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ENZYME STABILITY REACTION FOR *RHODOCOCCUS RHODOCHROUS* DAP 96253
ASPARAGINASE AS A POTENTIAL TREATMENT FOR ACUTE LYMPHOBLASTIC
LEUKEMIA.

By

YATHREB BAYAN

Under the Direction of George Pierce, PhD.

ABSTRACT

Asparaginase derived from *Eschericia coli* or *Erwinia crysanthum* is used in the treatment of juvenile Acute Lymphoblastic Leukemia (ALL). ALL cells cannot make asparagine due to the lack of L-asparagine synthetase gene. Asparaginase is therefore used as a chemotherapeutic treatment for ALL through hydrolysis of the L-asparagine to aspartic acid, which leads to starvation of the ALL tumor cells, then apoptosis. In addition, the asparaginase enzyme is used in the food industry. Asparaginase can be used to reduce acrylamide in baked food formed during the Milliard reaction. In this study, in this study, *Rhodococcus rhodochrous*

DAP 96253 asparaginase enzyme activity is measured at varying temperatures (27 °C and 37 °C) and substrate concentrations, using colorimetric reagents, to determine the enzyme reaction stability with respect to these variables. Asparaginase enzyme activity increases by increasing the temperature to a specific optimal point, after that it declines. This accelerates the hydrolysis of L-asparagine to L-aspartic acid. The goal is to determine the optimum temperature for the enzyme function.

INDEX WORDS Asparaginase activity, ALL, Glutaminase, *Rhodococcus rhodochrous* DAP 96253.

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Masters of Science
in the College of Arts and Sciences
Georgia State University
2019

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Yathreb Bayan Abdelsattar Mohamed
2019

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YATHREB BAYAN

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May 2019

DEDICATION

This thesis is dedicated to the memory of my beloved grandmother. Although she was always my supporter to be successful, she was unable to see me return with my degree. This is for her.

Thank you to my knowledgeable academic adviser Dr.Pierce who helped and guided me in this process, and the committee who kept me on track.

I dedicate this thesis to my amazing Mother and Father, without their encouragement I would not have made it to this level.

I dedicate this thesis to my dear siblings, faithful friends, and colleagues. You made my hard Journey easier by having my back and believing in me.

This thesis is dedicated to my great professors at Ain Shams University faculty of pharmacy and 6th October faculty of pharmacy, exclusively:

The Dean: Dr. Khaled Abouzid, Dr. Walid Faisal, Dr. Mohamed El Naggar, Dr. Sohier El Nahhas Dr.Walaa al sharief.

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Besides my advisor, I would like to thank the rest of my thesis committee: Prof. Dr. Eric Gilbert, and Prof. Dr. Sidney Crew, for their insightful comments and encouragement, but also for the hard question which incited me to widen my research from various perspectives.

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1 INTRODUCTION

1.1 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a blood and bone marrow cancer which affects white blood cells (Patra et al. 2014). Juvenile ALL is considered one of the most common forms of cancer among children and adolescents in the United States (Siegel et al. 2018). Around 6000 cases in the United States are diagnosed annually with acute lymphoblastic leukemia; half of these cases occur in children and teenagers (Stephen et al. 2015). The childhood ALL patients' treatment has advanced to the extent that 5 years event free survival rates reached around 80% (Evans, 1998). There are many factors that contribute to the poor outcome in adults' ALL treatment, and the increased frequency of high-risk leukemia, such as poor tolerance of and compliance with treatment, great drug resistance, less effective treatment regimens, and the changes in the physiology and organ function affecting the pharmacology of ALL therapy, in comparable with childhood ALL (Musa et al. 2017).

1.1.1 *Asparaginase mechanism of action*

Asparaginase can be isolated from different bacteria such as *Escherichia coli* (*E.coli*), *Erwinia crysanthum*, *Rhodococcus rhodochrous* and *Pseudomonas pseudocaligines* (Dalfard, 2016). Asparaginase formulations derived from *E. coli* and *Erwinia crysanthum* are currently used in the treatment of juvenile acute lymphoblastic leukemia. Asparaginase hydrolyzes L-asparagine to L-aspartic acid and ammonia in tumor cells that do not have L-asparagine synthetase. This results in starvation of the tumor cells due to depletion of L-asparagine and then the protein synthesis inhibition (Karl et al. 2017). Asparagine is considered critical for protein synthesis in leukemic cells that cannot synthesize this amino acid due to the deficient or the

absence of enzyme asparagine synthetase (Neil, 2009). Despite, the prominent role of L-asparaginase in treating acute lymphoblastic leukemia, the use is still limited due to the high adverse side effects associated with it such as pancreatitis, nephrotoxicity and neurological problems. These side effects of L-asparaginase usually occur as a result of the dual substrate specificity towards glutamine and asparagine (Ramia et al, 2012). The L- asparaginase leads to decrease the glutamine levels in circulation through the deamination of glutamine to glutamic acid (Grigoryan et al. 2004). The glutamine decreasing leads to depletion of tumor growth and enhancing cell apoptosis, as glutamine is believed to be a source of nitrogen, which plays a role in synthesis of DNA and RNA by the tumor cells (Cory et al.2006).

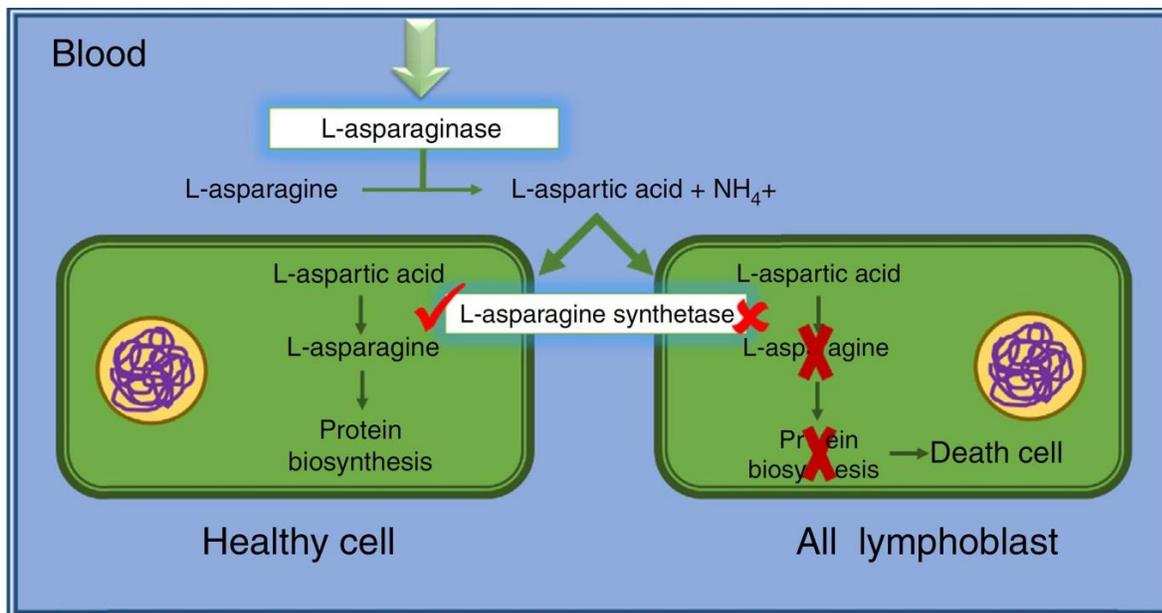


Figure 1 L-asparaginase mode of action in healthy and tumor cells.

1.1.1.1 Asparaginase role in treatment of Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is one of the most progressive and common cancers in children. Asparaginase is recognized for treating acute lymphoblastic leukemia with 90 % complete remission in children and adults (Piatkowska et al. 2008). Asparaginase leads to starvation of the tumor cells then subsequent apoptosis. Three asparaginase forms derived from different bacteria have the same mechanism of action, yet each asparaginase displays a uniquely different pharmacokinetics profile. These three forms include *Erwinia* asparaginase, *Escherichia coli* and pegylated form of the native *E.coli* asparaginase (pegaspargase) (Asselin, 1999). *Escherichia coli* PEGylated asparaginase is used as first line therapy for acute lymphoblastic leukemia. However, it displayed hypersensitivity in 30% of patients (Egler et al. 2016). The antibodies against *E.coli*-asparaginase lead to these hypersensitivity reactions and silent inactivation of *E.coli* asparaginase in up to 60% of cases (Rob et al.2011). The PEGylated asparaginase derived from *Escherichia coli* has a less immunogenic potential and a longer half-life than the native *E.coli*. In addition, it is more feasible with intravenous injection (Vrooman et al.2015). In European and US protocols, the asparaginase derived from *Erwinia chrysanthemi* acts as second or third line of therapy after *E.coli* hypersensitivity (Pieters et al. 2011). Patients switch to another asparaginase form to decrease the hypersensitivity derived from Gram-negative bacteria. One of the most frequent and serious complications of acute lymphoblastic leukemia the treatment are the thromboembolic events (TEs) (Sutor, 1992). In juvenile acute lymphoblastic leukemia, the timing of TEs occur after the chemotherapy with L- asparaginase immediately or during the treatment (kuknar et al. 1985). These thromboembolic events may lead to neurological sequela and significant morbidity in acute lymphoblastic leukemia patients (Vijaya et al. 2015).

2 BACKGROUND

2.1 *Rhodococcus rhodochrous* DAP 96253 bacteria

Rhodococcus rhodochrous is a Gram positive, aerobic, and non-motile bacterium (Warhurst, 1994). *Rhodococcus* classification is mainly based on its distinctive composition of mycolic acids (Larkin et al. 2010). All the *Rhodococcus* strains start their life as coccus or short rod shape, their morphology change upon growth into various forms: hyphae, short rods and filaments. Formation of new generation occurs at the end of the exponential growth stage via fragmentation of the matured individuals (Goodfellow et al, 1998). *Rhodococcus rhodochrous* has the capability of using a wide range of organic compounds as sources of carbon and energy for their growth (Goodfellow et al. 1998). *Rhodococcus rhodochrous* is known to be closely related to *Cornebacterium*, *Mycobacterium* and *Nocardia* by analysis of 16SrDNA (Bell et al. 1998).

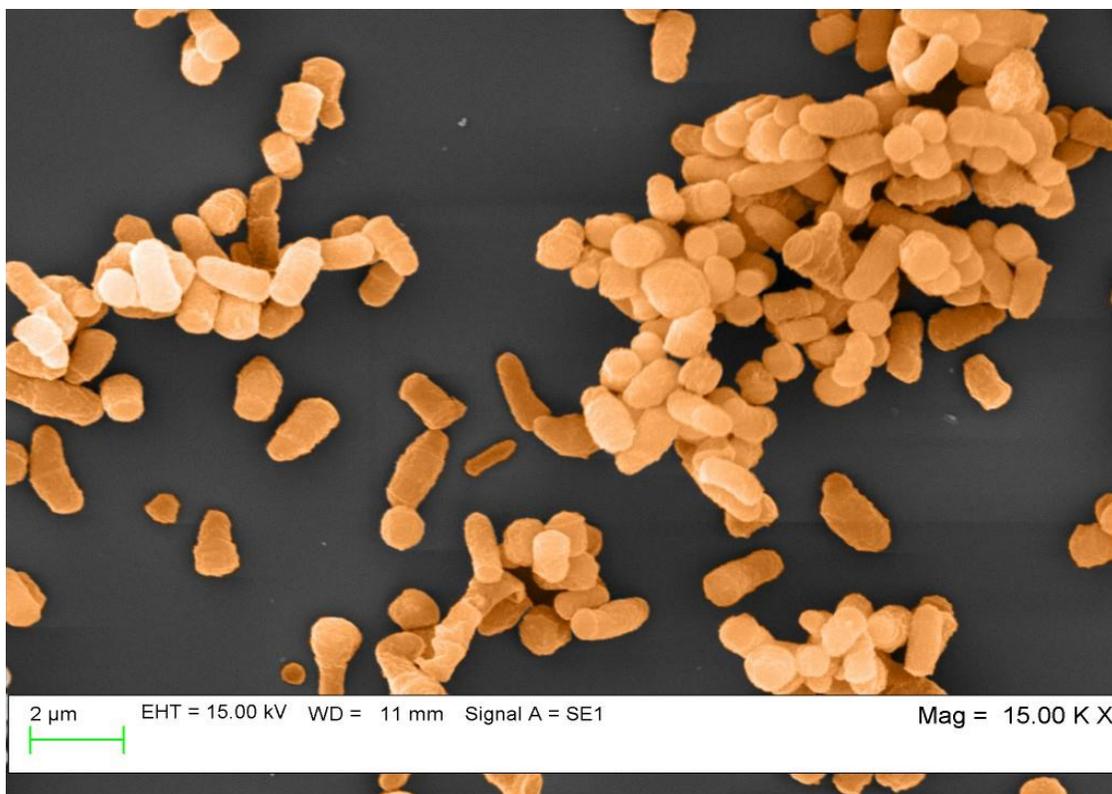


Figure 2 Rhodococcus rhodochrous Dap 96253 (AEM Pierce).

2.1.1 *Rhodococcus rhodochrous* strains and pathogenesis

Rhodococcus genus members inhabit diverse niches, cause pathogenesis in mammals and plants. However, *Rhodococcus rhodochrous*, which is isolated from contaminated soil, does not possess any pathogenesis (Larkin et al. 2010). *Some of the Rhodococcus rhodochrous* strains, like ATCC 17895, showed the optimal growth on cultural media at 26 °C within 3 to 4 days. That leads to dry opaque and pale orange colonies (Christopher et al. 2011). Many *Rhodococcus* strains are considered as a significant part of the soil microbial community due to the diversity of their biodegradation mechanisms (Larkin et al. 2010). In addition, some of the *Rhodococcus rhodochrous* strains are exploited in industrial production and pollutants degradation (Warhurst, 1994). *Rhodococcus* strain 11Y, which is isolated from explosives-contaminated land, uses RDX

(hexahydro-1,3,5-trinitro-1,3,5-triazine) as a sole nitrogen source for growth (Zhao et al. 2004). *Rhodococcus rhodochrous* has a diverse array of organic compounds degradation capabilities which contributed by its large genome size (Bell et al. 1998). There are a massive number of biodegradative, and catabolic genes stored in their large linear plasmids. *Rhodococcus* has generally increased in scientific research due to its highly diverse ability to biodegrade (Bell et al. 1998). Thus, revealing their commercial potentials *Rhodococcus rhodochrous* strains possess a variety of biotransformative capabilities. For example, the short chain hydrocarbons *Rhodococcus rhodochrous* E5, is able to degrade acetylene via acetaldehyde to acetyl CoA. While *Rhodococcus rhodochrous* ATCC21198 shows the ability to grow on propane with CO₂ presence as it contributes to the fixation of CO₂ (Warhurst,1994). Some strains of *Rodochochocus rhodochrous*, KUCC 8801 and KUCC 8802, demonstrate the ability to degrade n-alkanes; other strains of *R. rhodochrous* are specialist in long-chain hydrocarbons degradation (Warhurst, 1994). The biotransformation of nitriles would be a successful applied aspect of *R. rhodochrous*, as *R. rhodochrous* J1 produces nitrile hydratase that is used to produce more than 30,000 tons of acrylamide from acrylonitrile annually for commercial purposes (Bell et al. 1998). When catalyst derived from cells of *R. rhodochrous* strain DAP 96253 on YEMEA, which is supplemented with urea, cobalt, and Asparagine showed a great impact in delaying fruits ripening, in addition it was noted that it had the highest level of Nitrile hydratase (Pierce et al. 2011).

3 RATIONALE

Asparaginase is an enzyme that is purified from *Rhodococcus rhodochrous* DAP 96253 bacteria; it accelerates the hydrolysis of l-asparagine to l-aspartic acid and ammonia, which could lead to inhibition of protein synthesis in tumor cells through deprivation of amino acid asparagine (Karl et al, 2017). Asparaginase is also considered as a recognized part in treatment of acute lymphocytic leukemia (Neil, 2009). Since asparaginase is derived from a Gram positive bacteria *Rhodococcus rhodochrous* DAP 96253, this could be safer, and better than the asparaginase derived from Gram negative bacteria like *Escherichia coli* (*E.coli*) and *Erwinia crysanthum*, which possess hypersensitivity.

4 HYPOTHESIS

It is hypothesized that *Rhodococcus rhodochrous* DAP 96253 asparaginase enzyme activity increases by increasing the temperature to a specific optimal point after that it declines. This accelerates the hydrolysis of L-asparagine to L- aspartic acid and ammonia, and thus led to deprivation of the L- asparagine in the acute lymphoblastic leukemia tumor cells then cell death (apoptosis).

5 AIMS

5.1 Determine the effects of different temperatures on asparaginase activity.

Exposing *Rhodococcus rhodochrous* DAP 96253 asparaginase and the substrate concentrations to different temperatures 27 °C and 37 °C, and noticing the corresponding results and the effects on the asparaginase and glutaminase activity by using thermomixer R with different Substrate concentrations.

6 MATERIALS AND METHODS

1. 96 well plates u bottoms.
2. L-Asparagine, minimum 98% TLC.
3. Glutamine.
4. 4 N NaOH (dissolve 3.2g NaOH in 20ml of distilled H₂O)
5. 4N H₂SO₄ (Mix 4.4ml of 18N stock H₂SO₄ with 15.6 distilled H₂O)
6. 25% Sodium phenate (dissolve 25g of phenol in 800 ml distilled H₂O and add 78 ml of 4N NaOH, bring volume to 1000ml)
7. 0.01% sodium nitroprusside (mix 10 ml of 1% sodium nitroprusside stock solution with 990ml distilled H₂O)
8. 0.15% Sodium hypochlorite (mix 25 ml of 6% sodium hypochlorite with 975ml of distilled H₂O)
9. Stock Ammonium (1000 ppm Ammonium) made using NH₄Cl.
10. DDH₂O.
11. Thermomixer R.
12. Viktor plate Spectrophotometer.
13. Laser Grip 1080 (infrared thermometer).
14. Thermometer.

*All chemicals unless otherwise stated were from Sigma.

Measuring the purified *Rhodococcus rhodochrous* DAP 96253 asparaginase and glutaminase enzyme activity with varying temperatures. The temperatures selected include 27 °C and 37 °C (body temperature 37°C), these varying temperatures were maintained using thermomixer hot plate. Asparagine and glutamine substrate concentrations tested include 500ppm which is the

average concentration, and time points tested include 2 minutes to determine the enzyme stability with respect to these variables. The asparagine solution and the glutamine solution were made by adding L-asparagine to sodium phosphate buffer pH 7.2 to the desired concentration, and the glutamine solution was made by phosphate buffer 7.2 enzyme and substrates controls as well as ammonia standards were made. The ammonia standard were made from Stock Ammonium (1000 ppm Ammonium) using NH_4Cl . 100 μl of enzyme was added to triplicate Eppendorf tubes of 900 μl of asparagine substrate and 900 μl of glutamine substrate agitated to initiate the reaction for the desired time period, after which 10 μl of 2M sulfuric acid (Mix 4.4ml of 18N stock H_2SO_4 with 15.6 distilled H_2O) was added and then vortexed to stop the reaction. 10ul of 4N NaOH (dissolve 3.2g NaOH in 20ml of distilled H_2O) was added to the reaction to neutralize the acid, after which the samples were transferred to the test tubes. Colorimetric reagents of 2 ml of 25% Sodium phenate (dissolve 25g of phenol in 800 ml distilled H_2O and add 78 ml of 4N NaOH, bring volume to 1000ml), 3ml of 0.01% sodium nitroprusside (mix 10 ml of 1% sodium nitroprusside stock solution with 990ml distilled H_2O), and 3 ml of 0.15% Sodium hypochlorite (mix 25 ml of 6% sodium hypochlorite with 975ml of distilled H_2O) were added respectively and the solutions were vortexed. Test tubes were incubated at room temperature in the dark for 30 minutes at least. 200 μl reaction sample from each test tube was pipetted onto 96 well plates (u bottom) and optical density was measured at 630nm using a Viktor plate Spectrophotometer.

7 RESULTS AND DISCUSSION

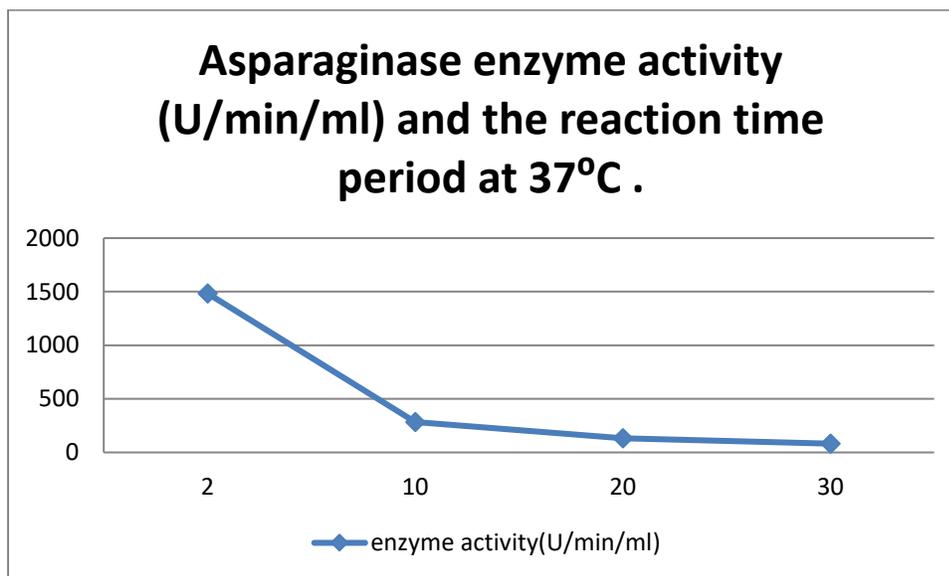


Chart 1 1000 ppm asparaginase enzyme activity (U/min/ml) at different reaction time points 2, 10, 20 and 30 minutes at 37°C.

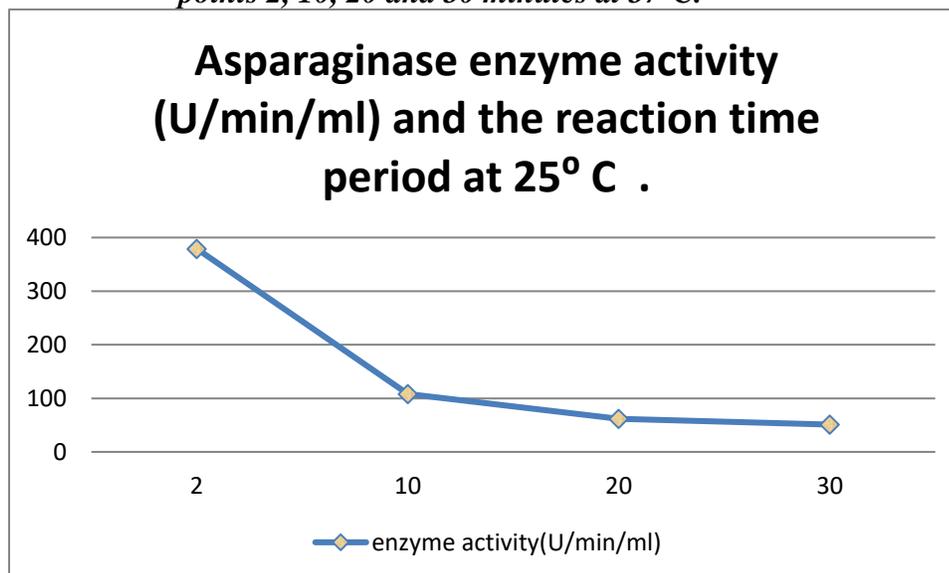


Chart 2 1000 ppm asparaginase enzyme activity (U/min/ml) at different reaction time points 2, 10, 20 and 30 minutes at 25°C.

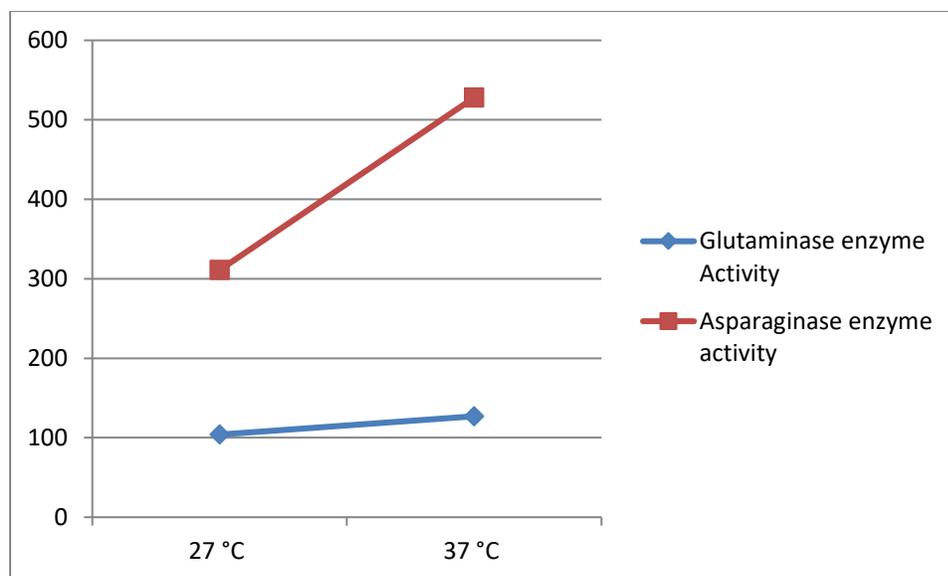


Chart 3 The asparaginase and the glutaminase enzyme activity (U/min/ml) at (2minutes) reaction at 27 °C and 37°C temperatures, 500 ppm asparagine and glutamine substrate concentration.

*Chart (1) and Chart (2) indicate that the enzyme activity of asparaginase substrate solution of 1000 ppm concentration at different time reaction periods (2, 10, 20 and 30 min), was decreasing with respect to the time periods at 10, 20 min and 30 min reaction. The Optimal enzyme activity was determined to occur at the 2 minutes time point at different temperatures 25 °C and 37°C.

* Chart (3) indicates that the enzyme activity of asparaginase substrate solution 500 ppm and the enzyme activity of glutaminase substrate solution 500 ppm concentration were increasing by increasing temperature from 27 °C to 37 °C at 2 min time reaction, which indicates that the enzyme activity for both the Asparaginase and Glutaminase increases by increasing temperature at 37 °C.

Table 1 The samples (2 minutes) reaction 27 °C and 37°C temperatures, 500ppm
Asp substrate concentration

Asparagine substrate conc.(ppm)	Temperatures	Enzyme activity(u/min/ml)
500 PPM	27°C	< 0.1
	37°C	3

Table 2 The samples (2minutes) reaction at 27 °C and 37°C temperatures, 500 ppm glutamine substrate concentration

Glutamine	Enzyme	
substrate	activity(u/min/ml)	
conc.(ppm)	Temperatures	
500 PPM	27°C	135
	37°C	90

* Table (1.1) and table (1.2) indicate that with (500ppm) asparagine substrate concentration the enzyme activity was increasing by increasing temperature at 37 °C. That shows that the asparagine enzyme activity increased by temperature. While, the enzyme activity of 500 ppm glutamine substrate, show a decrease in the enzyme activity at 37 °C temperature. As a result of unequal protein distribution in the sample that contain low protein concentration 0.002mg/ml. These results based on using 100ul of enzyme and 900ul of substrates concentration were related to the more color intensity showed during the reaction by using 100ul volume instead of other lower volumes that have faint color that make it hard to detect the enzyme activity with colorimetric reagents. In addition, if the sample of 100ul enzyme contains a high protein concentration equally distributed (homogenous) among the volume, this will result in a more consistent increase or decrease in the asparaginase and glutaminase enzyme activity

respectively with increasing temperatures to 27 °C at 37 °C at 2 min time reaction. While if the sample of 100ul contains a low protein concentration with uneven distribution that will result in fluctuation and inconsistent of the glutaminase enzyme activity results with increasing temperature.

8 CONCLUSION

This study is directly relevant to the enzyme stability reaction for *Rhodococcus rhodochrous* DAP 96253 asparaginase as a potential treatment for acute lymphoblastic leukemia. This study uses a new form of derived asparaginase, through switching from Gram negative bacteria (*Eschericia coli* and *Erwinia crysanthum*) asparaginase to a Gram-positive bacteria *Rhodococcus rhodochrous* DAP 96253 asparaginase. Asparaginase derived from *Rhodococcus rhodochrous* DAP 96253 could be safer, more stable, and less immunogenic to the patients. In this study, the asparaginase and glutaminase activity were increasing by increasing temperature from 27 °C to 37 °C at 2 min time reaction. That indicates it will be stable during storage at 4°C in refrigerator and active inside our body temperature 37 °C when used as a medication. Thus, asparaginase hydrolyzes L-asparagine to L-aspartic acid and ammonia in tumor cells that do not have L- asparagine synthetase, these results in starvation of the tumor cells due to depletion of L- asparagine and then the protein synthesis inhibition. Also, the L- asparaginase leads to decrease the glutamine levels in circulation through the deamination of glutamine to glutamic acid and the glutamine decreasing leads to depletion of tumor growth and enhancing cell apoptosis. Furthermore, this study will improve the quality of the therapeutic use, and maintain the optimal stability during storage. In general, translating this research into medical practice and meaningful health outcomes will enhance the human health and well-being of ALL patients. Due to the fact that, acute lymphoblastic leukemia is considered one of the most common forms of cancers in children in the US.so this study is considered patient-and population oriented. That means the public gets a significant return on investment with the clear objective of having a more positive impact on our health. Also, the stability of enzyme during storage and the therapeutic use means

it can be designed to reach the rural and urban communities without affecting the outcomes; this will improve the access to quality health care and service throughout the health care system.

9 FUTURE STUDIES

Asparaginase enzyme activity that are purified from *Rhodococcus rhodochrous* DAP 96253 are increasing by increasing temperatures from 27 °C to 37 °C at 2 min time reaction. However, *Rhodococcus rhodochrous* DAP 96253 asparaginase and glutaminase enzyme activity can be measured among further temperatures above 37 °C until reaching the optimal activity of the enzyme, which after that it declines. Moreover, further stability reactions can be done.

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