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Preliminary Report on Protein Preparations Exhibiting Both Asparaginase and Glutaminase Activity Purified from Rhodococcus Rhodochrous DAP 96253

Nosazena Amadasun

PRELIMINARY REPORT ON PROTEIN PREPARATIONS EXHIBITING BOTH ASPARAGINASE AND GLUTAMINASE ACTIVITY PURIFIED FROM *RHODOCOCCUS RHODOCHROUS D*AP 96253

by

NOSAZENA AMADASUN

Under the Direction of George E Pierce, Ph.D.

ABSTRACT

Bacterial L-Asparaginase has been utilized along with chemotherapy in the treatment of acute lymphoblastic leukemia. Current forms of treatment are fraught with undesirable outcomes in a significant number of cases. This has led to a continued investigation of other bacterial sources of L-asparaginase.

Rhodococcus rhodochrous strain DAP 96253 when induced on specialized media was found to present L-asparaginase and L-glutaminase activity. A purification scheme was developed evaluating the effect of the method of cell lysis, the use of a reducing agent and

dialysis on the final product. A protein preparation was purified from the crude extract employing dialysis, anion exchange and size exclusion chromatography using a Hitrap®Q HP and a Hiprep^{TM} 16/60 Sephacryl^{TM} S500HR column respectively. The purified protein exhibited 1492 and 2173 IU/mg of L-asparaginase and L-glutaminase respectively.

Further kinetic studies of the purified preparation demonstrated an optimum reaction pH at 7.6, maximal enzyme activity at 37°C and stability exceeding 60 minutes. The K_m for L-asparagine and L-glutamine were determined to be 13.6µM and 850µM respectively. The protein was determined to be stable in human serum at 37ºC with a $T_{1/2}$ of approximately 53 hours. Endotoxins were 771 times less than an *Escherichia coli* sample, with the endotoxins detected being introduced during the purification process, the three subunits of the protein were estimated to be ~72, 28 and 23kDa versus a homotetrameric protein in *E. coli*, and the protein was determined to be effective in reducing the cell number and viability of Jurkat E6-1 clone and Molt-4 cells exhibiting an IC_{50} of 0.123 and 1.982 IU/mg respectively. This is the first attempt to characterize an L-asparaginase from *R. rhodochrous.*

INDEX WORDS Asparaginase, Glutaminase, Acute lymphoblastic leukemia, *Rhodococcus rhodochrous*, Cancer

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May 2020

DEDICATION

This work is wholeheartedly dedicated to my dear friend Ike O Ike who has served a constant source of inspiration, strength and support.

ACKNOWLEDGEMENTS

This project would not have been possible without the consistent assistance of Dr G. E. Pierce, whose guidance has led to the completion of this dissertation. I would also like to acknowledge the contributions of Dr S. Crow and Dr E. Gilbert for their agreement to serve as members of my dissertation committee and for their continued guidance and assistance. I would also like to acknowledge the contributions of Dr B. Baumstark, Dr C. Robbins, Dr M. Ezeoke, Dr I. Rodriguez-Bussey and the rest of the Bio-Bus program especially for being my personal family at Georgia State University. Very special thanks to Dr K. Cannon for mentoring me and showing me how to be an efficient scientist. I would also like to acknowledge the massive contributions of Etna Sanchez to the completion of this project. I would also like to thank Yong Gyu Lee, Brianna Galbreath Chilton, Merhawi Mihreteab, Mariya Campbell, Dr B. Stubblefield, Dr C. Johnson and members of Dr. Baro's laboratory for their assistance and positive contribution to my time at Georgia State University.

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LIST OF ABBREVIATIONS

ALL……. Acute lymphoblastic leukemia

HSCs……Hematopoietic stem cells

ASNase……. L-Asparaginase

ASN……. L-Asparagine

ASNS……Asparagine synthetase

GLNase…….....L-Glutaminase

Nhase…….Nitrile hydratase

ACC …….....1 aminocyclopropane 1 carboxylic acid

1 HMO……...Monooxygenase and epoxide hydrolase

FBS…….Fetal bovine serum

βME …….....2- mercaptoethanol

CFL…….Cell free lysate

AEX……Anion exchange chromatography

SEC…….....Size exclusion chromatography

PB……Sodium phosphate buffer

RT……Room temperature

1 INTRODUCTION

1.1 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is the most prevalent form of childhood cancer in the United States of America, accounting for 80% of leukemias specifically and 25 to 26% of all cancers observed in children and about 8% in adolescents with peak incidence in individuals between the ages of 2 and 5[9,53,107]. Incidence rates are much lower in adults with ALL accounting for less than 1% of adult cancers[53]. Analysis of ALL occurrence rates in the USA from 2001 to 2014 showed an average occurrence rate of 34.1 per 1 million individuals, with the highest occurrence among Hispanics at 42.9 per million individuals. Caucasians had a slightly lower incidence rate at 34.2 per million individuals and African Americans had the lowest incidence rates at 18.7 per million individuals. Incidence rates are also slightly higher in male children when compared to females (~1.28 times higher in males)[95,7].

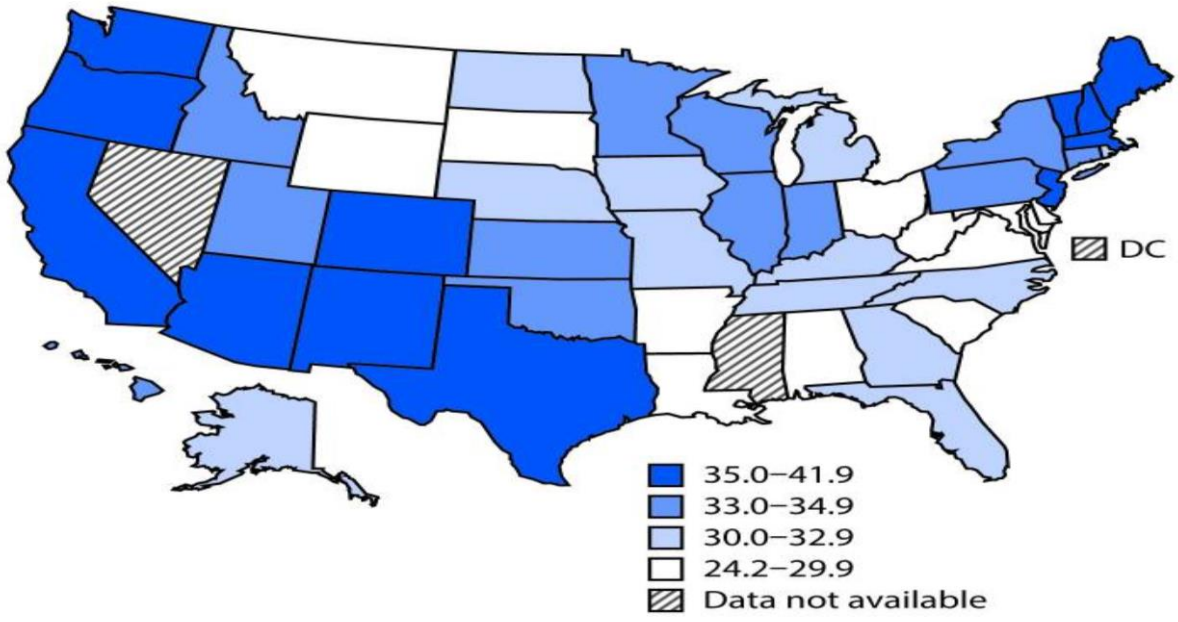
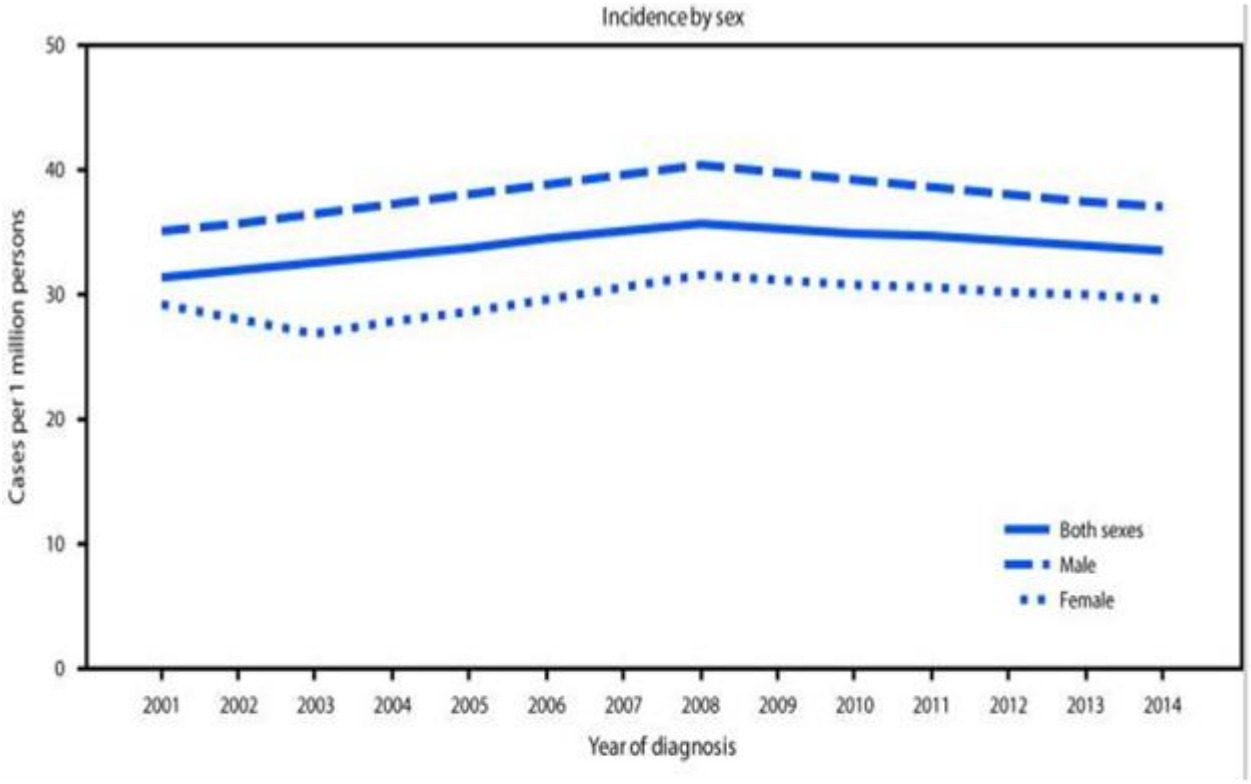


Figure 1: Annual age adjusted rates of ALL among children by State National Program of Cancer Registries, and Surveillance, Epidemiology and End Results Program, United States, 2001-2014 [Siegel et al., 2017].



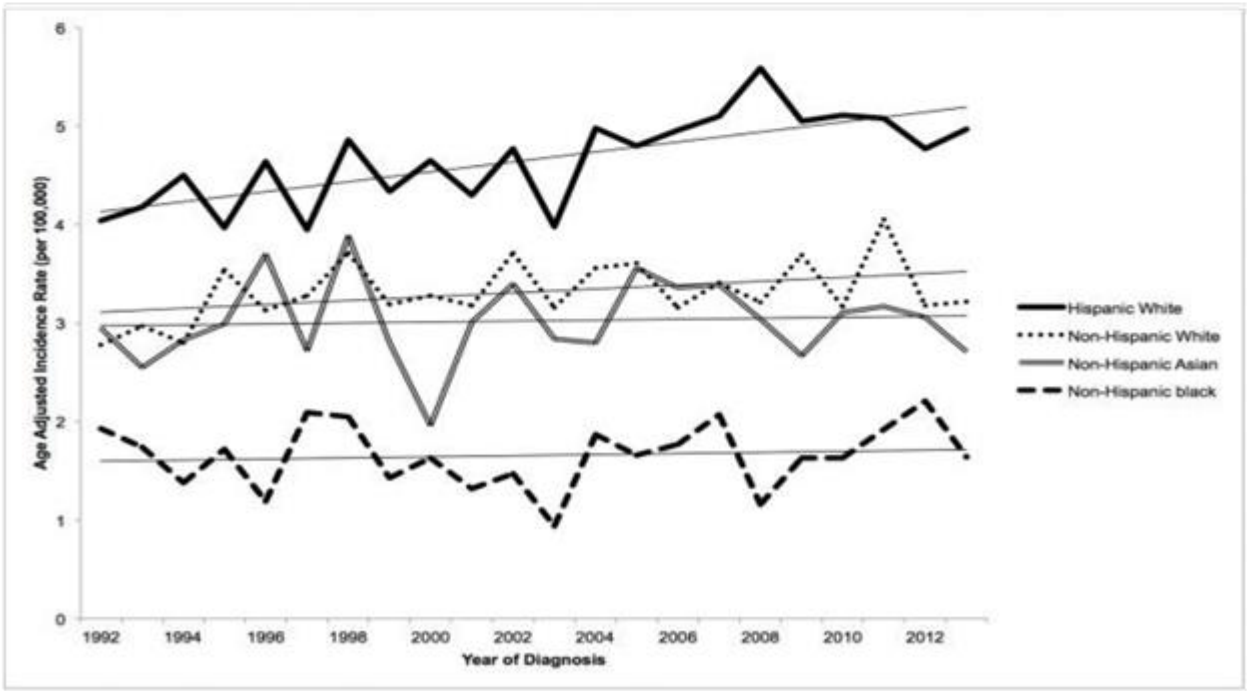


Figure 2: a: Trends in age-adjusted rates of acute lymphoblastic leukemia in children by sex and b; race/ethnicity [Siegel et al., 2017, Barrington-Trimis et al., 2017]

On a global scale, based on the data collected from 2003 to 2007, Incidence rates of ALL stand at 10.8 to 21.2 per million individuals with higher incidence rates in the Americas and Oceania and lower rates in Asia and Eastern Europe[53]. There are an estimated 6000 new cases of ALL accounting for 0.3% of new cancer cases in the USA and 1500 deaths, accounting for 0.2% of all cancer related deaths all in 2019 alone[81].

The pathogenesis of ALL is characterized by an unusual proliferation and differentiation of a clonal population of lymphoid progenitor cells which never achieve maturation and stymy the production of healthy lymphocytes. Predisposing conditions that can lead to this abnormal proliferation in a small number of cases include Fanconi anemia, Bloom syndrome, Down syndrome, Human immunodeficiency virus, Epstein Barr virus, ataxia telangiectasia and Nijmegen breakdown syndrome[94,41,10,14,44,40]. Ionizing rays, pesticides and to a lesser degree, electromagnetic fields and cigarette smoke are also included as other predisposing factors that influence the onset of ALL[8,46].

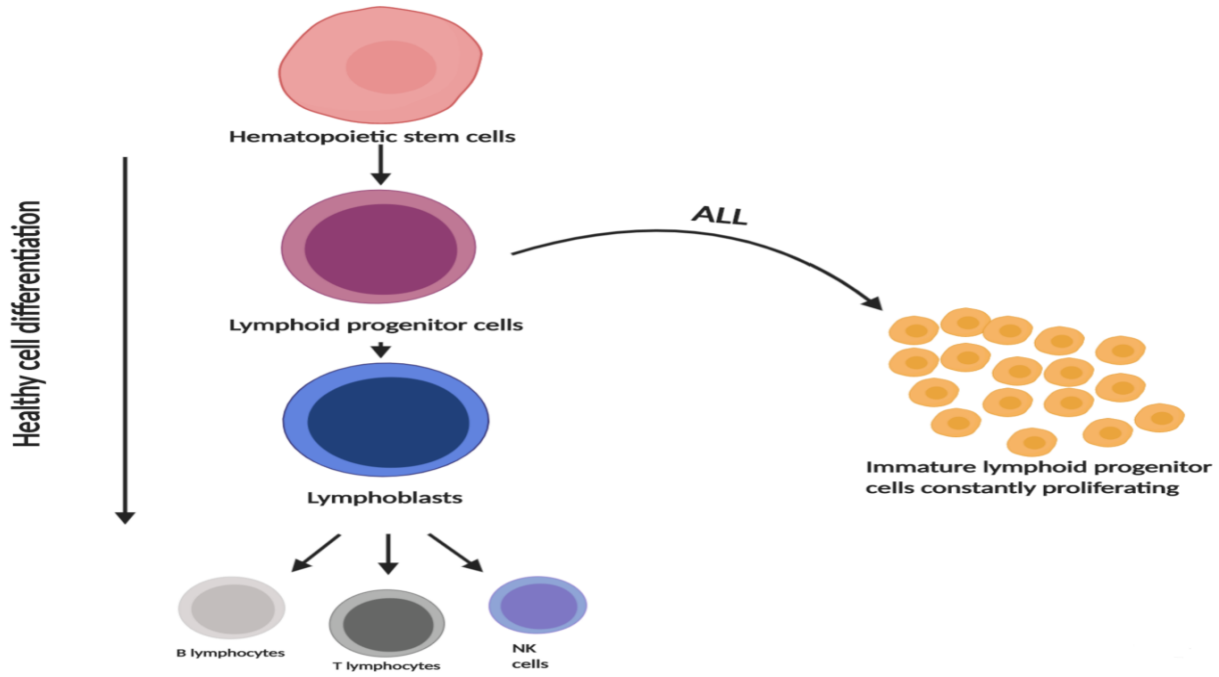


Figure 3: Demonstrates the normal path of differentiation of HSCs to become B lymphocytes, T lymphocytes or NK cells and the uncontrolled proliferation of lymphoid progenitor cells that occurs with ALL.

The disease condition is characterized by symptoms such as bone pain, osteolytic lesions, osteonecrosis, extremity and joint pain, fever, nausea, night sweats, weight loss, easy bruising, bleeding, dyspnea, dizziness and increased susceptibility to diseases[77,52,56,33]. Hematopoietic stem cells (HSCs) are progenitors for B and T lymphocytes as well as natural killer cells, all of which constitute an important part of the human immune system[108]. Throughout a human's life, HSCs provide a steady supply of B and T lymphocytes, with a massive production of lymphocytes during fetal and neonatal periods but lymphocyte production especially T lymphocytes slows down after adolescence[50]. The site of differentiation determines the fate of the progenitor HSCs with B lymphocytes formed from lymphoid progenitor cell differentiation in the bone marrow (liver in fetus) and T lymphocytes from lymphoid progenitor cell differentiation in the thymus. This means then that based on immunophenotypes, there are two subtypes of ALL; the B cell and T cell ALL[93,64,17].

A whole host of genetic factors has been implicated in the onset of ALL[97].Wiemels *et al.,* is believed to be the first to draw a correlation between ALL and genetic anomalies. The study identified TEL-AML1 gene fusion which when expressed results in the inhibition of B cell differentiation leading to massive accumulation of B cell progenitors and a correlated reduction in mature B lymphocytes[109]. Another example of a genetic anomaly associated with ALL is the Philadelphia chromosome; characterized by genetic mutations that activate several signaling pathways such as those concerned with cytokine receptors, tyrosine kinases and epigenetic modifiers. Philadelphia chromosome-like ALL constitutes 10% and 13% of standard and high-risk childhood B lineage ALL respectively. The frequency of incidence of the Philadelphia chromosome-like ALL also increases with age making for very poor survival in adult ALL[112,92]. In 40% of B lineage ALL cases, deletions, amplifications, mutations and structural rearrangements in key transcription factors that influence early lymphoid differentiation have been observed[112]. In particular, mutations of the IKZF1 gene encoding the IKAROS transcription factors which is vital for the induction of B lymphocyte differentiation has been associated with poor prognosis of ALL[39,79,112] and mutations in tumor suppressor genes have also been implicated in childhood ALL[54]. Ultimately, the fate of the affected cells is the same; massive proliferation of immature lymphoid progenitor cells which undercuts the number of healthy lymphocytes. The current form of treatment for ALL includes the use of L-asparaginase along with chemotherapy.

1.2 Asparaginase (ASNase)

L Asparaginase (ASNase) is an enzyme that catalyzes the hydrolysis of L-asparagine (ASN) to L-aspartic acid and ammonia[74,55]. Bacterial ASNase enzymes obtained from *Escherichia coli* have been classified into two subtypes based on their intrinsic location in a

bacterial cell as well as their substrate affinity. Subtype I isolated from the cell cytosol has a lower affinity for ASN while subtype II isolated from the periplasm has a much higher substrate affinity for ASN[74]. The enzymatic activity of ASNase was first discovered by Lang who observed ASNase activity in several beef tissues in 1904, this was confirmed in 1910 by Furth and Fredmanm. Clementi discovered in 1922 that ASNase was active in all herbivore tissues (particularly in guinea pig serum), omnivore livers and absent in tissues of carnivores, amphibians and reptiles[74,60]. The anticancer capabilities of guinea pig serum against mouse and rat lymphomas were first reported by Kidd et al., in 1953 and this particular capability was later attributed to the ASNase present in guinea pig serum[13]. Shortly after, an ASNase isolated from *E. coli* was shown to possess significant anti-tumor capabilities and this spurred a flurry of research into *E. coli* ASNases and eventually led to its approval for clinical trials for anticancer treatment, making it the first ASNase used as an antileukemic treatment.[91,12,47,20,82].

ASNase is employed as an antileukemic because of its ability to hydrolyze ASN to L aspartate and ammonia. ASN is a vital amino acid required by the leukemic lymphoid progenitor cells for their survival. Leukemic lymphoid progenitor cells are usually incapable of synthesizing this amino acid and utilize blood serum ASN for their metabolism. Normal cells expressing the asparagine synthetase gene are capable of synthesizing ASN and are not negatively impacted by ASN deficit brought about by the use of ASNase[86,63,32]. The absence of ASN results in inability of the leukemic cells to synthesize proteins and this arrests their development and leads to their subsequent apoptosis[100]

There are three ASNases currently approved to be used as part of chemotherapeutic cocktail. Native *E. coli* ASNase trademarked under the name Elspar, a pegylated form of native *E. coli* ASNAse also known Oncospar and ASNase derived from *Erwinia chrysanthemi* also

known as Erwinaze[35]. Erwinaze is approved for use only in Europe and is used in the USA only under compassionate recourse

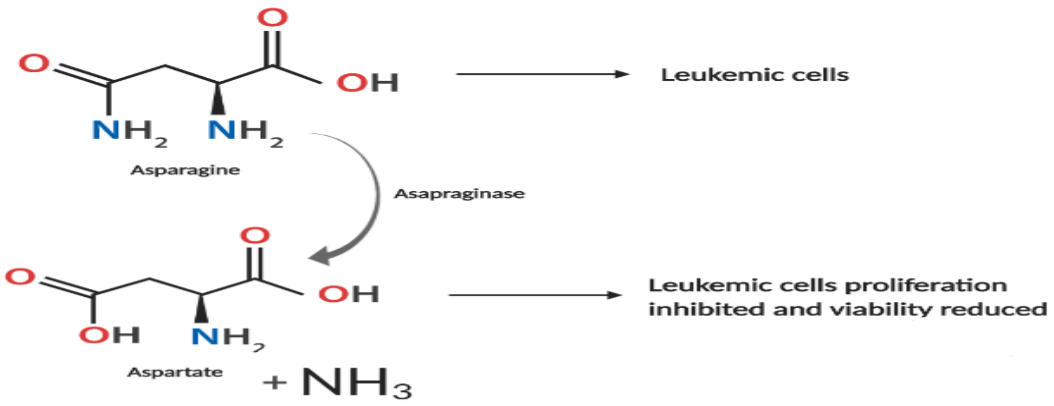


Figure 4: The hydrolytic effect of ASNase on ASN and its subsequent antileukemic effect [adapted from Elshafei et al.,2012]

The use of ASNase has significantly improved the outcome of treatment of ALL. Since the introduction of ASNase as a form of treatment, the survival rate of individuals with this disease condition has progressively increased from almost universally fatal to about 90% in children[28] and 78% in young adults[58]. These numbers, however, are only applicable to individuals who do not develop a treatment preventing condition. A study showed that patients treated using chemotherapy incorporating ASNAse had increased survivability when compared to patients treated using chemotherapy not incorporating ASNase, indicating that ASNase chemotherapy is significant for the successful treatment of ALL[28].

The use of ASNase as a form of treatment for ALL is not without its risks, studies have shown toxicities in up to 30% of treated patients with the most prevalent form of toxicity being hypersensitivity[90,106], other toxicities include pancreatitis, liver dysfunction, diabetes, coagulation abnormalities and thrombosis which is particularly common in adult patients with

ALL[58,26]. Both organisms used as sources for ASNase are Gram-negative bacteria which therefore possess lipopolysaccharide Endotoxins as integral membrane structures and lipopolysaccharides have been implicated in human hypersensitivity to Gram-negative bacterial cells and their products.[38,37] A study showed 41% of observed patients treated with *E. coli* ASNase showed an allergic reaction, 12.5% of these patients with allergic reactions who were then switched to *Erwinia* derived ASNase showed an allergic reaction while 33.3% showed an allergy to pegylated *E. coli* ASNase indicating increased toxicity to *E. coli* when compared to *Erwinia* ASNase[66,28]. It is noteworthy that *E. coli* ASNase has been shown to have a higher efficacy than *Erwinia* ASNase. Comparative toxicity and efficacy of the two ASNases showed that when patients are treated with the same dosage (10,000 IU,m^2), ASNase obtained from *E. coli* had higher coagulation abnormalities but similar toxicities to those treated with *Erwinia* ASNase, while a higher number of patients on *Erwinia* ASNase failed to achieve complete remission[26] indicating that ASNase derived from *E. coli* are more effective but with more side effects when compared to ASNase derived from *E. chrysanthemi*[27]. Elspar derived from *E. coli* has a half-life of 8 to 30 hours, Oncospar, the polyethylene conjugated ASNase formulation also prepared using *E. coli* derived ASNase has a half-life of about 6 days while Erwinaze derived from *E. chrysanthemi* has a half-life of 16 hours[111]. The reduced half-life of Erwinaze is probably tied to its reduced efficacy when compared to the *E. coli* formulations. Other problems associated with ASNase therapy are susceptibility to proteases and the development of serum antibodies that inactivate the protein, and this has been shown to occur both in patients with and without hypersensitivity to the protein[104,15,84].

Another major problem associated with the use of currently approved ASNases are their inherent L-Glutaminase (GLNase) activity. This activity has been linked to several side effects

associated with ASNase therapy, side effects such as hepatotoxicity, hyperglycemia, and dyslipidemia[80]. Some studies suggest that this inherent GLNase activity is vital to induce apoptosis in lymphoblasts especially those expressing the Asparagine synthetase (ASNS) gene and are therefore capable of synthesizing ASN from serum Glutamine (GLN)[18,85].

ASNase has been isolated from a wide variety of lifeforms such as animal tissue, fungi and plants[76,13,96], however, only bacterial ASNases have been approved for use in the treatment of leukemia. The source and growth conditions fundamentally influence the properties of the isolated ASNases such as substrate affinity, optimum temperature, optimum pH, and structural diversity. The problems associated with the currently approved ASNases have led to increased investigations of other bacterial ASNases as potential alternatives to the currently approved ones in the hope of circumventing the problems associated with their use for cancer therapy.

1.3 *Rhodococcus rhodochrous*

Members of the genus *Rhodococcus* are aerobic, non-spore forming, non-motile, mycolate containing, Gram-positive bacteria that are largely ubiquitous and classified along with *Corynebacterium*, *Mycobacterium* and *Nocardia* under the phylum *Actinobacteria* due largely to morphological similarities[36,103,6]. The genus '*Rhodococcus'* was settled upon after *Mycobacterium, Nocardia and Corynebacterium* were offered as possible genera under which to classify microbes that possessed the "Rhodochrous effect"[43]. The rhodochrous effect was used to describe bacteria from which mycolic acids have been isolated[75]. The mycolic acids isolated from bacteria within the *Rhodochrous complex* are composed of hydrocarbon chain lengths centered around C_{40}, which separates them from the genera *Corynebacterium* and *Norcadia* that have mycolic acids with chain length around C_{20} to C_{30} and C_{50} respectively[3].

Members of this genus are characterized by high guanine/ cytosine DNA content[6], cell wall components that include A-acetylglucosamine, N-acetyl glucomuramic acid, D-and L-alanine, D-glutamic acid, mesodiaminopimelic acid, arabinose, galactose, cardiolipin, phosphatidylinositol mannosides, and mycolic acids with 34-52 carbon atoms[36] and the presence of carotenoids which are believed to be responsible for the characteristic red, orange, pink and yellow pigmentation of most *Rhodococci*[98].

Another notable characteristic of the members of the genus *Rhodococcus* is their unusually large genome, A study by McLeod et al., in 2006 identified a 9.7 mbp genome with a 67% guanine/ cytosine content arranged in a linear chromosome and three linear plasmids (pRHL1, pRHL2, and pRH3) in *Rhodococcus sp* RHA1, the largest genome observed in a prokaryote to date[70]. This unusually large genome size is credited for the massive enzyme suite typically observed in members of the genus *Rhodococcus*, of particular interest are the large number of genes that were identified that were involved with secondary metabolism, including genes for polyketide and peptide synthase indicating prolific antibiotic production[31]. Members of this genus have also been demonstrated to produce biosurfactants and bioflocculants[36,62]. Biosurfactants specifically have been demonstrated to be indispensable for waste cleanup as they increase the surface area of hydrophobic insoluble substances, essentially functioning as bio-emulsifiers[83] and facilitating the degradation of these substances by water based enzymes. Moreover, the hydrophobic surface area of *Rhodococci* has been implicated in their adhesion to hydrocarbons essentially enhancing their biodegradative capabilities against hydrocarbons specifically[73]. Flocculants aid in the collection of suspended matter such as solids and colloid particles and is vital in water purification process, wastewater treatment, activated sludge treatment and food fermentation[1]. The biosurfactant and bioflocculant production by members

of the genus *Rhodococcus* suggests that these organisms can be utilized broadly for industrial processes. In addition to waste management, some members of the genus *Rhodococcus* have also been demonstrated to bioaccumulate heavy metals such as lead, mercury and copper[5,25].

R. rhodochrous strain DAP 96253 when induced by growth on media incorporating Urea and Cobalt (II) chloride are capable of increased production of certain enzymes of interest. Some of the enzymes upregulated by media incorporating urea and cobalt (II) chloride include Nitrile hydratase (NHase), ASNase, GLNase, and Amidase. Past and ongoing studies at the Pierce lab have shown that a catalyst derived from induced cells of *R. rhodochrous* DAP 96253 can delay the ripening process of climacteric fruits. An elaborate scheme explaining the pathways utilized by the catalyst to impede the Yang cycle which is responsible for the ripening of climacteric fruits was put forward and it included the activities of enzymes such as NHase, amidase, cyanidase, 1 aminocyclopropane 1 carboxylic acid deaminase (ACC deaminase), β cyanoalanine synthase (β CAS), isocitrate lyase, monooxygenase and epoxide hydrolase (1-HMO).[87,88]

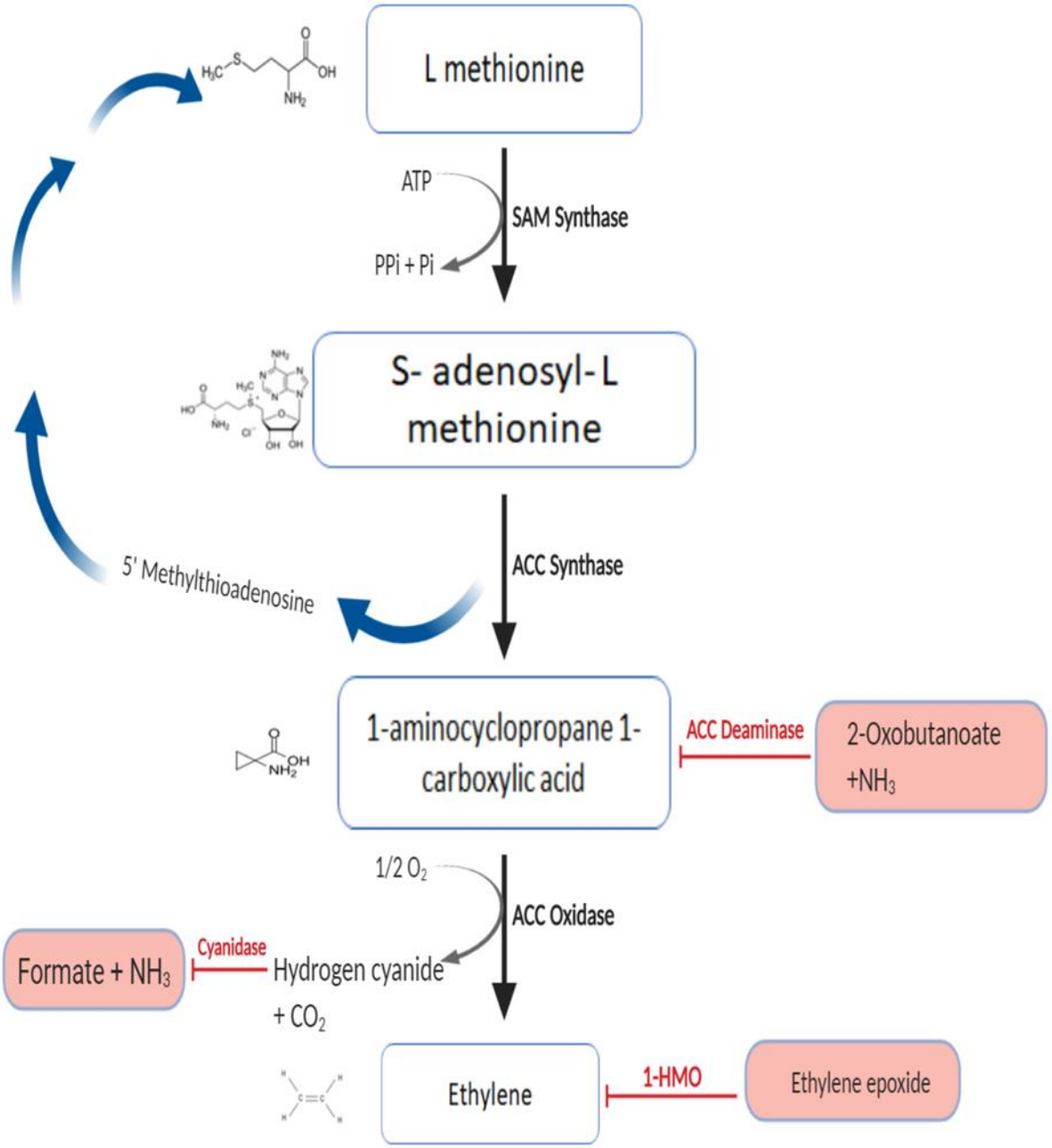


Figure 5: The Yang cirlce describing the conversion of methionine to ethylene and HCN and CO2 which have been implicated in the ripening process and the effect of selected enzymes (highlighted in red) and their impact on delaying the ripening of climacteric fruits [adapted from Pierce et al., 2014]

Along with the ability to delay the ripening of fruits, *R. rhodochrous* DAP 96253 has also been found to fungicidal and fungistatic. Closed airspace, contact independent setups using induced cells of *R. rhodochrous* DAP 96253 against *Pseudogymnoascus destructans*, the fungus

responsible for the fatal white nose syndrome in North American bats showed that the *R. rhodochrous* totally inhibited the conidial growth of *P destructans* at 15ºC and was strongly fungistatic at 4ºC[22].

Induced cells of *R. rhodochrous* DAP 96253 have also been demonstrated to be to fungicidal against the fungus *Ophidiomyces ophiodiicola* which has been implicated in fatality of snakes in 15 American states[21]. The delayed ripening and fungicidal and fungistatic properties observed in *R. rhodochrous* strain DAP 96253 and other interesting metabolic capabilities associated with other species of *Rhodococcus* is typically attributed to their unusually large genome[103]. The metabolic capabilities of *R. rhodochrous* DAP 96253 coupled with it being a Gram-positive organism suggests that ASNase and GLNase purified from this organism could circumvent some of the issues plaguing currently approved ASNases.

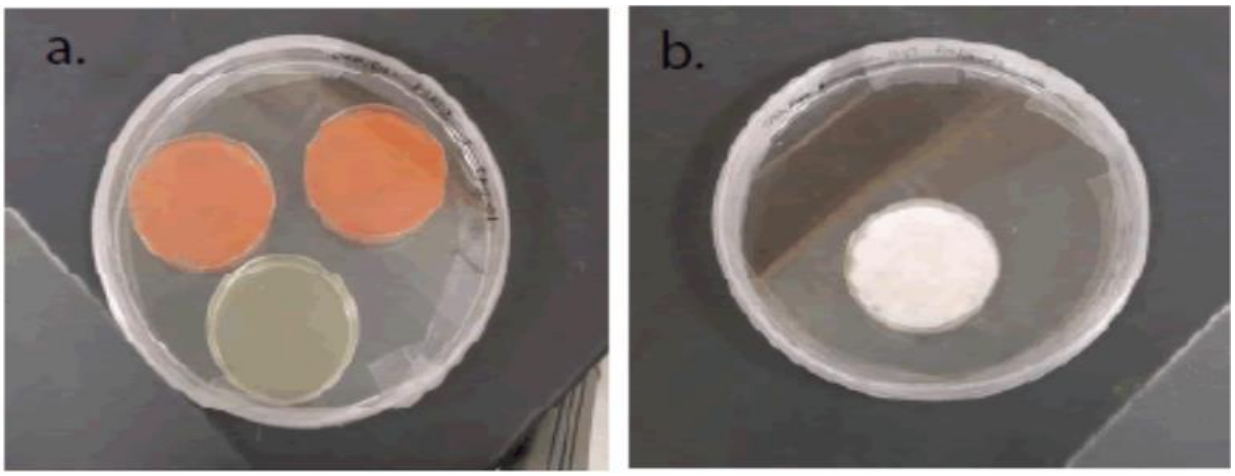


Figure 6: a; Closed headspace setup showing complete inhibition of O. ophiodiicola by packed cells of Induced R. rhodochrous DAP 96253 and b; growth of O. ophiodiicola in the absence of R. rhodochrous [Cornelison et al., 2016]

1.4 Objectives

The main objective of this research project was to investigate classical methods of protein purification to determine optimum protein purification methods resulting in a reproducible protein preparation exhibiting stable ASNase and GLNase activity obtained from induced cells of *R. rhodochrous* strain DAP 96253 that can serve as alternative antileukemic treatment for ALL.

Upon completion of this objective, other studies were performed to determine

a. The levels of enzyme production by induced cells of *R. rhodochrous* DAP 96253 employing various induction media
b. The optimum buffer pH for protein purification,
c. The stability of the purified protein at a defined pH and temperature range.
d. The stability of the purified protein in sterile human serum
e. long term storage stability at subzero temperatures,
f. endotoxin analysis, and
g. determination of the efficacy of the purified protein against selected leukemia expressing cell lines.

2 MATERIALS AND METHODS

2.1 Media and organisms

1. Yeast extract, malt extract agar (YEMEA/ Co/ U); 4g dextrose anhydrous (Fisher chemical), 4g Yeast extract (BD Bacto™), 10g Malt extract (BD Bacto™), 0.1g Cobalt II chloride (Sigma Aldrich), 20g Technical agar (BD Difco™), dissolved in 850 mL of deionized water. More deionized water is added to reach a final volume of 0.95L and autoclaved. 7.5g of Urea (Fischer chemical) was filter sterilized and added to the molten agar after it had cooled to below 50ºC to reach a final volume of 1L. Plates are made by aseptically pipetting 25 mL of molten agar into sterile petri dishes.

   Yeast extract, malt extract broth (YEMEA/ Co/ U); 4g dextrose anhydrous (Fisher chemical), 4g Yeast extract (BD Bacto™), 10g Malt extract (BD Bacto™), 0.1g Cobalt II chloride (Sigma Aldrich), all dissolved in 850 mL of deionized water and autoclaved. 7.5g of Urea (Fischer chemical) was dissolved into 150 mL of deionized water and filter sterilized into the broth when it had cooled to room temperature (RT).
2. Modified R3A media (mR3A); 3g Proyield cotton (FrieslandCampina), 1.5g soluble starch (BD Difco™), 1.5g yeast extract (BD Bacto™), 0.15g Magnesium sulfate anhydrous (J.T. Baker), 0.90g potassium phosphate dibasic (EMD Millipore), and 0.1g Cobalt II chloride (Sigma-Aldrich), dissolved in 1L of deionized water and autoclaved. 10g dextrose anhydrous (Fisher chemical), 16g Urea (fisher chemical) and 0.76g Sodium pyruvate (Sigma-Aldrich) are dissolved and filter sterilized into the autoclaved media after it has cooled to room temperature.
3. Complete media 1 (for cultivation of Jurkat, clone E6-1 ATCC® TIB-152™): 225 mL of sterile 1x RPMI 1640 with L-glutamine (Corning), 25 mL Fetal bovine serum (FBS)

(Millipore Sigma), 2.5 mL Penicillin-Streptomycin-Glutamine 100x (Gibco). This solution is filter sterilized using a 0.2µm vacuum filter (Thermo Scientific™Nalgene™ Rapid-Flow™). The sterilized filtrate referred to as complete media is aliquoted in 10 mL volumes into sterile 15 mL Falcon® tubes and stored at 4ºC or -20ºC.

4. Complete media 2 (for the cultivation of Molt-4 ATCC® CRL-1582™): 225 mL of sterile 1x RPMI 1640 (Gibco), 25 mL of FBS (Millipore Sigma), 2.5 mL Penicillin-Streptomycin (Gibco). The complete media is filter sterilized using a 0.2µm vacuum filter (Thermo Scientific™Nalgene™ Rapid-Flow™). The sterilized filtrate referred to as complete media is aliquoted in 10 mL volumes into sterile 15 mL Falcon tubes® and stored at 4ºC or -20ºC.
5. All samples of *Rhodococcus rhodochrous* strain DAP 96273 utilized for this project were obtained from stocks made by Dr. Kelly Cannon.

2.2 Chemicals and Solutions

2.2.1. Sodium nitroprusside solution

1% stock solution was prepared by dissolving 1g of sodium nitroprusside dihydrate (Honeywell) in 99 mL of deionized water. The stock solution was kept at 4ºC in the dark and working solutions were prepared by mixing the stock solution with deionized water in 1:100 ratio and was stable for up to a month with storage at 4ºC in the dark.

2.2.2 Sodium phenate solution

Sodium phenate solution was prepared by dissolving 25g of Phenol (≥ 99.5% GC grade Sigma -Aldrich) in 800 mL of deionized water, 78 mL of 4N Sodium hydroxide (Sigma-Aldrich)

was stirred in and deionized water was added in to reach a final volume of 1L. The solution was kept at 4ºC in the dark and is stable for up to 2 months.

2.2.3 Sodium hypochlorite

Sodium hypochlorite working solution was prepared by mixing 25 mL of commercial Clorox bleach with deionized water to reach a final volume of 1L. This solution is typically prepared fresh before an assay but will keep for up to 1 month at 4ºC

2.2.4 Buffer Solutions

The volume of the acid and conjugate base component of all the buffers utilized for this study were determined using the Michaelis Menten equation. Buffers were utilized for washing cells, cell lysis, dialysis, purification, protein storage and pH studies.

2.3 Methods

2.3.1 Bacterial cell cultivation

Cells utilized for the study were either obtained from fermentation by either Dr Kelly Cannon or Dr Maurice de la Croix. In cases where cells weren't obtained from fermentation in a bioreactor, 1 mL of stock cells preserved at -80ºC were transferred aseptically to 100 mL of sterile nutrient broth (BD Difco ^{TM}) and incubated at 30ºC in an incubator shaker for 48 hours, after which the contents were transferred to 1L of YEMEA broth or mR3A broth or spread on YEMEA plates. Broth samples were incubated at 30ºC in an incubator shaker (New Brunswick Scientific Edison NJ USA) for 5 days before harvesting by centrifugation at 13,000 rpm for 5 minutes using a Beckman Avanti^{TM} J-25 centrifuge with the JLA-16.250 rotor. When grown on solid media, cells were grown for 9 days before harvesting by scraping the surface of the agar.

Figure 7: Cell scraper utilized in the harvest of cells from solid media.

2.3.2 Cell lysis

Induced cells of *R. rhodochrous* DAP 96253 were suspended in three different lysis buffers: 25mM Sodium phosphate buffer (PB), 50 mM MOPS or 50 mM Tris-HCl, varying pH and with or without 5mM 2- mercaptoethanol supplementation. Cell suspensions were made using a ratio of 1 g of whole cells (packed wet weight) per 1 mL of lysis buffer (w/v) and vortexed until cells were entirely suspended. Cells were lysed either by high-pressure homogenization or sonication. High pressure homogenization was performed at 4ºC using an APV- 1000 (homogenizer) at 700 bar. Sonication was conducted on ice for 20 minutes at 1 sec on 1 sec off cycling using a Fisher® 550 Sonic Dismembrator [with Minonix incorporated convertor and a horn with a ½" diameter tip]

Sonicated or homogenized cells were centrifuged for 30 minutes at 13,000 RPM at 4°C using a Beckman Avanti™ J-25 centrifuge with the JA-25-50 rotor or a Beckman Coulter™

Allegra™ 64R centrifuge with the Conical C1015 rotor. The centrifugation was repeated three times recovering the supernatant, termed as the cell free lysate (CFL).

2.3.3 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% $MgSO_4.7H_20$, and 5mM 2-mercaptoethanol).

2.3.4 Anion exchange chromatography

Anion exchange chromatography (AEX) was performed using a GE AKTAExplorer™ 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.

2.3.5 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.

2.3.6 ASNase and GLNase activity

ASNase and GLNAse were quantified using 1000ppm ASN (Sigma Life Science) and GLN (Sigma) solution (10mg of ASN or GLN dissolved in 10 mL of 25mM PB pH 7.6). 10µL of CFL or purified protein was reacted with 990µL of ASN or GLN solution for 2 minutes. The reaction was stopped by the addition of 10µL freshly prepared 2M H_2SO_4 and vortexed for 1 minute, 10µL of 4N NaOH (Sigma-Aldrich) was added and also vortexed for 1 minute to neutralize the acid.

The solution was transferred to a clean test tube and the amount of ammonia generated was determined by a modification of the method by Fawcett and Scott[34]. Colorimetric reagents were added in this order; 2 mL sodium phenate, 3 mL of sodium nitroprusside and 3 mL of sodium hypochlorite. The test tubes are vortexed after the addition of each reagent for 1 minute and incubated at RT in the dark for 30 minutes. The ASNAse and GLNase assay for each sample was performed in triplicates. 200µL of each reaction solution was loaded into an untreated 96

well plate (Carplugs/ Evergreen u bottomed) and absorbance was read at a wavelength of 630nm using a PW Victor 3 V (Wallac1420) plate reader. The amount of ammonia generated was extrapolated by comparing to an ammonia standard of 0-20 ppm. One unit of ASNase or GLNase is defined as the amount of enzyme required to convert 1µM of ASN or GLN into 1µM of aspartic acid or glutamic acid and 1µM of NH_3 per minute per mg of protein at RT, at pH 7.6.

2.3.7 NHase activity

NHase activity was determined using 1000ppm Acrylonitrile as the substrate (12.3µL of acrylonitrile mixed with 10 mL of 25mM PB pH 7.6). 10µL of CFL or purified protein was stopped by reacted with 990µL of acrylonitrile solution for 2 minutes. The reaction was stopped by the addition of 10µL freshly prepared 2M H2SO4 and vortexed for 1 minute, 10µL of 4N NaOH (Sigma-Aldrich) was added and vortexed for 1 minute to neutralize the acid. 1 mL of the reaction solution is transferred to a clean Eppendorf tube and 10µL of a working solution of Amidase (Amidase from *Pseudomonas aeruginosa* ≥ 100 units Sigma-Aldrich, made by mixing 1 part Amidase with 49 parts sterile deionized water) is added to the solution. The tubes were incubated at 37ºC for 30 minutes.

The solution was transferred to a clean test tube and the amount of ammonia generated was determined by a modification of the method by Fawcett and Scott[34]. Colorimetric reagents were added in this order; 2 mL sodium phenate, 3 mL of sodium nitroprusside and 3 mL of sodium hypochlorite. The test tubes are vortexed after the addition of each reagent for 1 minute and incubated at RT in the dark for 30 minutes. The NHase assay for each sample was performed in triplicates. 200µL of each reaction solution was loaded in triplicates into a 96 well u bottomed plate and absorbance is measured at wavelength of 630nm using a PW Victor 3 V (Wallac1420)

plate reader. The amount of ammonia generated was extrapolated after color development by comparing to an ammonia standard of 0-20 ppm.

One unit of NHase is defined as the amount of enzyme required to convert 1µM of acrylonitrile into 1µM of acrylic acid per minute, per mg of protein at RT, at pH 7.6.

2.3.8 Protein quantification

Proteins obtained from the cell lysis and purification were quantified using the Bradford or BCA protein quantification assay.

When performing the Bradford protein quantification, A standard protein dilution of 0 to 2000 µg/mL of protein was made using an Albumin standard (Thermo Scientific), multiple dilutions of the samples to be quantified were also made to fall within the 10 to 200 µg/mL protein range. 25µL of the protein standard and diluted samples were loaded in triplicates into an untreated 96 well plate (Carplugs/ Evergreen flat bottomed) and 250µL of the Bradford reagent (ThermoFisher Scientific) was added to each well and the plate was incubated at RT for 10 minutes. Absorbance was measured at 595nm using a PW Victor 3 V (Wallac1420) plate reader.

With the BCA quantification, a standard protein dilution of 0 to 2000 µg/mL of an Albumin standard (Thermo Scientific) and multiple dilutions of the samples to be quantified were also made and loaded in triplicates into an untreated 96 well plate (Carplugs/Evergreen flat bottomed). 200µL of BCA working solution (49 parts Pierce™ protein assay reagent A plus 1-part Pierce™ protein assay reagent B) was added to each well. The well plate was incubated at 37ºC for 30 minutes to allow for complete color development. Absorbance was measured at 562nm using a PW Victor 3 V (Wallac1420) plate reader. Protein concentration of the samples were extrapolated by comparing to the standard curve calculated from the standard dilutions.

2.3.9 Mammalian cell culture

Jurkat, clone E6-1(ATCC® TIB-152™) and Molt-4 (ATCC® CRL-1582™), T lymphoblasts expressing acute T cell leukemia and acute lymphoblastic leukemia respectively, obtained from ATCC were revived using complete media. All the steps for the revival and culture of mammalian cells were performed aseptically in a Biosafety cabinet and using 70% alcohol solution. The vials of mammalian cells obtained from ATCC were brought up to 37ºC using a water bath. 1 mL of the contents from the vial was transferred to a falcon tube containing 9 mL of complete media which had also been brought up to 37ºC. After very slight agitation to homogenize the solution, the falcon tube was centrifuged at 120g for 7 minutes using a Beckman Coulter Allegra® X15R Centrifuge. The supernatant now containing the cryopreservant used for preservation of cells was discarded and the cells which were in a pellet were resuspended in 2 mL of complete media and transferred to a T25 mammalian cell culture flasks (Corning) containing 8 mL of complete media. The flask was incubated at 37°C at 5% CO_2 saturation in a Queue Stabiltherm CO_2 incubator. After 72 hours of growth, a cell count was performed by staining 20µL of cells with 20µL of 0.4% Trypan blue solution (Sigma®). 20µL of this suspension was loaded into Nexcelom cell counting chamber and the cell number and viability was determined using a Nexcelom Biosciences cellometer® auto 2000 cell viability counter. This count was performed to determine if the cells are confluent (cell density of $3x10^6$ cells/mL for Jurkat Clone E6-1 and $2x10^6$ cells/mL for Molt-4). Protein efficacy studies and stock samples of cells were made after cells were determined to have reached confluence. Stocks were made by suspending $5x10^5$ cells/mL in freeze media (Complete media supplemented with 5% v/v Dimethyl sulfoxide (Fisher Bioreagents™) stored at 4ºC). This suspension was stored in 1 mL

volumes in cryovials for 1 hour at 4°C, another hour at -20°C and overnight at -80°C. The vials were then transferred to liquid nitrogen for storage indefinitely.

2.3.10 Molecular weight determination

Samples exhibiting ASNase and GLNase activity were diluted to 10mg/mL of protein using 2.5µL of NuPAGE™ LDS sample buffer (4x), 1µL of NuPAGE™ reducing agent (10x) and deionized water up to a final volume of 15µL. These samples were heat treated at 70ºC for 10 minutes or 95ºC for 5 minutes using a heat block (Benchmark MyBlock™). These heat treated samples as well as PageRuler™ plus prestained protein ladder (10 to250 kDa) were loaded onto a precast NuPAGE™ Novex Bis-tris gels. The gel was run employing an SE 600 Ruby system (Amersham Sciences), Invitrogen NuPage® Novex® gel chamber, and NuPage® Novex® 1x MOPS SDS running buffer, voltage was set to 200V, and power was set to 50W. After a run time of ~45 minutes, the protein bands were visualized by staining using Coomassie brilliant blue staining solution (0.25g Coomassie brilliant blue R-250/ 90 mL of Methanol (Sigma-Aldrich): deionized water, 1:1 v/v, and 10 mL of glacial acetic acid (Fisher chemical)) overnight and destained by suspending the gel in destaining solution (deionized water/ Methanol (Sigma-Adrich)/ glacial acetic acid (Fisher chemical) in 50/40/10, v/v/v) for up to 4 hours. Gels images were captured using a UVP BioDoc-It™ imaging system.

2.3.11 pH and Thermal stability of protein purification

CFL obtained from induced cells of *R. rhodochrous* DAP 96253 was purified using AEX and SEC described above at pH ranging from 6 to 9 to determine the optimum pH for purification. 25mM sodium phosphate buffer was employed for purifications from pH 6-8 and 25mM Tris HCl buffer was employed for purifications at pH 8 and 9. After determining the optimum pH for purification, purified protein samples exhibiting stable ASNase and GLNase

activity were also incubated for 25 hours at pH 3, 4, 5, 6, 7, 7.2, 7.4, 7.6, 8, 9 and 10 to determine the protein stability at varying pH. 100mM Citrate buffer was employed for pH ranging from 3-5, 50mM sodium phosphate buffer for pH ranging from 6-8 and Glycine-NaOH for pH 9 and 10. Post incubation, samples were analyzed for ASNAse and GLNase activity using the method described above.

Purified protein samples obtained from induced cells of *R. rhodochrous* DAP 96253 exhibiting stable ASNase and GLNAse activity were also incubated at 4, 10, 15, 25, 30, 37, 42, and 50ºC. The samples were incubated at these temperatures for up to 60 minutes and assayed for ASNase and GLNase activity at the 15, 30 and 60-minute timepoints.

2.3.12 Effect of selected chemicals on ASNase and GLNase activity of protein preparations

The purified protein preparation obtained from induced cells of *R. rhodochrous* DAP 96253 were incubated for 30 minutes with selected chemicals at varying concentrations to determine their effect on the ASNase and GLNase activity. The effectors tested and the concentrations are listed in table 1. The sample was incubated for up to 1 hour at RT before the samples were analyzed for ASNase and GLNase activity.

Table 1: List of chemicals evaluated for their effect on ASNase and GLNase activity of protein preparation obtained from induced R. rhodochrous DAP 96253

	Name	*Concentration (mM)*
1	Zinc chloride ($ZnCl_2$)	7.4
2	Magnesium chloride ($MgCl_2$)	4.9
3	Silver nitrate ($AgNO_3$)	8.9
4	2- Mercaptoethanol	12.8
5	Iron II sulfate ($FeSO_4$)	3.6
6	Copper sulfate ($CuSO_4$)	4.0
7	Hydrogen peroxide (H_2O_2)	29.4
8	Ammonium persulfate ($(NH_4)_2S_2O_8$)	4.4
9	Calcium chloride ($CaCl_2$)	11.0
10	Cobalt chloride ($CoCl_2$)	1.0
11	Sodium phosphate buffer	50.0
12	Manganese sulfate ($MnSO_4$)	1.0

2.3.12 Stability of ASNase in human serum

O.5 mL of purified protein preparation obtained from induced cells of *R. rhodochrous* DAP 96253 was mixed with 2.5 mL of sterile human serum (Sigma) to a obtain a final enzyme concentration of 50 IU/mL and the mixture was incubated at 37ºC. ASNase activity was

determined every 5 hours for up to 55 hours to determine the level of deterioration of ASNase in human serum.

2.3.13 Endotoxin determination

The endotoxin concentrations of CFL and purified protein preparations obtained from *R. rhodochrous* DAP 96253 were determined using a Charles River LAL Endosafe Endochrome kit. A standard was prepared using lyophilized *E. coli* 055: B5 endotoxin ranging from 50 endotoxin units (EU) per mL to 0.005 EU/mL. Samples analyzed for endotoxins included *R. rhodochrous* DAP 96253 CFL, CFL purified using AEX and CFL purified AEX and SEC. A positive control (*E. coli* K12 CFL prepared using the same method for lysis employed with *R. rhodochrous* DAP 96253) was also tested. 100µL of standards and samples were loaded in duplicates into a pyrogen free 96 well plate (Endosafe®) and incubated at 37ºC for 5 minutes. 100µL of reconstituted lyophilized limulus amebocyte lysate was added to each well, and the intensity of color formation based on the concentration of endotoxin in samples and standards was measured using a PW Victor 3 V (Wallac1420) multiplate reader at a wavelength of 405nm

2.3.14 Efficacy of protein preparation against leukemia expressing cell lines

Jurkat Clone E6-1 and Molt-4 were the two-leukemia expressing cell lines utilized for this test. Approximately 1.2 $x10^4$ cells/well for Jurkat E6-1 cells and 3.0 $x10^5$ cells/ well for the Molt-4 were subcultured in sterile 96 well plate in duplicates and treated with varying amounts of the purified protein preparation obtained from induced cells of *R. rhodochrous* DAP 96253 (table 2) and appropriate controls were performed using purification buffers. The inoculated well plate was incubated at 37°C in Queue Stabiltherm CO_2 incubator at 5% CO_2 saturation and 37°C for up to 96 hours. Cell count and viability were determined at 24-hour timepoints by staining

using 0.4% trypan blue solution (Sigma®) and counting using a Nexcelom Bioscience cellometer® auto 2000 viability counter.

Table 2: Depicts the dose, volume, ASNase and GLNase concentration (IU/mL and IU/mg) of the protein preparations utilized for the leukemia expressing cell line treatment.

	Jurkat E6-1 cell line					Molt-4 cell line			
Dose	Volume (µL)	ASNase (IU/mL)	GLNase (IU/mL)	ASNase (IU/mg)	GLNase (IU/mg)	ASNase (IU/mL)	GLNase (IU/mL)	ASNase (IU/mg)	GLNase (IU/mg)
Control	100 PB	0	0	0	0	0	0	0	0
1x	5	27	125	22	102	60	9	74.8	7
2x	0	54	250	44	203	119.5	17	149.4	14
3x	5	81	375	61	305	239	26	299	21
4x	00	108	500	81	407	478	35	597.5	28

3 RESULTS

3.1 Enzyme profile of *R. rhodochrous* DAP 96253 in different media

After growth of and induction of *R. rhodochrous* DAP 96253 in mR3A broth, YEMEA broth or on YEMEA plates incorporating Urea and Cobalt II chloride, cells grown in broth were harvested by centrifugation and cells grown on agar were harvested by carefully scraping the agar. These whole cells were analyzed for a number of select enzymes. The enzyme activities are defined in international units per mg of cell dry weight (IU/mg CDW). One international unit is the amount of enzyme required to catalyze the conversion of one µmole of the substrate into one µmole of the product per minute per mg of cell dry weight at pH 7.6, at RT.

Table 3: Enzyme activity of induced cells of R. rhodochrous DAP 96253 grown on YEMEA plates

Enzyme activity (IU/mgCDW)	**NHase**	**ASNase**	**GLNase**	**ACC deaminase**	**Cyanidase**	**Urease**
Induced with Urea and Cobalt II chloride	412.5±34.7	4.7±1.4	22.8±2.2	2.1±0.1	11.2±0.3	10.6±0.3
W/O Urea and Cobalt II chloride	3.1±0.1	0.1	0.1	0.9± 0.1	0.1	5.0±0.6

Table 4: Enzyme activity of induced cells of R. rhodochrous DAP 96253 grown in YEMEA broth

Enzyme activity (IU/mgCDW)	**NHase**	**ASNase**	**GLNase**	**ACC deaminase**	**Cyanidase**	**Urease**
	491.5±56.7	9.5±2.5	17.5±3.2	3.8±0.7	3.9±0.1	ND

Table 5: Enzyme activity of induced cells of R. rhodochrous DAP 96253 grown in mR3A media

Enzyme activity (IU/mgCDW)	**NHase**	**ASNase**	**GLNase**	**ACC deaminase**	**Cyanidase**	**Urease**
	352.9±44.6	16.4±0.4	22.8±2.2	ND	ND	ND

In the development of a purification scheme to obtain an ASNase/ GLNase preparation that was reproducible, a number of variables were explored such as the impact of the method of lysis, use of reducing agents and duration of dialysis. The goal was to develop a method of purification that resulted in reproducible protein preparations exhibiting stable ASNase and GLNase activity. The impact of the method employed for lysis, reducing agents (2-mercaptoethanol) and the duration of dialysis was investigated on the ASNase and GLNase activity of the purified protein. In all cases of analysis, induced cells were lysed and purified, and the variables were incorporated and compared to negative controls. Samples from the lysis and purification were analyzed for ASNase and GLNase activity using the modified Fawcett and Scott method[34].

3.2 Cell lysis exploring homogenization and sonication

After growth and induction, cells of *R. rhodochrous* DAP 96253 were suspended in lysis buffer and cells were lysed using homogenization and sonication. The cell free lysate obtained from both processes were purified using AEX and the fractions obtained analyzed for ASNase and GLNase activity.

Table 6: Compares the ASNase and GLNase activity of CFL and CFL purified using AEX. The CFL were obtained using 2 different means of cell lysis.

	Homogenization			**Sonication**		
	NHase (mg/mL)	ASNase (mg/mL)	GLNase (mg/mL)	NHase (mg/mL)	ASNase (mg/mL)	GLNase (mg/mL)
CFL	33.1± 2.9	0.0	1.4 ± 0.2	ND	136.3 ± 30.2	156.7 ± 2.6
AEX	229.2 ± 45.6	0.0	0.0	ND	204.5 ± 27.6	324.6 ± 35.2

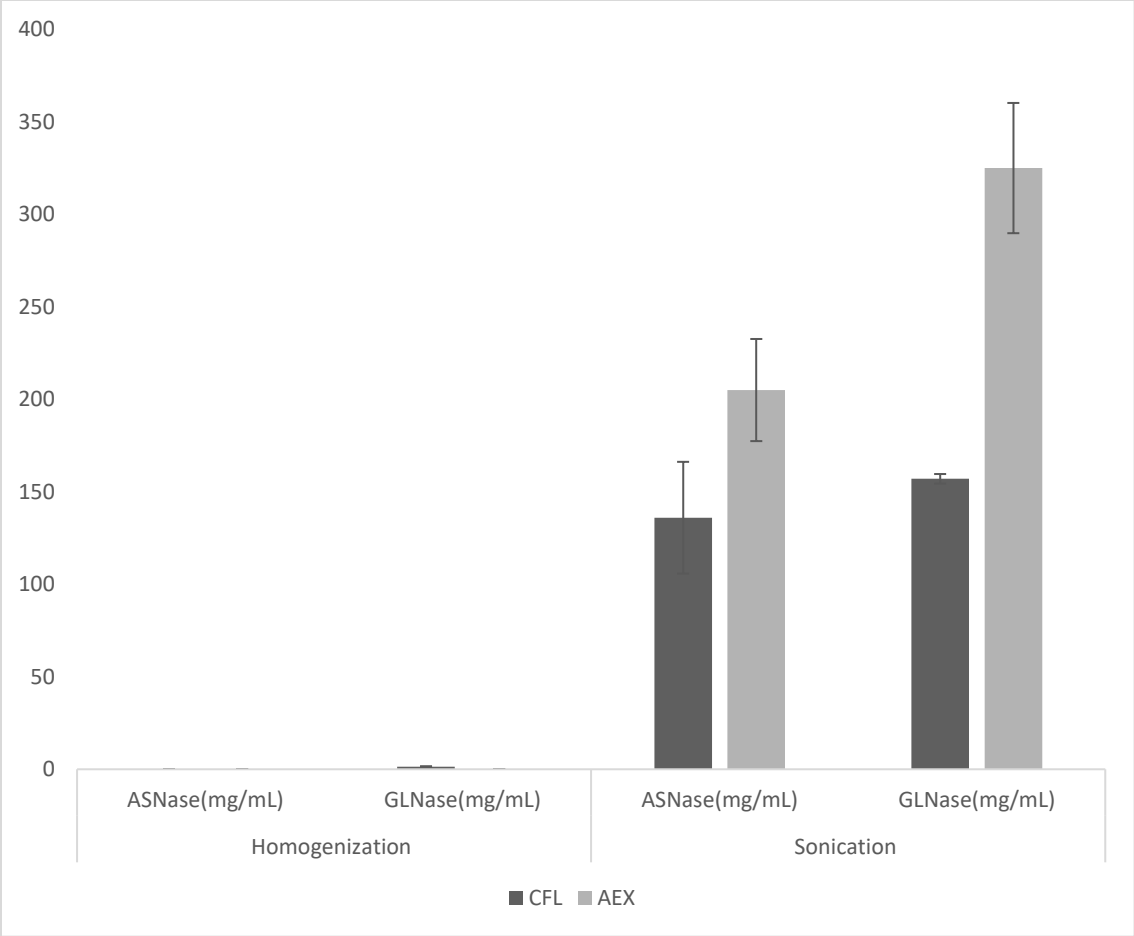


Figure 8: Comparison of the methods of homogenization and sonication for cell lysis and their impact on ASNase/GLNase activity

3.3 Cell lysis w/wo the incorporation of 2-mercaptoethanol

Having established the superiority of sonication as the better means of cell lysis, the effect of a reducing agent, specifically 2- mercaptoethanol on the ASNase and GLNase activity was investigated. 2-mercaptoethanol was incorporated into the lysis buffer in up to a 5mM concentration and cells were lysed and purified using AEX. Both the CFL and fractions obtained from the purification were analyzed for ASNase and GLNase activity.

Table 7: Compares the ASNase activity of samples obtained from lysis incorporating 2-mercaptoethanol and Samples lysed without 2-mercaptoethanol

	2-mercaptoethanol	
	PRESENT	ABSENT
	ASNase (mg/mL)	ASNase (mg/mL)
CFL	443.8 ± 24.6	12.4 ± 1.3
AEX	1280 ± 38.9	39.6 ± 2.8

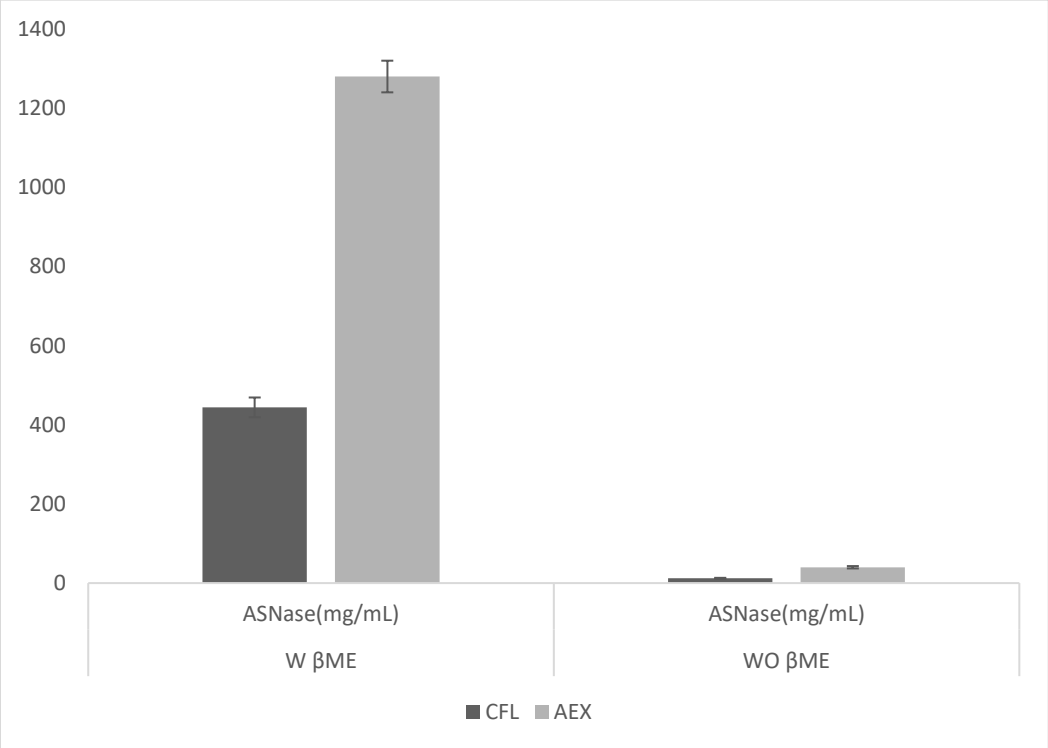


Figure 9: Evaluation of the effect of incorporating βME into the lysis process on ASNase activity of samples obtained from induced cells of R. rhodochrous DAP 96253

3.4 Effect of the duration of dialysis on ASNase activity

Protein dialysis employing a dialysis membrane with a 3.5kDa molecular weight cutoff was utilized to get rid of the 2-mercaptoethanol post cell lysis. The duration of the dialysis and its impact on the stability of the ASNase was also investigated. Samples were dialyzed for up to 40 hours and analyzed for ASNase activity. These dialyzed samples were also purified using AEX and analyzed for ASNase activity too.

Table 8: Comparison of ASNase activity of CFL and AEX purified samples obtained after varying lengths of dialysis.

	No dialysis	**24 hour Dialysis**	**40 hour Dialysis**
	ASNAse (mg/mL)	ASNase(mg/mL)	ASNase (mg/mL)
CFL	12.7 ± 2.9	38.4 ± 3.8	65.9 ± 4.8
AEX	49.3 ± 7.8	163 ± 5.6	682 ± 87.6

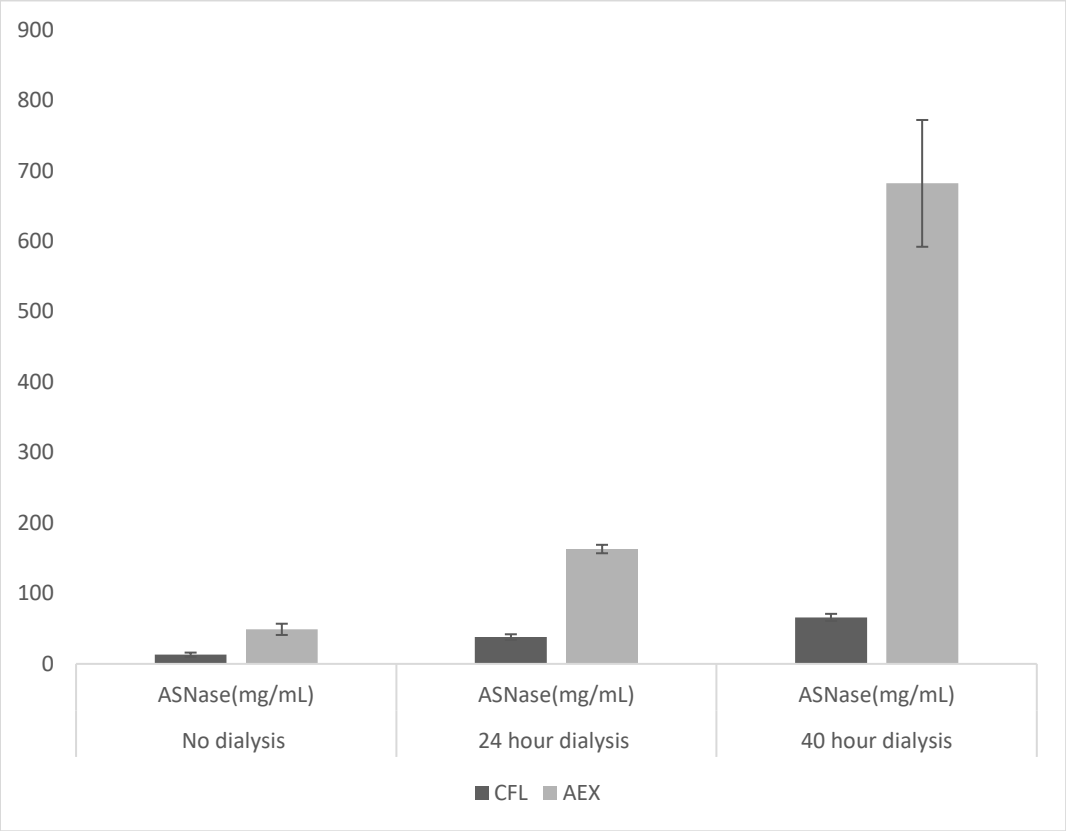


Figure 10: Evaluation of the duration of dialysis on the ASNase activity of CFL and protein purification obtained from induced cells of R. rhodochrous DAP 96253.

3.5 Determination of optimum pH to obtain protein preparation exhibiting stable ASNase activity from induced cells of *R. rhodochrous* DAP 96253.

With the determination of the sonication as the better of the two means of cell lysis, the incorporation of 2-merceptoethanol at 5mM concentration into the lysis buffer as well as dialysis for up to 40 hours significantly improved the ASNase and GLNase activity of the CFL obtained from induced cells of *R. rhodochrous* DAP 96253 and the subsequently purified CFL via AEX also exhibited improved ASNase and GLNase activity. Purifications of ASNase were then performed at pH 6, 7, 8, and 9 to determine the optimum pH for the puifirication process. 25mM

PB was selected for pH ranging from 6 to 8 and 25mM Tris-HCl was selected for pH 9. The lysis process incorporated 2-mercaptoethanol up to 5mM and sonication was utilized for cell lysis and the CFL was dialysed for up to 40 hours before AEX. SEC was investigated and also incorporated into the purofication process to obtain a purer, more active protein.

Table 9: Comparison of ASNase activity of protein preparation lysed and purified at pH 6, 7, 8 and 9

	pH 6.0	**pH 7.0**	**pH 8.0**	**pH 9.0**
	ASNase (IU/mg)	ASNase (IU/mg)	ASNase (IU/mg)	ASNase (IU/mg)
CFL	12.9 ± 3.7	32.6 ± 5.3	24.6 ± 3.8	10.3 ± 4.3
AEX	123.6 ± 15.8	200.6 ± 16.8	49.75 ± 4.9	5.2 ± 1.8
SEC	0.1	342.25 ± 13.7	151.25 ± 21.5	0.1

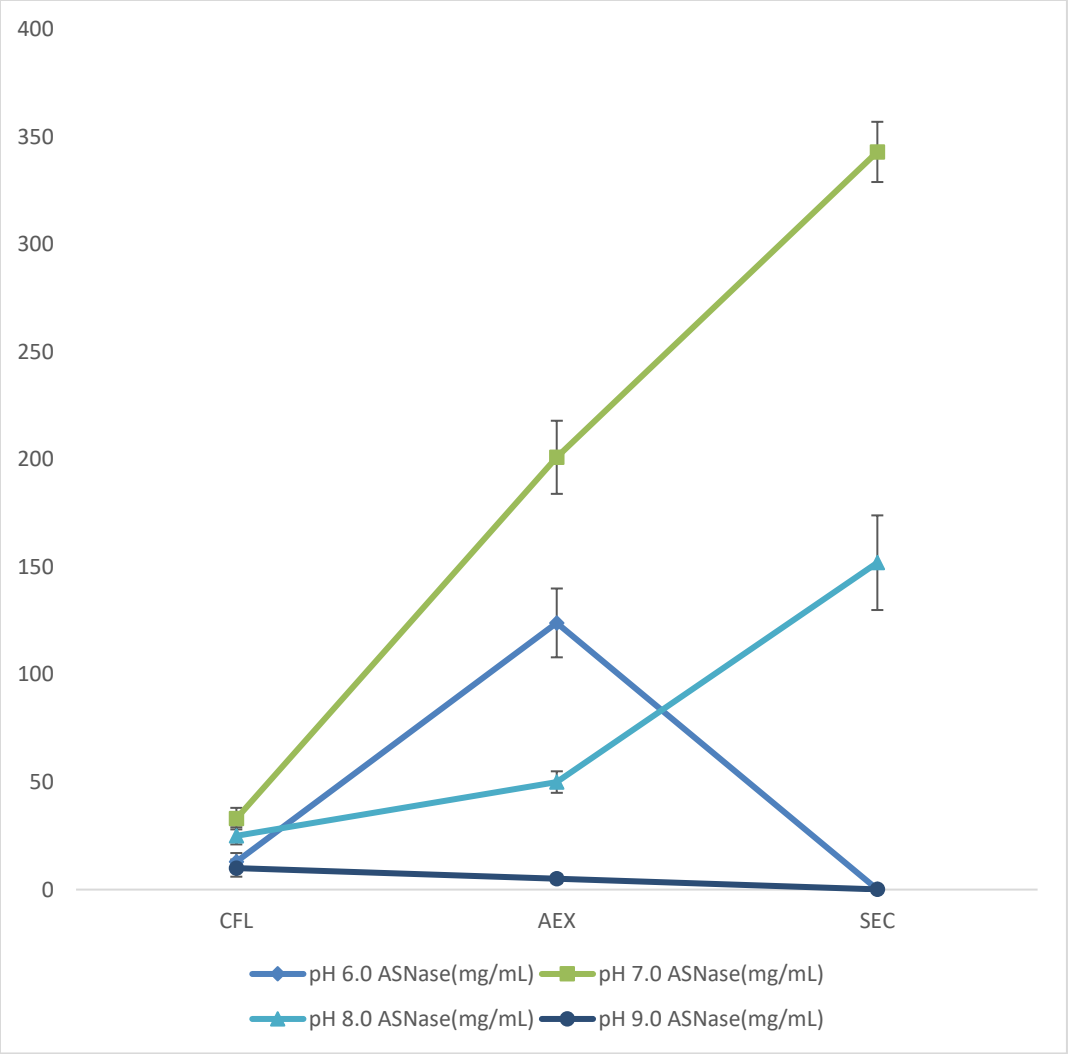


Figure 11: Comparison of ASNase activity of protein preparations obtained from induced cells of R. rhodochrous DAP 96253 lysed and purified using AEX and SEC at varying pHs.

,3.6 Protein purification incorporating sonication as a means of lysis, 2-mercaptoethanol into the lysis buffer, dialysis for 40 hours and AEX and SEC at pH 7.6

Under the conditions employed, it was established based on these results that the optimum pH for purification lay around pH 7.0. Etna Sanchez investigated the optimum pH, focusing on pH 6.8, 7.0, 7.2, 7.4, 7.8, and 8.0. The data she obtained allowed for us to conclude that purification at pH 7.6 showed the highest and most stable ASNase and GLNase activity. Subsequent purifications were then performed at pH 7.6, incorporating 5mM mercaptoethanol

into the lysis buffer and using sonification as a means of cell lysis. Size exclusion chromatography was also incorporated into the purification process because the purer protein obtained showed improved ASNase and GLNase activity. Using the data garnered thus far, a purification using AEX and SEC was performed at pH 7.6, incorporating 5mM 2-mercapethanol into the lysis buffer and utilizing sonication as the means of cell lysis and the elution obtained was analyzed for ASNase and GLNase activity.

Table 10: ASNase and GLNase activity of protein preparation obtained from the purification of CFL obtained from induced cells of R. rhodochrous DAP 96253. The cells were lysed using PB incorporating 5mM βME and sonication and the purification process involved AEX and SEC

	Total activity (IU/mL)		**Specific activty (IU/mg)**		**Fold purification**		**Percentage yield**	
	ASNase	GLNase	ASNase	GLNase	ASNase	GLNase	ASNase	GLNase
CFL	2308	3107	136	183	1	1	100	100
AEX	2008	3032	386	583	4	3	87	98
SEC	1790	2607	1492	2173	23	20	78	84

3.7 Protein characterization (Temperature)

The purification obtained using the variables analyzed above was characterized to determine the stability of the protein preparation at temperature range of 4, 10, 15, 25, 30, 37, 42 and 50°C. The protein prepration was aliquoted into small fractions and incubated at these temperatures for up to an hour. The ASNase and GLNase activity of the samples were analyzed peridoically as a measure of protein stability.

Table 11: Shows the varying ASNase/ GLNase activity of the purified protein obtained from induced cells of R. rhodochrous DAP 96253 after incubation for 15 minutes at varying temperatures.

Temperature (°C)	ASNase (IU/mg)	GLNase (IU/mg)
4	251.4 ± 33.5	999.5 ± 23.2
10	190.9 ± 31.2	878.47 ± 28.8
15	105.23 ± 16.95	219.96 ± 5.1
25	244.87 ± 29.01	290.1 ± 12.39
30	420.27 ± 20.82	873.64 ± 53.1
37	466.7 ± 12.58	1291.22 ± 219.6
42	303.25 ± 27.63	518.74 ± 21.76
50	223.4 ± 2.79	470.18 ± 58.8

Table 12: Shows the varying ASNase/ GLNase activity of the purified protein obtained from induced cells of R. rhodochrous DAP 96253 after incubation for 30 minutes at varying temperatures.

Temperature (°C)	ASNase (IU/mL)	GLNase (IU/mL)
4	552.4 ± 73.7	1122.5± 48.64
10	498.2 ± 87.28	1376.58 ± 92.78
15	554.04 ± 3.1	766.94 ± 61.26
25	572.14 ± 7.4	775.4 ± 68.9
30	590.03 ± 90.74	853.56 ± 88.48
37	632.15 ± 47.2	1454.6 ± 135.7
42	488.2 ± 59.9	776.5 ± 47.7

50	416.61 ± 78.66	475.1 ± 31.86

Table 13: Shows the varying ASNase/ GLNase activity of the purified protein obtained from induced cells of R. rhodochrous DAP 96253 after incubation for 60 minutes at varying temperatures.

Temperature ℃)	**ASNase (IU/mL)**	**GLNase (IU/mL)**
4	417.14 ± 11.7	849.23 ± 147.9
10	191.1 ± 8.9	477.34 ± 78.8
15	292. 82 ± 2.81	1230.71 ± 68.9
25	349.33 ± 25.7	1340.41± 58.64
30	368.9 ± 21.8	2016.6 ± 10.57
37	406.84 ± 4.96	2154.59± 251.34
42	196.7 ± 40.1	1299.52 ± 8.91
50	177.3 ± 28.7	640.1 ± 15.95

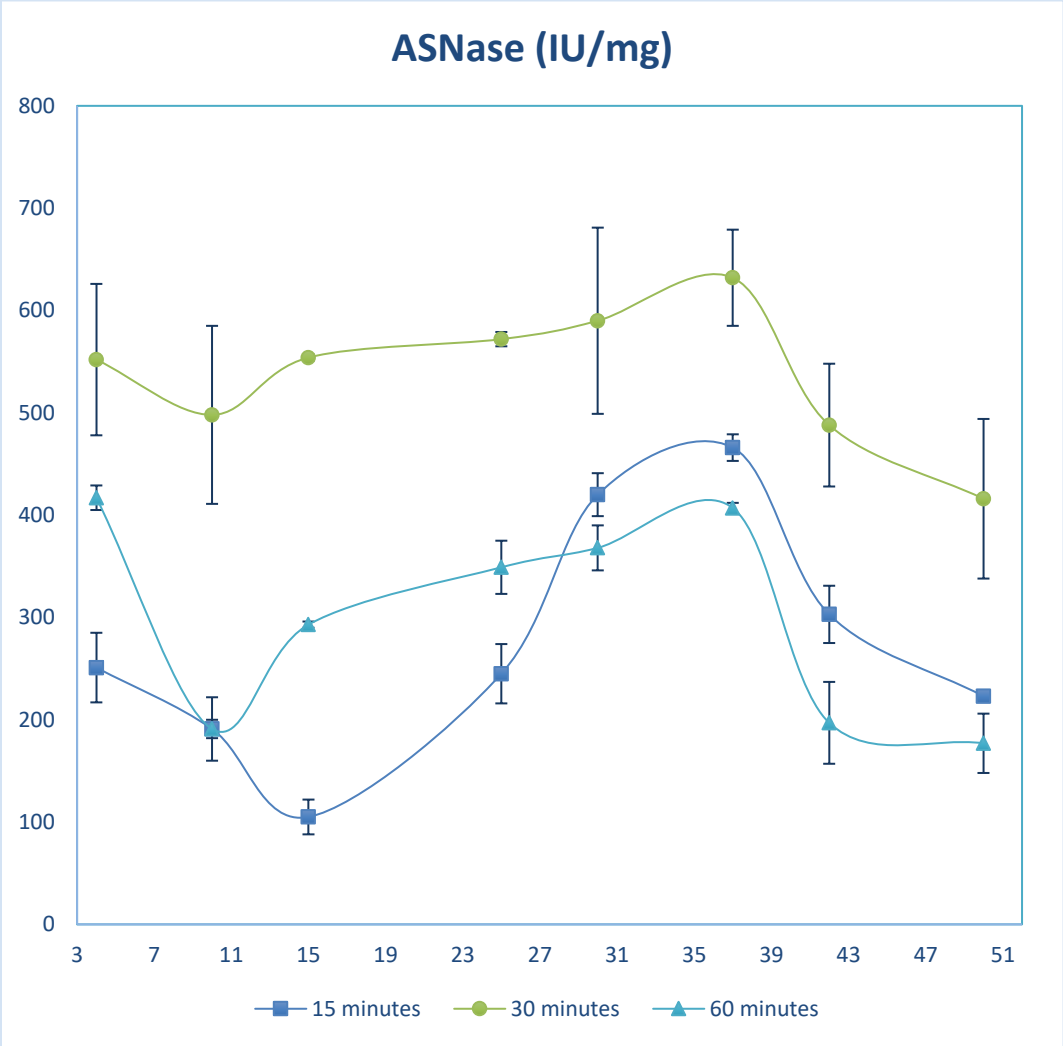


Figure 12: Shows stability of ASNase purified from induced cells of R. rhodochrous DAP 96253 at varying temperatures ranging from 4℃ to 50℃

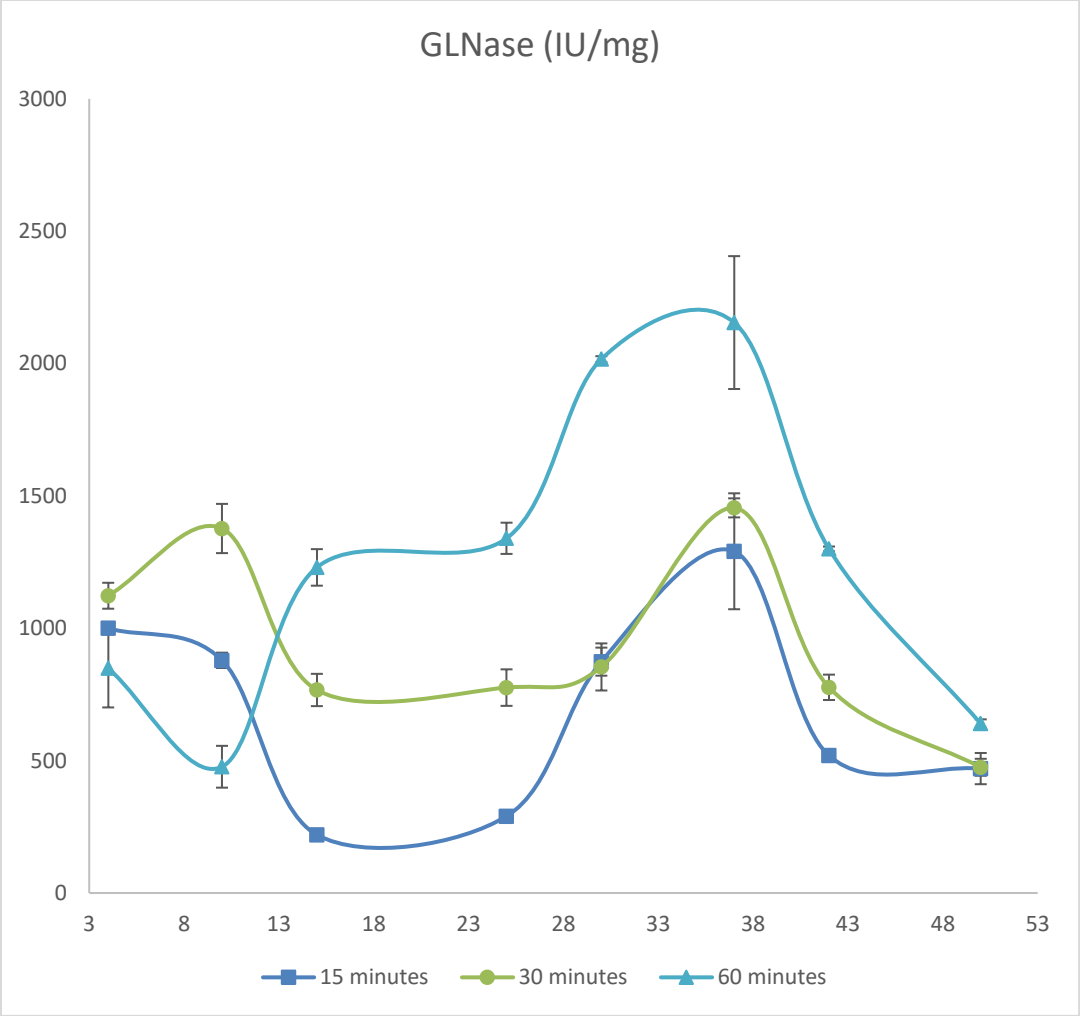


Figure 13: Shows stability of ASNase purified from induced cells of R. rhodochrous DAP 96253 at varying temperatures ranging from 4℃ to 50℃

3.8 Protein characterization (pH)

The purification obtained using the variables analyzed above was characterized to determine the stability of the protein preparation at pH range of 3, 4, 5, 6, 7, 8, 9, and 10. pH 7.2, 7.4, 7.6, and 7.8 were also analysed because previous studies showed the protein to be most stable at pH ranging frrom 7 to 8. The protein prepration was aliquoted into small fractions and incubated at these pHs for up to 25 hours. The ASNase and GLNase activity of the samples were analyzed as a measure of protein stability.

Table 14: Shows the varying ASNase/ GLNase activity of the purified protein after incubation at varying pHs for 25 hours

pH	ASNase (IU/mg)	GLNase (IU/mg)
3	195 ± 34	1199 ± 148
4	497 ± 62	1667 ± 257
5	553 ± 99	1910 ± 107
6	803 ± 77	2347 ± 115
7	966 ± 18	2502 ± 148
7.2	967 ± 20	2505 ± 145
7.4	1226 ± 133	2654 ± 133
7.6	2491 ± 115	3995 ± 86
7.8	762 ± 28	2310 ± 54
8	143 ± 20	1753 ± 258
9	48 ± 10	1173 ± 200
10	0	44 ± 11

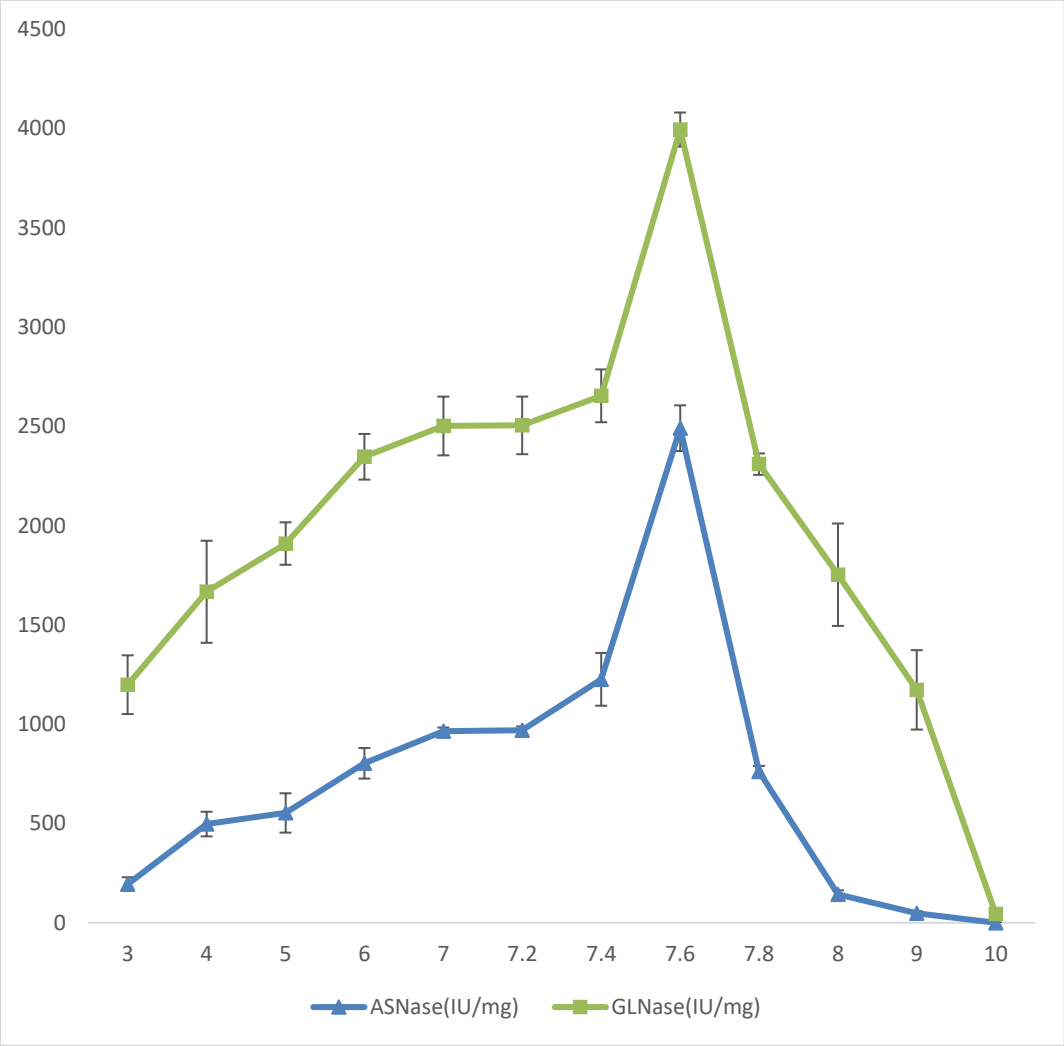


Figure 14: Shows the relative ASNase and GLNase activity of protein preparation purified from induced cells of R. rhodochrous DAP 96253 at pH ranging from 3 to 10 with optimum ASNase and GLNase activity observed at pH 7.6

3.9 Molecular weight determination of the purified protein sample

Samples from the lysis, AEX and SEC were analyzed for purity and molecular weight determination using an SDS-PAGE. Samples were loaded on the gel and after the run was completed, the gels were stained using Coomassie brilliant blue dye and destained. The gel image was developed and analyzed to determine protein purity with each successive purification.

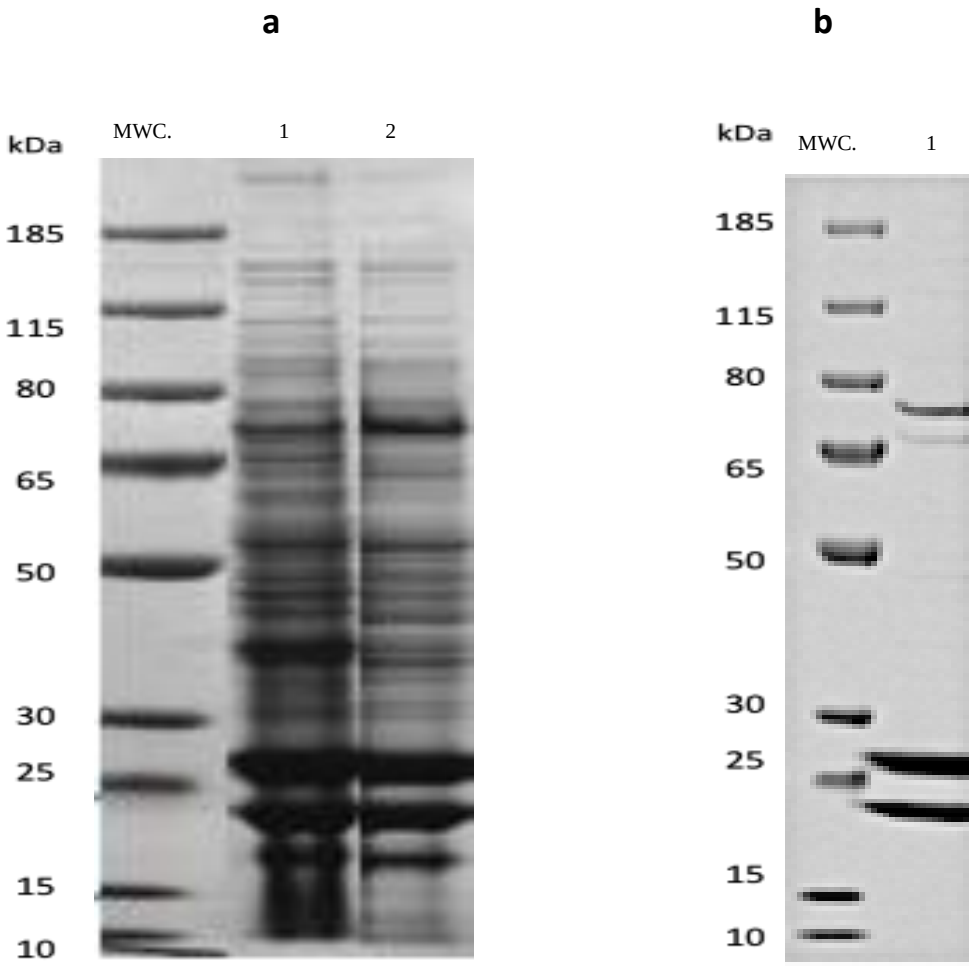


Figure 15: SDS- PAGE for protein preparation from induced cells of R. rhodochrous DAP 96253. (a) shows the CFL in lane 1 and the AEX fraction in lane 2. (b) shows protein bands from SEC fraction with ASNase and GLNase activity.

3.10 Stability of protein preparation in sterile human serum

The purified protein samples were incubated in sterile human serum at a 50 IU/ml concentration and analyzed for ASNase activity using the modified Fawcett and Scott method periodically until a 50% reduction in initial enzyme activity had been determined.

Table 15: Demonstrates the stability of ASNAse activity over time in sterile human serum

Time (hours)	ASNasse (IU/mL)
0	50.35 ± 0.5
5	48.34 ± 0.3
10	42.8 ± 1.7
12	39.27 ± 1.2
24	32.73 ± 0.8
29	31.72 ± 0.4
34	30.71 ± 0.6
48	29.2 ± 1.6
53	26.68 ± 0.2
56	23.66± 1.4

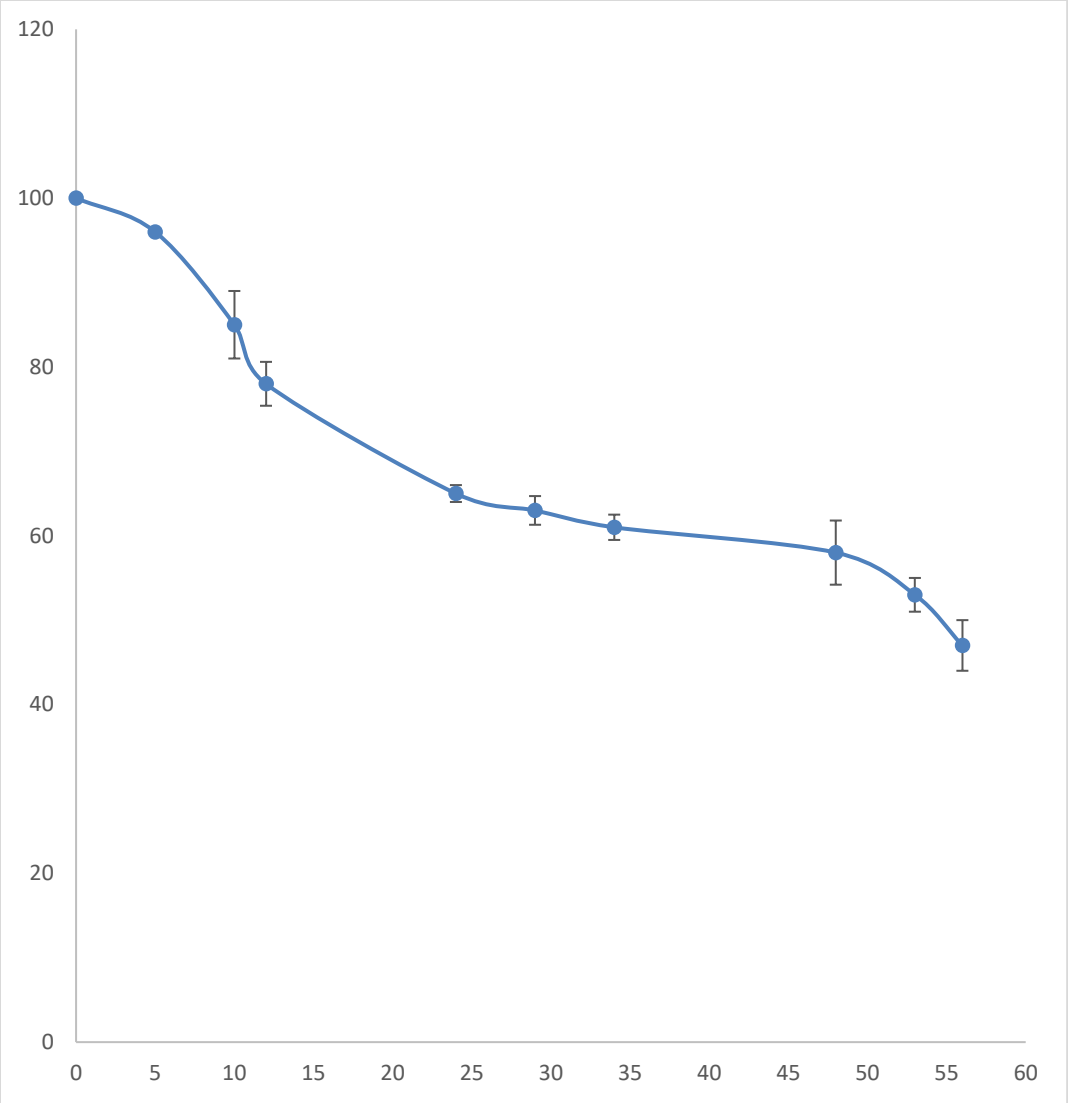


Figure 16: Shows the relative stability of ASNase purified from the induced cells of R. rhodochrous DAP 96253 in sterile human serum over time.

3.11 Determination of endotoxin concentrations of *R. rhodochrous* DAP 96253 and its purified products

R. rhodochrous DAP 96253 is a Gram-positive bacteria and therefore lacking in LPS. One of the proposed benefits to using a an ASNase/ GLNase protein preparation obtianed from *R. rhodochrous* DAP discussed earlier is the absence of endotoxins and thus none of the negative immunological responses associated with Gram-negative bacterial products.

Table 16: Shows the endotoxin concentration levels of samples obtained from the lysis of induced cells of R. rhodochrous DAP 96253 and from the purification using AEX and SEC as well as an endotoxin control using E. coli strain K12

Sample	**Endotoxin concentration (EU/mL)**
***R. rhodochrous* DAP 96253 CFL**	4.20 ± 0.67
***R. rhodocrous* DAP 96253 CFL(pruified using AEX)**	21.01 ± 2.43
***R. rhodochrous* DAP 96253 CFL (purified using AEX and SEC)**	107.63 ± 11.72
***E. coli* K12 CFL**	3239.68 ± 95.86

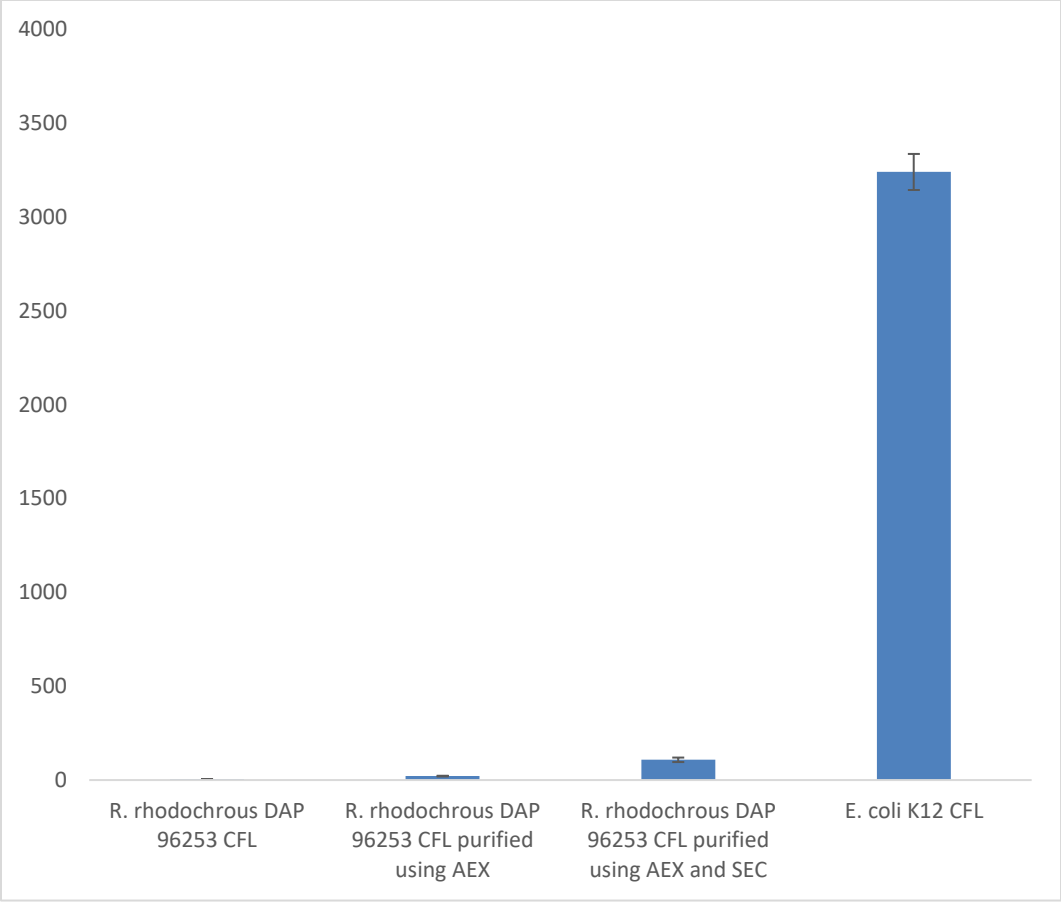


Figure 17: Comparison of the endotoxin levels of the CFL obtained from induced cells of R. rhodochrous DAP 96253 before and after purification using just AEX and AEX and CFL and the CFL obtained from E. coli K12

3.12 Substrate affinity and maximal velocity of protein preparation.

The substrate affinity of the protein preparation exhibiting stable ASNase and GLNase activity was determined using the substrate ASN and GLN in varying concentrations. This study was performed to determine how effective the protein preparation will be as a treatment measure for ALL. Substrate concentrations analyzed ranged from 0.05 to 26.6mM. The substrate affinity (*Km*) for ASN was determined based on the graph below (fig 17) to be 0.0136mM and the maximal velocity (V_{max})was determined to be 5µmoles/ minute.

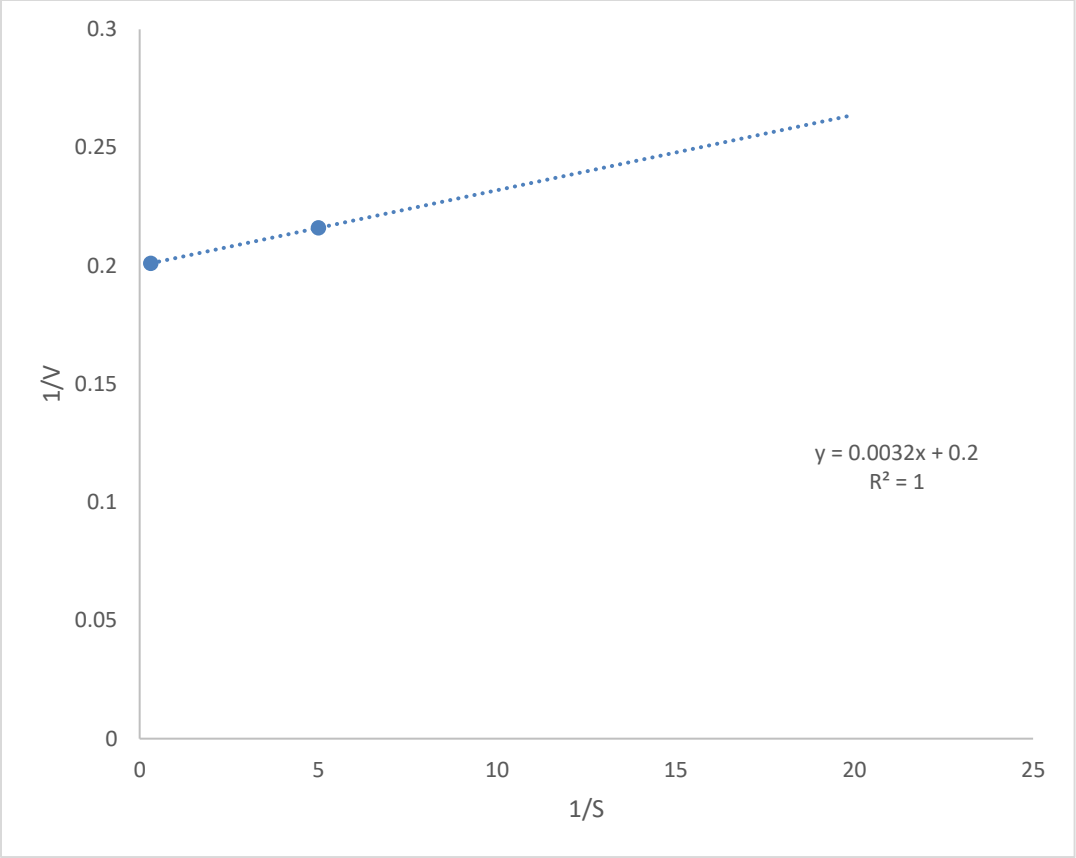


Figure 18: Line-Weaver Burke plot utilized in the determination of substrate affinity and maximal velocity for ASN of the protein preparation obtained from induced cells of R. rhodochrous DAP 96253 exhibiting ASNAse activity.

The *Km* for GLN was determined to be signficantly higher than the Km observed for ASN at 0.85mM and a V_{max} of 61.3µmoles per minute.

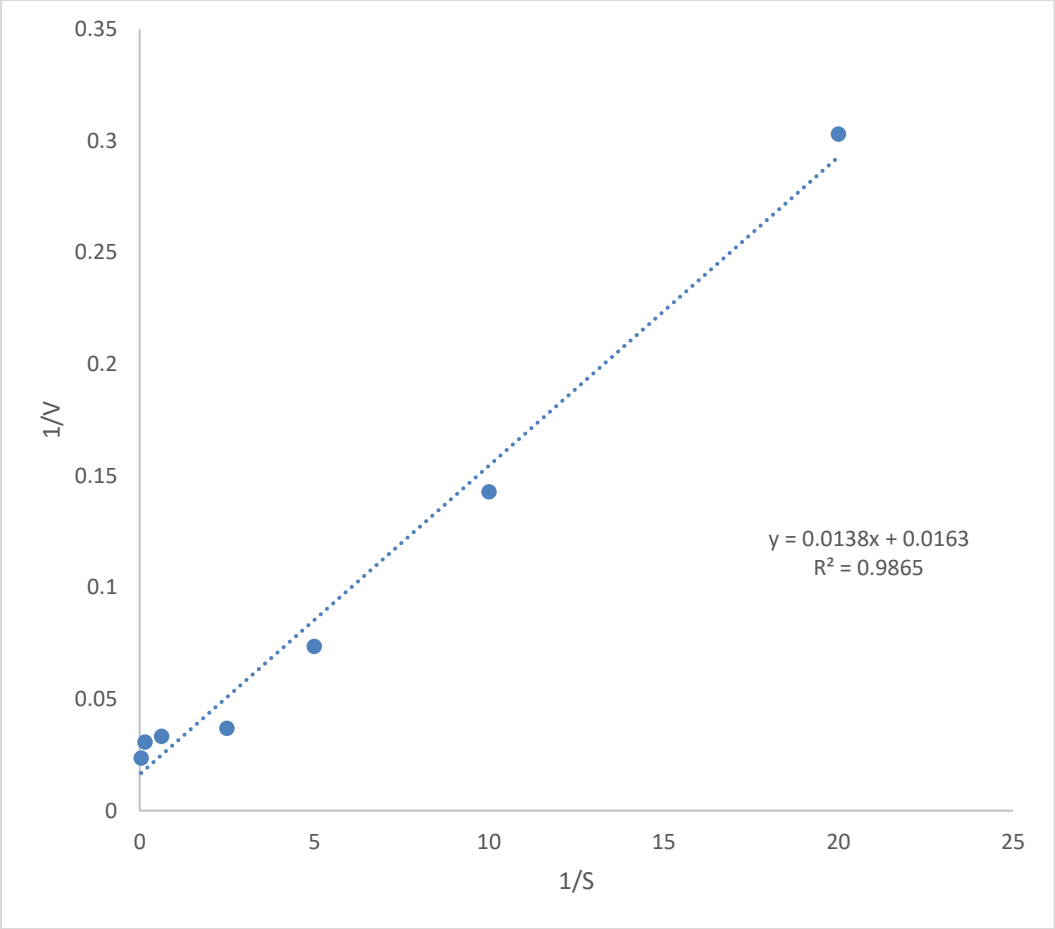


Figure 19: Line-Weaver Burke plot utilized in the determination of substrate affinity and maximal velocity for GLN of the protein preparation obtained from induced cells of R. rhodochrous DAP 96253 exhibiting GLNAse activity.

3.13 Effect of selected chemicals on ASNase activity of protein preparations purified from induced cells of *R. rhodochrous* DAP 96253

The effect of selected chemical substances in varying concentrations (table 1) was evaluated. The effect of some of these chemicals had already been evaluated in other studies and comparisons were made.

Table 17: Shows the ASNase activities of the untreated purification and samples treated with selected chemicals to demonstrate the effect of these chemicals on the purified protein.

	Name	**ASNase activity (IU/mg)**
1	Untreated enzyme	1219.35 ± 24.38
2	Zinc chloride ($ZnCl_2$)	914.51 ± 14.63
3	Magnesium chloride ($MgCl_2$)	341.42 ± 48.77
4	Silver nitrate ($AgNO_3$)	524.32 ± 12.32
5	2- Mercaptoethanol (βME)	951.09 ± 19.75
6	Iron II sulfate ($FeSO_4$)	1560.77 ± 32.92
7	Copper sulfate ($CuSO_4$)	158.51 ± 3.65
8	Hydrogen peroxide (H_2O_2)	877.89 ± 17.07
9	Ammonium persulfate ($(NH_4)_2S_2O_8$)	1024.25 ± 20.97
10	Calcium chloride ($CaCl_2$)	829.16 ± 11.94
11	Cobalt chloride ($CoCl_2$)	755.99 ± 13.95
12	Manganese sulfate ($MnSO_4$)	963.29 ± 13.66
13	Sodium phosphate buffer	1877.80 ± 30.48

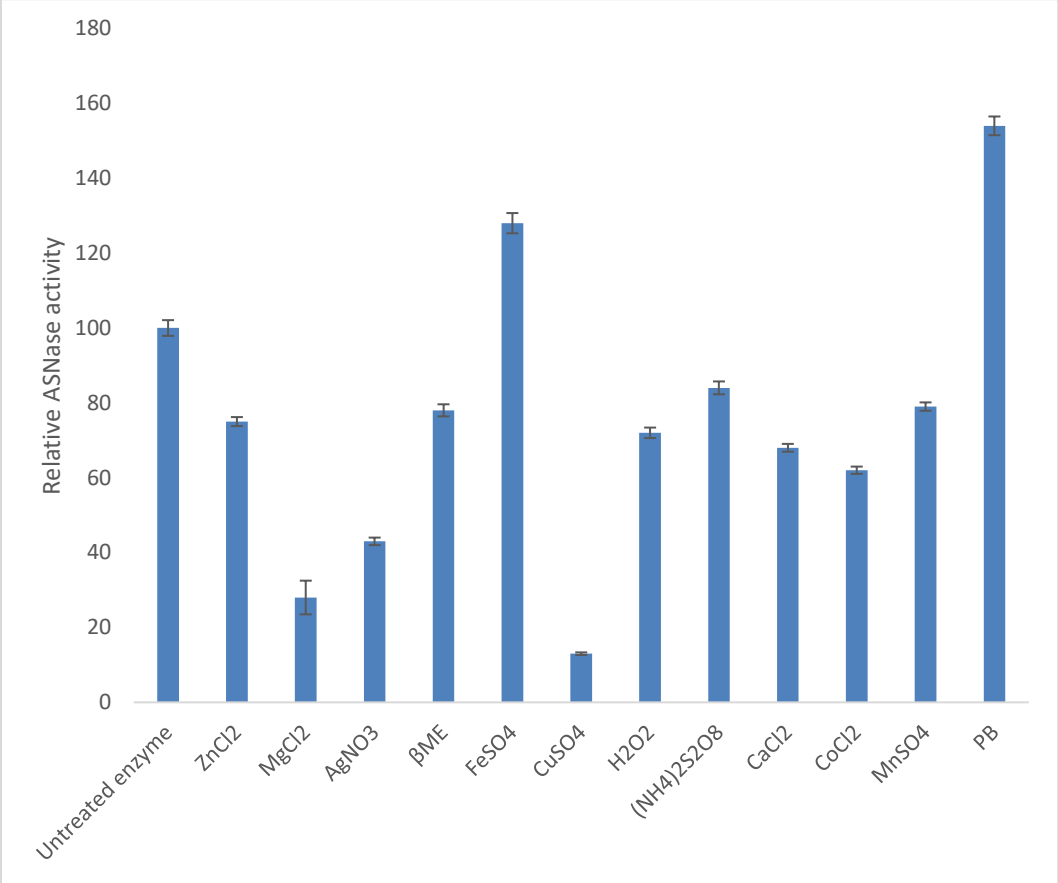


Figure 20: Evaluation of the effect of selected chemicals on the ASNase activity of protein preparation purified from induced cells of R. rhodochrous DAP 96253

3.14 The effect of protein preparations exhibiting ASNase and GLNase activity on leukemia expressing cell lines

Having determined that stability of the purified protein preparation obtained from induced cells of *R. rhodochrous* DAP 96253 in sterile human serum, at varying pHs, temperatures, the substrate affinity with ASN and GLN and the maximal velocities, the purified samples were utilized in varying doses to treat leukemia expressing cells. The setup was

performed in sterile well plates and treated in doses varying from 1x to 4x with controls incorporating up to 100µL of sterile purification buffers (Table 2). The rate of cell growth, death or survivability was determined by staining cell samples with trypan blue solution and counting using a nexcelom cell counter every 24 hours post incubation. After 96 hours, the cell count was discontinued, and images of the wells were taken at 100x magnification using an inverted icroscope.

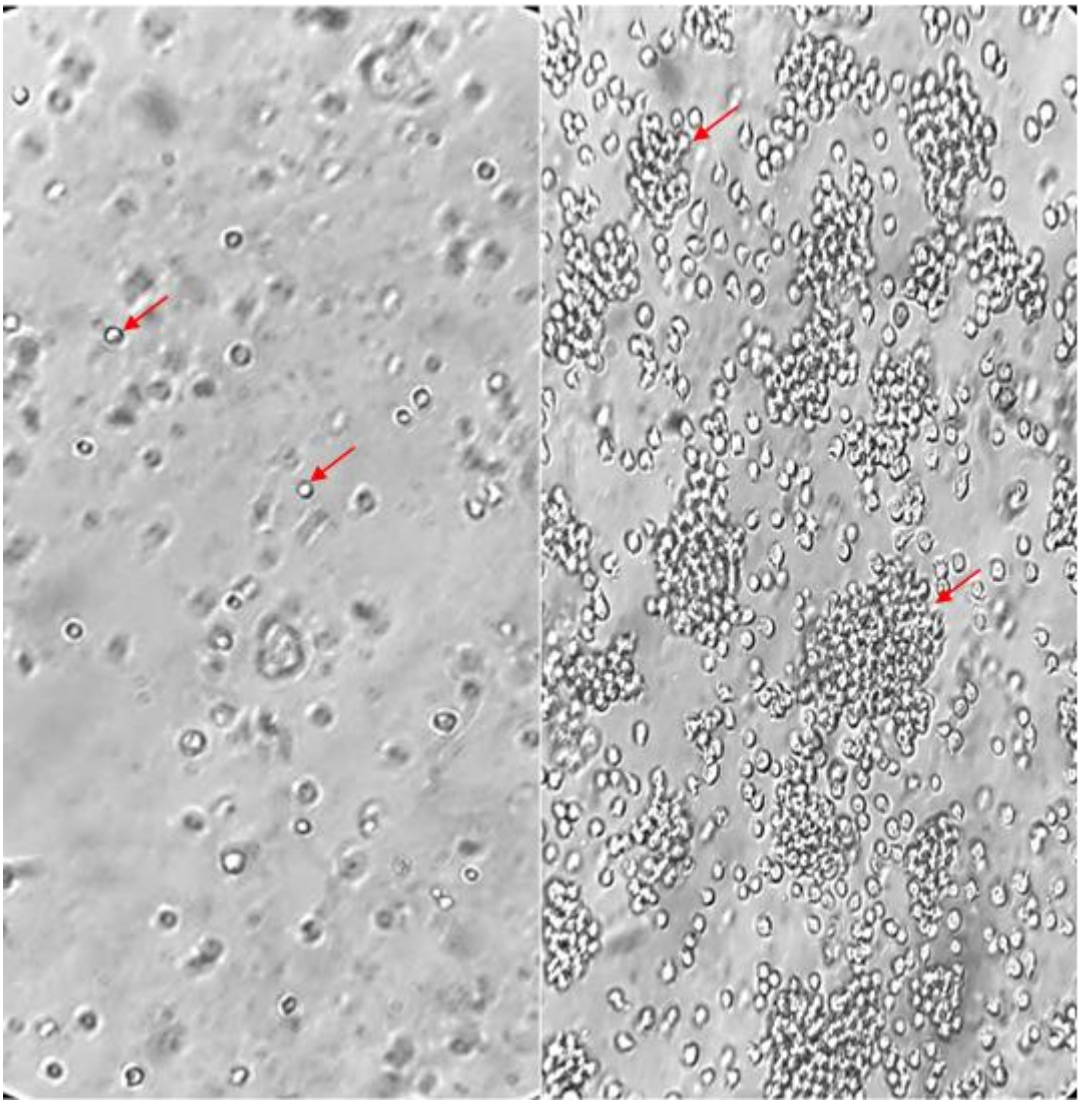


Figure 21: a: Jurkat E6-1 cells shortly after incubation at a concentration of 1x105 cells/mL in complete media. b: depicts the increased cell density after 72 hours of incubation demonstrating the typical clusters observed with the Jurkat E6-1 cell line. Image of cells in a culture flask was observed using an inverted microscope at 1000x magnification

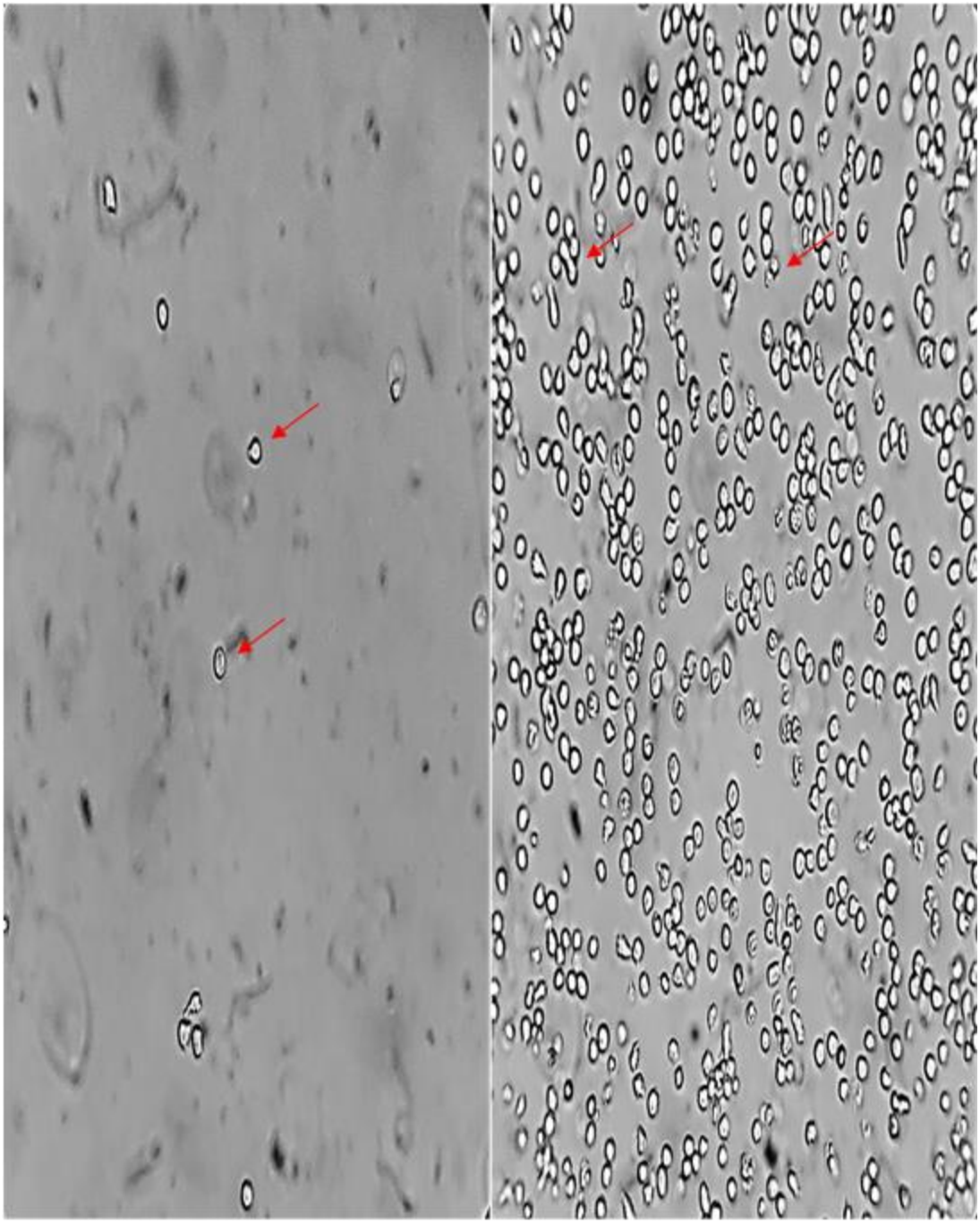


Figure 22: Molt-4 cells shortly after incubation in complete media. b; depicts the increased cell density after 72 hours of incubation. Image was observed using an inverted microscope at 10000x magnification.

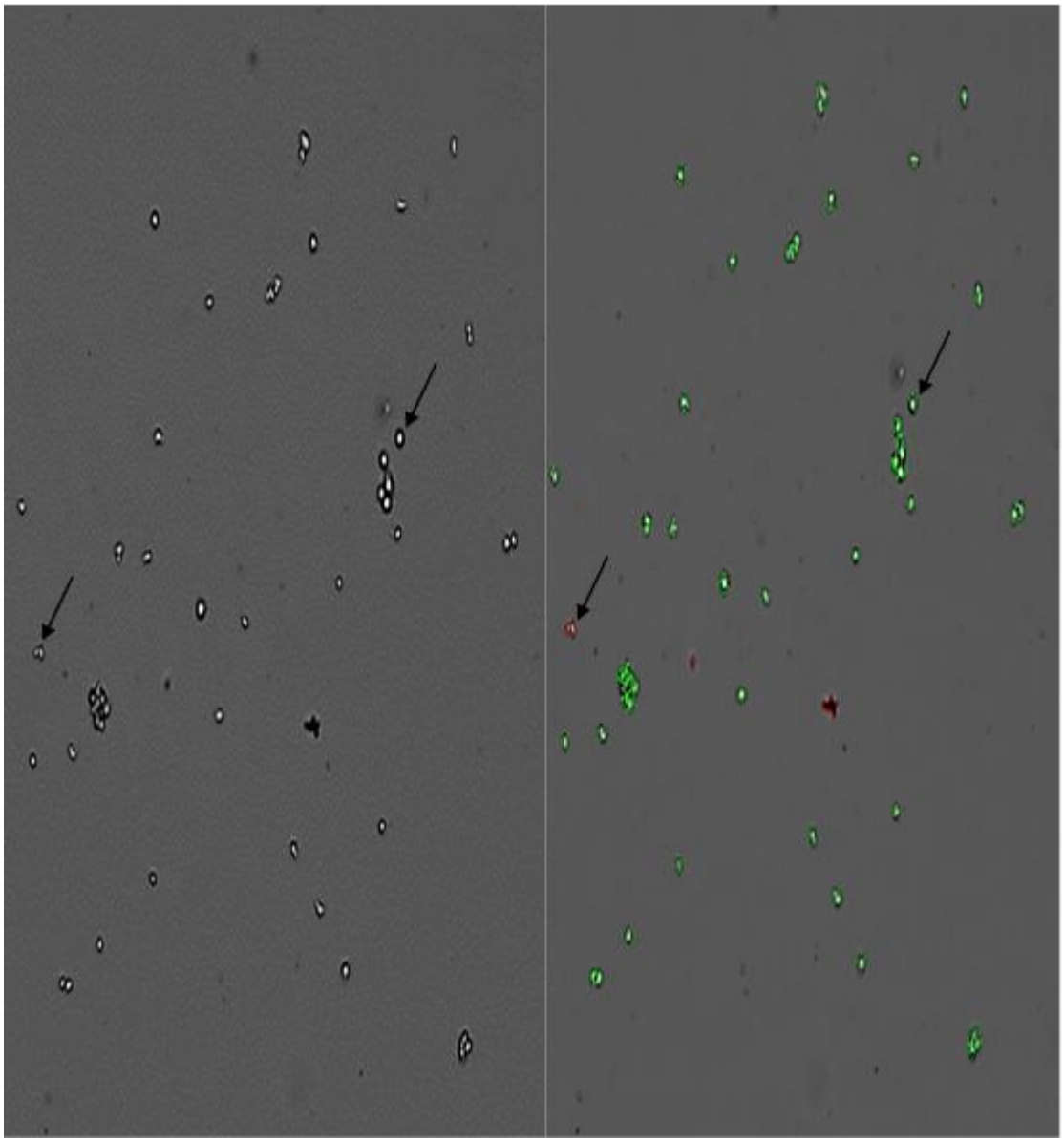


Figure 23: Depiction of the views observed using the Nexcelom Bioscience cellometer® auto 2000 viability counter. a; describes the cell number and b; depicts the cells that are viable and still capable of replicating as green and non-viable cells as red.

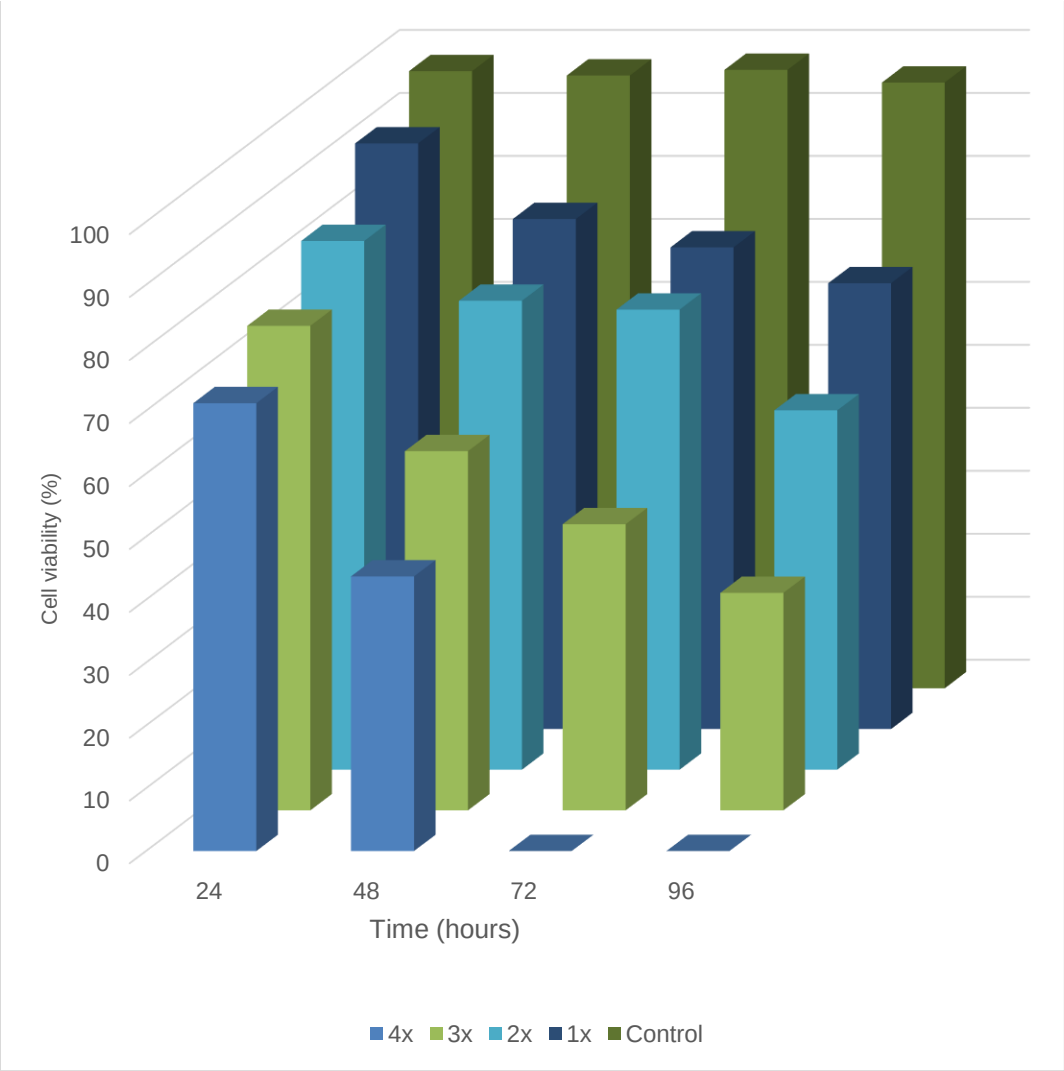


Figure 24: Demonstrates the retardation of Jurkat E6-1 cell viability by the protein preparation obtained from induced cells of R. rhodochrous DAP 96253. Jurkat E6-1 cell viability reduces over time and increased protein dose.

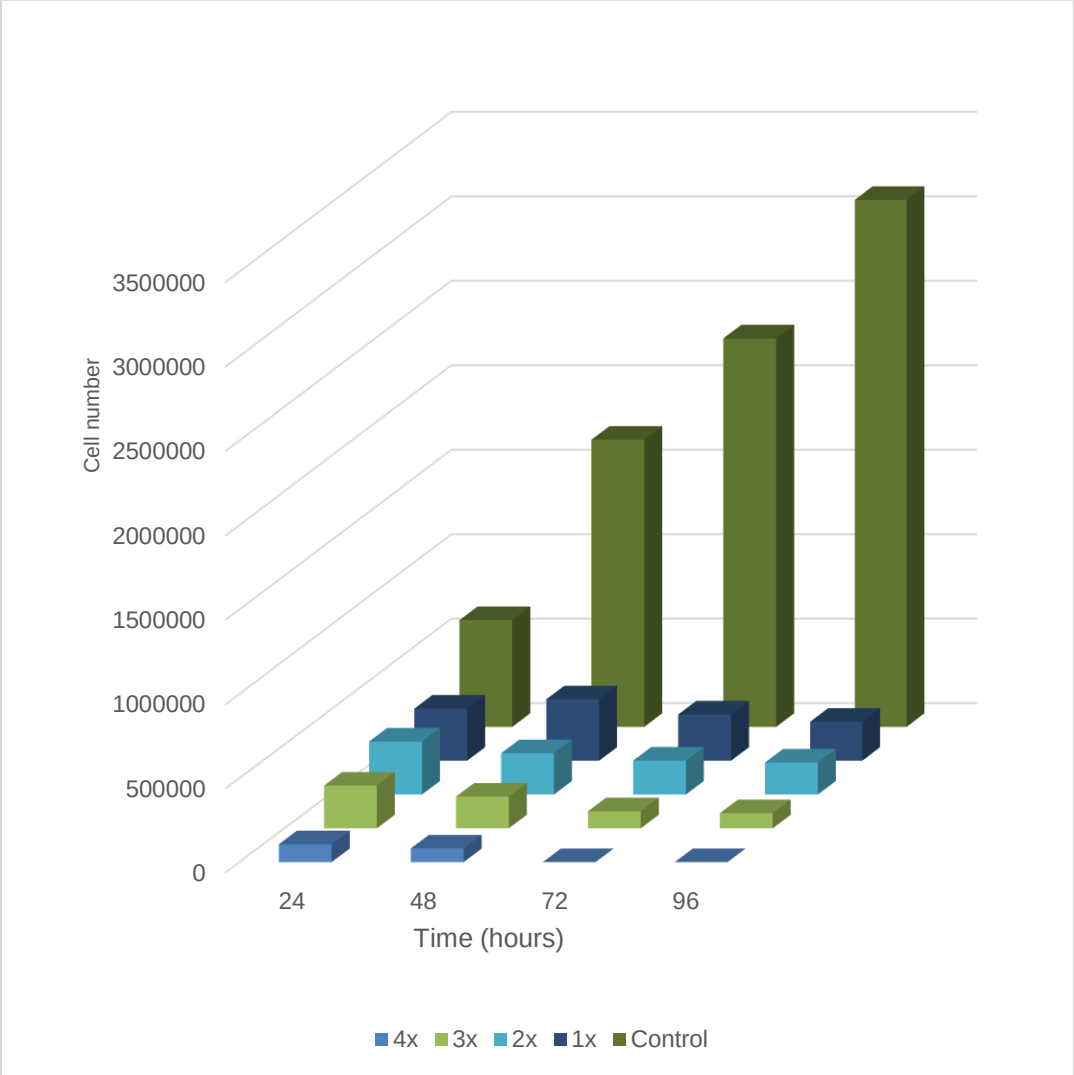


Figure 25: Depicts the cytotoxicity of the purified protein obtained from induced cells of R. rhodochrous DAP 96253 against the Jurkat E6-1 cell line over a 96-hour time period.

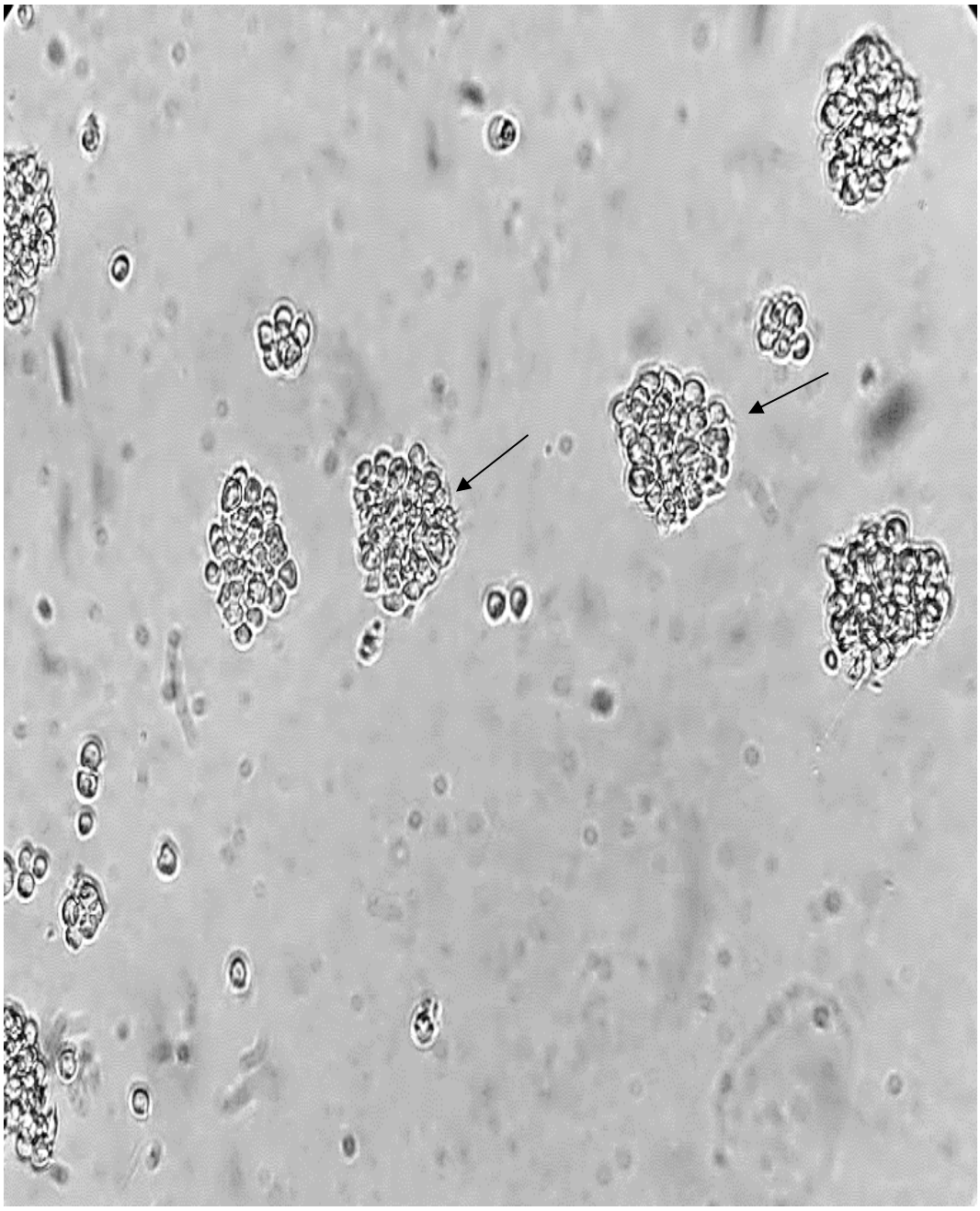


Figure 26: Control setup created by treating Jurkat E6-1 cells with just 100µL of purification buffer. The well was incubated for up to 96 hours with 24-hour periodic cell counts and this image was captured using an inverted microscope.

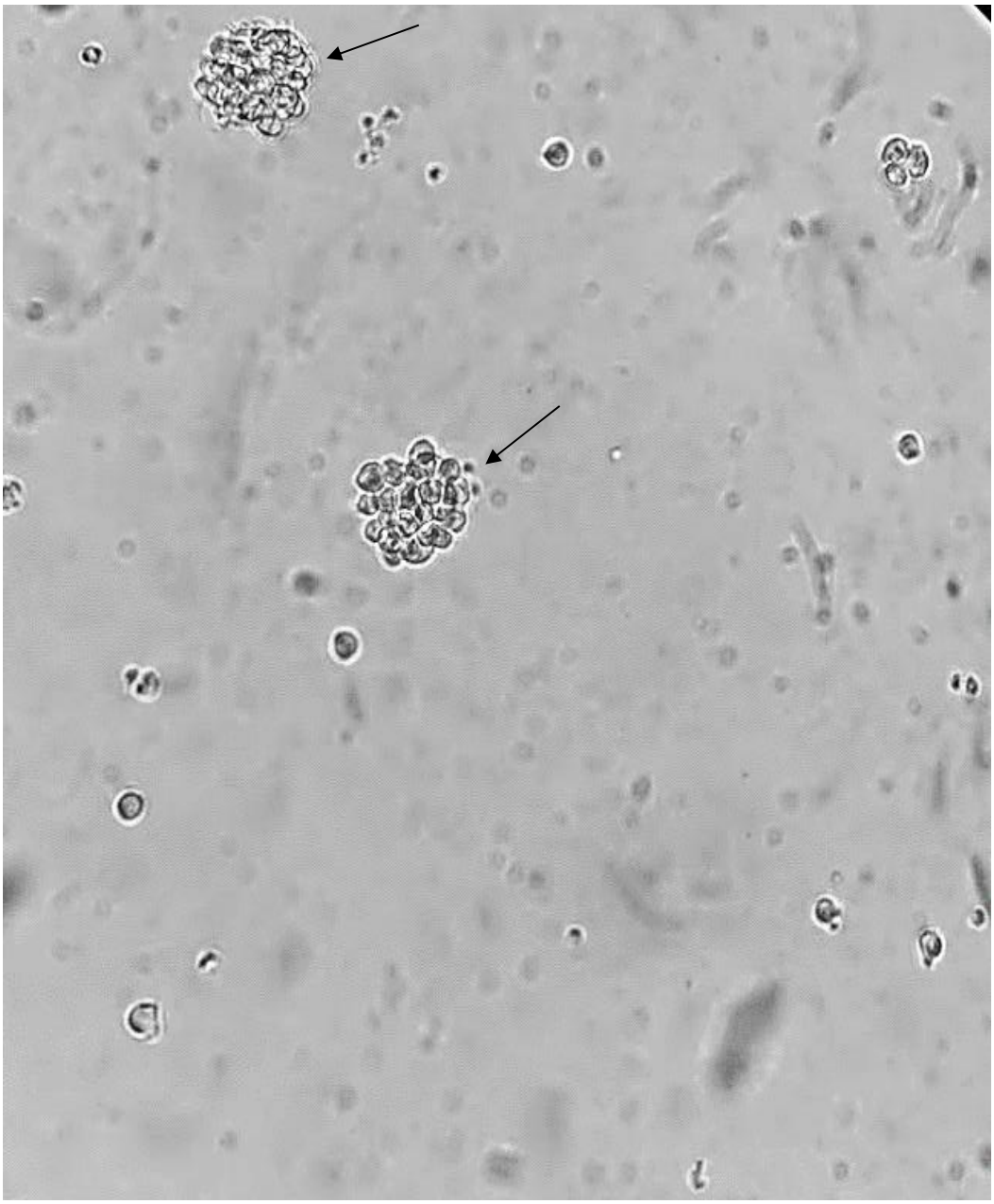


Figure 27: Ix treatment setup demonstrating fewer Jurkat E6-1 cell clusters and reduced cluster size with treatment using the lowest tested dose of purified protein obtained from induced cells of R. rhodochrous DAP 96253.

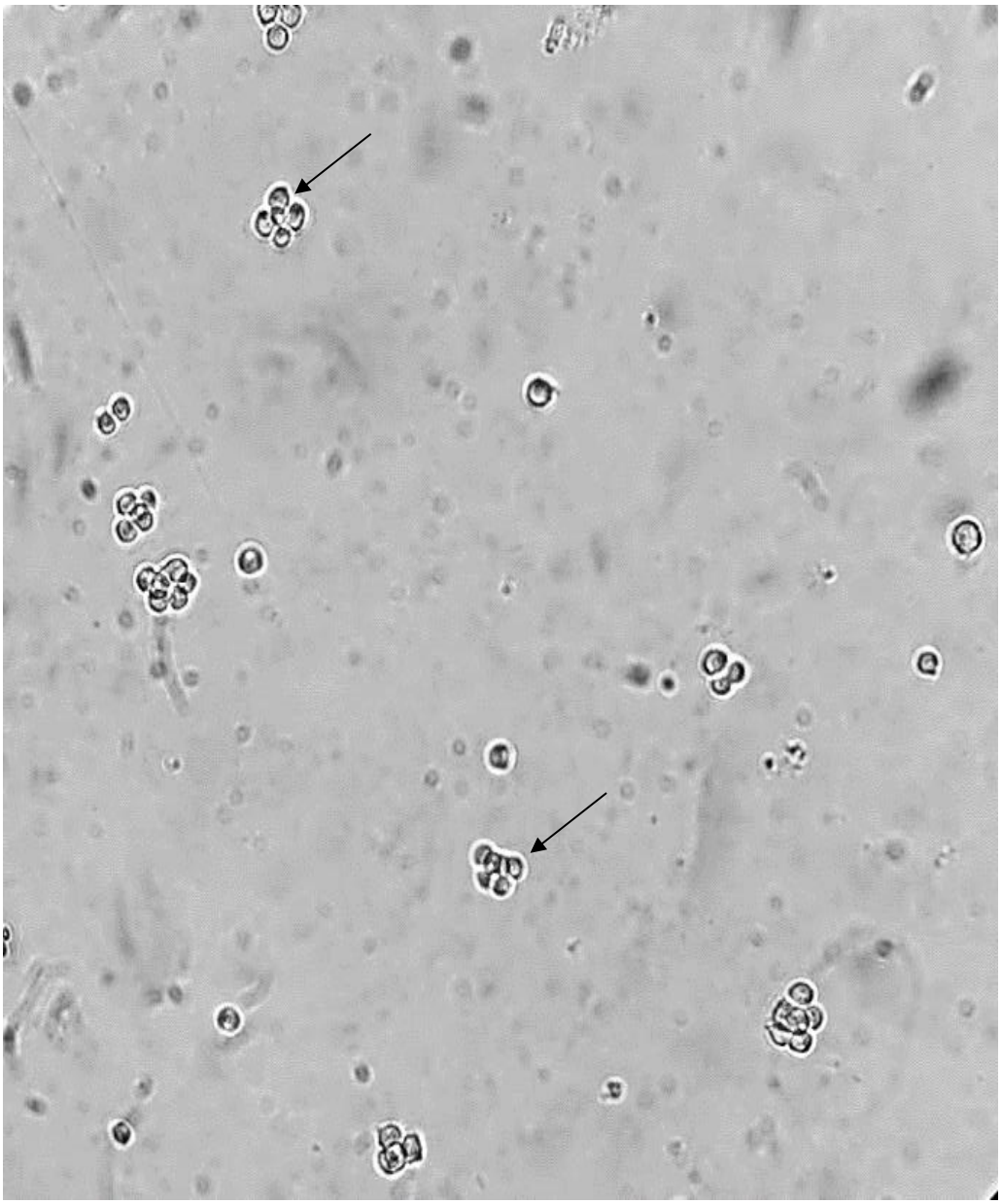


Figure 28: 2x treatment setup demonstrating much smaller Jurkat E6-1 cell clusters with treatment using the purified protein obtained from induced cells of R. rhodochrous DAP 96253.

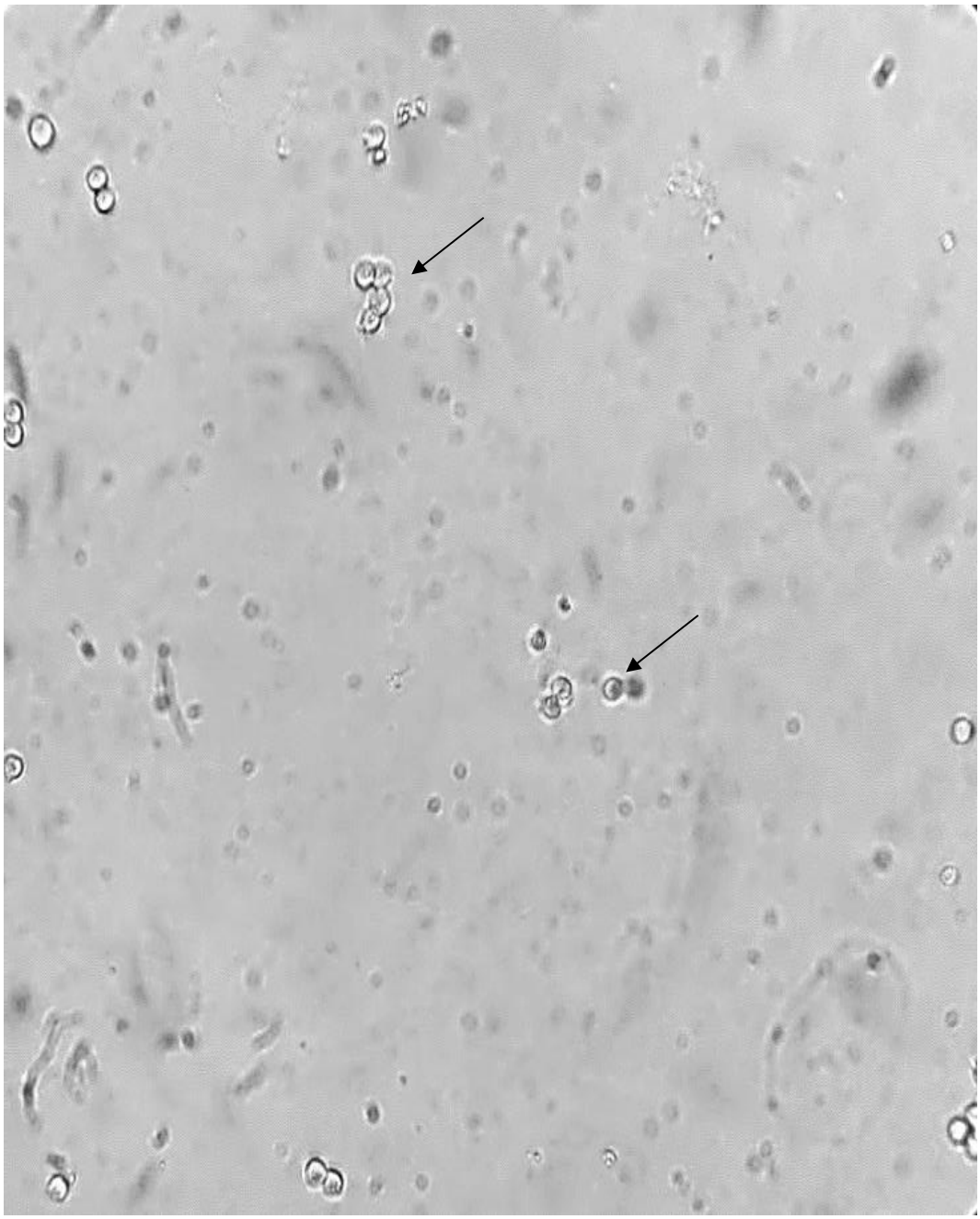


Figure 29: 3x treatment setup demonstrating much smaller and less tightly packed Jurkat E6-1 cell clusters with treatment using the purified protein obtained from induced cells of R. rhodochrous DAP 96253.

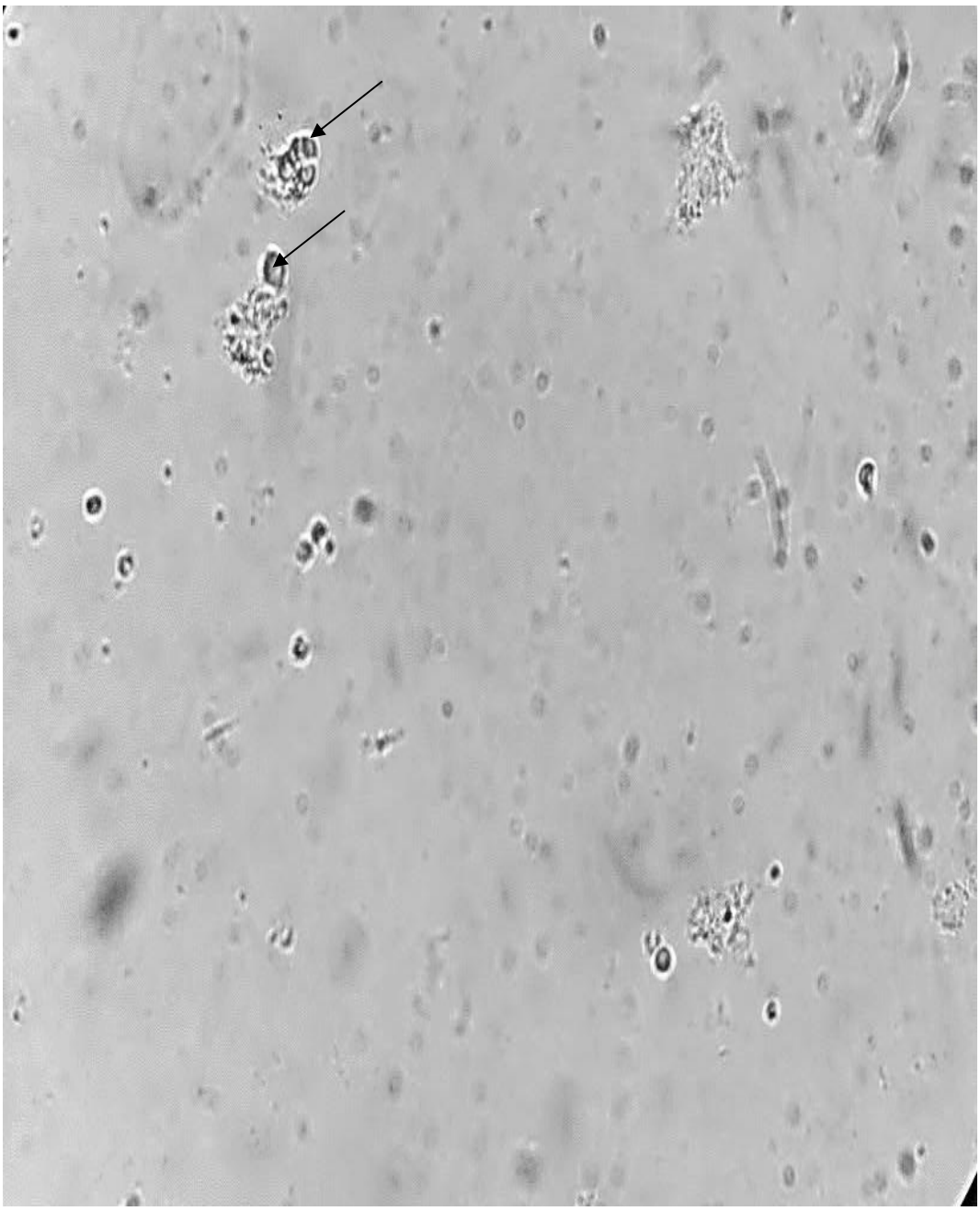


Figure 30: 4x treatment setup demonstrating complete absence of the typical Jurkat E6-1 cell clusters with treatment using the purified protein obtained from induced cells of R. rhodochrous DAP 96253.

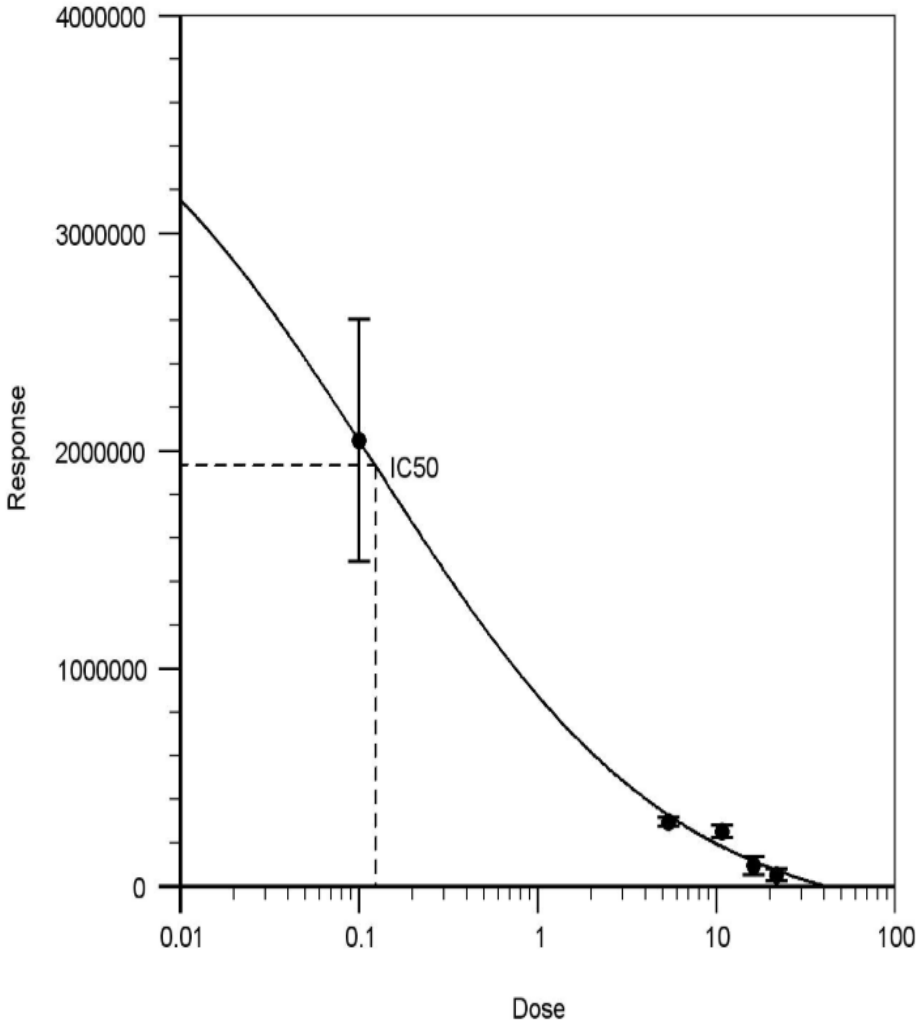


Figure 31: Demonstrating the IC_{50} of the protein preparation obtained from induced cells of R. rhodochrous DAP 96253 utilized in the treatment of Jurkat E6-1 cells

The IC_{50} of the protein preparation purified using AEX and SEC and obtained from induced cells of *R. rhodochrous* DAP 96253 against Jurkat E6-1 cells expressing acute leukemia was determined to be 0.123 IU/mg.

The entire process was repeated utilizing a different cell line, Molt-4 cell line expressing ALL was utilized in a similar protein setup using different protein concentrations detailed in table 2 above.

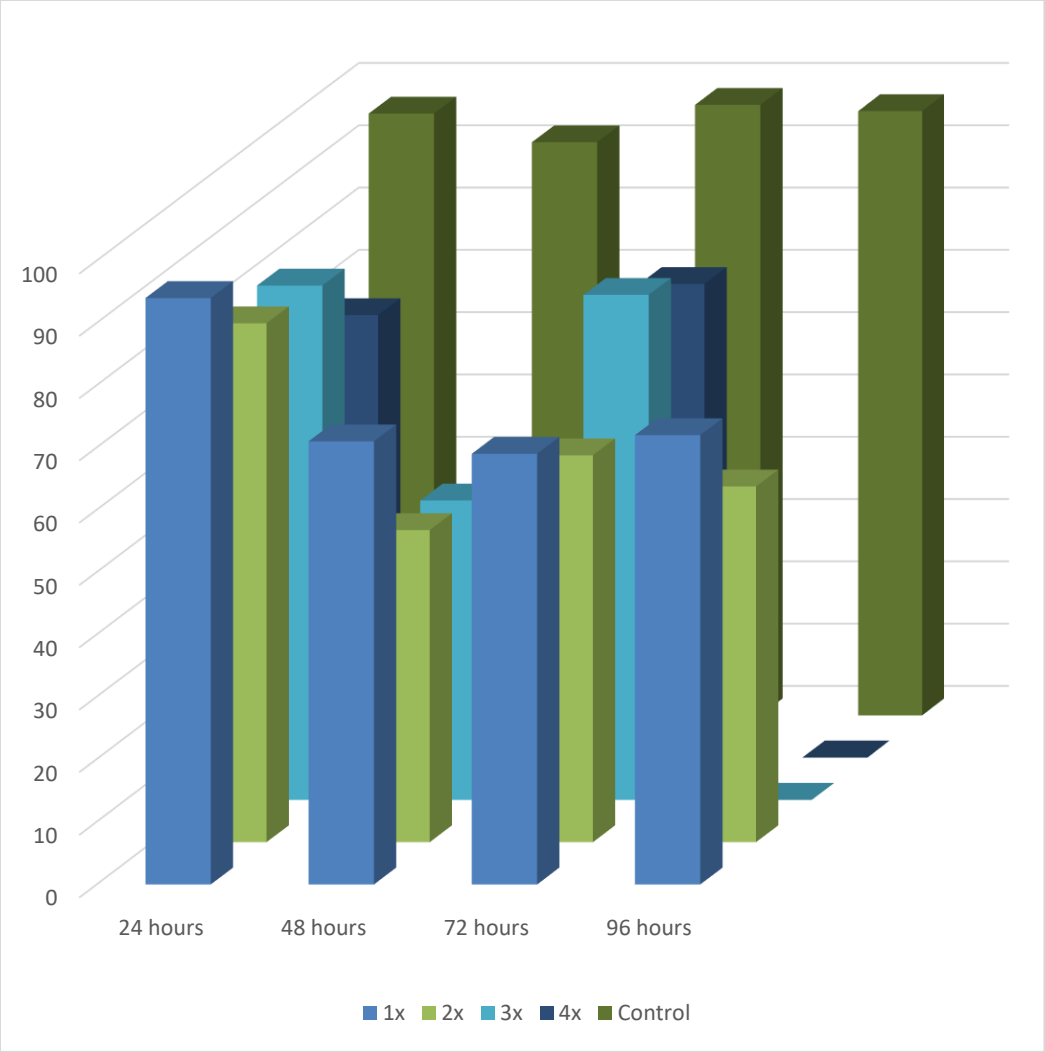


Figure 32: Demonstrates the effect on Molt-4 cell viability of the protein preparation obtained from induced cells of R. rhodochrous DAP 96253. Jurkat E6-1 cell viability reduces over time and increased protein dose.

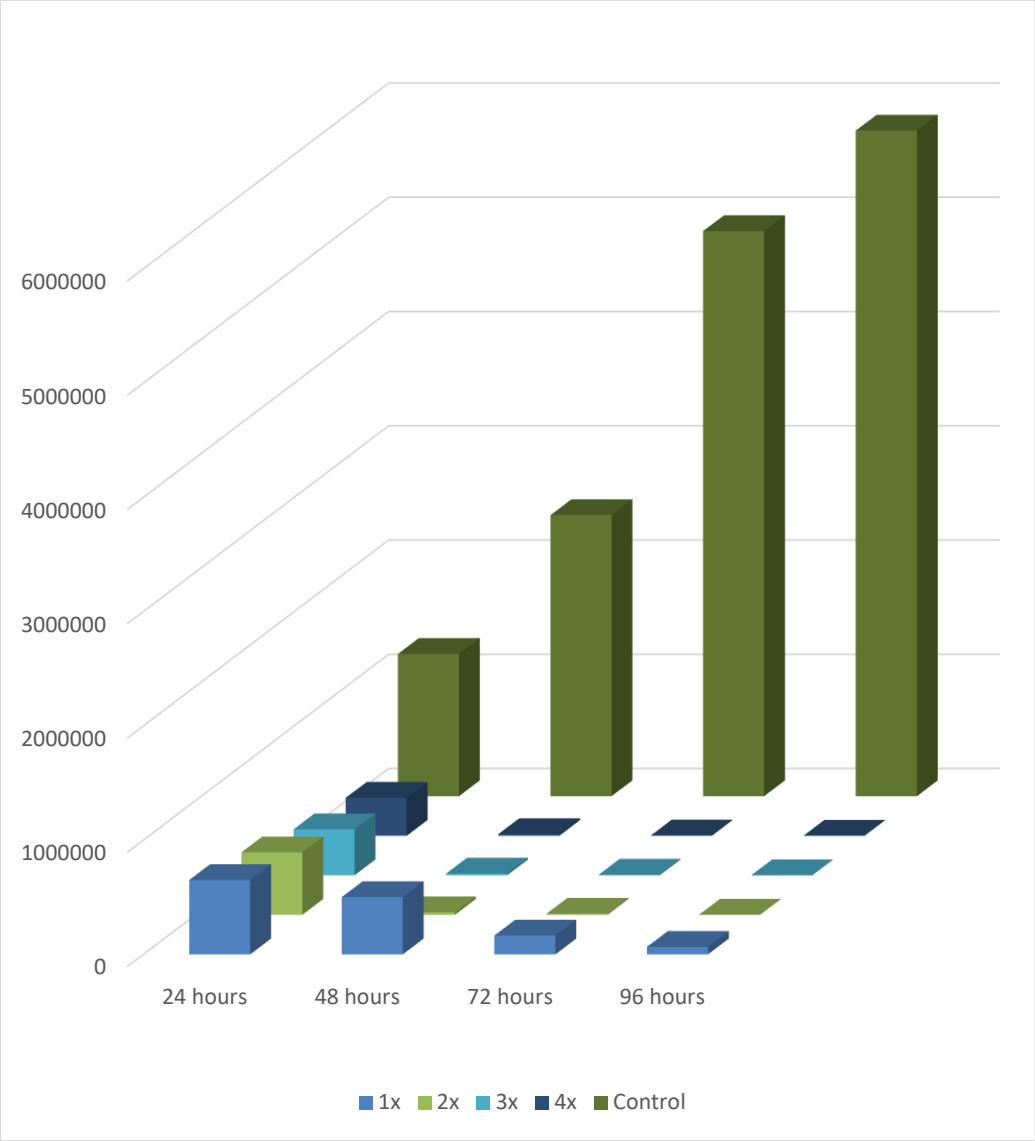


Figure 33: Depicts the cytotoxicity of the purified protein obtained from induced cells of R. rhodochrous DAP 96253 against the Molt-4 cell line over a 96-hour time period.

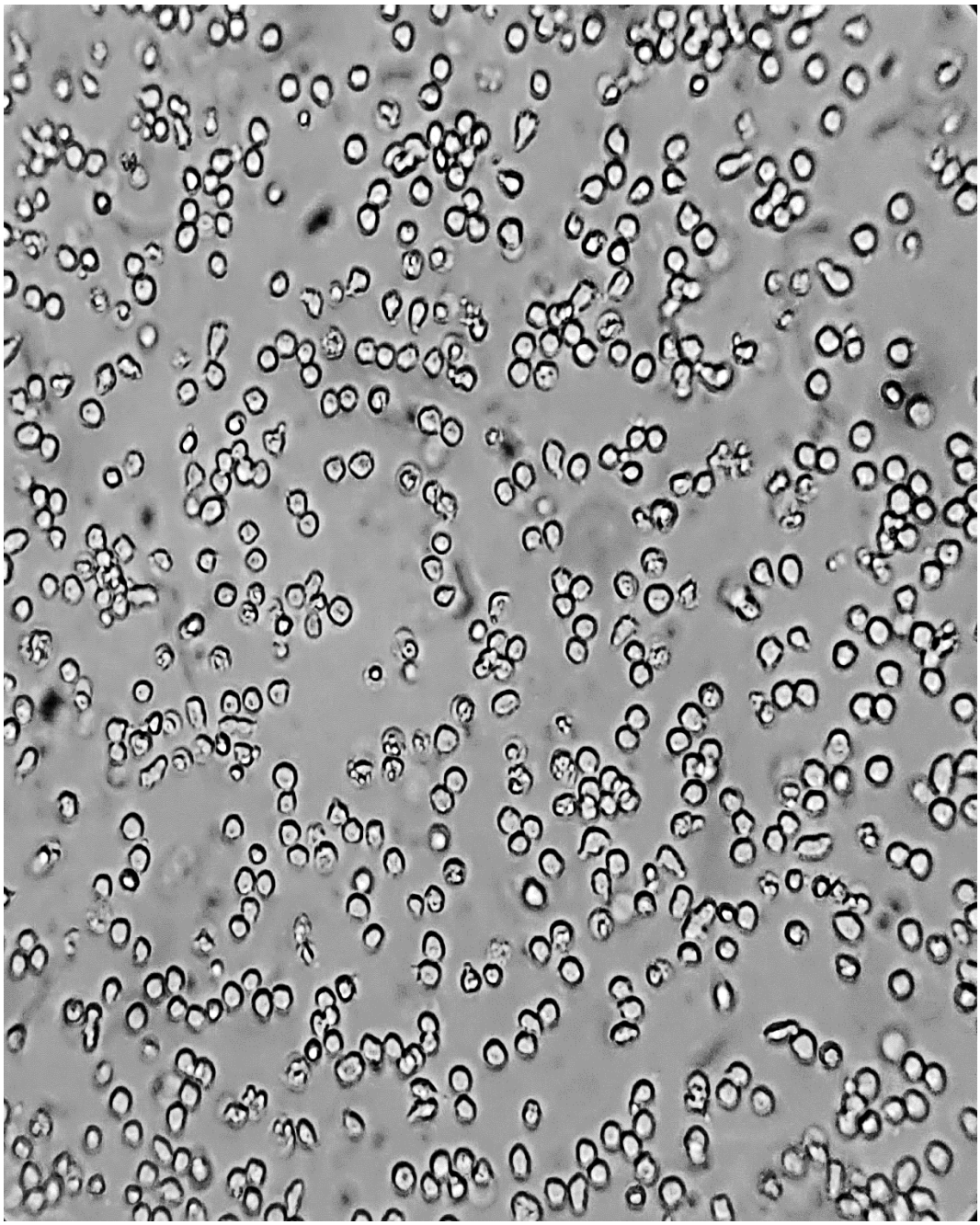


Figure 34: Control setup created by treating Molt-4 cells with just the purification buffer demonstrating the robust cell density. Image was captured after 96 hours of incubation using an inverted microscope

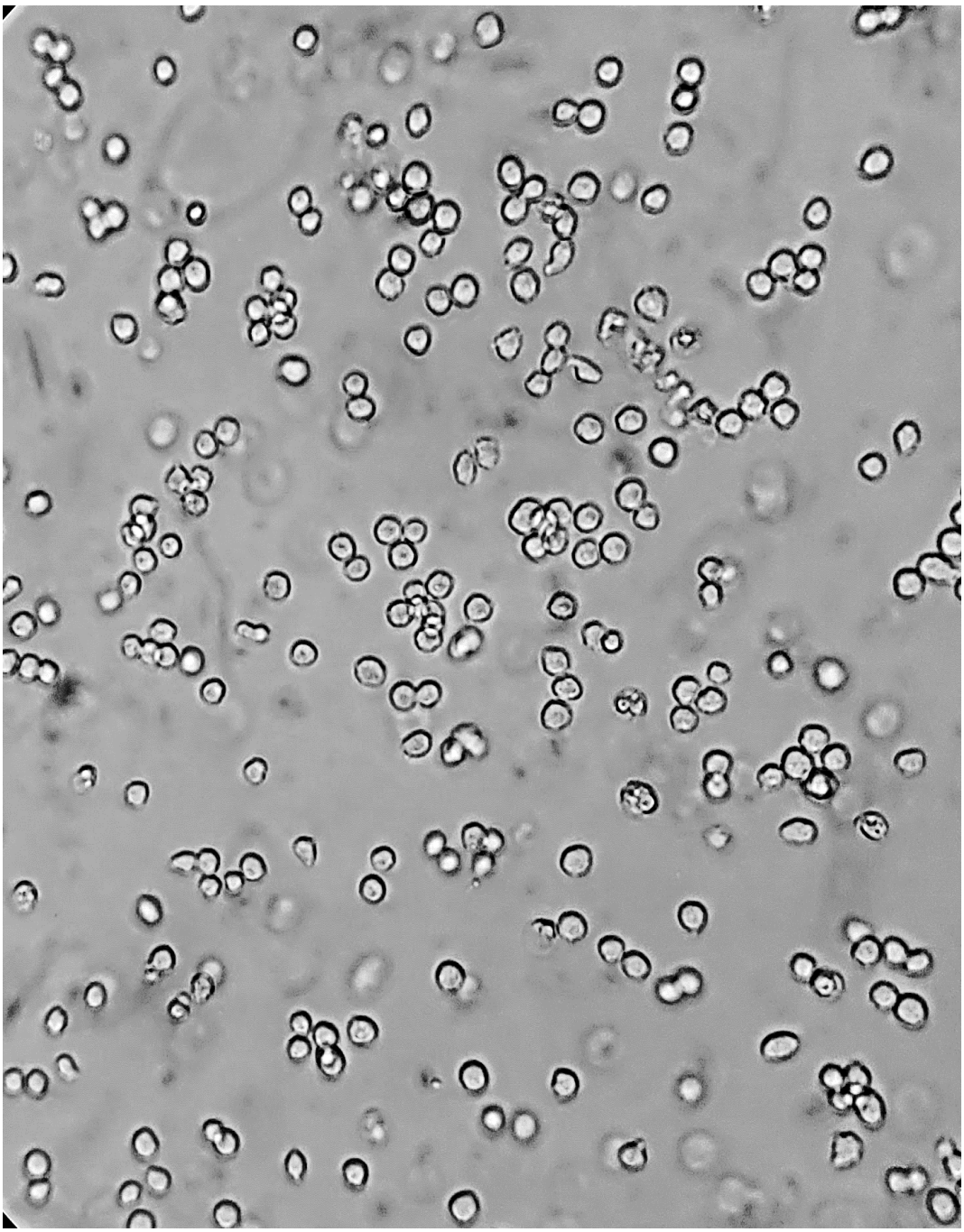


Figure 35: Ix protein treatment setup utilizing purified protein obtained from induced cells of R. rhodochrous DAP 96253 demonstrating reduced Molt-4 cell density when compared to the untreated control

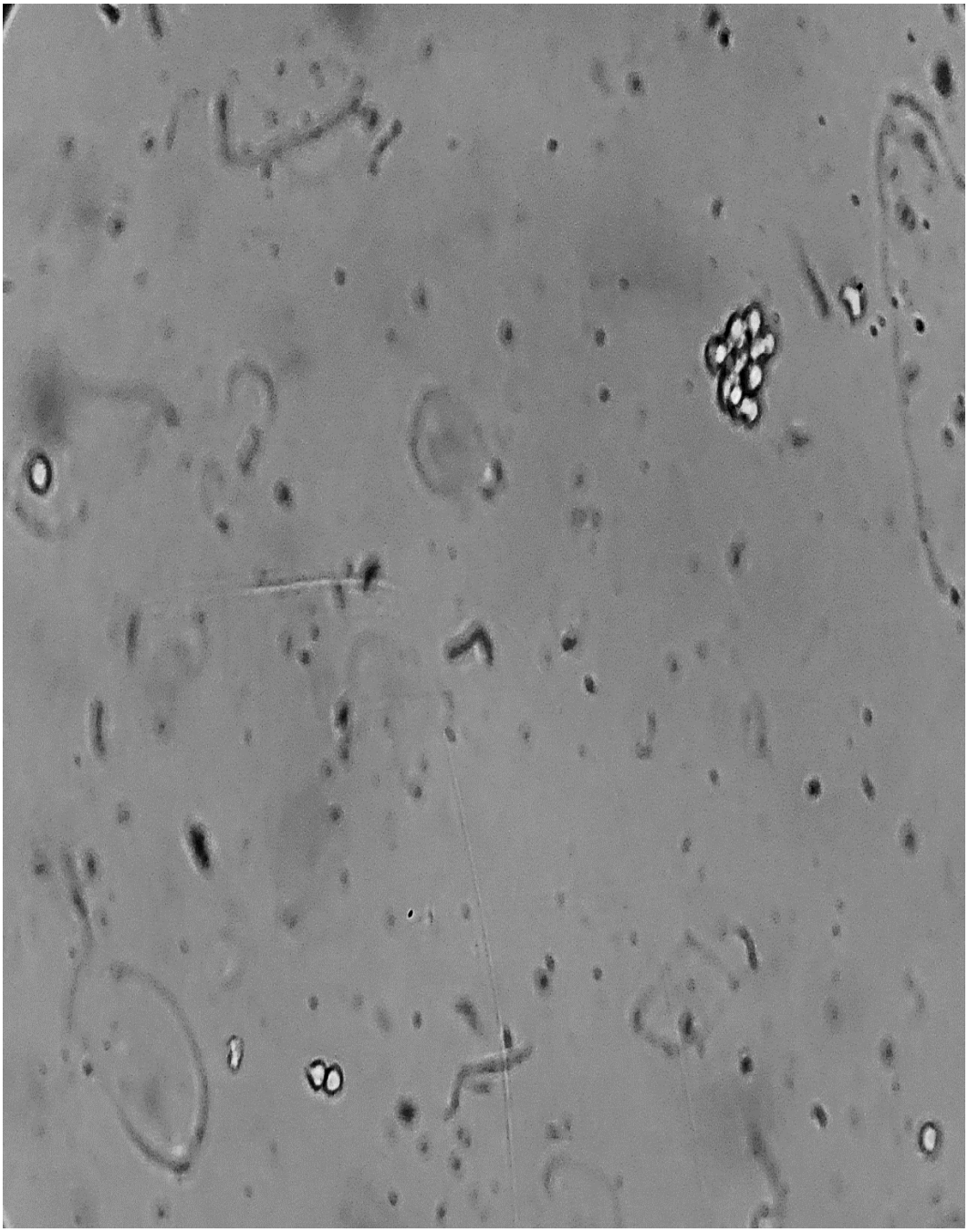


Figure 36: 2x Protein treatment setup obtained from induced cells of R. rhodochrous DAP 96253. The image demonstrates a significant reduction in cell number after 96 hours of incubation.

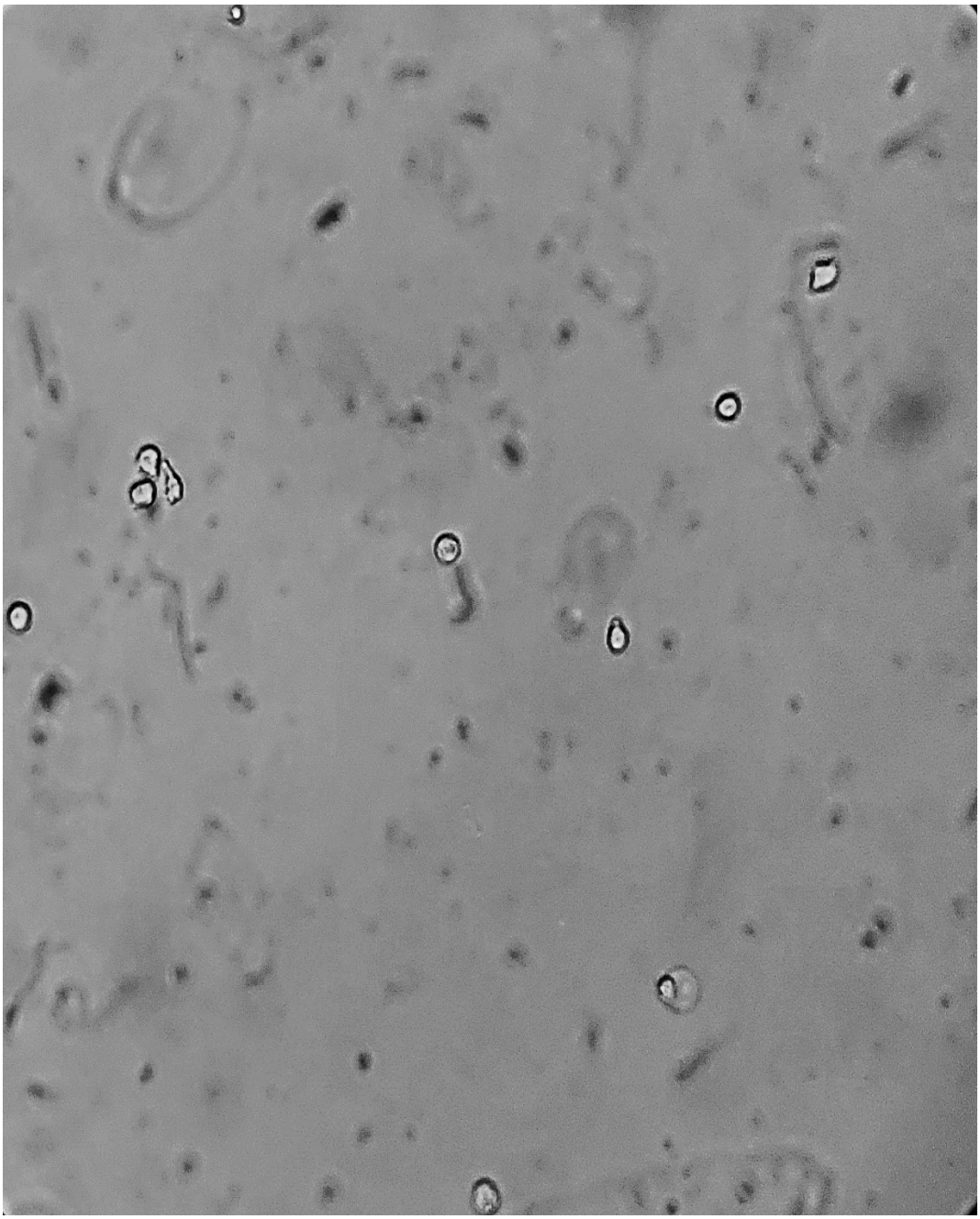


Figure 37: 3x Protein treatment setup obtained from induced cells of R. rhodochrous DAP 96253. The image demonstrates a significant reduction in cell number after 96 hours of incubation.

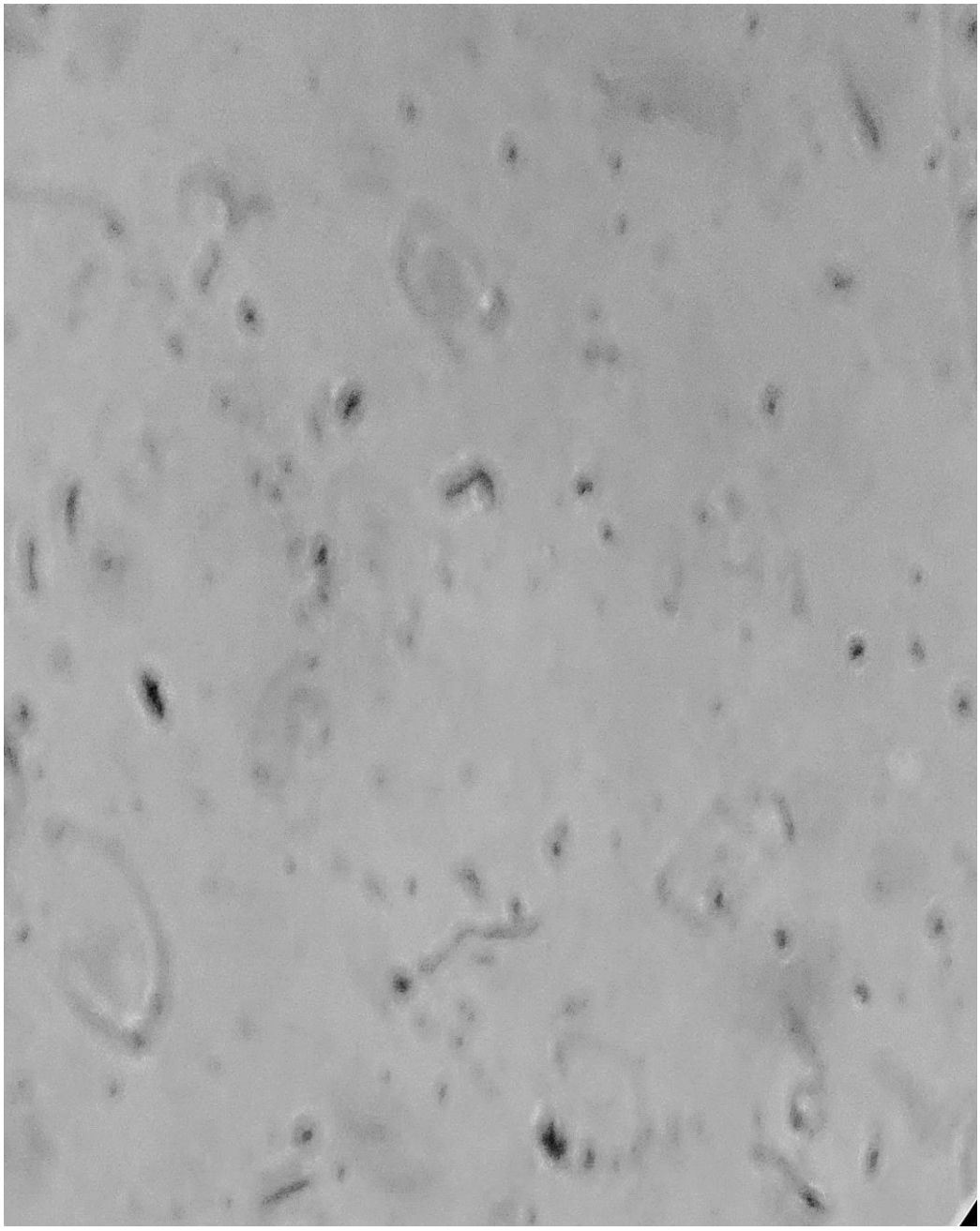


Figure 38: 4x Protein treatment setup obtained from induced cells of R. rhodochrous DAP 96253. The image demonstrates a complete absence of cells after 96 hours of incubation.

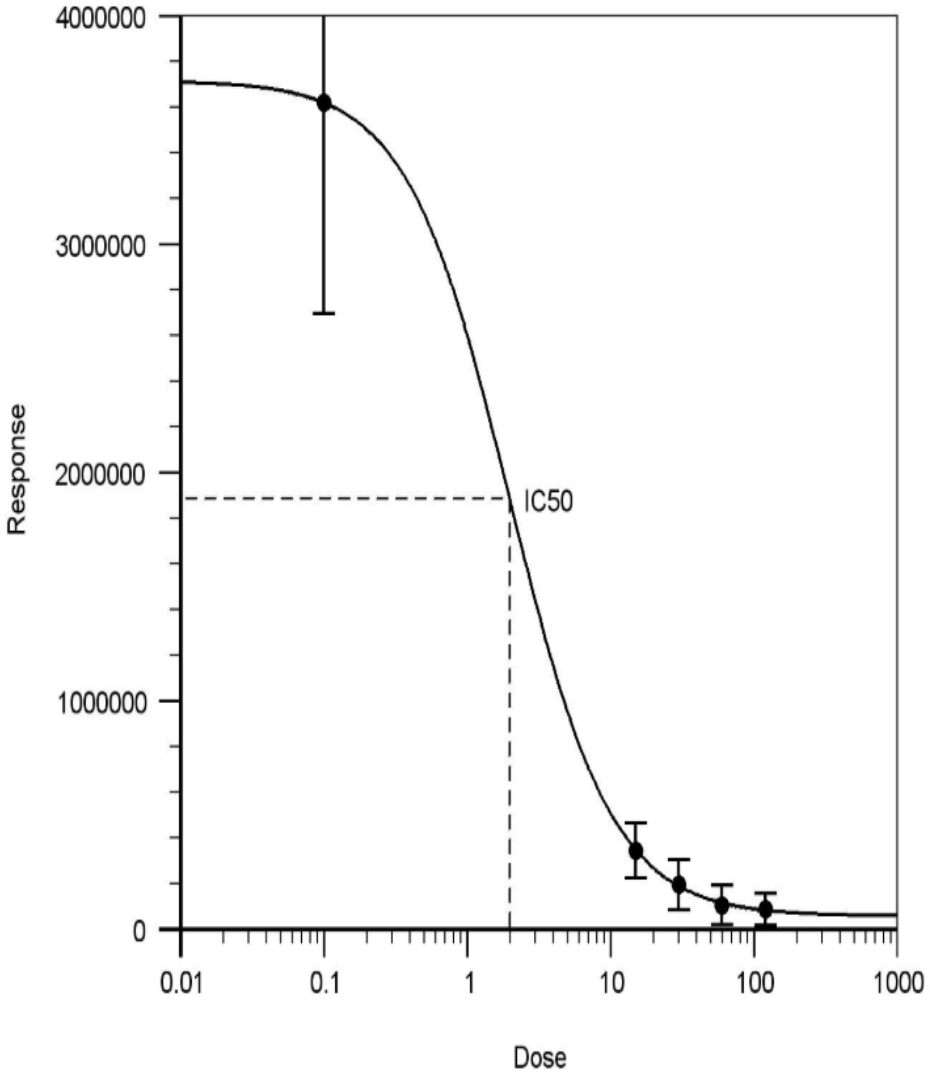


Figure 39: Demonstrating the IC_{50} of the protein preparation obtained from induced cells of R. rhodochrous DAP 96253 utilized in the treatment of Molt-4 cells

The IC_{50} of the purified protein preparation obtained from the induced cells of *R. rhodochrous* DAP 96253 when utilized in the treatment of Molt-4 cell line expressing ALL was determined to be 1.982 IU/mg.

4 DISCUSSIONS

4.1 Enzyme production on varying induction media

R. rhodochrous DAP 96253 enzyme activity on varying induction media were investigated. All the media investigated incorporated Urea and Cobalt II chloride. Previous studies conducted in Dr G. E. Pierce's laboratory at Georgia State University suggests a massive increase in the production of the NHase enzyme specifically and an increase in the production of enzymes such as ASNase and GLNase with the incorporation of Urea and Cobalt II chloride into the growth media. Comparatively, *R. rhodochrous* DAP 96253 grown on YEMEA not incorporating Urea and Cobalt II chloride shows no detectable ASNase or GLNase activity and very low NHase activity (Table 3). This data suggests that induction to upregulate the production of ASNase and GLNase is vital to obtain a stable protein preparation. The cells utilized for this study were obtained via fermentation conducted by Dr. K. Cannon or Dr. M. de la Croix or induced using the media listed above. After growth and induction, the cells are harvested, typically by centrifugation at 4℃ for about 10 minutes at speeds between 10,000 to 13,000rpm for cells cultivated and induced in broth and scraped using a cell scraper when the cells are grown on solid agar (fig 7). The harvested cells are typically utilized immediately after the harvest is complete or otherwise stored in sterile 50mL Falcon® tubes at 4℃ until the commencement of the purification process.

4.2 Effect of the methodology of cell lysis: homogenization and sonication

To start, the cells are typically suspended in a lysis buffer (see appendix) in a 1:1 ratio (w/v in g/mL) and then lysed using the homogenization or sonication. When the cells were homogenized, a minimum volume of 125 mL of the final volume of the cell suspension was required for proper cell lysis using the APV homogenizer, making small scale studies using

suspended volumes less than 150 mL impossible. During the lysis process, steps were taken to keep the suspension at or below 4℃ to protect protein integrity. However, when lysing *R. rhodochrous,* pressure around 700 Bar was required which results in the suspension heating up with successive passes. Typically, the suspension was allowed to cool to 2℃ before resuming the homogenization process. This cooling process depending on the volume of the suspension takes anywhere from 30 to 60 minutes per pass resulting in a total lysis period of close to 4 hours. Despite the efforts to keep the suspension cool, the lysis process has been noted to drive the temperature of the suspension up to 40℃. Subsequent purifications of the CFL obtained from the homogenization resulted in protein preparations with low ASNase and GLNase activity. When sonication was explored, the use of the Fisher® 550 Sonic Dismembrator [with Minonix incorporated convertor and a horn with a ½" diameter tip] allowed for volumes varying from as little as 10 mL to as much as 100 mL. The suspension sits in ice throughout the lysis process and the volume of ice can be adjusted as needed. This process allowed for more stable temperature control during the lysis process. With the one second on one second off pulsing, cell lysis was typically completed in about 40 minutes, and enzyme analysis of the CFL obtained after sonication and fractions obtained from the purification process also showed improved ASNase and GLNase activity (Table 6). Based on the data obtained, sonication was adopted as the preferred means of cell lysis for induced cells of *R. rhodochrous* DAP 96253 targeting a purification with stable ASNase and GLNase activity.

4.3 Cell lysis incorporating 2-mercaptoethanol

The incorporation of a reducing agent into the lysis process for induced cells of *R. rhodochrous* DAP 96253 was investigated. Several studies have extoled the virtues of using reducing agents for protein stabilization [57,99]. When present in high concentrations, proteins

aggregate, and this aggregation negatively impacts their native conformation and thus their function.[42]. Proteins typically aggregate around a cystine amino acid. Cystine is an oxidized dimer formed by a double linkage between two sulfur residues or a disulfide bridge on separate cysteine units[105]. It is important to note that this dimerization only occurs under oxidative conditions. To prevent this protein aggregation, reducing agents such as Dithiothrietol (DTT) or 2-mercaptoethanol (βME) are utilized in the cell lysis process. The reducing agents negate the oxidizing conditions necessary for the dimerization reaction to form the cystine residue around which proteins aggregate thus preventing protein aggregation and maintaining the protein structure and function. βME was utilized as the reducing agent of choice. Cells were lysed using lysis buffer incorporating βME at a 5mM concentration and compared to a cell lysis not incorporating βME. Enzyme analysis of the CFL obtained using lysis buffer incorporating βME and the subsequent purification showed ASNase activity about 35 times higher when compared to CFL and purifications obtained without incorporating a reducing agent into the lysis buffer (Table 7, Fig 8). This data suggests that a reducing condition induced using a reducing agent is necessary for the stability of ASNase obtained from induced cells of *R. rhodochrous* DAP 96253.

4.4 Impact of the duration of dialysis on ASNase activity.

The sample obtained after the lysis of induced cells of *R. rhodochrous* DAP 96253 utilizing a buffer incorporating βME in up to 5mM concentration was dialyzed. The dialysis membrane selected for this study had a molecular cutoff weight of 3.5KDa. Samples from the dialysis membrane were analyzed after 24 and 40 hours for ASNase activity. The utilization of dialysis as a component of the purification process was explored due to the utilization of ammonium sulfate for protein precipitation. Ammonium sulfate has been used in varying

concentrations for the purification process as it significantly increases the hydrophobicity of proteins and can be used to “salt out” or separate proteins based on hydrophobicity[110]. This procedure was not adopted as part of the final purification process because one of the products of the enzyme assay utilized for analysis is ammonia. Samples precipitated using ammonium sulfate showed a much higher (false positive) ammonia concentration when analyzed. The dialysis process was adopted to eradicate the ammonium sulfate post precipitation during the initial stages of the purification process development. Effective ammonium sulfate eradication usually took up to 48 hours with at least 3 dialysis buffer changes. After dropping the precipitation using ammonium sulfate, it was also noticed during the purifications that samples that were dialyzed immediately showed improved ASNase activity post lysis. It was hypothesized that dialysis concentrated the CFL and increased enzyme activity. This data made it necessary then to incorporate dialysis into the purification process even if ammonium sulfate precipitation was not employed and determine how the period of dialysis impacted the ASNase and GLNase activity. The analysis of the CFL at two timepoints (24 and 40 hours) suggests that the longer the sample is dialyzed, the more improved the ASNase and GLNase activity recovered (Table 8, Fig 9). Based on this data, dialysis for up to 40 hours under the conditions used was incorporated into the purification protocol.

4.5 Optimum purification pH

At the commencement of this project, sample purifications and enzyme assays were performed at pH 7.2. The impact of purification pH on ASNase activity was investigated. The pH range investigated include 6, 7, 8 and 9. The resulting ASNase activity from each of the purifications is detailed in table 9 and figure 10, with the highest ASNase activity observed at pH 7. Investigations into pH ranges between 7 and 8 showed pH 7.6 to be particularly suitable for a

purification with stable ASNase activity. All the variables highlighted thus far were incorporated into a purification and the resulting purified fraction showed ASNase and GLNase activity of 1492 IU/mg and 2173 IU/mg respectively.

4.6 Protein characterization (Temperature)

A purification obtained from induced cells of *R. rhodochrous* DAP 96253 was characterized to determine its maximal activity and stability at temperatures ranging from 4℃ to 50℃. The results presented in tables 11, 12 and 13 as well as figure 11 and 12 suggest the ASNase to be stable at all the analyzed temperatures ranging from 4℃ to 50℃ and the highest activity was observed at human physiological pH (37℃). As this study was performed over a 60-minute period and the samples were tested at 15, 30 and 60 minutes timepoint, ASNase activity at 37℃ was noted to be highest at the 30-minute timepoint but activity declined by 36% when analyzed at the 60-minute timepoint.

The GLNase activity like the ASNase was determined to be highest at 37℃ but was significantly more stable for a longer amount of time, with the GLNase activity progressively increasing from the 15 to 30 to 60-minute timepoint. In both cases, there was a progressive decline in ASNase and GLNase activity after 37℃. The increased ASNase and GLNAse activity at human physiological temperature suggest that the protein preparation will be a suitable treatment option for ALL.

Studies into temperature-based stability of other ASNases showed that a fungal ASNase isolated from *Rhizomucor meihei* showed optimum activity at 45℃ but was only stable for 30 minutes[48], ASNases isolated from *Streptomyces gulbargensis, S. fradiae* NEAE-82 and *Erwinia sp*. were noted to possess maximal ASNase activity at 40℃[4,30,11] and an ASNase sourced from the plant *Vigna unguiculata* was also observed to possess maximal enzyme activity

at 40°C[2]. Bacterial ASNases that have been shown to possess maximal activity comparable to the ASNase isolated from induced cells of *R. rhodochrous* DAP 96253 include *E. coli* ASNase, *S. brollosae* NEAE-155 ASNase, and *Nocardius alba* ASNase. Studies performed suggest that the vast majority of characterized ASNases from bacterial and fungal sources possess optimal activity north or south of the physiological human temperature[71,72,16,30].

4.7 Protein characterization (pH)

A characterization was also performed on a purification obtained from induced cells of *R. rhodochrous* DAP 96253 checking maximal enzyme activity and stability at pH ranging from 3 to 10. The data obtained (Table 14, Fig 13) suggest that the protein is stable for over 25 hours when incubated at pH ranging from 3 to 10. The maximal enzyme activity of the protein purification lay between pH 7 and 8. Investigation of pH points 7.2, 7.4, 7.6, 7.8 and 8.0 showed the pH with maximal enzyme activity to be pH 7.6. These data are supported by previous purification studies. It is noteworthy that at physiological pH, the purified protein exhibited about 50% less ASNase and 66% less GLNase activity. These numbers however were significantly higher than the activity observed at pH below 7 or above 8. This suggests therefore that at physiological pH, the protein preparation will still serve as a suitable therapeutic against ALL.

ASNases obtained from *Streptomyces noursei, S. parvulus, S. ginsengisoli* and *Bacillus subtilis* all had maximal ASNase activity close to the activity observed with the purification from induced cells of *R. rhodochrous* DAP 96253 at pH 7.5[23,102,51]. In most other characterized ASNases such as ASNase isolated from *Phaseolus vulgaris* seeds[76], as well as ASNase isolated from *S. thermoluteus* and *B. licheniformis* optimal pH for ASNase activity was observed

at pH 8 and above and ASNase sourced from *E. coli* has been demonstrated to possess maximal enzyme activity between pH 6 to 8 and stability from pH 5.5 to pH 9.[45,67,78].

4.8 Molecular weight determination of the purified protein sample

After cell lysis, anion exchange and size exclusion chromatography, samples from each step of the purification process containing a total of 10µg of protein was loaded on a premade 4-12% polyacrylamide gel according to the method detailed above. After destaining the gel, the purest sample observed in figure 14 above suggests that the protein exhibiting ASNAse and GLNase activity is a multimeric protein of approximately 123 kDa comprising subunits of ~72, ~28 and ~23 kDa. It is noteworthy that previous studies of purified NHase obtained from induced cells of *R. rhodochrous* DAP 96253 also show protein bands in the 28 and 23 kDa location on an SDS PAGE. This has led to the conclusion that either the NHase obtained from purified cells of *R. rhodochrous* DAP 96253 possess unique ASNase and GLNAse activity or induced cells of *R. rhodochrous* DAP 96253 produced so much NHase, that NHase is present in the purified preparation exhibiting ASNase and GLNase activity.

When compared to other characterized ASNases, there were distinct differences. Studies report that Gram-negative bacterial ASNases are typically homo-tetrameric with subunits around the 33 and 36.5 kDa and a native size of ~146 kDa. More specifically, recombinant ASNase isolated from *E. coli* was found to be ~150 kDa, ASNase isolated from *Pseudomonas stutzeri* MB-405 and *Thermus thermophilus* when run on an SDS PAGE was shown to have subunits about 33 kDa[89,69,101].

Gram-positive bacterial ASNase subunits on the other hand have been showed to be ~85 kDa in the case of *S. gulbargensis*, and a 144.4 kDa homotetramer in *Pectobacterium carotovorum* MTCC 1428[4,61].

4.9 Stability of protein preparation in sterile human serum

The results obtained from the studies performed on the stability of the protein preparation measured the ASNase activity over a specified amount of time. The studies were performed until the ASNase activity dipped below the 50% of the initial activity. The data obtained shown in Table15 and Fig 15 suggests that the Rhodococcal ASNase is significantly stable in human serum, losing 50% of its initial activity after incubation for over 50 hours at 37℃.

A comparison was made to a similar study performed on ASNase isolated from *Enterobacter cloacae* and this ASNase was demonstrated to possess a half-life of 39 hours[49]. When compared to currently approved ASNases on the market, *E. coli* ASNase was found to possess a halflife of 8 to 30 hours after injection intravenously and *E. chryansthemi* ASNase was found to be stable for only 16 hours after injection intramuscularly[111].

4.10 Endotoxin levels of the protein products of induced cells of *R. rhodochrous* DAP 96253

The currently approved ASNases are sourced from *E. coli* and *E. chrysanthemi*. Both organisms are lipopolysaccharide (LPS) possessing Gram-negative organisms. Products sourced from Gram-negative organisms utilized for therapeutic go through an expensive cleanup process to remove bacterial LPS[68]. The effectiveness of this cleanup process however is debatable as several side effects associated with the use of these products have also been tied to the immunological response to the presence of LPS.

It was predicted that as a Gram-positive organism, the products obtained from induced cells of *R. rhodochrous* DAP 96253 should be endotoxin free. This confers the benefit of reduced production costs as the expense tied with endotoxin cleanup of products obtained from Gram-negative organism is circumvented, and the side effects associated with the use of contaminated Gram-negative bacterial products are avoided all together.

The CFL and purified products obtained from induced cells of *R. rhodochrous* DAP 96253 were analyzed for endotoxin concentration at varying levels of the purification process. The results of the analysis are shown in Table 16 and Fig 16. The data obtained suggests that the purest sample obtained from *R. rhodochrous* DAP 96253 showed the highest endotoxin concentration. This concentration however was found to be 30 times lower than the endotoxin concentration present in an *E. coli* K12 control utilized for the study. The data also suggests that with successive purifications, the endotoxin levels of the purified sample from *R. rhodochrous* DAP 96253 spiked. An analysis of the column utilized for the purification process showed that the column was not endotoxin free and could well be responsible for contaminating the purified sample. A direct comparison of the CFL obtained from induced cells of *R. rhodochrous* DAP 96253 and an *E. coli* K12 control showed that the Rhodococcal sample had an endotoxin concentration 771 times lower than the *E. coli* sample. Evidence from the study suggests that the endotoxin present in the protein preparation was introduced from an external source and not from the *R. rhodochrous*. It is safe therefore to state that an ASNase sourced from *R. rhodochrous* would be preferable for cancer therapy.

4.11 K_m and V_{max} for ASN and GLN by protein preparation obtained from induced cells of *R. rhodochrous* DAP 96253

Analysis of the substrate affinity utilizing ASN and GLN was performed using purified protein obtained from induced cells of *R. rhodochrous* DAP 96253 exhibiting stable ASNase and GLNase activity. The protein was reacted with varying concentrations of the substrate and the enzyme activity was plotted against substrate concentration in both cases using the Line- Weaver Burke plot (see appendix).

The affinity of the protein for ASN was determined to be high with reactions occurring in concentrations of ASN as low as 13.6µM and higher concentrations of GLN required for a reaction with a Km of 850µM. Blood ASN concentration in humans are typically between 50 to 100µM while blood GLN concentrations are typically between 500 to 800µM[59], with the Km displayed by the ASNase isolated from *R. rhodochrous* DAP 96253, efficient substrate binding can be expected in the relatively low substrate concentrations present in human blood. Several studies point to the fact that GLNase activity is not required for the antitumor activity of ASNase and some studies have linked the side effects associated with the use of ASNase therapy directly to the inherent GLNase activity[49,19]. There are other studies that point to GLNase activity expressed by ASNase as being responsible for the effective treatment of ALL especially with respect to ALL cell lines expressing the ASNS gene and are therefore capable of synthesizing ASN using serum GLN[18,85,24]. With these opposing points of view about the ASNase/ GLNase activity and anticancer capabilities, it was determined that an ideal protein preparation was one that exhibited a much stronger affinity for ASN over GLN.

With the relatively low substrate affinity displayed by the purified protein for GLN, it is expected that if the protein is utilized as for ALL treatment, it will deplete serum ASN and reduce the concentration of serum GLN to a point where the leukemic cells are negatively impacted without necessarily bringing on the negative side effects tied to inherent GLNase activity.

4.12 Effect of selected chemicals on ASNase activity of protein preparations purified from induced cells of *R. rhodochrous* DAP 96253

A selected number of chemicals in varying concentrations were analyzed for their impact on the ASNase and GLNase activity expressed by the purified protein obtained from induced

cells of *R. rhodochrous* DAP 96253. Of all the evaluated chemicals, only $FeSO_4$ was found to positively influence the ASNase activity of the protein purification with almost 30% bump in enzyme activity. The other chemicals were found to negatively impact the protein's ASNase activity with $(NH_4)_2S_2O_8$ having the least negative effect on ASNase activity and $CuSO_4$ having the most negative impact on ASNase activity (Fig 19). These findings are in line with a study performed on ASNase obtained from *B. lichenformis* showed that treatment with $ZnCl_2$, $CaCl_2$ and βME negatively impacted the protein. *S. brollosae* NEAE-115 ASNase was found to be unaffected by Na and Fe but improved by Mg and Co[29,67]

Interestingly, all the selected chemicals were found to completely inhibit the GLNase activity of the purified protein. This phenomenon has been proposed as a means of obtaining GLNase free protein preparation from induced cells of *R. rhodochrous* DAP 96253 which exhibits ASNase activity.

4.13 The effect of protein preparations exhibiting ASNase and GLNase activity on leukemia expressing cell lines

The two leukemia expressing cells that were selected to be utilized for this study were Jurkat clone E6-1 (ATCC® TIB-152™) and Molt-4 (ATCC® CRL-1582™) cell lines. The Jurkat cells were selected because they have been shown to express ASNS and are believed to be resistant to GLNase free ASNase protein preparations[65], and the Molt-4 cells were selected because they are ALL expressing cell line isolated from a patient who underwent chemotherapy to treat ALL.

96 well plates were incubated with 1.2×10^4 cells/ well for the case of Jurkat cells and 3.0×10^5 cells/ well in the case of Molt-4 cells. The increase in the number of Molt-4 cells utilized for the study was prompted by the results from an initial setup using 1.2×10^4 cells/ well. It was

observed that after 24 hours of growth, all the cells present in the 2x through 4x dose treatments were dead and after 48 hours, all the treated cells were dead (results not shown).

The results from the study indicate that the protein preparation exhibiting ASNase and GLNase activity was cytotoxic against both cell lines tested, decreasing cell numbers and viability with protein dose and time. Jurkat cells typically grow in clusters (Fig 20 and Fig 26), with the increasing protein dose and treatment time, Jurkat cell clusters were found to reduce in size. The IC_{50} for Jurkat E6-1 clone was determined to be 0.123 IU/mg and 1.982 IU/mg for Molt-4 cells.

In conclusion, the purified protein preparation obtained from induced cells cells of *R. rhodochrous* DAP 96253 has been demonstrated to be functional at physiological human pH and temperature, endotoxin free, possessing a high affinity for ASN and antileukemic against Jurkat clone E6-1 and Molt-4 cell lines. All of these results suggest that the protein preparation will serve as a suitable alternative for the treatment of ALL.

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APPENDICES

Appendix A

Lysis buffers; Sodium phosphate (pH 5.8, 6.0, 7.2, 7.4, 7.6, 7.8) Tris-HCl (pH 8.0, 9.0), typically incorporating 2-mercaptoethanol at 5mM concentration and glycerol at 5% concentration

Dialysis buffers; Sodium phosphate (pH 7.0, 7.2 and 7.6) incorporating 2-mercaptoethanol at 5mM concentration, glycerol at 5% concentration and Magnesium sulfate heptahydrate (J.T. Baker) at 1% concentration.

Purification buffers; Sodium phosphate buffer (pH 6.0, 7.0, 7.2, 7.4, 7.6, 8.0), potassium phosphate buffer (pH 7.6), MOPS buffer (pH 7.2), Tris-HCl buffer (pH 8.0, 9.0). AEX elution buffers incorporate 1M NaCl

pH Study buffers: Citrate buffer (pH 3.0, 4.0, 5.0), Sodium phosphate buffer (pH 6.0, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0), Tris HCl buffer (pH 8.0, 9.0, 10.0), Glycine-NaOH buffer (pH 9.0, 10.0).

Appendix B

- Total enzyme activity =µmols of the product/ (reaction time x enzyme volume)
- Specific activity determined by dividing the international units of the enzyme by the total protein concentration expressed in IU/mg.
- Fold purification of the protein is determined by dividing the specific activity of the purified fraction by the specific activity of the CFL.
- Percentage yield is determined by dividing the total activity of the purified fraction by the total activity of the CFL.

Appendix C

Lineweaver-Burk plot equation

$1/V = K_m/V_{max} \times 1/[S] + 1/V_{max}$

Based on the linear plot

$y = mx + b$

$y = 1/V$

$m = K_m/V_{max}$

$x = 1/[S]$

$b = 1/V_{max}$

$V_{max} = 1/b$

$Km = (V_{max}) \times (m)$