initial model proposed by Kobayashi, et al.)

1.2 Acrylamide

Acrylamide (AMD) is an important commodity chemical that is used in the formation of the polymer, polyacrylamide (PAM) [32]. PAM is used primarily in water treatment and in various other industries as coagulators, soil conditioners, mineral refiners, stock additives for paper treatment and sizing, adhesives, paints, and petroleum recovering agents [8]. As of 1995, worldwide demand for AMD was estimated at 200,000 metric tons per year [33]. As of 2011, that current demand exceeded 470,000 metric tons per year [34]. Acrylamide has been traditionally produced via chemical hydrolysis of AN using either sulfuric acid or copper salt catalyst. However, these methods were both complicated and environmentally unfriendly [35]. In 1976, Commeyras, et al. patented the first potentially commercial biological conversion of

acrylonitrile to acrylamide [36]. A comparison of the chemical and microbial processes can be seen below in Figure 3 [8].

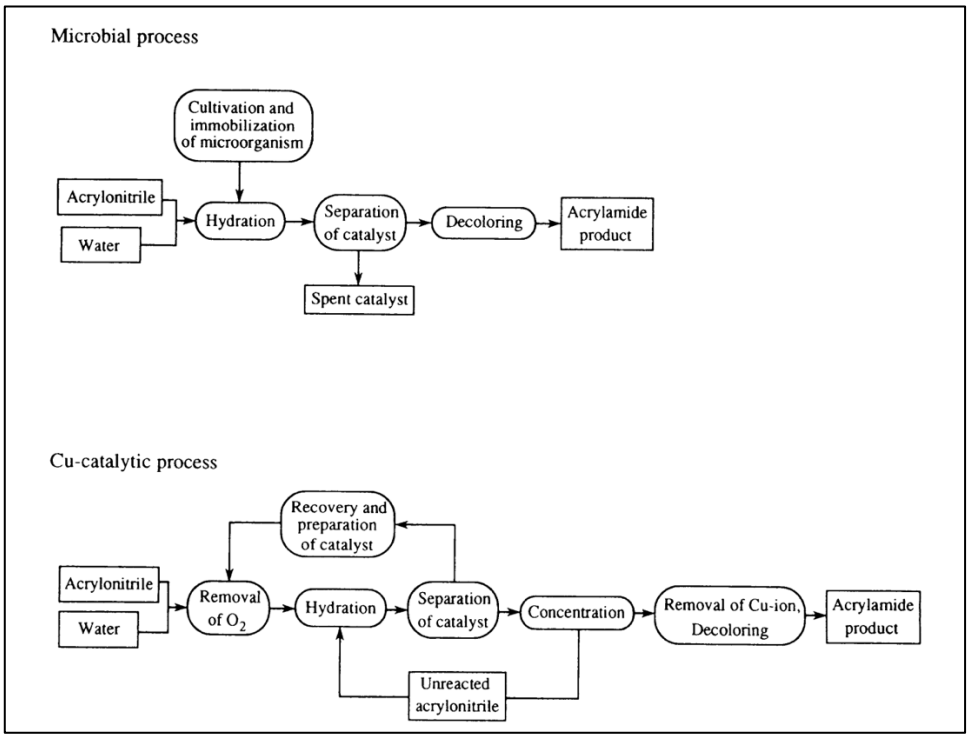


Figure 3: Comparison diagram of the chemical (Cu-catalytic) and microbial processes for the production of acrylamide from the conversion of acrylonitrile [8].

Since then, the microbial bioconversion process has become of greater interest due to its economic and technical advantages [35]. Acrylamide is often shipped as an aqueous solution of at least 20% and most typically at 40%w/w AMD. To bypass the costs which occur with shipping a solution that is 60-80%w/w water, one can locally convert AN, which is shipped as 100% AN and no water, to AMD and polymerize the AMD on-site, if needed. The amount needed does not often justify the cost of building a plant for the continuous production using a chemical catalyst but can be cost effective to use for smaller facilities to produce cheaper acrylamide. Other problems with using a chemical catalyst involve the high rate of formation of acrylic acid instead of AMD, the production of byproducts such as nitrylotrispropionamide,

ethylene cyanohydrin, and bis-acrylamide, and the polymerization that can occur at the double bond of both the substrate and the product. Further complications arise from these impurities, such as the compounds limiting the molecular weight achieved in PAM due to increased branching. While the use of the microbial process can still create unwanted by-products, like the further conversion of acrylamide to acrylic acid via the enzyme amidase, using microorganisms to catalyze the hydrolysis of acrylonitrile to bioacrylamide works in smaller, readily-available settings and solves some of the problems with the chemically catalyzed procedure [8]. *Rhodococcus rhodochrous* J1 has been used for years as the third-generation catalyst for the microbial bioconversion of acrylonitrile to acrylamide [37].

1.3 Nitrile Hydratase

There are two recognized enzymatic pathways by which a microorganism can degrade a nitrile for metabolic use. The first uses the enzyme nitrilase which directly converts a nitrile into its corresponding acid and ammonia. The second uses the enzymes nitrile hydratase and amidase to catalyze the step-wise hydrolysis of nitriles to their corresponding amides and then to their corresponding acids with the release of ammonia for metabolic use [38]. Nitrile hydratase being adequately named for its ability to hydrate a nitrile to an amide [39]. These two processes are compared below in Figure 4 [40, 41]:

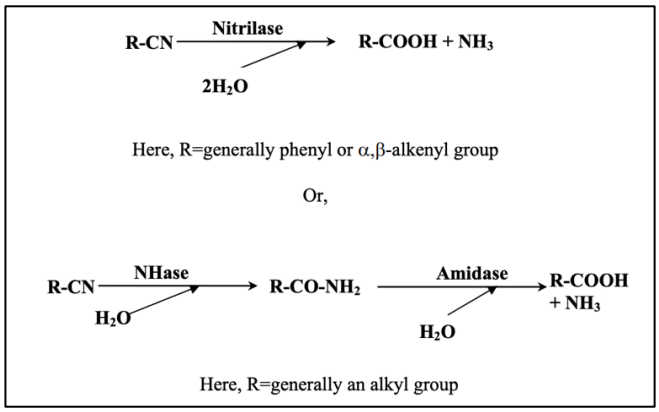


Figure 4: Comparison diagram of the two different enzymatic pathways for the microbial breakdown of nitriles into respective acids and ammonia [40].

While the nitrilase enzymatic pathway has not been formally studied yet in *Rhodococcus rhodochrous* DAP 96253, the nitrile hydratase and amidase enzymatic pathway is documented as being used in this microbe. Its ability to produce an intermediate amide from a nitrile in a separate reaction step, using nitrile hydratase, makes *R. rhodochrous* DAP 96253 a suitable choice for the industrial bioconversion of AN to AMD. This bacterial strain is also characterized as having much lower amidase levels than nitrile hydratase levels; thus, producing lower levels of unwanted by-products, like acrylic acid and ammonia, and higher levels of the product, acrylamide [40]. When growing *R. rhodochrous* DAP 96253, enhanced expression of the levels of nitrile hydratase produced by the bacteria can be accomplished by inducing the cells with cobalt (Co), urea (U), and nickel (Ni). *R. rhodochrous* DAP 96253 cells induced with Co/U were shown to produce the highest levels of nitrile hydratase [42]. This enhanced production has been seen before with cobalt(+/-urea)-induced and ferric-containing nitrile hydratase in *Pseudomonas chlororaphis* B23 and with cobalt(+/-urea)-induced and cobalt-containing nitrile hydratase in *Rhodococcus rhodochrous* J1. These nitrile hydratases have been evaluated and used industrially for the bioproduction of acrylamide [39]. It was first discovered in *R. rhodochrous* J1 that there

are two nitrile hydratases present in this organism: (1) high-molecular-mass, 520 kDa, nitrile hydratase (H-NHase) and (2) low-molecular-mass, 130 kDa, nitrile hydratase (L-NHase). The α-subunit of 26 kDa and β-subunit of 29 kDa of H-NHase (lane 2) and the difference in concentrations between inducing with Co/U (lane 4) and without (lane 3) can be seen below in Figure 5 (lane 1 is a molecular mass standard) [8]. The same purification and isolation was performed on *Rhodococcus rhodochrous* DAP 96253 in Dr. George Pierce's lab where the same α-subunit and β-subunit monomers were expressed, along with a novel 50 kDa αβ-subunit dimer and a 20 kDa protein band fragment (seen in Figure 6 below) [42].

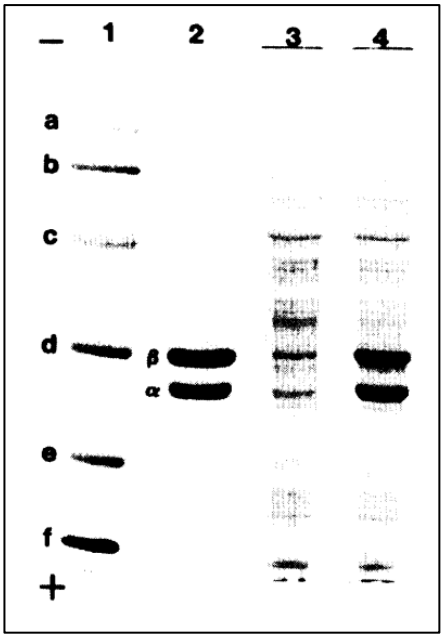


Figure 5: Protein expressions of (U)-induced cells of Rhodococcus rhodochrous J1 on SDS-PAGE. Lane 1 shows a molecular mass standard. Lane 2 shows the α- and β-subunits of H-Nase. Lane 3 and 4 show cell free lysate from non-Co-induced and Co-induced cells respectively. [8]

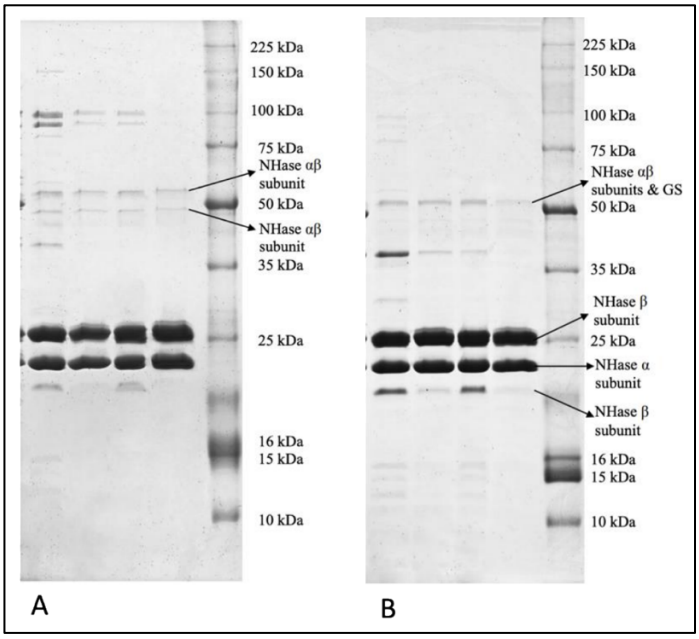


Figure 6: Protein expressions of Co/U-induced cells of Rhodococcus rhodochrous DAP 96253 showing the α- and β-subunits of H-Nase, a novel 50 kDa αβ-subunit dimer, and a 20 kDa protein band fragment. [42]

When induced with Co/U, it is found that over 50% of cell-free extract, proteins usable outside of the cell, is H-NHase. This high-molecular-mass nitrile hydratase is found to have higher heat stability and to have higher resistance to the toxicity of acrylonitrile and acrylamide. These characteristics make it very easy to set up a controlled production facility. This makes *Rhodococcus rhodochrous* strains highly valuable as a catalyst for the industrial bioconversion of acrylonitrile to bioacrylamide [8]. In relation to these findings and improvements in the levels and stability of nitrile hydratase, it is hypothesized that *R. rhodochrous* DAP 96253 will produce comparable bioconversion results to that of *R. rhodochrous* J1; thus, making it a desirable substitution for a catalyst in the microbial production of acrylamide.

1.4 Diverse Uses of Nitrile Hydratase

1.4.1 *Cellular Immobilization*

For industrial production purposes, cells that are reusable and maintain enzymatic activity are preferred for cost and time benefits. The immobilization of bacterial cells in a matrix can aid in the sustainability of the enzymatic function over time and the ability to reuse cells multiple times. Entrapping the cells via a variety of procedures can be used to create sustainability nitrile hydratase in *R. rhodochrous* DAP 96253. Most immobilization techniques create an initial decrease in enzymatic activity and, for some, a gradual decrease in overall activity levels over time. Consistent practice of enzymatic techniques shows increased results in the retention of initial enzymatic activity. Polyacrylamide (PAM) immobilization involves a polymerization that is created by a chemical reaction with the bacterial cells and acrylamide to form small cubes of cells. After the initial decrease in enzyme levels, PAM immobilized cells maintain most enzymatic activity for approximately 1-year while being stored at 30˚C. Calcium alginate (Ca-Alg) immobilization traps the cells in a calcium alginate gel and forms small, round beads. To better preserve the beads during the conversion process, polyethyleneimine (PEI) can be added during the last step of the procedure to "harden" these beads (Ca-Alg_hard). Past studies in the lab showed proper storage for the beads to be thorough washing and storage at 4˚C for maintaining consistent nitrile hydratase enzymatic levels. Polyethyleneimine-glutaraldehyde (PEI-glu) immobilization involves entrapping the cells in a cross-linkage of PEI and glutaraldehyde. Because the immobilized cells produced in this procedure are dry, rinsing the cells with phosphate buffer and storing them at 4˚C should provide proper storage [40]. Figure 7 below shows prepared immobilized whole cells compared to un-immobilized whole cells.

Figures 8, 9, and 10 show prepared immobilized cells compared to scanning election microscopic pictures depicting the cells in their respective immobilization matrices. Studies have shown these prepared immobilized cells have retained >90% of NHase activity after 1.5 years of storage, as shown in Figure 11 below [40]. Immobilized cells are commonly used in the biological production of acrylamide due to the numerous advantages they provide, such as the reduction of undesirable by-products from the cells, the ease of removing the cells from the final product, the ability to reuse the cells, and the increased stability in nitrile hydratase, as shown in Figure 11. These characteristics, along with the ability to produce acrylamide in quantities directly comparable to the production using free cells (as shown in Table 1 below), make immobilized cells a cost-effect option for production. It is hypothesized that immobilized cells will be the preferred catalyst version of *R. rhodochrous* DAP 96253 as a result of their bioconversion rates and ease of removing the cells.

Figure 7: Comparison images showing from left to right: free whole cells, PAM immobilized whole cells, PEI-Glu immobilized whole cells, and Ca-Alg immobilized cells (including left: non-hardened and right: hardened).

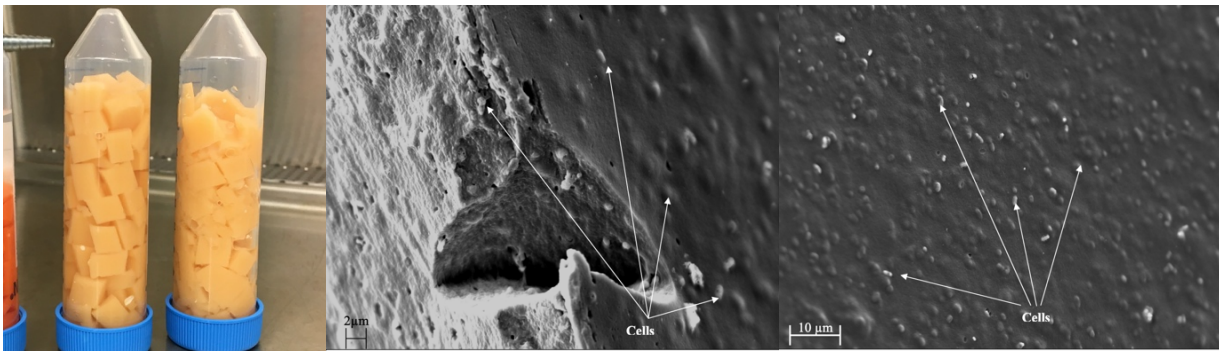


Figure 8: Comparison images showing from left to right: PAM immobilized whole cells, SEM image of the edge of a PAM cube, and SEM image of the inside of a PAM cube. The last two images show RrDAP96253 cells suspended in the PAM matrix [39].

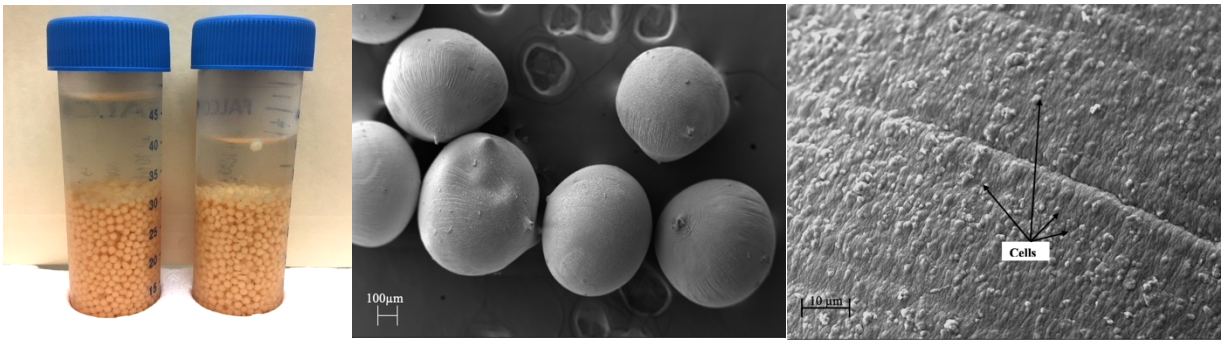


Figure 9: Comparison images showing from left to right: Ca-Alg immobilized whole cells, SEM image of Ca-Alg beads, and SEM image of the inside of a Ca-Alg bead. The last two images show RrDAP96253 cells suspended in the alginate matrix [39].

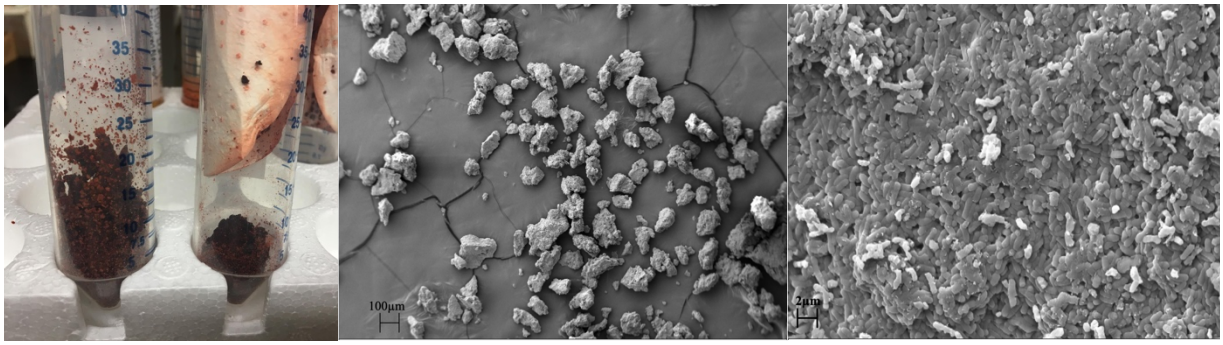


Figure 10: Comparison images showing from left to right: PEI-Glu immobilized whole cells, SEM image of PEI-Glu particles, and SEM image of the inside of a PEI-Glu particles. The last two images show RrDAP96253 cells immobilized in PEI [39].

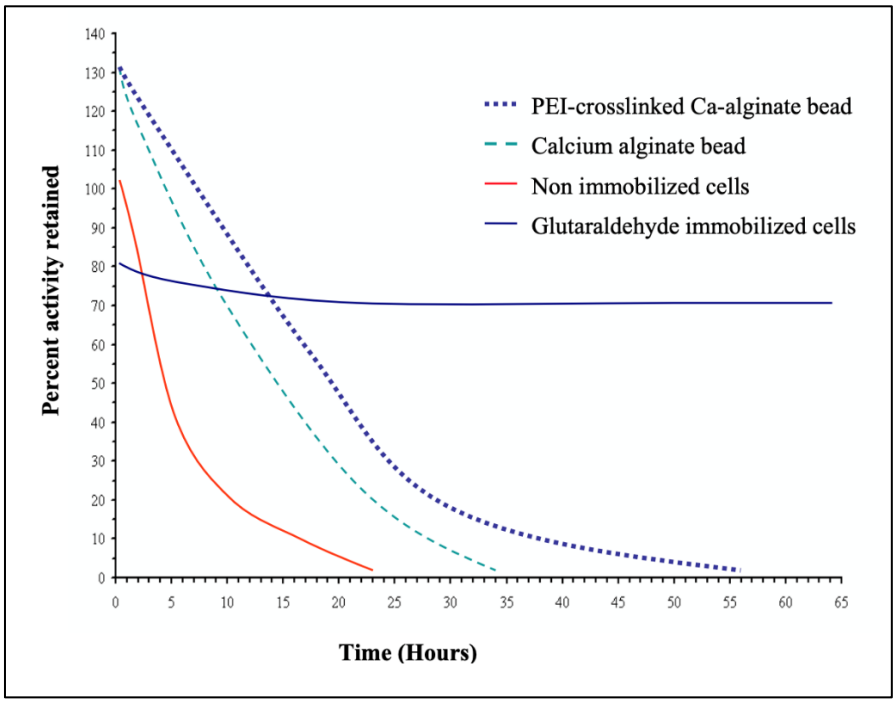


Figure 11: Chart showing the increased stabilized activity of nitrile hydratase in RrDAP96253 in PEI-Glut and Ca-Alg (hardened and non-hardened) immobilized cells versus free whole cells [40].

Table 1: AMD production (%w/w) with comparison for %(w/w) for whole vs immobilized, color, turbidity, conductivity, and foam test [9].

Item	Unit	Acrylamide produced by	
		Free cells	Immobilized cells
Concentration	%(w/w)	40	40
Color	APHA	40-50	10
Turbidity	NTU	3.1	1.5
Conductivity	µs/cm	180	30
Foam test	mL	Overflow	50-100

1.4.2 *Enzyme and Enzyme Immobilization*

By purifying and isolating the nitrile hydratase enzyme and using it either in free form or immobilizing it, a cleaner product can be produced than with using a whole or immobilized cell. This is due to the absence of amidase to further the conversion of acrylamide to acrylic acid and ammonia once it is formed. The absence of other cellular components is also credited to creating a product of increased quality [9]. Even with the already lowered amidase enzymatic activity of *Rhodococcus rhodochrous*, there is still enough present in cellular form to produce residual acrylic acid and ammonia. However, small amounts of acrylic acid can help stabilize AMD solutions [40]. The addition of cobalt during cell growth to increase nitrile hydratase activity is also proven to decrease amidase activity 20-90%; however, some activity is still present and can produce small amounts of acrylic acid and ammonia [43]. The advantages of using pure enzyme can lead to increased yields and significant cost benefits. Isolated enzymes come with disadvantages too [9]. The monetary cost of purifying and isolating enzymes is much higher than using either free or immobilized cells. This process is also quite costly time wise. Purification and isolation both happen after the cells have been fermented; thus, delaying the bioconversion process. Proteins are often water soluble which means retrieving the free enzyme after production is physically impossible or extremely expensive and time-consuming [44]. Purified enzymes are also far less stable than whole cells. This consequence has been remedied by the immobilization of enzymes, which also aids in the retrieval of the enzyme from the final product. Immobilized enzymes are considered more stable and robust than free-form enzymes [44, 45]. The usability, stability, and activity of the enzyme after immobilization all depend on the enzyme being used, pH, extant of crosslinking, the procedure performed, and the material to which the enzyme is attached [9]. Immobilization of enzymes also add more cost and time to the

production process [44]. These are all factors that must all be evaluated when assessing the use of free or immobilized enzyme in industry and lead to the hypothesis that immobilized whole cells will be the preferred catalyst over free or immobilized enzyme when using microbes to convert acrylonitrile to acrylamide. Despite this, the use of free or immobilized enzymes in acrylonitrile bioconversion has not been studied but hypothesized on and will be reported here.

1.5 L-ASPARAGINASE

Acute lymphoblastic leukemia (ALL) is a rare blood cancer of the bone marrow in which lymphoblast, immature white blood cells that normally develop into lymphocytes, maturation is blocked at an early stage. Instead of dying, as they should, the cells proliferate rapidly, explaining the ‘acute’ part of the name, and replace mature lymphocytes. While ALL is less than 1% of adulthood cancers, it comprises 25% of childhood cancers and 80% of leukemia in children with the peak infection age being between ages 1-4 years old [46].

In 1953, Dr. John Kidd showed the regression of two kinds of lymphomas, acute lymphoblastic leukemia and non-Hodgkin lymphoma, in mice by injecting normal guinea pig serum [47]. This regression was later linked to the enzyme L-asparaginase that is present in guinea pig serum. This enzyme catalyzes the degradation of L-asparagine, an amino acid, into aspartic acid and ammonia. While this enzyme is found in bacteria, plants, and certain animals, it is not found in humans but can be synthesized via alternative synthesis. The cancerous cells of ALL rely on the asparagine from human cells to supplement their metabolism in order to grow and divide. Adding L-asparaginase to the plasma and depleting L-asparagine leads to DNA, RNA, and protein synthesis inhibition, which consequently causes cell apoptosis. Thus, killing the cancerous, immature, lymphoblast cells. In the early 1970s, L-asparaginase was added to

modern chemotherapy protocols. In juvenile patients with ALL and in children with non-Hodgkin lymphoma, treatment outcomes and remission rates improved greatly to 5-year event-free 70% survival rate. While this rate is still the current rate for all patients, the survival rate for patients 14 and younger has increased to >90%. However, 50% of patients showing recurrence within 10 years with the majority of those patients showing relapse within the first 2 years [48].

Modern chemotherapy treatments involve medicines that use L-asparaginase purified from the Gram-negative bacteria *Escherichia coli*, like Oncaspar® and Elspar®, and *Erwinia chrysanthemi,* like Erwinase® [49]. However, Erwinase® is not currently approved by the FDA for use in the U.S., except on a compassionate basis [50]. Native forms of the enzyme from both bacteria, Oncaspar® and Erwinase®, and a derivative form solely from *E. coli*, Elspar®, are used in various treatment protocols. While treatment shows inhibitory effects on the infected lymphoblasts, there are concerns about its use due to the enzyme's side effects, premature inactivation, rapid elimination by the patient's body, and short duration of effect. This creates the need for frequent and numerous injections to keep the desired level in the bloodstream as asparaginase therapy works best when prolonged and intensified; leading to the rapid and thorough depletion of asparagine [12, 51]. Given the foreign relationship between the human body and the enzyme being injected, treatment is often discontinued due to toxic and/or immune response side effects, like hypersensitivity, thrombosis, pancreatitis, hyperglycemia, hepatotoxicity, and in some cases, anaphylactic shock. These side effects, most commonly hypersensitivity, are found in up to 30% of patients who are administered any form of the drug and result in the termination of the use of the drug in its current treatment [51]. Hypersensitivity, presenting as flush, rash, fever, renal impairment, pulmonary infiltrates, or death, is a common response to the lipopolysaccharide (LPS) endotoxins found in Gram-negative bacteria and the

products developed from those bacteria. Both Gram-negative bacteria, *Escherichia coli* and *Erwinia chrysanthemi*, present the challenge of removing endotoxins during the purification process given the heightened levels found in their membranes [52]. To decrease the side effects, to enhance the half-life from hours to days, which then increases the days between injections, the derivative form is modified by covalently conjugating the asparaginase to monomethoxypolyethylene glycol (PEG). This process leads to pegylated asparaginase, i.e. PEG-asparaginase or PEG-asp [12]. PEG-asparaginase is shown to have the same anti-leukemic efficacy as non-pegylated asparaginase [53].

In past research, *Rhodococcus rhodochrous* DAP 96253 has shown asparaginase activity, as shown below in Table 2 [54].

Table 2:Asparaginase activity present in R. rhodochrous DAP 96253 free whole cells [54].

Enzyme activity (IU/mgCDW)	**NHase**	**ASNase**	**GLNase**
	352.9±44.6	16.4±0.4	22.8±2.2

Given *R. rhodochrous* DAP 96253 is Gram-positive, has significantly lower levels of endotoxins, and exhibits commercially comparable asparaginase activity, further research on the expression, purification, and pegylation of nitrile hydratase with asparaginase activity will be conducted with the immediate goal of preserving enzymatic activity in pegylated, i.e. immobilized, cells [54]. This immediate goal supports the long-term goal of extending the half-life of asparaginase in the bloodstream and to decrease the side effects associated with this

therapeutic treatment. It is hypothesized that asparaginase purified from *R. rhodochrous* DAP 96253 and pegylated will produce improved and stable enzymatic activity making it a viable source for the production of therapeutic treatments.

1.6 PURPOSE OF STUDY

The purpose of this study has been divided into three aims, two major and one minor:

1.6.1 *Major Aim #1*

The evaluation of enzyme formulations from induced cells of *Rhodococcus rhodochrous* DAP 96253 for industrial uses.

1.6.2 *Major Aim #2*

The continued evaluation of the bioconversion capabilities of *Rhodococcus rhodochrous* DAP 96253, with focus on the bioconversion of acrylonitrile to bioacrylamide.

1.6.3 *Minor Aim #1*

The evaluation of immobilized fully-isolated asparaginase or nitrile hydratase with asparaginase-like activity from induced cells of *Rhodococcus rhodochrous* DAP 96253 to be used for possible novel treatments.

1.7 HYPOTHESIS

The proposed results are as follows:

1.7.1 *Major Aim #1 Hypothesis*

Improvement in the levels of nitrile hydratase and nitrile hydratase with asparaginase-like activity (i.e. increased specific activity and stability) will be expressed, purified, and immobilized using a variety of different techniques. The evaluation and outcome of differing levels of enzymatic activity and enzymatic stability from varying methodology will provide useful insight for developing practical enzymes from *Rhodococcus rhodochrous* DAP 96253 for various novel uses, such as bioconversion, bioremediation, and therapeutic treatments. Stability and activity of isolated enzyme(s) of interest should be different from that seen for whole cells establishing a path forward for the most promising use of these enzymes.

1.7.2 *Major Aim #2 Hypothesis*

Past and ongoing research has been used to define a process capable of the consistently high production of bioacrylamide via the bioconversion of acrylonitrile using either whole or immobilized cells of *Rhodococcus rhodochrous* DAP 96253. The use of cell-free lysate and purified and/or immobilized nitrile hydratase will be investigated to clarify the practical utility of the most promising mode to employ *R. rhodochrous* DAP 96253 derived catalyst to produce bio-acrylamide. It is hypothesized that immobilized cells of *Rhodococcus rhodochrous* DAP 96253 will be deemed the most consistent, productive, and cost-valuable of the methods conducted.

1.7.3 *Minor Aim #1 Hypothesis*

The immobilization of purified asparaginase and/or nitrile hydratase with asparaginase-like activity from *Rhodococcus rhodochrous* DAP 96253 should provide a catalyst with significantly improved half-life versus the naked enzyme of *R. rhodochrous* DAP 96253 and of comparable activity levels of L-asparaginase from other bacteria, such as *Escherichia coli* and *Erwinia chrysanthemi,* in its use for the therapeutic treatment of acute lymphoblastic leukemia (ALL). It is hypothesized that pegylated asparaginase from *Rhodococcus rhodochrous* DAP 96253 will give useable and longer lasting activity levels for the treatment of ALL.

2 METHODS

2.1 Culture

2.1.1 *Acquisition*

Rhodococcus rhodochrous DAP 96253 was initially derived from *Rhodococcus* sp. strain ATCC 55899 (which was deposited with the ATCC at the time of the initial patent filings, No. 5863750, involving *R. rhodochrous* DAP 96253). Several generations of the cell line have been produced throughout the years and were utilized for this research.

2.1.2 *Fermentation*

Induced cell paste was obtained via fermentation using a Biostat C 20L fermentor by members of the Pierce Lab at Georgia State University using the following method:

Inocula were prepared from *Rhodococcus rhodochrous* DAP 96253 cultures started from 30% glycerol stocks stored at -80˚C and grown in nutrient broth (NB). After 2 days, 100mL of the cell suspension was transferred to a 2L with 1x mR3A broth and incubated at 30˚C while shaking at 250rpm for 3 days. This cell suspension is then used as the inoculum for the seed bioreactor. The fermentation vessel also contained 6.6L of dd H_2O, 2L of 10X mR3A (16g/L urea for 10L batch) and 20ml of 20% antifoam 204. Initial glucose concentration was 1.58g/L. The feed was 8L YED broth (0g Maltose + 80g/L Glucose, 16g/L Urea, 50mg/L $CoCl_2$) via a planned rate. Temperature was maintained at 30°C. pH values were maintained at 7. Dissolved oxygen was maintained at 30%. Complete details are shown below in Appendix A [55].

2.1.3 *Storage*

Cell paste was collected from the sterile Carr-powerfuge bowl and immediately wrapped in aluminum foil for sterile transfer to the biosafety cabinet. Within the biosafety cabinet, cell paste was aliquoted out into 50gm portions and stored in sterile 50mL FalconTM (ThermoFisher

Scientific, Waltham, MA) conical centrifuge tubes. The remaining cell paste was kept in aluminum foil and stored in 4.4L polypropylene containers (Tupperware, Leominster, MA) at 4°C for long-term storage. [55]

2.2 Chemical Reagents

AN and all chemicals used to perform various immobilization techniques were obtained through Sigma Aldrich unless otherwise stated.

2.3 Whole Cell Immobilization Techniques

2.3.1 *Polyacrylamide Immobilization (PAM Immobilization)*

5g (wet weight) of fermentation paste were suspended in 40 ml DI H2O. This suspension was set up on ice with constant stirring. 40 ml of thoroughly mixed acrylamide-bisacrylamide solution (4.5 g AMD and 0.5 g N-methylbisacrylamide in DI water) was added. 5 ml of 3-dimethylaminoproprionitrile and 10 ml of 2.5% potassium persulfate solution were prepared next. Respectively, both were added in rapid succession to the original 80ml solution mixing on ice. This mixture is then quickly poured into empty petri dishes at a thickness of 2mm, which was marked on the dish. The dishes were incubated at room temp for 60 min for complete polymerization. The gel was cut into thin strips and then further cut into small cubes of 2-3-5 mm with a large knife to ensure the cubes do not tear. The cubes were washed 3x with DI H2O to remove residual monomers and stored at 4˚C in DI H2O. 5g of fermentation paste produces 50g of PAM immobilized cubes. Comparison to past method used in lab is shown below in Appendix B [40].

2.3.2 *Calcium Alginate Immobilization (Ca-Alg and Ca-Alg w/ PEI Immobilization)*

5g cells were suspended in 15ml 50mM Tris-HCl buffer. The cell suspension was mixed with an equal volume of 2.5% sodium alginate (alginic acid). The alginate-cell mixture was added dropwise to a 0.5M calcium chloride solution by pumping through 1/8 ID Teflon tubing (C-Flex) and 20-gauge needle. Beads were filtered using steel mesh. Non-hardened beads were then stored. Hardened beads were then transferred to 0.5M calcium chloride with 0.5% PEI (w/v) for crosslinking. Both were then stored at 4˚C. Comparison to past method used in lab is shown below in Appendix C [40].

2.3.3 *PEI-Glutaraldehyde Immobilization*

5g cells were suspended in 41.7g of ddH2O in a 50ml centrifuge tube. Approximately 833.3mg of PEI was added into the tube by using a glass rod. Allow the PEI to drip into the tube that is balanced on a scale. Vortex until homogeneous. The tube was placed on ice to cool at 4˚C for 15 minutes. 1.7ml of 25% glutaraldehyde solution was added and the mixture was inverted several times and then centrifuged at 10,000rpm for 5 minutes. Filter paper, Buchner funnel, and a suction flask was set up in the fume hood. The supernatant was discarded, and the cell pellet was spread out on the filter paper. Suction was turned on until the cells were visibly dried. The filter paper was left overnight at room temperature in the hood. The cells were stored the following morning at 4˚C. Comparison to past method used in lab is shown below in Appendix D [40].

2.4 Cell Lysis

Cell lysis was conducted with 5gm of whole-cells of *R. rhodochrous*, which were suspended in 5mL of PB buffer, pH 7.2 for nitrile hydratase and pH 7.6 for asparaginase, with 5mM of 2-mercaptoethanol supplementation. The cell suspension was vortexed, using the Vortex Genie 2 (Avantor, Radnor, PA) until complete homogenization and sonicated on ice to maintain enzyme integrity. Sonication was conducted with a 550 Sonic Dismembrator (Fisher Scientific, Hampton, NH), containing a CL4 Ultrasonic Convertor (Misonix, Farmingdale, NY) and a ½" diameter tip. The cell solution was sonicated for 20min, using a 1-second on and 1-second off cycle. Following sonication, the lysed cells were centrifuged for 30min at 4°C at 13,000rpm using the Avanti Centrifuge J-25 (Beckman Coulter, Brea, CA) with the JA-25.50 rotor. The resulting supernatant was recovered, and the process was repeated two more times [54]. 5gm of whole cells + 5ml of PB buffer lysed normally yields around 7.5mL of cell free lysate (CFL) after centrifuged [40, 54].

2.5 Enzyme Assays

Enzyme assays on whole-cells, lysate, and purified enzyme of *R. rhodochrous* were conducted for nitrile hydratase (NHase) and asparaginase (ASNase). All enzyme assays were conducted in 50mM phosphate buffer (PB), pH 7.2, and as triplicates. [55]

2.5.1 *Nitrile Hydratase*

For the determination of NHase activity, 40mg/ml of whole cells and 1µL of the cell free lysate or purified enzyme was suspended in 999µL of 1000 parts per million (ppm) of Acrylonitrile +99%, ACROS Organics (#AC149631000). The mixture was mixed for two minutes and terminated with 10µL of 4N H2SO4 and neutralized with 10µL of 4N NaOH.

Following the neutralization, 10µL of Amidase from *Pseudomonas aeruginosa*, Sigma-Aldrich (#A6691) was added to the reaction and incubated for 30min at 37°C. The ammonium concentration was determined by the addition of 2mL 2.5% sodium phenate, 3mL 0.01% sodium nitroprusside, and 0.15% sodium hypochlorite; these colorimetric reagents were added to a glass test tube containing 1mL of the previously incubated NHase samples. Additionally, an ammonium standard was created utilizing the same concentrations of colorimetric reagents and 1mL of ammonium standard ranging from 0ppm to 20ppm. The glass test tubes were vortexed for 30sec and incubated in the dark at room temperature (RT) for 30min before transferring 200µL of each sample to the Evergreen Scientific Polystyrene 96-Well Microplate (Caplugs, Buffalo, NY) in triplicates. The absorbance was measured using a Multilabel Counter Victor3V (PerkinElmer, Waltham, MA) plate reader at 630nm using established protocol. One unit of nitrile hydratase is defined as 1 µm/ml/min.

2.5.2 *Asparaginase*

For the determination of ASNase activity, 10mg/ml of whole cells and 100µL of the CFL or purified enzyme was suspended in 900µL of 100 ppm of L-asparagine ≥98%, Sigma-Aldrich (#A0884). The mixture was mixed for two minutes and terminated with 10µL of 4N H2SO4 and neutralized with 10µL of 4N NaOH. The ammonium concentration was determined and measured following the NHase protocol (See 2.5.1). One unit of asparaginase is defined as 1 µm/ml/min. [54-57]

2.6 **Nitrile Hydratase Purification** (using the CFL obtained from section 2.4)

The following purification is carried out using a Millipore Sigma HiTrap™ Fast Flow 5ml column. Wash the column by running 20mL of 20% ethanol through the column at a rate of

2mL/min. Equilibrate the column by running 20mL of equilibration buffer (150mM phosphate buffer at pH 7.2) through the column at a rate of 2mL/min. Run the diluted sample (1:2 dilution; 7.5mL of CFL + 7.5mL equilibration buffer) through the column at a rate of 400µL/min. Wash any unbound proteins from the column by running 10mL of equilibration buffer through the column at a rate of 400µL/min. Next, elute the protein by using a gradient of elution buffers of 0.1M, 0.25M, 0.5M, and 1M NaCl in equilibration buffer. Run 5mL of each elution buffer starting from lowest molarity to highest at 400µL/min. Collect the elutions in their own respective falcon tubes. (protocol modified from Nimna Wijewantha, adapted from Shirley Belshazzar) [58]

2.7 Enzyme Immobilization

2.7.1 *Calcium Alginate Immobilization*

5mL of purified enzyme (use the elution from the purification process with the highest enzymatic activity) were suspended in 15ml 50mM Tris-HCl buffer. The cell suspension was mixed with an equal volume of 2.5% sodium alginate (alginic acid) (this percentage can vary depending on the stability and accessibility needed). The alginate-cell mixture was added dropwise to a 0.5M calcium chloride solution by pumping through 1/8 ID Teflon tubing (C-Flex) and 20-gauge needle (needle size can vary depending on the size of bead needed). Beads were filtered using steel mesh. Non-hardened beads were then stored. Hardened beads were then transferred to 0.5M calcium chloride with 0.5% PEI (w/v) for crosslinking. Both were then stored at 4˚C.

2.8 Bioconversion of AN to AMD Using Various Forms of *R. rhodochrous* DAP 96253

5g of whole cell fermentation paste or the equivalent measurement of 5g of whole cells in various forms (i.e. immobilized cells, lysate, purified enzyme, or immobilized enzyme) are suspended in 50ml of DI H2O in a Falcon centrifuge tube. The suspension was then added to a small-scale bioreactor, pictured below in Figure 12, along with 200ml of DI H2O. The bioreactor was set up on a stir plate and left stirring for the remainder of the time at 30˚C. A syringe pump (Havard Apparatus, Ca. No. 55-5920) was programmed to pump AN into the bioreactor at variable rates using a glass syringe containing variable amounts of AN. Due to the toxic nature of this chemical, extreme care was taken when measuring out and transferring the AN. The syringe was connected to the bioreactor via appropriate tubing and a glass sampling pipette. Once the syringe pump was started, samples were taken every 30 minutes for variable run times to track the production of AMD. 1ml of solution was taken via a sampling port by using a glass pipette, appropriate tubing, and a 3ml syringe and transferred to a microcentrifuge tube. 10µl of H2SO4 were added immediately to stop the any further conversion. An image of the system while running is provided in Figure 13. The sample was then prepared to be analyzed via gas chromatography by centrifugation to remove the cells and dilutions of either 1:10, 1:20, or 1:40, as to not oversaturate the machine, into a glass autosampler vial. All samples were prepared in triplicate. Once finished, the solution is then centrifuged and placed in a glass storage vessel, covered in aluminum foil to shield from light, and stored at 2-8˚C.

Figure 12: Small-scale bioreactor with sampling port, addition port, and air relief filter port.

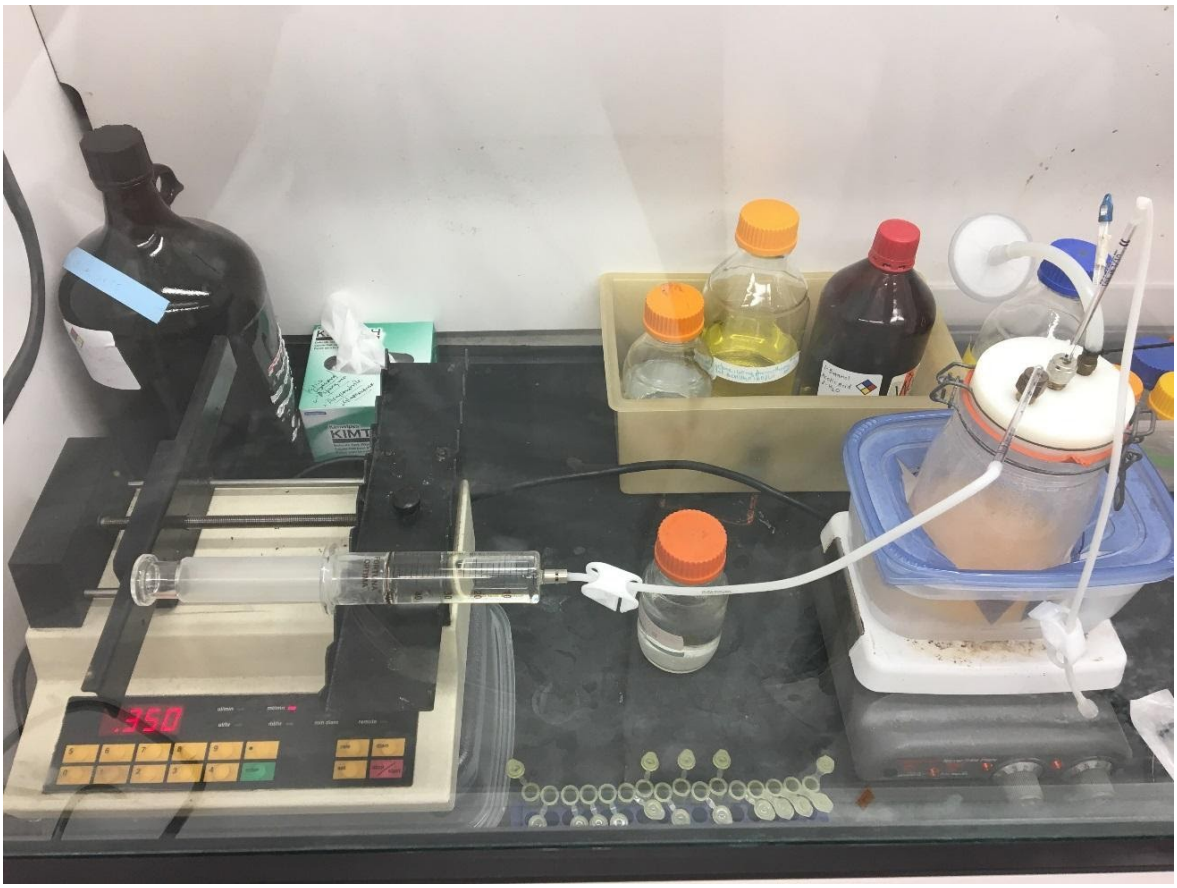


Figure 13: Bioconversion of AN to AMD process completely set up and running.

2.9 GC-FID Analysis of AN Production (along with AN and AA)

1 ul samples were analyzed using a Perkin Elmer Clarus 400 Autosystem XL Gas Chromatograph (Perkin Elmer, Shelton, CT) equipped with an FID detector and a ZB-WAX DB 642 capillary column. The following chromatographic conditions were used and are stored on the system under the file name "Acrylamide assayZB Wax" under the folder "Trudy" under the folder "S200methods" [59]:

Table 3: GC-FID chromatographic conditions for the detection of acrylonitrile, acrylamide, and acrylic acid in water.

Chromatographic Conditions	
Column	ZB Wax Column DB 642
Initial Isotherm	145°C for 1.00 min
Rate 1	20°C/min to 210°C, hold 6.00 min
Rate 2	50°C/min to 260°C
Final Isotherm	260°C
Run Time	10.25 min
Flow	2 mL/min
Injector Mode	Splitless, 1µL
Injector Temp	260°C
Transfer Line Temp	260°C

2.9.1 *GC-FID Standard Curve and Retention Times*

Solutions containing 0.1%, 0.25%, 0.5%, 0.75%, and 1% AMD in water were used to create a standard curve and regression equation for the analysis of the production of AMD as seen in Figure 14. This should be redone every few months with an R^2 value of 0.97 or better. Solutions containing 1% AN and 1% AA were analyzed. Subsequent retention times, shown below in Table 4, were used to identify the substrate, the product, and the unwanted by-product

by peak formation. After each bioreactor run, all samples were run on the GC to analyze the production of AMD over time using the regression equation from the most current standard curve.

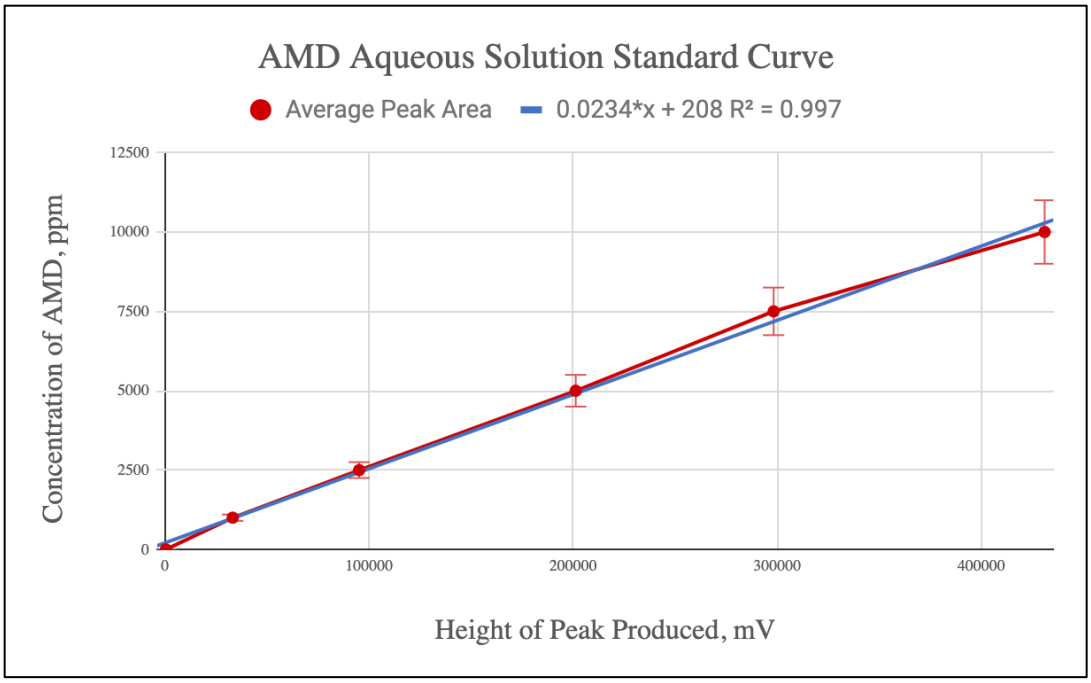


Figure 14: GC-FID standard curve for AMD. The red line depicts the trendline through the averaged sample points. The blue line depicts the statistical regression line.

Table 4: GC-FID retention times of AN, AMD, AA

Chemical	Retention Time
Acrylonitrile	1-2 minutes
Acrylamide	4.6-4.7 minutes
Acrylic Acid	<1 minute

2.10 GC-MS Analysis of AN Production

1 ul samples were analyzed using an Agilent 7890A Gas Chromatograph/Mass Spectrometer (Agilent, Santa Clara, CA) and a DB-5MS UI capillary column. The following chromatographic conditions were used and are stored under on the system under the file name "Acrylamide_ver1_02-2019" under the folder "Brie Galbreath" under the folder "methods" under then folder "GCMS" [60]:

Table 5: GC-MS chromatographic conditions for the detection of acrylamide in methanol.

Chromatographic Conditions	
Column	DB-5MS UI
Initial Isotherm	50°C for 2.00 min
Rate 1	45°C/min to 250°C
Rate 2	30°C/min to 260°C
Final Isotherm	260°C for 1.22 min
Run Time	8.00 min
Flow	1mL/min
Injector Mode	Pulsed Splitless, 30psi/0.5min, 1µL
Injector Temp	250°C
Transfer Line Temp	260°C

2.10.1 *GS-MS Standard Curve and Retention Times*

Solutions containing 0.015%, 0.0175%, 0.02%, 0.0225%, and 025% AMD in methanol were used to create a standard curve for the analysis of the production of AMD as seen in Graph 1. The retention time for acrylamide is shown below in Table 5. The MS is used for the detection of acrylonitrile or acrylic acid. After each bioreactor run, all samples were run on the GC to analyze the production of AMD over time.

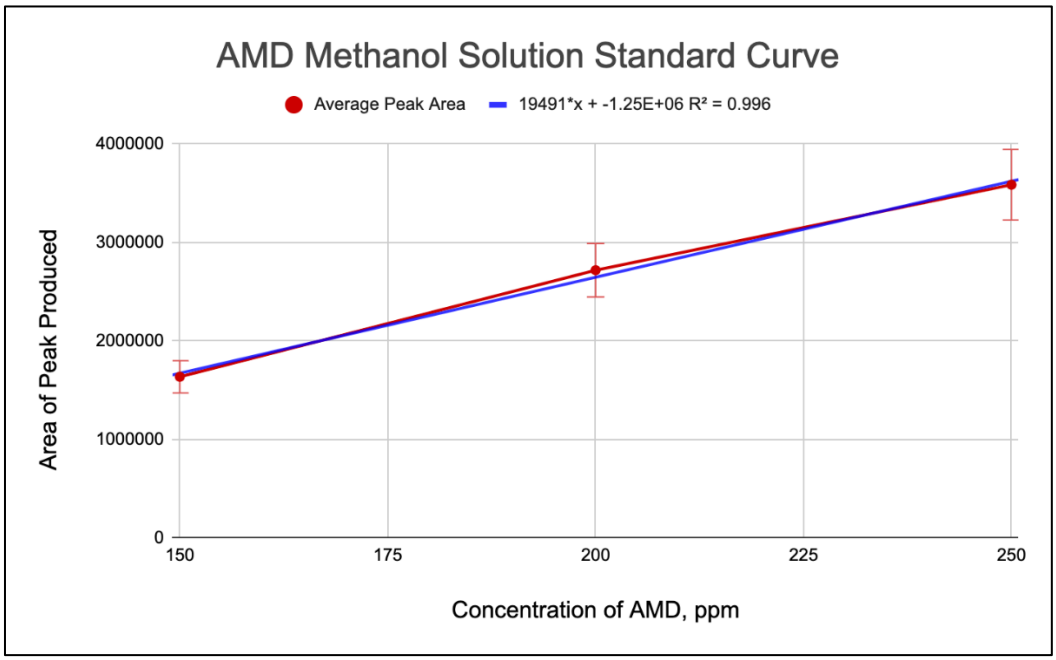


Figure 15: GC-MS standard curve for AMD. The red line depicts the trendline. The blue line depicts the statistical regression line.

Table 6: GC-MS retention time of AMD

Chemical	Retention Time
Acrylamide	4.6-4.7 minutes

2.11 Asparaginase Purification

2.11.1 *Post-lysis Dialysis*

All CFLs containing 2-mercaptoethanol were dialyzed in the following manner. 7 mL of CFL was loaded into an untreated Spectra/Por®_3 dialysis membrane (Standard RC Tubing of 3550 MWCO) and was dialyzed for up to 40 hours with constant stirring at 4°C against 300 mL of dialysis buffer. (50mM PB, 1.5% glycerol, 1% MgSO4.7H20, and 5mM 2-mercaptoethanol) [57].

2.11.2 *Purification*

2.11.2.1 Anion Exchange Chromatography

Anion exchange chromatography (AEX) was performed using a GE AKTAExplorerTM model 100 (Amersham Pharmacia, Piscataway, NJ) fast protein liquid chromatography (FPLC) controlled using the UNICORN 5.11 application. Protein peaks were detected using GE UV-900 detector at wavelength UV280, protein elutions were collected using the GE Frac 950 autosampler. AEX was executed using a Hitrap®Q HP packed with 34µm Q Sepharose ammonium anion exchange resin. 50 mM Tris-HCl, 50 mM MOPS or 25 mM PB at several pHs were evaluated as equilibration buffers. The column was equilibrated using 5 column volumes of equilibration buffer at a flowrate of 1 mL per minute, after which the dialyzed sample, diluted at a 1:1 ratio to a final volume of 10 mL using the equilibration buffer was loaded onto the AEX column at a flowrate of 0.5 mL per minute. Unbound proteins were washed from the column using 4 to 5 column volumes of equilibration buffer or until a baseline was reestablished. Elution of bound proteins was accomplished using a 1M NaCl linear gradient in assigned volumes of 5 mL at a flow rate of 1 mL/minute. These collected volumes were analyzed for ASNase and

GLNase activity. Samples exhibiting enzyme activity were pooled together and purified further using size exclusion chromatography [54].

2.11.2.2 Size Exclusion Chromatography

Size exclusion chromatography (SEC) was conducted post AEX. This process was executed using a GE AKTAExplorer™ model 100 (Amersham Pharmacia, Piscataway, NJ) fast protein liquid chromatography (FPLC) controlled using the UNICORN app 5.11 application, employing a Hiprep™ 16/60 Sephacryl™ S500 HR, a propylene column packed with dextran acrylamide particle platform allyl dextran and N, N methylenebisacrylamide. The column was first equilibrated using 2 column volumes of PB at pH 7.6 at a flow rate of 0.5 mL/minute, partially purified protein samples were then loaded onto the column at a flow rate of 0.5 mL/minute and the increasingly purified proteins were collected using the FRAC950 in 5 mL fractions. The fractions were analyzed for ASNase and GLNase activity [54].

2.11.3 *Pegylation*

The 4 fractions with the highest asparaginase activity from purification were combined and then divided into two 20 ml falcon tubes and mixed with .2ml-20mM 2-picoline borane (making up 1% of the final volume). Each tube was mixed with a different %PEG solution, shown below:

Table 7: PEG solution compositions and names

PEG Solution	Total % in Lysate	Referred to as
80% PEG solution	40% PEG Solution	PEG80
40% PEG solution	20% PEG Solution	PEG40

In addition, 2ml of CFL mixed with .02ml of 20mM 2-picoline borane (making up 1% of the final volume) was created in a falcon tube as a control. All the PEG/lysate samples were placed in a shaking incubator for 4 hours at 25°C. The duration of shaking will change as the best method to obtain mono-pegylated enzyme is established. The samples were then run through a size exclusion column (HiPrep Sephracyl 500) and proteins were sorted into 2ml fractions by size. (protocol provided by Palmer Amos, Georgia State University and is currently under further experimentation) [61]

3 RESULTS

3.1 Acrylamide Results

The following results are centered around the bioconversion of acrylonitrile to acrylamide using the various formulations of *Rhodococcus rhodochrous* DAP 96253 including free whole cells, immobilized whole cells, cell free lysate, purified nitrile hydratase, and immobilized nitrile hydratase. The conversion of AN to AMD is 1:1 conversion. To assess the production of AMD, the theoretical yield of AMD, i.e. the number of moles of AN pumped into the bioreactor as determined by the amount of AN added, will be compared to the actual number of moles of acrylamide produced. As AN to AMD is a 1:1 conversion, by adding 1 mole of water per mole of AN added, theoretical AMD can be calculated and then compared with actual AMD produced.

3.1.1 *Determination of R. rhodochrous DAP 96253 Nitrile Hydratase Activity via Different Growth Techniques*

Whether *R. rhodochrous* DAP 96253 cells are grown via media plates or fermentation and whether or not the fermentation paste is washed with buffer+Urea after being harvested, these variables result in different starting enzymatic activities. Plate grown cells showed nitrile hydratase activity of 106.38 um/mL/min while fermentation cells showed a nitrile hydratase activity of 744.52 um/mL/min when unwashed and 2298.27 um/mL/min when washed with buffer following their harvest. The results are shown below in Table 8. Raw data and calculations are as shown in Appendix E.

Table 8: Average nitrile hydratase activity for R. rhodochrous DAP 96253 depending on mode of growth, plate or fermentation, and whether the fermentation cells were washed after or not.

Growth Mode	NHase Avg Units (um/ml/min)
Plate	106.38 ± 19.80
Ferm Unwashed	744.52 ± 76.85
Ferm Washed	2298.27 ± 33.77

3.1.2 *Determination of R. rhodochrous DAP 96253 Nitrile Hydratase Activity via Different Fermentation Time Lengths*

When *R. rhodochrous* DAP 96253 fermentation paste is harvested, it is harvested at two times points, 48hrs and 62hrs, the nitrile hydratase activity is taken at both time points to determine what age of cells are best to use for the bioconversion process. The activity is also taken for the 'seed', the flask growth used to inoculate the fermenter. The seed is shown as having nitrile hydratase activity of around 210 um/mL/min. During fermentation, the cells harvested at the 48-hour mark as shown to have activity around 2387 um/mL/min while the cells harvested at the 62-hour mark are shown to have activity around 945 um/mL/min. These results are shown below in Table 9. Raw data and calculations are shown in Appendix F:

Table 9: Average nitrile hydratase activity depending on mode and duration of fermentation for R. rhodochrous DAP 96253.

Sample	NHase Avg Units (um/ml/min)
Nhase seed (4th pass WJ)	210 ± 0.6
Nhase 48 H	2387 ± 103
Nhase 62 H	945 ± 120

3.1.3 *R. rhodochrous DAP 96253 Whole Cell Bioconversion of Acrylonitrile to Acrylamide, including manipulation of bioconversion parameters*

Multiple runs of the bioconversion process were carried out using whole cells while the manipulation of multiple variables was carried out and assessed for the resulting effects on acrylamide production. The standard used for these initial runs is found in Appendix G.

The first variable experimented with was temperature. The first whole cell run, whole cells #1, was carried out using previously stored *R. rhodochrous* DAP 96253 whole cells and a temperature of 55°C, accomplished by using a stirring hot plate. The second run carried out, whole cells #2, used the same stored *R. rhodochrous* DAP 96253 whole cells but was conducted at room temperature (~20°C). All other variables were consistent, with 100 mL of AN being added at a rate of 0.33 mL/min, and the maximum theoretical amount of acrylamide able to be produced was 1.53 moles. The percentage of actual production compared to theoretical production, known as conversion percentage from here on out, is calculated from these numbers. Whole cells run #1 was shown to produce 0.15 moles of acrylamide which gives it a conversion percentage of 9.80%. Whole cells run #2 produced 0.94 moles of acrylamide which gives it a conversion percentage of 61.4%. This comparison is shown below in Table 10.

Table 10: Production of acrylamide using selected temperatures.

	Temperature (°C)	Acrylonitrile Added (g)	Acrylamide Produced (moles)	Theoretical Acrylamide (moles)	Conversion Percentage (%)
Whole Cells #1	55°C	81g	0.15	1.53	9.80%
Whole Cells #2	20°C	81g	0.94	1.53	61.4%

The actual production over time in comparison to the theoretical amount of acrylamide able to be produced over time, showing conversion comparison over time, for the room temperature run (Whole Cells #2) is shown below in Figure 16. At 240 minutes, it is seen that the conversion percentage was at its highest, having 0.88 moles of acrylamide produced out of a theoretical 1.21 moles of acrylamide. Thus, making the conversion percentage 72.1%. However, production then slows; bringing the conversion down to 61.4% to give a final production of 0.94 moles acrylamide out of a possible 1.53 moles. Production calculations for Whole Cells #2 are shown in Appendix H.

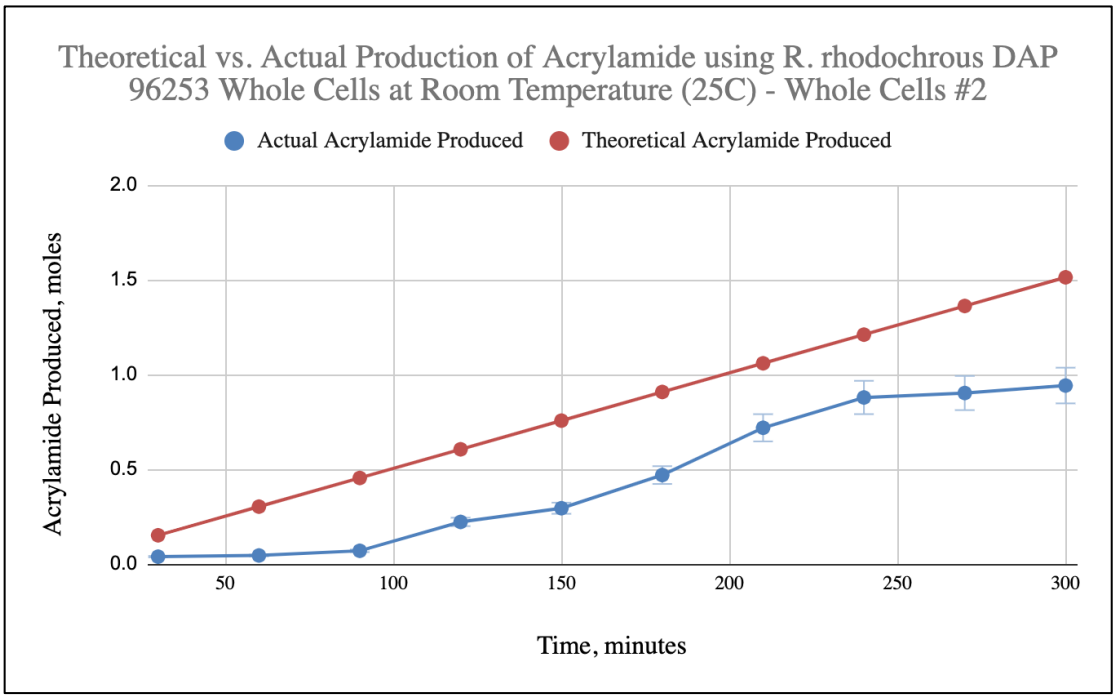


Figure 16: Theoretical vs. Actual Production of Acrylamide using R. rhodochrous DAP 96253 Whole Cells at Room Temperature (25 ℃) (Whole Cells #2).

All bioconversion runs from here on out will be run with *R. rhodochrous* DAP 96253 cells from fresh fermentation paste and at room temperature unless noted elsewise.

The next variable experimented with was the rate at which the acrylonitrile added to the bioreactor. For whole cells run #2, shown above in figure 16, the original rate of 0.33 mL/min for a total of 100 mL of AN was initially chosen. This equates to 9.9 mL, or .15 moles, of AN being added every 30mins, which is when samples are taken. For whole cells runs #3, the same amount of AN, 100 mL, was ran at a slightly faster rate of 0.38mL/min to observe acrylamide production. This equates to 11.4mL, or .17 moles, of AN being added every 30mins. For both runs, the final amount of AN added was 1.53 moles. Whole cells #2 produced 0.94 moles of AMD giving the run a conversion rate of 61.4%. Whole cells #3 produced 1.07 moles of AMD giving the run a conversion rate of 69.9%. This data is shown below in Table 11. Production calculations are shown for whole cells #2 in Appendix H and for whole cells #3 in Appendix I.

Table 11: Production of acrylamide using variable amounts of acrylonitrile.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)
Whole Cells #2	0.33	100mL	1.53	0.94	61.4%
Whole Cells #3	0.38	100mL	1.53	1.07	69.9%

Whole cells #2 run was carried out for 300 minutes and whole cells #3 run was carried out for 270 minutes. These times varied due to the faster input rate for whole cells #3. The comparison for the conversions over time of both runs is shown below in Figure 17.

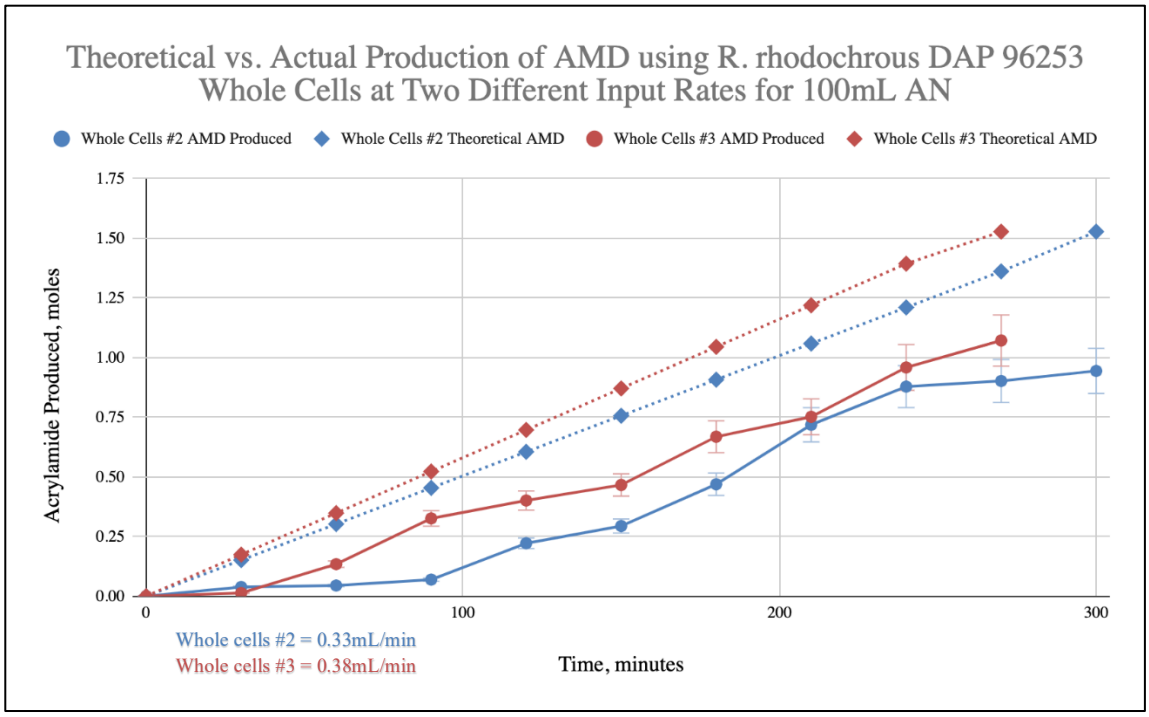


Figure 17: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 Whole Cells at Two Different Input Rates. The blue lines represent whole cells #2 with an input rate of 0.33 mL/min and the red lines represent whole cells #3 with an input rate of 0.38 mL/min. The solid lines with circular sample points represent the actual amount of AMD produced. The dashed lines with diamond sample points represent the theoretical yield of AMD possible.

After this, whole cells #4 run was conducted utilizing *R. rhodochrous* DAP 96253 with an input rate of 0.35 mL/min for 130 mL of AN. The theoretical yield of AMD was 1.99 moles and the actual acrylamide produced was 1.79 moles of AMD, giving whole cells #4 a conversion percentage of 89.9%. The final solution of acrylamide produced was 36.80%w/v. This data is shown below in Table 12. Production calculations are shown for whole cells #4 in Appendix J.

Table 12: Production of acrylamide using 130 mL of AN at a rate of 0.35 mL/min.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)	Final Solution (AMD %w/v)
Whole Cells #4	0.35	130mL	1.99	1.79	89.9%	36.80%

The conversion comparison over time of whole cells #4 run is shown below in Figure 18. The run took around 370 minutes in order to pump in 130 mL at 0.35 mL/min.

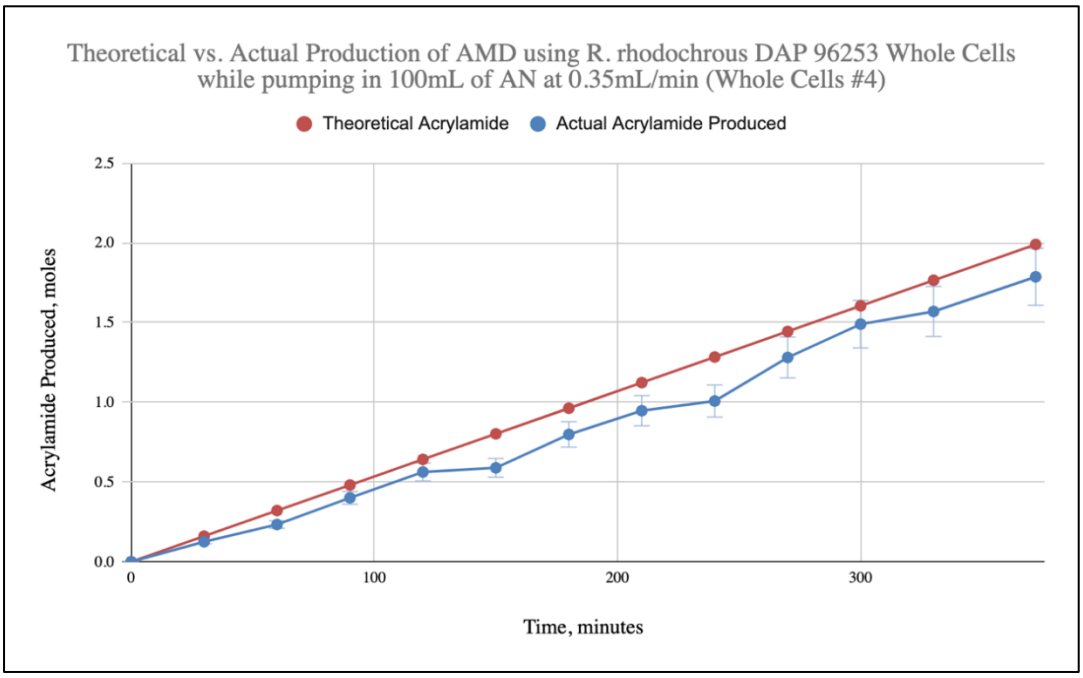


Figure 18: Theoretical vs. Actual Production of Acrylamide using R. rhodochrous DAP 96253 Whole Cells while pumping in 100mL of AN at 0m35mL/min (Whole Cells #4).

The next variable experimented with was the amount of acrylonitrile added to the bioreactor while using the same input rate, 0.38 mL/min. For whole cells run #3, depicted in table 11 and figure 17, 100 mL of AN was added to the bioreactor. This equates to 11.4 mL, or 0.17 moles, of AN being added every 30mins. This run lasted for around 270 minutes to add all the AN. Whole cells #5 run ran for longer, 420 minutes, in order to add 160 mL of AN to the bioreactor. While the amount of AN, mL and moles, added to the bioreactor is the same per 30 minutes, the total amount varies. Whole cells #3 run ended with 1.53 moles of AN added while whole cells #5 ended with 2.45 moles of AN being added. Whole cells #3 produced 1.14 moles of AMD giving the run a conversion rate of 74.5%. Whole cells #5 produced 2.32 moles of AMD giving the run a conversion rate of 94.7% This data is shown below in Table 13. Production calculations are shown for whole cells #3 in Appendix I and for whole cells #5 in Appendix K.

Table 13: Production of acrylamide using varying amounts of acrylonitrile.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)
Whole Cells #3	0.38	100mL	1.53	1.07	69.9%
Whole Cells #5	0.38	160mL	2.45	2.32	94.7%

The conversion comparisons over time of both runs are shown below in Figure 19 and Figure 20.

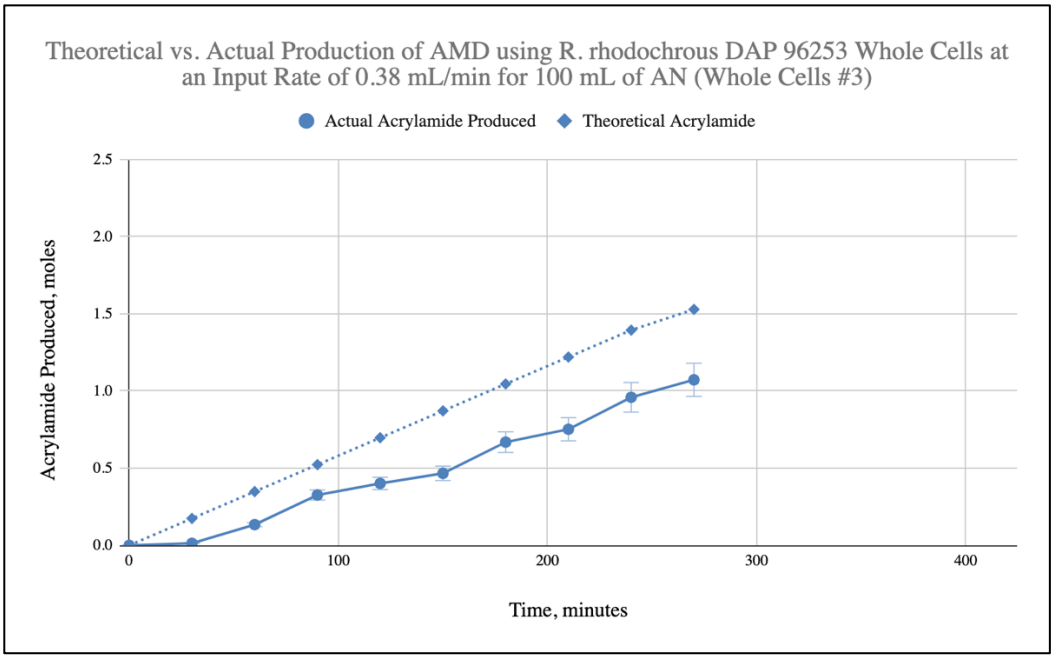


Figure 19: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 Whole Cells at an Input Rate of 0.38 mL/min for 100 mL of AN (Whole Cells #3).

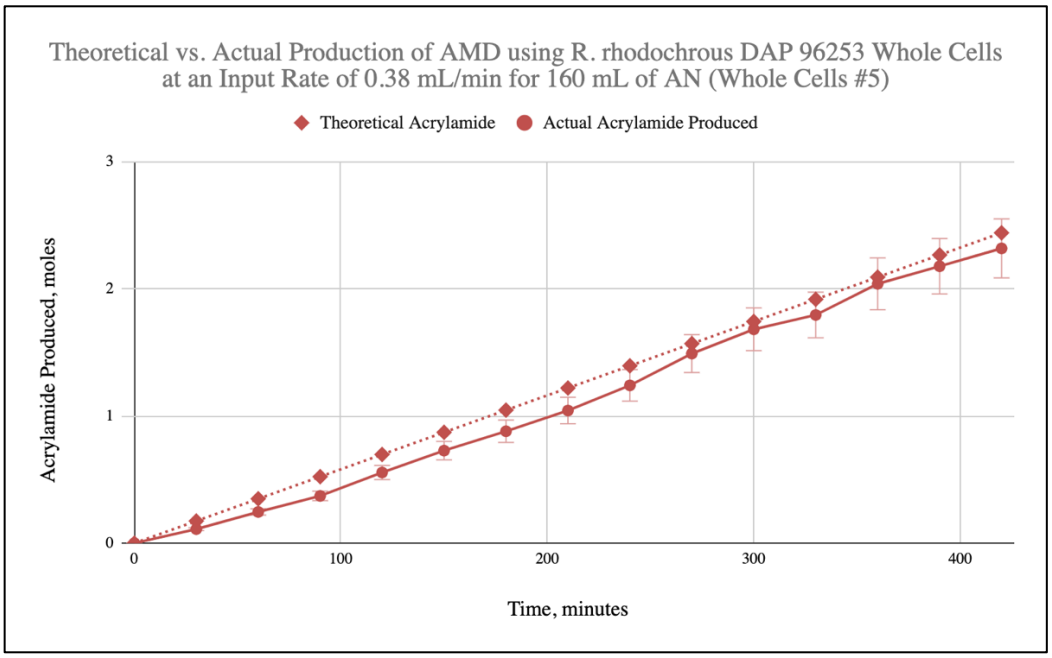


Figure 20: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 Whole Cells at an Input Rate of 0.38 mL/min for 160 mL of AN (Whole Cells #5).

The next comparison of whole cells runs was conducted using 160mL of AN at the selected input rates of 0.38, 0.6, and 0.8 mL/min. Whole cells run #5 used an input rate of 0.38 mL/min which equates to 11.4 mL, or 0.17 moles, of AN being added every 30mins. This run took 420 minutes to add the acrylonitrile into the bioreactor. Whole cells run #6 used an input rate of 0.6 mL/min, which equates to 18 mL, or .28 moles, of AN being added every 30mins. This run took approximately 270 minutes to finish adding all the AN. Whole cells run #7 used an input of 0.8 mL/min, which equates to 24 mL, .37 moles, of AN being added every 30mins. This run lasted for around 210 minutes. All runs added the same amount of AN; thus, having 2.45 of moles of theoretical AMD able to be created. This data is shown below in Table 14. Production calculations are shown for whole cells #5, whole cells #6, and whole cells #7 in Appendix K, L, and M respectfully.

Table 14: Production of acrylamide using varying input rates of acrylonitrile.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)	Final Solution (AMD%w/v)
Whole Cells #5	0.38	160mL	2.45	2.32	94.7%	45.37%
Whole Cells #6	0.6	160mL	2.45	2.12	86.5%	49.92%
Whole Cells #7	0.8	160mL	2.45	2.30	93.9%	44.9%

The comparisons of conversion over time for the 3 runs (Whole Cells #5, #6, and #7) can be found below in Figures 21, 22, and 23 respectively.

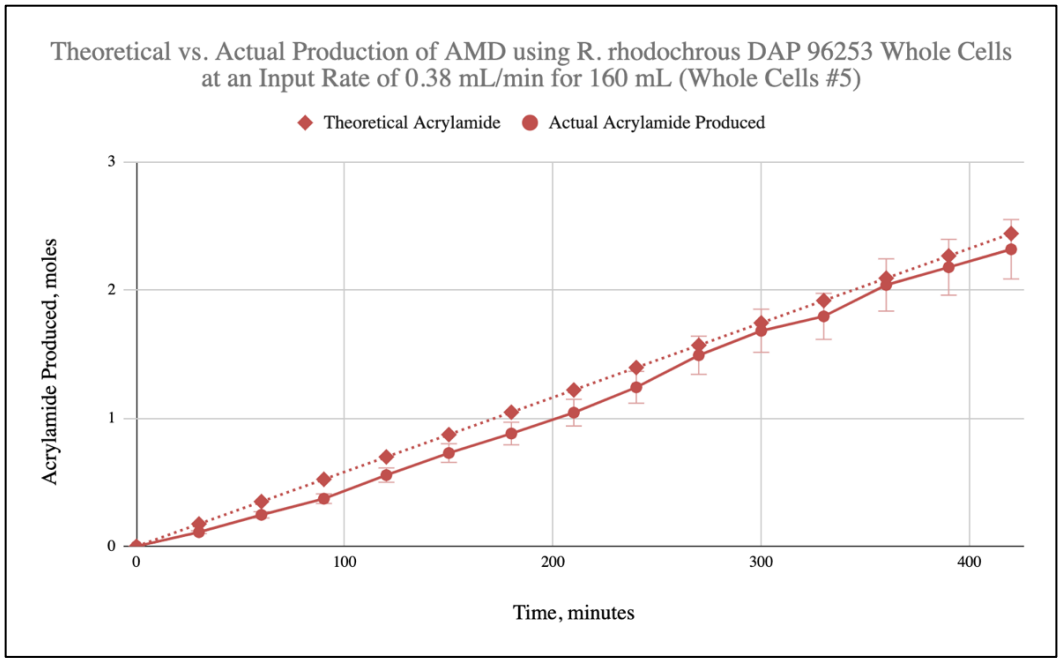


Figure 21: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 Whole Cells at an Input Rate of 0.38 mL/min for 160 mL of AN (Whole Cells #5).

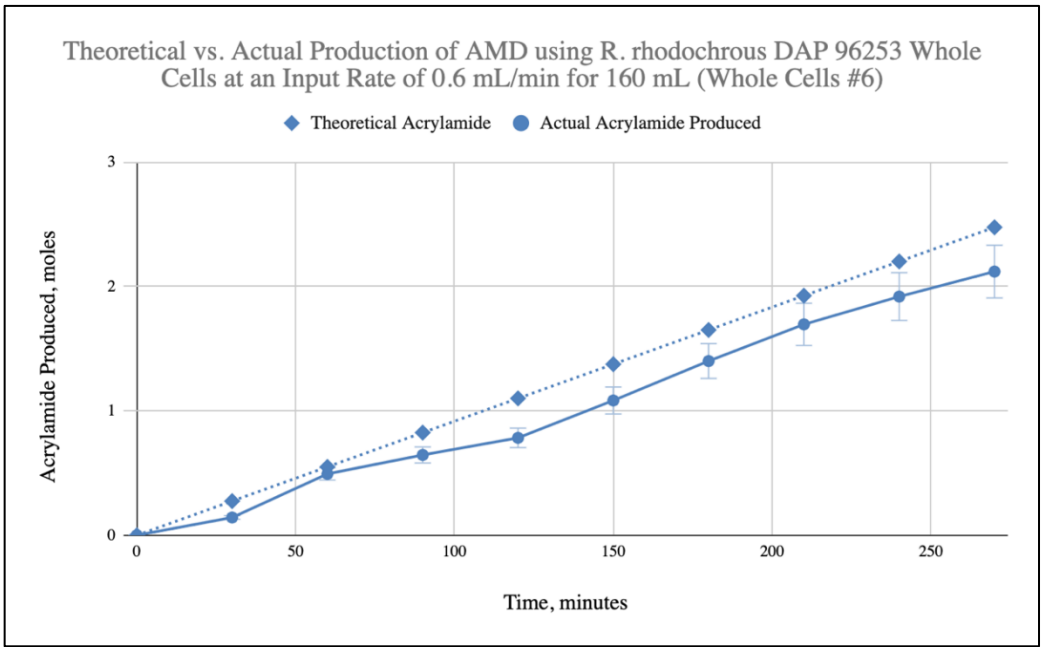


Figure 22: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 Whole Cells at an Input Rate of 0.6 mL/min for 160 mL of AN (Whole Cells #6).

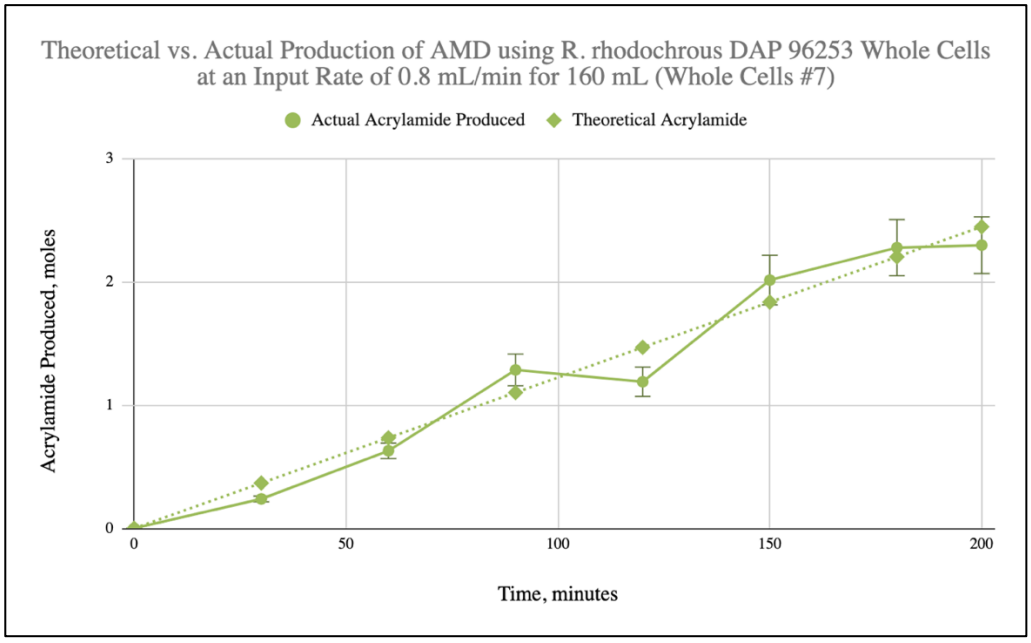


Figure 23: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 Whole Cells at an Input Rate of 0.8 mL/min for 160 mL of AN (Whole Cells #7).

When comparing the two runs with the highest conversion percentage of the 3 runs using 160mL of AN, whole cells #5 and #7, both runs converted 94-95% of the acrylonitrile added into the bioreactor. Both runs also ended with 45%w/v AMD solutions. A comparison of the acrylamide production over time of the two runs is shown below in Figure 24.

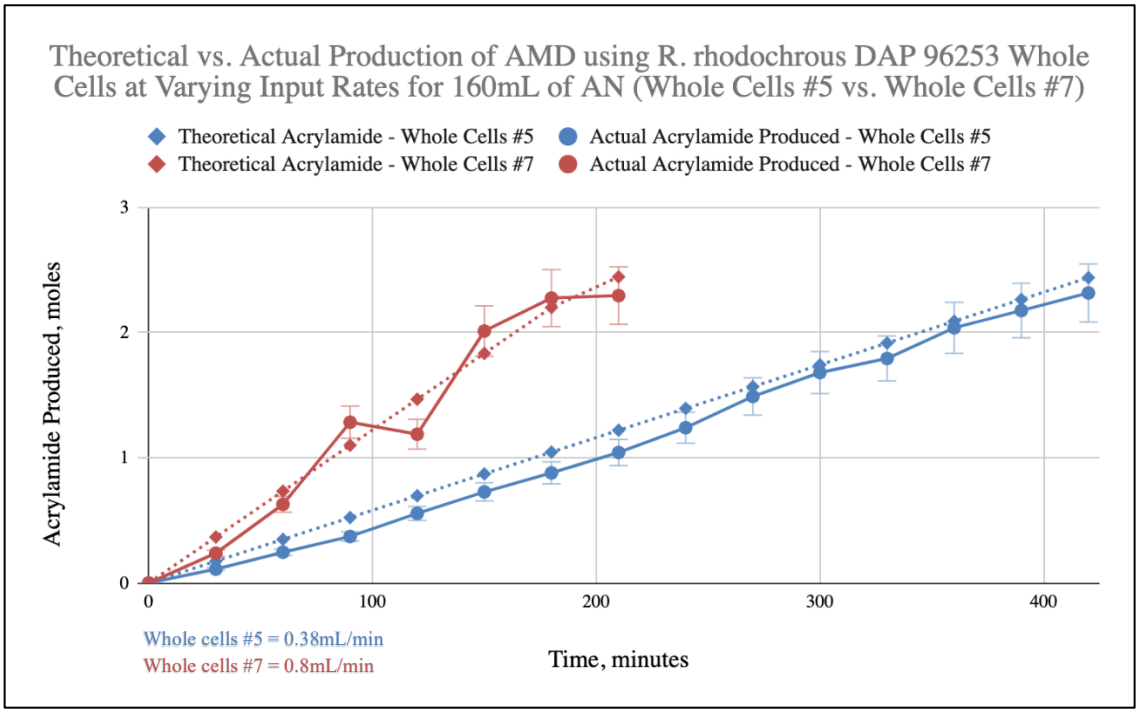


Figure 24: Theoretical vs. Actual Production of AMD using R. rhodochroud DAP 96253 Whole Cells at Varying Input Rates for 160mL (Whole Cells #5 vs. Whole Cells #7)

An overview of all the runs conducted while manipulating various bioconversion parameters (Whole cells #2-7) can be found below in Table 15. This table includes the comparison of the %w/v AMD of the final solutions produced.

Table 15: Production of acrylamide across various parameter manipulations.

Whole Cells (#)	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)	Final Solution (AMD%w/v)
#2	0.33	100mL	1.53	0.94	61.4%	20.19%
#3	0.38	100mL	1.53	1.07	69.9%	23.13%
#4	0.35	130mL	1.99	1.79	89.9%	36.80%
#5	0.38	160mL	2.45	2.32	94.7%	45.37%
#6	0.6	160mL	2.45	2.12	86.5%	49.92%
#7	0.8	160mL	2.45	2.30	93.9%	44.9%

The pH values of the solution starting out as solely DI water and resulting from the input of acrylonitrile and the production of acrylamide over time were taken at each sample time point during whole cells run #7. Despite the timepoint of the bioconversion run, the pH registered as 7.3. The values over time are shown below in Table 16. The conversion comparison for the production of acrylamide during this run is shown above in Figure 24. Raw data and calculations are shown in Appendix M.

Table 16: pH values for the Production of Acrylonitrile using R. rhodochrous DAP96253 Whole Cells

Minutes	0	30	60	90	120	150	180	210
pH	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3

A second strain of *Rhodococcus rhodochrous* was used to analyze the whole cell production of acrylamide. *Rhodococcus rhodochrous* DAP 96622 was used to convert acrylonitrile to acrylamide in comparison to *Rhodococcus rhodochrous* DAP 96253. Fresh grown fermentation paste of both strains was used to produce AMD using an input rate of 0.75 mL/min for approximately 135mL of AN. This equates to 22.5 mL, or 0.34 moles, of AN being added every 30 minutes. The total possible amount of acrylamide produced from each run in 2.06 moles. The acrylamide production and conversion percentages for both strains are shown below in Table 17. The run using RrDAP96622 ended up converting 15% of the acrylonitrile added to acrylamide. This run was stopped at 180 minutes due to the bilayer accumulation shown below in Figure 26. The run using RrDAP96253 converted 81.7% of the acrylonitrile added to acrylamide. During this run, a sample for minute 180 was missed however a sample was taken at minute 210 to get final conversion percentage. The comparison chart for the conversion comparisons of both runs is shown below in Figure 25. Raw data and calculations for the *R. rhodochrous* DAP 96253 (whole cells #8) and for the *R. rhodochrous* DAP 96622 (whole cells #9) run are shown in Appendix N and Appendix O respectively.

Table 17: Production of Acrylamide using Whole Cells of both R. rhodochrous DAP96253 (Whole Cells #8) and R. rhodochrous DAP96622 (Whole Cells #9).

	Minutes →	0	30	60	90	120	150	180	210
Whole Cells #8 96253	AMD produced (moles)	0	.28	.62	.93	1.44	1.64	n/a*	1.97
	Theoretical AMD (moles)	0	.34	.69	1.03	1.38	1.71	2.06	2.41
	Conversion Percentage (%)	0%	82.4%	89.9%	90.3%	104%**	95.9%	n/a*	81.7%
Whole Cells #9 96622	AMD produced (moles)	0	0.09	0.18	0.23	0.26	0.29	0.31	n/a***
	Theoretical AMD (moles)	0	.34	.69	1.03	1.38	1.72	2.06	2.41
	Conversion Percentage (%)	0%	26.5%	26.1%	22.3%	18.8%	16.9%	15%	n/a***

*this sample point was missed
**this conversion percentage is unobtainable
**the bioconversion run was stopped after 180mins due to bilayer accumulation shown below in figure 25

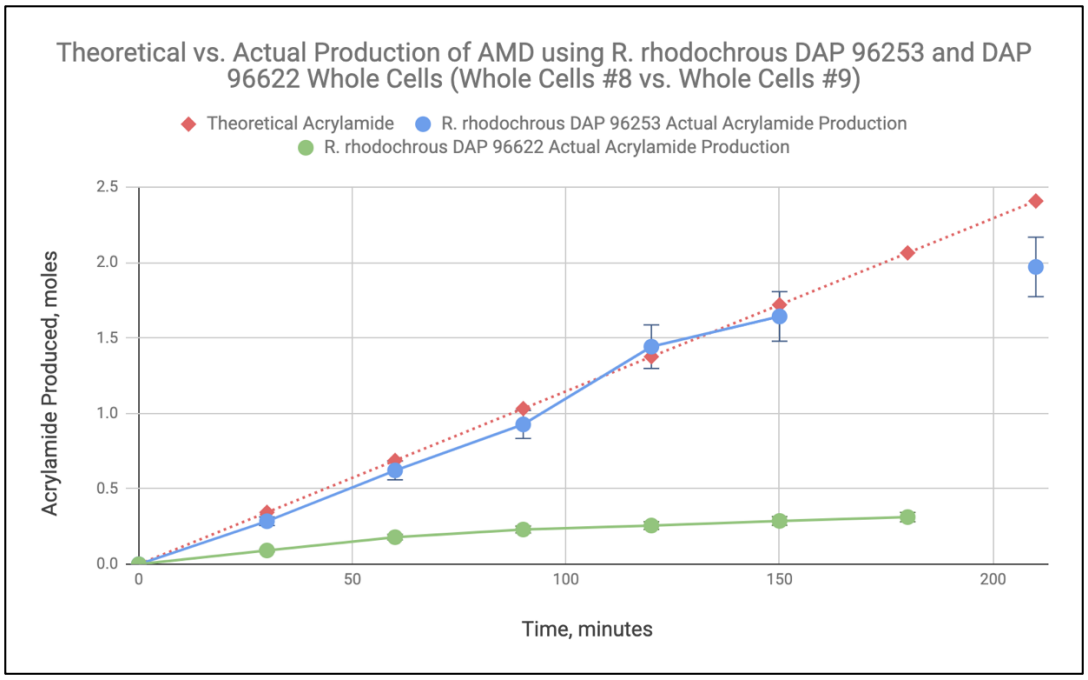


Figure 25: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 (shown in blue) and DAP 96622 Whole Cells (shown in green) (Whole cells #8 vs. Whole cells #9).

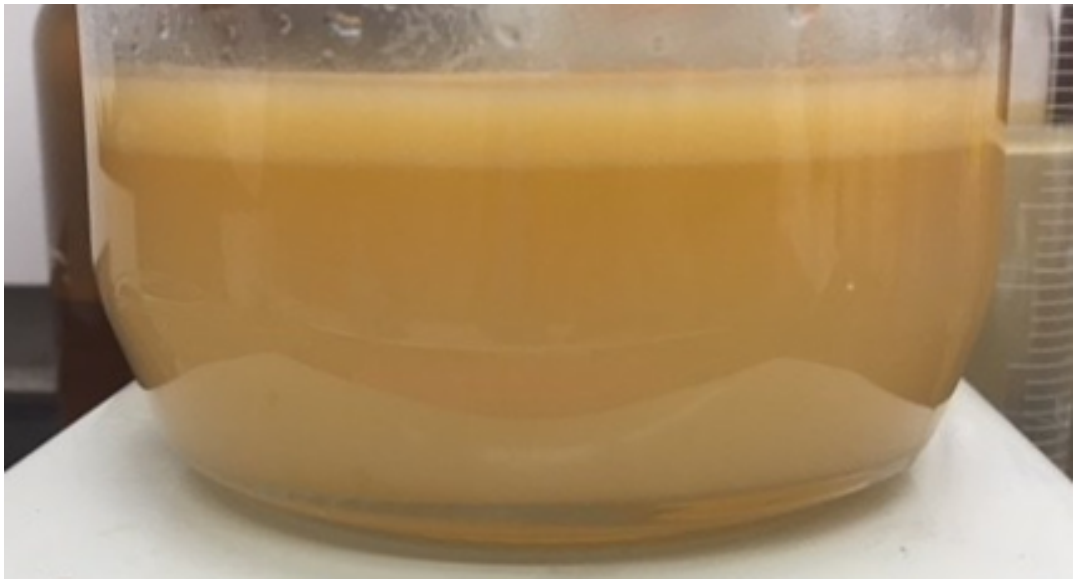


Figure 26: Bilayer Accumulation seen during the Production of Acrylamide using R. rhodochrous DAP 96622 Whole Cells (Whole cells #9).

3.1.4 *Polyacrylamide Immobilized Cells Bioconversion of Acrylonitrile to Acrylamide*

Initial PAM immobilized cell bioconversion runs were conducted using *R. rhodochrous* DAP 96253 cells that were grown via plates and that were grown via fermentation. Both runs were conducted using an input rate of 0.38 mL/min for a total of 100 mL of AN. Each run took 270 minutes to complete. The theoretical acrylamide yield for both runs was 1.53 moles. For the plate grown cells, the run produced 0.03 moles of AMD, giving the run a conversion percentage of 1.96%. For the fermentation grown cells, the run produced 0.80 moles of AMD, giving the run a conversion percentage of 52.3%. Nitrile hydratase enzyme assays conducted for both plate grown and fermenter grown cells, as seen in Table 8, show plate grown cells to have an activity level of 106.38 ± 19.80, while ferm grown cells have an activity level of 2298.27 ± 33.77. This is an approximate difference of 2191.89 units of NHase activity. These data are shown below in Table 18. The comparison of the conversion comparisons for both the plate grown cells and the fermentation grown cells are shown below in Figure 27. Raw data and calculations are shown in Appendix P and Q respectively.

Table 18: Production of Acrylamide using PAM Immobilized Cells of R. rhodochrous DAP96253 Either Grown on Plates (PAM Run #1) or via Fermentation (PAM Run #2)

	Nitrile Hydratase Activity (um/ml/min)	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)
Plate Grown	106.38 ± 19.80	0.38	100	1.53	0.03	1.96%
Ferm Grown	2298.27 ± 33.77	0.38	100	1.53	0.80	52.3%

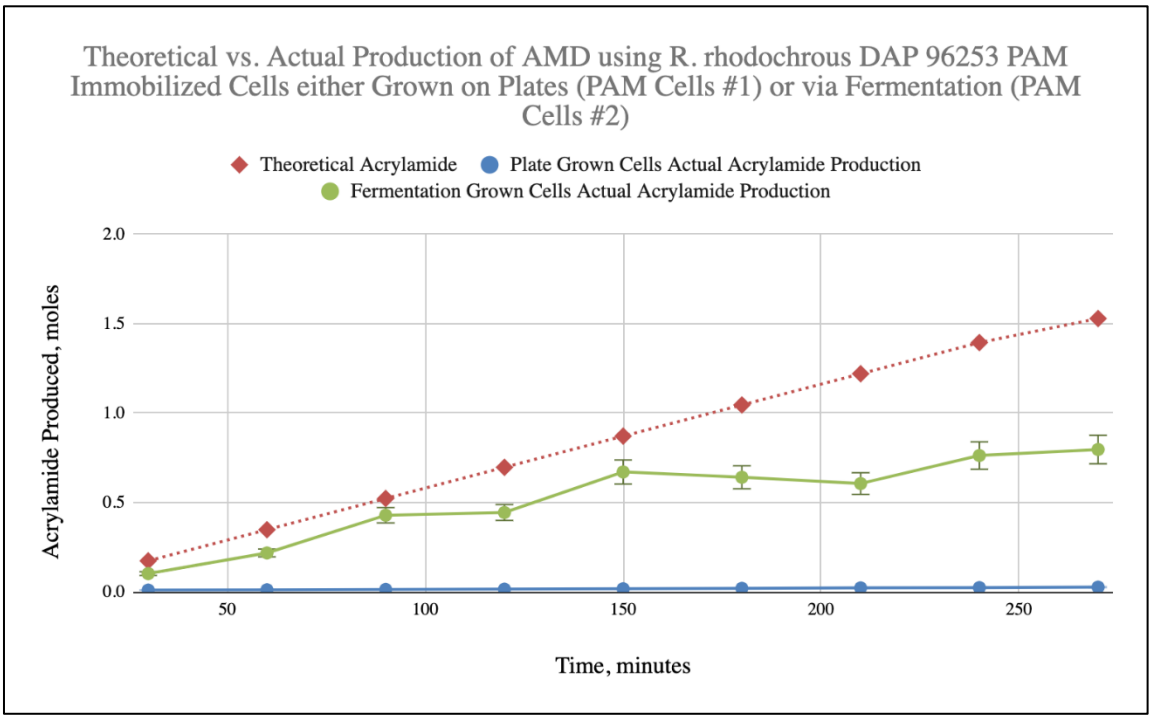


Figure 27:Theoretical vs. Actual Production of Acrylamide using PAM Immobilized Cells of R. rhodochrous DAP 96253 Grown on Plates (shown in blue) vs Grown via Fermentation (shown in green).

Further PAM immobilized cell runs were conducted using *R. rhodochrous* DAP 96253 cells for repetition while also varying the amount of acrylonitrile added and by changing the input rate. The amount of acrylamide produced is compared to the highest possible amount produced. This data can be found below in Table 19. Further information follows.

Table 19: Production of Acrylamide of 3 Repetitions of Runs using PAM Immobilized Cells of RrDAP96253

PAM Cells #	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)	Final Solution (AMD%w/v)
Run #2	100mL	0.38	1.53	0.80	52.3%	16.88%
Run #3	100mL	0.38	1.53	0.80	52.3%	16.95%
Run #4	100mL	0.33	1.53	1.07	69.9%	23.13%
Run #5	160mL	0.60	2.45	1.11	45.3%	19.19%
Run #6	160mL	0.75	2.45	0.82	33.5%	14.89%

For PAM cells runs #2 and #3, freshly harvested fermentation paste was immobilized and used for the bioconversion process by adding 100mL of AN at a rate of 0.38mL/min. Both runs had a theoretical yield of 1.53 moles of AMD and both actually produced 0.80 moles of AMD, giving both runs a conversion percentage of 52.3%. The final percentages of the solutions produced varied slightly at 16.88% and 16.95% w/v AMD. This data is shown above in Table 19. The comparison of the acrylamide production for these two runs can be found below in Figure 28. Raw data and calculations can be found in Appendix Q and Appendix R respectively.

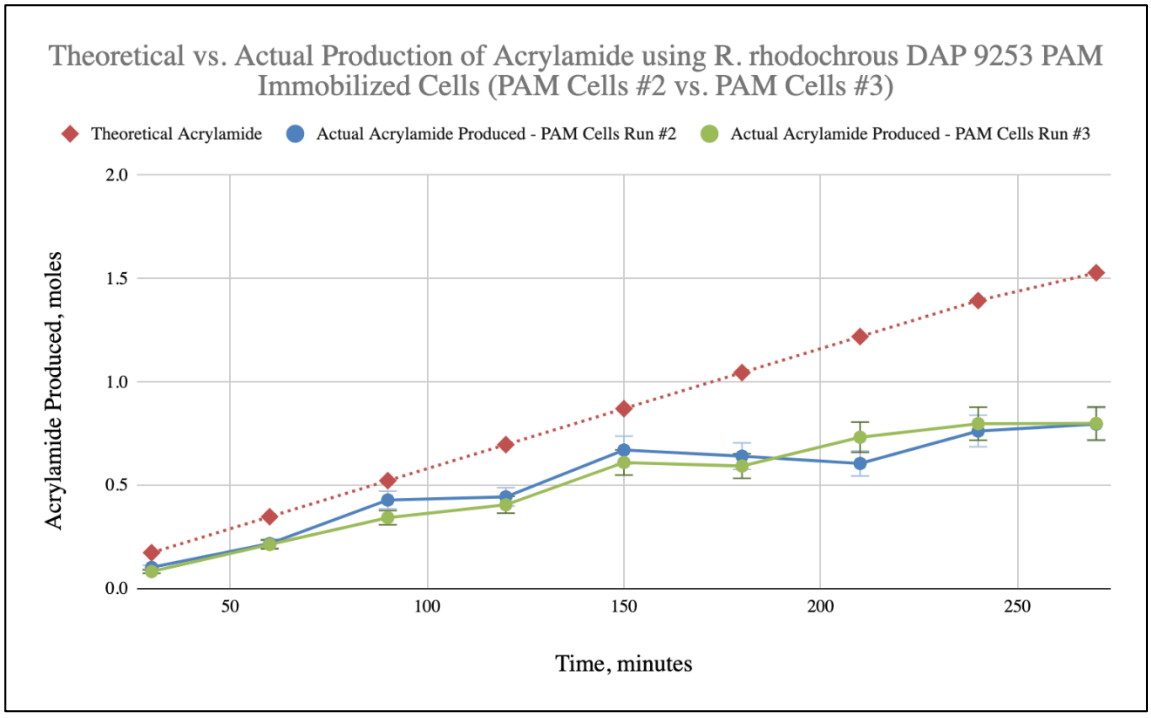


Figure 28: Theoretical vs. Actual Production of Acrylamide using R. rhodochrous DAP 96253 PAM Immobilized Cells (PAM Cells #2 [shown in blue] vs. PAM Cells #3 [shown in green]). Both runs were carried out by adding 100 mL of AN at a rate of 0.38 mL/min for 270 minutes.

For run #4, the input rate was slightly decreased to 0.33 mL/min but the same amount of AN, 100mL, was added as in run #3. This means the theoretical yield stayed the same at 1.53 moles of AMD. Run #4 produced 1.07 moles of AMD, giving it a conversion percentage of 69.9% while run #3 produced 0.80 mole of AMD and had a conversion percentage of 52.3%. These data can be found above in Table 19. The comparison of the conversion of run #4 to a slightly faster run using the same amount of acrylamide, run #3, can be found below in Figure 29. Raw data and calculations can be found in Appendix R and Appendix S respectively.

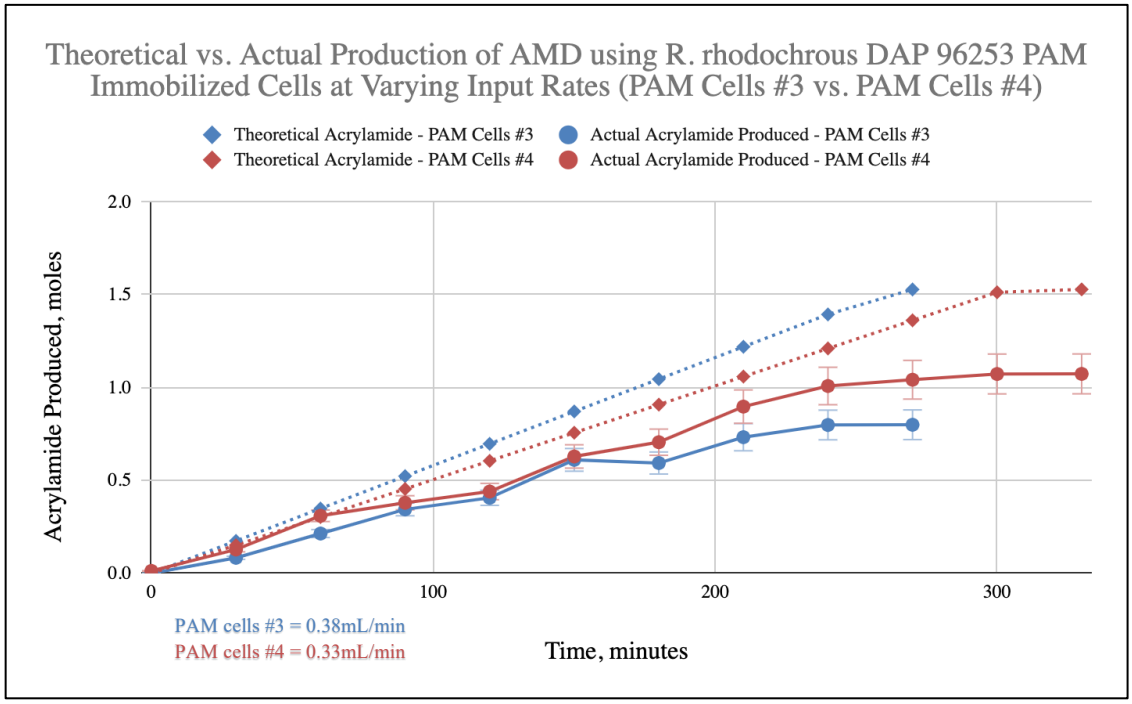


Figure 29: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 PAM Immobilized Cells at Varying Input Rates (0.38mL/min – PAM Cells #3 vs. 0.33mL/min – PAM Cells #4)

For run #5, the input rate was increased to 0.60 mL/min to add in 160mL of AN to the bioreactor. The theoretical yield increased to 2.45 moles of AMD. This run produced 1.11 moles of AMD, giving it a conversion percentage of 45.2% This data can be found above in Table 19. The conversion comparisons of run #5 can be found below in Figure 30. Raw data and calculations can be found in Appendix T.

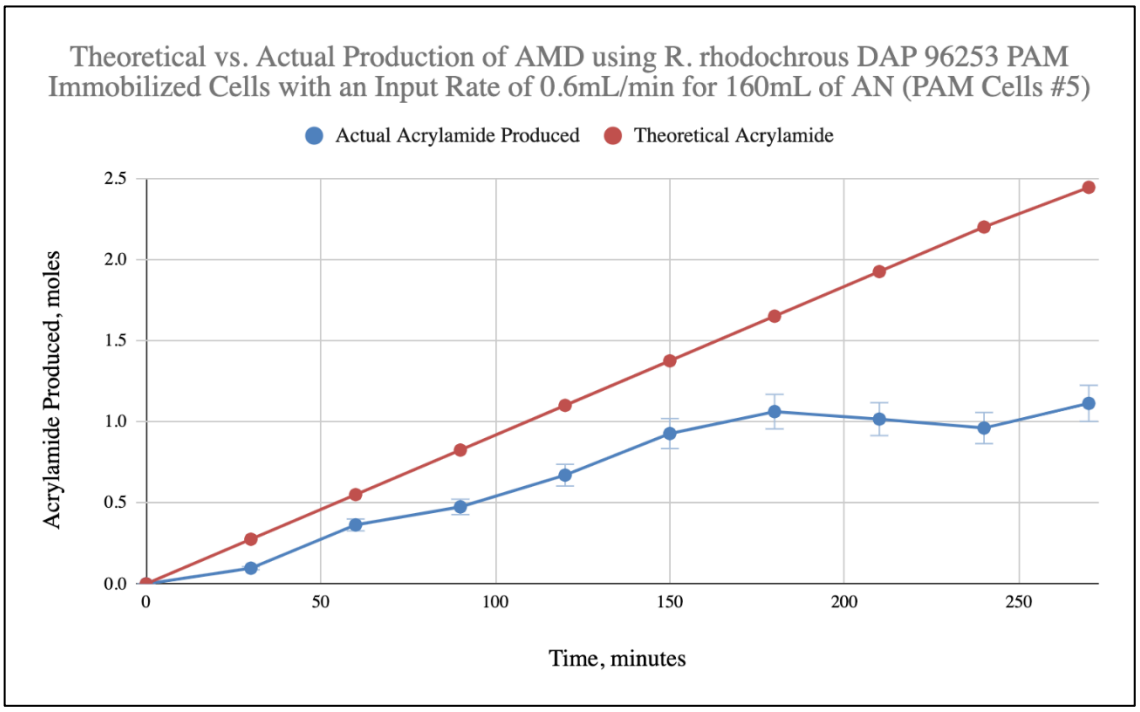


Figure 30: Theoretical Acrylamide vs. Actual Acrylamide Produced using R. rhodochrous DAP 96253 PAM Immobilized Cells with an Input Rate of 0.6mL/min for 160mL of AN

For run #6, the input rate was increased from 0.6mL/min in run #5 to 0.75 mL/min to add in 160mL of AN to the bioreactor. The theoretical yield stayed the same at 2.45 moles of AMD. PAM run #6 produced 0.82 moles of AMD, giving it a conversion percentage of 33.5%. In comparison, PAM run #5 produced 1.11 moles of AMD, giving it a conversion percentage of 45.3%. This data can be found above in Table 19. The comparison of the conversions of run #5 and run #6 can be found below in Figure 31. Raw data and calculations can be found in Appendix T and U respectively.

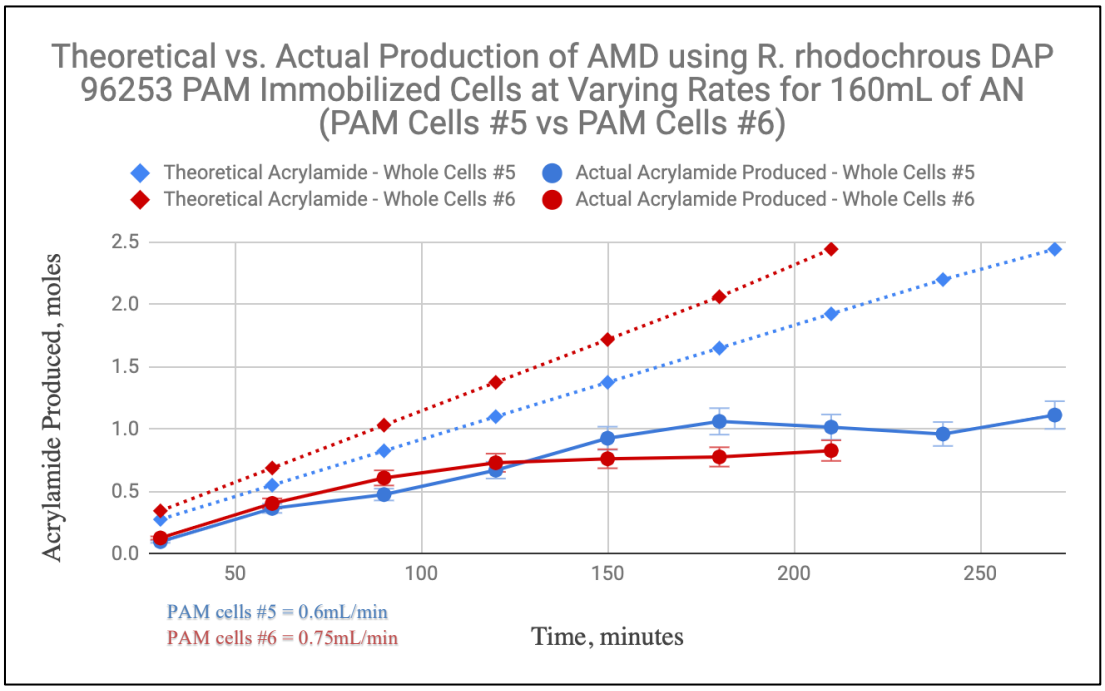


Figure 31: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 PAM Immobilized Cells at Varying Rates for 160mL of AN (PAM Cells #5 [0.6mL/min] vs. PAM Cells #6 [0.75mL/min]).

Further PAM immobilized cell runs were conducted using *R. rhodochrous* DAP 96253 cells to test the reusability of the immobilized cells. The used immobilized cells from Run #5 were washed with DI water, stored, and used to conduct a bioconversion run the next day. For the first run, the process produced 1.11 moles of AMD out of a possible 2.45 moles, giving the run a conversion percentage of 45.3%. After the cells were washed and stored overnight, the appearance of the cells had changed from before the run. Instead of being coral/orange well-formed squares, as seen in the first picture of Figure 8, they were now opaquer in color, almost clear, and seemed to be bloated and soggy. Also, they were sheared from the constant stirring and appeared to now be smaller, amorphous pieces that were clustered together. For the second run of the same cells, the process produced 0.12 moles of AMD out of a possible 1.38 moles, giving the run a conversion percentage of 8.7%. This run was stopped after 150 minutes due to a similar bilayer formation as seen in Figure 20. The PAM immobilized cells now looked to be disintegrating. The production results are shown below in Table 20. The conversion comparisons for both runs of PAM immobilized cells are shown below in Figure 32. Raw data and calculations are shown in Appendix T and V respectively.

Table 20: Production of Acrylamide using PAM Immobilized Cells of R. rhodochrous DAP 96253 for Multiple Runs using PAM Cells #5.

PAM Cells #5	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)	Final Solution (AMD%w/v)
1st Run	0.6	160mL	2.45	1.11	45.3%	20.32%
2nd Run	0.6	90mL	1.38	0.12	8.7%	2.47%

*2nd run was cancelled after 150minutes due to bilayer formation

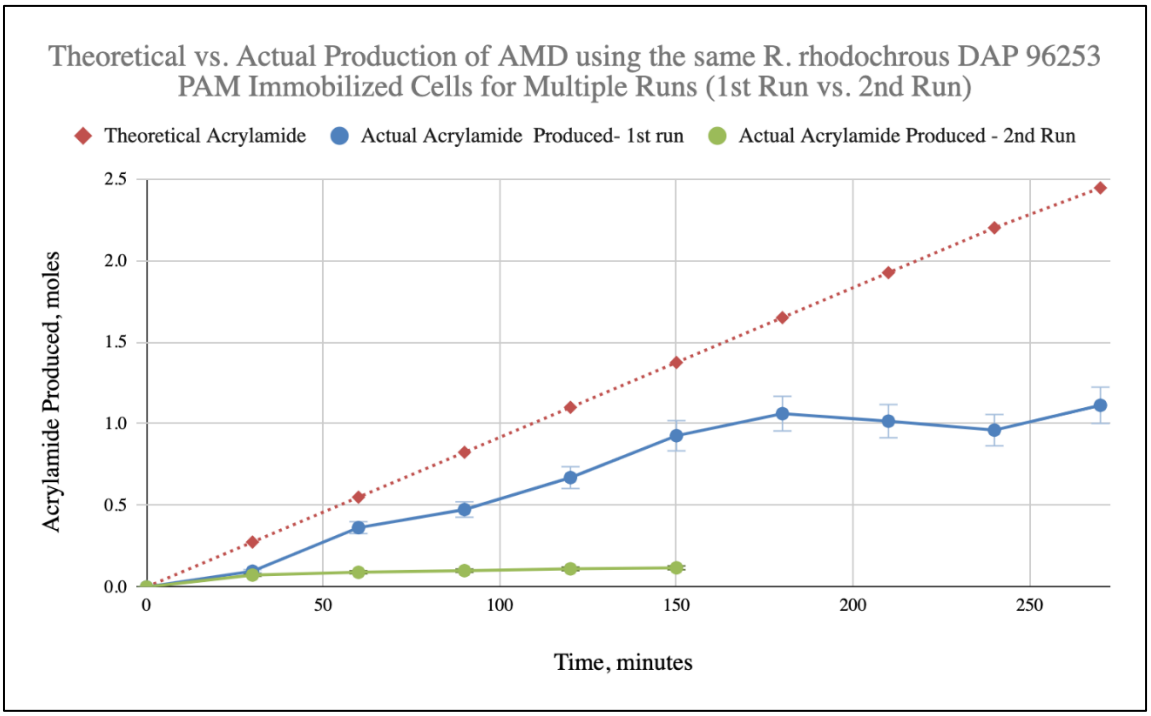


Figure 32: Theoretical vs. Actual Production of Acrylamide using the same R. rhodochrous DAP 96253 PAM Immobilized Cells from PAM Cells Run #5 for Multiple Runs – 1st Run vs. 2nd Run.

3.1.5 *Polyethyleneimine-Glutaraldehyde Immobilized Cells Bioconversion of Acrylonitrile to Acrylamide*

PEI-Glu immobilized cell runs were conducted using *R. rhodochrous* DAP 96253 cells. The amount of acrylamide produced is compared to the highest possible amount produced.

For numerous PEI-Glu immobilized cell runs, AMD production numbers held consistent. The data from two of these bioconversion runs are shown below in Table 21. For PEI-Glu Cells #1, the run produced 0.78 moles of AMD out of a possible 1.65 moles of AMD, giving the run a conversion percentage of 47.3%. The final solution was 16.1%w/v AMD. For PEI-Glu Cells #2, the run produced 0.81 moles of AMD out of a possible 1.65 moles of AMD, giving the run a conversion percentage of 49.1%. The final solution was 16.7%. At the end of bioconversion runs using PEI-Glu immobilized cells, the original clumped together nature, as seen in the first two pictures of Figure 10, were dissolved into powder suspended in the final solution. The conversion comparisons for both runs are shown respectively in Figures 32 and 33 below. The conversions for both runs are compared to each other in Figure 34. Raw data and calculations are shown in Appendix W and X respectively.

Table 21: Production of Acrylamide using PEI-Glu Immobilized Cells of R. rhodochrous DAP96253 – PEI-Glu Cells #1 and PEI-Glu Cells #2.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage, (%)	Final Solution (AMD%w/v)
PEI-Glu #1	0.6	108mL	1.65	0.78	47.3%	16.1%
PEI-Glu #2	0.6	108mL	1.65	0.81	49.1%	16.7%

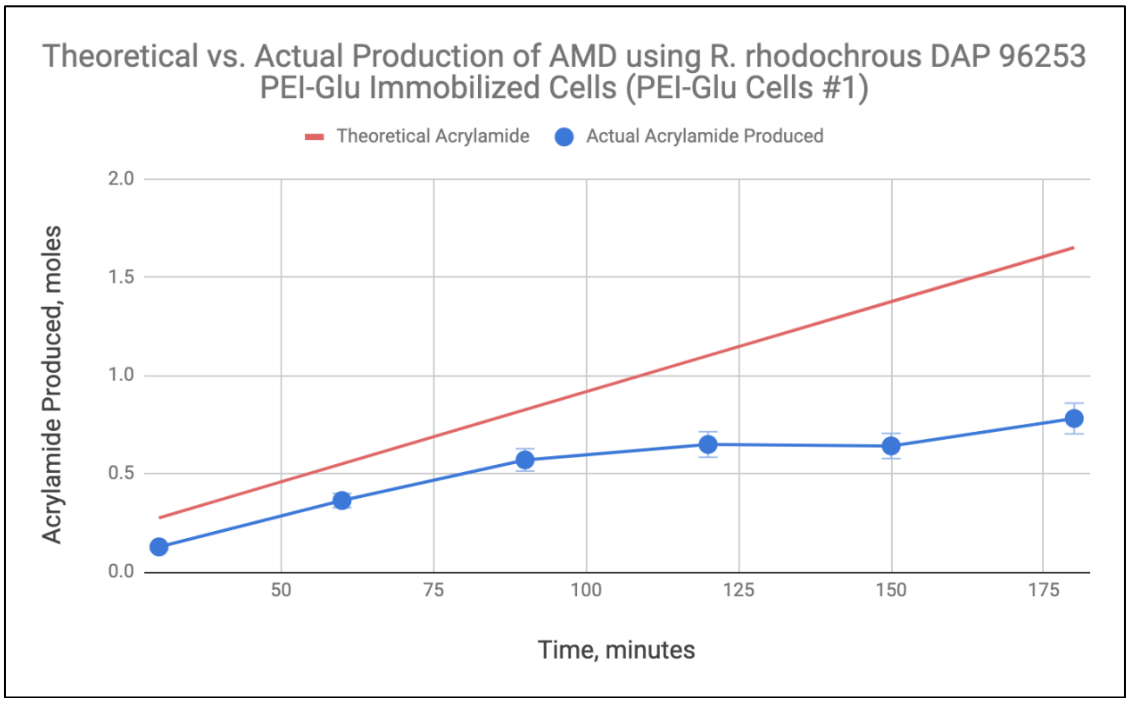


Figure 33: Theoretical vs. Actual Production of Acrylamide using R. rhodochrous DAP 96253 PEI-Glu Immobilized Cells (PEI-Glu Cells #1).

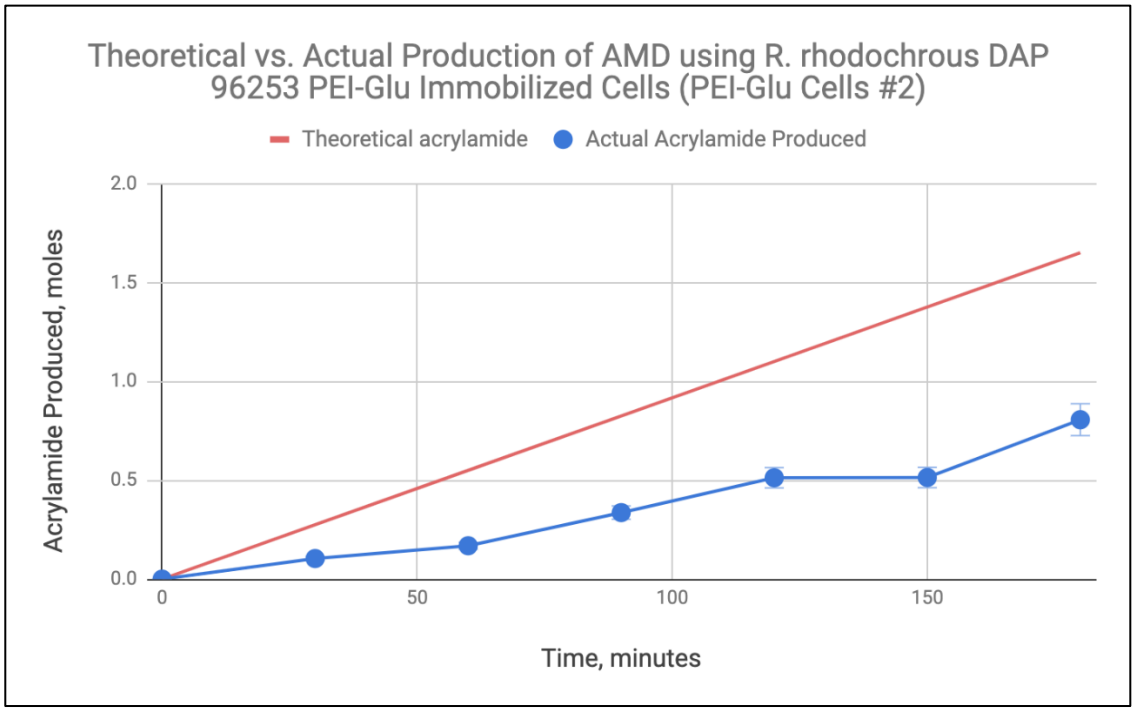


Figure 34: Theoretical vs. Actual Production of Acrylamide using R. rhodochrous DAP 96253 PEI-Glu Immobilized Cells (PEI-Glu Cells #2).

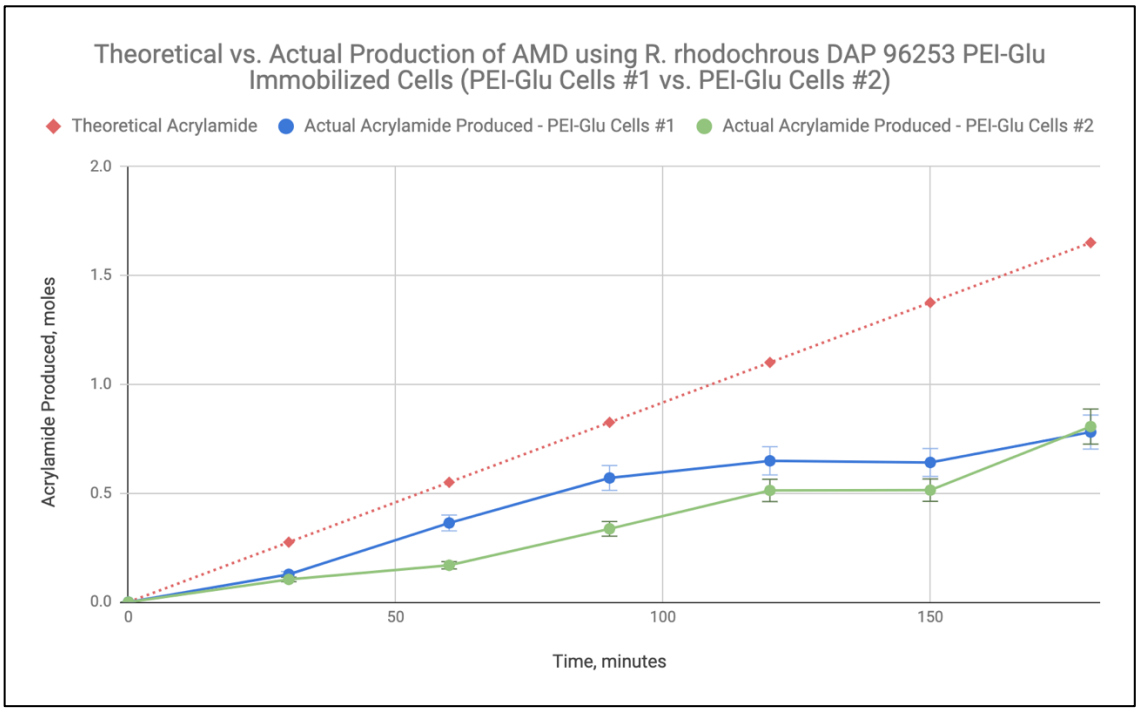


Figure 35: Theoretical vs. Actual Production of AMD using R. rhodochrous DAP 96253 PEI-Glu Immobilized Cells (PEI-Glu Cells #1 vs. PEI-Glu Cells #2)

3.1.6 *Calcium Alginate, Non-hardened and Hardened, Immobilized Cells Bioconversion of Acrylonitrile to Acrylamide*

Non-hardened and hardened immobilized cell runs were conducted using *R. rhodochrous* DAP 96253 cells. The amount of acrylamide produced is compared to the highest possible amount produced.

Initial bioconversion runs were used to assess the different conversion capabilities between the hardened and non-hardened immobilized cells of the same fermentation paste. For both runs, 126 mL of AN were added at a rate of 0.6 mL/min. This led to a theoretical production of 1.93 moles of AMD. For NH-CaAlg Cells #1, 1.68 moles of AMD were produced, giving the run a conversion percentage of 87%. The final solution of the run was 34.8%w/v AMD. For H-CaAlg #1, 1.93 moles of AMD were produced, giving the run a conversion percentage of 100%. The final solution of the run was 40.6%. Samples of the H-CaAlg final solution will be stored for storage testing. This data is shown below in Table 22. The comparison of the conversion of the two runs is shown below in Figure 36. Raw data and calculations are shown in Appendix Y and Appendix Z respectively.

Table 22: Production of Acrylamide using Hardened and Non-hardened Ca-Alg Immobilized Cells of R. rhodochrous DAP96253 – NH-CaAlg #1 vs. H-CaAlg #1

Ca-Alg Imm. Cells:	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Final Solution (AMD%w/v)
NH-CaAlg #1	0.6	126mL	1.93	1.68	87%	34.8%
H-CaAlg #1	0.6	126mL	1.93	1.93	100%	40.6%

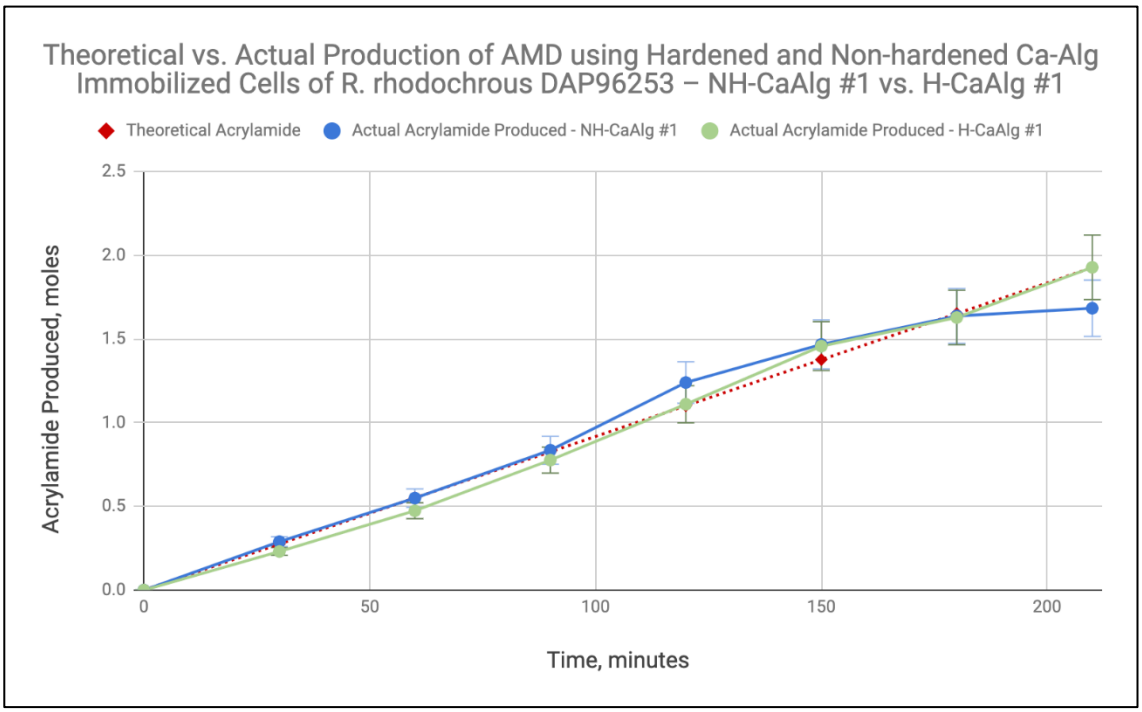


Figure 36: Theoretical vs. Actual Production of Acrylamide using Hardened and Non-hardened Ca-Alg Immobilized Cells of R. rhodochrous DAP96253 – NH-CaAlg #1 vs. H-CaAlg #1

Both sets of cells from the initial hardened vs. non-hardened bioconversion comparison runs were washed with DI water, stored, and used again the following day after their initial use.

For the testing of multiple uses of NH-CaAlg #1, the data is found below in Table 23. For the 1st run, 126mL of AN were added at a rate of 0.6 mL/min. This created the possibility for 1.93 moles of AMD to be produced. A total of 1.68 moles were created, giving the run a conversion percentage of 87% and a final solution of 34.8%w/v AMD. For the 2nd run, the plan was to add 126mL of AN at a rate of 0.6 mL/min. However, after 90mL of AN was added at the rate of 0.6 mL/min (150 minutes), conversion had stalled. This was observable by the bilayer accumulation of AN. The highest possible acrylamide production for the 2nd run was 1.38 moles of AMD and 0.15 moles were produced, giving the run a conversion percentage of 10.9%. The final solution was 3.25%w/v AMD. To best compare the 2nd run of NH-CaAlg #1 to the 1st run, the first 150 minutes of the conversions will be compared. For the abridged 1st run, 1.47 moles of AMD were produced out of a possible 1.38 moles, giving the run a conversion percentage of 106.5%. In Figure 36 above, it is seen that at minute 150 the data would suggest that more AMD has been produced than is possible. However, by comparing the conversion percentages of the time points 120 minutes and 180 minutes, it is proposed that the conversion percentage at minute 150 is 100% and will be used for this comparison. It is observed that, after 180 minutes, the conversion rate slowed down and the percentage dropped to 87% for the final time point, 210 minutes. The NH-CaAlg #1 abridged 1st run and 2nd run are shown below in Figure 37. Raw data and calculations for NH-CaAlg #1 – 2nd Run are shown in Appendix AA.

Table 23: Production of Acrylamide using Ca-Alg Immobilized Cells of R. rhodochrous DAP 96253 for Multiple Runs using NH-CaAlg #1. The unabridged and abridged data from the 1st run is present for comparison purposes.

NH-CaAlg Imm. Cells #1:	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Final Solution (AMD %w/v)
1st Run – unabridged	0.6	126mL	1.93	1.68	87%	34.8%
1st Run – 150 minutes	0.6	90mL	1.38	1.47	106% *100%	33.47%
2nd Run – 150 minutes	0.6	90mL	1.38	0.15	10.9%	3.25%

*proposed conversion percentage of 100% replacing the erroneous data point of 106%.

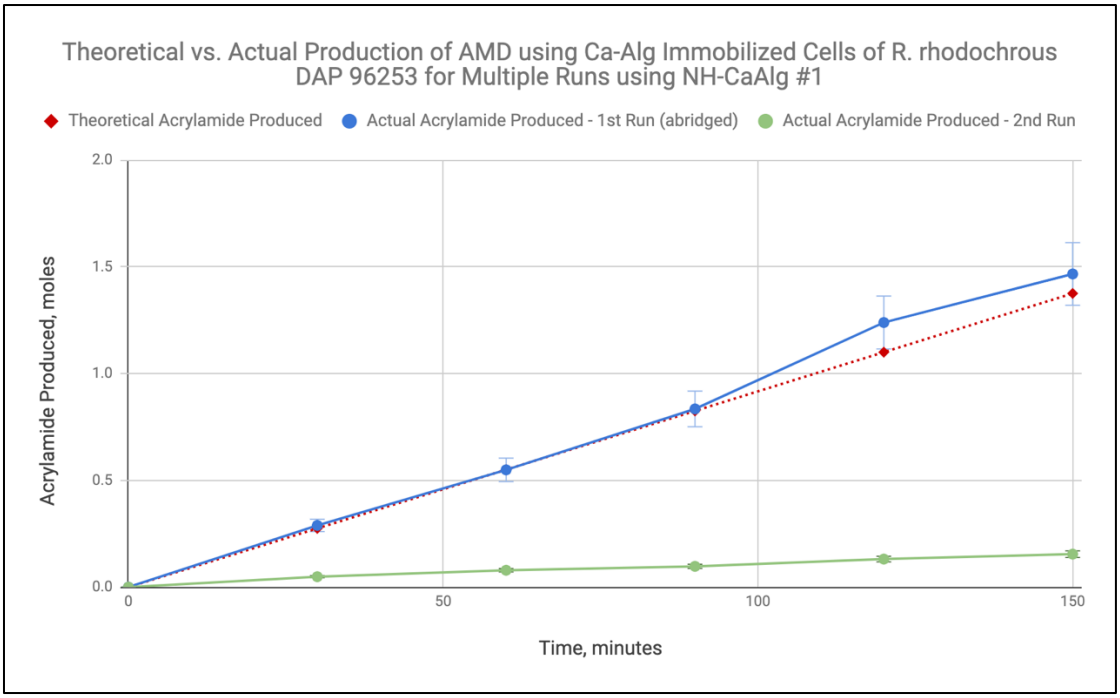


Figure 37: Theoretical vs. Actual Production of Acrylamide using Ca-Alg Immobilized Cells of R. rhodochrous DAP 96253 for Multiple Runs (1st Run vs. 2nd Run) using NH-CaAlg #1. The abridged version of the 1st run is represented here.

For the testing of multiple uses of H-CaAlg #1, the data is found below in Table 24. For the 1st and 2nd runs, 126mL of AN were added at a rate of 0.6 mL/min. This created the possibility for 1.93 moles of AMD to be produced for both trials. For the 1st run, a total of 1.93 moles were created, giving the run a conversion percentage of 100% and a final solution of 40.6%w/v AMD. For the 2nd run, a total of 0.69 moles of AMD was produced, giving the run a conversion percentage of 35.8% and a final solution of 13.5%w/v AMD. Both conversions are compared in Figure 38 below. Raw data and calculations for NH-CaAlg #1 – 2nd Run are shown in Appendix BB.

Table 24: Production of Acrylamide using Ca-Alg Immobilized Cells of R. rhodochrous DAP 96253 for Multiple Runs using H-CaAlg #1.

H-CaAlg Imm. Cells #1:	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Final Solution (AMD %w/v)
1st Run	0.6	126mL	1.93	1.93	100%	40.6%
2nd Run	0.6	126mL	1.93	0.69	35.8%	13.5%

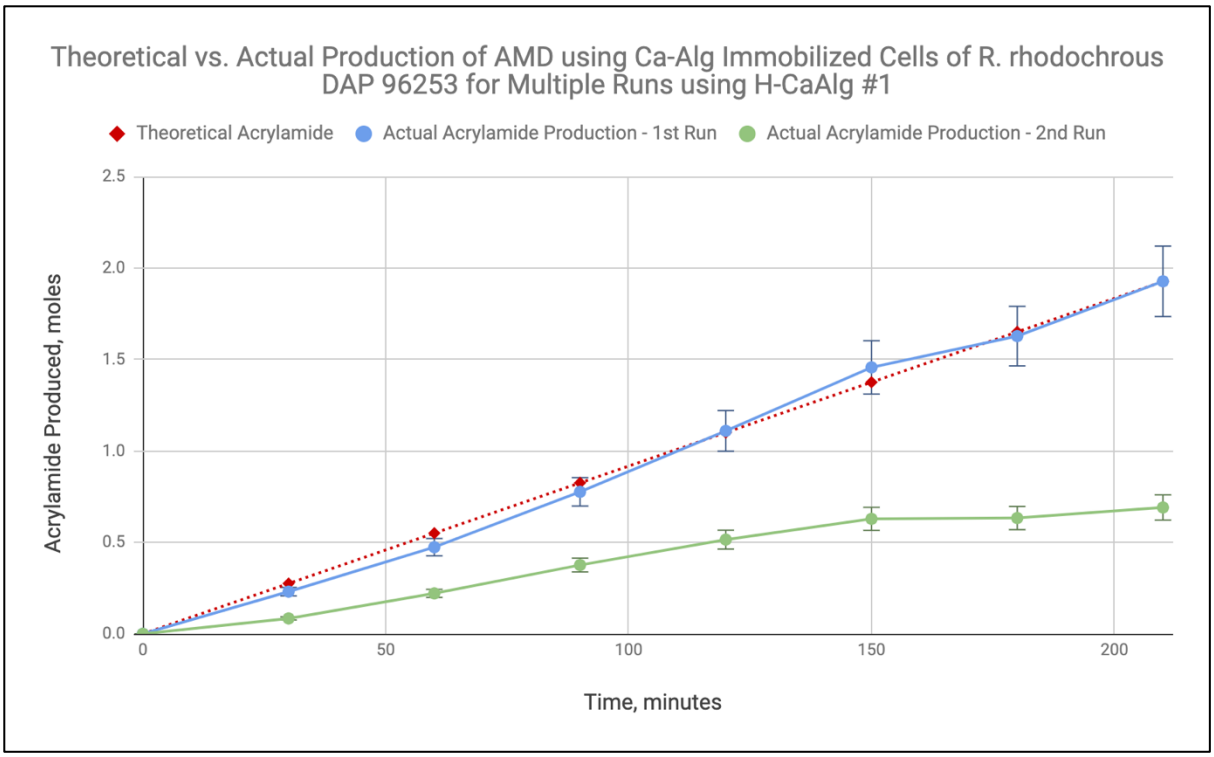


Figure 38: Theoretical vs. Actual Production of AMD using Ca-Alg Immobilized Cells of R. rhodochrous DAP 96253 for Multiple Runs (1st Run vs. 2nd Run) using H-CaAlg #1.

3.1.7 *Cell Free Lysate Bioconversion of Acrylonitrile to Acrylamide*

Initial studies researching the bioconversion capabilities of cell free lysate (CFL) focused on the verification of nitrile hydratase activity in the CFL and the comparison of the activity between the *R. rhodochrous* DAP 96253 whole cells used to obtain the CFL and the CFL. To equate the activity of whole cells to the activity in CFL (or purified enzyme later), 40 mg/mL of whole cells and 1μL or CFL/purified enzyme are used in the assay reaction [57]. Nitrile hydratase enzyme assays showed nitrile hydratase activity present in both whole cells and cell free lysate. While the whole cells assayed had nitrile hydratase activity of 450 um/ml/min, the lysate showed nitrile hydratase activity of 6658 um/ml/min. These results are shown below in Table 25. Raw data and calculations are shown in Appendix CC.

Table 25: Comparison of Nitrile Hydratase Activity Found in Cell Free Lysate and Whole Cells of RrDAP96253

Sample	Nitrile Hydratase Activity (um/ml/min)
Whole Cells	450
Lysate	6658

To confirm the presence of nitrile hydratase, outside of the enzyme assay, within the CFL post-lysis, SDS-Page gels were run with lysate from *R. rhodochrous* DAP 96253. Figure 39, found below, shows the expression of both the α- and β-subunits of H-Nase in Lane 2 of the gel. This gel seems to be absent of numerous other proteins often found in CFL produced from *R. rhodochrous* DAP 96253. [62].

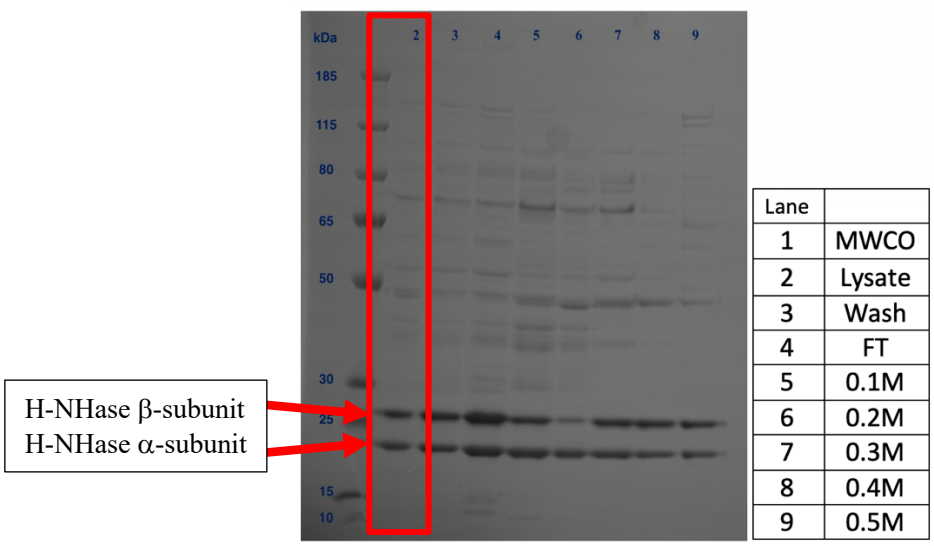
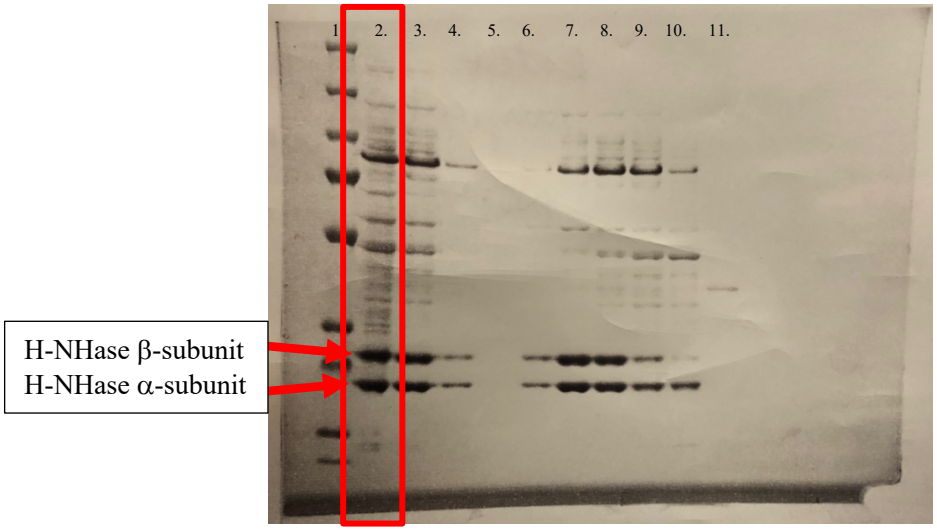


Figure 39: SDS-Page Ran with Cell Free Lysate from R. rhodochrous DAP96253 shows the expression of both the α- and β-subunits of H-Nase, shown in Lane 2.

Another SDS-Page gel ran with CFL is shown below in Figure 40. This gel also shows the expression of both the α- and β-subunits of H-NHase in Lane 2 of the gel while also showing numerous other proteins present in the CFL.



Lane	
1	MWCO
2	Lysate
3	Flowthrough
4	Wash
5	Blank
6	0.1M
7	0.2M
8	0.3M
9	0.4M
10	0.5M
11	EtOH

Figure 40: A Second SDS-Page Ran with Cell Free Lysate from R. rhodochrous DAP96253 shows the expression of both the α- and β-subunits of H-Nase, shown in Lane 2.

During the first bioconversion run conducted with cell free lysate, CFL #1, the bioreactor was placed in an ice bath. The process was run for 90 minutes until bilayer formation occurred and small, string-like objects appeared in the solution. The run was suspended at this time. These observations can be seen in Figure 42 below. Until it was stopped, the process produced 0.02 moles of AMD out of a possible 0.73 moles, giving the run a conversion percentage of 2.74%. Further research led to the bioreactor being used at room temperature (~25ºC) without any bilayer or small, string-like objects forming. These observations can be seen below in Figure 44. This run, CFL #2, produced 1.14 moles of AMD out of a possible 2.2 moles, giving the run a conversion percentage of 51.8%. Both runs were conducted at an input rate of 0.8 mL/min. These results are shown below in Table 26. The conversion comparison for both runs can be found below in Figures 41 and 43 respectively. A comparison of the two runs, CFL #1 vs. CFL

#2, can be found below in Figure 45. Raw data and calculations are shown in Appendix DD and EE respectively.

Table 26: Production of Acrylamide using Cell Free Lysate from R. rhodochrous DAP 96253 while at Varying Temperatures.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Final Solution (AMD%w/v)
CFL #1 Ice Bath	0.8	48mL	0.73	0.02	2.74%	0.64%
CFL #2 Room Temp	0.8	144mL	2.2	1.14	51.8%	21.82%

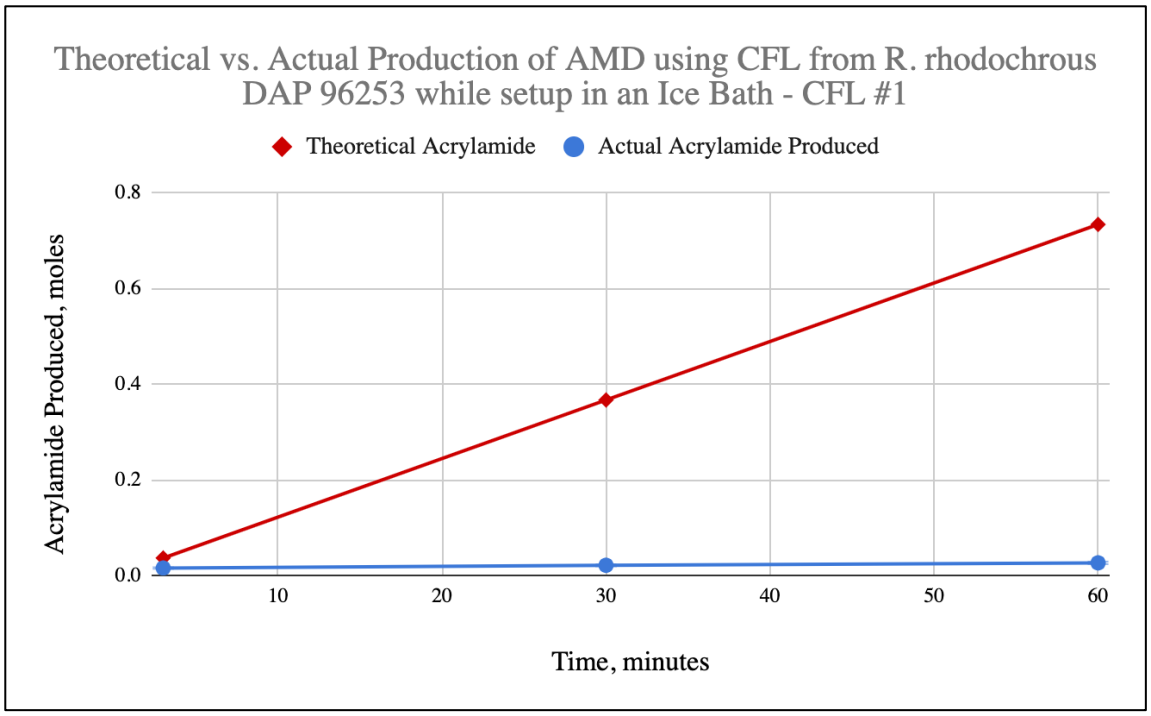


Figure 41: Production of Acrylamide using Cell Free Lysate from R. rhodochrous DAP 96253 while being setup in an Ice Bath, CFL #1

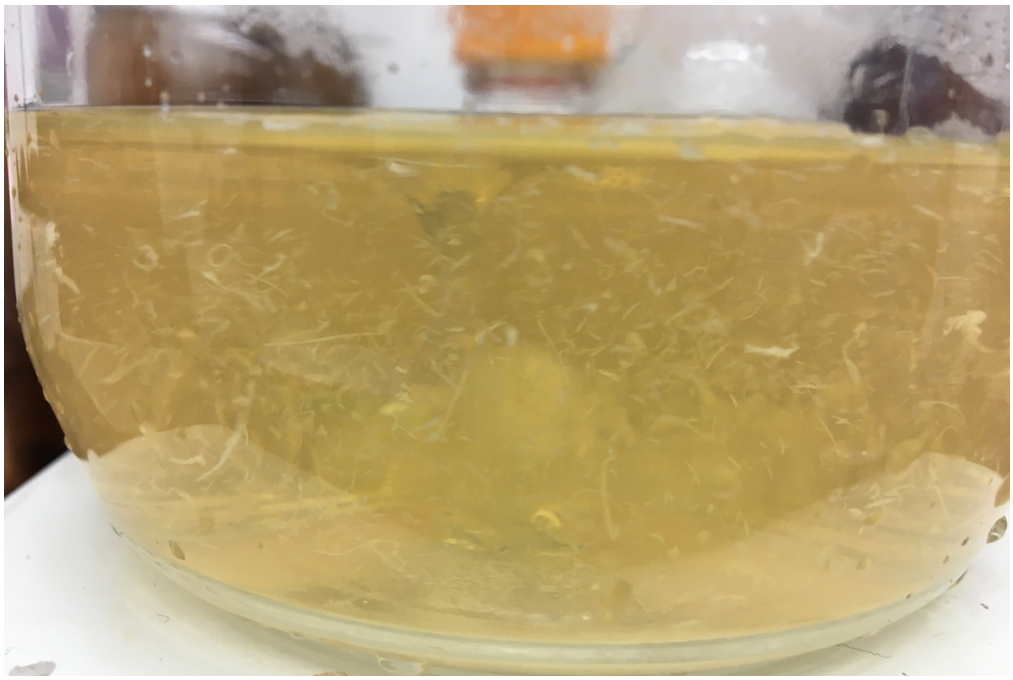


Figure 42:AN Bilayer and Small, String-like Objects Present during the Production of AMD using CFL from R. rhodochrous DAP96253 while being Housed in an Ice Bath, CFL #1

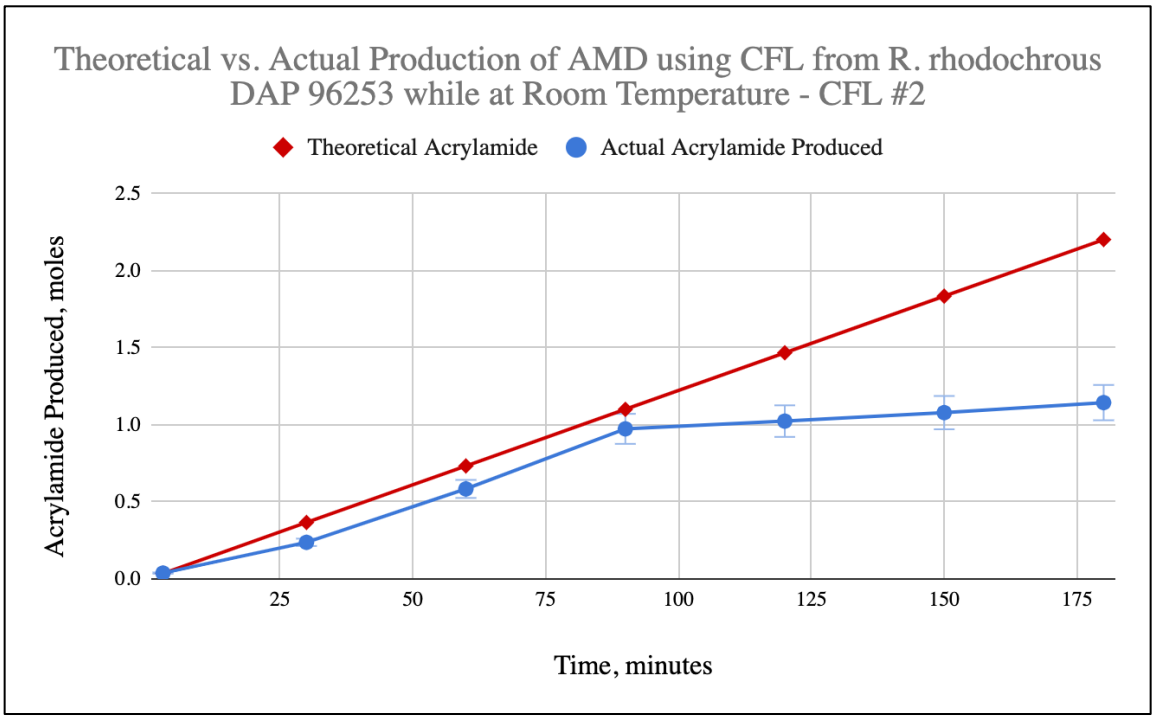


Figure 43: Production of AMD using CFL from R. rhodochrous DAP 96253 while at Room Temperature, CFL #2

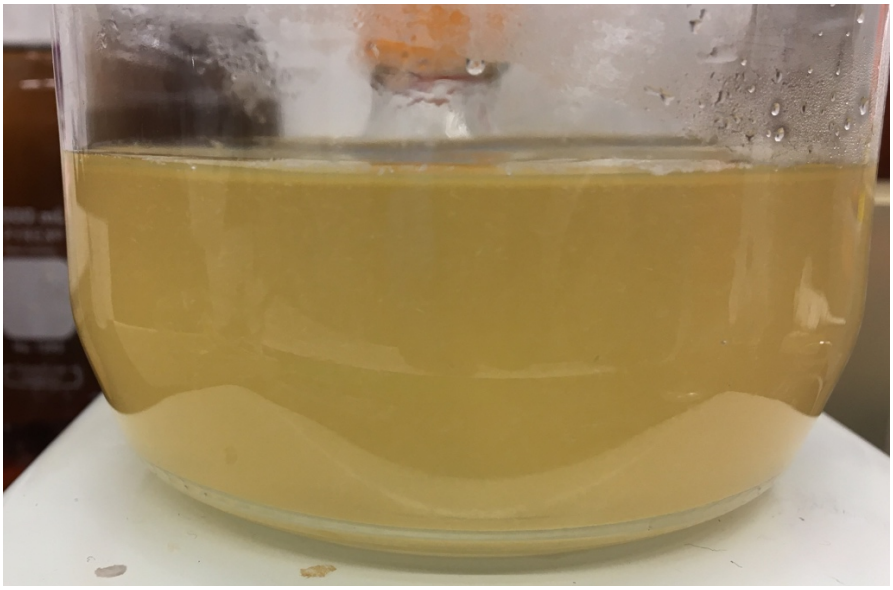


Figure 44: Absence of AN Bilayer and Small, String-like Objects during the Production of AMD using CFL from R. rhodochrous DAP 96253 at Room Temperature, CFL #2

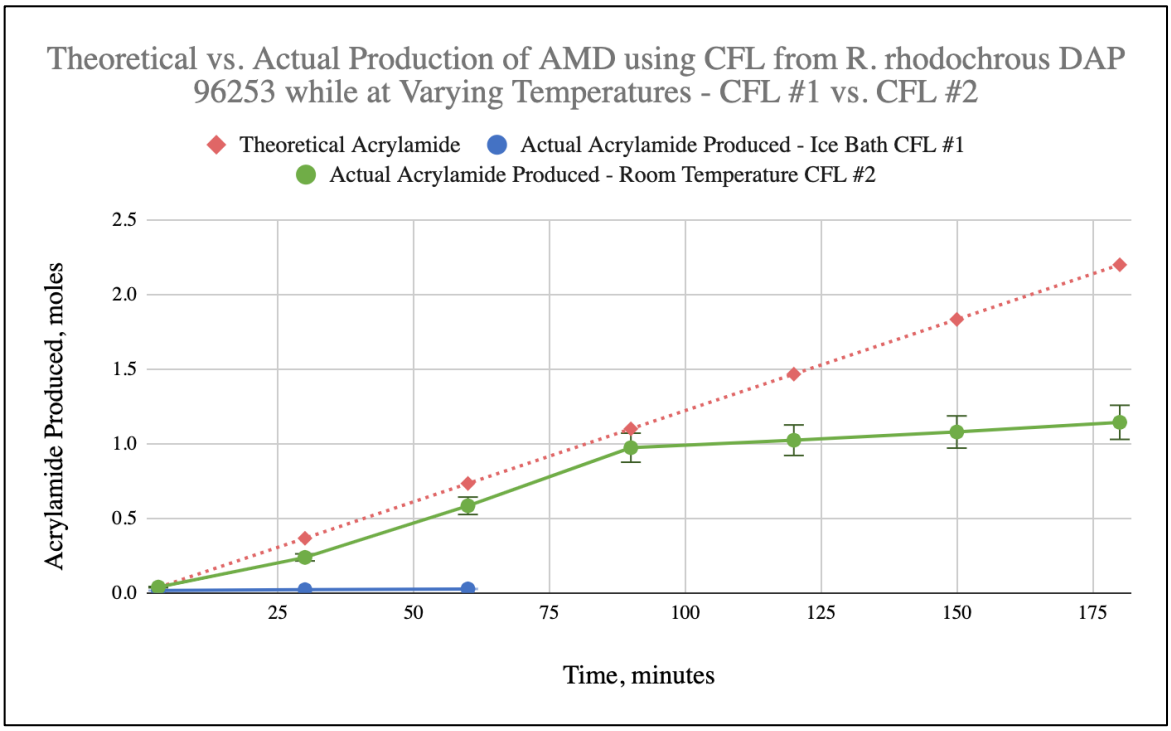


Figure 45: Production of AMD using CFL from R. rhodochrous DAP 96253 while at Varying Temperatures – Ice Bath (CFL #1) vs. Room Temperature (CFL #2).

A replicate run of CFL #2 was carried out using the same parameters. The third CFL bioconversion run, CFL #3, was ran using a rate of 0.8 mL/min for 180 minutes. By adding 144 mL of AN, a total of 2.2 moles of AMD was able to be produced. The data for CFL #3 is shown below in comparison to CFL #2. Out of a total of 2.2 moles, CFL #3 run produced 1.69 moles of AMD, giving it a conversion percentage of 76.8%. The final solution was 33.26%*w/v AMD compared to CFL #2 final solution percentage AMD of 21.82%w/v. The comparison of these two runs' conversions can be found below in Figure 46. Raw data and calculations can be found in Appendix EE and FF respectively.

Table 27: Production of Acrylamide using Two Different Samples of Cell Free Lysate from R. rhodochrous DAP 96253 under the Same Run Conditions.

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Acrylamide Produced (AMD%w/v)
CFL #2	0.8	144mL	2.2	1.14	51.8%	21.82%
CFL #3	0.8	144mL	2.2	1.69	76.8%	33.26%*

*Sample point '180 minutes' was not run in triplicates on the GC-MS. A single sample was evaluated.

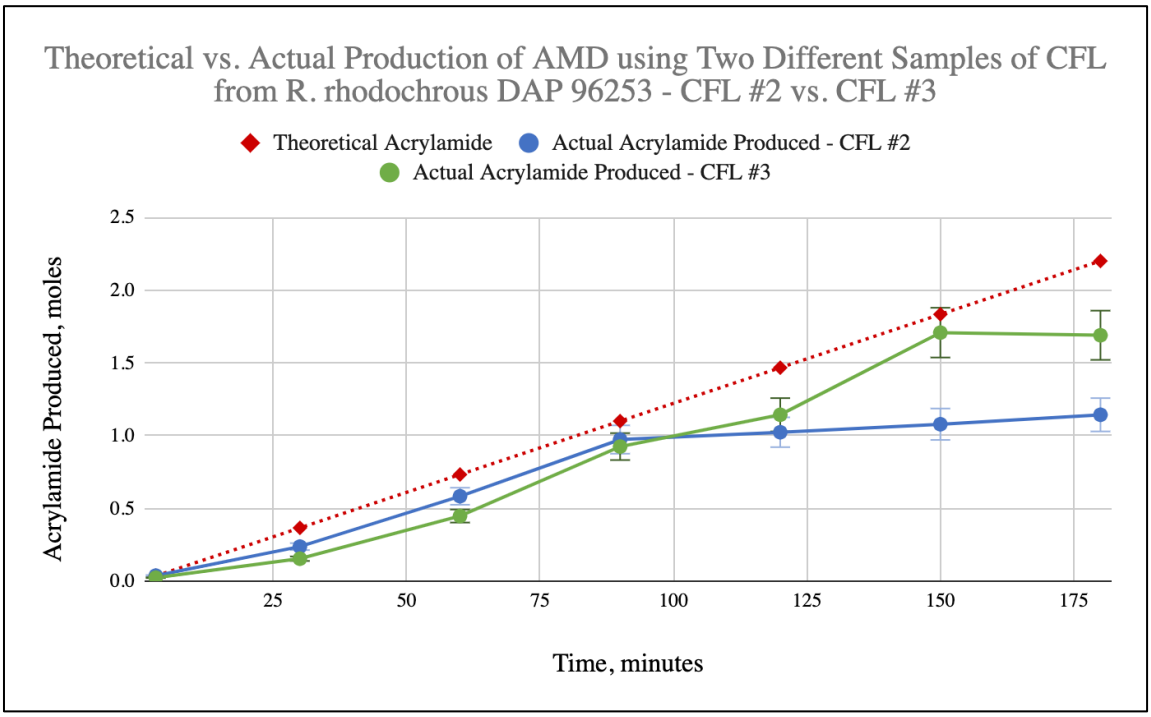


Figure 46: Production of AMD using Two Different Samples of CFL from R. rhodochrous DAP 96253 under the Same Run Conditions – CFL #2 vs. CFL #3.

3.1.8 *Purified Nitrile Hydratase Bioconversion of Acrylonitrile to Acrylamide*

The standard used for the remaining bioconversion runs can be found in Appendix GG. This standard marks the change in final product evaluation being performed on the GC-FID to the GC-MS.

Initial studies researching the bioconversion capabilities of purified nitrile hydratase focused on the verification of nitrile hydratase activity in the final purified samples. The first step to this process was verifying the effectiveness of a weak anion exchange column media, i.e. Sephacel® and Sepharose®, to attract nitrile hydratase out of the CFL. A hand-packed Sephacel® column was first used to test the retention of nitrile hydratase. After running the sample through the column, the CFL, the flowthrough (collected CFL after running through the column), the wash (buffer used to wash any unbound proteins from the column after the sample has been run through), and the packed Sephacel, pulled from the column before the CFL was ran through, were assayed for NHase activity. The CFL possessed NHase activity of 8246.02 ± 1017.19. The flowthrough possessed NHase activity of 4724.28 ± 1797.91. The wash buffer possessed NHase activity of 3581.38 ± 1354.69. The Sephacel® column media possessed a NHase activity of -2 ± 0.886. Table 28 below shows the nitrile hydratase activity of each sample. Raw data and calculations can be found in Appendix HH.

Table 28: Evaluation of the Ability of Sephacel® to Bind Nitrile Hydratase Proteins to the Column Media by Assessing the Nitrile Hydratase Activity of the CFL, Flowthrough, Wash, and Column Media during an Initial Purification Process.

Sample	Nitrile Hydratase Activity (um/ml/min)
Cell Free Lysate	8246.02 ± 1017.19
Flowthrough	4724.28 ± 1797.91
Wash	3581.38 ± 1354.69
Sephacel	-2 ± 0.886

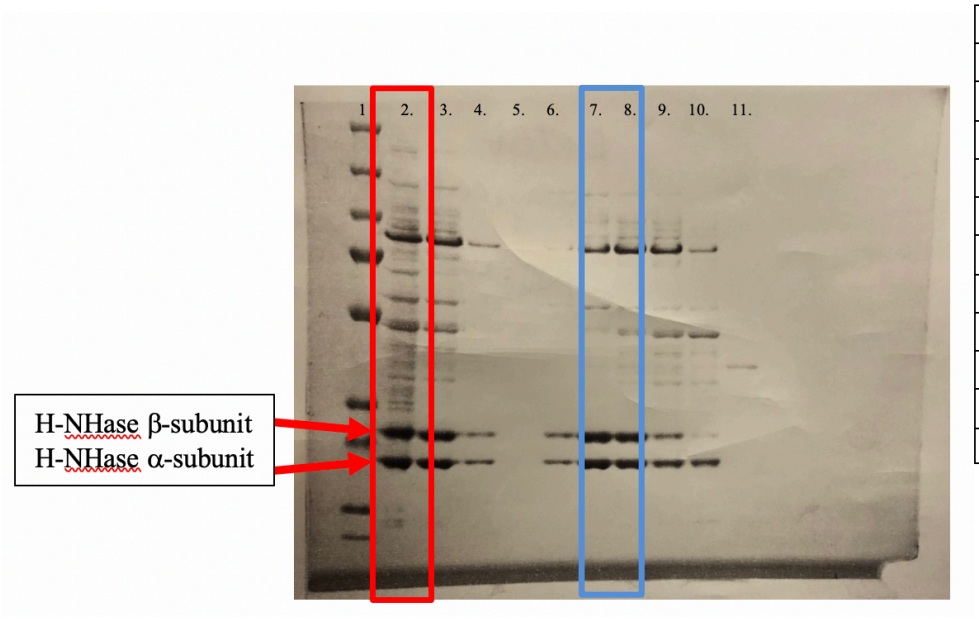
After this evaluation, a prepacked column, DEAE FF column containing Sepharose®, was used for the purification process. The effectiveness of both a 1mL and 5mL column were tested and evaluated for the amount of protein in each step using the bicinchoninic acid assay (BCA) [62]. The evaluates steps were CFL, flowthrough, wash, 0.1M elution, 0.25M elution, 0.5M elution, 1.0M elution. Cell free lysate from the same lysing procedure was used for both purification runs. Thus, it has the same amount of protein, 15.05 mg/mL. The amount of protein per sample was measured in mg/mL. The data for the experiment can be found below in Table 29. The 1mL column had more protein retained in the column initially, 2.87 mg/mL, but more protein was lost in the wash, 4.05. The 5mL column had more protein contained in the flowthrough, 7.25 mg/mL, but had more retained on the column during the wash, 2.43 mg/mL. The 5mL had more protein present in all 4 elutions buffer with protein concentrations of 0.40, 0.99, and 0.12 mg/mL in order of 0.1M, 0.25M, and 0.5M concentrations. The 1mL column had

0.18, 0.32, and 0.06 mg/mL of protein in order of increasing elution concentration. Both columns showed no protein elution with the 1M elution buffer. Nitrile hydratase activity was not assayed for this experiment. Raw data and calculations can be found below in Appendix II.

Table 29: Evaluation of the Purification Efficiency of Two Different Size Anion Exchange Column, 1mL vs. 5mL, by Assessing the Protein Concentration (mg/mL) during each Step of the Purification Process.

Column	CFL	Flowthrough	Wash	0.1M Elution	0.25M Elution	0.5M Elution	1M Elution
1mL	15.05	2.87	4.05	0.18	0.32	0.06	0.00
5mL	15.05	7.25	2.43	0.40	0.99	0.12	0.00

The higher protein concentrations found in Table 29 above were consistent with Figure 40 above, shown below as Figure 47. The purification process depicted in Figure 47 was produced using the 5mL DEAE FF column used in the comparison in Table 29. The highest concentration of proteins, in this case verified as expressed H-NHase, was present between the 0.1M and 0.25M concentrations. Figure 47's purification process used elutions with the concentrations 0.1M, 0.2M, 0.3M, 0.4M, and 0.5M of NaCl but the darker NHase bands are located in lane 7, depicting the 0.2M elution, and lane 8, depicting the 0.3M elution. These lanes are highlighted in blue on the figure below.



Lane	
1	MWCO
2	Lysate
3	Flowthrough
4	Wash
5	Blank
6	0.1M
7	0.2M
8	0.3M
9	0.4M
10	0.5M
11	EtOH

Figure 47: A SDS-Page showing the Highest Expression of both the α- and β-subunits of H-Nase in Lanes 6 and 7.

A bioconversion run using the 0.25M elution of purified nitrile hydratase was conducted using a rate of 0.6mL for 180 minutes at room temperature. This resulted in 108 mL of AN being added to the reactor over time. Thus, giving a theoretical yield of 1.65 moles of AMD. Bioconversion run PU #1 produced a total of 0.45 moles of AMD, giving the run a conversion percentage of 27.3%. This data is shown below in Table 30. The conversion over time is shown below in Figure 48. Raw data and calculations can be found below in Appendix JJ. An outlier data point was thrown out for the 30min timepoint sample.

Table 30: Production of Acrylamide using Purified Nitrile Hydratase from R. rhodochrous DAP 96253 (PU #1).

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Acrylamide Produced (AMD%w/v)
PU #1	0.6	108mL	1.65	0.48	29.1%	9.17%

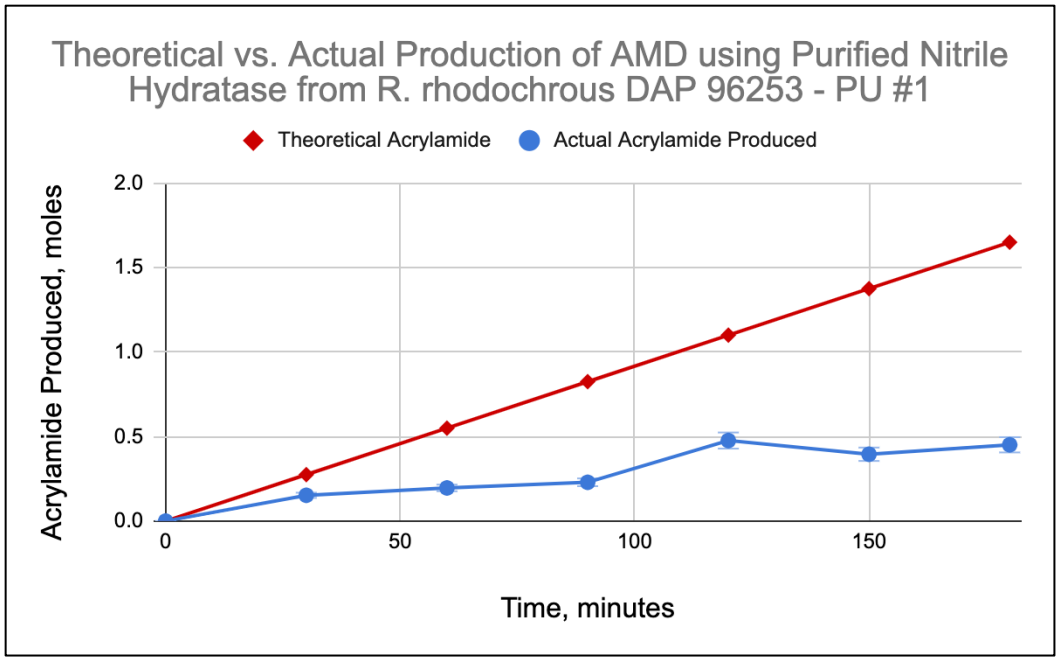


Figure 48: Production of Acrylamide using Purified Nitrile Hydratase from R. rhodochrous DAP 96253.

A second bioconversion run using a 0.25M elution of purified nitrile hydratase was conducted using a rate of 0.6mL for 180 minutes at room temperature. This resulted in 108 mL of AN being added to the reactor over time. Thus, giving a theoretical yield of 1.65 moles of AMD. Bioconversion run PU #2 produced a total of 0.56 moles of AMD, giving the run a conversion percentage of 33.9%. This data is shown below in Table 31. The conversion over

time is shown below in Figure 49. Raw data and calculations can be found below in Appendix KK. Outlier data points were thrown out for samples 30A and 180C.

Table 31: Production of Acrylamide using Purified Nitrile Hydratase from R. rhodochrous DAP 96253 (PU #2).

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Acrylamide Produced (AMD%w/v)
PU #2	0.6	108mL	1.65	0.56	33.9%	10.6%

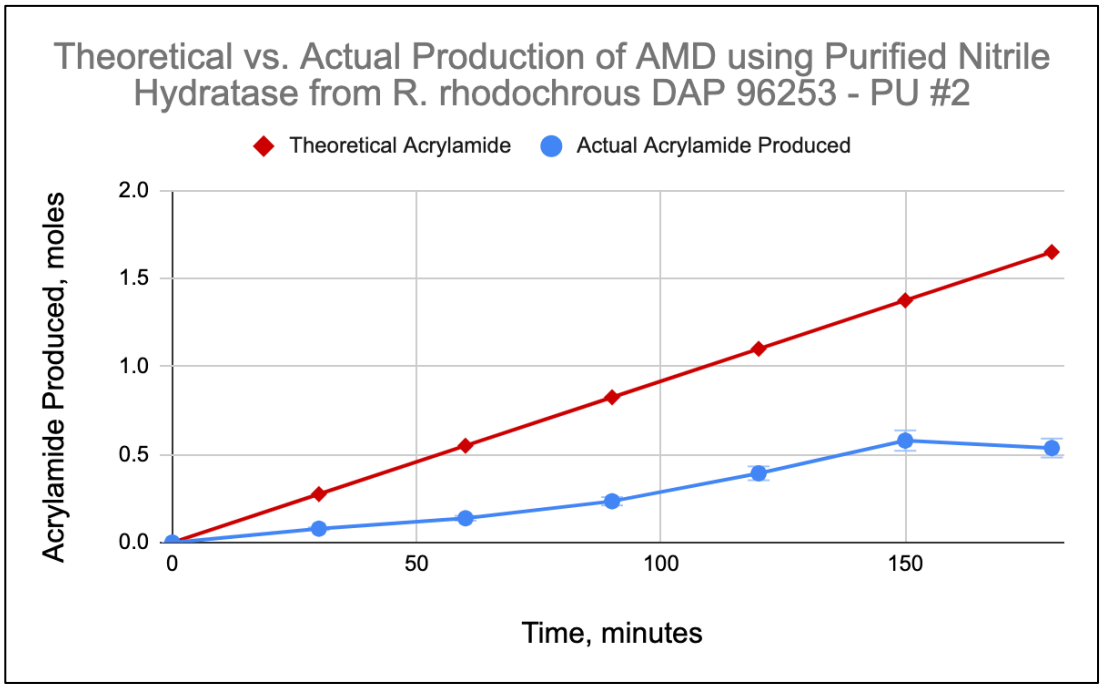


Figure 49: Production of Acrylamide using Purified Nitrile Hydratase from R. rhodochrous DAP 96253

3.1.9 *Immobilized Purified Enzyme Bioconversion of Acrylonitrile to Acrylamide*

Initial work dealing with immobilized nitrile hydratase started with immobilizing nitrile hydratase purified from *R. rhodochrous* DAP 96253. The 0.25M elution was used for this purpose due to its activity describe in the prior section. Final calculations ended on using the same method employed for whole cells of *R. rhodochrous* DAP 96253 as described in the methods sections. The procedure produced the immobilized enzyme seen below in Figure 49.

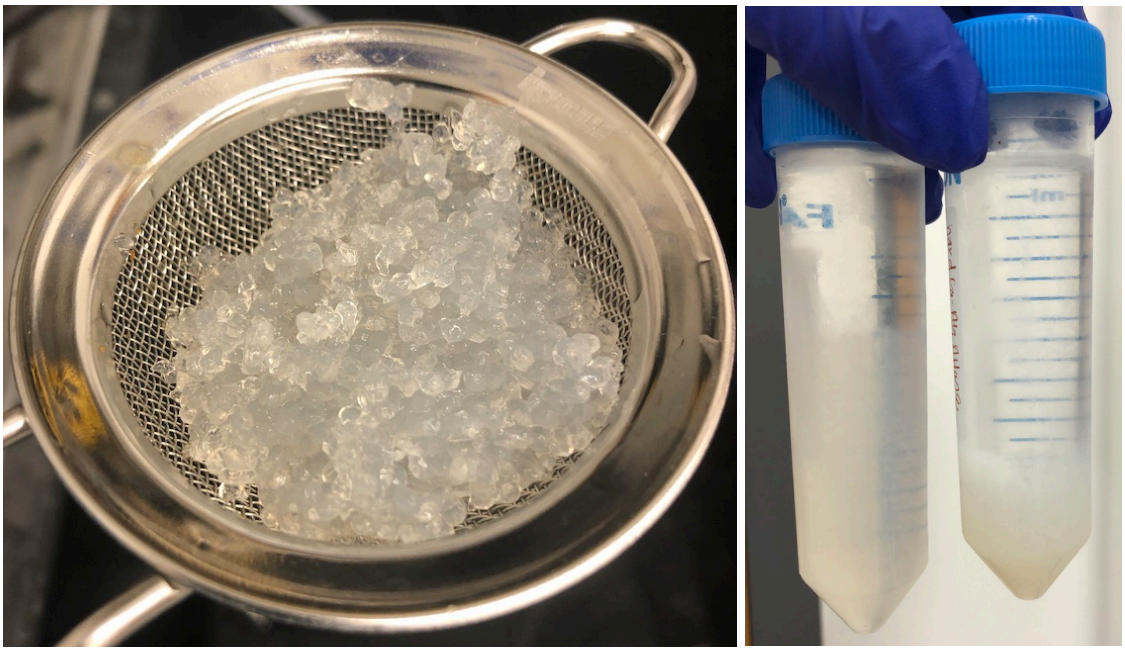


Figure 50: (L) Calcium Alginate Immobilized Nitrile Hydratase purified from R. rhodochrous DAP 96253 (R) Non-hardened and Hardened Calcium Alginate Immobilized Nitrile Hydratase purified from R. rhodochrous DAP 96253.

A bioconversion run using hardened calcium alginate immobilized 0.25M elution of purified nitrile hydratase was conducted using a rate of 0.6mL for 180 minutes at room temperature. This resulted in 108 mL of AN being added to the reactor over time. Thus, giving a theoretical yield of 1.65 moles of AMD. Bioconversion run H-CaAlg-PU #1 produced a total of 0.39 moles of AMD, giving the run a conversion percentage of 23.6%. Raw data and calculations can be found below in Appendix LL.

A bioconversion run using non-hardened calcium alginate immobilized 0.25M elution of purified nitrile hydratase was conducted using a rate of 0.6mL for 180 minutes at room temperature. This resulted in 108 mL of AN being added to the reactor over time. Thus, giving a theoretical yield of 1.65 moles of AMD. Bioconversion run NH-CaAlg-PU #1 produced a total of 0.30 moles of AMD, giving the run a conversion percentage of 18.2%. Raw data and calculations can be found below in Appendix MM. An outlier data point was thrown out for sample 180B.

This data is shown below in Table 32.

Table 32: Production of AMD using Hardened and Non-hardened Purified Nitrile Hydratase from R. rhodochrous DAP 96253 (H-CaAlg-PU vs. NH-CaAlg-PU).

	Input Rates (mL/min)	Acrylonitrile Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Acrylamide Produced (AMD%w/v)
H-CaAlg-PU	0.6	108mL	1.65	0.39	23.6%	5.8%
NH-CaAlg-PU	0.6	108mL	1.65	0.30	18.2%	5.14%

The conversion comparisons of actual acrylamide produced compared to theoretical acrylamide produced for both H-CaAlg-PU and NH-CaAlg-PU are both shown below in Figure 51. Both bioconversion runs' data start at minute 60 due to GC-MS data not identifying acrylamide until the minute 60 samples.

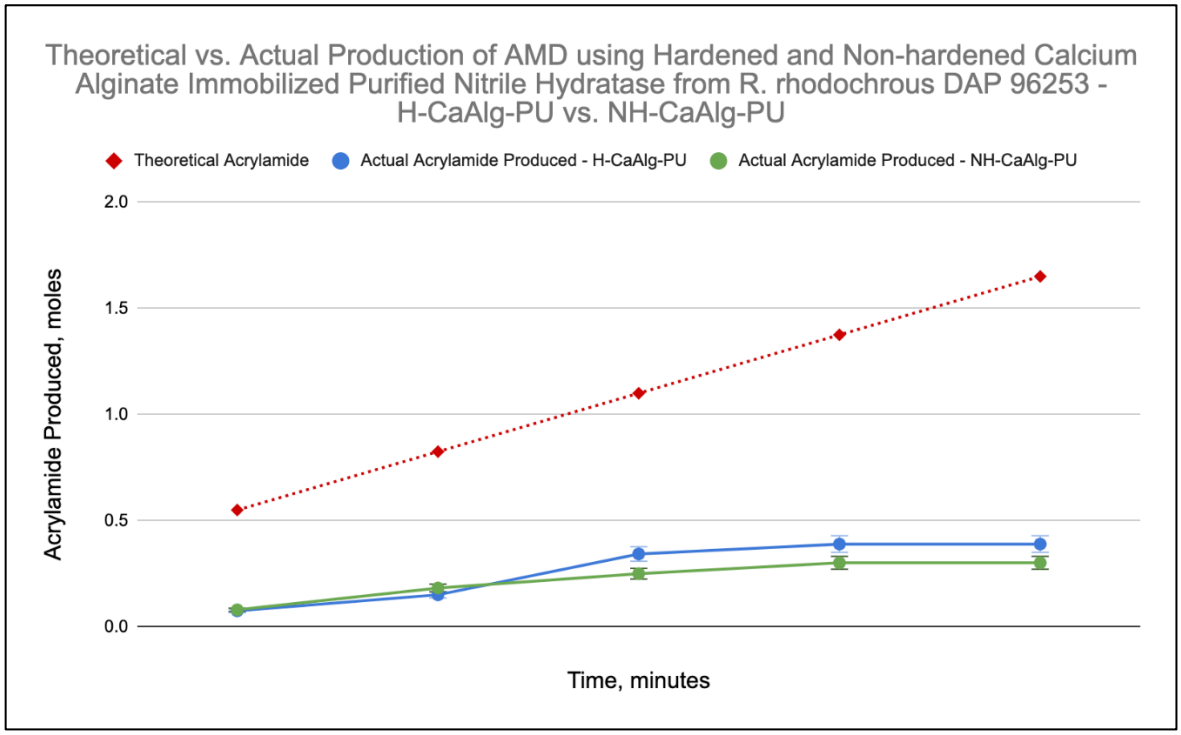


Figure 51: Production of Acrylamide using Hardened and Non-hardened Calcium Alginate Immobilized Purified Nitrile Hydratase from R. rhodochrous DAP 96253. (H-CaALg-PU vs. NH-CaAlg-PU)

3.1.10 *Overall Comparison of the Bioconversion of Acrylonitrile to Acrylamide using Whole Cells, Immobilized Cells, Cell Free Lysate, Purified Enzyme, and Immobilized Enzyme of RrDAP96253*

The best conversion run data were used to compare the overall production of acrylamide across all different forms of microbial catalysts derived from *R. rhodochrous* DAP 96253. Whole cells produced a total of 2.12 moles out of a possible 2.45 moles, giving a conversion percentage of 86.5%. Polyacrylamide immobilized whole cells produced a total of 1.07 moles of acrylamide out of a possible 1.53 moles, giving a conversion percentage of 69.9%. Polyethyleneimine-glutaraldehyde immobilized whole cells produced a total of 0.81 moles of acrylamide out of a possible 1.65 moles, giving a conversion percentage of 49.1%. Hardened calcium alginate whole cells produced a total 1.93 moles of acrylamide out of a possible 1.93 moles, giving a conversion percentage of 100%. Non-hardened calcium alginate whole cells produced a total 1.68 moles of acrylamide out of a possible 1.93 moles, giving a conversion percentage of 87%. Cell free lysate produced a total of 1.14 moles of acrylamide out of a possible 2.2 moles, giving a conversion percentage of 51.8%. Purified nitrile hydratase produced a total of 0.56 moles of acrylamide out of a possible 2.2 moles, giving a conversion percentage of 33.9%. Hardened calcium alginate immobilized purified nitrile hydratase produced a total of 0.39 moles of acrylamide out of a possible 1.65 moles, giving a conversion percentage of 23.6%. Non-hardened calcium alginate immobilized purified nitrile hydratase produced a totally of 0.30 moles of acrylamide out of a possible 1.65 moles, giving a conversion percentage of 18.2%. This data can be found in Table 33 below.

Table 33: Comparison of the Production of Acrylamide across all forms of R. rhodochrous DAP 96253 used.

	Input Rates (mL/min)	AN Added (mL)	Theoretical Acrylamide (moles)	Acrylamide produced (moles)	Conversion Percentage (%)	Acrylamide Produced (AMD%w/v)
Whole Cells	0.8	160mL	2.45	2.30	93.9%	44.9%
PAM Imm. Cells	0.33	100mL	1.53	1.07	69.9%	23.13%
PEI-Glu Imm. Cells	0.6	108mL	1.65	0.81	49.1%	16.7%
H-CaAlg Whole Cells	0.6	126mL	1.93	1.93	100%	40.6%
NH-CaAlg Whole Cells	0.6	126mL	1.93	1.68	87%	34.8%
CFL	0.8	144mL	2.2	1.14	51.8%	21.82%
Purified Enzyme	0.6	108mL	1.65	0.56	33.9%	10.6%
H-CaAlg-PU	0.6	108mL	1.65	0.39	23.6%	5.8%
NH-CaAlg-PU	0.6	108mL	1.65	0.30	18.2%	5.14%

3.1.11 *Storage Study of BioAcrylamide*

In order to study the commercial acrylamide storage effects and the lasting capabilities of the bioacrylamide being produced, the acrylamide produced from run #1 using hardened Ca-Alg immobilized cells of RrDAP96253 from Figure 32 was placed in a glass container with a screwed tight lid, wrapped in aluminum foil, and placed in a 4ºC refrigerator. The acrylamide quantities were then checked periodically over the course of 6 months. The initial concentration of the solution after production was 40.56%. When it was checked 1 week later, the solutions were an average of 41.6%(w/v). Almost two months later, the solution showed on average 36.74%(w/v) AMD. Fifteen days later, the concentration of AMD showed on average 39.95%(w/v). Almost 4 months later, the concentration showed an average of 45.81%(w/v) AMD. These results are shown in Table 24 below. Raw data and calculations are shown in Appendix NN.

Table 34: Storage Study depicting the Concentration over Time of Bioacrylamide Produced

Date Produced	**%(w/v) Acrylamide**
03-05-2018	40.56%
Date Checked	**%(w/v) Acrylamide**
03-12-2018	41.60%
05-07-2018	36.74%
05-22-2018	39.95%
09-13-2018	45.81%

3.2 Pegylated Asparaginase Preliminary Results

3.2.1 *Initial Enzymatic Activity of Freeform vs. Pegylated Purified Asparaginase from R. rhodochrous DAP 96253*

The initial experiment conducted to research the effects of pegylation on the enzymatic activity level of purified asparaginase involved tracking and comparing asparaginase enzyme activities of the various pegylated fractions collected from the size exclusion column to a control sample of free-form purified asparaginase set aside before the rest of the sample was divided and pegylated either as PEG40 or PEG80.

For both the PEG40 and PEG80 fractions, the same control sample of free-form purified asparaginase was assayed and retained. Its initial asparaginase activity was 1916.61 um/ml/min. For the PEG40 samples, the pegylated samples retained an average of 49.2% of the enzymatic activity exhibited prior to pegylation, with fractions #6, #7, and #8 showing the highest retentions of activity. This data is shown below in Table 35. For the PEG80 samples, the pegylated samples retained an average of 45.9% of the enzymatic activity exhibited prior to pegylation, with fractions #4, #5, and #6 showing the highest retentions of activity. This data is shown below in Table 36. While the initial asparaginase activity of the freeform purified protein was 1916.61 um/ml/min, the PEG40 samples showed an average of 942.24 um/ml/min of asparaginase activity and the PEG80 samples showed an average of 879.53 um/ml/min of asparaginase activity. The same samples for PEG40 and PEG80 exhibited the highest initial activities post-pegylation. The comparison of activity in the freeform protein versus the pegylated protein for both the PEG40 and PEG80 samples can be visually seen below in Figures 52 and 53. The raw data for these results can be found in Appendix OO.

Table 35: Comparison of Enzymatic Activity of Freeform Purified Asparaginase versus PEG40 Pegylated Purified Asparaginase Fractions

Samples	Initial Asparaginase Activity (um/ml/min)	Activity retained (%)
Control – purified asparaginase	1916.61	---
PEG40 #2	831.68	43.4%
PEG40 #3	875.25	45.7%
PEG40 #4	617.63	32.2%
PEG40 #5	881.95	46%
PEG40 #6	1160.89	60.6%
PEG40 #7	1182.77	61.7%
PEG40 #8	1342.80	70.1%
PEG40 #9	1059.85	55.3%
PEG40 #10	854.08	44.6%
PEG40 #11	793.66	41.4%
PEG40 #12	891.65	46.5%
PEG40 #13	814.67	42.5%
Average Asparaginase Activity Retained:		49.2%

Table 36: Comparison of Enzymatic Activity of Freeform Purified Asparaginase versus PEG80 Pegylated Purified Asparaginase Fractions

Samples	Initial Asparaginase Activity	Activity retained (%)
Control – purified asparaginase	1916.61	---
PEG80 #1	576.14	30.1%
PEG80 #2	553.15	28.9%
PEG80 #3	722.65	37.7%
PEG80 #4	1205.93	62.9%
PEG80 #5	1167.91	60.9%
PEG80 #6	1126.15	58.8%
PEG80 #7	904.73	47.2%
PEG80 #8	779.54	40.7%
Average Asparaginase Activity Retained:		45.9%

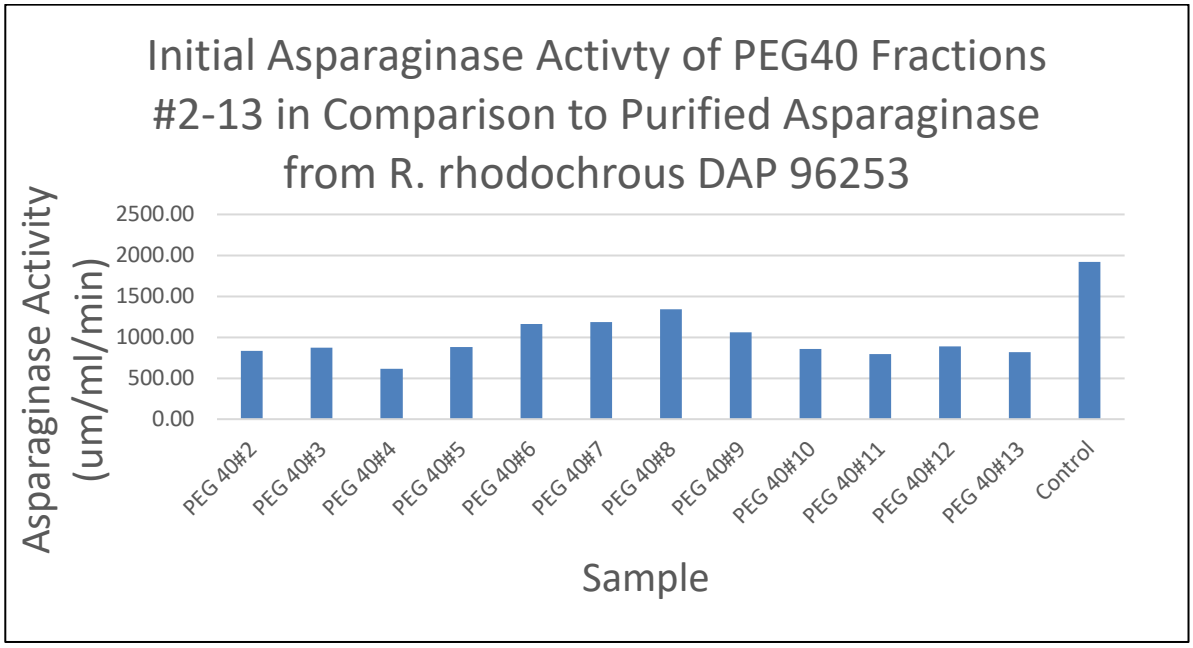


Figure 52: Comparison of Initial Asparaginase Activity of Freeform Asparaginase to PEG40 Asparaginase Fractions Purified from R. rhodochrous DAP 96253

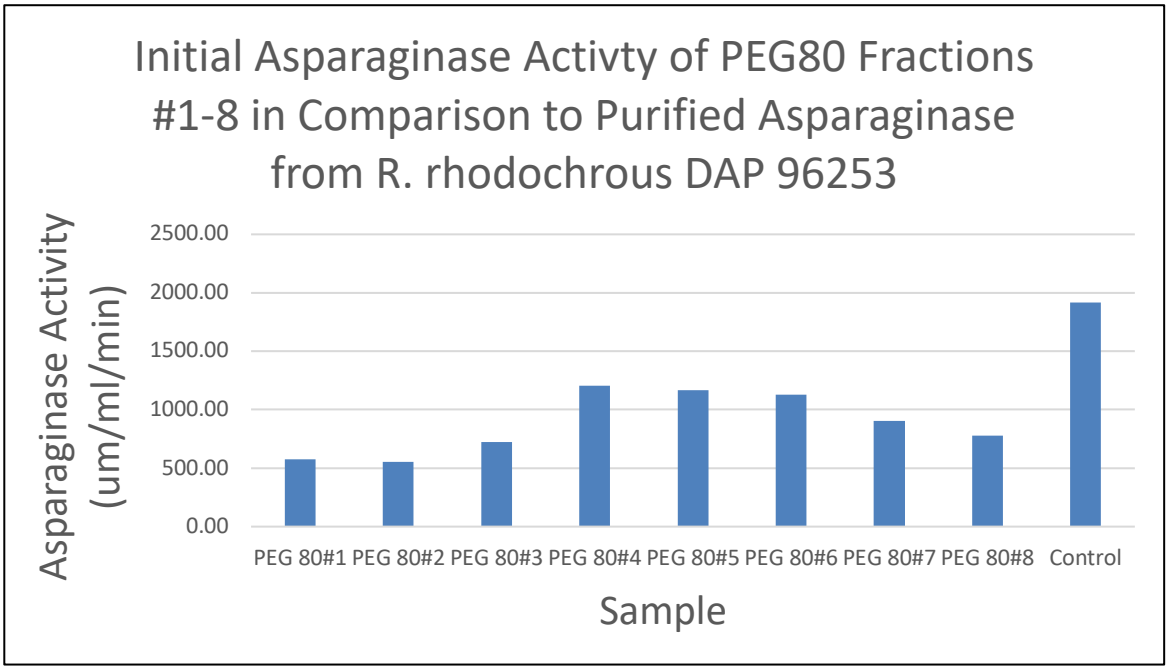


Figure 53: Comparison of Initial Asparaginase Activity of Freeform Asparaginase to PEG80 Asparaginase Fractions Purified from R. rhodochrous DAP 96253

3.2.2 *Storage Study of Asparaginase Activity over Time in Freeform and Pegylated Purified Asparaginase from R. rhodochrous DAP 96253 stored at 4℃*

After the initial loss of activity data was analyzed, all samples were stored at 4°C for the evaluation of asparaginase activity over time. After 7 days, asparaginase assays were conducted to analyze the loss of enzymatic activity over time in the freeform protein versus the pegylated protein. The control sample, freeform asparaginase, retained 91.6% of its enzymatic activity after 7 days. For all the PEG40 samples, an average of 77% of enzymatic activity was retained. However, these values ranged from 47.3-97.6%. PEG40 fractions #4 and #5 retained the highest amount of activity with 97.6% and 97.2% retention rates respectively. PEG40 fractions #6 and #8 retained the least amount of activity with 56.3% and 47.3% respectively. For the PEG80 samples, an average of 76% of enzymatic activity was retained. These samples exhibited a tighter range of 59.2-93.7%. PEG80 fractions #7 and #4 retained the highest amount of activity with 93.7% and 86.6% respectively. For PEG80 fractions #6 and #3 retained the least amount of activity with 67.6% and 59.2% respectively. For both PEG40 and PEG80, fraction #4 exhibited high enzymatic retention. For PEG80 samples, fractions #1 and #2 were not observed here as their asparaginase activities both increased after 7 days of incubation. Data for the comparison of the retention of enzymatic activity in freeform versus pegylated asparaginase after 7 days of storage at 4°C is shown below in Tables 37 and 38 respectively. The raw data for these results can be found in Appendix OO.

Table 37: Comparison of Enzymatic Activity of Freeform Purified Asparaginase Versus PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 after 7 Days of Storage at 4℃

Samples	Initial Asparaginase Activity (um/ml/min)	Asparaginase Activity 1 Week	Activity retained (%)
Control – purified asparaginase	1916.61	1756.04	91.6%
PEG40 #2	831.68	651.85	78.4%
PEG40 #3	875.25	608.37	69.5%
PEG40 #4	617.63	602.53	97.6%
PEG40 #5	881.95	857.39	97.2%
PEG40 #6	1160.89	653.54	56.3%
PEG40 #7	1182.77	862.93	73%
PEG40 #8	1342.80	635.66	47.3%
PEG40 #9	1059.85	752.78	71%
PEG40 #10	854.08	523.96	61.4%
PEG40 #11	793.66	705.15	88.9%
PEG40 #12	891.65	818.36	91.8%
PEG40 #13	814.67	746.16	91.6%
Average PEG-Asparaginase Activity Retained:			77%

Table 38: Comparison of Enzymatic Activity of Freeform Purified Asparaginase Versus PEG80 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 after 7 Days of Storage at 4℃

Samples	Initial Asparaginase Activity (um/ml/min)	Asparaginase Activity 1 Week	Activity retained (%)
Control – purified asparaginase	1916.61	1765.54	91.6%
PEG80 #1	576.14	665.77	---
PEG80 #2	553.15	576	---
PEG80 #3	722.65	427.88	59.2%
PEG80 #4	1205.93	1044.63	86.6%
PEG80 #5	1167.91	822.70	70.4%
PEG80 #6	1126.15	760.84	67.6%
PEG80 #7	904.73	847.34	93.7%
PEG80 #8	779.54	611.69	78.5%
Average PEG-Asparaginase Activity Retained:			76%

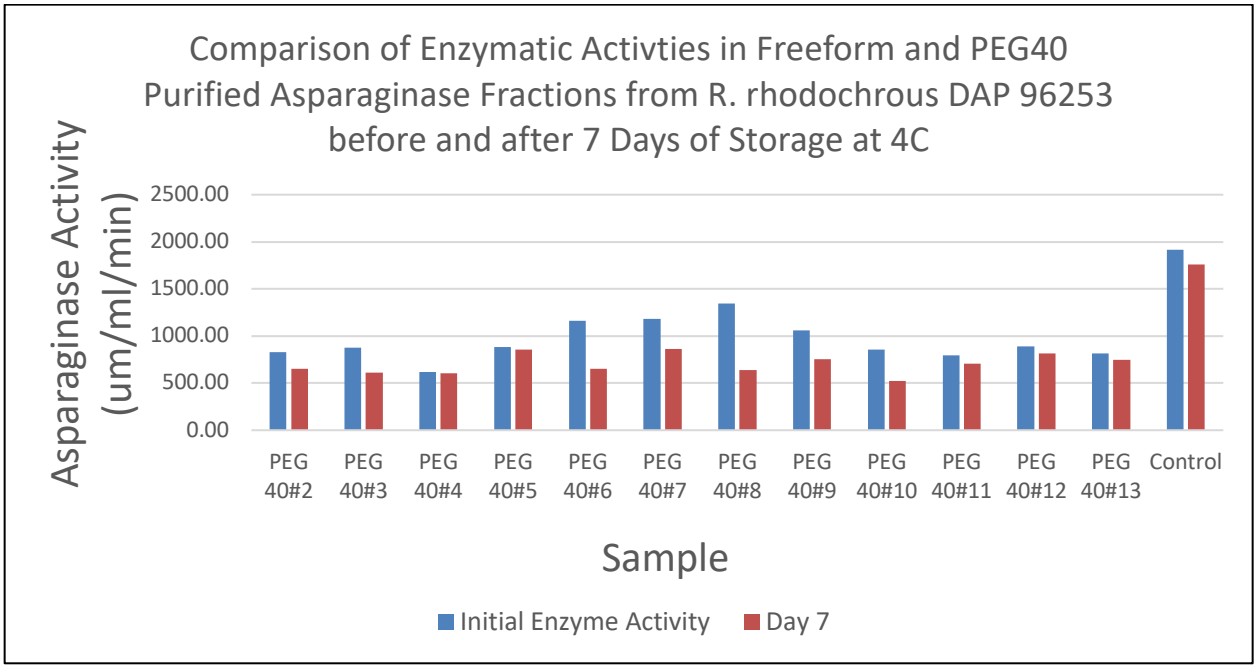


Figure 54: Comparison of Enzymatic Activities in Freeform and PEG40 Purified Asparaginase Fractions from R. rhodochrous DAP 96253 before and after 7 Days of Storage at 4℃

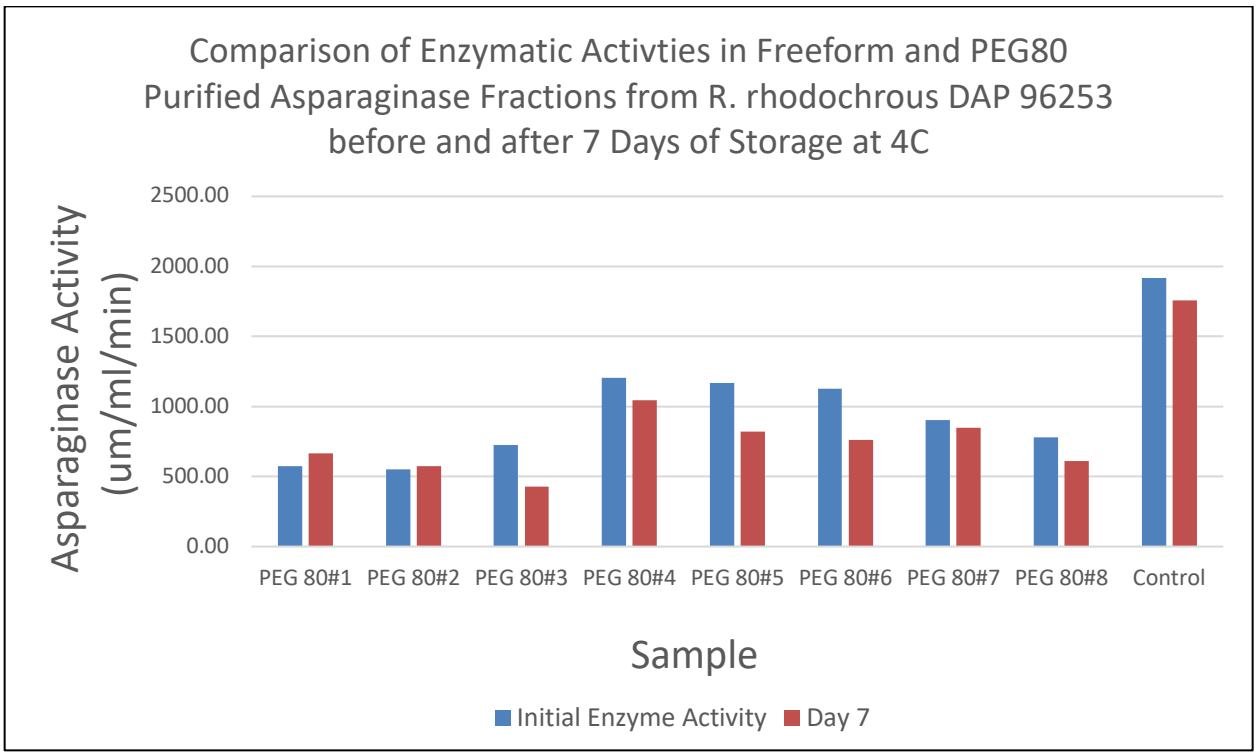


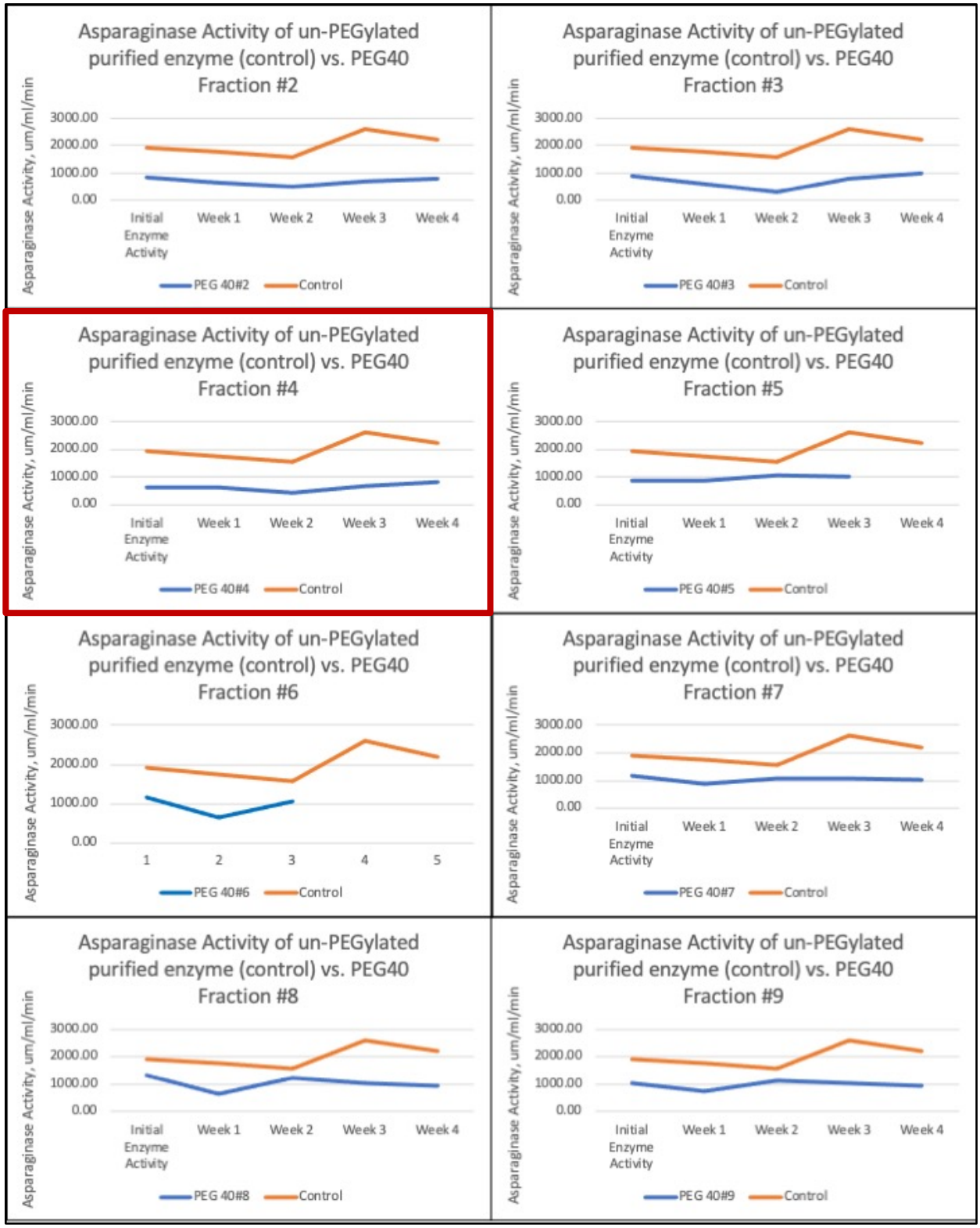
Figure 55: Comparison of Enzymatic Activities in Freeform and PEG40 Purified Asparaginase Fractions from R. rhodochrous DAP 96253 before and after 7 Days of Storage at 4℃

Enzymatic activity data were collected every 7 days for the following 3 weeks after the Day 7 data was collected for all samples available. These are shown in 5 different sample points labeled ‘initial enzyme activity’, ‘week 1’, ‘week 2’, ‘week 3’, and ‘week 4’.

The comparison of the enzymatic activities over time between the control sample and PEG40 fractions are shown individually over time below in Figure 56. Unfortunately, PEG40 fractions #5, #6, and #13 ran out of sample to complete the entire run of data collection. Fractions are distributed in different volumes dependent on size exclusions properties detected by the AKTA. This lack of data is present in the graphs below in Figure 56. The comparison of the enzymatic activities over time between the control sample and PEG80 fractions are shown individually over time below in Figure 57.

The graphs depicted below in Figures 56 and 57 show irregular data during the duration of the 4-week study. Most samples showed an increase in protein activity from week 2 to week 3. This is noticeable in the control sample as well as numerous other pegylated fraction samples, like PEG40 #2, #3, #4, #7, #12, and #13 and PEG80 #1, #2, #3, #4, #5, #6, and #7. For the fractions not mentioned in the prior statement, PEG40 #5, #6, #8, #9, #10, and #11 and PEG80 #8, while the enzymatic decreased from week 2 to week 3, the enzymatic activity increased at some point during the 4-week study time. It is also noted that for the control sample and for samples PEG40 #3, #4, #5, #10, #11, #12, and #13 and PEG80 #1, #2, #3, #5, #7, and #8, the samples’ enzymatic activity was higher at the end of the study than what was started with. Fraction PEG40 #4 stands out as exhibiting a consistent slow decrease in activity through week 2. Fraction PEG80 #5 stands out as exhibiting a gradual decrease in activity through week 2 in a mimicking trend of the control sample but with overall decreased activity. Both of these

noteworthy graphs are outlined in red below in Figured 56 and 57. The raw data for these results can be found in Appendix OO.



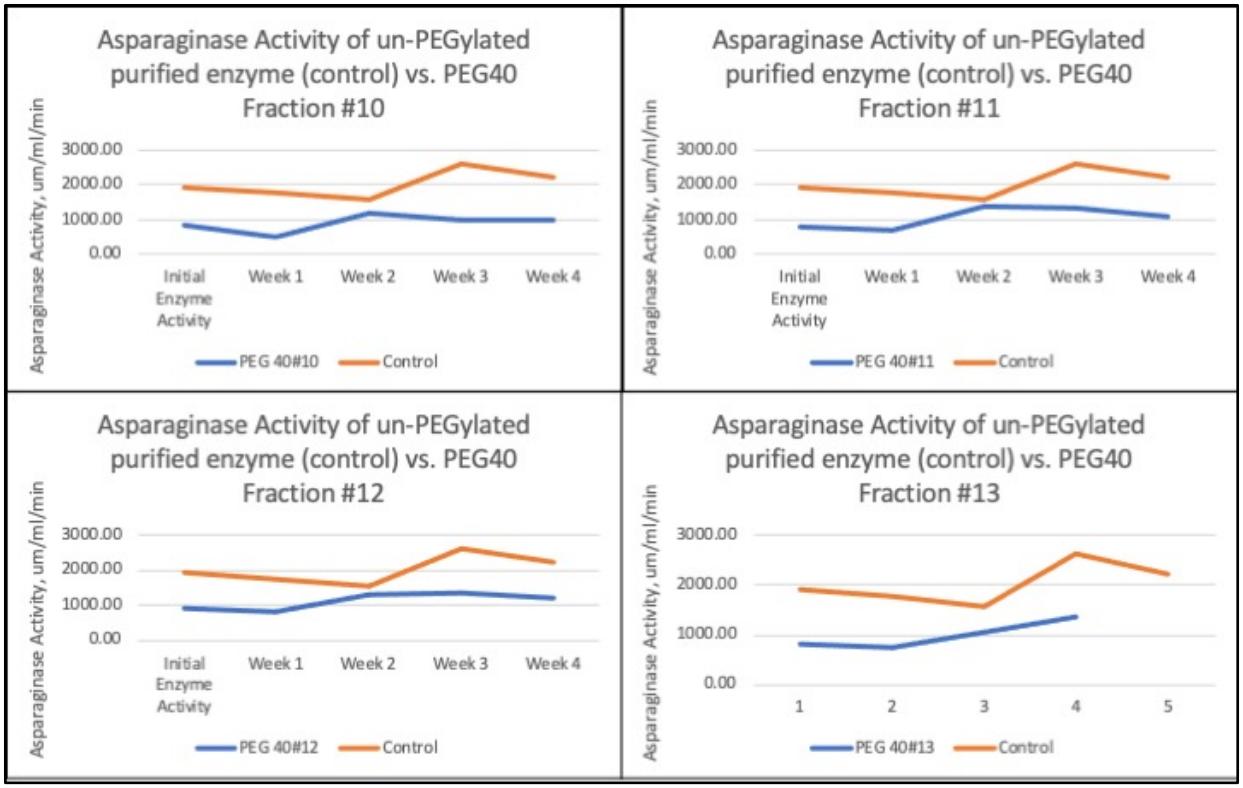


Figure 56: Graphs A-L (left-right from top-bottom): Comparison of Enzymatic Activity of Freeform Purified Asparaginase vs. PEG40 Pegylated Fractions Over 4 Weeks while Stored in 4℃

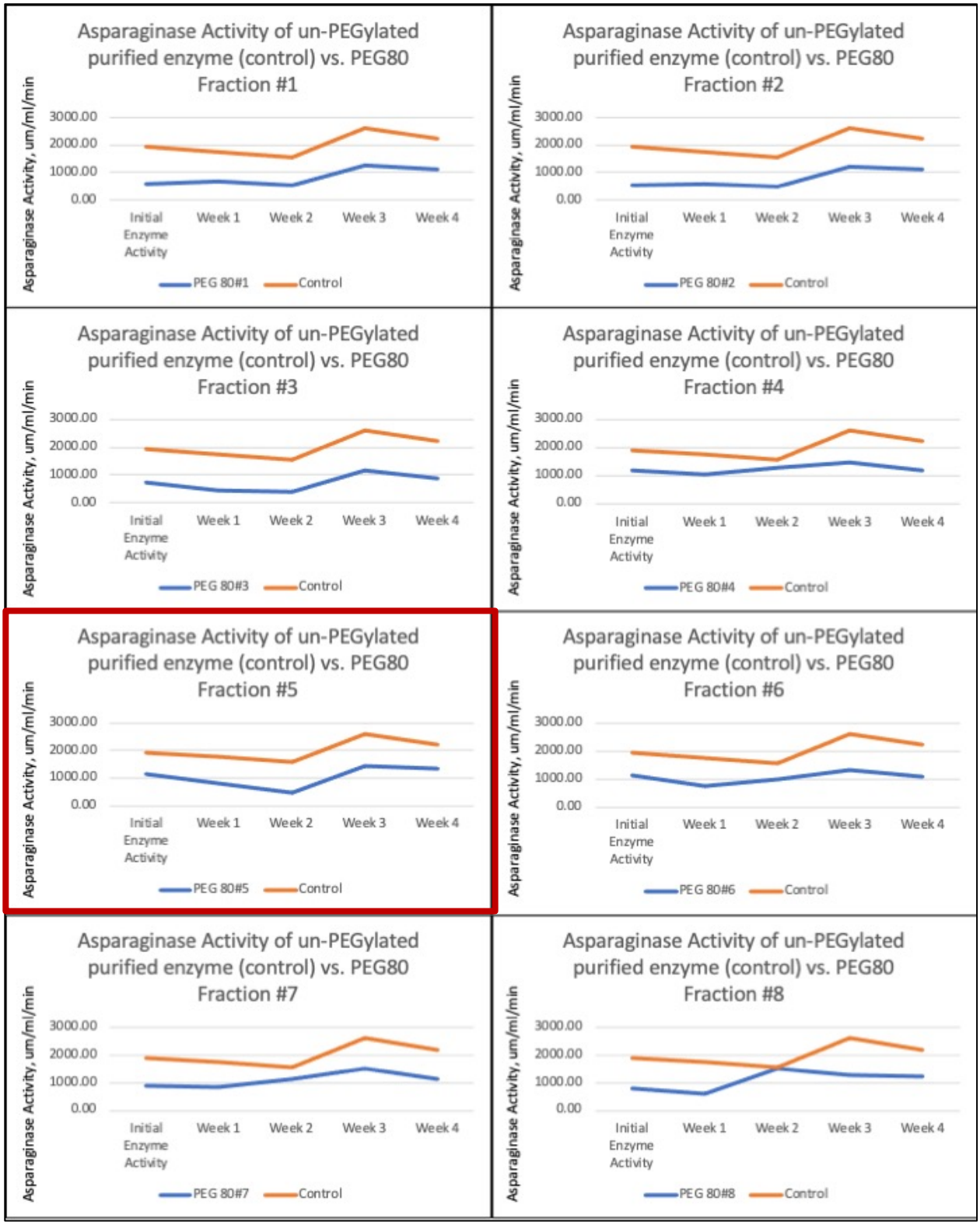


Figure 57: Graphs A-H (left-right from top-bottom): Comparison of Enzymatic Activity of Freeform Purified Asparaginase vs. PEG80 Pegylated Fractions Over 4 Weeks while Stored in 4℃

3.2.3 *Initial Enzymatic Activity of Freeform vs. Pegylated Purified Asparaginase from R. rhodochrous DAP 96253*

Further experimentation was carried out testing the enzymatic activity of freeform vs. pegylated fraction samples. Fractions with the highest asparaginase levels from the purification process were used for the control freeform samples. Instead of looking at all of the fractions from the two different pegylation concentrations (PEG40 and PEG80) used, three different syringes of PEG40 pegylated asparaginase were run through the size exclusion column. The fractions with the highest asparaginase activity levels were used for initial enzymatic activity comparisons shown below in Table 39 and Figure 58.

Fractions 4 and 5 of the freeform purified asparaginase were used as the control samples. Their initial enzymatic activity was 1632.98 and 1554.44 um/ml/min respectively. For comparison, the average of the two was calculated at 1593.71 um/ml/min. Syringe 1 of pegylated asparaginase produced two fractions, fractions 4 and 5, with the highest enzymatic activity at 1475.03 and 1479.23 um/ml/min respectively. The samples showed enzymatic retention rates of 92.6% and 92.8% respectively. Syringe 2 produced 4 fractions, fractions 4, 5, 6, and 7, with the highest enzymatic activity at 1690.97, 1648.81, 1743.94, and 1322.17 um/ml/min respectively. Fractions 4, 5, and 6 exhibited higher enzymatic activity than the purified enzyme samples. Fraction 7 exhibited an enzymatic retention rate of 83%. Syringe 3 of pegylated asparaginase produced three fractions, fraction 4, 5, and 6, with the highest enzymatic activity at 1337.92, 1292.32, and 1348.01 um/ml/min. The samples showed enzymatic retention rates of 84%, 81.1%, and 84.6% respectively. The raw data for these results can be found in Appendix PP.

Table 39: Initial Enzymatic Activity of Freeform vs. Pegylated Purified Asparaginase from R. rhodochrous DAP 96253

Samples	Fractions	Initial Asparaginase Activity (um/ml/min)	Average Activity (um/ml/min)	Activity retained (%)
Control – purified asparaginase	F4	1632.98	1593.71	---
	F5	1554.44		---
Syringe 1	F4	1475.03		92.6%
	F5	1479.23		92.8%
Syringe 2	F4	1690.97		---
	F5	1648.81		---
	F6	1743.94		---
	F7	1322.17		83%
Syringe 3	F4	1337.92		84%
	F5	1292.32		81.1%
	F6	1348.01		84.6%

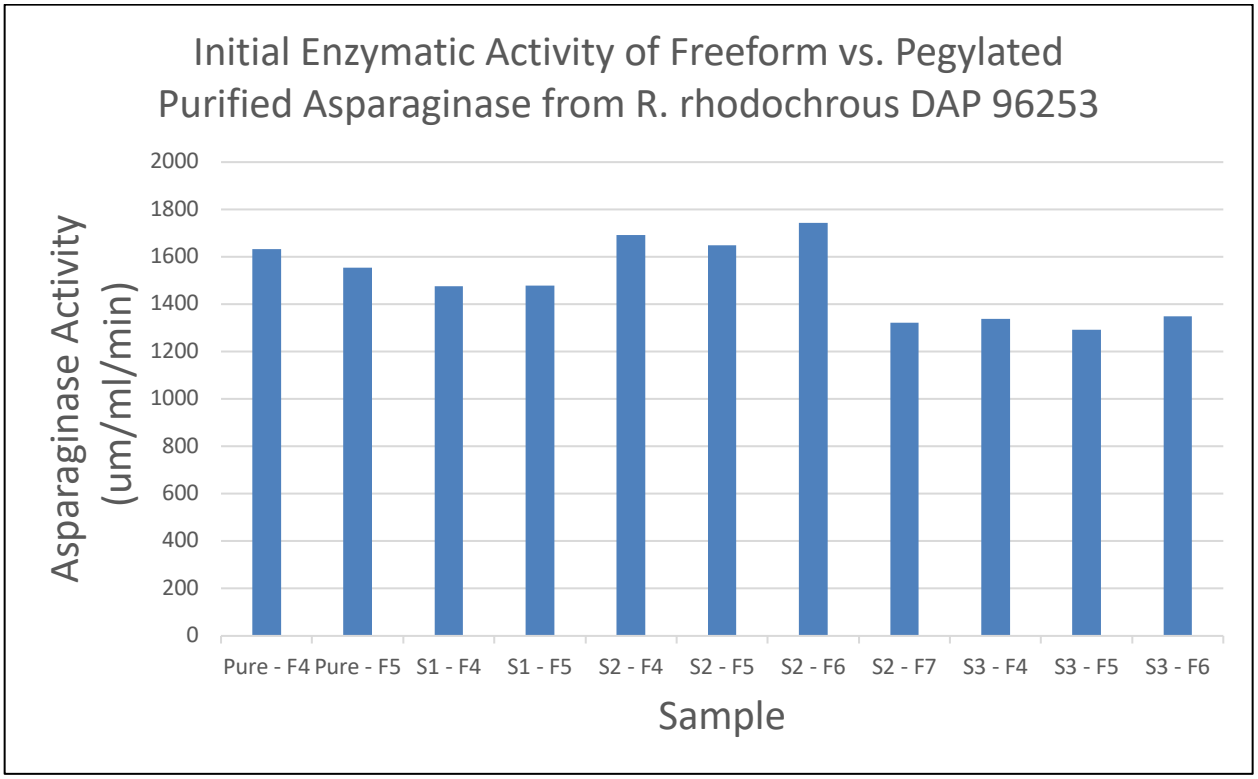


Figure 58: Comparison of Initial Asparaginase Activity of Freeform Asparaginase to Pegylated Asparaginase Fractions Purified from R. rhodochrous DAP 96253

3.2.4 *Storage Study of Asparaginase Activity over Time in Freeform and Pegylated Purified Asparaginase from R. rhodochrous DAP 96253 stored in 37℃*

After the initial loss of activity data was analyzed, all samples were stored at 37°C for the evaluation of asparaginase activity over time at a temperature mimicking that of the average body temperature. Asparaginase assays were conducted after 26 hours and 49 hours (1 and 2 days) and then again at Day 12 to analyze the loss of enzymatic activity over time at 37°C.

The control fractions of freeform asparaginase retained 66.1% and 72.9% of their enzymatic activity after 1 day in storage. Fractions 4 and 5 from syringe 1 retained 73.8% and 76.8% after 1 day of storage. Fractions 4, 5, 6, and 7 from syringe 2 retained 58.2%, 61.8%, 57.7%, and 91.3% of their enzymatic activity after 1 day of storage. Fractions 4, 5, and 6 from syringe 3 retained 71.6%, 74.1%, and 89.5% of their enzymatic activity after 1 day in storage. Syringe 2 and 3 had the samples with the highest retention of activity taking place in the last fractions of the separation process. S2-Fraction 7 had a retention rate of 91.3% while S3-Fraction 6 had a retention rate of 89.5%. Syringe 2 had the samples with the lowest retention of activity with fractions 4 and 6 showing 58.2% and 57.7% respectively. All retention rates are shown below in Table 40. Graphical comparison of the retention rates is shown below in Figure 59. The raw data for the results can be found in Appendix PP.

Table 40: Comparison of Enzymatic Activity of Freeform Purified Asparaginase Versus PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 after 26 Hours of Storage at 37℃

Samples	Fractions	Initial Asparaginase Activity (um/ml/min)	24hrs Activity (um/ml/min)	Activity retained (%)
Control – purified asparaginase	F4	1632.98	1079.71	66.1%
	F5	1554.44	1133.91	72.9%
Syringe 1	F4	1475.03	1089.03	73.8%
	F5	1479.23	1135.9	76.8%
Syringe 2	F4	1690.97	983.35	58.2%
	F5	1648.81	1019.52	61.8%
	F6	1743.94	1007.02	57.7%
	F7	1322.17	1206.98	91.3%
Syringe 3	F4	1337.92	958.24	71.6%
	F5	1292.32	957.05	74.1%
	F6	1348.01	1206.98	89.5%

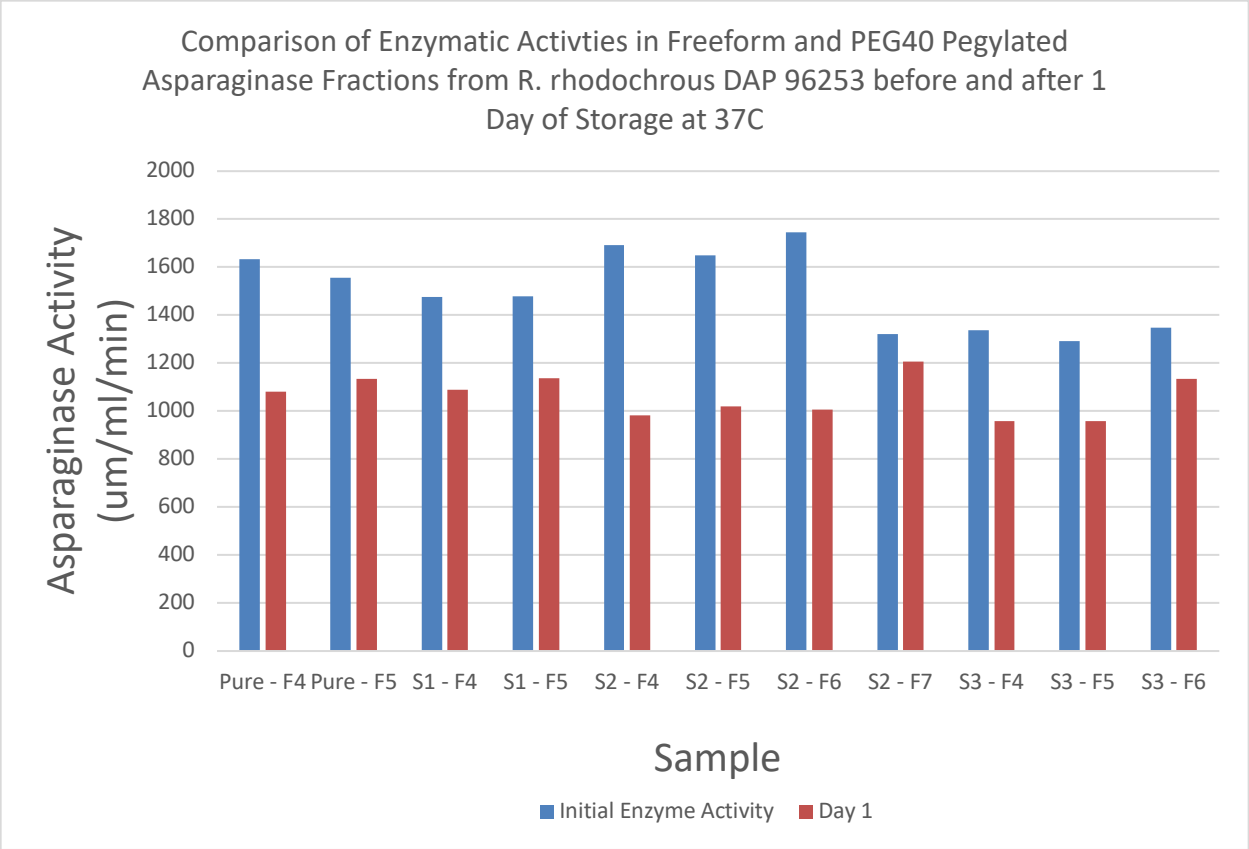


Figure 59: Comparison of Freeform and PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 before and after 26 Hours of Storage at 37℃

All samples were stored at 37°C and asparaginase assays were conducted after 49 hours (Day 2) and compared to Day 1's activity as well as the initial enzymatic activity to analyze the loss of enzymatic activity over time at the average human body temperature.

Numerous retention rates were not calculated from Day 1 to Day 2 due to an increase in enzymatic activity over the 23 hours of storage. Fraction 4 of the control sample, fractions 4 and 5 from syringe 1, and fractions 4 and 5 from syringe 2 all showed an increase in activity. For the rest of the samples, retention rates varied. Fraction 5 showed an enzymatic retention rate of 95.8%. This rate along with syringe 2-fraction6's rate of 96% and syringe3-fraction4's rate of 95% were the highest retention rates seen from Day 1 to Day 2. Syringe2-fractions 7 showed a retention rate of 77% and syringe3-fraction6 showed a retention rate of 62.9% and showed the lower end of retention rates from Day 1 to Day 2. Syringe 3-fraction 5 landed in the middle of retention rate with 84.1% of activity retained.

Overall activity was measured by looking at the initial enzymatic activity compared to the Day 2 activity. Syringe 1-fractions 4 and 5 along with syringe 2-fraction 4 showed the highest retention rates with 79.3%, 81.1%, and 82.3% of activity retained after 2 days respectively. Syringe 2-fraction 6 and syringe 3-fraction 6 exhibited the lowest retention with rates of 55.4% and 56.3% respectively. All other samples fell in between the highest and lowest numbers.

All retention rates are shown below in Table 41. Graphical comparison of the retention rates is shown below in Figure 60. The raw data for the results can be found in Appendix PP.

Table 41: Comparison of Enzymatic Activity of Freeform Purified Asparaginase Versus PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 from Day 1 to Day 2 and after 49 Hours of Storage at 37℃

Samples	Fractions	Initial Asparaginase Activity (um/ml/min)	Day 1 (26 hours) Activity (um/ml/min)	Day 2 (49 hours) Activity (um/ml/min)	Activity retained Day 1 →2 (%)	Overall activity retained (%)
Control – purified asparaginase	F4	1632.98	1079.71	1105.72	---	67.7%
	F5	1554.44	1133.91	1085.90	95.8%	69.9%
Syringe 1	F4	1475.03	1089.03	1170.25	---	79.3%
	F5	1479.23	1135.9	1200.31	---	81.1%
Syringe 2	F4	1690.97	983.35	1392.48	---	82.3%
	F5	1648.81	1019.52	1060.70	---	64.3%
	F6	1743.94	1007.02	966.71	96%	55.4%
	F7	1322.17	1206.98	929.75	77%	70.3%
Syringe 3	F4	1337.92	958.24	910.63	95%	68.1%
	F5	1292.32	957.05	804.59	84.1%	62.3%
	F6	1348.01	1206.98	758.81	62.9%	56.3%

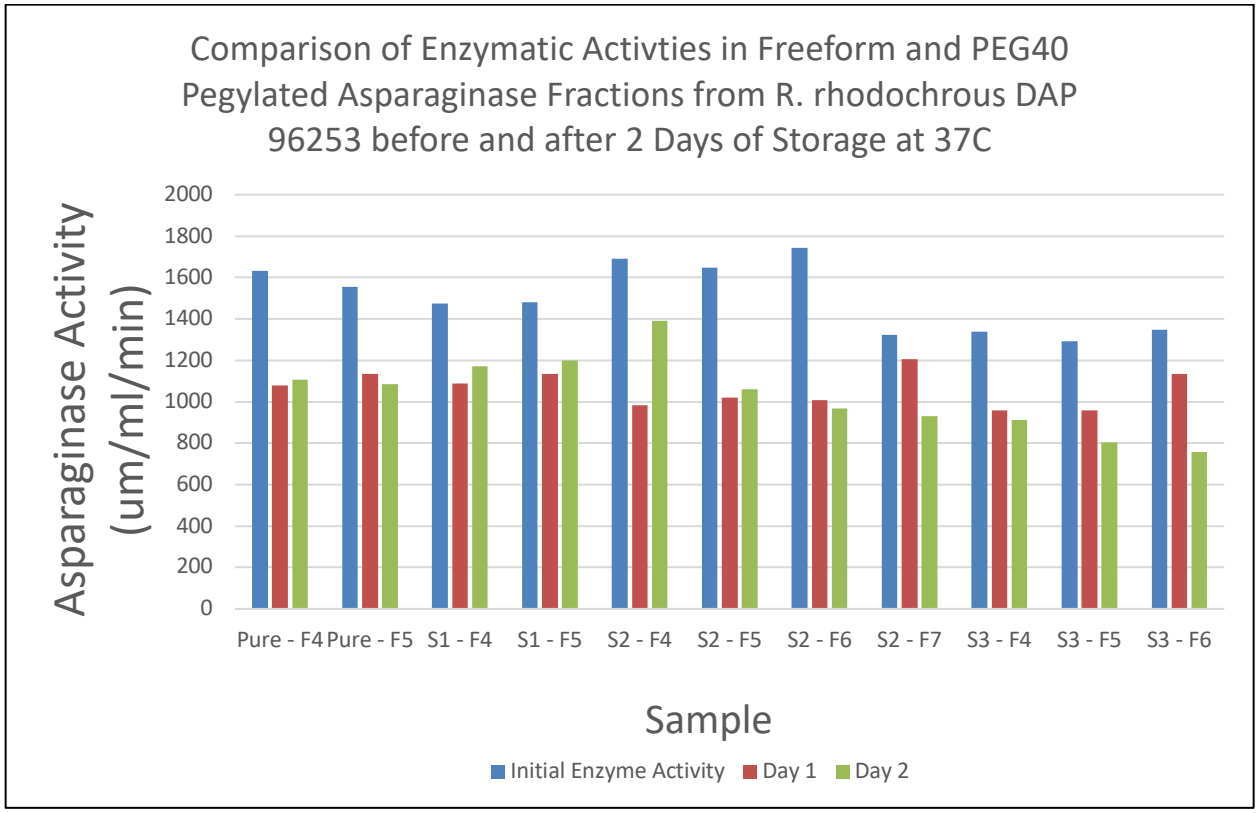


Figure 60: Comparison of Freeform and PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 before and after 26 and 49 Hours of Storage at 37℃

All samples were stored at 37°C and asparaginase assays were conducted after 12 days and compared to the initial enzymatic activity to analyze the loss of enzymatic activity over time at the average human body temperature.

Fraction 4 and 5 of the control sample showed an enzymatic retention rate of 47% and 49.8%. From syringe 1, fractions 4 and 5 showed enzymatic retention rates of 38.6% and 35.9%. From syringe 2, fractions 4, 5, 6, and 7 showed enzymatic retention rates of 28.3%, 25%, 19.2%, and 23.5%. From syringe 3, fractions 4, 5, and 6 showed enzymatic retention rates of 38.4%, 53.5%, and 48.1%. While fraction 5 of the control sample showed one of the highest retention rates, fractions 5 and 6 from syringe 3 also showed the highest rates with 53.5% and 48.1%. Syringe 2-fractions 5, 6, and 7 showed the lowest retention rates of 25.0%, 19.2%, and 23.5%.

All retention rates are shown below in Table 42. Graphical comparison of the retention rates is shown below in Figure 61 and in Figure 62. The raw data for the results can be found in Appendix PP.

Table 42: Comparison of Enzymatic Activity of Freeform Purified Asparaginase Versus PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 after 12 days of Storage at 37℃

Samples	Fractions	Initial Asparaginase Activity (um/ml/min)	Day 12 Activity (um/ml/min)	Activity retained (%)
Control – purified asparaginase	F4	1632.98	768.00	47.0%
	F5	1554.44	774.11	49.8%
Syringe 1	F4	1475.03	569.03	38.6%
	F5	1479.23	530.86	35.9%
Syringe 2	F4	1690.97	478.28	28.3%
	F5	1648.81	412.17	25.0%
	F6	1743.94	335.30	19.2%
	F7	1322.17	310.26	23.5%
Syringe 3	F4	1337.92	514.42	38.4%
	F5	1292.32	691.80	53.5%
	F6	1348.01	648.29	48.1%

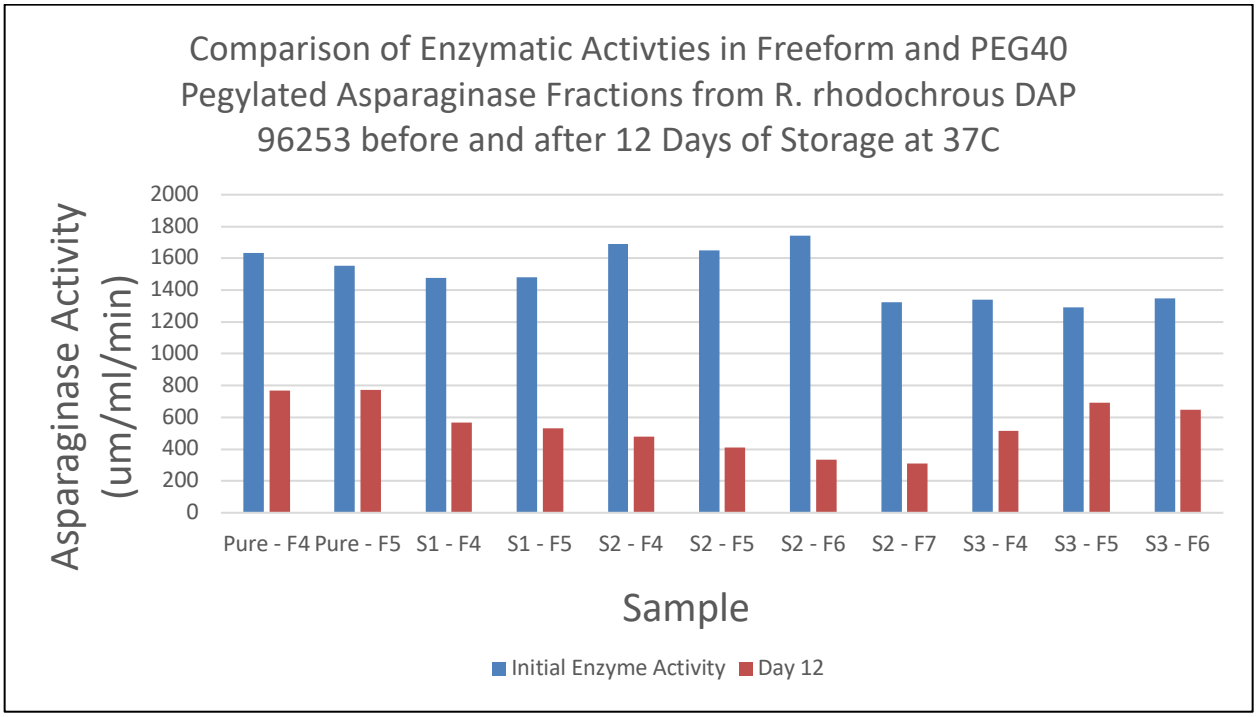


Figure 61: Comparison of Freeform and PEG40 Pegylated Purified Asparaginase Fractions from R. rhodochrous DAP 96253 before and after 12 Days of Storage at 37℃

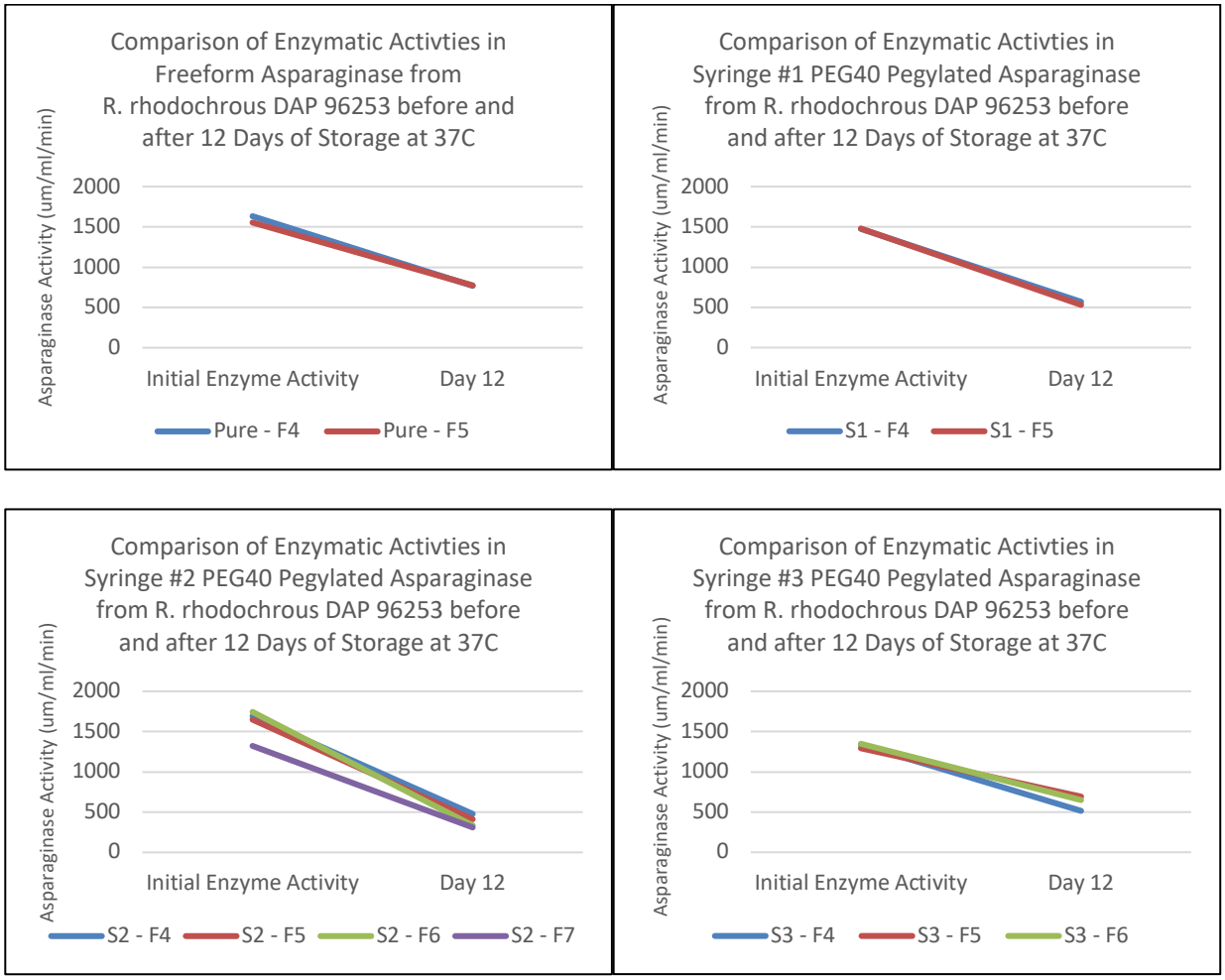


Figure 62: Graphs A-D (left-right from top-bottom): Comparison of Enzymatic Activity of Freeform Purified Asparaginase vs. PEG40 Pegylated Syringes 1-3 Fractions Before and After 12 Days while Stored in 37℃

4 DISCUSSION

4.1 Bioconversion

While nitrile hydratase has been found in many different genera of bacteria, such as Proteobacteria, Actinobacteria, Cyanobacteria, and Firmicutes, it was first discovered in *Rhodococcus rhodochrous* J1, known then as *Arthrobacter* sp. J1, 40 years ago [11, 63]. Despite the existence in other bacteria, *Rhodococcus* species are of significance in the bioconversion of acrylonitrile to acrylamide given their high activity of nitrile hydratase, ability to withstand the heat given off in the conversion reaction, and ability to remain viable despite the toxicity of acrylonitrile and acrylamide. While the Fe-type and Co-type nitrile hydratases have both been found in *Rhodococcus* species, the Co-type nitrile hydratase in certain species, such as *Rhodococcus rhodochrous* DAP 96253, is desired due to its increased thermal stability [11, 27]. The evaluation of the bioconversion capabilities of *R. rhodochrous* DAP 96253 was conducted in response to the bacteria's desirable traits as a fourth-generation biocatalyst. In addition to whole cells and immobilized whole cells, the use of extracellular enzymes procured from bacterial cells, such as cell-free lysate, purified enzymes, and immobilized purified enzymes, are of significant interest given the industrial advantages they can produce, such as a product with increased purity or of higher concentration. Thus, leading to significant cost benefits [9]. However, the use of extracellular enzymes come with disadvantages also, such as decreased stability, expensive catalyst retrieval, and increased production costs and time [44]. While most enzymatic background research reports the pros and cons of using extracellular enzymes, there is a lack of experimental data to conclude the best enzymatic formulations to use for the bioconversion of acrylonitrile to acrylamide.

4.1.1 *Determination of Whole Cells used for Bioconversion based on Nitrile Hydratase Activity as a Product of Growth Variations*

Rhodococcus rhodochrous DAP 96253 cells show heightened nitrile hydratase activity when induced during growth with Cobalt [27]. The amount of heightened activity varies dependent upon how the cells are grown and other factors carried out in relation to harvesting cells grown via fermentation. While cells grown on media plates exhibit nitrile hydratase activity around 106 um/ml/min, producing cells via batch-fed fermentation exhibits higher nitrile hydratase activity ranging from 744-2298 um/ml/min, as seen in Table 8. Cells being prepped for fermentation inoculation, known as the 'seed', are also induced with Cobalt and exhibit nitrile hydratase activity of around 210 um/ml/min, as shown in Table 9. The decreased enzymatic activity levels are shown to correlate to the stunted biomass growth also seen with cells grown on media plates and in flask cultures. It is hypothesized this is related to the Co outcompeting Fe during the synthesis of critical proteins needed for cell growth. The amount of Cobalt used in fermentation, 75 ppm, was determined to show a desired maximum increase in nitrile hydratase activity of $3.3x10^5$ while also having the lowest biomass yield due to the increased Co levels. Post-harvest, cells washed with buffer+Urea showed a heightened activity of 2298 um/ml/min over those not washed post-harvest, which showed activity of 744 um/ml/min, as shown in Table 8. It is hypothesized that *R. rhodochrous* DAP 96253 shows increased NHase production with the addition of Urea and the depletion of freely available Cobalt [55].

Harvest time also produced varying amounts of nitrile hydratase activity. Harvesting at 48hrs of growth produced around 2387 um/ml/min in comparison to harvesting at 62hrs of growth, which produced around 945 um/ml/min, as shown in Table 9. Twenty-four hours of growth has been shown to produce maximum nitrile hydratase activity for *R. rhodochrous* NHB-

2 while forty-eight hours of growth has been shown to produce maximum nitrile hydratase activity in other genera, such as *Agrobacterium tumefaciens* [64]. For *Rhodococcus rhodochrous* DAP 96253, 48hrs is determined as maximum nitrile hydratase production based on a 62hr fermentation growth timeline in which the feed media, containing Cobalt, is depleted at the 42hr mark. Thus, leading to a drop off in nitrile hydratase production shortly thereafter [55]. This timeline makes it a competitive alternative to the longer incubation time of 72hrs for the world-dominantly used third-generation catalyst, *R. rhodochrous* J1 [65]. Bioconversion runs conducted using whole cells or immobilized whole cells of *R. rhodochrous* DAP 96253 are best conducted with whole cells grown via fermentation, harvested at 48hrs, and washed with buffer+Urea post-harvest. These cells should also be used for any post-harvest production, such as lysing or purifying.

4.1.2 *Evaluation of the Bioconversion of Acrylonitrile to Acrylamide using Whole Cells of R. rhodochrous DAP 96253*

Initial experimentation carried out using whole cells for the bioconversion process experimented with the temperature at which the process was carried out at. While *Bacillus* sp. exhibit optimal nitrile hydratase activity at 55ºC, *Rhocococcus erythropolis* shows optimal nitrile conversion at 25ºC and *Rhodococcus rhodochrous* NHB-2 has shown optimal acrylonitrile conversion at 50ºC. Thus, the bioconversion process was experimented with whole cells being carried out at both 20ºC (room temperate with no temperature manipulation) and 55ºC (accomplished by using a stirring hot plate) [66-68]. As seen in Table 10, the bioconversion process carried out at room temperature converted 61.4% of the acrylonitrile added compared to the process carried out at 55ºC, which converted only 9.8% AN. Given the exothermic nature of the conversion reaction and heightened thermostability of the Co-type nitrile hydratase found in

Rhodococcus rhodochrous DAP 96253, the cells are able to perform at ideal enzymatic levels at room temperature due to the increased temperature of the solution inside the bioreactor [68]. Besides converting a higher percentage of the AN added, running the process at room temperature creates the advantage of not having to monitor or manipulate the temperature of the system in an industrial setting.

While the bioconversion process carried out at room temperature converted a higher percentage of AN, there was still room to increase the conversion rate to a more favorable number. The next variable manipulated was the input rate of acrylonitrile. Given the toxic nature of the chemical, there was concern about the amount being added having a negative effect on the efficiency of the cells/enzyme by creating a toxic environment due to buildup of AN if the cells did not convert it close to the rate at which it was being added. For the first experiment, 100mL of AN was added at 0.33 ml/min and 0.38 ml/min. As shown in Table 11, the faster rate of 0.38 ml/min converted 69.9% of the AN added in comparison to the 61.4% conversion at the rate of 0.33 ml/min. During the next experiment, a higher volume, 160mL, was pumped into the reactor at a rate of 0.38 ml/min. Besides pumping in more substrate to be converted, this gave the 30.1% of unconverted AN from the prior 0.38 ml/min run more time and opportunity to be converted. As seen in Table 13, 160mL of AN pumped in at a rate of 0.38 ml/min led to 94.7% of AN being converted versus 100mL of AN pumped in at a rate of 0.38 ml/min resulting in 69.9% of AN, as shown in Table 11. This amount of added AN was then added at two faster rates to evaluate the conversion. 160mL of AN was added at a rate of 0.6 ml/min and 0.8 ml/min. In comparison to the 94.7% conversion seen in the conversion run of 160mL at 0.38 ml/min, the 0.6 ml/min rate converted 86.5% of AN while the 0.8 ml/min rate converted 93.9%, as seen in Table 14. While a higher conversion rate, such as 93.9%, is preferable over a lower conversion rate, such as 86.5%,

it is noteworthy to point out the inconsistent nature with which the higher conversion occurred. When observing the conversion over time in Figure 24, the conversion happening at 0.6 ml/min shows a more consistent pattern than that of the 0.8 ml/min conversion. Further observation shows that the conversion with the 0.38 ml/min showed a more consistent conversion than the other two varying rates' runs. While all three increased rates of 0.38, 0.6, and 0.8 ml/min of 160mL of AN converted an average of >90% of AN resulting in final solutions averaging >40% AMD, the 0.6 ml/min rate produced more consistent conversion than the 0.8 ml/min at a time of 4-5 hours versus 7 hours for the 0.38 ml/min. It is noted here that these runs all produced final solutions comparable to that of commercially bought acrylamide with the 0.38 ml/min and the 0.8 ml/min runs having very little acrylonitrile by-product present in the final solution.

During the conversion run at a rate of 0.8 ml/min, the pH was monitored throughout the entire 210 minutes of the run. The pH remained consistent at 7.3 throughout the run. This level is consistent with the findings that *Rhodococcus* species convert nitriles optimally at "near physiological pH", which is 7.4 [67]. This also shows that no manipulations or controlling of the pH needs to be carried out in an industrial setting.

4.1.3 *Evaluation of the Bioconversion of Acrylonitrile to Acrylamide using Immobilized Whole Cells of R. rhodochrous DAP 96253*

In further validation of the use of fermentation-grown cells over media plate-grown cells, polyacrylamide immobilized cells were produced using both the plate-grown and fermentation-grown cells previously analyzed for the enzymatic activity comparison shown in Table 8 and mentioned in the prior section. Using an input rate of 0.38 ml/min, plate-grown PAM cells converted 1.96% of AN added while the fermentation-grown PAM cells converted 52.3% AN

added, as seen in Table 18. Figure 27 shows the lack of conversion of the plate-grown immobilized cells but of more interest is the conversion of the fermentation-grown immobilized cells which shows consistent conversion up until minute 150 when the conversion activity seems to plateau.

Various input rates, of 0.33, 0.38, 0.60, and 0.75 ml/min, and AN quantity, 100mL and 160mL, were further tested on PAM immobilized cells. In contrast to the whole cells, the slowest rate of less AN volume produced the highest conversion of AN at 69.9%, as seen in Table 19. In comparison to Figure 28, where PAM immobilized cell runs using an input rate of 0.38 ml/min are shown to plateau at minute 150, Figure 29 shows the slower conversion rate of 0.33 ml/min maintaining consistent conversion activity past minute 150. Figures 30 and 31 show the same plateauing conversion activity occurrence with the faster input rates of 0.6 and 0.75 ml/min. While 69.9% conversion isn't low, it comes with the added disadvantages of longer immobilization production time, around 3-4hours, and longer run time when compared to the highest production whole cell run from the previous section. It is hypothesized that the uptake of acrylonitrile is happening at a decreased rate in PAM-immobilized cells in comparison to whole cells. This could be due to the immobilization matrix. This slower uptake leads to build up of acrylonitrile in the bioreactor solution which accumulates in heightened exposure to a toxic substance that can affect the stability of the immobilization matrix and the expressed enzymatic levels. Another disadvantage to PAM cells is that, during the bioconversion process, the PAM cells seem to break apart and become 'mushed' together, possibly due to the heightened acrylonitrile levels and the gelatin-like compound the immobilization matrix is made of. However, they are still able to be removed easily from the final solution [69].

Polyethyleneimine-glutaraldehyde immobilized cells show decreased conversion activity in comparison to PAM immobilized cells. They reported an average of 48% conversion of AN added in comparison to the almost 70% of conversion with PAM immobilized cells, as shown in Table 21. As seen in Figure 33 and 34, PEI-Glu immobilized cells seem to lose conversion activity earlier in the process, somewhere between 50-100 minutes, than PAM immobilized cells, which lose activity around 150 minutes. This is why runs with PEI-Glu were often ended earlier, after 108mL of AN, due to the build-up of the bilayer of acrylonitrile, as seen for example in Figure 26 with the use of a different *R. rhodochrous* strain. Thus, making the final product less desirable. For this research, equal amounts of catalyst were used in bioconversion runs but, due to the heightened loss of enzymatic activity that occurs with PEI-Glu immobilization, it is hypothesized that increased amount of catalyst should be used to obtain increased acrylamide production [69]. The PEI-Glu immobilization process takes overnight to cure, significantly increasing the cell production time to well over 24hrs. Also, in production, the cells seem to dissolve during the bioconversion process, making them much harder to remove from final solution. Even with centrifugation, the dust-like quality of the cells makes them easily go back into solution when removing.

Non-hardened and hardened calcium alginate immobilized cells exhibited the highest conversion activity with 87% and 100% conversion rates respectively. In comparison to PEI-Glu and PAM cells, Figure 36 shows the consistent production of acrylamide over time in response to a consistent input of acrylonitrile. Alginate-immobilized cells are characterized as having one of the highest retention rates of enzymatic activity amongst immobilized cells [69]. Besides having higher conversion activity, non-hardened calcium alginate cells are usable immediately after immobilization, which takes roughly an hour. In contrast to having the highest conversion rate of

100%, hardened calcium alginate cells are left to harden overnight, making the cells not usable till the following morning. Both forms come with the extreme ease of removability from the final solution and keep their form during the bioconversion process.

The immobilized cells with the highest and most consistent conversion percentages and ease of removal with the matrix integrity still intact, NH-Ca-Alg and H-Ca-Alg cells, were chosen to study the reusability of immobilized cells. PAM cells were also tested but given their lack of form retention and poor conversion rates, as seen in Table 20, they were not experimented with for long. While the non-hardened calcium alginate cells converted 87% of substrate during the 150 minutes of the first usage run, their activity significantly decreased, exhibiting a conversion percentage of 10.9% for the second usage run of 150 minutes. As seen in Figure 37, while the initial run showed consistent conversion, the subsequent run expressed little to no enzymatic activity. While the hardened calcium alginate cells converted 100% of the 126mL added during the first usage run, it converted 35.8% of the AN added during the second usage run. As seen in Figure 38, while the first run showed consistent conversion, there was less activity during the second run which seems to drop off at minute 150. It seems the hardening of the matrix of the immobilized cells offer added protection from the AN than in the non-hardened cells [69].

While freeform whole cells are not reusable and take centrifuging to remove, they convert 94-95% of AN added consistently at rates of 0.38 ml/min and 0.8 ml/min and are ready to use once harvested. However, hardened immobilized cells convert 100% of AN added and are able to be reused, with decreased returns. They are extremely easy to be removed but they take additional production process and must cure overnight. While each of these cellular forms have

pros and cons, they are both established modes in the industrial bioconversion of acrylonitrile to acrylamide which show the best results when using *Rhodococcus rhodochrous* DAP 96253.

4.1.4 *Evaluation of the Bioconversion of Acrylonitrile to Acrylamide using Enzymatic Formulations Produced from R. rhodochrous DAP 96253*

The first product in the process of developing extracellular enzymatic formulations is cell-free lysate. While breaking down the cell walls of the bacterial cell and removing cellular debris leaves the cellular machinery that is left more vulnerable, the activity of the desired enzyme is increased while the activity of the undesired enzymes is still present and increased just like that of the desired enzyme [70]. The nitrile hydratase activity of CFL reports as 6658 um/ml/min compared to 450 um/ml/min as reported in the whole cells prior to lysing, shown in Table 25. Further justification to show the presence of nitrile hydratase in the lysate is present with the protein bands being visible in the SDS-page gels shown in Figure 39, this gel showing very few and low concentrations of other enzymes present, and in Figure 40, this gel showing larger amounts of other enzymes present. Due to the protocoled production and storage of lysate to be kept on ice or refrigerated at -20-4ºC to prevent denaturing the proteins, two bioconversion runs, one in an ice/water bath and one at room temperature, were compared with the thought of cooling down the solution during the conversion to prolong the life of the lysate enzymes [70]. The ice/water bath run, CFL#1, showed extremely low enzymatic activity by converting only 2.74% of AN added. Besides the accumulation of an AN bilayer on top of the water, small, string-like objects appeared as if the cellular machinery, i.e. proteins, were denaturing and coming out of solution, as shown in Figure 42. This is thought to be consist with the findings from the temperature study conducted with whole cells due to the thermal stability of the enzyme [11]. The bioconversion run conducted at room temperature converted 51.8% of AN added at a

consistent rate before plateauing off around minute 90. This run was conducted again and reported a conversion rate of 76.8% of AN added. While this run began to plateau around minute 125, it seemed to pick back up before tailing off again at minute 150. Conversion at minute 150 was just below 100% of AN added. It is hypothesized that the heat from the conversion reaction affected the activity of the enzyme of this point in the procedure. However, denatured proteins were not observed. The process of lysing and precipitating the lysate adds ¾ of a workday onto the preparation timeline before the enzyme can be used for bioconversion. Further research conducted on a continuous bioconversion process in which new lysate is added in at minute 150 while AN is pumped in at a continuous rate could create a cycle in which 100% conversion is consistent.

The process of purifying nitrile hydratase resulted in higher protein concentration using a 5mL ion exchange column rather than a 1mL column as previously used in lab, as seen in Table 29. Figure 47 shows the darkest/thickest nitrile hydratase bands with less presence of other proteins eluting with 0.2M and 0.3M NaCl solution. Thus, purified nitrile hydratase evaluated for its use in the bioconversion process will be the 0.25M NaCl elution from a 5mL column while being conducted with the same parameters as the cell-free lysate [62]. Bioconversion runs using 0.25M purified enzyme samples converted 29.1-33.9% of AN added. As seen in Figures 48 and 49, production begins slower than with other enzymatic formulations and plateaus off even despite a bump in activity seen around minute 125-150. The act of purifying and eluding the protein results in a loss of protein to the column and to the other elution fractions so, while a purer catalyst is achieved, some activity will be lost. After lysing, purification adds on another 1/3 of a workday to the preparation process. Thus, making the entire process take over a 8-hr workday before it’s able to be used for acrylamide production.

Purified enzyme immobilization using calcium alginate was chosen given its advantages over the other immobilization techniques evaluated with whole cells. Most notably, the stability that immobilizing with alginate gives to its substrate, in this case delicate free-form proteins [69]. Hardened and non-hardened techniques were both evaluated. H-CaAlg-PU converted 23.6% of AN added while NH-CaAlg-PU converted 18.2% of AN added. As seen in Figure 51, graphical presentation of the conversion shows the activity of the different immobilized purified enzyme started off extremely slow while seeming to plateau before even starting to convert with any efficiency. While calcium alginate is noted as having the least decrease, it is still characterized as causing an initial decline in enzymatic activity. This decline could account for the lower conversion rate seen with immobilized enzyme over free-form enzyme. It makes sense that the conversion would start off slower before it would with freeform purified enzyme. By starting off slower, there is more opportunity for the build-up of AN to be affecting the immobilization matric as well. It is noted that the immobilized beads shown in Figure 50 for the purified enzyme were not as structurally sound as the immobilized beads shown in Figure 9 for the whole cells. Despite different formulations for the alignate concentration used, it seems as if the lack of cellular walls gives the matrix less of a rigid and protective matrix. This lack of protectiveness could lead to deactivation of the internal enzyme.

4.1.5 *Evaluation of the Bioconversion of Acrylonitrile to Acrylamide using All Forms of Nitrile Hydratase of R. rhodochrous DAP 96253*

When comparing the manipulated runs with the best production using whole cells, immobilized whole cells, cell-free lysate, purified enzyme, and immobilized purified enzyme, conversion rates ranged from 18.2-100% with stable process temperature but variability in input rate and amount of acrylonitrile added. Manipulating input rate and amount of AN directly affect

the overall time in which it takes to make a certain amount of acrylamide, which is the final product. As shown in Table 33, whole cells ran at an input rate of 0.8ml/min for 160mL, taking about 3.5hrs, converted around 93.9% of AN added. PAM immobilized cells ran at an input rate of 0.33 ml/min for 100mL, taking about 5hrs, converted 69.6% of AN added. PEI-Glu immobilized cells ran at an input rate of 0.6 ml/min for 108mL, taking about 5 hrs, converted 49.1% of AN added. H-CaAlg immobilized cells ran at an input rate of 0.6 ml/min for 126mL, taking about 3.5hrs, converted 100% of AN added. NH-CaAlg immobilized cells ran at an input rate of 0.6 ml/min for 126mL, taking about 3.5hrs, converted 87% of AN added. Cell-free lysate ran at an input rate of 0.8mL for 144mL, taking about 3hrs, converted 51.8% of AN added. Purified nitrile hydratase ran at an input rate of 0.6 ml/min for 108mL, taking about 3hrs, converted 33.9% of AN added. Hardened CaAlg immobilized enzyme ran at an input rate of 0.6 ml/min for 108mL, taking about 3hrs, converted 23.6% of AN added. Non-hardened CaAlg immobilized enzyme ran at an input rate of 0.6 ml/min for 108mL, taking about 3 hrs, converted 18.2% of AN added.

For final production numbers, whole cells and H-CaAlg whole cells are deemed the best use of the enzyme nitrile hydratase for the bioconversion of acrylonitrile to acrylamide for use in industrial settings.

4.1.6 *Evaluation of the Storage Capabilities Bio-acrylamide*

Once acrylamide is created, the storage capabilities of bioacrylamide were evaluated using current protocoled storage conditions for commercially bought acrylamide. Conditions include storing the liquid in either a dark bottle or a bottle covered from light at room temperature (~25ºC). However, for long term storage (characterized as longer than 3 weeks), it is recommended to store at 2-8ºC. For this study, the acrylamide was stored at room temperature

for one week and then stored at 4ºC for the duration of 6 months. The beginning concentration of the bioacrylamide being stored was 40%, mimicking that of commercially bought acrylamide. While the number varied slightly due to GC-MS sensitivity, the solution maintained a constant concentration of 40-45% bioacrylamide. Thus, making microbially-catalyzed produced acrylamide a viable option for use after storage in an industrial setting.

4.2 Pegylation

In addition to expressing nitrile hydratase activity, *Rhodococcus rhodochrous* DAP 96253 expresses asparaginase activity [27]. Since the evidence showing that the regression of acute lymphoblastic leukemia was linked to the enzyme asparaginase, the enzyme has been added to modern chemotherapy treatments for ALL. The addition of asparaginase to the treatment adds asparaginase to the plasma of the patient leading to the depletion of L-asparagine. This depletion leads to DNA, RNA, and protein synthesis inhibition and causes cell apoptosis. Thus, killing the cancerous, immature, lymphoblast cells [49]. While current treatment plans use asparaginase drugs stemming from either *Escherichia coli* or, outside of the US, *Erwinia chrysanthemi, R. rhodochrous* DAP 96253 is of novel interest as a form of therapeutic treatment due to its significantly decreased levels or absence of endotoxins [54]. Given the negative side effects of giving freeform asparaginase to patients, a form of immobilization using monomethoxypolyethylene glycol (PEG), known as pegylation, is performed to decrease the side effects, to enhance the half-life, and increase the time between injections [12]. While freeform purified asparaginase from *R. rhodochrous* DAP 96253 has shown activity levels of 1790, 1226+/-133, 2491+/-115, and 1219+/-24.38, asparaginase activity needs to be retained in the pegylated-asparaginase to move forward with further testing the efficacy of this bacterium's asparaginase [54].

4.2.1 *Evaluation of Initial Retention of Asparaginase Activity Post-Pegylation using both PEG40 and PEG80 Concentrations*

As discussed with immobilization, initial enzymatic levels are decreased compared to that of the freeform purified enzyme they originally were [69]. The objective of these initial studies is to look at the initial enzymatic activities of pegylated asparaginase in comparison to the freeform purified asparaginase of *R. rhodochrous* DAP 96253. The retention rate of enzymatic activity will be looked at for both PEG40 and PEG80 pegylated samples, two different PEG:ASP concentrations. As seen in Table 35, the initial asparaginase activity retention percentage for PEG40 samples was an average of 49.2%. This included a range of 32.2-70.1% retention rate depending on the fraction that came off the size exclusion column. As seen in Table 36, the initial asparaginase activity retention percentage for PEG80 samples was an average of 45.9%. This included a range of 28.9-62.9%. While the numbers were close, the PEG40 concentrated samples had an overall higher average of activity retained, 49.2%, while also having the highest retention rate per sample, 70.1%. This percentage is competitive with the average of 62.84% activity retained with pegylating asparaginase from other bacteria [71]. Further initial activity retention studies were carried out with another set of PEG40 pegylated samples. This pegylation process consisted of running 3 different syringes of pegylated samples through the size exclusion column and studying the fractions with the highest asparaginase activity. Syringe 2 will not be used in this initial retention study due to its enzymatic levels having increased. This data is inconsistent with common findings about initial enzymatic activity in immobilized samples. However, for syringes 1 and 3, initial retention levels ranged from 81.1-92.8%. This further supports that a lower PEG:ASP concentration leads to higher initial enzymatic activity retention. It is suggested in recent literature that even lower concentrations of PEG:ASP, such as 25:1,

should be tested given its heightened enzymatic stability due to its larger production of mono-pegylation. Further testing should be carried out with lower concentrations of PEG:ASP [72].

4.2.2 *Evaluation of Storage Study of Freeeform vs. Pegylated Purified Asparaginase at 4℃*

In following proper storage protocol for purified enzymes, all PEG40 and PEG80 samples were stored at 4ºC and assayed 1 week later. As seen in Table 37, the PEG40 samples retained an average of 77% of asparaginase activity after 1 week in storage. As seen in Table 38, the PEG80 samples retained an average of 76% of activity after 1 week in storage. The numbers are very similar. Again, the highest retention numbers are seen with the PEG40 samples. The two highest retention rates, fractions #4 and #5, showed a retention of 97.6% and 97.2%. The two highest PEG80 fractions, fraction #4 and #7, were below at 86.6% and 93.7%. Further investigation into the storage study was not carried out due to the increase in activity seen in weeks 2, 3, and 4 for various samples. This is believed to be due to ammonia being released and skewing the assay results. Further studies on the ammonia levels in storing samples are needed to validate this hypothesis. However, Figure 56 and 57 show the overall retention rates over 1 month of storage at 4ºC.

4.2.3 *Evaluation of Storage Study of Freeform vs. Pegylated Purified Asparaginase at 37ºC*

In order to further test the enzymatic activity retention of pegylated asparaginase vs. freeform asparaginase, the PEG40 samples from the second initial activity experiment were stored at body temperature (37ºC) and tested 1 day, 2 days, and 12 days later. After 1 day of

storage, PEG40 samples retained 57.7-91.3% with the two highest retention rates being from later eluded fractions, fraction #7 from syringe 2 and fraction #6 from syringe 3. The retention rates were 91.3% and 89.5% respectively. The freeform asparaginase retained 66.1% and 72.9% of their activity after 24hrs. These pegylated samples exhibited higher activity retention. This data is shown in Table 40. In day 2's asparaginase assays, pegylated asparaginase activity retention rates were 62.9-96% compared to the 95.8% retention rate of the control sample #2. While a control sample and two pegylated samples showed the highest retention from Day 1 to Day 2, the overall enzymatic activity from the initial pegylation to the 49hr mark was retained more successful by S1-F4, S1-F5, and S2-F4 with 79.3%, 81.1%, and 82.3% retention rates compared to the 67.7% and 69.9% retention rates of the control samples. This data is shown in Table 41. Further testing using PEG40 and lower concentrations are needed to confirm the increased stability and half-life of pegylated asparaginase over the free-form enzyme. Confirmation of the degree of pegylation is needed in order to determine whether or not the fractions with the highest retention are mono-pegylated as previous research would suggest. This work is still ongoing in the research lab [72].

The overall retention rate from initial activity to the Day 12 activity varied from 19.2-53.5%. While the second control retained 49.8% of its activity, S3-F5 and S3-F6 both retained 48.1% and 53.5% of their initial activity.

5 SUMMARY

Rhodococcus rhodochrous DAP 96253 is a bacterium of significance in both the industrial and the pharmaceutical world. While its heightened nitrile hydratase activity makes it a viable source for use in small-scale yet large quantity acrylamide production, its expression of asparaginase activity with low endotoxin presence also makes it a viable source for use in the treatment of acute lymphoblastic leukemia. Both of these settings are great opportunities to create safer and more efficient ways of producing important products.

The bioconversion of acrylonitrile to acrylamide is a safe and green alternative to the chemical production of acrylamide. While there are other microbes that produce competitive products, *R. rhodochrous* DAP 96253 produces bioacrylamide at high efficiency while being able to be used at settings that require no manipulation or monitoring, such as temperature, pressure, and pH. Despite the in-depth look at the various enzymatic formulations, current research suggests whole cells and calcium alginate, both hardened and non-hardened, immobilized cells represent the best catalyst with which to produce bioacrylamide. While other formulations prove to work in the bioconversion process, such as cell-free lysate and purified nitrile hydratase, further investigation into continuously pumping fresh CFL or PU could be used to produce bioacrylamide in a concentration viable to whole cells or immobilized cells but the money and time that goes into creating the catalyst would still be a hinderance.

While the documentation of endotoxin-free asparaginase activity in *R. rhodochrous* DAP 96253 is of great importance, the refinement of a pegylation process that retains a significant percentage of the purified enzyme's initial activity while remaining effective in the human body is the next step in making a drug that is market competitive. Further investigation into lowering the ratio of PEG:ASP could be deemed beneficial if it led to higher retention of activity as

hypothesized. This must be balanced with the level at which the peglyation carries out the advantages offered by the process in the first place. The confirmation of what elution fractions consist of mono-pegylated asparaginase is of great importance to the next step in this project given the increased stability and bio-life this conjugated form delivers. Once defined, the pegylation procedure should be modified to create competitive amounts of mono-pegylated asparaginase, as previously seen around 42%. After a more-narrow range of pegylation concentration is determined, the next step is to test the pegylated samples on tissue cultures in comparison to the freeform asparaginase purified from *R. rhodochrous* DAP 96253 and compared to the previous studies conducted with free-form asparaginase.

REFERENCES

1. Sicard, D. and J.-L. Legras, *Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex.* Comptes Rendus Biologies, 2011. **334**(3): p. 229-236.
2. McGovern, P.E., et al., *Fermented beverages of pre- and proto-historic China.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(51): p. 17593.
3. Houtzager, H.L., *Antonie van Leeuwenhoek.* Eur J Obstet Gynecol Reprod Biol, 1983. **15**(3): p. 199-203.
4. Geison., L.P.T.b.G.L., *Memoire Sur La Fermentation Alcoolique.* 1860 (1981).
5. Eduard Buchner. Translated by Friedmann, H.C., *Alkoholische Gährung Ohne Hefezellen.* European Journal of Inorganic Chemistry, 1897 (1997). **30**(1): p. 117-24.
6. Bud, R.C., Mark F. , *The Uses of Life: A History of Biotechnology (1st ed.).* 1994.
7. Thackray, A., *Private Science: Biotechnology and the Rise of the Molecular Sciences.* Philadelphia: University of Pennsylvania Press., 1998: p. 6–8.
8. Yamada, H. and M. Kobayashi, *Nitrile hydratase and its application to industrial production of acrylamide.* Bioscience, biotechnology, and biochemistry, 1996. **60**(9): p. 1391-1400.
9. De Regil, R., and Sandoval, G., *Biocatalysis for Biobased Chemicals.* Biomolecules, 2013. **3**: p. 812-847.
10. Bhatia, S., *History, scope and development of biotechnology*, in *Introduction to Pharmaceutical Biotechnology, Volume 1*. 2018, IOP Publishing. p. 1-1-1-61.
11. Jiao, S., et al., *Advances in acrylamide bioproduction catalyzed with Rhodococcus cells harboring nitrile hydratase.* Applied Microbiology and Biotechnology, 2020. **104**(3): p. 1001-1012.
12. Shrivastava, A., et al., *Recent developments in L-asparaginase discovery and its potential as anticancer agent.* Crit Rev Oncol Hematol, 2016. **100**: p. 1-10.
13. *4. W. Zopf: Ueber Ausscheidung von Fettfarbstoffen (Lipochromen) seitens gewisser Spaltpilze.* Berichte der Deutschen Botanischen Gesellschaft, 1891. **9**(1): p. 22-28.
14. Goodfellow, M. and G. Alderson, *The actinomycete-genus Rhodococcus: a home for the "rhodochrous" complex.* J Gen Microbiol, 1977. **100**(1): p. 99-122.
15. Embley, T.M. and E. Stackebrandt, *The molecular phylogeny and systematics of the actinomycetes.* Annu Rev Microbiol, 1994. **48**: p. 257-89.
16. Goodfellow, M., *Genus Rhodococcus.* In Bergey's Manual of Systematic Bacteriology, 1989. **4th edition**: p. 2362-2371.
17. Zakrzewska-Czerwinska, J., M. Mordarski, and M. Goodfellow, *DNA Base Composition and Homology Values in the Classification of Some Rhodococcus Species.* Journal of general microbiology, 1988. **134**: p. 2807-13.
18. Koch, C., Klatte, S., Schumann, P., Burghardt, J., Kroppenstedt, R.M. and Stackebrandt, E., *Transfer of Arthrobacter picolinophus to Rhodococcus erythropolis.* International Journal of Systematic Bacteriology, 1995. **45**: p. 576-577.
19. Majidzadeh, M. and M. Fatahi-Bafghi, *Current taxonomy of Rhodococcus species and their role in infections.* European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 2018. **37**(11): p. 2045-2062.
20. Finnerty, W.R., *THE BIOLOGY AND GENETICS OF THE GENUS RHODOCOCCUS.* Annual Review of Microbiology, 1992. **46**(1): p. 193-218.
21. Larkin, M. and L. Kulakov, *Genomes and Plasmids in Rhodococcus*. 2010. p. 73-90.
22. Chen, B.-S., et al., *Draft genome sequence of Rhodococcus rhodochrous strain ATCC 17895.* Standards in Genomic Sciences, 2013. **9**(1): p. 175-184.

23. McLeod, M.P., et al., *The complete genome of Rhodococcus sp. RHA1 provides insights into a catabolic powerhouse.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(42): p. 15582-15587.
24. Blattner, F.R., Plunkett, G. 3rd, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J. et al., *The complete genome sequence of Escherichia coli K-12.* Science, 1997. **277**: p. 1453-1474.
25. Pierce, G.E., et al., *Preliminary report on a catalyst derived from induced cells of Rhodococcus rhodochrous strain DAP 96253 that delays the ripening of selected climacteric fruit: bananas, avocados, and peaches.* J Ind Microbiol Biotechnol, 2011. **38**(9): p. 1567-73.
26. Pierce, G.E., Tucker, T.A., Wang, C., Swensen, K., and Crow Jr., S.A., *Delayed Ripening of Climacteric Fruit by Catalysts Prepared from Induced Cells of Rhodococcus rhodochrous DAP 96253: A Case for the Biological Modulation of Yang-Cycle Driven Processes by a Prokaryote.* Industrial Biotechnology, 2014. **10**(5): p. 354-362.
27. Pierce, G.E., Drago, G.K., and Ganguly, S., *Patent No. 9,605,241 B2.* 2017.
28. Neville, J., *Lipid Utilization of Pseudogymnoascus Destructans and Accompanying Physiological Factors that Influence White-Nose Syndrome.* Unpublished, 2017. **Doctoral Dissertation**(Georgia State University).
29. Kobayashi, M. and S. Shimizu, *Metalloenzyme nitrile hydratase: Structure, regulation, and application to biotechnology.* Nature Biotechnology, 1998. **16**(8): p. 733-736.
30. Zhou, Z., Y. Hashimoto, and M. Kobayashi, *Self-subunit swapping chaperone needed for the maturation of multimeric metalloenzyme nitrile hydratase by a subunit exchange mechanism also carries out the oxidation of the metal ligand cysteine residues and insertion of cobalt.* J Biol Chem, 2009. **284**(22): p. 14930-8.
31. Zhou, Z., et al., *Unique biogenesis of high-molecular mass multimeric metalloenzyme nitrile hydratase: intermediates and a proposed mechanism for self-subunit swapping maturation.* Biochemistry, 2010. **49**(44): p. 9638-48.
32. Smith, E.A. and F.W. Oehme, *Acrylamide and polyacrylamide: a review of production, use, environmental fate and neurotoxicity.* Rev Environ Health, 1991. **9**(4): p. 215-28.
33. Yamada, H., and Magasawa, T., *Microbial Production of Commodity Chemicals.* Pure and Applied Chemistry, 1995. **67**(7): p. 1241-1256.
34. Du, C., and Webb, C., *Engineering Fundamentals of Biotechnology.* Comprehensive Biotechnology, 2011. **2**: p. 11-23.
35. Zheng, R.C., Zheng, Y.G., and Shen, Y.C., *Acrylamide, Microbial Production by Nitrile Hydratase*, in *Encyclopedia of Industrial Biotechnology*. 2010. p. 1-39.
36. Commeyras, A., Arnaud, A., Galzy, P., and Jallageas, J.C., *Process for the Production of Organic Acids by Biological Hydrolysis.* USP 3,940,316, 1976: p. Filed 09/17/1974. Issued 02/24/1976.
37. Nagasawa, T., H. Shimizu, and H. Yamada, *The superiority of the third-generation catalyst, Rhodococcus rhodochrous J1 nitrile hydratase, for industrial production of acrylamide.* Applied Microbiology & Biotechnology, 1993. **40**(2/3): p. 189-195.
38. Kobayashi, M., T. Nagasawa, and H. Yamada, *Nitrilase of Rhodococcus rhodochrous J1. Purification and characterization.* Eur J Biochem, 1989. **182**(2): p. 349-56.
39. Nagasawa, T., K. Takeuchi, and H. Yamada, *Occurrence of a cobalt-induced and cobalt-containing nitrile hydratase in RhodococcusRhodochrous J1.* Biochemical and Biophysical Research Communications, 1988. **155**(2): p. 1008-1016.
40. Ganguly, S., *Enhanced Stabilization of Nitrile Hydratase Enzyme from Rhodococcus Sp. DAP 96253 and Rhodococcus rhodochrous DAP 96622.* Unplublished, 2005. **Doctoral Dissertation**: p. Georgia State University.
41. Nagasawa, T., and Yamada, H., *Large-scale bioconversion of nitriles into useful amides and acids.* Biocatalysis, 1990: p. 277-318.

42. Du, F., *Comparison of Nitrile Hydratases in Rhodococcus Rhodochrous DAP 96253 and DAP 96622 Growing on Inducing and Non-Inducing Media.* Unplublished, 2013. **Doctoral Dissertation**: p. Georgia State University.
43. Kobayashi, M., et al., *Occurrence of Amidases in the Industrial Microbe Rhodococcus rhodochrous J1.* Bioscience, Biotechnology, and Biochemistry, 1993. **57**(11): p. 1949-1950.
44. Virgen-Ortíz, J.J., et al., *Polyethylenimine: a very useful ionic polymer in the design of immobilized enzyme biocatalysts.* Journal of Materials Chemistry B, 2017. **5**(36): p. 7461-7490.
45. Prasad, S., J. Raj, and T. Bhalla, *Purification of a hyperactive nitrile hydratase from resting cells of Rhodococcus rhodochrous PA-34.* Indian journal of microbiology, 2009. **49**: p. 237-42.
46. Katz, A.J., et al., *Acute lymphoblastic leukemia: an assessment of international incidence, survival, and disease burden.* Cancer Causes Control, 2015. **26**(11): p. 1627-42.
47. Kidd, J.G., *Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum.* J Exp Med, 1953. **98**(6): p. 565-82.
48. UK, C.R., *Acute Lymphoblastic Leukaemia (ALL).*
49. Müller, H.J. and J. Boos, *Use of L-asparaginase in childhood ALL.* Crit Rev Oncol Hematol, 1998. **28**(2): p. 97-113.
50. Enzon, I., *Form 10-K Annual Report.* Annual Report Pursuant to Section 13 or 15(d) of the Securities Exchange Act of 1934., 1995.
51. Van Thu, H. and S. Bergeron, *Asparaginase Toxicities: Identification and management in patients with acute lymphoblastic leukemia.* Clinical Journal of Oncology Nursing, 2017. **21**(5): p. E248-E259.
52. Freudenberg, M.A., et al., *Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity.* Immunobiology, 2008. **213**(3-4): p. 193-203.
53. Young-A, H., Yahiya, S., and Keam, S., *Pegaspargase: A Review in Acute Lymphoblastic Leukaemia.* Drugs, 2019. **79**: p. 767-777.
54. Amadasun, N., *Preliminary Report on Protein Preparations Exhibiting both Asparaginase and Glutaminase Activity Purified from Rhodococcus Rhodochrous DAP 96253.* Unpublished, 2020. **Doctoral Dissertation**: p. Georgia State University.
55. de la Croix, M., *Pre-Pilot Fed-Batch Fermentation of Rhodococcus Rhodochrous DAP 96253 with Emphasis on Production of Therapeutic Enzymes and Post-Harvest Agricultural Applications.* 2019. **Unpublished**: p. Georgia State University.
56. Fawcett, J.K. and J.E. Scott, *A rapid and precise method for the determination of urea.* J Clin Pathol, 1960. **13**(2): p. 156-9.
57. Sanchez-Castelblanco, E.M., *Further Purification of an Isolated Nitrile Hydratase Fraction with Asparaginase and Glutaminase Activity from Rhodococcus Rhodochrous and Evaluation of its Kinetics and Properties as a Potential Treatment for Human Leukemia.* 2019. **Unpublished**: p. Georgia State University.
58. Belshazzar, B., *Process Improvements for Purification, Characterization, and Identification of a Novel 1-Hexene Monooxygenase and a Unique Asparaginase Activity contained within the Cobalt High Mass Nitrile Hydratase from Induced Cells of Rhodococcus rhodochrous DAP 96253.* Doctoral Dissertation, 2017. **Unpublished**: p. Georgia State University.

59. Tucker, T.A.M., *The Effect of Media Composition on Nitrile Hydratase Activity and Stability, and on Cell Envelope Components of Rhodococcus DAP 96253.* Unpublished, 2008. **Doctoral Dissertation**(Georgia State University).
60. Pascali, J.P., and Pozzebon, M., *Direct GC Determination of Acrylamide in Water using the Agilent 7000B Triple Quadrupole GC/MS.* Environmental Analysis, 2014.
61. Tegner, F., *Optimization of a PEGylation Process: A combined reaction and separation with Size Exclusion Reaction Chromatography.* Doctoral Dissertation, 2015. **Unpublished**: p. Lund University.
62. Momin, N., *Analysis of Nitrile Hydratase, Asparaginase, and Glutaminase Activity of Rhodococcus rhodochrous DAP 96253, Growin in YEMEA Media Supplemented with Iron.* Unpublished, 2019. **Master's Non-thesis**: p. Georgia State University.
63. Asano, Y., et al., *Aliphatic Nitrile Hydratase from Arthrobacter sp. J-1 Purification and Characterization.* Agricultural and Biological Chemistry, 1982. **46**(5): p. 1165-1174.
64. Singh, R., et al., *Bioconversion of acrylonitrile using nitrile hydratase activity of Bacillus sp. APB-6.* 3 Biotech, 2018. **8**(5): p. 225.
65. Lan, Y., et al., *Overexpression and characterization of two types of nitrile hydratases from Rhodococcus rhodochrous J1.* PLOS ONE, 2017. **12**(6): p. e0179833.
66. Padmakumar, R. and P. Oriel, *Bioconversion of acrylonitrile to acrylamide using a thermostable nitrile hydratase.* Appl Biochem Biotechnol, 1999. **77-79**: p. 671-9.
67. Kamble, A.L., et al., *Nitrile hydratase of Rhodococcus erythropolis: characterization of the enzyme and the use of whole cells for biotransformation of nitriles.* 3 Biotech, 2013. **3**(4): p. 319-330.
68. Sankhian, U., et al., *Nitrile hydratase of Rhodococcus rhodochrous NHB-2: Optimisation of conditions for production of enzyme and conversion of acrylonitrile to acrylamide.* Asian Journal of Microbiology, Biotechnology and Environmental Sciences, 2003. **2**.
69. Homaei, A.A., et al., *Enzyme immobilization: an update.* J Chem Biol, 2013. **6**(4): p. 185-205.
70. Burns, B., G. Mendz, and S. Hazell, *Methods for the Measurement of a Bacterial Enzyme Activity in Cell Lysates and Extracts.* Biological procedures online, 1998. **1**: p. 17-26.
71. Vasudev, S.S., et al., *Formulation of PEG-ylated L-asparaginase loaded poly (lactide-co-glycolide) nanoparticles: influence of Pegylation on enzyme loading, activity and in vitro release.* Pharmazie, 2011. **66**(12): p. 956-60.
72. Meneguetti, G.P., et al., *Novel site-specific PEGylated L-asparaginase.* PloS one, 2019. **14**(2): p. e0211951-e0211951.
73. Reasoner, D.J. and E.E. Geldreich, *A new medium for the enumeration and subculture of bacteria from potable water.* Applied and environmental microbiology, 1985. **49**(1): p. 1-7.
74. Wu, Z.-L. and Z.-Y. Li, *Enhancement of enzyme activity and enantioselectivity via cultivation in nitrile metabolism by Rhodococcus sp. CGMCC 0497.* Biotechnology and Applied Biochemistry, 2002. **35**(1): p. 61-67.

APPENDICES

Appendix A

Complete Details for the Fermentation of *R. rhodochrous* DAP 96253 [55]:

Stock Preparation

A clean 250mL flask was filled with 100mL of ddH_2O. While stirring, 0.8gm of Nutrient Broth, BD Difco™ (#23400) was added and autoclaved at 121°C for 30 minutes. Following the cooling of the nutrient broth (NB) to room temperature, the media was inoculated appropriately. The culture was incubated at 30°C at 225 rotations per minute (RPM) in a G-25KC New Brunswick Scientific Gyratory Shaker (Edison, NJ) for two days. A sterile 60% stock solution of glycerol was prepared using 100% Glycerol, Fisher Chemical (#G33-4), and DI water. Stocks were created by the addition of 500µL of culture and 500µL of glycerol stock solution into a 2mL Nalgene® Cryoware Cryogenic Vial (#5000-0020) resulting in a solution that was 30% v/v glycerol. The 30% glycerol stock culture was flash-frozen in liquid nitrogen for five minutes using a Taylor Wharton Cryogenic Storage Dewar (35 VHC). Following the flash-freeze process, the newly formed bacterial stocks were stored at -80°C until future use in an Isotemp® (Fisher Scientific, Hampton, NH) low-temperature freezer.

Nutrient Broth and 1X mR3A Seed

Rhodococcus rhodochrous strain DAP 96253 stored at -80°C in 1mL glycerol stocks were transferred to 100mL of NB and incubated at 30°C at 225 RPM in a gyratory shaker for two days at which time the entire content was transferred to a 2L modified animal protein-free R3A medium seed (mR3A) based on Reasoner and Geldreich [73]. Modifications include the addition of Urea, Fisher Chemical (#U15-3), and Cobalt (II) Chloride Hexahydrate, Sigma-Aldrich (#255599-500G) at varying concentrations determined by experiment parameters. Additionally,

the animal protein sources, such as proteose peptone and casamino acids, were replaced with Proyield Cotton® Friesland Campina (#2497813) on an equivalent basis (shown below).

Component	*Manufacture*	*Concentration (g/L)*
Proyield Cotton, UI	FrieslandCampina	3.00
Soluble Starch	BD Difco™	1.50
Yeast Extract Technical	BD Bacto™	1.50
Magnesium Sulfate, Anhydrous	J.T. Baker	0.15
Potassium Phosphate, Dibasic	EMD Millipore	0.90
Dextrose (D-Glucose), Anhydrous	Fisher Chemical	10.00
Sodium Pyruvate (ReagentPlus®)	Sigma-Aldrich	0.76
Urea, Certified ACS	Fisher Chemical	16.00
Cobalt (II) Chloride Hexahydrate	Sigma-Aldrich	0.05

The inoculated 2L 1X mR3A seed was incubated at 30°C at 225 RPM in a gyratory shaker for four days. All media was autoclaved for 30min at 121°C, with the exception of Dextrose (D-Glucose) Anhydrous, Fisher Chemical (#D16-3), Sodium Pyruvate, Sigma-Aldrich (#P2256), and urea; these temperature-sensitive materials were filter sterilized using a 250mL 0.2µm Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable CN Membrane (Waltham, MA) filter. Following four days of growth, the 1X mR3A seed culture was transferred to a sterilized 2L bottle, which was then aseptically connected to the reactor.

pH Adjustment and Anti-foaming

To maintain a stable pH, a 2N NaOH, Sigma-Aldrich (#S8045), and 1N HCl, Fisher Scientific (#A114SI) solutions were created at the final volumes of 1.5L and 3L respectively. In 2019 the 1N HCl was replaced with 1N H_2SO_4, Sigma-Aldrich (#339741). Additionally, 800mL of Antifoam 204®, Sigma-Aldrich (#A6426) at 20% by volume, was prepared for foam control.

Fermentation

Fermentation runs conducted with *R. rhodochrous* strain DAP 96253 were performed in a sterilizable in place (SIP) 20L stainless steel BioStat® C (Sartorius Stedim, Göttingen, Germany) stirred-tank reactor (STR).

Pre-Fermentation

The nutrient broth was inoculated with *R. rhodochrous* DAP 96253 glycerol stock six days before fermentation and incubated for two days. Following incubation, the entire 100mL of the culture was transferred to the 1X mR3A seed and grown for an additional four days. The 5X mR3A batch and YECFM were prepared one day before the fermentation run, in addition to the corrective solutions. Bottle tops, each containing a Midistart® 2000 (Sartorius Stedim, Göttingen, Germany) filter, were assembled one day before the fermentation run and autoclaved on the dry cycle in Duo-Check® (Crosstex International, Little Falls, NJ) self-sealing sterilization pouches. Six feet of Masterflex® tubing C-flex tubing 1/8" x 1⁄4" (Cole-Parmer, Vernon Hills, IL) was attached to each 3-way addition port needle (bbi-biotech, Berlin, Germany) and sacova valve standard port 3-way (bbi-biotech, Berlin, Germany) and were sterilized using self-sealing sterilization pouches and attached to each media and corrective solution in the biosafety cabinet.

Vessel Setup

The fermentation of *R. rhodochrous* was conducted utilizing the BioStat® C 20L bioreactor. The bioreactor was equipped with factory temperature, airflow, and foam sensors in addition to these attachable sensors: EasyFerm Plus K8 B120 (pH) (Hamilton, Reno, NV), InPro® 6050 (Mettler Toledo, Columbus, OH) dissolved oxygen sensor (DO), Optek Fermenter Control with a real-time optical density (OD) probe (Optek-Danulat, Germantown, WI). The pH probe was calibrated to pH 7.0 and pH 4.01, using standard buffer solutions, before attaching it to the vessel

port. The DO probe was attached to the vessel port and connected to the controller to maintain a consistent electrical current for optimal polarization of the probe.

The inlet air was set to “sterilization” mode and the Sartofluor® Junior (Sartorius Stedim, Göttingen, Germany) inlet air filter and the Sartofluor® Mini (Sartorius Stedim, Göttingen, Germany) outlet air filter was checked for integrity and replaced as needed. The vessel was filled with 6.3L of ddH_2O, and all remaining open side ports were sealed. The sacova valve standard port 3-way was screwed into the top reserve port, while the remaining reserve ports were sealed using the 19mm membrane (Sartorius Stedim, Göttingen, Germany), membrane holder, and blind plug (bbi-biotech, Berlin, Germany). By restricting the outlet air, by disconnecting the quick release, the pressure of the vessel was raised to 1 bar at room temperature for ten minutes to verify the vessel is airtight; this allows for proper sterilization and prevents contamination due to the negative pressure within the reactor during operation.

The vessel underwent a SIP protocol, which was set to 121°C for 45min by the use of plant steam generated by the building boiler. The cooling valve of the condenser was closed to allow for proper heating of the outlet air unit, and the sampling and collection ports were actively steamed for 45 minutes using pharmaceutical grade steam from the Mueller Pure Steam Generator (Paul Mueller, Springfield, MO). Once the internal vessel temperature reached 121°C, the 0% DO was calibrated for the sensor. Following 45min of sterilization, the temperature was automatically set to 30°C, and the cooling valve of the condenser was reopened once the vessel temperature reached 80°C. The inlet air was switched back over to the “fermentation” mode.

Once the vessel reached 30°C, the 3-way addition port needles were pierced through the diaphragm on the top reserve ports and firmly hand tightened. The number of triport inlet connector needles used will vary depending on the number of bottles of media utilized for each

fermentation run. The majority of the fermentation runs had the batch, feed, and base on the triport inlet sacova valve, and the seed, acid, and antifoam on the triport inlet connector needle.

The YECFM, antifoam, base, and acid masterflex tubing were directly connected to their respective peristaltic programmable pumps on the controller and primed to remove any air bubbles within the line. The 5X mR3A batch was pumped into the reactor using a Cole-Parmer peristaltic pump in a pulse-batch fashion. The pH was set to 7.0, and the DO probe was calibrated to 100% before the inoculation. The 1x mR3A seed inoculum, containing the *R. rhodochrous* cells, was pumped into the vessel with the Cole-Parmer peristaltic pump, and the fermentation run was started on the Biopat® MFCS/WIN (Sartorius Stedim, Göttingen, Germany).

Fermentation Parameters

Using the Biostat C controller, the temperature was maintained at 30°C and a pH of 7.0. The pH was controlled by the incremental addition of the acid (HCl or H_2SO_4) and the base (NaOH). The airflow was kept between 0.5 L/V min^{-1}, utilizing filtered building air. Dissolved oxygen (DO) was configured to maintain a dissolved oxygen concentration of ≥30% saturation by the use of agitation running on a cascade control loop. Agitation was conducted by the rotation of Rushton turbines with a minimum value of 150rpm and a maximum value of 350rpm. Supplemental air (gas mix) was added once the maximum stir rate was achieved by the use of pure O_2 to maintain optimal dissolved oxygen concentration. The *R. rhodochrous* cells were fed-batch the YECFM to stimulate growth and maintain metabolic activity. This was achieved by the programming of a feed profile, which was adjusted depending on biomass, glucose concentration, dissolved oxygen concentration, and enzyme activity. A 100% feed profile indicated a fed-batch profile of 20mL/min of the YECFM. The Biopat® MFCS/WIN software

monitored the conditions of the fermentation run. It recorded real-time data of the elapsed fermentation time (EFT), temperature, stir rate, dissolved oxygen, pH, airflow, and feed profile.

Appendix B

Comparison Method for PAM Immobilization of Whole Cells [40]:

10 g (packed wet weight) cells were suspended in 40 ml DI H2O and thoroughly mixed with 40 ml of acrylamide-bisacrylamide solution (4.5 g AMD and 0.5 g N-methylbisacrylamide in DI water) on ice. To the 80 ml of admixed solution, first 5 ml of 3- dimethylaminoproprionitrile and then 10 ml of 2.5% potassium persulfate solution were added in rapid succession with vigorous mixing on ice, and then quickly poured between two glass plates to form a gel slab of 2 mm thickness. The gel was allowed to incubate at room temp for 60 min for complete polymerization. After complete polymerization, the gel was cut into thin strips and then further cut into small cubes of 2-3 mm. The cubes were washed 3 X with DI H2O to remove residual monomers and stored at 4 C in DI H2O till used.

Appendix C

Comparison Method for non-hardened and hardened Ca-ALG Immobilization of Whole Cells [40]:

Cells immobilized in Ca-alginate beads (2-3 mm in dia.) were prepared according to a modification of the method of Wu and Li (2002). Cells (5 g packed wet wt) were resuspended in 50 ml of 50 mM TRIS, pH 7.2, mixed thoroughly with a 25 g of 4% sodium alginate (in 50 mM DI water) and the mixture was passed through a hypodermic needle (27 gauge) drop wise into a solution of 0.1 M calcium chloride. The beads were cured in the calcium chloride solution for 60 min, washed and stored at 4 C until used.

Hardened Calcium alginate: Hardened Ca-alginate beads were prepared by adding 50% w/w PEI (in 1 M CaCl2 solution, to achieve 0.5% solution) to previously prepared ca-alginate beads. The beads were incubated in the PEI solution for 24 h at room temperature, washed once with DI water and stored in 50 mM TRIS buffer, pH 7.2 at 4 C until used [74].

Appendix D

Comparison Method for PEI-Glu Immobilization of Whole Cells [40]:

Cells 10 g (packed wet weight) were suspended in 100 ml DI H_2O, to which a 25% solution (in DI water) of glutaraldehyde (GA) was added with constant stirring, to achieve a 0.5% final concentration of GA. The cell mixture was left stirring for 60 min at room temperature, at which time 400 ml DI H_2O was added to the mixture with stirring, followed by the slow addition of PEI solution (50% w/v in DI water). PEI was added until flocculation was achieved. The flocculated material was harvested by filtering (0.45 μ nalgene bottletop filter, Nalge Nunc International, Rochester, NY). The packed flocculate was extruded through a syringe and dried for 24 hours. It was cut into small pieces (2-3 mm length) and stored at room temperature till used.

Appendix E

Raw Calculations of *R. rhodochrous* DAP 96253 Nitrile Hydratase Activity via Different Growth Techniques.

	Absorbance	Absorbance	Absorbance	Average	Adjusted Avg	y=	uM NH3 per minute	per mg CWW	per mg CDW	**Avg Units**	**Std Dev**
Plate	0.173	0.173	0.168	0.171	0.168	0.63	18.48	18.48	92.39	**106.38**	**19.80**
	0.184	0.183	0.185	0.184	0.185	0.82	24.08	24.08	120.38		
Ferm	0.534	0.529	0.531	0.531	0.531	4.69	138.03	138.03	690.17	**744.52**	**76.85**
	0.598	0.596	0.597	0.597	0.597	5.43	159.77	159.77	798.86		
Ferm Wash	1.521	1.52	1.522	1.521	1.522	15.79	464.43	464.43	2322.15	**2298.27**	**33.77**
	1.479	1.501	1.493	1.491	1.493	15.47	454.88	454.88	2274.39		

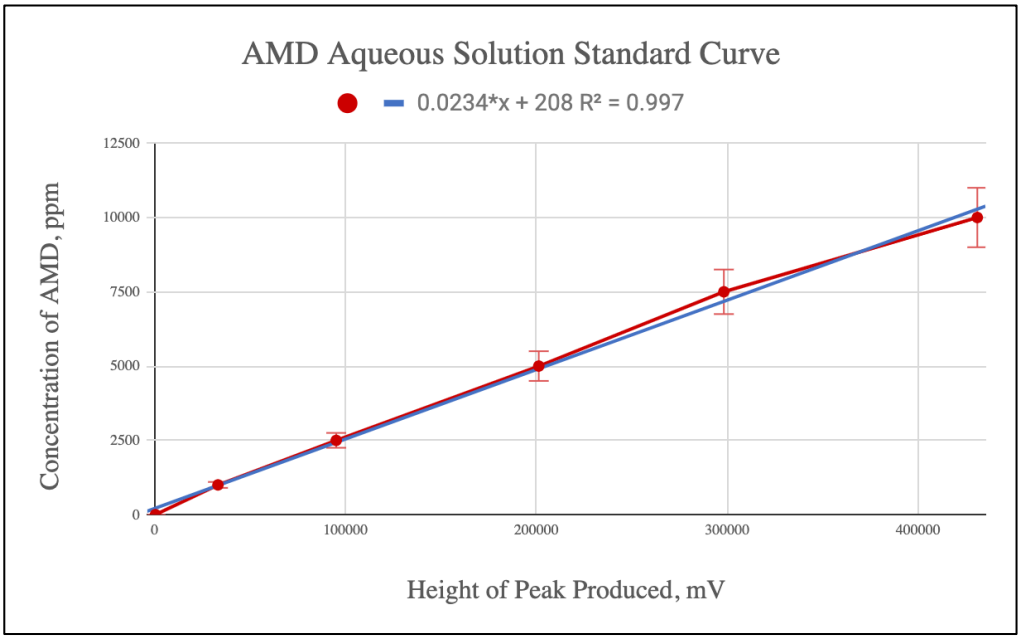
Appendix F

Raw Calculations of *R. rhodochrous* DAP 96253 Nitrile Hydratase Activity via Different Fermentation Time Lengths.

	Absorbance	Absorbance	Absorbance	Average	Adjusted Avg	y=	um NH3 per minute	um/ min/ml	average
Ferm Paste 62H	0.935	0.935	0.924	0.931	0.883	9.477	557.5	1114.900	945
	0.747	0.773	0.699	0.740	0.692	7.412	436.0	872.050	
	0.720	0.728	0.718	0.722	0.674	7.221	424.8	849.521	
Ferm Paste 48H	1.689	1.925	1.929	1.848	1.800	19.341	1137.7	2275.451	2387
	1.764	2.168	2.196	2.043	1.995	21.448	1261.7	2523.311	
	1.892	1.928	1.927	1.916	1.868	20.076	1181.0	2361.916	

Appendix G

Acrylamide Standard Curve using GC-FID for Fall 2017 Bioacrylamide Production.



Appendix H

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 100mL total of Acrylonitrile at a rate of 0.33mL/min. (Whole Cells #2)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	.0207(X)+246.13	Dilution	%AMD	Avg %	Std. Dev.
30	3.085417	30minA	38486.40	1042.80	10427.98	1.04	1.05	0.02
		30minB	39517.13	1064.13	10641.35	1.06		
		30minC	37222.06	1016.63	10166.27	1.02		
60	5.944403	60minA	44135.51	1159.74	11597.35	1.16	1.18	0.03
		60minB	46339.22	1205.35	12053.52	1.21		
		60minC	44382.93	1164.86	11648.57	1.16		
90	8.601001	90minA	68857.80	1671.49	16714.86	1.67	1.77	0.09
		90minB	74311.53	1784.38	17843.79	1.78		
		90minC	77968.60	1860.08	18600.80	1.86		
120	11.075967	120minA	240000.83	5214.15	52141.47	5.21	5.52	0.62
		120minB	234830.04	5107.11	51071.12	5.11		
		120minC	289267.72	6233.97	62339.72	6.23		
150	13.387312	150minA	345539.11	7398.79	73987.90	7.40	7.10	1.02
		150minB	276129.56	5962.01	59620.12	5.96		
		150minC	371987.31	7946.27	79462.67	7.95		
180	15.550743	180minA	524079.76	11094.58	110945.81	11.09	11.08	1.28
		180minB	461162.45	9792.19	97921.93	9.79		
		180minC	584844.46	12352.41	123524.10	12.35		
210	17.580019	210minA	830118.47	17429.58	174295.82	17.43	16.69	0.70
		210minB	789494.75	16588.67	165886.71	16.59		
		210minC	763270.63	16045.83	160458.32	16.05		
240	19.487242	240minA	955680.66	20028.72	200287.20	20.03	19.96	0.51
		240minB	975466.72	20438.29	204382.91	20.44		
		240minC	926246.79	19419.44	194194.39	19.42		
270	21.283102	270minA	897498.32	18824.35	188243.45	18.82	19.89	0.93
		270minB	976072.71	20450.84	204508.35	20.45		
		270minC	974018.78	20408.32	204083.19	20.41		
300	22.977077	300minA	964677.55	20214.96	202149.55	20.21	20.19	0.22
		300minB	973650.10	20400.69	204006.87	20.40		
		300minC	952356.55	19959.91	199599.11	19.96		

Appendix I

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 100mL total of Acrylonitrile at a rate of 0.38mL/min. (Whole Cells #3)

Minutes	Actual Minutes*	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5	0.61	30minA	6492.07	360.07	3600.74	0.36	0.36	0.00
			30minB	6714.49	365.28	3652.79	0.37		
			30minC	6012.49	348.85	3488.52	0.35		
60	35	4.09	60minA	136944.95	3412.67	34126.72	3.41	3.53	0.16
			60minB	146852.90	3644.52	36445.18	3.64		
			60minC	127545.34	3192.72	31927.21	3.19		
90	70	7.79	90minA	341508.26	8199.45	81994.53	8.20	8.32	0.24
			90minB	340395.03	8173.40	81734.04	8.17		
			90minC	358465.95	8596.26	85962.63	8.60		
100	80	8.78	100minA	450695.66	10754.44	107544.38	10.75	9.88	1.11
			100minB	429761.58	10264.58	102645.81	10.26		
			100minC	360082.35	8634.09	86340.87	8.63		
120	110	11.60	120minA	554072.56	13173.46	131734.58	13.17	11.10	1.94
			120minB	389789.38	9329.23	93292.31	9.33		
			120minC	452023.53	10785.51	107855.11	10.79		
150	140	14.21	150minA	646220.42	15329.72	153297.18	15.33	15.52	0.76
			150minB	626655.49	14871.90	148718.98	14.87		
			150minC	690121.47	16357.00	163570.02	16.36		
180	170	16.63	180minA	739649.41	17515.96	175159.56	17.52	16.92	0.79
			180minB	726636.76	17211.46	172114.60	17.21		
			180minC	675688.00	16019.26	160192.59	16.02		
210	200	18.88	210minA	982507.06	23198.83	231988.25	23.20	21.08	2.11
			210minB	801976.70	18974.41	189744.15	18.97		
			210minC	891413.33	21067.23	210672.32	21.07		
240	230	20.98	240minA	982364.70	23195.49	231954.94	23.20	23.10	0.09
			240minB	974459.91	23010.52	230105.22	23.01		
			240minC	977888.85	23090.76	230907.59	23.09		
270	260	22.94	270minA	983427.94	23220.37	232203.74	23.22	23.18	0.03
			270minB	981490.69	23175.04	231750.42	23.18		
			270minC	980612.07	23154.48	231544.82	23.15		
300	290	24.78	300minA	980119.57	23142.96	231429.58	23.14	23.15	0.01
			300minB	980693.16	23156.38	231563.80	23.16		
			300minC	980701.23	23156.57	231565.69	23.16		
330	290	24.78	330minA	980381.90	23149.10	231490.96	23.15	23.13	0.01
			330minB	979358.45	23125.15	231251.48	23.13		
			330minC	979240.88	23122.40	231223.97	23.12		

Appendix J

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 130mL total of Acrylonitrile at a rate of 0.38mL/min. (Whole Cells #4)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	3.26	30minA	139700.98	3477.16	34771.63	3.48	3.45	0.04
		30minB	137360.64	3422.40	34223.99	3.42		
		30minC	151490.02	3753.03	37530.26	3.75		
60	6.28	60minA	248984.43	6034.40	60343.96	6.03	6.22	0.26
		60minB	264686.49	6401.82	64018.24	6.40		
		60minC	266721.76	6449.45	64494.49	6.45		
90	9.06	90minA	449757.64	10732.49	107324.89	10.73	10.39	0.95
		90minB	466094.25	11114.77	111147.65	11.11		
		90minC	389244.61	9316.48	93164.84	9.32		
120	11.65	120minA	586920.23	13942.09	139420.93	13.94	14.20	0.57
		120minB	580796.64	13798.80	137988.01	13.80		
		120minC	625632.79	14847.97	148479.67	14.85		
150	14.06	150minA	518996.27	12352.67	123526.73	12.35	14.34	2.10
		150minB	595427.39	14141.16	141411.61	14.14		
		150minC	697972.19	16540.71	165407.09	16.54		
180	16.30	180minA	848372.13	20060.07	200600.68	20.06	19.03	1.95
		180minB	708046.98	16776.46	167764.59	16.78		
		180minC	856389.02	20247.66	202476.63	20.25		
210	18.40	210minA	983675.51	23226.17	232261.67	23.23	22.02	1.26
		210minB	936042.89	22111.56	221115.64	22.11		
		210minC	876196.72	20711.16	207111.63	20.71		
240	20.37	240minA	981791.4	23182.08	231820.79	23.18	22.74	0.75
		240minB	925542.15	21865.85	218658.46	21.87		
		240minC	980810.71	23159.13	231591.31	23.16		
270	22.22	270minA	608588.35	14449.13	288982.55	28.90	28.46	2.50
		270minB	541596.87	12881.53	257630.54	25.76		
		270minC	647259.03	15354.02	307080.43	30.71		
300	23.96	300minA	704997.64	16705.10	334102.10	33.41	32.47	1.42
		300minB	650143.08	15421.51	308430.16	30.84		
		300minC	699891.28	16585.62	331712.32	33.17		
330	25.60	330minA	661869.49	15695.91	313918.12	31.39	33.30	2.15
		330minB	752473.02	17816.03	356320.57	35.63		
		330minC	693628.05	16439.06	328781.13	32.88		
360	27.14	360minA	842627.08	19925.63	398512.67	39.85	36.80	3.39
		360minB	790555.44	18707.16	374143.15	37.41		
		360minC	699348.32	16572.91	331458.21	33.15		

Appendix K

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 160mL total of Acrylonitrile at a rate of 0.38mL/min. (Whole Cells #5)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	3.53	30minA	113368.49	2860.98	28609.83	2.86	3.02	0.23
		30minB	127085.77	3181.97	31819.67	3.18		
		30minC	120064.31	3017.66	30176.65	3.02		
60	6.77	60minA	277352.40	6698.21	66982.06	6.70	6.49	0.29
		60minB	259914.66	6290.16	62901.63	6.29		
		60minC	243937.44	5916.30	59162.96	5.92		
90	9.75	90minA	376931.51	9028.36	90283.57	9.03	9.51	0.51
		90minB	420051.47	10037.36	100373.64	10.04		
		90minC	395530.65	9463.58	94635.77	9.46		
120	12.50	120minA	620583.18	14729.81	147298.06	14.73	13.83	0.79
		120minB	567936.14	13497.87	134978.66	13.50		
		120minC	557404.72	13251.43	132514.30	13.25		
150	15.04	150minA	739054.19	17502.03	175020.28	17.50	17.61	1.40
		150minB	686255.71	16266.54	162665.44	16.27		
		150minC	805473.72	19056.25	190562.45	19.06		
180	17.40	180minA	868892.13	20540.24	205402.36	20.54	20.70	2.10
		180minB	968425.93	22869.33	228693.27	22.87		
		180minC	789687.21	18686.84	186868.41	18.69		
210	19.60	210minA	561173.70	13339.62	266792.49	26.68	23.89	3.54
		210minB	527049.36	12541.12	250822.30	25.08		
		210minC	416475.70	9953.69	199073.83	19.91		
240	21.65	240minA	580010.87	13780.41	275608.29	27.56	27.76	0.33
		240minB	580536.96	13792.72	275854.50	27.59		
		240minC	592342.71	14068.98	281379.59	28.14		
270	23.57	270minA	700233.15	16593.62	331872.31	33.19	32.72	3.26
		270minB	616119.61	14625.36	292507.18	29.25		
		270minC	754169.08	17855.72	357114.33	35.71		
300	25.37	300minA	799445.80	18915.19	378303.83	37.83	35.96	1.84
		300minB	758521.32	17957.56	359151.18	35.92		
		300minC	720661.45	17071.64	341432.76	34.14		
330	27.06	330minA	895422.36	21161.04	423220.86	42.32	37.48	4.33
		330minB	716945.86	16984.69	339693.86	33.97		
		330minC	763505.70	18074.19	361483.87	36.15		
360	28.65	360minA	853728.73	20185.41	403708.25	40.37	41.86	1.65
		360minB	923261.43	21812.48	436249.55	43.62		
		360minC	879381.73	20785.69	415713.85	41.57		
390	30.15	390minA	475175.75	11327.27	453090.90	45.31	43.63	1.65
		390minB	456735.56	10895.77	435830.88	43.58		
		390minC	439845.19	10500.54	420021.50	42.00		
410	31.10	410minA	503126.07	11981.31	479252.40	47.93	45.37	4.83
		410minB	508123.75	12098.26	483930.23	48.39		
		410minC	416379.11	9951.43	398057.25	39.81		

Appendix L

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 160mL total of Acrylonitrile at 0.6mL/min. (Whole Cells #6)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5.44	30minA	72950.29	1915.20	38303.94	3.83	3.83	0.00
		30minB	73026.17	1916.97	38339.45	3.83		
		30minC	84882.86	2194.42	43888.38	4.39		
60	10.20	60minA	248543.72	6024.08	120481.66	12.05	12.65	0.86
		60minB	274442.23	6630.11	132602.16	13.26		
		60minC	220397.49	5365.46	107309.23	10.73		
90	14.39	90minA	350088.42	8400.23	168004.58	16.80	15.71	1.28
		90minB	333226.39	8005.66	160113.15	16.01		
		90minC	296746.78	7152.03	143040.69	14.30		
120	18.11	120minA	347913.18	8349.33	166986.57	16.70	18.10	2.04
		120minB	357729.91	8579.04	171580.80	17.16		
		120minC	427968.07	10222.61	204452.26	20.45		
150	21.44	150minA	516494.73	12294.14	245882.73	24.59	24.10	1.90
		150minB	461286.44	11002.26	220045.25	22.00		
		150minC	540510.40	12856.10	257122.07	25.71		
180	24.44	180minA	666584.64	15806.24	316124.81	31.61	30.07	2.81
		180minB	564279.33	13412.30	268245.93	26.82		
		180minC	670027.62	15886.81	317736.13	31.77		
210	27.14	210minA	876139.49	20709.82	414196.48	41.42	35.15	5.62
		210minB	644487.02	15289.16	305783.13	30.58		
		210minC	705771.05	16723.20	334464.05	33.45		
240	29.60	240minA	787771.61	18642.02	372840.31	37.28	38.30	3.39
		240minB	890300.66	21041.20	420823.91	42.08		
		240minC	750142.05	17761.48	355229.68	35.52		
270	31.85	270minA	937420.60	22143.80	442876.04	44.29	40.74	3.10
		270minB	831714.54	19670.28	393405.60	39.34		
		270minC	815669.55	19294.83	385896.55	38.59		
300	33.91	300minA	540987.03	12867.26	514690.26	51.47	49.92	1.38
		300minB	512629.14	12203.68	488147.28	48.81		
		300minC	519536.73	12365.32	494612.78	49.46		

Appendix M

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 160mL total of Acrylonitrile at 0.8mL/min. (Whole Cells #7)

Minutes	AN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	7.09	30minA	124807.62	3128.66	62573.17	6.26	6.27	0.02
pH= 7.3		30minB	125416.41	3142.90	62858.08	6.29		
		30minC	125543.21	3145.87	62917.42	6.29		
60	13.05	60minA	342933.89	8232.81	164656.26	16.47	15.59	0.76
pH= 7.3		60minB	315595.68	7593.10	151861.98	15.19		
		60minC	314221.29	7560.94	151218.76	15.12		
100	19.64	90minA	661315.44	15682.94	313658.83	31.37	30.71	0.83
pH= 7.3		90minB	627207.23	14884.81	297696.18	29.77		
		90minC	653140.91	15491.66	309833.15	30.98		
120	22.47	120minA	712776.94	16887.14	337742.81	33.77	26.11	18.35
pH= 7.3		120minB	832770.45	19694.99	393899.77	39.39		
		120minC	101715.60	2588.31	51766.10	5.18		
150	26.27	150minA	845667.22	19996.77	399935.46	39.99	43.39	2.96
pH= 7.3		150minB	948533.74	22403.85	448076.99	44.81		
		150minC	960642.21	22687.19	453743.75	45.37		
180	29.60	180minA	984583.68	23247.42	464948.36	46.49	46.49	0.02
pH= 7.3		180minB	984073.38	23235.48	464709.54	46.47		
		180minC	984705.26	23250.26	465005.26	46.50		
210	32.56	210minA	887120.28	20966.77	419335.49	41.93	44.85	2.53
pH= 7.3		210minB	978184.80	23097.68	461953.69	46.20		
		210minC	983283.80	23217.00	464340.02	46.43		

Appendix N

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96253 Whole Cells at Room Temperature while adding in 135mL total of Acrylonitrile at 0.75mL/min. (Whole Cells #8)

Minutes	ACN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	6.69	30minA	149624.85	3709.38	74187.63	7.42	7.58	0.22
		30minB	156378.82	3867.42	77348.49	7.73		
		30minC	156544.86	3871.31	77426.19	7.74		
60	12.36	60minA	312984.49	7532.00	150639.94	15.06	15.58	0.73
		60minB	335084.13	8049.13	160982.57	16.10		
		60minC	382717.16	9163.74	183274.83	18.33		
90	17.22	90minA	498755.44	11879.04	237580.75	23.76	21.94	1.73
		90minB	424989.67	10152.92	203058.37	20.31		
		90minC	456206.67	10883.40	217667.92	21.77		
120	21.44	120minA	583203.39	13855.12	277102.39	27.71	32.85	4.52
		120minB	730381.29	17299.08	345981.64	34.60		
		120minC	765378.5	18118.02	362360.34	36.24		
150	25.14	150minA	724431.45	17159.86	343197.12	34.32	35.33	2.02
		150minB	717871.39	17006.35	340127.01	34.01		
		150minC	795748.62	18828.68	376573.55	37.66		
180	28.40	180minA		208.16	4163.20	0.42	0.42	0
*		180minB		208.16	4163.20	0.42		
		180minC		208.16	4163.20	0.42		
210	31.31	210minA	803932.06	19020.17	380403.40	38.04	37.99	5.78
		210minB	678659.98	16088.80	321776.07	32.18		
		210minC	925661.9	21868.65	437372.97	43.74		

*the converter was left for a meeting and the last sample wasn't taken until a few hours later

Appendix O

Raw Calculations for the Production of Acrylamide using *R. rhodochrous* DAP 96622 Whole Cells at Room Temperature while adding in 135mL total of Acrylonitrile at 0.75mL/min. (Whole Cells #9)

Minutes	ACN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	6.69	30minA	41451.23	1178.12	23562.38	2.36	2.40	0.06
		30minB	43156.28	1218.02	24360.34	2.44		
		30minC	45694.75	1277.42	25548.34	2.55		
60	12.36	60minA	83571.04	2163.72	43274.45	4.33	4.37	0.05
		60minB	85197.8	2201.79	44035.77	4.40		
		60minC	88687.52	2283.45	45668.96	4.57		
90	17.22	90minA	91005.73	2337.69	46753.88	4.68	5.22	0.53
		90minB	113743.58	2869.76	57395.20	5.74		
		90minC	103054.33	2619.63	52392.63	5.24		
120	21.44	120minA	104799.89	2660.48	53209.55	5.32	5.43	0.28
		120minB	102765.28	2612.87	52257.35	5.23		
		120minC	114009.23	2875.98	57519.52	5.75		
150	25.14	150minA	96854.4	2474.55	49491.06	4.95	5.70	0.65
		150minB	120613.56	3030.52	60610.35	6.06		
		150minC	121506.58	3051.41	61028.28	6.10		
180	28.40	180minA	108133.16	2738.48	54769.52	5.48	5.85	0.33
		180minB	118731.23	2986.47	59729.42	5.97		
		180minC	121707.5	3056.12	61122.31	6.11		
210 exp stopped after 180mins due to bilayer formation								

Appendix P

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253 Cells Grown via Plates. (PAM Cells #1)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	.0207(X)+246.13	Dilution	%AMD	Avg %	Std. Dev.
30	3.09	30minA	2654.07	301.07	3010.69	0.3	0.29	0.01
		30minB	2054.74	288.66	2886.63	0.29		
		30minC	2032.65	288.21	2882.06	0.29		
60	5.94	60minA	3498.34	318.55	3185.46	0.32	0.33	0.01
		60minB	4241.00	333.92	3339.19	0.33		
		60minC	3751.36	323.78	3237.83	0.32		
90	8.60	90minA	4980.01	349.22	3492.16	0.35	0.36	0.02
		90minB	5041.35	350.49	3504.86	0.35		
		90minC	6520.46	381.10	3811.04	0.38		
120	11.08	120minA	7012.61	391.29	3912.91	0.39	0.38	0.01
		120minB	6297.53	376.49	3764.89	0.38		
		120minC	6258.28	375.68	3756.76	0.38		
150	13.39	150minA	8581.72	423.77	4237.72	0.42	0.42	0.01
		150minB	8643.35	425.05	4250.47	0.43		
		150minC	7680.67	405.12	4051.20	0.41		
180	15.55	180minA	10197.61	457.22	4572.21	0.46	0.46	0.02
		180minB	9193.79	436.44	4364.41	0.44		
		180minC	11298.36	480.01	4800.06	0.48		
210	17.58	210minA	10052.18	454.21	4542.10	0.45	0.5	0.04
		210minB	13715.53	530.04	5300.41	0.53		
		210minC	12546.12	505.83	5058.35	0.51		
240	19.49	240minA	13359.25	522.67	5226.66	0.52	0.51	0.02
		240minB	11895.43	492.37	4923.65	0.49		
		240minC	13583.93	527.32	5273.17	0.53		
270	21.28	270minA	13863.41	533.10	5331.03	0.53	0.56	0.03
		270minB	14530.00	546.90	5469.01	0.55		
		270minC	16504.18	587.77	5877.67	0.59		
300	22.98	300minA	14423.76	544.70	5447.02	0.54	0.55	0.03
		300minB	16554.15	588.80	5888.01	0.59		
		300minC	13766.47	531.10	5310.96	0.53		
330	24.58	330minA	17321.69	604.69	6046.89	0.6	0.63	0.03
		330minB	19945.85	659.01	6590.09	0.66		
		330minC	18211.10	623.10	6231.00	0.62		

Appendix Q

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253 Cells Grown via Fermentation. (PAM Cells #2)

Minutes	AN (%w/v)	AMD (%w/v)
30	3.53	2.82
60	6.77	5.77
90	9.75	11.03
120	12.50	10.98
150	15.04	16.18
180	17.40	14.86
210	19.60	13.51
240	21.65	16.57
270	23.57	16.88

Appendix R

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253. (PAM Cells #3)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	.0207(X)+246.13	Dilution	%AMD	Avg %	Std. Dev.
30	3.53	30minA	82574.93	1955.43	19554.31	1.96	2.27	0.45
		30minB	113263.36	2590.68	25906.82	2.59		
		30minC	109777.86	2518.53	25185.32	2.52		
60	6.77	60minA	245162.58	5321.00	53209.95	5.32	5.65	0.46
		60minB	276648.74	5972.76	59727.59	5.97		
		60minC	299481.63	6445.40	64454.00	6.45		
90	9.75	90minA	375630.79	8021.69	80216.87	8.02	8.79	0.81
		90minB	408603.87	8704.23	87042.30	8.70		
		90minC	453616.74	9636.00	96359.97	9.64		
120	12.50	120minA	470020.39	9975.55	99755.52	9.98	10.01	0.94
		120minB	517716.48	10962.86	109628.61	10.96		
		120minC	427321.60	9091.69	90916.87	9.09		
150	15.04	150minA	743677.68	15640.26	156402.58	15.64	14.67	1.39
		150minB	726859.46	15292.12	152921.21	15.29		
		150minC	619472.83	13069.22	130692.18	13.07		
180	17.40	180minA	664563.11	14002.59	140025.86	14.00	13.71	0.26
		180minB	646298.83	13624.52	136245.16	13.62		
		180minC	640846.27	13511.65	135116.48	13.51		
210	19.60	210minA	809906.06	17011.19	170111.85	17.01	16.46	0.80
		210minB	738718.97	15537.61	155376.13	15.54		
		210minC	801445.85	16836.06	168360.59	16.84		
240	21.65	240minA	726137.48	15277.18	152771.76	15.28	17.38	2.47
		240minB	797984.36	16764.41	167644.06	16.76		
		240minC	959071.74	20098.92	200989.15	20.10		
270	23.57	270minA	735258.98	15465.99	154659.91	15.47	16.95	1.86
		270minB	777352.55	16337.33	163373.28	16.34		
		270minC	908010.69	19041.95	190419.51	19.04		

Appendix S

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253. (PAM Cells #4)

Minutes	Actual Minutes*	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5	0.61	30minA	6492.07	360.07	3600.74	0.36	0.36	0
			30minB	6714.49	365.28	3652.79	0.37		
			30minC	6012.49	348.85	3488.52	0.35		
60	35	4.09	60minA	136944.95	3412.67	34126.72	3.41	3.53	0.16
			60minB	146852.9	3644.52	36445.18	3.64		
			60minC	127545.34	3192.72	31927.21	3.19		
90	70	7.79	90minA	341508.26	8199.45	81994.53	8.2	8.32	0.24
			90minB	340395.03	8173.40	81734.04	8.17		
			90minC	358465.95	8596.26	85962.63	8.6		
100	80	8.78	100minA	450695.66	10754.44	107544.38	10.75	9.88	1.11
			100minB	429761.58	10264.58	102645.81	10.26		
			100minC	360082.35	8634.09	86340.87	8.63		
120	110	11.60	120minA	554072.56	13173.46	131734.58	13.17	11.1	1.94
			120minB	389789.38	9329.23	93292.31	9.33		
			120minC	452023.53	10785.51	107855.11	10.79		
150	140	14.21	150minA	646220.42	15329.72	153297.18	15.33	15.52	0.76
			150minB	626655.49	14871.90	148718.98	14.87		
			150minC	690121.47	16357.00	163570.02	16.36		
180	170	16.63	180minA	739649.41	17515.96	175159.56	17.52	16.92	0.79
			180minB	726636.76	17211.46	172114.60	17.21		
			180minC	675688	16019.26	160192.59	16.02		
210	200	18.88	210minA	982507.06	23198.83	231988.25	23.2	21.08	2.11
			210minB	801976.7	18974.41	189744.15	18.97		
			210minC	891413.33	21067.23	210672.32	21.07		
240	230	20.98	240minA	982364.7	23195.49	231954.94	23.2	23.1	0.09
			240minB	974459.91	23010.52	230105.22	23.01		
			240minC	977888.85	23090.76	230907.59	23.09		
270	260	22.94	270minA	983427.94	23220.37	232203.74	23.22	23.18	0.03
			270minB	981490.69	23175.04	231750.42	23.18		
			270minC	980612.07	23154.48	231544.82	23.15		
300	290	24.78	300minA	980119.57	23142.96	231429.58	23.14	23.15	0.01
			300minB	980693.16	23156.38	231563.80	23.16		
			300minC	980701.23	23156.57	231565.69	23.16		
330	290	24.78	330minA	980381.9	23149.10	231490.96	23.15	23.13	0.01
			330minB	979358.45	23125.15	231251.48	23.13		
			330minC	979240.88	23122.40	231223.97	23.12		

*problems with the syringe pump turning off during the run were accounted for here

Appendix T

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253. (PAM Cells #5, 1st Run)

Minutes	Acrylonitrile Added (%w/v)	Sample*	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5.44	30minA	49706.02	1371.28	27425.62	2.74	2.58	0.24
		30minB	42548.62	1203.80	24075.95	2.41		
		30minC	42767.94	1208.93	24178.60	2.42		
60	10.20	60minA	199874.19	4885.22	97704.32	9.77	9.24	0.75
		60minB	177313.56	4357.30	87145.95	8.71		
		60minC	194202.14	4752.49	95049.80	9.50		
90	14.39	90minA	214430.26	5225.83	104516.56	10.45	11.41	0.87
		90minB	239953.09	5823.06	116461.25	11.65		
		90minC	250640.76	6073.15	121463.08	12.15		
120	18.11	120minA	305170.30	7349.15	146982.90	14.70	15.38	3.19
		120minB	393986.84	9427.45	188549.04	18.85		
		120minC	260010.60	6292.41	125848.16	12.58		
150	21.44	150minA	415379.02	9928.03	198560.58	19.86	20.41	0.61
		150minB	441084.06	10529.53	210590.54	21.06		
		150minC	425286.23	10159.86	203197.16	20.32		
180	24.44	180minA	502429.84	11965.02	239300.37	23.93	22.33	2.34
		180minB	410965.11	9824.74	196494.87	19.65		
		180minC	491303.38	11704.66	234093.18	23.41		
210	27.14	210minA	394941.38	9449.79	188995.77	18.90	20.23	1.15
		210minB	437900.87	10455.04	209100.81	20.91		
		210minC	436947.26	10432.73	208654.52	20.87		
240	29.60	240minA	351649.15	8436.75	168735.00	16.87	18.15	1.33
		240minB	376914.74	9027.96	180559.30	18.06		
		240minC	408274.62	9761.79	195235.72	19.52		
270	31.85	270minA	414017.96	9896.18	197923.61	19.79	20.32	1.38
		270minB	403309.44	9645.60	192912.02	19.29		
		270minC	458810.84	10944.33	218886.67	21.89		
300	33.91	300minA	428895.54	10244.32	204886.31	20.49	19.19	2.96
		300minB*	328739.80	7900.67	158013.43	15.80		
		300minC*	445704.39	10637.64	212752.85	21.28		

*GC had error so vials were not analyzed until 4 days later

**between minutes 270 and 300 no AN was added

Appendix U

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253. (PAM Cells #6)

Minutes	AN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	6.69	30minA	58809.17	1584.29	31685.89	3.17	3.31	0.2
		30minB	64983.44	1728.77	34575.45	3.46		
		30minC	65160.91	1732.93	34658.51	3.47		
60	12.36	60minA	199403.03	4874.19	97483.82	9.75	9.96	0.3
glass syringe popped out and broke		60minB	208370.87	5084.04	101680.77	10.17		
		60minC	197230.47	4823.35	96467.06	9.65		
90	17.22	90minA	269343.38	6510.80	130215.90	13.02	14.1	1.28
		90minB	322651.31	7758.20	155164.01	15.52		
		90minC	285437.11	6887.39	137747.77	13.77		
120	21.44	120minA	333412.76	8010.02	160200.37	16.02	15.89	0.63
		120minB	315811.29	7598.14	151962.88	15.20		
		120minC	342360.07	8219.39	164387.71	16.44		
150	25.14	150minA	323162.38	7770.16	155403.19	15.54	15.54	1.44
		150minB	353796.98	8487.01	169740.19	16.97		
bilayer production occurred		150minC	292420.55	7050.80	141016.02	14.10		
180	28.40	180minA	288162.13	6951.15	139023.08	13.90	14.89	0.97
		180minB	310186.39	7466.52	149330.43	14.93		
		180minC	329442.53	7917.12	158342.30	15.83		
210	31.31	210minA	285174.00	6881.23	137624.63	13.76	14.89	0.98
		210minB	322305.09	7750.10	155001.98	15.50		
		210minC	320553.46	7709.11	154182.22	15.42		

Appendix V

Raw Calculations for the Production of Acrylamide using PAM Immobilized *R. rhodochrous* DAP 96253. (PAM Cells #5, 2nd Run)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30.00	5.44	30minA	10719.87	459.00	18360.20	1.84	1.94	0.15
		30minB	13045.78	513.43	20537.25	2.05		
		30minC	10629.78	456.90	18275.87	1.83		
60.00	10.20	60minA	13605.49	526.53	21061.14	2.11	2.25	0.20
		60minB	16578.16	596.09	23843.56	2.38		
		60minC	13499.44	524.05	20961.88	2.10		
90.00	14.39	90minA	17241.65	611.61	24464.58	2.45	2.35	0.17
		90minB	14039.42	536.68	21467.30	2.15		
		90minC	17251.11	611.84	24473.44	2.45		
120.00	18.11	120minA	18848.56	649.22	25968.65	2.60	2.46	0.20
		120minB	18420.29	639.19	25567.79	2.56		
		120minC	15021.73	559.67	22386.74	2.24		
150.00	21.44	150minA	18661.59	644.84	25793.65	2.58	2.47	0.19
*		150minB	15171.95	563.18	22527.35	2.25		
		150minC	18604.77	643.51	25740.46	2.57		

*run stopped after 150mins due to bilayer formation layer

Appendix W

Raw Calculations for the Production of Acrylamide using PEI-Glu Immobilized *R. rhodochrous* DAP 96253. (PEI-Glu Cells #1)

Minutes	ACN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5.35	30minA	57294.23	1548.84	30976.90	3.10	3.41	0.44
		30minB	70546.33	1858.94	37178.88	3.72		
		30minC	62787.82	1677.39	33547.90	3.35		
60	9.88	60minA	192083.17	4702.91	94058.12	9.41	9.24	0.24
		60minB	184947.45	4535.93	90718.61	9.07		
		60minC	192488.66	4712.39	94247.89	9.42		
90	13.78	90minA	275991.11	6666.35	133327.04	13.33	13.8	0.4
		90minB	290106.38	6996.65	139932.99	13.99		
		90minC	291596.64	7031.52	140630.43	14.06		
120	17.15	120minA	315844	7598.91	151978.19	15.20	14.86	1.21
		120minB	330211.29	7935.10	158702.08	15.87		
		120minC	280104.35	6762.60	135252.04	13.53		
150	20.11	150minA	303194.12	7302.90	146058.05	14.61	13.88	1.25
		150minB	256737.91	6215.83	124316.54	12.43		
		150minC	303054.64	7299.64	145992.77	14.60		
180	22.72	180minA	358856.17	8605.39	172107.89	17.21	16.15	0.92
		180minB	323789.4	7784.83	155696.64	15.57		
		180minC	325654.96	7828.49	156569.72	15.66		

Appendix X

Raw Calculations for the Production of Acrylamide using PEI-Glu Immobilized *R. rhodochrous* DAP 96253. (PEI-Glu Cells #2)

Minutes	AN Added (%w/v)	Sample	Peak Area	Peak 1	Peak 2	Peak 3	LR Equation	Dilution	%AMD	Avg %	Std. Dev.
30	5.32	30minA	82134	82134.00			17.46	27938.43	2.79	2.78	0.01
		30minB	76587	76587.00			17.33	27722.68	2.77		
		30minC	78798	78798.00			17.38	27808.67	2.78		
60	9.79	60minA	328801	328801.00			23.46	37532.63	3.75	4.24	0.48
		60minB	576080	576080.00			29.47	47150.64	4.72		
		60minC	458254	458254.00			26.60	42567.76	4.26		
90	13.58	90minA	1326202	1298644.00	27558.00		47.70	76326.92	7.63	8.02	0.64
		90minB	1336815	1336815.00			47.96	76739.71	7.67		
		90minC	1615259	1615259.00			54.73	87569.89	8.76		
120	16.86	120minA	2277539	2277539.00			70.83	113329.52	11.33	11.65	1.31
		120minB	2072210	1272470.00	799740.00		65.84	105343.17	10.53		
		120minC	2729103	2729103.00			81.81	130893.27	13.09		
150	19.70	150minA	1487947	760658.00	727289.00		51.64	82618.05	8.26	11.05	2.49
		150minB	2717910	1529698.00	1188212.00		81.54	130457.92	13.05		
		150minC	2404674	1595666.00	809008.00		73.92	118274.48	11.83		
180	22.20	180minA	4095427	4095427.00			115.02	184036.95	18.40	16.69	2.41
		180minB	2946011	1772918.00	1173093.00		87.08	139329.99	13.93		
		180minC	3926819	3926819.00			110.92	177478.88	17.75		

Appendix Y

Raw Calculations for the Production of Acrylamide using non-hardened Ca-Alg Immobilized *R. rhodochrous* DAP 96253. (NH-CaAlg #1)

Minutes	ACN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5.35	30minA	151878.40	3762.11	75242.29	7.52	7.81	0.41
		30minB	164234.04	4051.24	81024.73	8.10		
		30minC	147122.86	3650.83	73016.70	7.30		
60	9.88	60minA	286988.35	6923.69	138473.75	13.85	14.14	0.42
		60minB	299627.62	7219.45	144388.93	14.44		
		60minC	241987.92	5870.68	117413.55	11.74		
90	13.78	90minA	409814.08	9797.81	195956.19	19.60	20.57	0.93
		90minB	432259.21	10323.03	206460.51	20.65		
		90minC	449617.28	10729.20	214584.09	21.46		
120	17.15	120minA	600274.05	14254.57	285091.46	28.51	29.54	0.89
		120minB	632449.81	15007.49	300149.71	30.01		
		120minC	633995.08	15043.64	300872.90	30.09		
150	20.11	150minA	742204.19	17575.74	351514.76	35.15	33.47	2.80
		150minB	739228.12	17506.10	350121.96	35.01		
		150minC	637253.45	15119.89	302397.81	30.24		
180	22.72	180minA	824479.09	19500.97	390019.41	39.00	35.68	3.01
		180minB	698759.19	16559.13	331182.50	33.12		
		180minC	737434.22	17464.12	349282.42	34.93		
210	25.05	210minA	687747.21	16301.44	326028.89	32.60	34.85	3.04
		210minB	709954.15	16821.09	336421.74	33.64		
		210minC	809560.27	19151.87	383037.41	38.30		

Appendix Z

Raw Calculations for the Production of Acrylamide using hardened Ca-Alg Immobilized *R. rhodochrous* DAP 96253. (H-CaAlg #1)

Minutes	Acrylonitrile Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	5.35	30minA	128593.03	3217.24	64344.74	6.43	6.20	0.33
		30minB	118752.94	2986.98	59739.58	5.97		
		30minC	122500.62	3074.67	61493.49	6.15		
60	9.88	60minA	279565.24	6749.99	134999.73	13.50	12.13	1.93
		60minB	221140.16	5382.84	107656.79	10.77		
		60minC	297601.54	7172.04	143440.72	14.34		
90	13.78	90minA	369909.18	8864.03	177280.70	17.73	19.05	1.72
		90minB	439513.26	10492.77	209855.41	20.99		
		90minC	384828.58	9213.15	184262.98	18.43		
120	17.15	120minA	584226.66	13879.06	277581.28	27.76	26.21	1.36
		120minB	538391.94	12806.53	256130.63	25.61		
		120minC	530632.82	12624.97	252499.36	25.25		
150	20.11	150minA	747612.01	17702.28	354045.62	35.40	33.21	2.05
		150minB	660570.75	15665.52	313310.31	31.33		
		150minC	694238.70	16453.35	329066.91	32.91		
180	22.72	180minA	751398.89	17790.89	355817.88	35.58	35.47	1.02
		180minB	726068.85	17198.17	343963.42	34.40		
		180minC	769291.57	18209.58	364191.65	36.42		
210	25.05	210minA	821010.14	19419.80	388395.95	38.84	40.56	1.72
		210minB	894436.15	21137.97	422759.32	42.28		
		210minC	857783.32	20280.29	405605.79	40.56		

Appendix AA

Raw Calculations for the Production of Acrylamide using non-hardened Ca-Alg Immobilized *R. rhodochrous* DAP 96253. (NH-CaAlg #1 – 2nd Run)

Minutes	ACN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	6.69	30minA	17273.83	612.37	12247.35	1.22	1.30	0.11
		30minB	20582.91	689.80	13796.00	1.38		
		30minC	24400.96	779.14	15582.85	1.56		
60	12.36	60minA	32786.65	975.37	19507.35	1.95	1.97	0.03
		60minB	33725.22	997.33	19946.60	1.99		
		60minC	40303.21	1151.26	23025.10	2.30		
90	17.22	90minA	40045.07	1145.21	22904.29	2.29	2.29	0.06
		90minB	41220.44	1172.72	23454.37	2.35		
		90minC	38790.61	1115.86	22317.21	2.23		
120	21.44	120minA	54936.65	1493.68	29873.55	2.99	2.92	0.09
		120minB	54344.59	1479.82	29596.47	2.96		
		120minC	51220.38	1406.72	28134.34	2.81		
150	25.14	150minA	61971.37	1658.29	33165.80	3.32	3.25	0.12
		150minB	62154.84	1662.58	33251.67	3.33		
		150minC	57684.15	1557.97	31159.38	3.12		
180	28.40	180minA						
		180minB						
		180minC						
210	31.31	210minA						
		210minB						
		210minC						

*run was stopped after 150 minutes due to bilayer accumulation

Appendix BB

Raw Calculations for the Production of Acrylamide using hardened Ca-Alg Immobilized *R. rhodochrous* DAP 96253. (H-CaAlg #1 – 2nd Run)

Minutes	AN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
30	6.69	30minA	40933.45	1166.00	23320.05	2.33	2.24	0.13
		30minB	37091.83	1076.11	21522.18	2.15		
		30minC	40903.99	1165.31	23306.27	2.33		
60	12.36	60minA	109964.11	2781.32	55626.40	5.56	5.57	0.01
		60minB	110144.14	2785.53	55710.66	5.57		
		60minC	128626.76	3218.03	64360.52	6.44		
90	17.22	90minA	157676.54	3897.79	77955.82	7.80	8.99	1.03
		90minB	196008.12	4794.75	95895.00	9.59		
		90minC	195669.90	4786.84	95736.71	9.57		
120	21.44	120minA	218042.00	5310.34	106206.86	10.62	11.70	1.21
		120minB	236576.26	5744.04	114880.89	11.49		
		120minC	269009.18	6502.97	130059.50	13.01		
150	25.14	150minA	318195.30	7653.93	153078.60	15.31	13.59	1.55
		150minB	253672.07	6144.09	122881.73	12.29		
		150minC	272779.65	6591.20	131824.08	13.18		
180	28.40	180minA	272794.25	6591.55	131830.91	13.18	12.98	0.36
		180minB	273014.91	6596.71	131934.18	13.19		
		180minC	259663.96	6284.30	125685.93	12.57		
210	31.31	210minA	273964.62	6618.93	132378.64	13.24	13.50	0.75
		210minB	267203.27	6460.72	129214.33	12.92		
		210minC	297791.24	7176.48	143529.50	14.35		

Appendix CC

Raw Calculations for the Nitrile Hydratase Activity of Cell Free Lysate from *R. rhodochrous* DAP 96253

Samples				Average OD	Adjested OD (-	PPM	um NH3	tti ACT	Average Activity	Standard Deviation of Population
	0.056	0.056	0.055	0.056	0.005	0.051	3.009568627	150.4784314		
	0.053	0.053	0.053	0.053	0.003	0.020	1.175372549	58.76862745		
1	0.052	0.053	0.056	0.054	0.003	0.028	1.633921569	81.69607843	96.98104575	38.96917332
	0.054	0.054	0.053	0.054	0.003	0.028	1.633921569	81.69607843		
	0.058	0.057	0.058	0.058	0.007	0.075	4.385215686	219.2607843		
2	0.057	0.057	0.057	0.057	0.007	0.067	3.926666667	196.3333333	165.7633987	60.17697652
	0.069	0.07	0.069	0.069	0.019	0.187	11.00517647	550.2588235		
	0.067	0.076	0.068	0.070	0.020	0.199	11.693	584.65		
Whole Cells	0.06	0.06	0.059	0.060	0.009	0.074	4.356215686	217.8107843	450.9065359	165.4204952
	0.069	0.068	0.069	0.069	0.018	0.203	11.95127451	597.5637255		
	0.603	0.583	0.614	0.600	0.550	6.416	377.4148431	18870.74216		
CFL	0.067	0.067	0.064	0.066	0.016	0.172	10.11707843	505.8539216	6658.053268	8635.756292

Appendix DD

Raw Calculations for the Production of Acrylamide using Cell Free Lysate from *R. rhodochrous* DAP 96253 while being Housed in an Ice Bath. (CFL #1)

Minutes	AN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
3	0.58	30minA	493.38	219.71	4394.10	0.44	0.44	0.01
		30minB	699.52	224.53	4490.58	0.45		
		30minC	722.97	225.08	4501.55	0.45		
30	5.32	60minA	2533.44	267.44	5348.85	0.53	0.56	0.02
		60minB	3390.16	287.49	5749.79	0.57		
		60minC	3195.41	282.93	5658.65	0.57		
60	9.79	90minA	3858.47	298.45	5968.96	0.60	0.64	0.04
		90minB	4646.71	316.89	6337.86	0.63		
		90minC	5556.35	338.18	6763.57	0.68		

*bilayer formation and small stringy objects appeared around minute 30

Appendix EE

Raw Calculations for the Production of Acrylamide using Cell Free Lysate from *R. rhodochrous* DAP 96253 while at Room Temperature. (CFL #2)

Minutes	AN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
3	0.77	0minA	13510.78	524.31	10486.25	1.05	1.10	0.07
		0minB	15671.71	574.88	11497.56	1.15		
		0minC	15163.29	562.98	11259.62	1.13		
30	7.09	30minA	113756.77	2870.07	57401.37	5.74	6.27	0.87
		30minB	146569.82	3637.89	72757.88	7.28		
		30minC	115088.66	2901.23	58024.69	5.80		
60	13.05	60minA	251171.13	6085.56	121711.29	12.17	14.45	2.30
		60minB	299193.94	7209.30	144185.96	14.42		
		60minC	349420.18	8384.59	167691.84	16.77		
90	18.11	90minA	450841.52	10757.85	215157.03	21.52	22.79	1.27
		90minB	504914.11	12023.15	240463.00	24.05		
		90minC	478496.51	11404.98	228099.57	22.81		
120	22.47	120minA	412025.81	9849.56	196991.28	19.70	22.27	2.23
		120minB	492375.00	11729.74	234594.70	23.46		
		120minC	496379.15	11823.43	236468.64	23.65		
150	26.27	150minA	496644.20	11829.63	236592.69	23.66	21.92	3.64
		150minB	370272.14	8872.53	177450.56	17.75		
		150minC	511809.14	12184.49	243689.88	24.37		
180	29.60	180minA	385542.52	9229.85	184597.10	18.46	21.82	4.54
		180minB	418858.92	10009.46	200189.17	20.02		
		180minC	567727.80	13492.99	269859.81	26.99		

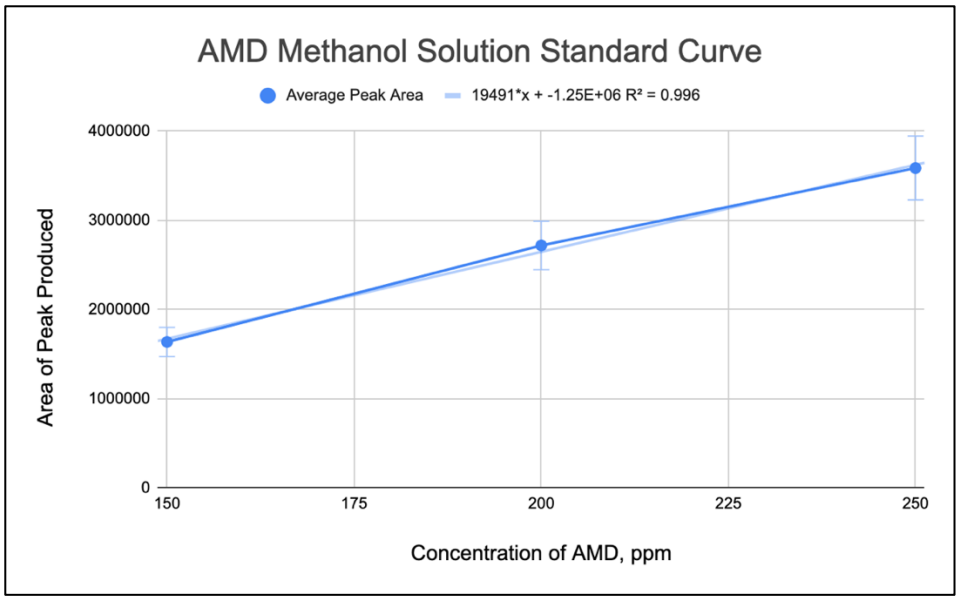
Appendix FF

Raw Calculations for the Production of Acrylamide using Cell Free Lysate from *R. rhodochrous* DAP 96253. (CFL #3)

Minutes	ACN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
3	0.77	3minA	4764.25	319.64	6392.87	0.64	0.70	0.08
		3minB	7285.25	378.63	7572.70	0.76		
		3minC	7887.23	392.72	7854.42	0.79		
30	7.09	30minA	58605.40	1579.53	31590.53	3.16	4.06	0.84
		30minB	81132.47	2106.66	42133.20	4.21		
		30minC	94002.72	2407.82	48156.47	4.82		
60	13.05	60minA	230031.34	5590.89	111817.87	11.18	10.99	1.58
		60minB	190244.38	4659.88	93197.57	9.32		
		60minC	257465.40	6232.85	124657.01	12.47		
90	18.11	90minA	483880.85	11530.97	230619.44	23.06	21.59	1.52
		90minB	419107.98	10015.29	200305.73	20.03		
		90minC	454064.77	10833.28	216665.51	21.67		
120	22.47	120minA	516743.44	12299.96	245999.13	24.60	25.07	0.48
		120minB	537295.42	12780.87	255617.46	25.56		
		120minC	526156.57	12520.22	250404.47	25.04		
150	26.27	150minA	688573.66	16320.78	326415.67	32.64	36.08	3.09
		150minB	781390.44	18492.70	369853.93	36.99		
		150minC	816175.38	19306.66	386133.28	38.61		
180	29.60	180minA	701825.49	16630.88	332617.53	33.26	11.36	18.96
		180minB		208.16	4163.20	0.42		
		180minC		208.16	4163.20	0.42		

Appendix GG

Acrylamide Standard Curve using GC-MS for 2020 Bioacrylamide Production.



Appendix HH

Raw Calculations for the Nitrile Hydratase Activity of CFL, Flowthrough, and Wash from *R. rhodochrous* DAP 96253 and Sephacel, a weak anion exchange column media.

Sample	OD	OD	OD	average	adjusted OD	ppm	um	um/ min/ml	average	st desv
	0.099	0.099	0.101	0.100	0.045	0.114	6.7	3346.971	8246.024	1017.188
	0.129	0.127	0.129	0.128	0.073	0.315	18.5	9263.212		
B	0.119	0.117	0.120	0.119	0.064	0.246	14.5	7228.836		
	0.068	0.069	0.070	0.069	0.014	-0.004	-0.2	-120.568	4724.275	1797.907
	0.101	0.100	0.101	0.101	0.046	0.222	13.0	6522.183		
A	0.083	0.084	0.084	0.084	0.029	0.099	5.9	2926.368		
	0.092	0.092	0.090	0.091	0.036	0.115	6.8	3394.402	3581.375	1354.688
	0.084	0.085	0.085	0.085	0.030	0.069	4.0	2023.633		
R	0.100	0.101	0.100	0.100	0.046	0.181	10.7	5326.089		
	0.061	0.062	0.062	0.062	0.007	-0.057	-3.4	-1.682	-2	0.896
	0.055	0.056	0.056	0.056	0.001	-0.099	-5.8	-2.924		
C	0.066	0.066	0.066	0.066	0.011	-0.026	-1.5	-0.763		

Key
B = "before" purified sample
A = "after" purified sample
R = flow through buffer
C = "bound DEAE Sephacel"
gray = disregarded outliner

Appendix II

Raw data for the Protein Concentration Evaluation of the Purification Steps for Two Different Size Purification Columns, 1mL vs 5mL

					adj avg	ug	df	avg [protein]	mg/ml
	lysate	0.909	0.908	0.920	0.856	502.719769	16087.0326	15050.18821	15.0501882
		1.507	1.516	1.504	1.453	875.833988	14013.3438		
5mL	Flowthrough	0.492	0.483	0.478	0.428	235.329249	7530.53596	7246.779633	7.24677963
		0.817	0.801	0.794	0.748	435.188957	6963.0233		
	Wash	0.228	0.224	0.224	0.169	73.2509305	2344.02978	2432.575245	2.43257525
		0.369	0.359	0.352	0.304	157.570045	2521.12071		
	0.1M	0.143	0.133	0.136	0.081	18.4523777	295.238044	401.0738915	0.40107389
		0.214	0.210	0.204	0.153	63.3637174	506.909739		
	0.25M	0.202	0.201	0.200	0.145	58.2179819	931.487711	985.2587055	0.98525871
		0.322	0.311	0.314	0.260	129.878712	1039.0297		
	0.5M	0.096	0.097	0.096	0.040	-7.3414283	0	119.1827063	0.11918271
		0.133	0.132	0.130	0.076	14.8978383	119.182706		
	1M	0.068	0.070	0.069	0.013	-24.361352	0.000	0.000	0.000
		0.078	0.079	0.079	0.023	-18.233927	0.000		
1mL	Flowthrough	0.254	0.239	0.241	0.189	85.5742178	2738.37497	2866.63869	2.86663869
		0.411	0.410	0.401	0.351	187.181401	2994.90241		
	Wash	0.312	0.309	0.310	0.254	126.648368	4052.74778	4052.222574	4.05222257
		0.520	0.507	0.513	0.457	253.231086	4051.69737		
	0.1M	0.101	0.103	0.105	0.047	-3.1763921	0	179.7028291	0.17970283
		0.144	0.143	0.145	0.088	22.4628536	179.702829		
	0.25M	0.131	0.129	0.131	0.074	13.9749998	223.599997	319.7153356	0.31971534
		0.193	0.188	0.192	0.135	51.9788343	415.830674		
	0.5M	0.087	0.087	0.090	0.032	-12.350727	0.000	55.03647447	0.05503647
		0.118	0.120	0.119	0.063	6.87955931	55.0364745		
	1M	0.080	0.083	0.083	0.026	-16.130999	0.000	0.000	0.000

Appendix JJ

Raw Calculations for the Production of Acrylamide using Purified Nitrile Hydratase from *R. rhodochrous* DAP 96253. (PU #1)

Minutes	ACN Added (%w/v)	Sample	Area	0.00005648(X) + 81.7	Dilution	%AMD	Avg %	Std. Dev.
1	0.19	0minA		81.70	1634.00	0.16	0.16	0.00
		0minB		81.70	1634.00	0.16		
		0minC		81.70	1634.00	0.16		
30	5.44	30minA	6366205.00	441.26	220631.63	22.06	4.09	10.38
		30minB	783.00	81.74	40872.11	4.09		
		30minC	783.00	81.74	40872.11	4.09		
60	10.20	60minA	251171.13	95.89	47943.07	4.79	4.93	0.14
		60minB	299193.94	98.60	49299.24	4.93		
		60minC	349420.18	101.44	50717.63	5.07		
90	14.39	90minA	450841.52	107.16	53581.76	5.36	5.44	0.08
		90minB	504914.11	110.22	55108.77	5.51		
		90minC	478496.51	108.73	54362.74	5.44		
120	18.11	120minA	412025.81	104.97	104971.22	10.50	10.81	0.27
		120minB	492375.00	109.51	109509.34	10.95		
		120minC	496379.15	109.74	109735.49	10.97		
150	21.44	150minA	16228.37	82.62	82616.58	8.26	8.26	0.00
		150minB	15829.11	82.59	82594.03	8.26		
		150minC	16473.54	82.63	82630.43	8.26		
180	24.44	180minA	165542.52	91.05	91049.84	9.10	9.17	0.07
		180minB	188858.92	92.37	92366.75	9.24		
		180minC	174837.80	91.57	91574.84	9.16		

*sample 30A was removed due to outlier data

Appendix KK

Raw Calculations for the Production of Acrylamide using Purified Nitrile Hydratase from *R. rhodochrous* DAP 96253. (PU #2)

Minutes	ACN Added (%w/v)	Sample	Area	0.00005648(X) + 81.7	Dilution	%AMD	Avg %	Std. Dev.
30	5.44	30minA	6366205.00	441.26	4412.63	0.44	2.08	0.98
		30minB	31101800.00	1838.33	18383.30	1.84		
		30minC	39775473.00	2328.22	23282.19	2.33		
60	10.20	60minA	77855960.00	4479.00	44790.05	4.48	3.42	0.92
		60minB	51605655.00	2996.39	29963.87	3.00		
		60minC	47950578.00	2789.95	27899.49	2.79		
90	14.39	90minA	179131843.00	10199.07	50995.33	5.10	5.48	1.76
		90minB	260414187.00	14789.89	73949.47	7.39		
		90minC	137794696.00	7864.34	39321.72	3.93		
120	18.11	120minA	319115796.00	18105.36	90526.80	9.05	8.67	1.00
		120minB	265216757.00	15061.14	75305.71	7.53		
		120minC	332391503.00	18855.17	94275.86	9.43		
150	21.44	150minA	442978195.00	25101.11	125505.54	12.55	12.09	1.31
		150minB	374390414.00	21227.27	106136.35	10.61		
		150minC	463009631.00	26232.48	131162.42	13.12		
180	24.44	180minA	445397449.00	25237.75	126188.74	12.62	10.65	4.64
		180minB	305919639.00	17360.04	86800.21	8.68		
		180minC	633039144.00	35835.75	179178.75	17.92		

*sample 30A and 180C was removed due to outlier data

Appendix LL

Raw Calculations for the Production of Acrylamide using Hardened Calcium Alginate Purified Nitrile Hydratase from *R. rhodochrous* DAP 96253. (H-CaAlg-PU)

Minutes	ACN Added (%w/v)	Sample	Area	0.00005648(X) + 81.7	Dilution	%AMD	Avg %	Std. Dev.
60	10.20	60minA	50028.00	84.53	16905.12	1.69	1.88	0.16
		60minB	319949.00	99.77	19954.14	2.00		
		60minC	273565.00	97.15	19430.19	1.94		
90	14.39	90minA	1791470.00	182.88	36576.45	3.66	3.56	0.10
		90minB	1609630.00	172.61	34522.38	3.45		
		90minC	1714695.00	178.55	35709.19	3.57		
120	18.11	120minA	5274386.00	379.60	75919.46	7.59	7.72	0.17
		120minB	5333358.00	382.93	76585.61	7.66		
		120minC	5563448.00	395.92	79184.71	7.92		
150	21.44	150minA	5299140.00	381.00	76199.09	7.62	8.14	0.46
		150minB	5953283.00	417.94	83588.28	8.36		
		150minC	6035548.00	422.59	84517.55	8.45		
180	24.44	180minA	1589572.00	171.48	34295.81	3.43	5.80	3.01
		180minB	2803289.00	240.03	48005.95	4.80		
		180minC	6683840.00	459.20	91840.66	9.18		

Appendix MM

Raw Calculations for the Production of Acrylamide using Non-hardened Calcium Alginate Purified Nitrile Hydratase from *R. rhodochrous* DAP 96253. (NH-CaAlg-PU)

Minutes	ACN Added (%w/v)	Sample	Area	0.00005648(X) + 81.7	Dilution	%AMD	Avg %	Std. Dev.
60	10.20	60minA	1530631.00	168.15	16815.00	1.68	2.00	0.30
		60minB	2557672.00	226.16	22615.73	2.26		
		60minC	2212353.00	206.65	20665.37	2.07		
90	14.39	90minA	7222492.00	489.63	48962.63	4.90	4.32	0.50
		90minB	5660947.00	401.43	40143.03	4.01		
		90minC	5733726.00	405.54	40554.08	4.06		
120	18.11	120minA	8366251.00	554.23	55422.59	5.54	5.61	0.10
		120minB	8673237.00	571.56	57156.44	5.72		
		120minC	8397280.00	555.98	55597.84	5.56		
150	21.44	150minA	9993247.00	646.12	64611.86	6.46	6.30	0.27
		150minB	9146847.00	598.31	59831.39	5.98		
		150minC	9969931.00	644.80	64480.17	6.45		
180	24.44	180minA	6254280.00	434.94	43494.17	4.35	5.14	92.37
		180minB	290895467.00	16511.48	1651147.60	165.11		
		180minC	9044604.00	592.54	59253.92	5.93		

Appendix NN

Raw Calculations for the Storage Study of Bioacrylamide produced by hardened Ca-Alg Immobilized Cells of *R. rhodochrous* DAP 96253

Minutes	AN Added (%w/v)	Sample	Height	0.0234(X) + 208.16	Dilution	%AMD	Avg %	Std. Dev.
3/12/2018		40%A	792074.63	18742.70634	374854.1268	37.49	41.60	3.89
		40%B	957346.69	22610.07255	452201.4509	45.22		
		40%C	890490.63	21045.64074	420912.8148	42.09		
5/7/2018		40%A	695709.29	16487.75739	329755.1477	32.98	36.74	3.29
		40%B	825744.47	19530.5806	390611.612	39.06		
		40%C	807072.17	19093.64878	381872.9756	38.19		
5/22/2018		40%A	724206.24	17154.58602	343091.7203	34.31	39.95	5.16
		40%B	940654.43	22219.47366	444389.4732	44.44		
		40%C	869272.17	20549.12878	410982.5756	41.10		
9/13/2018		40%A	946714.91	22361.28889	447225.7779	44.72	45.81	0.95
		40%B	978392.38	23102.54169	462050.8338	46.21		
		40%C	984708.81	23250.34615	465006.9231	46.50		

Appendix OO

Raw Calculations for PEG80 and PEG40 Week 4 Asparaginase Activity 4ºC Storage Study and Data Summary for Week 1-Week 4

	Initial Enzyme Activity	Day 7	Week 2	Week 3	Week 4
PEG 80#1	576.14	665.77	546.38	1236.40	1134.58
PEG 80#2	553.15	576.00	511.53	1210.19	1124.11
PEG 80#3	722.65	427.88	414.28	1179.36	859.55
PEG 80#4	1205.93	1044.63	1262.53	1462.17	1181.63
PEG 80#5	1167.91	822.70	489.51	1427.09	1356.99
PEG 80#6	1126.15	760.84	998.99	1298.59	1098.64
PEG 80#7	904.73	847.34	1113.90	1524.78	1132.00
PEG 80#8	779.54	611.69	1525.70	1264.24	1243.69
Control	1916.61	1756.54	1566.17	2614.50	2209.67

	Initial Enzyme Activity	Day 7	Week 2	Week 3	Week 4
PEG 40#1					
PEG 40#2	831.68	651.85	495.45	686.76	785.37
PEG 40#3	875.25	608.37	307.57	808.13	1006.84
PEG 40#4	617.63	602.53	404.19	694.61	833.70
PEG 40#5	881.95	857.39	1076.78	1008.97	
PEG 40#6	1160.89	653.54	1049.80	Run Out	
PEG 40#7	1182.77	862.93	1061.40	1097.76	1009.84
PEG 40#8	1342.80	635.66	1216.91	1062.35	951.71
PEG 40#9	1059.85	752.78	1160.52	1040.08	960.60
PEG 40#10	854.08	523.96	1165.67	999.99	1017.75
PEG 40#11	793.66	705.15	1376.57	1327.39	1089.08
PEG 40#12	891.65	818.36	1278.11	1359.95	1192.51
PEG 40#13	814.67	746.16	1050.40	1364.32	
Control	1916.61	1756.04	1566.17	2616.87	2209.67

Samples				Average OD	Adjested OD (	PPM	um NH3	Wet Weight	Purified Enyzme	Dry Weight		Average Activity	Standard Deviation of Population
	0.102	0.105	0.102	0.103	0.058	0.356	20.96835589	0	1048.417795	0			
	0.101	0.101	0.103	0.102	0.057	0.348	20.49392653	0	1024.696327	0			
activity SEC 1	0.113	0.119	0.117	0.116	0.072	0.452	26.61269335	0	1330.634668	0		1134.58293	138.9673583
	0.103	0.107	0.103	0.104	0.060	0.416	24.48183025	0	1224.091512	0			
	0.097	0.097	0.098	0.097	0.052	0.364	21.43738457	0	1071.869229	0			
activity SEC 2	0.096	0.098	0.098	0.097	0.053	0.366	21.52731879	0	1076.365939	0		1124.108894	70.72221793
	0.098	0.096	0.099	0.098	0.053	0.330	19.42098437	0	971.0492183	0			
	0.087	0.086	0.088	0.087	0.042	0.253	14.85725148	0	742.862574	0			
activity SEC 3	0.091	0.096	0.090	0.093	0.048	0.294	17.29469414	0	864.7347069	0		859.5488331	93.22895158
	0.092	0.092	0.090	0.091	0.047	0.381	22.43932889	0	1121.966445	0			
	0.093	0.094	0.088	0.092	0.047	0.383	22.53835164	0	1126.917582	0			
activity SEC 4	0.097	0.105	0.097	0.100	0.055	0.441	25.92033268	0	1296.016634	0		1181.633554	80.90630487
	0.092	0.086	0.087	0.088	0.044	0.360	21.17160321	0	1058.58016	0			
	0.115	0.099	0.100	0.105	0.060	0.475	27.96171523	0	1398.085762	0			
activity SEC 5	0.113	0.116	0.115	0.115	0.070	0.549	32.28606379	0	1614.30319	0		1356.989704	228.7264534
	0.097	0.096	0.096	0.096	0.052	0.417	24.50261354	0	1225.130677	0			
	0.083	0.088	0.085	0.085	0.041	0.338	19.87643184	0	993.8215919	0			
activity SEC 6	0.089	0.089	0.090	0.089	0.045	0.366	21.53944393	0	1076.972197	0		1098.641489	95.66658023
	0.090	0.091	0.090	0.090	0.045	0.371	21.83016252	0	1091.508126	0			
	0.091	0.092	0.090	0.091	0.046	0.377	22.20020259	0	1110.010129	0			
activity SEC 7	0.094	0.095	0.095	0.095	0.050	0.406	23.88941459	0	1194.470729	0		1131.996328	44.8171777
	0.103	0.102	0.102	0.102	0.058	0.459	27.0195622	0	1350.97811	0			
	0.099	0.097	0.097	0.098	0.053	0.427	25.10578415	0	1255.289208	0			
activity SEC 8	0.091	0.090	0.094	0.092	0.047	0.382	22.4960751	0	1124.803755	0		1243.690357	92.6988303

Samples				Average OD	Adjested OD (	PPM	um NH3	Wet Weight	Purified Enyzme	Dry Weight		Average Activity	Standard Deviation of Population
	0.084	0.081	0.085	0.083	0.038	0.237	13.92712772	0	696.3563861	0			
	0.087	0.086	0.088	0.087	0.042	0.265	15.58332999	0	779.1664995	0			
PEG 40 SEC 2	0.091	0.094	0.092	0.092	0.047	0.299	17.61146129	0	880.5730644	0		785.3653167	75.33376909
	0.089	0.092	0.085	0.089	0.044	0.299	17.60428643	0	880.2143214	0			
	0.090	0.095	0.096	0.094	0.049	0.336	19.75605333	0	987.8026667	0			
PEG 40 SEC 3	0.101	0.101	0.103	0.102	0.057	0.392	23.04980771	0	1152.490386	0		1006.835791	111.9680272
	0.088	0.093	0.090	0.090	0.045	0.307	18.03979436	0	901.9897182	0			
	0.084	0.087	0.083	0.085	0.040	0.267	15.70281973	0	785.1409864	0			
PEG 40 SEC 4	0.086	0.085	0.088	0.086	0.041	0.277	16.27944891	0	813.9724457	0		833.7010501	49.70124014
				0.087	0.042	#DIV/0!	#DIV/0!	0	#DIV/0!	0			
				0.085	0.040	#DIV/0!	#DIV/0!	0	#DIV/0!	0			
PEG 40 SEC 5				0.087	0.042	#DIV/0!	#DIV/0!	0	#DIV/0!	0		#DIV/0!	#DIV/0!
				0.083	0.038	#DIV/0!	#DIV/0!	0	#DIV/0!	0			
				0.083	0.038	#DIV/0!	#DIV/0!	0	#DIV/0!	0			
PEG 40 SEC 6				0.084	0.039	#DIV/0!	#DIV/0!	0	#DIV/0!	0		#DIV/0!	#DIV/0!
	0.085	0.089	0.087	0.087	0.042	0.350	20.56961829	0	1028.480915	0			
	0.083	0.083	0.089	0.085	0.040	0.334	19.63368385	0	981.6841924	0			
PEG 40 SEC 7	0.084	0.084	0.091	0.087	0.042	0.347	20.38729724	0	1019.364862	0		1009.843323	20.2563254
	0.081	0.083	0.084	0.083	0.038	0.319	18.77517798	0	938.7588989	0			
	0.082	0.083	0.084	0.083	0.038	0.322	18.94721115	0	947.3605574	0			
PEG 40 SEC 8	0.083	0.084	0.085	0.084	0.039	0.329	19.38026184	0	969.0130921	0		951.7108494	12.72852001
	0.077	0.080	0.081	0.079	0.034	0.293	17.22037098	0	861.0185489	0			
	0.081	0.081	0.084	0.082	0.037	0.315	18.53463264	0	926.7316322	0			
PEG 40 SEC 9	0.091	0.090	0.090	0.090	0.045	0.372	21.88071522	0	1094.035761	0		960.5953141	98.0962691
	0.088	0.089	0.088	0.088	0.044	0.360	21.15183829	0	1057.591915	0			
	0.081	0.082	0.080	0.081	0.036	0.305	17.92362612	0	896.1813059	0			
PEG 40 SEC 10	0.092	0.089	0.090	0.090	0.046	0.374	21.98937347	0	1099.468674	0		1017.747298	87.64372416
	0.095	0.090	0.089	0.091	0.046	0.379	22.26685236	0	1113.342618	0			
	0.089	0.094	0.089	0.091	0.046	0.377	22.16230676	0	1108.115338	0			
PEG 40 SEC 11	0.086	0.090	0.088	0.088	0.043	0.356	20.91593312	0	1045.796656	0		1089.084871	30.68369015
	0.101	0.103	0.106	0.104	0.059	0.468	27.51083966	0	1375.541983	0			
	0.094	0.092	0.093	0.093	0.048	0.392	23.07727988	0	1153.863994	0			
PEG 40 SEC 12	0.086	0.090	0.087	0.088	0.043	0.356	20.96244757	0	1048.122378	0		1192.509452	136.4331411
				#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	#DIV/0!	0			
				#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	#DIV/0!	0			
PEG 40 SEC 13				#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	#DIV/0!	0		#DIV/0!	#DIV/0!

Appendix PP

Raw Calculations for Day 12 of Various PEG40 Fractions Asparaginase Activity 37ºC Storage Study and Data Summary from over 12 Days

Samples				Average OD	Adjested OD (-0.0	PPM	um NH3	Wet Weight	Purified Enyzme	Dry Weight		Average Activity	Standard Deviation of Population
	0.135	0.145	0.140	0.140	0.090	0.601	35.34261688	0	1767.130844	0			
	0.145	0.139	0.152	0.146	0.096	0.641	37.72579436	0	1886.339718	0			
Pure 4	0.150	0.132	0.149	0.144	0.094	0.628	36.92506647	0	1846.253324	0		1833.241295	0
	0.086	0.086	0.087	0.087	0.037	0.247	14.50987386	0	725.4936931	0			
	0.087	0.091	0.089	0.089	0.039	0.262	15.38876483	0	769.4382413	0			
Pure 5	0.081	0.081	0.081	0.081	0.031	0.208	12.22261318	0	611.1306588	0		702.0208644	0
Samples				Average OD	Adjested OD (-0.0	PPM	um NH3	Wet Weight	Purified Enyzme	Dry Weight		Average Activity	Standard Deviation of Population
	0.080	0.079	0.080	0.080	0.030	0.202	11.85615724	0	592.8078622	0			
	0.081	0.078	0.081	0.080	0.030	0.204	11.99191599	0	599.5957995	0			
S1 #4	0.080	0.079	0.082	0.080	0.031	0.206	12.11782416	0	605.8912081	0		599.4316232	0
	0.083	0.080	0.081	0.081	0.031	0.213	12.5106998	0	625.5349898	0			
	0.077	0.077	0.077	0.077	0.027	0.185	10.85951476	0	542.9757382	0			
S1 #5	0.080	0.080	0.078	0.079	0.030	0.200	11.78788246	0	589.394123	0		585.9682836	0
Samples				Average OD	Adjested OD (-0.0	PPM	um NH3	Wet Weight	Purified Enyzme	Dry Weight		Average Activity	Standard Deviation of Population
	0.076	0.076	0.075	0.076	0.026	0.175	10.27462259	0	513.7311297	0			
	0.075	0.075	0.074	0.074	0.025	0.167	9.823516663	0	491.1758332	0			
S2 4	0.079	0.077	0.076	0.078	0.028	0.188	11.03263728	0	551.6318642	0		518.8462757	0
	0.075	0.072	0.072	0.073	0.023	0.156	9.165138119	0	458.256906	0			
	0.075	0.075	0.075	0.075	0.025	0.170	9.973466694	0	498.6733347	0			
S2 5	0.078	0.076	0.076	0.076	0.027	0.180	10.60127494	0	530.0637468	0		495.6646625	0
	0.073	0.076	0.074	0.074	0.024	0.165	9.716077958	0	485.8038979	0			
	0.086	0.085	0.086	0.085	0.035	0.238	14.00624334	0	700.3121668	0			
S2 6	0.071	0.073	0.077	0.074	0.024	0.160	9.4328691	0	471.643455	0		552.5865066	0
	0.079	0.079	0.081	0.080	0.030	0.203	11.96294779	0	598.1473894	0			
	0.065	0.065	0.065	0.065	0.015	0.103	6.069958312	0	303.4979156	0			
S2 7	0.076	0.076	0.077	0.077	0.027	0.182	10.67697075	0	533.8485373	0		478.4979475	0
Samples				Average OD	Adjested OD (-0.0	PPM	um NH3	Wet Weight	Purified Enyzme	Dry Weight		Average Activity	Standard Deviation of Population
	0.078	0.076	0.078	0.077	0.028	0.186	10.92112125	0	546.0560626	0			
	0.073	0.073	0.072	0.073	0.023	0.156	9.192567989	0	459.6283994	0			
S3 4	0.072	0.072	0.073	0.072	0.022	0.151	8.869517521	0	443.475876	0		483.053446	0
	0.080	0.078	0.083	0.080	0.030	0.205	12.06331134	0	603.1655672	0			
	0.067	0.064	0.062	0.064	0.015	0.100	5.871340958	0	293.5670479	0			
S3 5	0.090	0.090	0.091	0.090	0.041	0.273	16.03671957	0	801.8359786	0		566.1895312	0
	0.076	0.073	0.071	0.073	0.024	0.159	9.356922729	0	467.8461365	0			
	0.078	0.080	0.079	0.079	0.029	0.195	11.49204693	0	574.6023464	0			
S3 6	0.075	0.075	0.072	0.074	0.024	0.164	9.652280794	0	482.6140397	0		508.3541742	0

	Initial Enzyme	Day 1	Day 2			Initial Enzy	Day 12
Pure - F4	1632.97621	1079.711	1105.717		Pure - F4	1632.976	767.9994
Pure - F5	1554.44077	1133.911	1085.897		Pure - F5	1554.441	774.1117
S1 - F4	1475.03362	1089.027	1170.253		S1 - F4	1475.034	569.0344
S1 - F5	1479.22854	1135.904	1200.309		S1 - F5	1479.229	530.862
S2 - F4	1690.9732	983.3543	1392.475		S2 - F4	1690.973	478.2778
S2 - F5	1648.81271	1019.523	1060.699		S2 - F5	1648.813	412.1734
S2 - F6	1743.94155	1007.015	966.7137		S2 - F6	1743.942	335.2955
S2 - F7	1322.17031	1206.984	929.7465		S2 - F7	1322.17	310.2593
S3 - F4	1337.92161	958.2422	910.6341		S3 - F4	1337.922	514.4158
S3 - F5	1292.31838	957.0508	804.5918		S3 - F5	1292.318	691.804
S3 - F6	1348.01059	1134.225	758.805		S3 - F6	1348.011	648.2933