AMINO ACIDS PRODUCTION FOR *GALDIERIA SULPHURARIA* UNDER DIFFERENT STRESSORS

By

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ABSTRACT

Amino acids are building blocks for protein synthesis and are essential to every metabolic process. A well-balanced diet is one in which all required amino acids are present in the food that is eaten. Unfortunately, this is not always the case and nutritional supplements can be used to make up the balance of the required amino acids. Supplements can be produced from different sources including animals, plants and microbial organisms. Animal sources such as fish and beef can generally be complete but require lengthy periods for growth and harvesting. These sources, however, are also susceptible to diseases and contaminations. Amino acids derived from individual plants do not form the basis for a complete diet and a mixture of plants is required for a healthy diet. This requirement is because individual plant proteins do not contain all nine of the essential amino acids in sufficient amounts. Microorganisms that have been used to produce amino acids include bacteria, fungus, and algae. These microorganisms can be genetically engineered to over produce specific proteins or amino acids. Algae has received much less attention than bacteria, yeast and fungi. The goal of this thesis is to establish a base case for amino acid production from non-genetically modified algae by investigating the effect of different growth parameters. The selection of the specific strain of algae is based on choosing a strain that can be cultured efficiently and economically. Algae can be divided into freshwater and saltwater environments, and both approaches have issues researchers must handle in growing and harvesting algae for protein production. For example, freshwater algae can be contaminated by a large number of microorganisms and the procedure for maintaining a monoculture free from contaminations is very difficult for mass production. On the other hand, saltwater algae have fewer contaminants than the freshwater algae but the operator must contend with control and disposal of salts in the growth media. The expanding need for natural ways to produce high

amounts of amino acids is fueling the search for other strong and competitive microalgal strains. This study investigates *Galdieria sulphuraria,* a type of red algae that can thrive in harsh environments that not many microorganisms can tolerate. *G. sulphuraria* is an extremophile that requires both thermophilic and acidophilic conditions for proper growth; furthermore, *G. sulphuraria* can also be a halotolerant alga, which has the ability to grow in high salt concentration conditions. *G. sulphuraria* also has the ability to consume a wide variety of sugars and nitrogen sources. A Box-Behnken experimental design was used to investigate amino acid production in this algal strain with the manipulated parameters being sugar sources and concentrations of macronutrients (carbon, nitrogen and phosphate). The amino acids that are directly correlated to the concentration of these macronutrients are serine, histidine, arginine, valine, phenylalanine, isoleucine, leucine, and proline. A poor correlation was observed between the concentration of the manipulated macronutrients and the amino acids aspartic acid, glutamic acid, glycine, threonine, alanine, tyrosine, cysteine, methionine, and lysine. Different sugars influence amino acid productions, even with the same carbon number and molecular weight. *G. sulphuraria* fed with glucose has the capability to produce higher amino acid concentrations compared to the same medium with a substituted sugar type, mannitol, using equivalent molar mass. This response is strongly noticeable with a growth medium supplied with xylose, in which *G. sulphuraria* produces greater amino acid concentrations compared to arabinose. When *G. sulphuraria* is grown on xylose, after 48 hours, the culture contains more amino acids than was observed with the other three sugar types. The only exception was for the concentration of aspartic acid when compared to growth on glucose. In this case, when grown on xylose, the aspartic acid concentration decreased by 178%. The superior production of amino acids from a xylose containing medium, which has an increasing value of 70% for serine, 61% for histidine,

75% for arginine, 78% for valine, 71% for phenylalanine, 71% for isoleucine, 70% for leucine, 80% for proline, 48% for glutamic acid, 95% for glycine, 69% for threonine, 76% for alanine, 84 % for tyrosine, 74% for cysteine, 46% for methionine, and 80% for lysine, suggests that amino acid production is irrelevant to the number of carbons in sugars.

PREFACE

This thesis is an original work that has been done by Abdullah Fayadh. No part of this thesis has been previously published.

DEDICATION:

This thesis is dedicated to Mohammad bin Abdullah and his family, who guided me in this journey. In addition, I can never have achieved this stage without my father and mother constant encouragement, support, and belief. Both of you always teach me to believe in myself and my dreams. I also want to dedicate and mention my son, who I missed and suffered from his forced absence from me during the past two years.

ACKNOWLEDGEMENT

The completion of this work could not have been possible without the guidance and assistance of my supervisor, Dr. William McCaffrey. His contributions and involvements were genuinely appreciated. Without his guidance and understanding, this thesis will never have seen the light and can never be completed. I also would like to express my deep appreciation and indebtedness to all my family who in one way or another shared their support in many ways. I also would like to thank Elliot Cameron about the enjoyable discussions and sharing of information to enhance our research.

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CHAPTER ONE: INTRODUCTION

In this introductory chapter, the general rationale for the selection of an alga, *Galdieria sulphuraria*, for amino acid production will be given. This chapter will also describe the variety of types of algae and a more detailed review of the red algae classification that is related to *G. sulphuraria*. Chapter one also explains the structure and components of proteins along with amino acid groupings. In addition, the research project goals and overview of the thesis structure will be summarized.

1.1 GENERAL INFORMATION

Proteins are complexes of organic polymers consisting of individual amino acids and are essential for both human and animals. These amino acids contain two organic substances, an amino group and a carboxylic acid group, necessary for forming protein structures in all life forms. Nowadays, the world is experiencing a deficiency in amino acids that are an essential foundation for well-balanced diets for both humans and animals. Increasing populations of both humans and animals put pressure on the supply of amino acids that are part of the obligatory nutritional diets. Amino acids can come from a wide variety of food resources, and these resources can be broadly categorized into three groups. Traditionally, animal based protein sources for humans have largely been beef and fish, with some regional areas utilizing mainly plant based proteins. Additionally, domestic and wild animals consume amino acids from the same sources, which humans use for properly nutritional diet. Non-animal and non-plant based amino acids can be produced from both conventional and genetically modified microorganisms. Almost all amino acid sources have obstacles and difficulties in their production, which motivates scientists to look for other renewable sources to solve the amino acid supply issues. An

overview of these hindrances and difficulties for each amino acid source is discussed further in this section.

Animal based proteins have high nutritional values for both humans and animals. These organic substances are readily available and are absorbed faster within the animal kingdom's bodies due to their structural similarities. These proteins have very high concentration or ratio of sulphur containing amino acids to those that do not contain sulphur. These advantages promote a positive role for optimal health when animal based proteins are taken within the recommended dietary allowance. However, there are many difficulties to meet the supply demand. It is projected that the global food demand will double in the next 50 years, creating a huge challenge for the sustainability of food production in both terrestrial and aquatic ecosystems (Tilman, et al. 2002). Even though agri-industrial livestock production is highly dynamic and expanding, the increase in human population demands for animal based amino acid currently exceeds the livestock production rate. Furthermore, because constant increases in the human population leads to shortages in available livestock, the cost of animal protein increases and effectively reduces the availability of this type of protein. Animal production is negatively impacted by the depletion of natural resources, such as land and water, which are required for its continued expansion. Available resources vary considerably around the world, as do animal agricultural practices. Moreover, the undesired effects of animal production become important at the socio-economic level when human health, such as diseases, can be impacted by the increases in livestock numbers. All these factors impact availability of well-balanced animal feed. One example of these trends is the increasing prices of fishmeal and other traditional protein feedstuffs for fish culture. These costs have created an incentive to find other alternatives of protein meal (Olvera-Novoa, et al. 1998).

Plant based proteins are another alternative that can be utilized instead of animal proteins for animal feed. Vegetarian proteins can be considered a healthy nutritional diet when different kinds of amino acids present in balanced proportions and proper quantities in the diet. However, plant based protein will vary in composition, depending on the type of plants and the maturity stage of plants. Moreover, most of the plant proteins are concentrated in seeds more so than any other structures in plants. Vegetarian proteins are needed to be consumed from a variety of sources and in higher amounts to meet the daily requirements when compared to animal proteins (Pimentel and Pimentel 2003). A cow growing in sub-Saharan Africa, for example, consumes 10 times more feed, mainly in the form of grasses, than one growing in Europe or North America (M Herrero 2013). Plants are well known to have lower protein amounts than animal proteins for human's requirements (Massey 2003). From an animal perspective, plant based amino acids are imbalanced, such as in the ratio of cysteine to methionine, which is essential to meet human diet requirements (Massey 2003). Plant proteins from single species do not contain the required quantities of individual amino acids. As with the animal proteins, plant production can encounter many difficulties in the availability of natural resources, land and water, which are required for its continued existence. Furthermore, land degradation and desertification have a huge impact on plant production and future expansion. The prolonged use of land extensively to grow plants causes yearly production yields to decrease. Generally, the cost for plant protein is increasing yearly even with the frequent variation in agricultural commodities from year to year. Since plants and their proteins have all these complications, nutritional supplements must be used to support proper diet.

Genetic engineering techniques have been used to successfully modify the deoxyribonucleic acid (DNA) in many types of organisms: animals, plants, and microorganisms. While DNA

modification is outside the scope of this thesis, it is important to acknowledge the importance of genetic engineering. Genetically engineered organisms are associated with a high level of controversy where both researchers and the public are divided between benefits and risks. Importantly, this continuous debate has reduced the public acceptance and approval of any genetically modified organisms in foodstuffs. An example of this conflict is the production of biofortified rice. A genetically modified golden rice has been synthesized with an increased amount of vitamin A in the grain to alleviate blindness and death in children in developing countries (Potrykus 2003). Genetically modified animals and plants have identical issues that are encountered in the conventional animal and plant productions. In general, genetic engineering supporters claim that genetic modification is an enhanced process or at least equivalent to natural selection in genes, actively occurring for generations. However, the opponents of genetic engineering argue that artificial genetic modification has the following risks and issues:

1- Resistance genes of modified organisms, such as crops, can put animal and human under health risks due to horizontal gene transfer between organisms.
2- Biological and physiological responses from a wide variety of genetically modified organisms have not extensively studied or stimulated to exclude all possibilities.

1.2 Algae Potentials

Scientists must search for other natural and renewable sources to play a significant role for promoting protein production away from animals, plants, and genetically modified organisms. Furthermore, these renewable resources have desirably minimal impact on the environment and diversity of the biome and can have a short production cycle and economical costs. Wild algae are one of the best renewable resources, which researchers have not focused on and can be used to avoid many protein production concerns. Algae have been cultivated and used for many years

as alternative food sources. Algae have potential to produce desirable and valuable resources through careful cultivation to produce desirable characteristics, such as fatty acids, carbohydrates, proteins, or pigments for food colouring. Under stress mechanisms, algae are manipulated to produce these preferred substances, which are required for its survival. There are numerous types of algae, green, brown, and red, that can be used for amino acid production. In addition, there are several macro-algae species that are well known to produce high protein content such as seaweeds. Despite the diversity in these organisms, all algae can be divided in two major groups or categories: freshwater and saltwater. Large scale cultivation of algae is difficult to achieve. Freshwater algae can relatively grow slowly and must compete with other aquatic species. To maintain a freshwater algae culture free from contamination, researchers must employ complicated procedures to guarantee a monoculture especially, in mass production. Saltwater algae have an advantage in that there are fewer halotolerant microorganisms that can cause contamination when compared with fresh water algae. The increase in salt concentration in the growth media, however, can have a negative impact on the environment.

1.3 Red Algae Background

The red microalgae *Galdieria sulphuraria* is the main focus of this thesis due to its advantages over many other algae strains. Starting with red algae or Rhodophyta in general, this phylum has the largest collection of algae under one group name. The red algae can range from unicellular to multicellular organisms such as *Galdieria sulphuraria,* representing unicellular organisms, and seaweeds, representing multicellular organisms, in this phylum. Rhodophyta are a group of eukaryotic organisms that have distinctive characterization in which their chloroplasts contain phycoerythin, phycocyanin, and allophycocyanins as auxiliary photosynthetic pigments, and red algae also lack any centrioles and flagella (Freshwater, et al. 1994). The red algae can live in a

wide range of habitats such as freshwater, brackish, sea, or even higher concentrations for few species. Most rhodophytes, around 98%, inhabit marine environments (Dixon 1973). Rhodophyta are considered true plants because they are classified with the green lineage, which includes higher plants and green algae, all having one single common ancestor (Adl, et al. 2005). The red algae group has five distinguished characteristics from other eukaryotic algae groups. This first one is that red algae are lacking centrioles and flagellate phase (Woelkerling 1990). The second distinguished characteristic red algae having is that their chloroplasts, which lack an external endoplasmic reticulum, contain unstacked thylakoids. The third distinguished characteristic for Rhodophyta is that they have different types of display colors due to the varying proportions of chlorophylls a, several occurring carotenoids, and phycobiliproteins, which are accessory pigments (Grossman, et al. 1993). Rhodophyta are typically red in color due to the presence of phycobiliproteins, but they can have shades of different colors such as green, brown, and purple (Gantt, et al. 1979). phycobiliproteins and nonpigmented polypeptides produce phycobilisomes, which are the light harvesting complex attached on the surface of thylakoids (Van den Hoek, Mann and Jahns 1995). The forth distinguished characteristic for red algae is that they have an absence of parenchyma and presence of pit-connections between cells (Woelkerling 1990). The fifth distinguished characteristic for Rhodophyta is the existence of floridean starch as storage product (Viola, Nyvall and Pedersen 2001). Within the Rhodophyta phylum, algae can reproduce sexually or asexually and can be morphologically separated to three major groups (Sheath 2003):

A- A unicellular group reproducing only by binary cell division.

B- A multicellular group with well-developed carpogonial branches.

C- A multicellular group with a carpogonial branch being absent or incipient.

Red algae systematics are changing rapidly in recent years due to advancements in molecular biology and phylogenetics. In the past, the phylum Rhodophyta has been divided into two subclasses, Bangiophyceae and Florideophyceae (Cole and Sheath 1990). Florideophyceae has six orders: Nemalionales, Gelidiales, Cryptonemiales, Gigartinales, Rhodymeniales, and Ceramiales (Brodie and Lewis 2007). Bangiophyceae has also six orders: Bangiales, Cyanidiales, Compsopogonales, Erythropeltidales, Porphyridiales, and Rhodochaetales. The current classification of Rhodophyta now includes seven classes: Cyanidiophyceae, Bangiophyceae, Florideophyceae, Compsopogonopgyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae (Sechbach and Chapman 2010). Due to the concentration on *Galdieria sulphuraria* in this thesis, the focus will be on the class of Cyanidiophyceae. Cyanidiales are an abundant group of unicellular, small, and most primitive extant eukaryotic algae. This class forms a dominant feature in warm to hot acidic streams and pools (Schaechter 2009). Within this group, Cyanidiales consists of three genera Cyanidioschyzon, Cyanidium, and Galdieria, and there are six species *Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria maxima*, *Galdieria partita*, *Galdieria daedala*, and *Galdieria sulphuraria* (Barbier, et al. 2005). These species are documented to belong to at least four distinct lineages that integrate the three traditional genera (Cinigia, et al. 2004). These species have the ability to live in volcanic and extreme environments which combine following conditions: low pH level, from 0.2 to 4.0, high temperatures, from 40 to 56 °C, and high levels of heavy metals (Toplin, et al. 2008). *Cyanidium caldarum* and *Cyanidioschyzon merolae* are similar in that they contain a single nucleus, mitochondrion, and plastid. *Cyanidium* are round with cell walls and form four endospores while *Cyanidioschyzon* are club shaped without cell walls and divide by binary fission (Lee 2008). *Cyanidium caldarum* was used as a synonym for *G. sulphuraria* until 1981. *G. sulphuraria,*

however, grows autotrophically and heterotrophically while *Cyanidium caldarum* has been reported to grow only autotrophically (Gross, et al. 2001).

1.4 GALDIERIA

Galdieria is classified into four species, *G. maxima*, *G. partita*, *G. daedala*, and *G. sulphuraria,* based on cell morphology (Barsanti and Gualtieri 2014). *Galdieria* cells have a spherical shape with rigid cell walls, and propagate asexually through the formation of 4 to 32 small endospores, or daughter cells, within a mother cell (Barsanti and Gualtieri 2014). This class of algae is an extremophile requiring both thermophilic and acidophilic conditions, with a required temperature of 40 to 56 °C and pH from 0.2 to 4. In extreme habitats, it can represent up to 90 % of the biomass (Weber, et al. 2004). *G. sulphuraria* possesses extensive metabolic versatility with the ability to tolerate high salt and metal concentrations (Schonknecht, et al. 2013). Cultures can tolerate up to 10% (weight/volume) salinity (Albertano, et al. 2000). Growing both photoautotrophically and heterotrophically, *G. sulphuraria* can consume more than fifty carbon sources and polyols (Vanselow, et al. 2009). A distinctive feature of *G. sulphuraria* growth is that it can only uptake certain amino acids or ammonia as a source of nitrogen and cannot uptake nitrate (Albertano, et al. 2000). Due to gene transfer mechanisms, *G. sulphuraria* has acquired required genes to live in extreme environments and feeding mechanisms, through horizontal gene transfer, to survive in these severe conditions from archaea and bacteria, which is rare among eukaryotes (Schonknecht, et al. 2013).

1.5 PROTEIN

Proteins are one of the fundamental biomolecules and are one of the main nutrients necessary for all living organisms. Proteins are macromolecules containing one or more polypeptides (Walsh

2002). They contain carbon, hydrogen, oxygen, nitrogen, and sometimes phosphorous and sulphur in their structure. Proteins play a wide range of important roles within biological and food systems, such as being the building blocks of cells, biocatalysts, antibodies, protective proteins, storage, fuel sources, a regulator for cell function, for molecule transportation, or cell response feedback to stimuli (Damodaran 1997). The significance of proteins comes from their constituent building blocks. These building blocks define protein characteristics and interactions within the system of living beings. Proteins are very complicated biopolymers which are linked together by peptide bonds, also known as amide bonds. Any protein must contain at the minimum one long linear chain of building blocks, which is also called a polypeptide chain. The smallest protein consists of 20 polypeptides. Each peptide consists of two or more amino acids so each protein has at least a chain of amino acids linked together by peptide bonds (Walsh 2002). Amino acids are essential organic compounds for biological systems, each containing a carboxyl, COOH, and an amino group, H_2N, along with a side chain group, R.

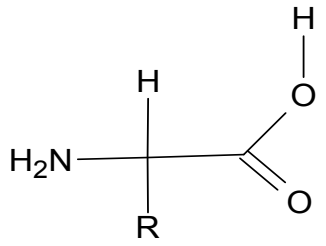


FIGURE 1: Amino Acids Generic Form

The reaction to form polypeptides can be done through bonding between the carboxyl group of one amino acid and the amino group of the next amino acid through condensation reaction involving a loss of water molecule to form a bond of -OC-NH-. The reverse reaction can be done with the same peptide bond linkage that had been formed during building the polypeptide via

hydrolysis. These reactions are dynamic and depend on variable system conditions and mediators to help achieve cellular growth, respiration and product formation.

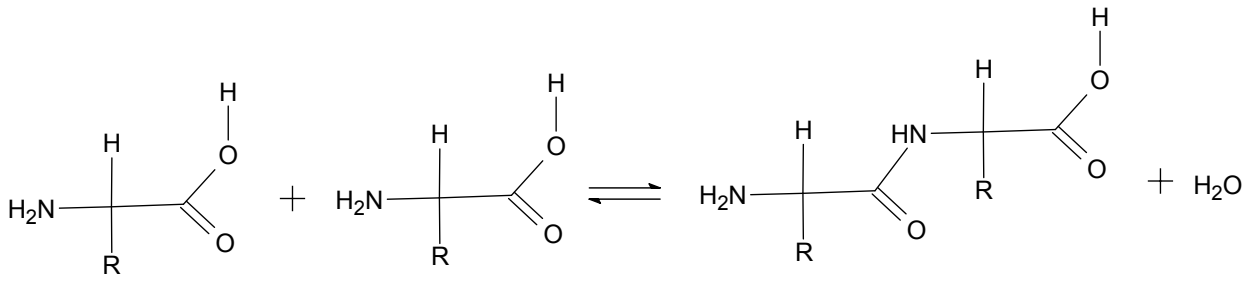
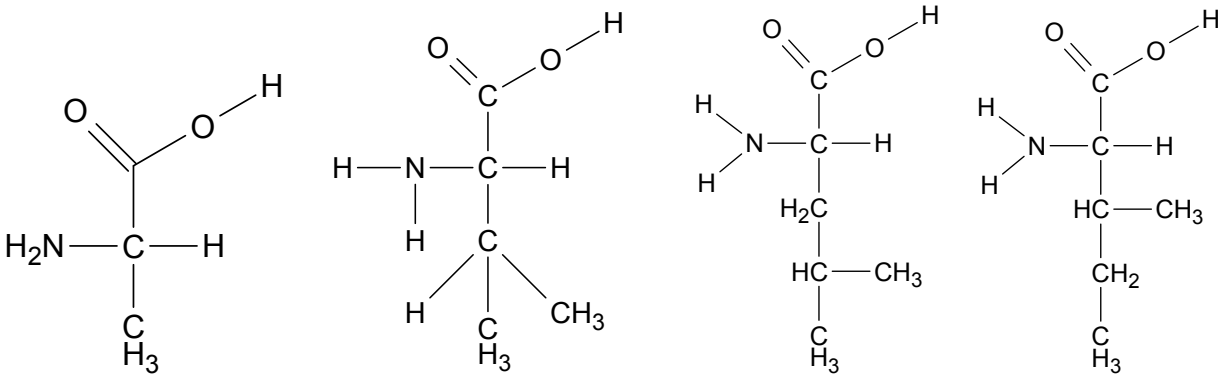


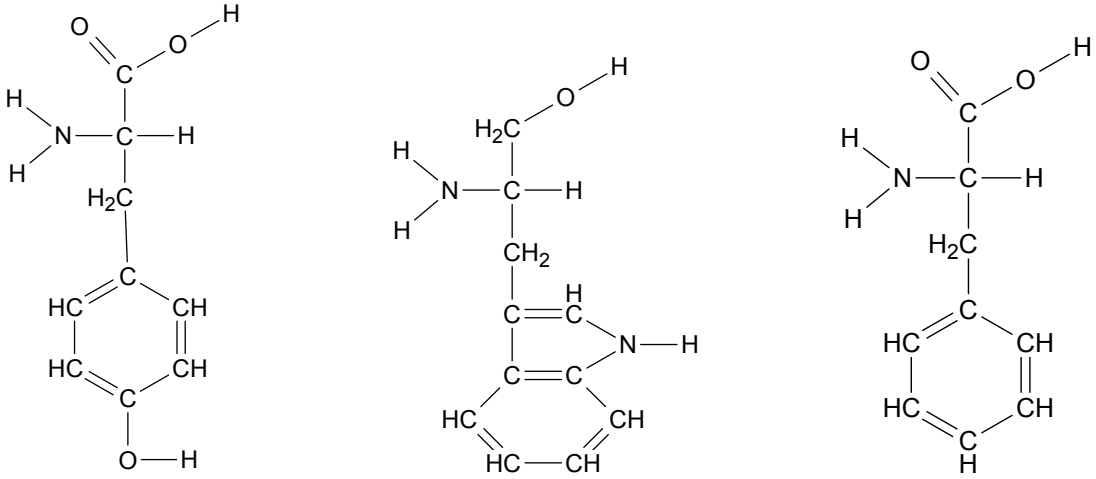
FIGURE 2: A Generic Condensation Reaction with Losing One Water Molecule to Form Polypeptides Chains

There are five hundred or so known naturally occurring amino acids (Wanger and Musso 1983). All proteins are synthesized from twenty common amino acids, with minor exceptions (Thompson, Morris and Smith 1969). The difference in amino acids comes from the chemical nature of the side groups that are attached to the alpha carbon. In amino acids, the side chain is key to specifying the amino acid chemical properties. These twenty amino acids can be classified in a variety of ways, such as by the structural functional group location, polarity, pH, or side-chain group type. Excluding asparagine and glutamine, eighteen amino acids found in proteins are presented in Figures 3 to 8. All amino acids can come with two forms of stereoisomers, which are called D and L, and most of these amino acids come in the L form. This difference in stereoisomers is related to the asymmetry of the alpha carbon of the amino acids with one exception, glycine which has a hydrogen in the R group.



Alanine Valine Leucine Isoleucine

FIGURE 3: Amino Acids with Hydrophobic Side Chains (Aliphatic)



Tyrosine Tryptophan Phenylalanine

FIGURE 4: Amino Acids with Hydrophobic Side Chains (Aromatic)

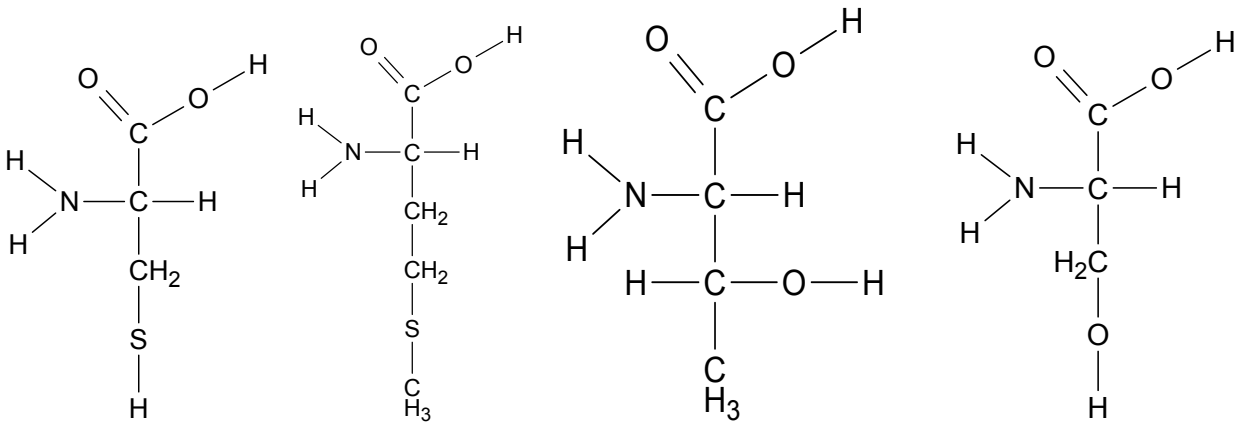


FIGURE 5: Amino Acids with Polar Neutral Side Chains

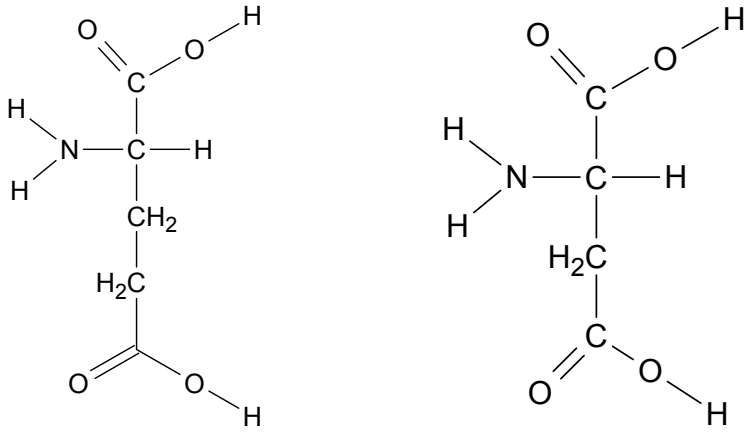


FIGURE 6: Amino Acids with Electrically Charged Side Chains (Acidic)

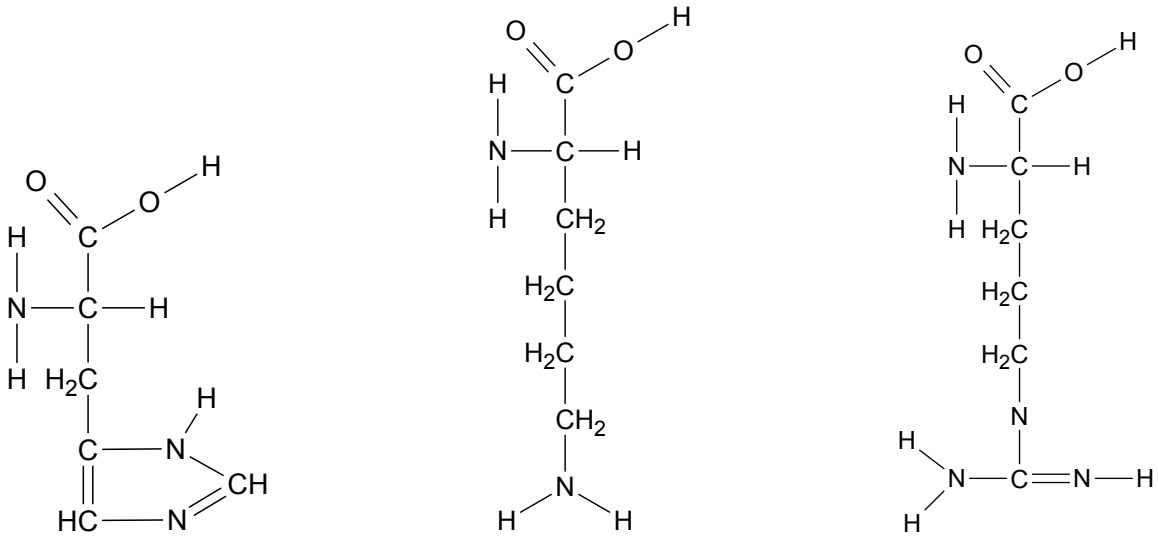


FIGURE 7: Amino Acids with Electrically Charged Side Chains (Basic)

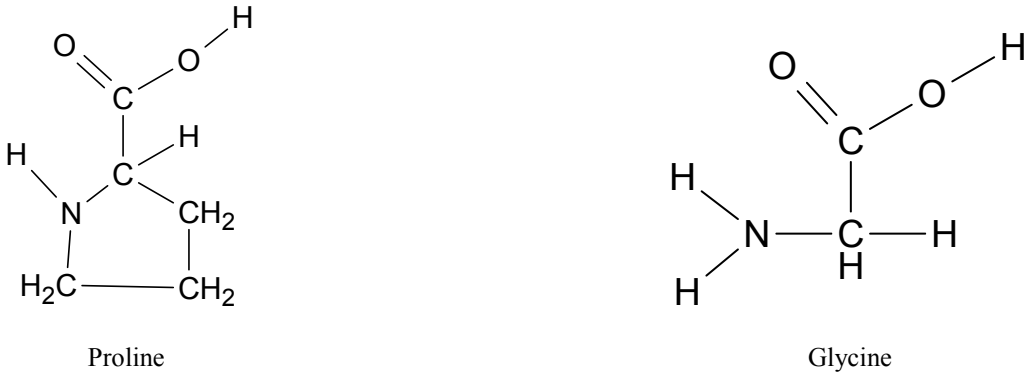


FIGURE 8: Unique Amino Acids

Since the difference in the amino acids composition is very broad, this variation leads to wide range of possibilities in both protein configuration and shape. Primary protein structure is defined by the linear sequence of covalent linkages of the amino acids into long chains.

Hydrogen bonding between the main chain peptide groups leads to the secondary structure of a protein. This secondary structure is highly organised into regular areas of folding or coiling inside the protein. Alpha helices and beta strands or sheets are also formed in the secondary structure due to hydrogen bonding between donors and acceptors in the peptide backbone of the protein. The tertiary structure is the three-dimensional structure of a protein that made from more compact amino acids interaction with each other due to forces other than hydrogen bonding in the secondary structure. These week interactions can be a result of hydrophobic or hydrophilic interaction of protein with its environment. The quaternary structure is also another non-covalent bonding interaction that binds different polypeptides into a single-large protein. These structures can be broken by heat treatment or acid treatment that proteins are subjected to during any experimental process.

1.6 Research Project Goal

The goal of this research is investigating the ability to control amino acid production profile for the red algae *G. sulphuraria* through environmental growth parameters. This research is needed because amino acids are used in a wide variety of products around the world; for example, amino acids supplements and food additives. A preliminary goal for this research is to understand the effect of the carbon, nitrogen, phosphorous on amino acid production at different concentrations. Another goal is to observe the impact of different carbon sources on amino acids production by *G. sulphuraria.*

1.7 Overview of The Thesis Structure

This chapter introduces the reader to the general research problem that is led to this project. In addition, chapter one focuses on red algae specifically, *G. sulphuraria,* and related classification.

Finally, the same chapter is also expended to describe protein and amino acids structures and classifications. In chapter two, materials and methods are summarized. The second chapter also describes the components of media formulations used to culture *G. sulphuraria* in this research project. Chapter two also includes information about the method used to extract amino acids and amino acid standards. Chapter three presents general information about the Box-Behnken experimental design. It also describes the experimental design and underlying assumptions used in this research. Chapter four presents the experimental results in detail. Chapter five covers the discussion of the results. The last chapter, six, presents conclusions and recommendations of the whole thesis.

CHAPTER TWO: MATERIALS AND METHODS

2.1 MICROALGAE STRAIN

The microalga *G. sulphuraria* was purchased from University of Texas at Austin UTEX 2919 in cyanidium medium. The alga was transferred aseptically on the same day of its arrival to 500 ml Erlenmeyer flasks, which contained 300 mL of modified Allen's cyanidium media according to algal culturing techniques (Andersen 2005).

2.2 ALLEN'S CYANIDIUM MEDIA

A modified form of Allen's cyanidium medium, which was mentioned in the algal culturing techniques (Andersen 2005), was used to grow the microalga *G. sulphuraria.* A modified Allen's cyanidium medium was only used for two weeks as a refreshing media (Table 1). This medium encompassed most of micro and macro nutrients for this microalga species growth for one liter of deionized water. The pH of the solution was adjusted to 2.5 by concentrated sulfuric acid

when all of components were added to the one liter of deionized water. The trace elements solution was also prepared in one liter of deionized water and stored at the room temperature until the day of media preparation, shown in Table 2. Since heterotrophic growth conditions were investigated in this study, 1% glucose was added to the media. The final media is autoclaved prior to inoculation, and the microalga *G. sulphuraria* was transferred aseptically.

TABLE 1: Modified Allen's Cyanidium Medium (for 1 Liter of Solution)

Component	***Quantity Used***
$(NH_4)_2SO_4$	**1.320 g**
KH_2PO_4	**0.272 g**
$MgSO_4 \cdot 7H_2O$	**0.247 g**
$CaCl_2$	**0.055 g**
Trace metals solution	**1 mL**

TABLE 2: Trace Metals Solution (for 1 Liter of Solution)

Component	***Quantity Used***
$Na_2EDTA \cdot 2H_2O$	**5.00 g**
$FeCl_3 \cdot 6H_2O$	**0.98 g**
$MnCl_2 \cdot 4H_2O$	**0.18 g**
$ZnSO_4 \cdot 7H_2O$	**0.11 g**
$CoCl_2 \cdot 6H_2O$	**0.020 g**
$Na_2MoO_4 \cdot 2H_2O$	**0.125 g**

2.3 GROSS AND SCHNARRENBERGER GROWTH MEDIA

After two weeks from healthy growth, all inoculua and experiments were cultured using Gross and Schnarrenberger growth media. This media encompassed all necessary micro and macro nutrients for this microalga species in one liter of deionized water (Table 3).

TABLE 3: Gross and Schnarrenberger Growth Media (for 1 Liter of Solution)

Component	*Quantity Used*
$(NH_4)_2SO_4$	**1.5 g**
$MgSO_4 \cdot 7H_2O$	**300 mg**
KH_2PO_4	**300 mg**
$CaCl_2 \cdot 2H_2O$	**20 mg**
NaCl	**20 mg**
Fe-EDTA-solution	**1.5 mL**
trace-element solution	**2 mL**

Iron sulfate stock solution was prepared with EDTA to form a complex to prevent iron precipitation (Table 4). The Fe-EDTA complex was obtained by boiling deionized water then adding both components together. The total volume was adjusted using deionized water addition to make yield 100 mL of solution after cooling. The Fe-EDTA complex solution must be allowed to cool in the room temperature before performing any media preparation, and this solution was also kept at room temperature.

TABLE 4: Fe-EDTA Solution (for 100 mL of Solution)

Components	*Quantity Used*
$FeSO_4$	**690 mg**
EDTA	**930 mg**

The trace-element solution was prepared in one liter of deionized water (Table 5). This trace-element solution was prepared and kept in room temperature.

TABLE 5: Trace-Element Solution (for 1 Liter of Solution)

Components	*Quantity Used*
H_3BO_3	**2.86 g**
$MnCl_2 \cdot 4H_2O$	**1.82 g**
$ZnSO_4 \cdot 7H_2O$	**220 mg**
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	**30 mg**
$CuSO_4 \cdot 5H_2O$	**80 mg**
$NaVO_3 \cdot 4H_2O$	**40 mg**
$CoCl_2 \cdot 6H_2O$	**40 mg**

Both solutions, Fe-EDTA complex and trace-element solution, did not show any sign of contamination or destabilization when stored at room temperature for a period of two years. Since microalgal growth was studied under heterotrophic conditions, 25mM of glucose was added to the media. The final media was adjusted to pH 1.8 by using concentrated sulfuric acid. After adjusting pH, the media solution was transferred to one liter Erlenmeyer flasks, which had been cleaned and rinsed thoroughly. Each Erlenmeyer flask contained around 300 mL of media

that was prepared for inoculation. All Flasks containing media were covered with cotton before being autoclaved. All chemicals that had been used to prepare the media were bought from Fisher Scientific and used as received.

2.4 Strain Maintaining and Inoculation

Before every batch-culture preparation, a fresh culture was prepared one week in advance to use for inoculation. Growing in a fresh media for 48 hours, the inoculum was 0.5 mL injected to the batch culture of 500 mL of media in 1 L Erlenmeyer flasks. The injection, which happened inside the bio-hood in a room temperature, contains algae in exponential phase and any equipment that had been used was sterilized at a temperature of 121 °C for 30 minutes.

Several cultures of 300 mL in one liter Erlenmeyer flasks used for strain maintenance. These cultures contained Gross and Schnarrenberger growth media, which had been mentioned above. These cultures were placed in the dark all times in a shaker.

2.5 Culture Growth Conditions

The microalga *G. sulphuraria* was grown in heterotrophic condition in the entire period since the time of its arrival to the lab. The shaker incubator operated at speed of 120 rpm with a temperature of 45.5 °C in the entire period. Since all Erlenmeyer flasks were covered with cotton wrapped with cheese-cloth to allow air exchange, cultures were not supplied with air. The shaker was opened every twenty-four hours to take out cultures for processing.

2.6 The Operation of Sampling

The sampling was performed every twenty-four hours per sample. Each sample consisted of 25 mL, that was equally divided into five falcon tubes with a capacity of 10 mL as replicates. All of

these tubes were immediately stored in a fridge at 4 °C for further analysis, and those investigations were done within two to three days.

2.7 Determination Algae Weight

The exact weight of the algae in any given sample was determined by using total cell material weight after washing in deionized water. The biomass content of each replicate, which contained 5 mL in 10 mL falcon tubes, was centrifuged in Thermofisher Scientific model Sorvall RC 6 plus at 5000 rpm (RCF= 3795 g) for fifteen minutes. After discarding the supernatant phase in all of the washes, sample pellets were washed three times with deionized water, vortexed for 1 minute with Fisher Vortex Genie 2 model G560, and centrifuged with the same speed mentioned above in each rinse. The final pellets were put in an Isotemp oven, which was made by Fisher Scientific model 637G, for twenty-four hours or until they reached constant weight. The temperature of this Isotemp oven was set to be 79 ±3 °C for the duration of drying. The dry weight of all replicates was measured, after being cooled on the counter to room temperature, using a Sartorius analytical balance model BL210S. All samples and their replicates were washed, dried, and weighted at the same time for each operation performed to minimize errors.

2.8 Sample Preparation for Amino Acids Extraction

After measuring the dry weights for all samples, microalga *G. sulphuraria* was soaked with 2 mL of 0.1 M hydrochloric acid to hydrolyse all amino acids (Perez-Palacios, et al. 2015). An acid concentration of 0.1 M HCl, instead of 6 M HCl, was used to prevent the reaction of hydrochloric acid with the Falcon tubes, which were made from polypropylene, was to minimize the risk of hazards. A comparison of cell lysis techniques between using HCl only and Polyton Homogenizer PT 1300D with water can be seen in Table 109. Polyton Homogenizer cannot be

used with HCl because the material of the Polyton reacts with the solution. This reaction prevented the use of HCl and promoted the use of distilled water. The results of these two methods were different from each other with advantages for the use of HCl only.

All Falcon tubes were tightly secured and sealed with caps to avoid any gases from leaking, which could affect amino acids reactions and concentrations in the end results. Furthermore, all samples were vortexed for 1 minute after the hydrochloric acid addition and before putting samples inside the Isotemp oven. The temperature of this Isotemp oven was set to be 110 ±3 °C during sample hydrolysis. All samples were put in the oven for a period of twenty-four hours, and all samples subjected to these conditions at the same time as one set. The change of time could cause a change in the amino acids concentrations and hydrolysis, so it was very important to keep a record of the time. When the time was reached the desired end point, all test tubes were taken out from the oven and allowed to cool to room temperature on the counter. The liquid, which was the hydrochloric acid left in the tubes, was allowed to evaporate in the fume hood.

2.9 Sample Preparation for HPLC Analysis

The dried residues in the tubes were dissolved in 2 mL of 0.1 M HCl and vortexed for 2 minutes with Fisher Vortex Genie 2 model G560. Each solution in every tube was transferred using a 3 mL syringe made by Covidien Monoject ref 1180300777. The solution in each syringe was filtered with a 0.2 µm PP micron filter from Sigma Aldrich when the amino acid solution was transferred into HPLC glass insert vials. The sample vials were put into the HPLC autosampler for amino acid analysis or kept in the fridge under 4 °C when the autosampler was full in vials.

2.10 Amino Acid Standards

Samples of amino acid standard were bought from Agilent Technologies with five different

concentrations. The concentrations of amino acid standards were as follow (Table 6):

TABLE 6: Different Concentration of Amino Acid Standards

Amino Acid Standard Concentration	*Item Number*	*Expiry Date*
10 pmol/µL in 0.1 M HCl	**5061-3334**	**05 Jun 2017**
25 pmol/µL in 0.1 M HCl	**5061-3333**	**05 Jun 2017**
100 pmol/µL in 0.1 M HCl	**5061-3332**	**10 Aug 2017**
250 pmol/µL in 0.1 M HCl	**5061-3331**	**15 Jul 2017**
1 nmol/µL in 0.1 M HCl	**5061-3330**	**10 Aug 2017**

Each of the ampoules of amino acid standard contained L-aspartic acid, glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-serine, L-threonine, L-tyrosine, L-proline, L-arginine, L-histidine, L-glutamic acid, L-cystine, L-phenylalanine, L-lysine, and L-methionine. Ampoules where stored at 4 °C and opened the same day of analysis and used within seven days from being opened. The amino acid standards were performed in triplicate at the beginning of samples test, after performing 50 samples, and in the end of all samples analysis test. Amino acid standard calibration curve was generated after all of standard tests had been done. The average of each concentration was used to plot the standard calibration curve.

2.11 Amino Acid Derivatization Reagents

There were two types of derivatization reagents used in this experiment. The first reagent was called OPA, which consisted of 10 mg/mL each of ortho-phthalaldehyde and 3-mercaptopropionic acid in 0.4 M borate buffer. The second reagent was named FMOC that contained 2.5 mg/mL 9-fluorenylmethylchloroformate in acetonitrile. Both reagents were bought from Agilent Technologies (Table 7).

TABLE 7: The Derivatization Reagents for Amino Acids

Reagents Name	*Item Number*	*Expiry Date*
OPA	**5061-3335**	**06 Apr 2017**
FMOC	**5061-3337**	**23 Sep 2016**

2.12 Pre-column Amino Acid Derivatization

All amino acid derivatizations took place in the pre-column stage in the HPLC automatic liquid sampler (ALS). The autosampler that had been used in this experiment was from Agilent Technologies G1329A. Every injection used the following steps:

1- Drawing 2.5 µL from borate buffer vial.
2- Drawing 1.0 µL from sample vial.
3- Mixing 3.5 µL in the air five times at the default speed.
4- Waited for 0.2 minutes.
5- Drawing 0.5 µL from OPA reagent vial.
6- Mixing 4.0 µL in the air ten times at the default speed.

7- Drawing 0.4 µL from FMOC reagent vial.

8- Mixing 4.4 µL in the air ten times at the default speed.

9- Drawing 32 µL from the injection diluent vial.

10- Mixing 20 µL in the air eight times.

11- Waited for 0.1 minutes.

12- Injected in the mainline of phase ingredients.

The borate buffer used in these steps was purchased from Agilent Technologies. The borate buffer had a pH 10.2 with 0.4 normality in water (Item number 5061-3339). The injection diluent was made from mobile phase A by taking 100 mL of the mobile phase A solution and added 0.4 mL of concentrated H_3PO_4. The injection diluent was stored at 4°C.

2.13 HPLC MOBILE PHASE PREPARATION

There were two different mobile phases running through the HPLC to perform the amino acid quantitative analysis. Mobile phase A contained disodium hydrogen phosphate, sodium tetraborate decahydrate, and sodium azide. These compounds were purchased from Sigma Aldrich (Table 8).

TABLE 8: Compound Quantity in 1L of Deionized Water

Compound Name	*Quantity*
Na_2HPO_4	**1.4 g**
$Na_2B_4O_7$	**3.8 g**
NaN_3	**0.032 g**

The sodium tetraborate decahydrate must be prepared alone to allow for a complete disassociation in the deionized water. The solution must be adjusted to pH 8.2 after mixing all

compounds together with concentrated HCl. The solution was filtered with 0.45 μm cellulose membrane discs that were purchased from Sartorius Stedim Biotech. Sodium azide is a very toxic compound that must be handled properly and with caution. The same compound must be naturalized under the fume-hood following a standard procedure.

Mobile phase B is made up from deionized water, methanol, and acetonitrile (Table 9). The methanol and acetonitrile were bought from Sigma Aldrich, and all solvents were HPLC grade.

TABLE 9: Compound Quantity in 1L of Deionized Water

Compound Name	*Quantity*
Deionized water	**100 g**
Methanol	**356.4 g**
Acetonitrile	**353.7 g**

The measurements were conducted at room temperature, and all of components were stored all the time at 25°C. All solutions were allowed to mix for couple of minutes to remove any bubbles that were formed from combining all compounds. Both solutions, mobile phase A and mobile phase B, were kept in HPLC amber type of bottles to minimize any type of organism's growth. The two mobile phases were prepared one day in advance of using the HPLC. All glassware, that had been used in these experiments were washed thoroughly and rinsed with deionized water three to four times, put in the oven to dry. The glassware were taken out from the oven only when it was needed for usage.

2.14 INSTRUMENTATION USAGE TYPE

The amino acids analysis was performed using high performance liquid chromatography (HPLC) Agilent 1200 series. This high performance liquid chromatography equipped with a degasser, model number G1222A, to remove any air in the lines going to the pump. A binary pump system, which had the model number G1312A, used to perform controlled phase ingredients during amino acids separation. In addition, this HPLC was equipped with an autosampler, model number G1329A, which was used to perform pre-column derivatizations for amino acid samples. Furthermore, the high performance liquid chromatography equipped with thermostatted column compartment model G1316A to control the temperature of the column and liquids entering into the column and the detector. In the case of the detector, diode array detector (DAD) model G1315C was used to analyse amino acids. In the following subsections, the setting or the method used to accomplish the end results for amino acids analysis described below.

2.14.1 PUMP PARAMETERS

There were two pumps in the HPLC instrument used at the same time during the analysis. The flowrate for these two pumps was 0.750 mL/min during and after performing each sample. Each trial lasted for 27.20 minutes with a post operation time of 5.00 minutes. Pump A used for controlling the flowrate of mobile phase A during the sample trial, and this was the same for pump B, which was used to control mobile phase B. The percentage range of mobile phase B was from 2 to 100 depending on the phase of the operating sample (Table 10).

TABLE 10: Mobile Phase B Percentage with the Time of the Trial

Time	*%B*
0.00	**2.0**
0.35	**6.0**

2.65	**19.0**
6.00	**27.0**
7.00	**30.0**
25.50	**57.0**
25.60	**100**
26.80	**100**
26.90	**2.0**

2.14.2 THERMOSTATED COLUMN COMPARTMENT

The temperature for the left and the right side of thermostated column compartment was set to 40 °C. The boundaries, which the analysis could be carried, were limited to ±0.5 °C during each trial. The temperature was allowed to stabilize for a couple of hours before analyzing any sample. Temperature stabilization was required in order to have reproducible results.

2.14.3 DIODE ARRAY DETECTOR

A diode array detector was used to record the ultraviolet and visible absorption spectra of a sample passing through the HPLC. A qualitative information about samples could be generated and collected using this detector by selecting the best wavelength for analysis. The diode array detector was calibrated to use two different wavelengths (Table 11).

TABLE 11: Diode Array Detector Wavelength Setting

Signal Number	*Wavelength (nm)*	*Bandwidth (nm)*	*Reference Wavelength*	*Reference (nm)*	*Bandwidth*

			(nm)	
Signal A	**338**	**10**	**390**	**20**
Signal B	**262**	**16**	**324**	**8**

The use of two different wavelengths for amino acid analysis was due to the presence of two different derivative agents that interacted differently with amino acids. OPA would interact with specific amino acids such as aspartate, that these amino acid derivative forms would be observable at the wavelength of 338 nm. On the other hand, FMOC would interact with different amino acids such as proline, which the derivative form of these amino acids and would be observable at a wavelength of 262 nm. Amino acids that interacted with OPA derivatives would elute earlier than FMOC derivatives, which were at the end of the amino acid separation process.

2.14.4 Column Type

There were two columns that were used for amino acids separation in this experiment. The first part was called the amino acid separation column that was the main segment to use for analysis. This column was purchased from Agilent Technologies, Poroshell HPH-C18 4.6 X 100 mm and 2.7 micron. The second component, which was attached to the amino acid separation column, was a UHPLC guard column to protect the main column and added length for better beak separation. The guard column Poroshell HPH-C18 was also bought from Agilent Technologies and had the measurements 4.6 X 5 mm and 2.7 micron. The column and guard column were washed at the end of every batch with 100 % of mobile phase B.

CHAPTER THREE: MODELING AND EXPERIMENTAL DESIGN

3.1 MODELING

Algae growth is a very complex process, but this growth behavior, or kinetics, is often describable using simple mathematical equations. The most common model is Monod equation which is commonly used to describe growth in microorganisms. The growth rate for three substrates, carbon, nitrogen, and phosphorous, can be expressed as below:

$$\mu = \mu_m \left(\left(\frac{C_c}{k_c + C_c} \right) \left(\frac{C_n}{k_n + C_n} \right) \left(\frac{C_p}{k_p + C_p} \right) \right) \quad (1)$$

μ is the specific growth rate of the algae, and μ_m is the maximum specific growth rate of the algae. C_c, C_n, C_p are the concentrations of carbon, nitrogen, and phosphorous substrates for growth. K_c, K_n, K_p are Monod constants for carbon, nitrogen, and phosphorous, respectively. In the growth curve, inhibition can often be seen, especially with the nitrogen concentration, which it can be described by Haldane equation as follows:

$$\mu = \mu_m \left(\frac{C_n}{K_n + C_n + \left(\frac{C_n^2}{K_{ni}} \right)} \right) \quad (2)$$

K_{ni} is the inhibition constant for nitrogen. Equation number 2 can be used to substitute the nitrogen relationship in equation number 1 as below:

$$\mu = \mu_m \left(\left(\frac{C_c}{k_c + C_c} \right) \left(\frac{C_n}{K_n + C_n + \left(\frac{C_n^2}{K_{ni}} \right)} \right) \left(\frac{C_p}{k_p + C_p} \right) \right) \quad (3)$$

Since biomass modeling is not the goal of this thesis, this is a qualitative overview for biomass modeling. The Monod/Haldane approach to bioprocess modeling is not applicable to protein production which is a complex process alternating between anabolism and catabolism depending on the state of cells.

3.2 Introduction To Experimental Design

In general, an experiment is a process in which one or more input variables or factors are manipulated that can have impact on the system output response or variable. On the other hand, experimental design is a procedure for planning a series of experiments through statistical methods to analyze the data for drawing valid and objective conclusions (Montgomery, Design and Analysis of experiments 2001). Every design of experiment starts with setting the objective for the experiment and choosing input factors that are determent by the researcher to generate the desired output response. An excellent design of experiment will act as a navigation map for the researcher to execute in the process of formulating meaningful conclusions based on results. Box-Behnken design has been chosen for the experimental design in this thesis. The Box-Behnken design was established in 1960 and developed three level designs which allow estimation of the general quadratic model (Eriksson, et al. 2008). Box-Behnken design comprise of 2^2 factorials in each pair of factors with all other factors that must be kept constant at their mid-level plus a few center points (Lawson 2015). There is no Box-Behnken design that can exist for only two factors (Lawson 2015). Box-Behnken design does not include any points at the vertices of the cubic region created by the upper and lower limits for each variable, which helps to reduce the number of required experimental trials to 15 experiments for this thesis (Mason, Gunst and Hess 2003). The Box-Behnken is a rotatable or near rotatable spherical design, and this kind of design is a one type of response surface methodology (Montgomery, Peck and

Vining, Introduction to Linear Regression Analsis 2012). A response surface methodology is a collection of statistical and mathematical techniques, which are useful for the modeling and analyzing of problems in that a response of interest is influenced by several factors or variables (Perincek and Colak 2013). The objective of the response surface methodology is to optimize the response of interest (Perincek and Colak 2013).

3.3 Experimental Design Goal

The objective for performing these experiments is to understand the impact of carbon, nitrogen, and phosphorous on the amino acids production, which are the three factors for the experimental design in this thesis. All three factors are categorized as macronutrients for algae growth and maintenance.

3.4 Hypothesis

In every experiment, a hypothesis, which is a speculated idea created by researchers that can be examined through experimentation, has been developed from pervious observations by a researcher. This research thesis is not different from any other research in this aspect.

Hypothesis # 1: It is hypothesized that using different concentrations for these three macronutrients will positively or negatively impact the amino acids production in *Galdieria sulphuraria*.

Hypothesis # 2: It is hypothesized amino acids production can be changed and altered with the type of carbon source that is used. Although using the highest amino acids production results from testing hypothesis number one above, the only change is the sugar type, keeping all other parameters at the same concentration. This modification can have dramatic change on the amino

acids production in *Galdieria sulphuraria*.

3.5 DESIGN

To rationally design the experimental series to investigate amino acid expression, Box-Behnken experimental design approach was selected. This approach was used rather than single factor experiments in order to decrease the total number of experiments, the expenditure of material resources, and to minimize the time of experimentation. Box-Behnken experimental design was chosen for this study to elucidate the relationship between the response functions (amino acids production) and variables (carbon, nitrogen, and phosphorous). To limit experimental costs, only three different concentrations were used for each variable (Table 12). In this experimental design with three factors and three levels, a total of fifteen experiments were required, including three center points to assess repeatability (Tables 13 & 14).

TABLE 12: Processes Variables and Levels for Box-Behnken Experimental Design

Variable	*Symbol*	*Coded Variable Level*		
		Low	**Center**	**High**
		-1	**0**	**+1**
Sugar (g/L)	**X_1**	**2.500**	**5.000**	**7.500**
Nitrogen (g/L)	**X_2**	**0.200**	**11.000**	**22.000**
Phosphorous (g/L)	**X_3**	**0.075**	**0.151**	**0.300**

TABLE 13:Coded Factor Levels for Box-Behnken Design of Three-Variable

Experiment Number	X_1	X_2	X_3
1	**-1**	**-1**	**0**
2	**+1**	**-1**	**0**
3	**-1**	**+1**	**0**
4	**+1**	**+1**	**0**
5	**-1**	**0**	**-1**
6	**+1**	**0**	**-1**
7	**-1**	**0**	**+1**
8	**+1**	**0**	**+1**
9	**0**	**-1**	**-1**
10	**0**	**+1**	**-1**
11	**0**	**-1**	**+1**
12	**0**	**+1**	**+1**
13 (central points)	**0**	**0**	**0**
14 (central points)	**0**	**0**	**0**
15 (central points)	**0**	**0**	**0**

TABLE 14: Actual Factor Levels for Box-Behnken Design of Three-Variable

Experiment Number	*Carbon (Glucose) g/L*	*Nitrogen (Ammonium Sulfate) g/L*	*Phosphorous (Monopotassium Phosphate) g/L*
1	**2.5005**	**0.2006**	**0.1516**
2	**7.5004**	**0.2002**	**0.1514**
3	**2.5003**	**22.0005**	**0.1510**
4	**7.5001**	**22.0002**	**0.1512**
5	**2.5005**	**11.0005**	**0.0750**
6	**7.5004**	**11.0004**	**0.0751**
7	**2.5002**	**11.0003**	**0.3004**
8	**7.5003**	**11.0004**	**0.3001**
9	**5.0003**	**0.2003**	**0.0754**
10	**5.0001**	**22.0003**	**0.0751**
11	**5.0003**	**0.2004**	**0.3001**
12	**5.0002**	**22.0005**	**0.3001**
13 (central points)	**5.0002**	**11.0003**	**0.1512**
14 (central points)	**5.0002**	**11.0005**	**0.1515**
15 (central points)	**5.0002**	**11.0005**	**0.1515**

3.6 VARIABLES

3.6.1 DEPENDENT VARIABLES

The dependent variables that are quantified in these experiments are the amino acid concentrations in each sample. In this work, seventeen amino acids were measured, as outlined in Table 15.

TABLE 15: Amino Acids Names and Chemical Formula

Amino Acids Names	*Chemical Formula*
Aspartic Acid	**$C_4H_7NO_4$**
Glutamic Acid	**$C_5H_9NO_4$**
Serine	**$C_3H_7NO_3$**
Histidine	**$C_6H_9N_3O_2$**
Glycine	**$C_2H_5NO_2$**
Threonine	**$C_4H_9NO_3$**
Arginine	**$C_6H_{14}N_4O_2$**
Alanine	**$C_3H_7NO_2$**
Tyrosine	**$C_9H_{11}NO_3$**
Cystine	**$C_6H_{12}N_2O_4S_2$**
Valine	**$C_5H_{11}NO_2$**
Methionine	**$C_5H_{11}NO_2S$**
Phenylalanine	**$C_9H_{11}NO_2$**
Isoleucine	**$C_6H_{13}NO_2$**

Leucine	**$C_6H_{13}NO_2$**
Lysine	**$C_6H_{14}N_2O_2$**
Proline	**$C_5H_9NO_2$**

The amino acids are measured by the HPLC with a Diode Array detector, which is detailed in the material and methods section.

3.6.2 INDEPENDENT VARIABLES

In this research, the three independent variables are carbon, nitrogen, and phosphorous. The chosen form for each source of carbon, nitrogen, and phosphorous is summarized in Table 16.

TABLE 16: The Form of the Independent Variables

Source of Carbon	***Source of Nitrogen***	***Source of phosphorous***
Glucose [$C_6H_{12}O_6$] ***Mannitol [$C_6H_{14}O_6$]*** ***Xylose [$C_5H_{10}O_5$]*** ***Arabinose [$C_5H_{10}O_5$]***	**Ammonium Sulfate [$(NH_4)_2SO_4$]**	**Monopotassium Phosphate [KH_2PO_4]**

Selected values for these macronutrients carbon, nitrogen, and phosphorous are chosen based on the molar ratio of three elements. The ratio has been such that each element will be a limiting factor at some point in the experimental design. Nitrogen concentration was the main pivot compared to the other two factors. It has been reported by many studies that photosynthetic organisms adapt to nitrogen starvation through similar and distinct mechanisms (Sinetova, Markelova and Los 2006). These mechanisms can be a reduction in cell division (Vladimirova, et al. 1968), an accumulation of nitrogen storage compound (Zhukova, et al. 1969), a reduction

of photosynthetic apparatus (Sinetova, Markelova and Los 2006), the maintenance of protein synthesis in cell (Gorl, et al. 1998), and cell survival (Sauer, et al. 2001). Although there are some studies suggested that nitrogen limitation causes decreasing protein content in algae, these results cannot be generalized due to the variation in algal responses (Sterner and Hessen 1994)**.** However**,** a study has shown that *G. sulphuraria* has ceased its growth and cell division under the influence of nitrogen starvation (Sinetova, Markelova and Los 2006). It has been reported that *G. sulphuraria* can tolerate an ammonium sulfate concentration up to 22 g/L (Schmidt, Wiebe and Eriksen 2005). The ratio of the middle point (center points) of carbon, nitrogen, and phosphorous 1:1:150 is used as a media preparation for growing this algal strain. For the carbon concentration, a typical batch cultures uses glucose concentration between 2- 10 g/L. Carbon limited batch culture in sufficient amount of nitrogen increases the production of phycocyanin, a pigment-protein complex. The phosphorous concentration has the highest phycocyanin production at 200 mg, and the other two selected values has lower production (Wan, et al. 2016).

3.7 ASSUMPTION

Almost all experiments must have assumptions, and these assumptions can be certain conditions to be approximately met for estimation or experimental purposes. There are several assumptions that have been made for this experimental design:

1- The first assumption is that there are no changes in concentrations of the compounds that are listed on Table 17 throughout the entire experiments. If a change occurs during the experiment, it is assumed that the change has a minimal to impact the amino acids production. These compounds do not act as a limiting substrate in amino acid production.

TABLE 17: List of Compounds in the Media

Component	*Quantity Used*
$MgSO_4 \cdot 7H_2O$	**300 mg**
$CaCl_2 \cdot 2H_2O$	**20 mg**
NaCl	**20 mg**
Fe-EDTA-solution	**1.5 mL**
trace-element solution	**2 mL**

2- The second assumption is the water volume and evaporation due to the high temperature have no influence on the algae response for amino acids synthesis.
3- The third assumption is the air exchange will not be affected with algae concentration or the volume of solution.
4- The fourth assumption due to sampling that the algae will not be affected from light and temperature change because the time for these shocks is very short to have impact on the algae.
5- The initial ratio of carbon, nitrogen, and phosphorous (C:N:P) from the start to the end of the experiment is constant in the media.
6- The pH has no influence on the algae behavior for synthesizing amino acids.

CHAPTER FOUR: RESULTS

4.1 GALDIERIA SULPHURARIA GROWTH

The biomass concentration of *G. sulphuraria* for each experiment listed in the design of

experiments, Table 13 and 14, is presented in Figure 9. The raw data for this figure are listed in Appendix A, Table 70 and 71.

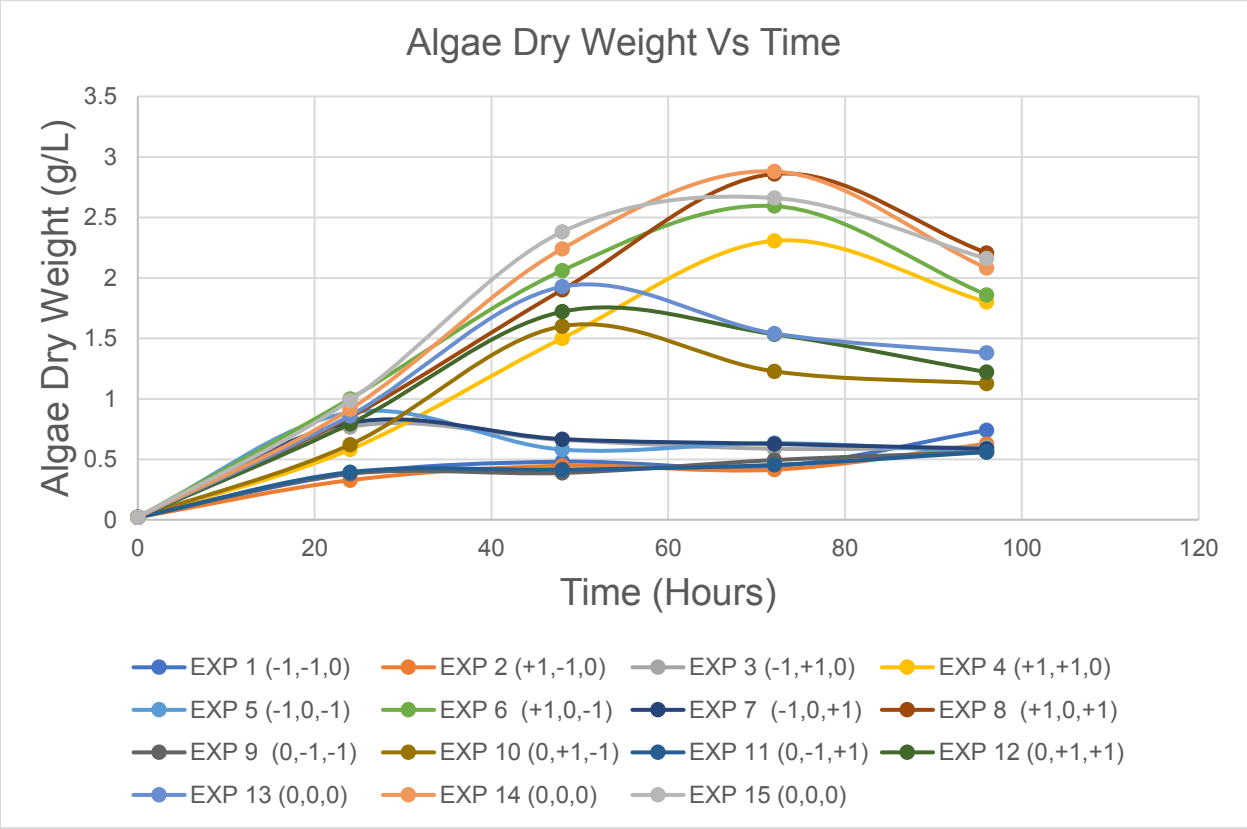


FIGURE 9: The Growth of *Galdieria sulphuraria* with Different Concentrations of Carbon (C), Nitrogen (N), and Phosphorous (P). Experiments 1 Through 15 Correspond to Conditions Listed in Table 13 & 14. Each Legend Contains the Experiment Number and (Carbon, Nitrogen, Phosphorous) Codes. Carbon Codes (+1,0,-1) represent the concentration (7.5000, 5.0000, 2.5000). Nitrogen Codes (+1,0,-1) signify the concentration (22.0000, 11.0000, 0.2000). Phosphorous Codes (+1,0,-1) symbolize the concentration (0.3000, 0.1515, 0.0750).

Experiment number thirteen (0,0,0) has been eliminated from the calculation because a lower dry weight of 1.93 g/L around 9% decrease from the other center points. This erroneous result is likely due to the sample injection that was used to inoculate the medium.

A regression is used to generate a relationship between algal biomass and the initial concentration of the three manipulated factors. The first day (24 H) value for R squared and adjusted R squared are 0.9403 and 0.8058, which indicates that the data are adequately represented by the regression line (Table 18 and 19). This model can explain only around 94.0

percent of the results for the first day. The full equations from the first day (24 H) to the fourth day (96 H) of growth can be seen below in Table 25.

TABLE 18: Full Equations of *G. sulphuraria* Growth Under the Influence of Three Factors and Three Levels

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.92	-0.01	0.16	-0.0075	-0.0325	-0.3725	-0.0025	-0.015	-0.035	0.04
48	2.18	0.044	0.4688	0.0088	-0.5692	-0.8417	-0.3117	-0.0625	0.2175	0.025
72	2.36	0.735	0.4838	0.0663	-0.335	-1.0875	-0.3475	0.0675	0.4375	0.085
96	1.8733	0.5013	0.2825	0.0588	-0.2467	-0.6842	-0.3217	0.08	0.3275	0.0225

TABLE 19: Regression Statistics for the First Day (24 H) with All Terms Included in the Equation

REGRESSION STATISTICS

MULTIPLE R	0.9696
R SQUARE	0.9402
ADJUSTED R SQUARE	0.8058
STANDARD ERROR	0.1063
OBSERVATION	14

TABLE 20: ANOVA Analysis for the First Day (24 H) Equation

	DF	**SS**	**MS**	**F**	**SIGNIFICANCE F**
REGRESSION	9	0.7121	0.0791	6.9944	0.0384
RESIDUAL	4	0.0453	0.0113		
TOTAL	13	0.7574			

For the first day (24 H), all terms have been shown in the equation, but they are not in equal importance or significant to be included in the equation (Table 21). The significance can be determined by looking at confidence level and p-values, which should be less than 0.05.

TABLE 21: Coefficient Analysis for the First Day (24 H) Equation

	COEFFICIENT	STANDARD ERROR	P-VALUE	LOWER 95%	UPPER 95%
INTERCEPT	0.92	0.0581	1.8289E-05	0.7706	1.0694
G	-0.01	0.03558	0.7900	-0.1015	0.0815
N	0.16	0.0356	0.0064	0.0685	0.2515
P	-0.0075	0.0356	0.8414	-0.0990	0.0840
G^2	-0.0325	0.0524	0.5621	-0.1671	0.1021
N^2	-0.3725	0.0524	0.0009	-0.5071	-0.2379
P^2	-0.0025	0.0524	0.9638	-0.1371	0.1321
GP	-0.015	0.0503	0.7776	-0.1444	0.1144
GN	-0.035	0.0503	0.5177	-0.1644	0.0944
NP	0.04	0.0503	0.4627	-0.0894	0.1694

The significant terms to be included in the equation for the first day are intercept, N, and N^2. The residual output was random for this equation (Number 4). The equation can be re-written as the following:

$$y = 0.9 + (0.16)N + (-0.37)N^2 \qquad (4)$$

For this equation, the date analysis has showed an improvement from the full equation for the same day (Table 22, 23, and 24). The residual output was also random for the first day (24 H) modified equation.

TABLE 22: Regression Statistics for the First Day (24 H) with Significant Terms Included in the Equation

REGRESSION STATISTICS	
MULTIPLE R	0.9553
R SQUARE	0.9127
ADJUSTED R SQUARE	0.8968
STANDARD ERROR	0.0775
OBSERVATIONS	14

TABLE 23: ANOVA Analysis for the First Day (24 H) Equation with Significant Terms Included in the Equation

	DF	**SS**	**MS**	**F**	**SIGNIFICANCE F**
REGRESSION	2	0.6912	0.3456	57.487	1.5001E-06
RESIDUAL	11	0.0661	0.0060		
TOTAL	13	0.7574			

TABLE 24: Coefficient Analysis for the First Day (24 H) Equation with Significant Terms Included in the Equation

	COEFFICIENT	**STANDARD ERROR**	**P-VALUE**	**LOWER 95%**	**UPPER 95%**
INTERCEPT	0.9067	0.0317	1.103E-11	0.8370	0.9763
N	0.16	0.02741	0.0001	0.0997	0.2202
N^2	-0.3767	0.0418	2.1076E-06	-0.4688	-0.2845

After preforming all these statistical analyses and applying selection criteria, the daily equations for dry weight growth of *G. sulphuraria* can be written individually as in Table 25.

TABLE 25: Growth Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H) for Dry Weight

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.9		0.16			-0.37				
48	1.99	0.44	0.47		-0.55	-0.82				
72	1.97	0.74				-1.04				
96	1.55	0.50				-0.64				

A comparison has been done for models of the same day using Akaike information criterion (AIC) equation (Number 5) and Bayesian information criterion (BIC) equation (Number 6) to help select the best fitting model with fewer terms (Sanchez, Ancheyta and McCaffrey 2007).

$$AIC = 2k + nln\left(\frac{RSS}{n}\right) \quad (5)$$

$$BIC = kln(n) + nln\left(\frac{RSS}{n}\right) \quad (6)$$

where the number of parameters is k, the number of observation is n, and the residual sum of squares is RSS. The lower result or score for AIC and BIC for a given model as the model becomes more preferred. The original model of the first day (24 H) has AIC of -65.36 and BIC of -58.28 while the modified one has AIC of -74.94 and BIC of -72.82. The same analysis has been performed for all days and equations to show the relationship between three factors (carbon, nitrogen, and phosphorous) with growth of this algae.

The second day (48 H) value for R square and adjusted R square 0.8331 and 0.7663. For the third day (72 H), R square and adjusted R square value are 0.6100 and 0.5450. The fourth day (96 H) equation of R square and adjusted R square is 0.5882 and 0.5195. Regression residuals

show no patterns and are close to zero and evenly distributed above and below the average. The significance F values are from day one to four as follow in order 4.261E-07, 0.0007, 0.0035, and 0.0049, less than 0.05 that means the probability of the regression output is not random. For the P-values of the y-intercepts and coefficients, they are lower than 0.05 on all days of measurements, which means that they are significant. For the second day (48 H), y-intercept, carbon, nitrogen, carbon square, and nitrogen square are 4.9805E-07, 0.0067, 0.0046, 0.0166, and 0.0015 respectively. The third day (72 H) for y-intercept, carbon, and nitrogen square has 4.7816E-06, 0.0089, and 0.011 respectively. For the fourth day (96 H), y-intercept, carbon, and nitrogen square have 1.1116E-06, 0.0090, and 0.0183. The original model of the second day (48 H) has AIC of -38.52 and BIC of -17.44 while the modified one has AIC of -26.26 and BIC of -22.72. The original model of the third day (72 H) has AIC of -11.63 and BIC of -4.55 while the modified one has AIC of -9.49 and BIC of -7.37. The original model of the fourth day (96 H) has AIC of -23.92 and BIC of -16.84 while the modified one has AIC of -20.94 and BIC of -18.81.

4.2 Amino Acid Standard Results from High Performance Liquid Chromatography (HPLC)

To separate and quantify amino acids, high performance liquid chromatography (HPLC) is used. The method was validated with standard amino acid concentrations. These standards have known concentrations that were purchased from Agilent Technologies. HPLC results for testing the method with the known amino acids standards can be seen in Figure 10. The raw data for Figure 10 are listed in Appendix A in Table 72.

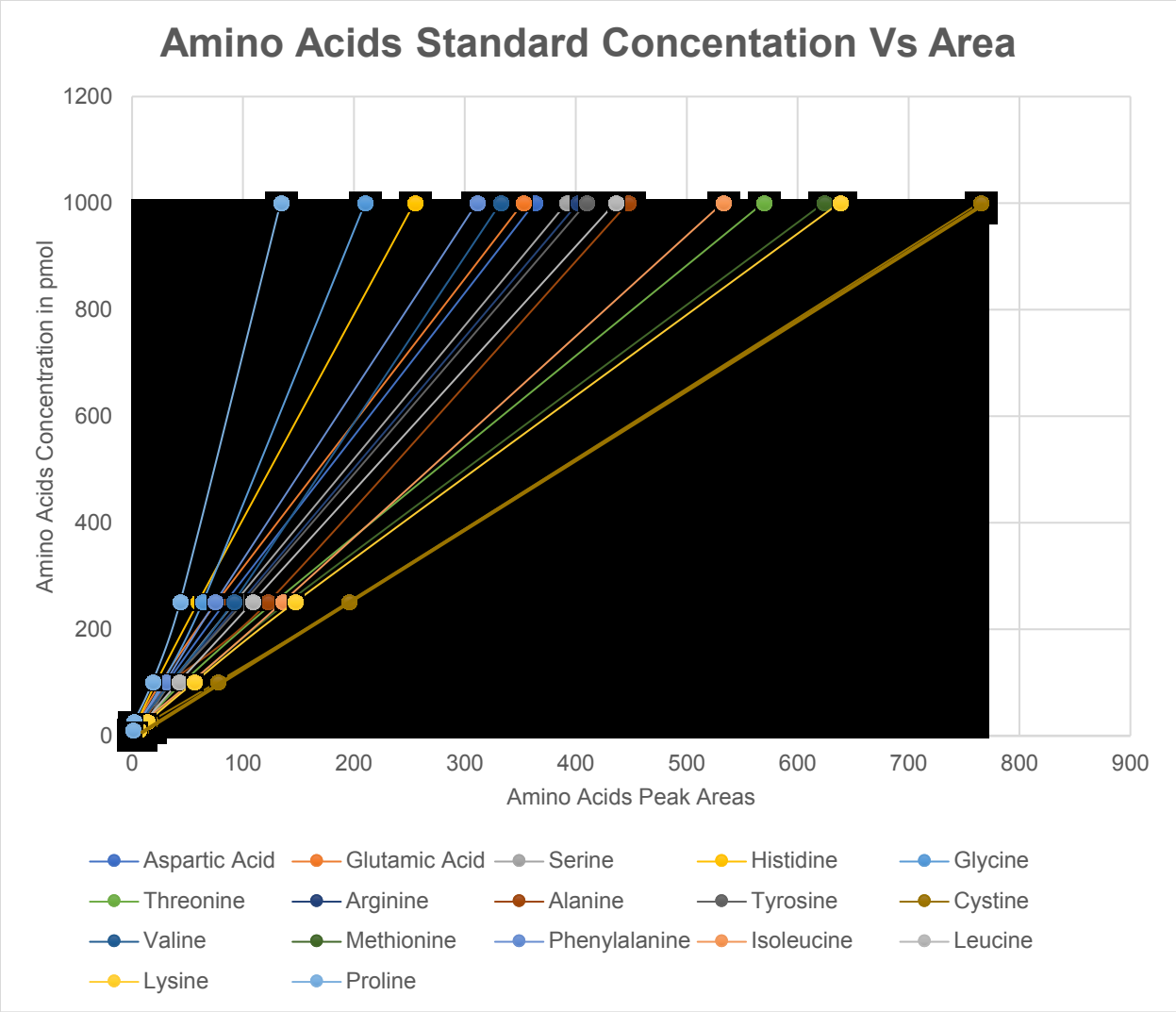


FIGURE 10: Standards of Seventeen Kinds of Amino Acid Consisting of Known Concentrations with Their Peak Areas in HPLC.

4.3 AMINO ACIDS PRODUCTION FOR *GALDIERIA SULPHURARIA*

Each amino acid was classified as being either directly or indirectly correlated with the three manipulated factors in the experimental design. Combining all *G. sulphuraria* amino acid results, experiments are listed in the design of experiments, Table 13 and 14. The raw data for these

figures are listed in Appendix A under *G. sulphuraria* amino acid production section. All amino acid weights are reported by gram per 100 grams of algae sample.

4.3.1 Direct Correlation Between Amino Acids Production and Carbon, Nitrogen, and Phosphorous Concentrations

Serine, histidine, arginine, valine, phenylalanine, isoleucine, leucine, and proline are directly linked to the concentration of these macronutrients (glucose, nitrogen, and phosphorous).

4.3.2 Serine

Equations for serine were different depending on the day of measurements, as was observed for all of the amino acids. The first day (24 H) value for R square and adjusted R square are 0.9330 and 0.8125, which means the data results are fitting the regression line (Table 27 and 28). This model can explain only around 93.3 percent of the results for the first day. The full equations from the first day (24 H) to the fourth day (96 H) of serine production can be seen below in Table 26.

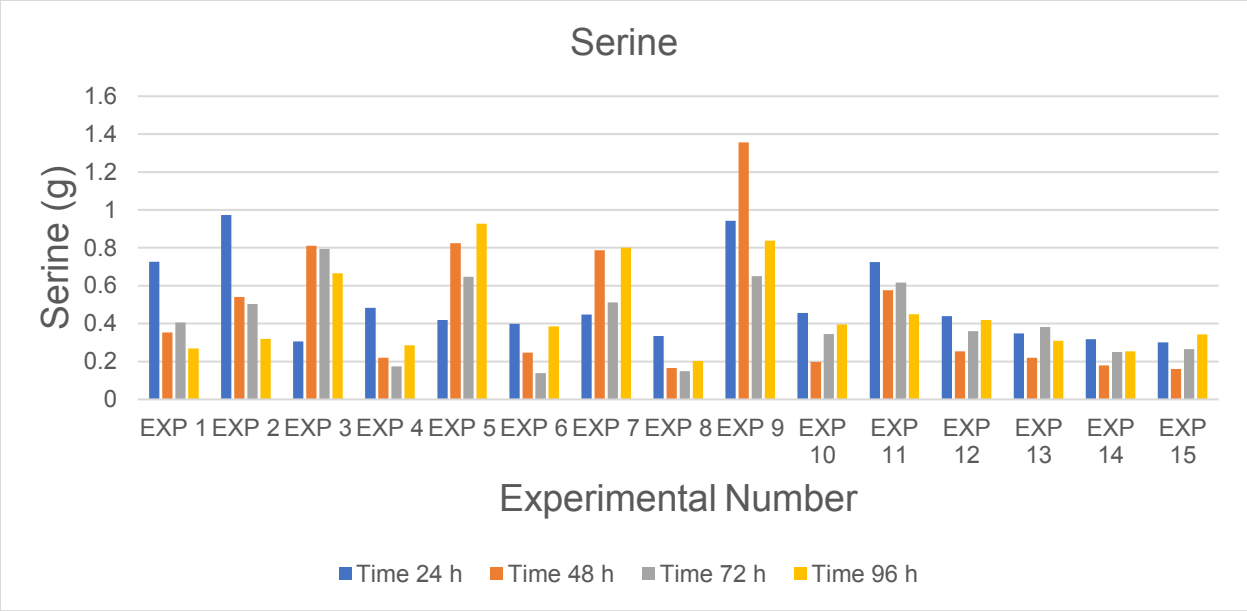


FIGURE 11:The Serine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

The equations for this amino acid, serine, were changing depending on the sampling time. As have been seen from Figure 11, serine production changed daily within each experiment.

TABLE 26: Serine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.3222	0.0362	-0.2104	-0.0339	0.0298	0.2705	0.0481	-0.0232	-0.0169	0.0498
48	0.1864	-0.2005	-0.1679	-0.1052	0.1021	0.1922	0.2176	-0.0105	-0.1948	0.2094
72	0.2991	-0.1740	-0.0630	-0.0181	0.0195	0.1510	0.0431	0.0358	-0.1793	0.012
96	0.3019	-0.1836	-0.0136	-0.0845	0.0680	0.0151	0.2090	-0.0139	-0.1078	0.1030

TABLE 27: Regression Statistics for the First Day (24 H) with All Terms Included in the Equation

REGRESSION STATISTICS

MULTIPLE R	0.9659
R SQUARE	0.9330

ADJUSTED R SQUARE	0.8125
STANDARD ERROR	0.0974
OBSERVATION	15

TABLE 28: ANOVA Analysis for the First Day (24 H) Equation

	DF	**SS**	**MS**	**F**	**SIGNIFICANCE F**
REGRESSION	9	0.6603	0.0734	7.7408	0.0182
RESIDUAL	5	0.0474	0.0095		
TOTAL	14	0.7077			

For the first day (24 H), all terms have been shown in the equation, but they are not in equal importance or significant (Table 29). As was done with the biomass, the significance of each coefficient can be determined by looking at confidence level and p-values, which should be less than 0.05.

TABLE 29: Coefficient Analysis for the First Day (24 H) Equation

	COEFFICIENT	**STANDARD ERROR**	**P-VALUE**	**LOWER 95%**	**UPPER 95%**
INTERCEPT	0.3222	0.0562	0.0023	0.1777	0.4667
G	0.0362	0.0344	0.3405	-0.0522	0.1247
N	-0.2104	0.0344	0.0017	-0.2989	-0.1220
P	-0.0339	0.0344	0.3701	-0.1224	0.0546
G^2	0.0298	0.0507	0.5823	-0.1005	0.1600

N^2	0.2705	0.0507	0.0031	0.1402	0.4007
P^2	0.0481	0.0507	0.3863	-0.0822	0.1783
GP	-0.0232	0.0487	0.6543	-0.1483	0.1020
GN	-0.0169	0.0487	0.7420	-0.1421	0.1082
NP	0.0498	0.0487	0.3534	-0.0753	0.1749

The significant terms to be included in the equation for the first day are intercept, N, and N^2. The residuals were random for this equation (Number 7). The equation can be re-written as the following:

$$y = 0.32 + (-0.21)N + 0.27N^2 \qquad (7)$$

For this equation, the date analysis has showed almost similar results from the full equation for the same day (Table 30, 31, and 32). The residual output was also random for the first day (24 H) of the modified equation.

TABLE 30: Regression Statistics for the First Day (24 H) with Significant Terms Included in the Equation of Serine

REGRESSION STATISTICS

MULTIPLE R	0.9332
R SQUARE	0.8709
ADJUSTED R SQUARE	0.8494
STANDARD ERROR	0.0872
OBSERVATIONS	15

TABLE 31: ANOVA Analysis for the First Day (24 H) Equation with Significant Terms Included in the Equation of Serine

	DF	SS	MS	F	SIGNIFICANCE F
REGRESSION	2	0.6163	0.3082	40.4835	4. 6251E-06
RESIDUAL	12	0.0913	0.0076		
TOTAL	14	0.7077			

TABLE 32: Coefficient Analysis for the First Day (24 H) Equation with Significant Terms Included in the Equation of Serine

	COEFFICIENT	STANDARD ERROR	P-VALUE	LOWER 95%	UPPER 95%
INTERCEPT	0.3667	0.0330	1.1258E-07	0.2948	0.4385
N	-0.2104	0.0308	1.8429E-05	-0.2777	-0.1432
N^2	0.2649	0.0452	7.6345E-05	0.1665	0.3633

The same analysis has been carried to all days (hours), and serine equations can be written as in Table 33.

TABLE 33: Serine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.37		-0.21			0.26				
48	0.19	-0.20	-0.17	-0.11	0.11	0.19	0.22	-0.01	0.19	0.21
72	0.41	-0.17							-0.18	
96	0.46	-0.18								

A comparison has been done for models of the same day using Akaike information criterion (AIC) equation and Bayesian information criterion (BIC) equation to select the best fitting

model. The original model of the first day (24 H) has AIC of -66.36 and BIC of -59.28 while the modified one has AIC of -70.52 and BIC of -68.39, indicating that the simplified model is superior. The same analysis has been performed for all days and amino acids to show the relationship between the three factors with amino acid production. The second day (48 H) value for R square and adjusted R square is 0.7369 and 0.2634. For the third day (72 H), R square and adjusted R square values are 0.6455 and 0.5864. The fourth day (96 H) equation of R square and adjusted R square is 0.3531 and 0.3033. Regression residuals show no patterns and close to zero by evenly distributed above and below the average. The significance F values are from day one to four as follow in order 4.6251E-06, 0.3259, 0.0020, and 0.0195 less than 0.05 that means the probability of the regression output is not random. For the P-values of the y-intercepts and coefficients, they are lower than 0.05 on all days of measurements except for the second day (48 H). The first day (24 H) of y-intercept, nitrogen, and nitrogen square is 1.1258E-07, 1.8429E-05, and 7.6345E-05 less than 0.05, which means they are significant. For the second day (48 H), all factors are higher than 0.05, which these values are random statistically. The third day (72 H) for y-intercept, carbon, and nitrogen with carbon has 3.7422E-08, 0.0026, and 0.018 respectively. For the fourth day (96 H), y-intercept and carbon have 5.3749E-07 and 0.0195. The second day (48 H) model has AIC of -32.46 and BIC of -25.38 without having any modification due to high P-values. The original model of the third day (72 H) has AIC of -59.37 and BIC of -52.29 while the modified one has AIC of -58.49 and BIC of -56.37. The original model of the fourth day (96 H) has AIC of -46.80 and BIC of -39.72 while the modified one has AIC of -47.19 and BIC of -45.77.

4.3.3 HISTIDINE

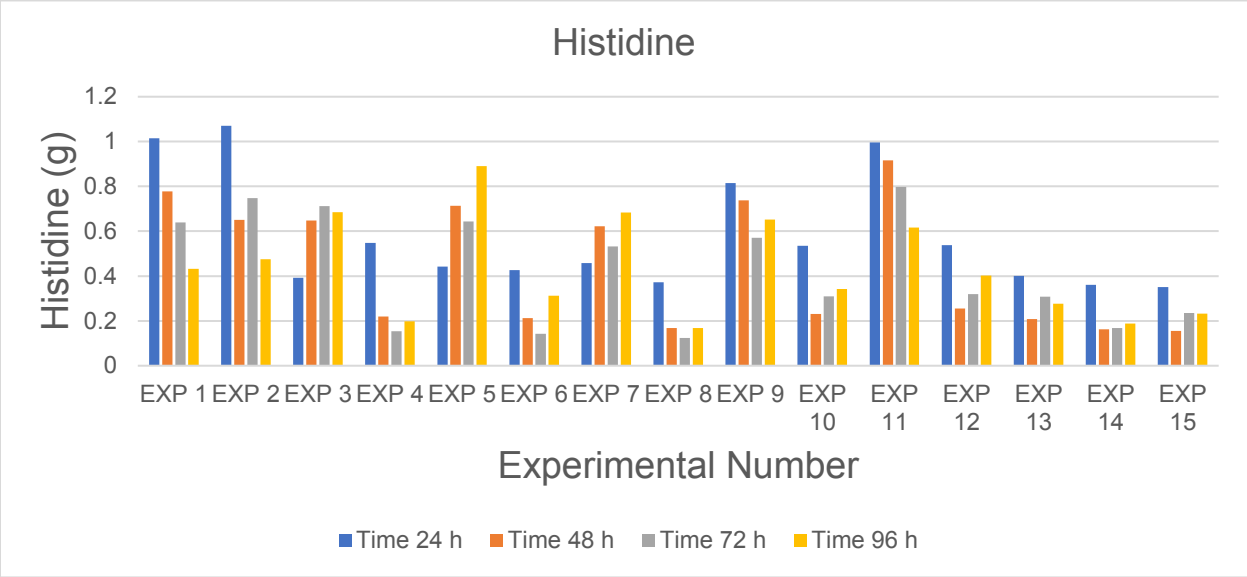


FIGURE 12:Different Concentrations of Carbon, Nitrogen, and Phosphorous, Histidine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

As with serine, the equations for histidine were varying due to sampling time. As have been seen from the above Figure 12, histidine production changed daily. Histidine equations for the first day (24 H) value for R square and adjusted R square are 0.9312 and 0.9197. The second day (48 H) value for R square and adjusted R square is 0.7929 and 0.7364. For the third day (72 H), R square and adjusted R square values are 0.8698 and 0.8177. The fourth day (96 H) equation of R square and adjusted R square is 0.4267 and 0.3826. Regression residuals show no patterns and close to zero by evenly distributed above and below the average. The significance F values are from day one to four as follow in order 1.0627E-07, 0.0004, 0.0002, and 0.0083 less than 0.05 that showing the probability of the regression output is not random. For P-values of y-intercepts and coefficients, they are lower than 0.05 on all days of measurements. All models from day one to day four can be seen in Table 34. The first day (24 H) of y-intercept, nitrogen, and nitrogen

square is 5.5411E-09, 9.7593E-07, and 1.2228E-06 less than 0.05, which they are significant. For the second day (48 H), factors lower than 0.05 are for y-intercept, carbon, nitrogen, and nitrogen square 9.9089E-05, 0.0033, 0.0013, and 0.0091. The third day (72 H) for y-intercept, carbon, nitrogen, nitrogen square, and nitrogen with carbon has 1.4642E-05, 0.0010, 0.0016, 0.0020, and 0.0100 respectively. For the fourth day (96 H), y-intercept and carbon have 2.5786E-07 and 0.0083. The first day (24 H) model has AIC of -68.66 and BIC -61.58 whereas the modified model has AIC -75.85 and BIC -73.72. The second day (48 H) model has AIC of -58.19 and BIC of -51.11 while the modified one has AIC -55.00 and BIC -52.16. The original model of the third day (72 H) has AIC of -63.07 and BIC of -55.99 while the modified one has AIC of -63.90 and BIC of -60.36. The original model of the fourth day (96 H) has AIC of -54.38 and BIC of -47.30 as the modified one has AIC of -50.50 and BIC of -49.09. All these data allow to write serine equations as in Table 34.

TABLE 34: Histidine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.40		-0.24			0.34				
48	0.32	-0.19	-0.22			0.23				
72	0.31	-0.17	-0.16			0.22			-0.17	
96	0.44	-0.19								

4.3.4 ARGININE, VALINE, PHENYLALANINE, ISOLEUCINE, LEUCINE, AND PROLINE

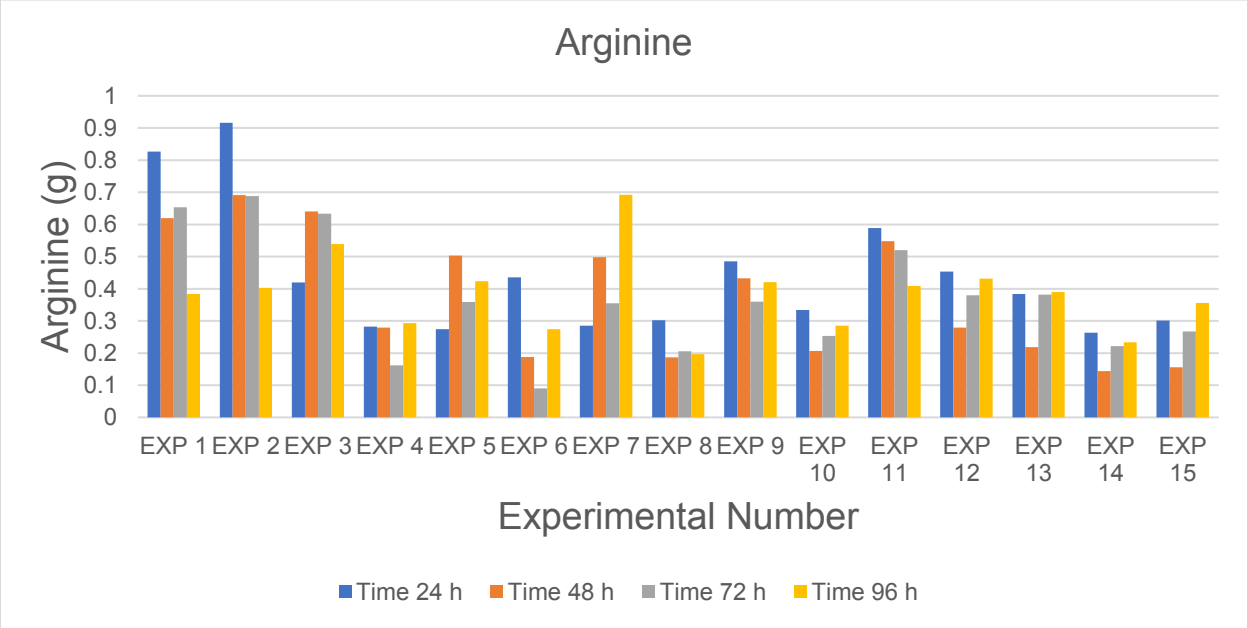


FIGURE 13: The Arginine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for this amino acid, arginine, were different dependent on the time of sampling, and calculations were determined on the concentration of this amino acid at that given time. As have been seen from Figure 13 and Table 35, arginine production changed daily.

TABLE 35: Arginine Production Equations of *G. Sulphuraria* for All Days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.43		-0.14			0.26				
48	0.31		-0.16			0.23				
72	0.31	-0.13	-0.12			0.20			-0.11	
96	0.40	-0.13								

4.3.5 VALINE

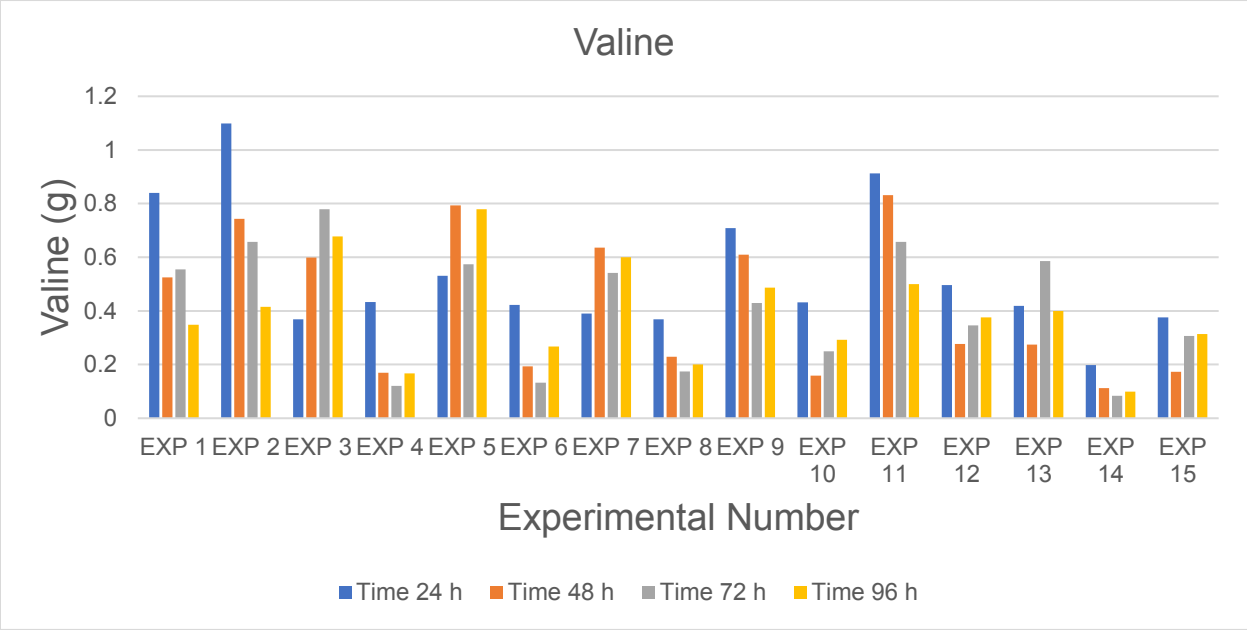


FIGURE 14: The Valine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Valine equations were changing according to the sampling time. The concentrations and calculations were changing every day for this amino acid. As have been seen from Figure 14 and Table 36, valine production changed daily.

TABLE 36: Valine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.39		-0.23			0.27				
48	0.42		-0.19							
72	0.41	-0.17								
96	0.39	-0.17								

4.3.6 PHENYLALANINE

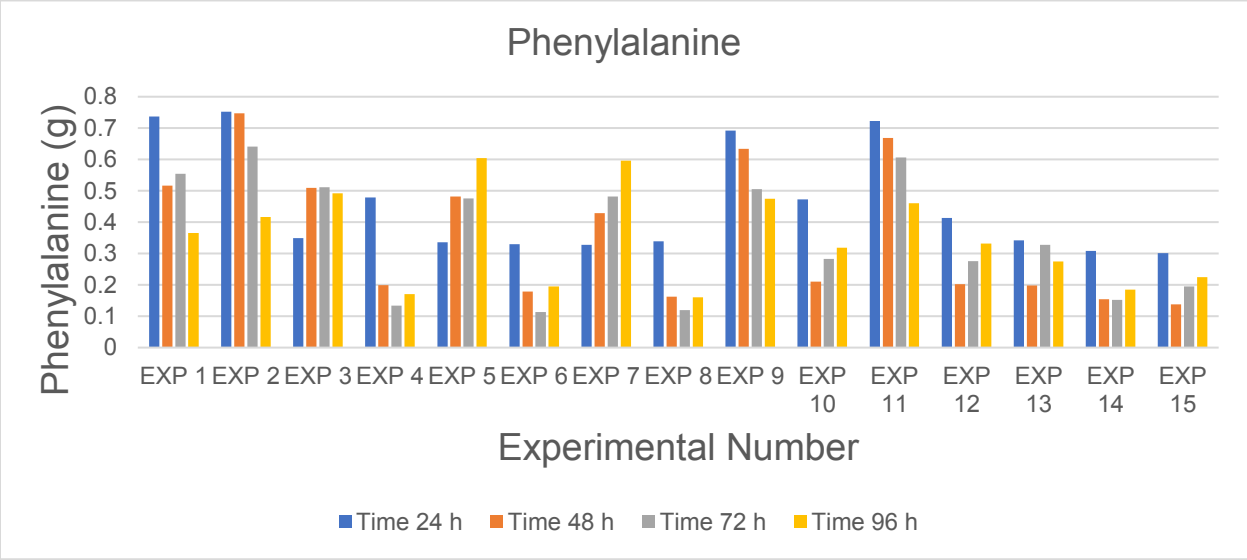


FIGURE 15:The Phenylalanine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for this amino acid, phenylalanine, were different depending on the sampling time (Table 37). As have been seen from Figure 15, phenylalanine production changed daily within each experiment.

TABLE 37: Phenylalanine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.33		-0.15			0.25				
48	0.25		-0.18			0.21			-0.14	
72	0.27	-0.13	-0.14			0.17				
96	0.35	-0.14								

4.3.7 ISOLEUCINE

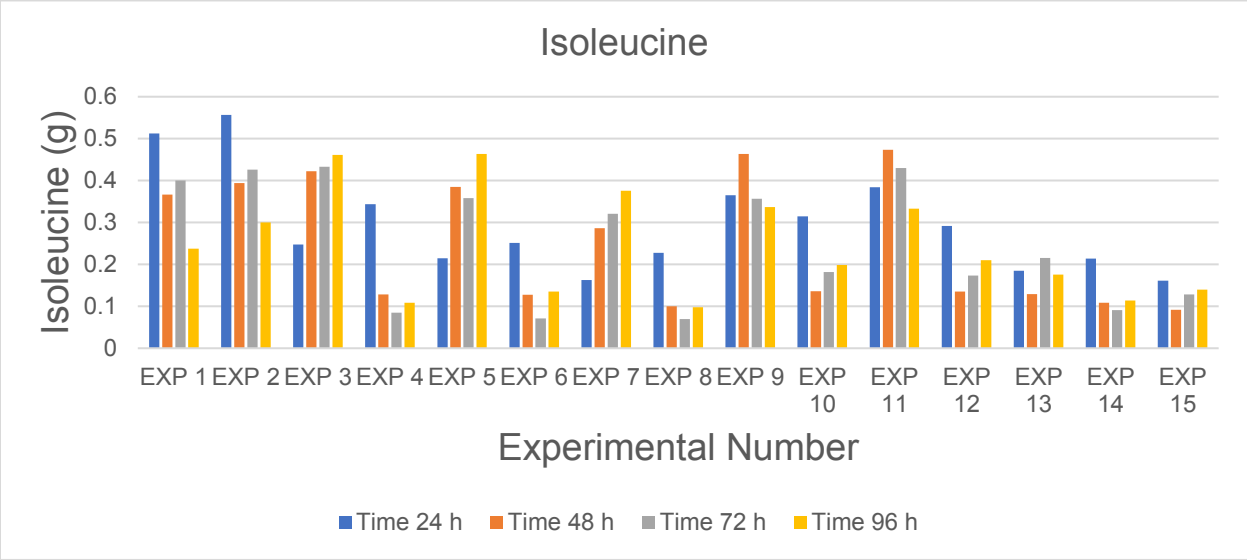


FIGURE 16:Different Concentrations of Carbon, Nitrogen, and Phosphorous, the Isoleucine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to The Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for isoleucine were different depending on the period of sampling due to calculations dependency on the concentration of this amino acid (Table 38). As have been seen from Figure 16, the isoleucine production changed daily.

TABLE 38: Isoleucine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.20		-0.08			0.17				
48	0.18	-0.09	-0.11			0.14				
72	0.18	-0.11	-0.09			0.13			-0.09	
96	0.25	-0.11							-0.10	

4.3.8 LEUCINE

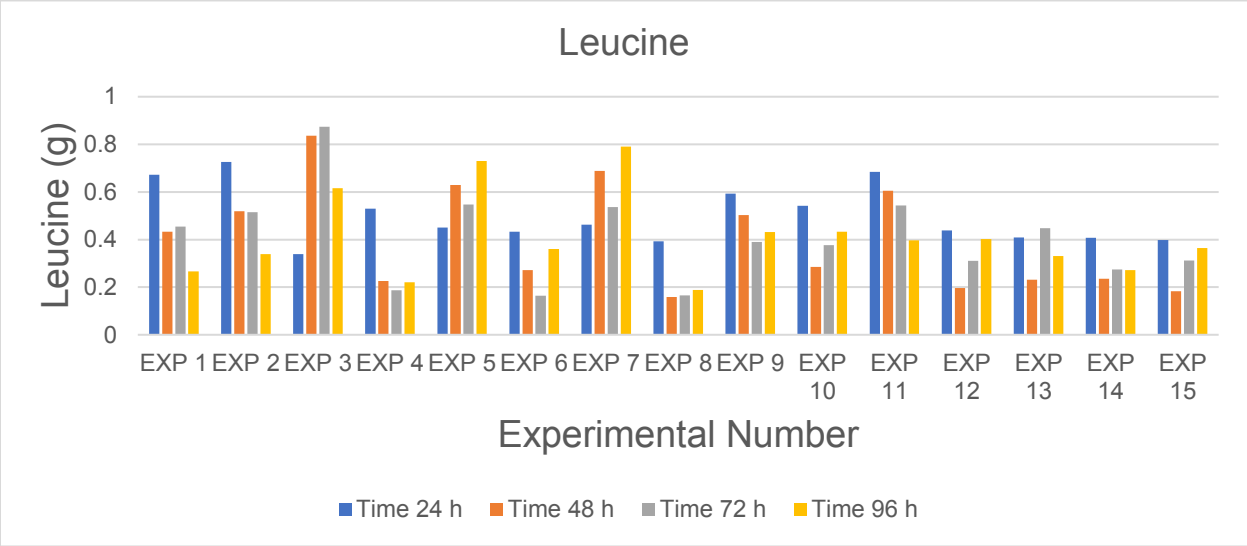


FIGURE 17:The Leucine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for this amino acid, leucine, were different depending on the sampling period (Table 39). Calculations were also changing due to the production of this amino acid at that given time. As have been seen from figure 17, leucine production changed daily.

TABLE 39: Leucine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.42		-0.10			0.14				
48	0.40	-0.18							-0.17	
72	0.41	-0.17							-0.19	
96	0.41	-0.16								

4.3.9 PROLINE

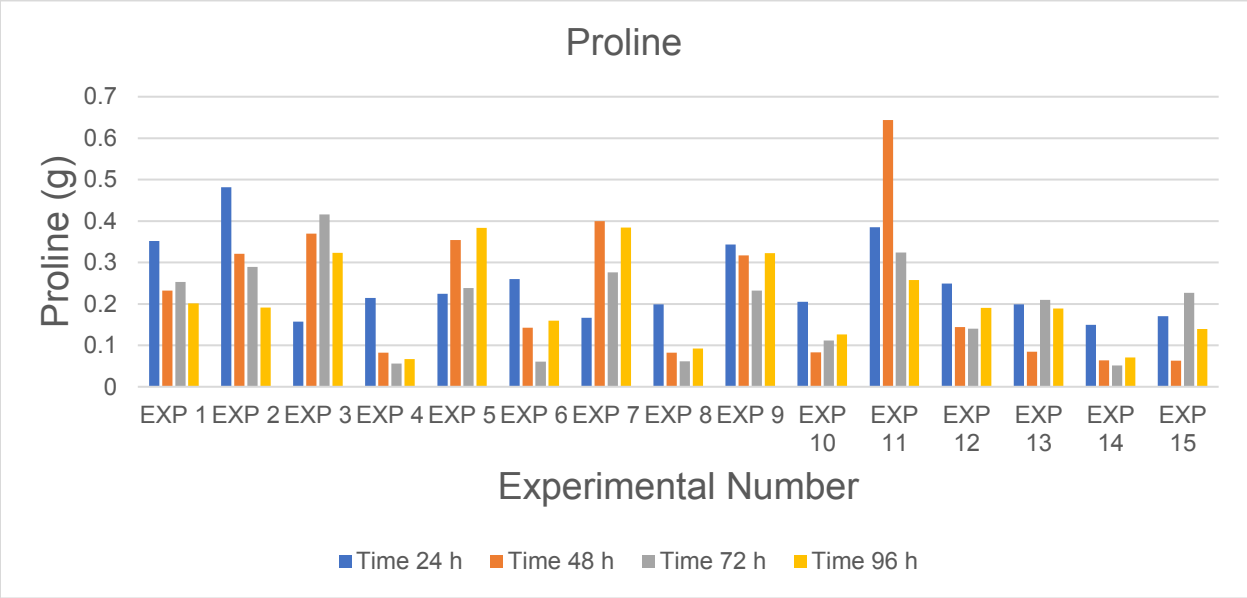


FIGURE 18:THE PROLINE PRODUCTION FOR *G. SULPHURARIA* WITH DIFFERENT CONCENTRATIONS OF CARBON, NITROGEN, AND PHOSPHOROUS. EXPERIMENTS 1 THROUGH 15 CORRESPOND TO CONDITIONS LISTED IN TABLE 2. THE CODED FACTORS FOR EACH EXPERIMENT IS (CARBON, NITROGEN, PHOSPHOROUS). EXP 1 (-1,-1,0), EXP 2 (+1,-1,0), EXP 3 (-1,+1,0), EXP 4 (+1,+1,0), EXP 5 (-1,0,-1), EXP 6 (+1,0,-1), EXP 7 (-1,0,+1), EXP 8 (+1,0,+1), EXP 9 (0,-1,-1), EXP 10 (0,+1,-1), EXP 11 (0,-1,+1), EXP 12 (0,+1,+1), EXP 13 (0,0,0), EXP 14 (0,0,0), EXP 15 (0,0,0).

Equations for this amino acid, proline, were changing due to the period of sampling (Table 40). Calculations were based on the concentration of this amino acid at that given time. As have been seen from figure 18, proline production changed daily.

TABLE 40: Proline Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.20		-0.09			0.10				
48	0.07	-0.09	-0.10	-0.05	0.06	0.12	0.11	-0.03	-0.09	-0.07
72	0.20	-0.09								
96	0.21	-0.10								

4.3.10 ARGININE VALINE, PHENYLALANINE, ISOLEUCINE, LEUCINE, AND PROLINE STATISTICAL ANALYSIS

For arginine, valine, phenylalanine, isoleucine, leucine, and proline, all these amino acids have similar patterns (Table 41 - 44). The same analysis has been done to them as in the above amino acids, serine and histidine with AIC and BIC (Table 45 - 48).

TABLE 41: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with R Square, Adjusted R Square, and Significance F for First Day (24 H)

	AMINO ACIDS					
	Arginine	Valine	Phenylalanine	Isoleucine	Leucine	Proline
R SQUARE	0.8629	0.8267	0.9663	0.8079	0.8012	0.8120
ADJUSTED R SQUARE	0.8401	0.7978	0.9607	0.7759	0.7681	0.7807
SIGNIFICANCE F	6.634E-06	2.7088E-05	1.4721E-09	5.019E-05	6.1728E-05	4.416E-05

TABLE 42: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with R Square, Adjusted R Square, and Significance F for First Day (48 H)

	AMINO ACIDS					
	Arginine	Valine	Phenylalanine	Isoleucine	Leucine	Proline
R SQUARE	0.5797	0.2962	0.7730	0.7300	0.5640	0.7932
ADJUSTED R SQUARE	0.5097	0.2420	0.7111	0.6564	0.4913	0.4211
SIGNIFICANCE F	0.0055	0.0360	0.0007	0.0018	0.0069	0.2094

TABLE 43: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with R Square, Adjusted R Square, and Significance F for First Day (72 H)

	AMINO ACIDS					
	Arginine	Valine	Phenylalanine	Isoleucine	Leucine	Proline
R SQUARE	0.8756	0.3238	0.7777	0.8950	0.7687	0.3674
ADJUSTED R SQUARE	0.8259	0.2718	0.7171	0.8530	0.7302	0.3188
SIGNIFICANCE F	0.0002	0.0268	0.0007	6.9763E-05	0.0002	0.0166

TABLE 44: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with R Square, Adjusted R Square, and Significance F for First Day (96 H)

	AMINO ACIDS					
	Arginine	Valine	Phenylalanine	Isoleucine	Leucine	Proline
R SQUARE	0.4736	0.4570	0.4870	0.6529	0.4856	0.4860
ADJUSTED R SQUARE	0.4331	0.4152	0.4475	0.5951	0.4460	0.4465
SIGNIFICANCE F	0.0046	0.0057	0.0038	0.0017	0.0039	0.0039

TABLE 45: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with AIC and BIC scores for First Day (24 H)

AMINO ACIDS	**ORIGINAL MODEL**		**MODIFIED MODEL**	
	AIC	BIC	AIC	BIC
ARGININE	-72.00	-64.92	-76.01	-73.88
VALINE	-54.49	-47.41	-63.39	-61.27
PHENYLALANINE	-98.26	-91.18	-98.21	96.09
ISOLEUCINE	-82.50	-75.42	-83.49	-81.37
LEUCINE	-78.89	71.81	-82.82	-80.69

PROLINE	-86.21	-79.13	-90.04	-87.92

TABLE 46: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with AIC and BIC scores for Second Day (48 H)

AMINO ACIDS	**ORIGINAL MODEL**		**MODIFIED MODEL**	
	AIC	BIC	AIC	BIC
ARGININE	-56.35	-49.27	-53.29	-51.16
VALINE	-48.92	-41.84	-42.57	41.15
PHENYLALANINE	-66.44	-59.36	-61.34	-58.51
ISOLEUCINE	-71.87	-64.79	-69.49	-66.66
LEUCINE	-57.29	-50.21	-53.37	-51.25
PROLINE	-57.76	-50.68		

TABLE 47: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with AIC and BIC scores for Third Day (72 H)

AMINO ACIDS	**ORIGINAL MODEL**		**MODIFIED MODEL**	
	AIC	BIC	AIC	BIC
ARGININE	-77.06	-69.97	-72.06	-68.52
VALINE	-49.02	-41.94	-47.46	-46.04
PHENYLALANINE	-68.54	-61.46	-65.46	-62.63
ISOLEUCINE	-81.56	-74.48	-82.94	-79.41
LEUCINE	-71.30	-64.23	-67.24	-65.12
PROLINE	-74.80	-67.72	-69.67	-68.26

TABLE 48: Amino Acids of Arginine, Valine, Phenylalanine, Isoleucine, Leucine, And Proline with AIC and BIC scores for Fourth Day (96 H)

AMINO ACIDS	ORIGINAL MODEL		MODIFIED MODEL	
	AIC	BIC	AIC	BIC
ARGININE	-58.05	-50.97	-65. 75	-64.33
VALINE	-53.71	-46.63	-56.10	-54.69
PHENYLALANINE	-65.94	-58.86	-63.73	-62.31
ISOLEUCINE	-74.63	-67.55	-73.25	-71.12
LEUCINE	-61.92	-54.84	-59.22	-57.80
PROLINE	-76.64	-69.56	-74.31	-72.89

4.3.11 Weak Correlation between Amino Acids Production and Carbon, Nitrogen, and Phosphorous Concentrations

Not all of the amino acids could be correlated to the three factors manipulated in this study. A poor correlation was observed between the concentration of the manipulated macronutrients (glucose, nitrogen, and phosphorous) and the amino acid production for aspartic acid, glutamic acid, glycine, threonine, alanine, tyrosine, cysteine, methionine, and lysine.

4.3.12 ASPARTIC ACID

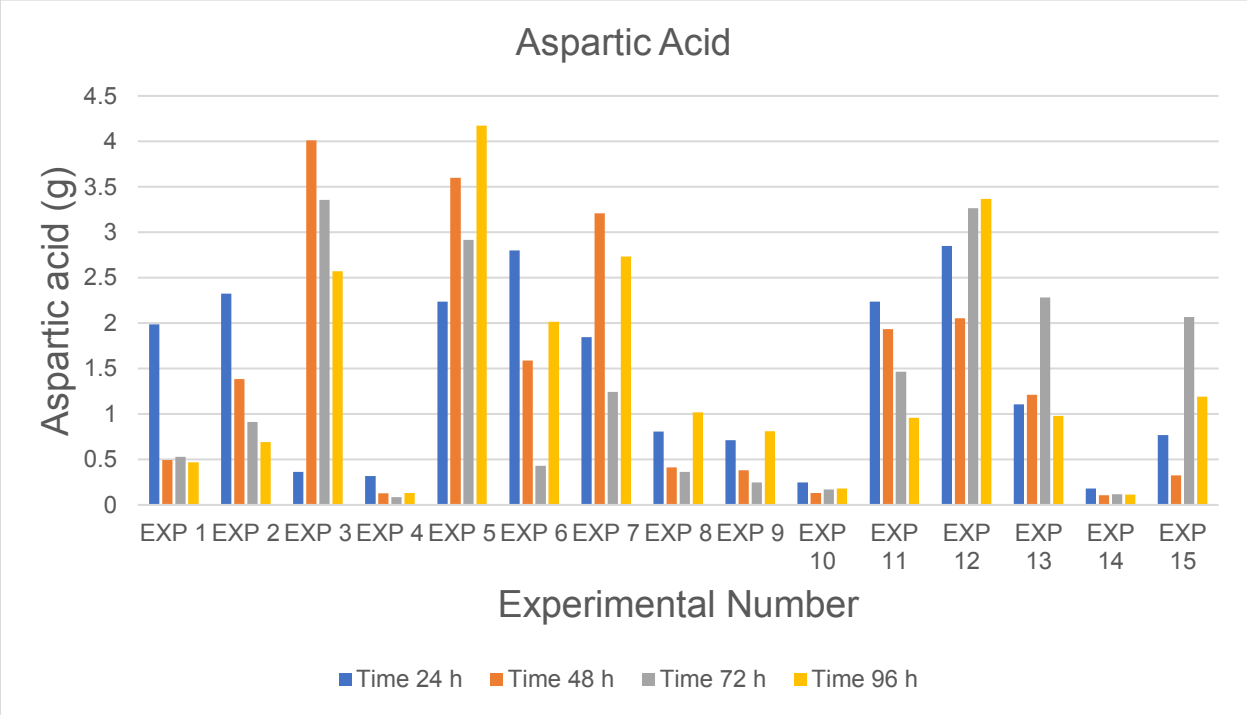


FIGURE 19:The Aspartic Acid Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Aspartic acid equations were different depending on the time of the sampling (Table 49). Calculations were also depending on the concentration of this amino acid at that given time. As have been seen from Figure 19, aspartic acid production changed daily. The first day (24 H) value for R square and adjusted R square is 0.4257 and -0.6081, which means the data results are not well described by regression line (Table 50 and 51). This model can explain only around 42.57 percent of the results for the first day. The full equations from the first day (24 H) to the fourth day (96 H) of this amino acid production can be seen below in equation 49.

TABLE 49: Aspartic Acid Production Equations of *G. sulphuraria* For All Days, The First (24 H), Second (48 H), Third (72 H), And Fourth (96 H) with all terms included.

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.6856	-0.0220	-0.4351	0.2183	0.4865	0.0760	0.7503	-0.4018	-0.0959	0.2692
48	0.5468	-0.9750	0.2663	0.2380	1.0171	-0.0605	0.6381	-0.1965	-1.1930	0.0928
72	1.4880	-0.7814	0.4655	0.3214	-0.1587	-0.1093	-0.0912	0.4024	-0.9131	0.4696
96	0.7627	-0.7621	0.4150	0.1117	0.6790	-0.4764	1.0425	0.1105	-0.6646	0.7595

TABLE 50: Regression Statistics for the First Day (24 H) with All Terms Included in the Equation

REGRESSION STATISTICS

MULTIPLE R	0.6524
R SQUARE	0.4257
ADJUSTED R SQUARE	-0.6081
STANDARD ERROR	1.2330
OBSERVATION	15

TABLE 51: ANOVA Analysis for the First Day (24 H) Equation

	DF	SS	MS	F	SIGNIFICANCE F
REGRESSION	9	5.6336	0.6260	0.4117	0.8830
RESIDUAL	5	7.6012	1.5202		
TOTAL	14	13.2348			

For the first day (24 H), all terms have been shown in the equation, but they are not in equal importance or significant to be included in the equation (Table 52). As was done in the previous

sections, the significance can be determined by looking at confidence level and p-values, which should be less than 0.05.

TABLE 52: Coefficient Analysis for the First Day (24 H) Equation

	COEFFICIENT	STANDARD ERROR	P-VALUE	LOWER 95%	UPPER 95%
INTERCEPT	0.6856	0.7119	0.3797	-1.1443	2.5155
G	-0.0220	0.4359	0.9617	-1.1426	1.0986
N	-0.4351	0.4359	0.3640	-1.5557	0.6855
P	0.2183	0.4359	0.6378	-0.9023	1.3389
G^2	0.4865	0.6417	0.4825	-1.1630	2.1359
N^2	0.0760	0.6417	0.9103	-1.5735	1.7254
P^2	0.7503	0.6417	0.2950	-0.8992	2.3997
GP	-0.4018	0.6165	0.5434	-1.9865	1.1830
GN	-0.0959	0.6165	0.8824	-1.6807	1.4888
NP	0.2692	0.6165	0.6806	-1.3156	1.8539

There are not any significant terms to be included in the equation for the first day due to the upper and lower values including zero in the two ranges. The residual output was random for the full equation. The equation can be written with all terms shown; however, more levels and other controlled parameters must be performed to see the relationship between carbon, nitrogen, and phosphorous. The residual output was also random for the first day (24 H) modified equation. The same kind of analysis has been done to second (48 H, third (72 H), and fourth (96 H), as can be seen in the Table 53.

TABLE 53: Aspartic Acid Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.69	-0.02	-0.44	0.22	0.49	0.08	0.75	-0.40	-0.10	0.27
48	1.40	-0.98								
72	1.49	-0.78	0.47	0.32	-0.16	-0.11	-0.09	0.40	-0.91	0.47
96	0.76	-0.76	0.42	0.11	0.68	-0.48	1.04	0.11	-0.66	0.76

The second day (48 H) value for R square and adjusted R square 0.3092 and 0.2560. For the third day (72 H), R square and adjusted R square value are 0.6255 and -0.0486. The fourth day (96 H) equation of R square and adjusted R square is 0.7667 and 0.3467. Regression residuals show no patterns and close to zero by evenly distributed above and below the average. The significance F values are from day one to four as follow in order 0.8830, 0.0314, 0.5668, and 0.2628 less than 0.05 that showing the probability of the regression output is not random for the second day (48 H) only. For the P-values of the y-intercepts and coefficients, they are higher than 0.05 on all days of measurements except for the second day (48 H).

4.3.13 GLUTAMIC ACID

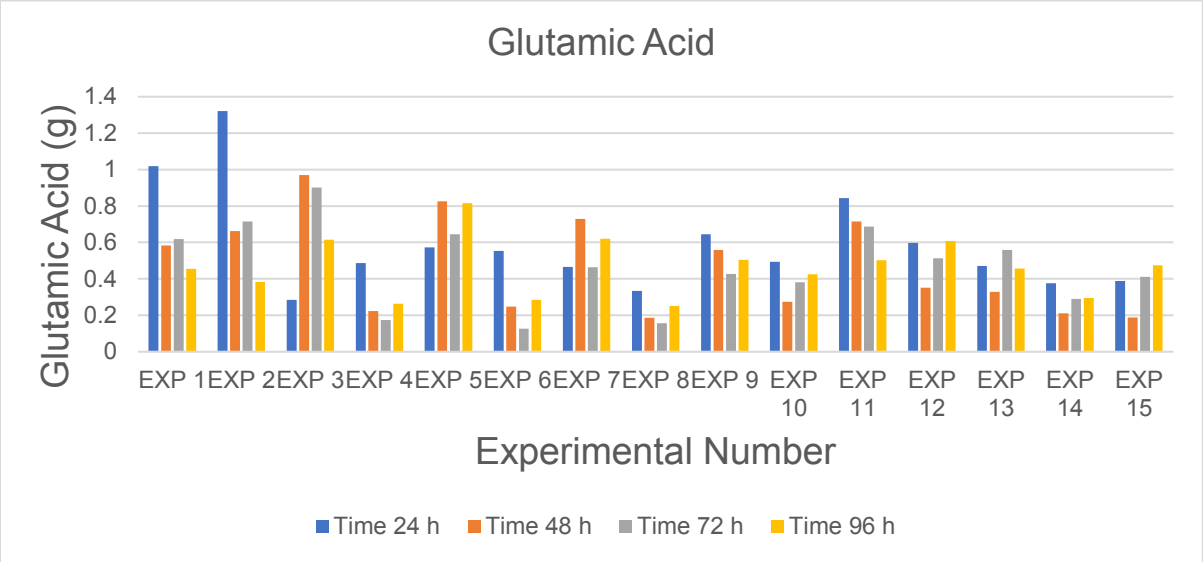


FIGURE 20:The Glutamic Acid Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for glutamic acid were changing with the time of sampling. As have been seen from Figure 20, glutamic acid production changed daily. Glutamic acid equations for the first day (24 H) value for R square and adjusted R square are 0.4460 and 0.4034. The second day (48 H) value for R square and adjusted R square 0.5876 and 0.5189. For the third day (72 H), R square and adjusted R square value are 0.7760 and 0.7149. The fourth day (96 H) equation of R square and adjusted R square is 0.6277 and 0.5991. Regression residuals show no patterns and close to zero by evenly distributed above and below the average. The significance F values are from day one to four as follow in order 0.0065, 0.0049, 0.0007, and 0.0004 less than 0.05 that meaning the probability of the regression output is not random. For the P-values of the y-intercepts and coefficients, they are lower than 0.05 on all days of measurements. All the models can be seen in Table 54. The first day (24 H) of y-intercept and nitrogen is 8.7872E-08 and 0.0065 less than

0.05, which they are significant. For the second day (48 H), all factors are lower than 0.05 for y-intercept, carbon and nitrogen interaction, and carbon 3.7128E-07, 0.0431, and 0.0047. The third day (72 H) for y-intercept, carbon, nitrogen square, carbon and nitrogen interaction has 4.2211E-06, 0.0013, 0.0173, and 0.0055 respectively. For the fourth day (96 H), y-intercept and carbon have 1.4632E-10 and 0.0004.

TABLE 54: Glutamic Acid Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.59		-0.25							
48	0.47	-0.22							-0.21	
72	0.38	-0.18				0.17			-0.21	
96	0.46	-0.17								

4.3.14 LYSINE

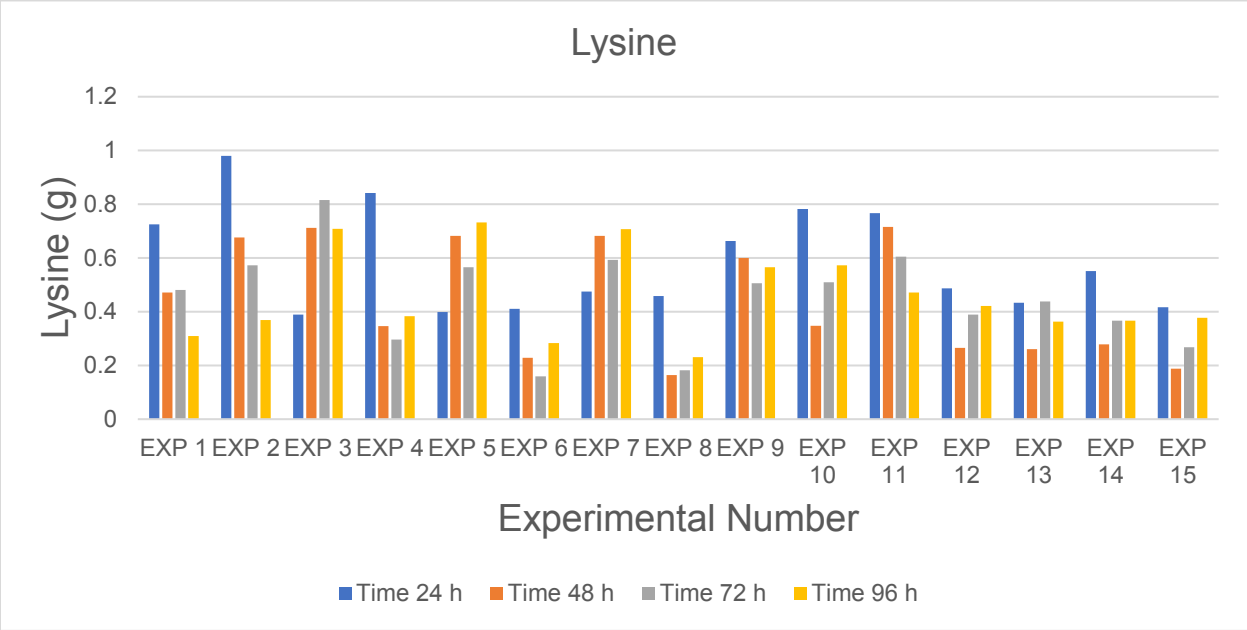


FIGURE 21:Different Concentrations of Carbon, Nitrogen, and Phosphorous, the Lysine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Lysine equations were different depending on the sampling time, and all calculations were depending on this amino acid production. As have been seen from the above Figure 21, the change in lysine production is daily. Lysine equation for the first day (24 H) value for R square and adjusted R square is 0.4737 and 0.4332. The second day (48 H) value for R square and adjusted R square is 0.7885 and 0.4079. For the third day (72 H), R square and adjusted R square values are 0.8586 and 0.8200. The fourth day (96 H) equation of R square and adjusted R square is 0.4820 and 0.4422. Regression residuals show no patterns and close to zero by evenly distributed above and below the average. The significance F values are from day one to four as follow 0.0046, 0.2187, 5.6329E-05, and 0.0041 less than 0.05 that showing the probability of the regression output is not random except for the second day (48 H). For the P-values of the y-

intercepts and coefficients, they are lower than 0.05 on all days of measurements except for the second day (48 H). All models can be seen in Table 55. The first day (24 H) of y-intercept and nitrogen square is 1.6139E-06 and 0.0046 less than 0.05, that they are significant. For the second day (48 H), all factors are higher than 0.05 for p-values. The third day (72 H) for y-intercept, carbon, nitrogen square, carbon and nitrogen interaction has 5.2719E-08, 0.0001, 0.0022, and 0.0018 respectively. For the fourth day (96 H), y-intercept and carbon have 1.891E-09 and 0.0041.

TABLE 55: Lysine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.45					0.26				
48	0.24	-0.14	-0.10	-0.004	0.13	0.18	0.06	-0.02	-0.14	-0.05
72	0.37	-0.16				0.15			-0.15	
96	0.46	-0.15								

4.3.15 GLYCINE, THREONINE, ALANINE, TYROSINE, CYSTEINE, AND METHIONINE

4.3.16 GLYCINE

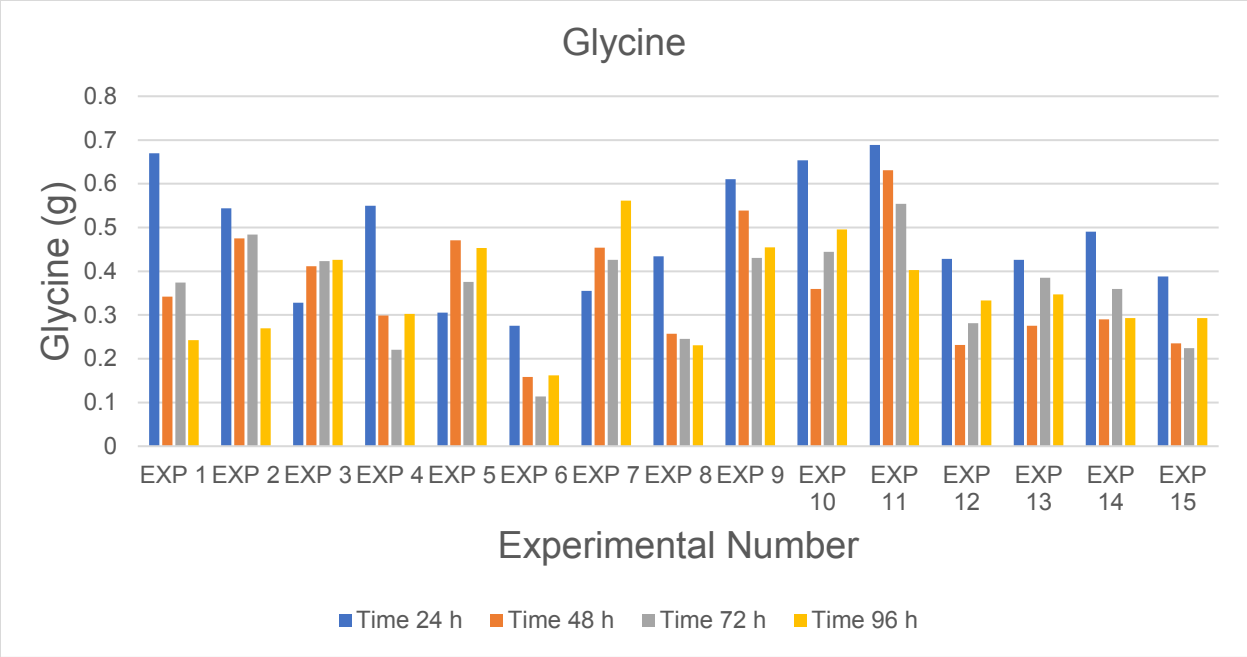


FIGURE 22:Glycine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for this amino acid, glycine, were changing daily, and calculations were depending on the concentration of this amino acid at that given time. All information for glycine production can be seen in Figure 22 and Table 56.

TABLE 56: Glycine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.38					0.18				
48	0.27	-0.06	-0.09	0.01	0.004	0.11	0.06	0.03	-0.06	-0.05
72	0.32	-0.06	-0.06	0.02	-0.04	0.10	0.01	0.02	-0.08	-0.07
96	0.31	-0.09	0.02	-0.004	-0.04	0.03	0.08	-0.01	-0.04	-0.03

4.3.17 THREONINE

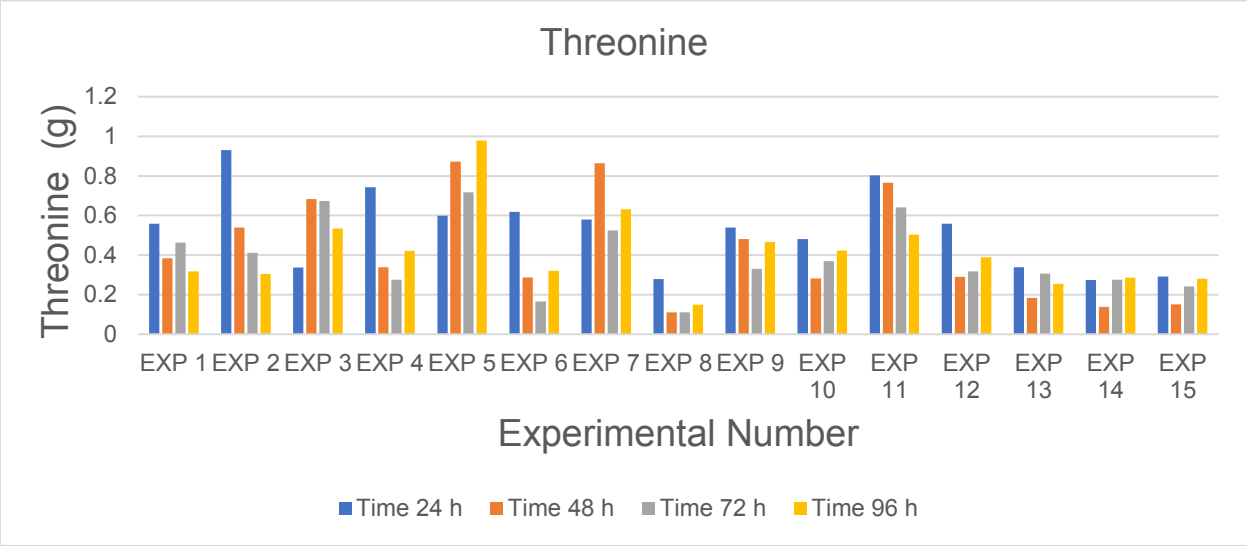


FIGURE 23:Different Concentrations of Carbon, Nitrogen, and Phosphorous, Threonine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Threonine equations were different depending on the sampling time due to the production change in this amino acid (Table 57). As seen from the above Figure 23, threonine production changed daily.

TABLE 57: Threonine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.30	0.06	-0.09	-0.002	0.13	0.21	0.09	-0.08	0.01	-0.05
48	0.16	-0.19	-0.07	0.01	0.20	0.12	0.17	-0.04	-0.12	-0.07
72	0.39	-0.18								
96	0.27	-0.16	0.02	-0.06	0.10	0.02	0.15	0.04	-0.03	-0.02

4.3.18 ALANINE

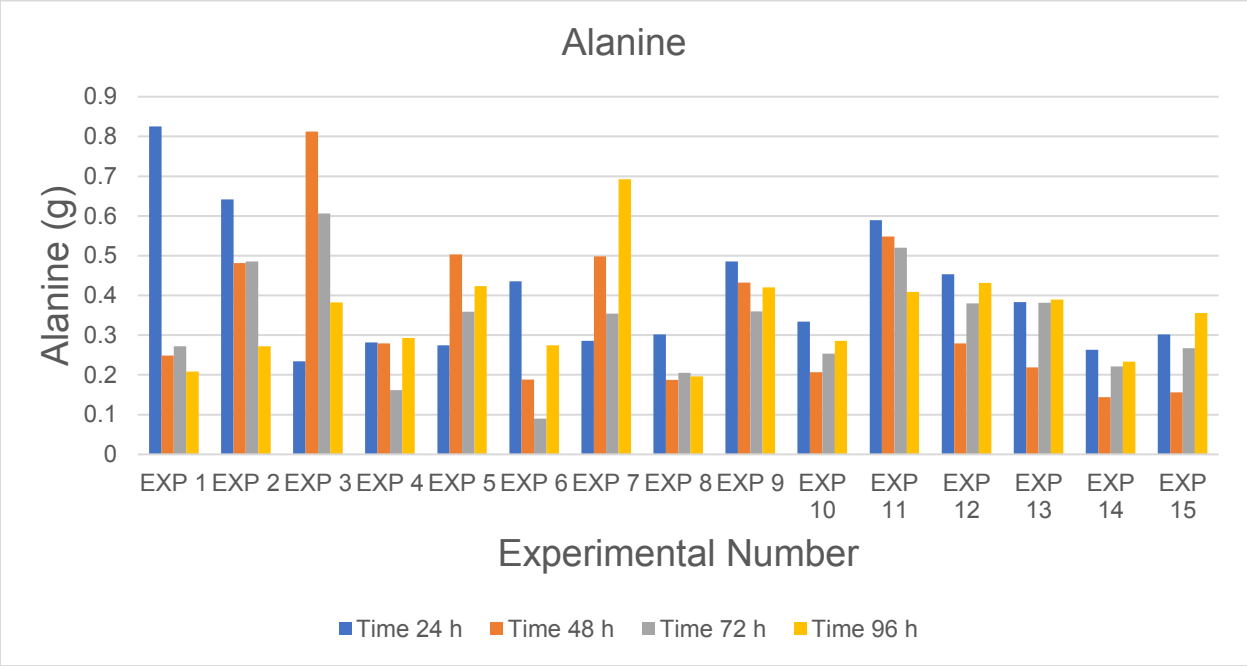


FIGURE 24:Different Concentrations of Carbon, Nitrogen, and Phosphorous, the Alanine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for alanine were changing with the sampling period (Table 58). Calculations were depending on the concentration of this amino acid as alanine production changed daily (Figure 24).

TABLE 58: Alanine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.41		-0.15			0.18				
48	0.17	-0.12	-0.02	0.02	0.13	0.15	0.04	0.001	-0.19	-0.01
72	0.27	-0.08				0.11			-0.16	
96	0.33	-0.08	0.01	0.04	-0.01	-0.02	0.08	-0.09	-0.04	0.04

4.3.19 TYROSINE

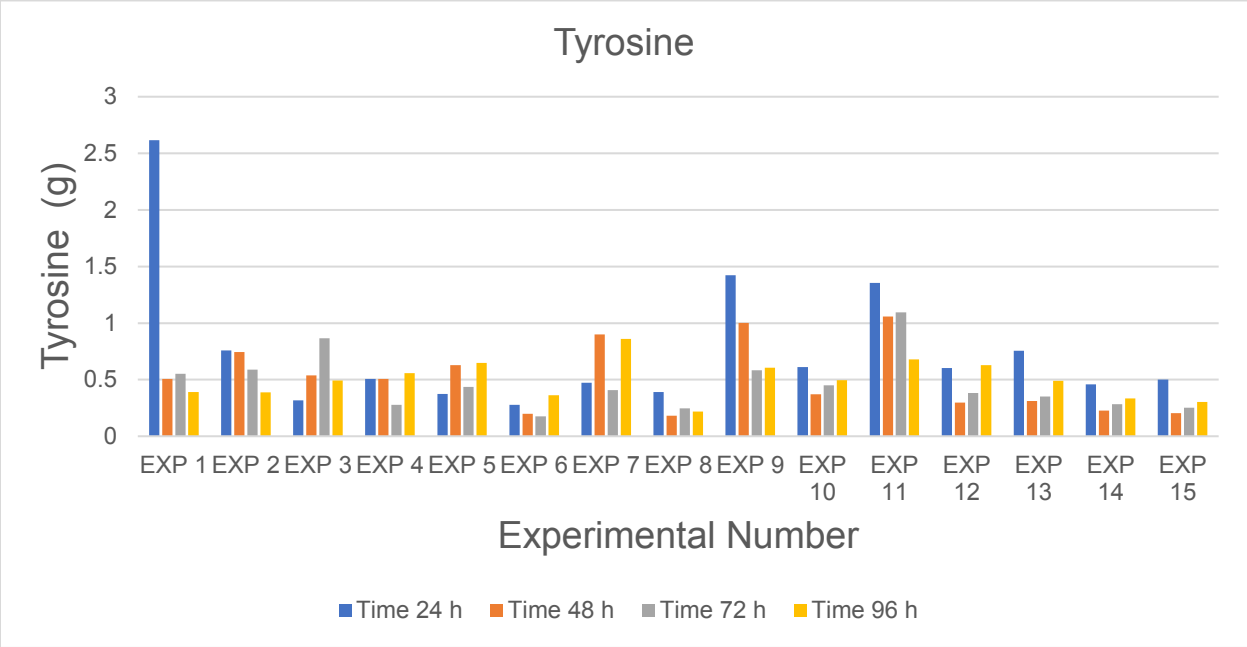


FIGURE 25:The Tyrosine Production for *G. sulphuraria* with Different Concentrations of Carbon, Nitrogen, and Phosphorous. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Tyrosine equations were different depending on the sampling time. All calculations were a result of this amino acid concentration at that given time as seen in figure 26.

TABLE 59: Tyrosine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.76		-0.51							
48	0.25	-0.12	-0.20	0.03	0.06	0.27	0.17	-0.07	-0.07	-0.03
72	0.30	-0.12	-0.11	0.06	-0.02	0.29	0.04	0.02	-0.16	-0.14
96	0.38	-0.11	0.01	0.03	0.0003	0.08	0.15	-0.09	-0.02	0.01

4.3.20 CYSTEINE

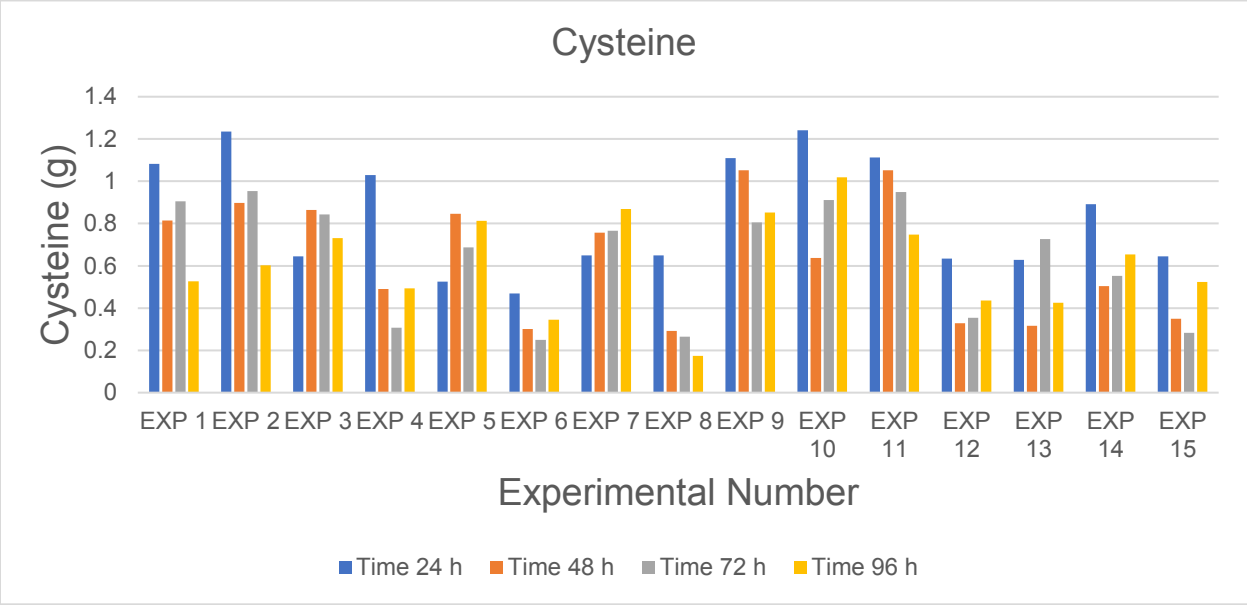


FIGURE 26:Different Concentrations of Carbon, Nitrogen, and Phosphorous, The Cystine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Equations for cysteine were changing with the sampling period because calculations were based on the concentration of this amino acid (Table 60). As seen from the above Figure 26, the cysteine production changed daily.

TABLE 60: Cysteine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.64					0.37				
48	0.48		-0.19			0.29				
72	0.50	-0.18				0.25				
96	0.53	-0.17	-0.01	-0.10	-0.08	0.13	0.10	-0.06	-0.08	-0.12

4.3.21 METHIONINE

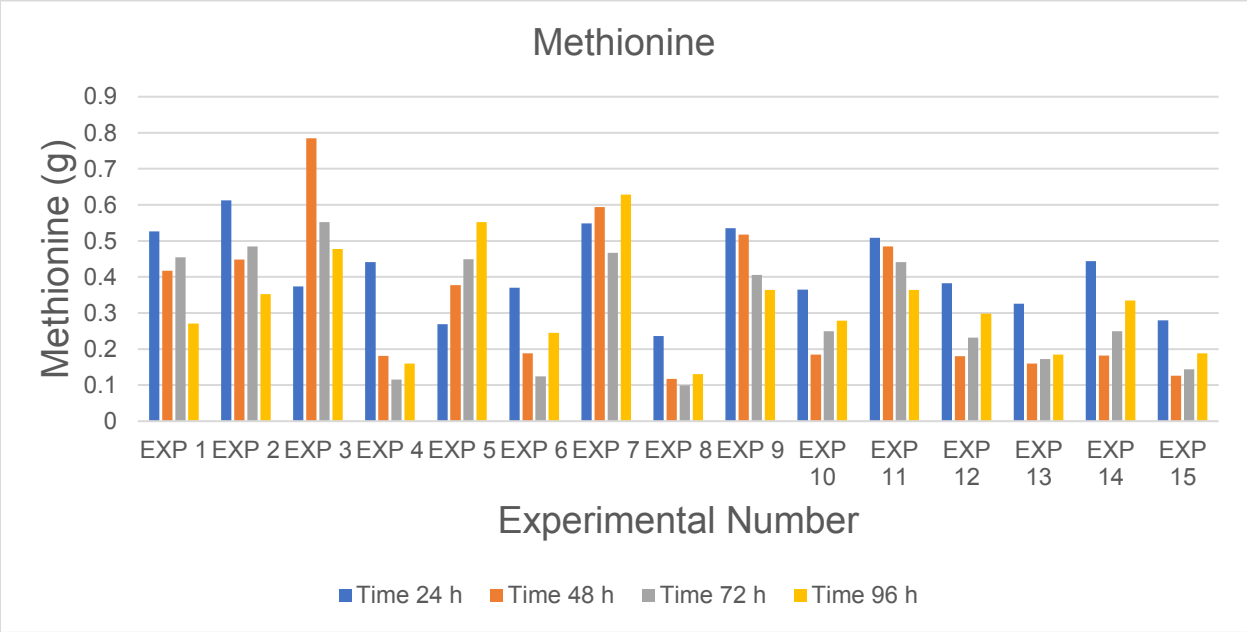


FIGURE 27:Different Concentrations of Carbon, Nitrogen, and Phosphorous, the Methionine Production for *G. sulphuraria* Can Be Seen in This Figure. Experiments 1 Through 15 Correspond to Conditions Listed in Table 2. The Coded Factors for Each Experiment is (Carbon, Nitrogen, Phosphorous). Exp 1 (-1,-1,0), Exp 2 (+1,-1,0), Exp 3 (-1,+1,0), Exp 4 (+1,+1,0), Exp 5 (-1,0,-1), Exp 6 (+1,0,-1), Exp 7 (-1,0,+1), Exp 8 (+1,0,+1), Exp 9 (0,-1,-1), Exp 10 (0,+1,-1), Exp 11 (0,-1,+1), Exp 12 (0,+1,+1), Exp 13 (0,0,0), Exp 14 (0,0,0), Exp 15 (0,0,0).

Methionine equations were different depending on the sampling time, and all calculations were established on this amino acid concentration (Table 61). As seen from the above Figure 27, methionine production changed daily.

TABLE 61: Methionine Production Equations of *G. sulphuraria* for all days, The First (24 H), Second (48 H), Third (72 H), and Fourth (96 H)

$$Y = b_0 + b_1G + b_2N + b_3P + b_4G^2 + b_5N^2 + b_6P^2 + b_7GP + b_8GN + b_9NP$$

TIME (H)	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9
24	0.35		-0.08			0.11				
48	0.33	-0.15								
72	0.20	-0.14	-0.08		0.08	0.13			-0.12	
96	0.33	-0.13								

4.3.22 GLYCINE, THREONINE, ALANINE, TYROSINE, CYSTEINE, AND METHIONINE STATISTICAL ANALYSIS

In glycine, threonine, alanine, tyrosine, cysteine, and methionine, a similar pattern can be seen for these amino acids (Table 62 - 65).

TABLE 62: Amino Acids of Glycine, Threonine, Alanine, Tyrosine, Cysteine, And Methionine with R Square, Adjusted R Square, and Significance F for First Day (24 H)

	AMINO ACIDS					
	Glycine	Threonine	Alanine	Tyrosine	Cysteine	Methionine
R SQUARE	0.4447	0.6328	0.4803	0.3989	0.5063	0.5391
ADJUSTED R SQUARE	0.4020	-0.0283	0.4403	0.3526	0.4683	0.4623
SIGNIFICANCE F	0.0066	0.5517	0.0042	0.0116	0.0029	0.0096

TABLE 63: Amino Acids of Glycine, Threonine, Alanine, Tyrosine, Cysteine, And Methionine with R Square, Adjusted R Square, and Significance F for First Day (48 H)

	AMINO ACIDS					
	Glycine	Threonine	Alanine	Tyrosine	Cysteine	Methionine
R SQUARE	0.7265	0.7226	0.7805	0.6629	0.5301	0.3288
ADJUSTED R SQUARE	0.2343	0.2231	0.3854	0.0561	0.4518	0.2771
SIGNIFICANCE F	0.3485	0.3571	0.2346	0.4876	0.0108	0.0254

TABLE 64: Amino Acids of Glycine, Threonine, Alanine, Tyrosine, Cysteine, And Methionine with R Square, Adjusted R Square, and Significance F for First Day (72 H)

	AMINO ACIDS					
	Glycine	Threonine	Alanine	Tyrosine	Cysteine	Methionine
R SQUARE	0.8091	0.5358	0.7668	0.8649	0.4621	0.9463
ADJUSTED R SQUARE	0.4654	0.5001	0.7032	0.6216	0.3724	0.9165
SIGNIFICANCE F	0.1794	0.0019	0.0008	0.0881	0.0242	1.8572E-05

TABLE 65: Amino Acids of Glycine, Threonine, Alanine, Tyrosine, Cysteine, And Methionine with R Square, Adjusted R Square, and Significance F for First Day (96 H)

	AMINO ACIDS					
	Glycine	Valine	Alanine	Tyrosine	Cysteine	Methionine
R SQUARE	0.6349	0.6509	0.6605	0.5814	0.7229	0.4725
ADJUSTED R SQUARE	-0.0224	0.0226	0.0493	-0.1721	0.2242	0.4319
SIGNIFICANCE F	0.5473	0.5133	0.4928	0.6542	0.3563	0.0046

4.4 AMINO ACIDS PRODUCTION UNDER DIFFERENT SUGAR TYPES

By changing the carbon source, a comparison can be made for the dynamic activity of amino acid productions for *G. sulphuraria.* Four different sugar types have been chosen to perform these experiments, as was outline in the experimental design chapter, Table 16. The raw data for these figures are listed in Appendix A under amino acid production under by changing sugar types section. All amino acid weights are reported by gram per 100 grams of algae sample.

Biomass production can be seen in Figure 28 for all sugars. The variation in amino acid production by holding all media components constant and only changing sugar sources (glucose, mannitol, arabinose, xylose) can be seen in Tables (29 – 31).

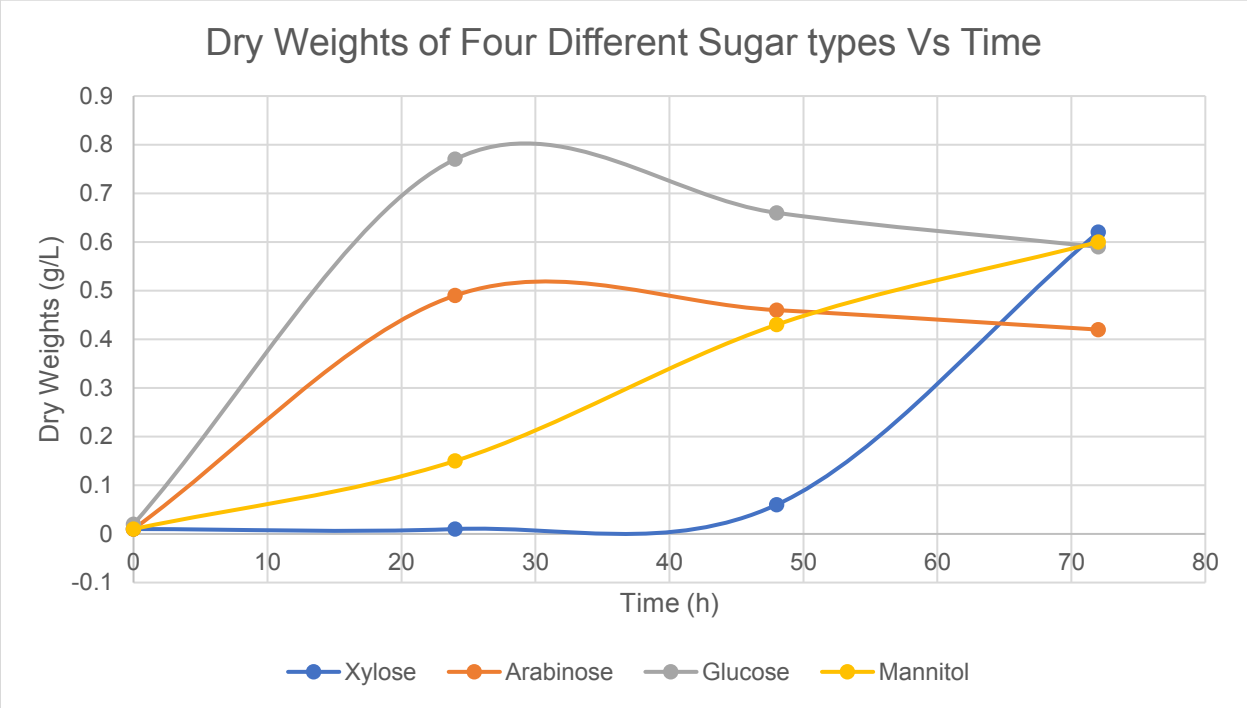


FIGURE 28: The Dry Weight of *G. sulphuraria* for Different Carbon Sources, Glucose, Mannitol, Xylose, and Arabinose with the Same Number of Moles. The Concentration of All Components Is the Same for Each Sugar Type. These Concentrations Are the Same.

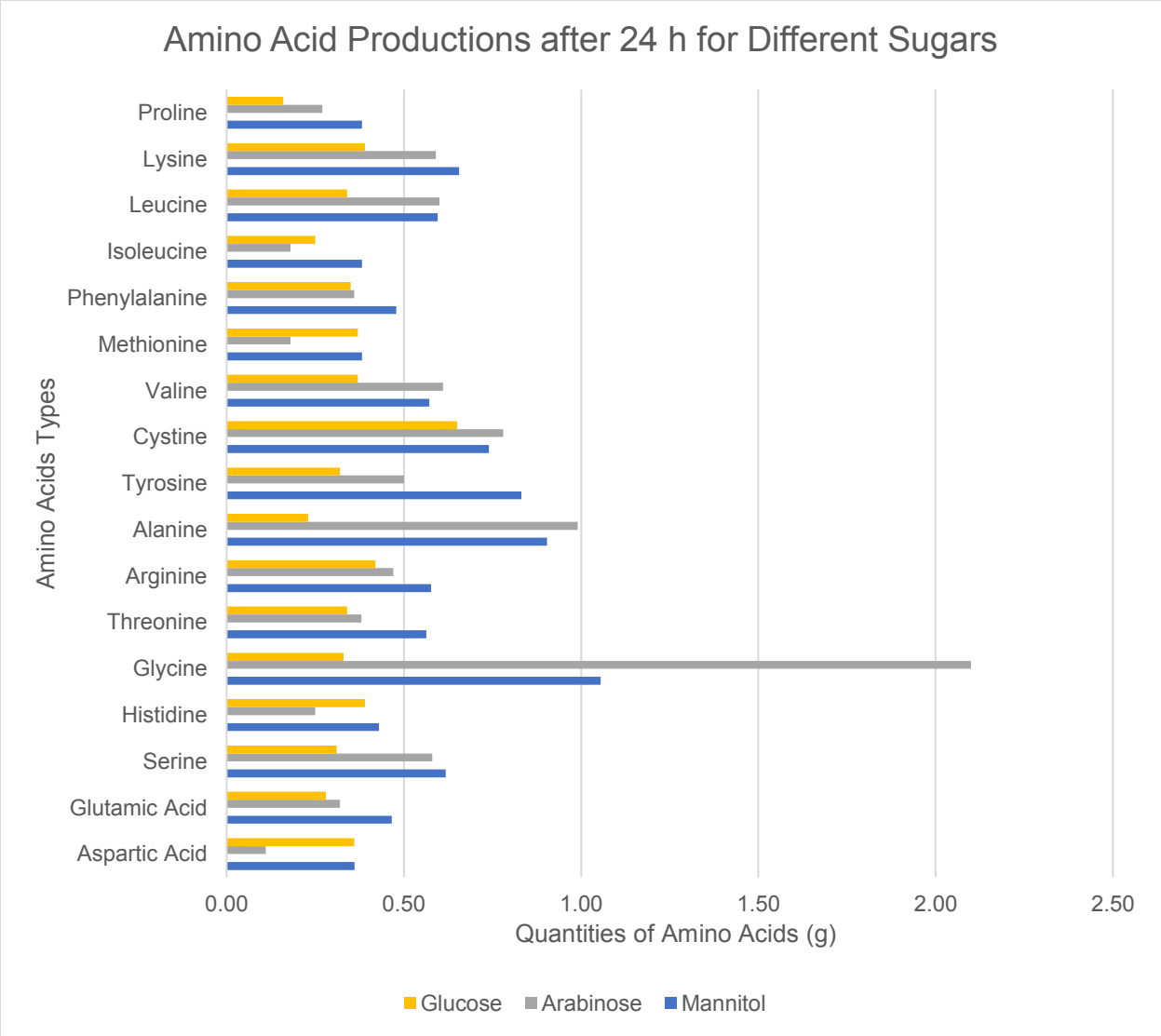


FIGURE 29: Amino Acid Productions for *G. sulphuraria* Feeding on Four Different Sugars, Glucose, Mannitol, Xylose and Arabinose with the Same Number of Moles. Xylose Not Shown in this Graph Due to no Biomass. The Concentration of All Components in Equal Amount among All Sugar Types.

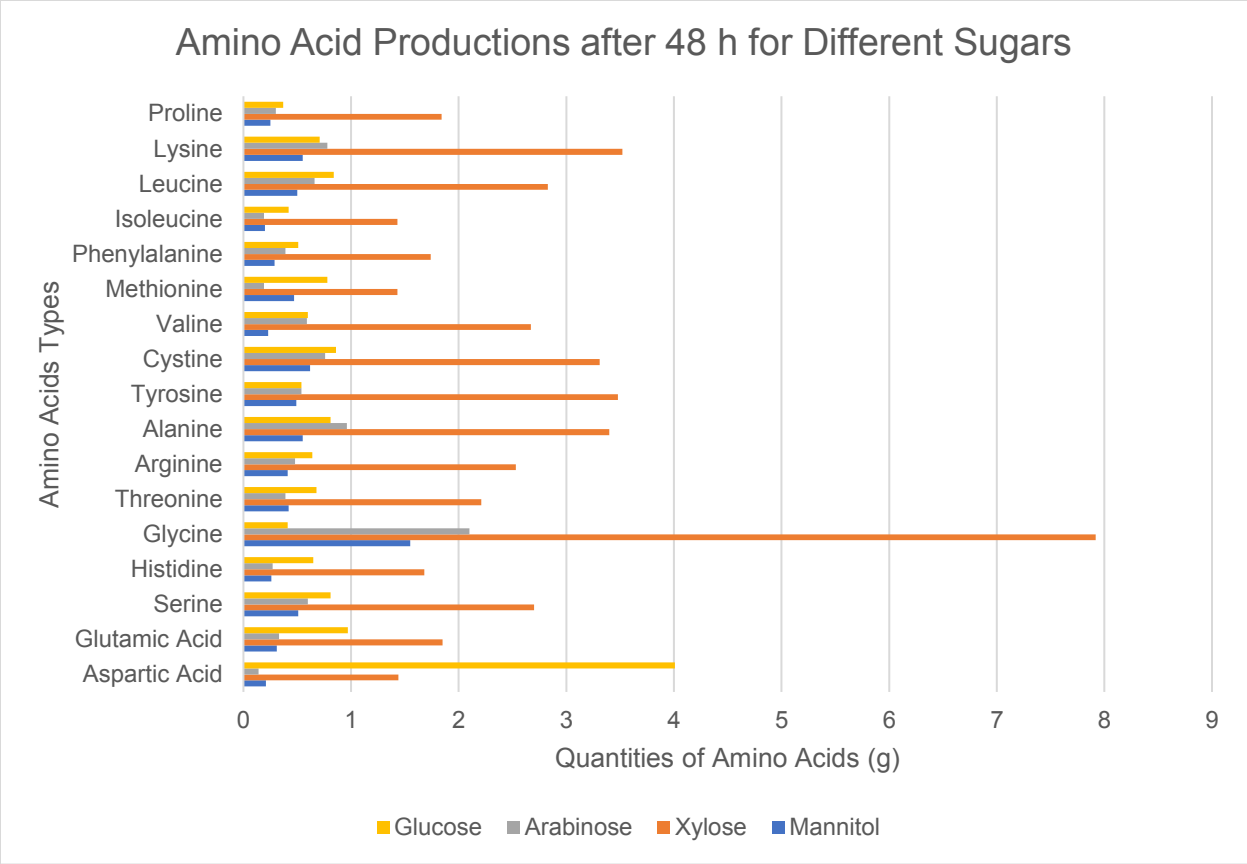


FIGURE 30: Four Different Sugars, Glucose, Mannitol, Xylose, And Arabinose with the Same Number of Moles Was Fed for *G. sulphuraria* to Observe Its Amino Acid Production. The Concentration of All Components in Equal Amount among All Sugar Types.

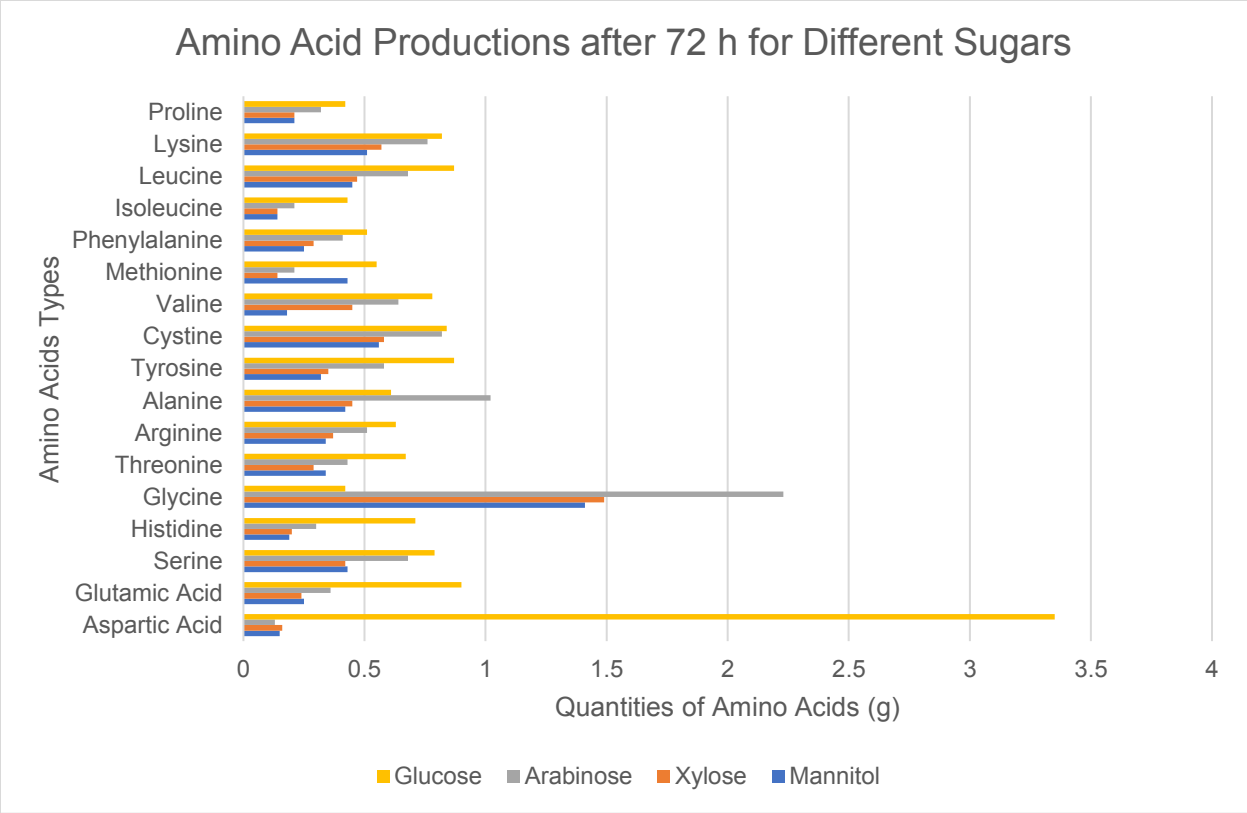


FIGURE 31: Amino Acid Productions for *G. sulphuraria* Feeding on Four Different Sugars, Glucose, Mannitol, Xylose, And Arabinose with the Same Number of Moles. The Concentration of All Components in Equal Amount among All Sugar Types.

CHAPTER FIVE: DISCUSSION

5.1 GALDIERIA SULPHURARIA GROWTH

The biomass growth curve is a very important element or tool that is used to shine a light on various processes inside the cell such as amino acid production and cell division. The growth of the extremophile algae used in this study is not different from any other algae, where five distinctive phases of growth were seen for *G. sulphuraria*, presented in Figure 9. Each experiment had different timing to show all five stages due to the amount of nutrients (carbon,

nitrogen, phosphorous) that were presented in the culture. The five growth phases are as follows:

1- The lag or induction phase, which can be long or short depending on the prior inoculation state, is the period for cells to adapt with the environment (medium) to start increasing enzymes and metabolites for cell division. This phase was not pronounced in Figure 9 because the inoculum was growing in the exponential phase.
2- The exponential phase is the stage for maximum rate of cell division where the cell density increases rapidly. Experiments had explicitly showed this exponential stage in Figure 9 and had started from the time zero. The phase was very short for some experiments such as experiment eleven (0,-1,+1) or nine (0,-1,-1). However, the same phase was long in other experiments, as this behavior can be seen in experiment fourteen (0,0,0).
3- The declining growth rate phase is where cell division slows, due to the decrease in elements and components needed for growth. On average, experiments had experienced or displayed this declining phase around 45 hours of growth.
4- The stationary phase is the period of cell division and death are balanced, which shall lead to slow cell growth. Furthermore, stationary stage started around 60 hours in average, Figure 9.
5- The death phase is the stage where cell density decreases due to dying cells for lacking resources. From Figure 9, experiments have showed death stage around the time of 70 hours of growth in average.

Biomass production has dramatically changed depending on concentration of the three factors (carbon, nitrogen, phosphorous) and their ratios. The highest dry weight biomass is for experiments number fourteen (0,0,0) and fifteen (0,0,0), which have dry weight of 2.86 g/L and

2.66 g/L, respectively (Appendix A1 in Table 66 - 67). These two experiments are center points in Box-Behnken design, with a 1:1:150 ratio of C:N:P. Experiments, number three (-1,+1,0), five (-1,0,-1), seven (-1,0,+1), and ten (0,+1,-1), have similar behavior in that they have reached the stationary phase earlier with lower biomass. On the other hand, the least dry weight is for experiments number nine (0,-1,-1) and eleven (0,-1,+1), which both have dry weights of 0.56 g/L. The ratio C:N:P of these two experiments 1:55.5:5.5 and 1:55.5:1.4 for experiments nine and eleven, respectively. The rest of experiments have values between these two boundaries. The percentage increase or decrease in the different experiments is very diverse compared to the average of the center point experiments (0,0,0); for instance, experiment number two (+1,-1,0) has the lowest average dry weight in the third day with a value of - 475.61% in all experimental numbers and days. Within the third day, experiment number eight (+1,0,+1) has the highest dry weight in the entire experimental numbers and days with a value of 17.48%.

Carbon, nitrogen, phosphorous have different influences on the biomass production on this algal strain. The concentration of nitrogen has more of an impact on the production of *G. sulphuraria* dry weight than carbon and phosphorous. Additionally, phosphorous has less effect on *G. sulphuraria* growth when compared to the influence of carbon or nitrogen. Furthermore, the concentration of nitrogen in the medium dictates the impact of phosphorous on *G. sulphuraria* growth; for example, the percentage difference between two experiments, that have constant carbon and low nitrogen concentration, is around 2.5 to 8 percent between high and low phosphorous concentration. As the nitrogen concentration increases in the medium, the difference in the dry weight of *G. sulphuraria* percentage increases between high and low in phosphorous concentration. The change is increasing from 2.5 to 8% for the low nitrogen concentration to a 7 to 27% difference in the dry weight for the high nitrogen experiment. This

behavior is only observed for changes in the nitrogen concentration. By holding the concentration of nitrogen constant between high and low, differences in carbon concentration does not impact *G. sulphuraria* dry weight percentage difference when the phosphorous concentration is varied between high and low. However, the change in carbon concentration does alter the biomass production for *G. sulphuraria,* regardless of the concentration of the phosphorous in the growth media. For instance, the average difference between high and low carbon and phosphorous concentrations, like in experiment five (-1,0,-1) or seven (-1,0,+1) and six (+1,0,-1) or eight (+1,0,+1) with medium nitrogen concentration, is 9%, 68%, 77%, and 72% respectively from day one to day four. The only influence phosphorous concentration can have on *G. sulphuraria* is accelerating the growth curve, as can be seen between experiments six (+1,0,-1) and eight (+1,0,+1), the highest growth happened between time zero to forty-eight hours for experiment six (+1,0,-1) and zero to seventy-two for experiment eight (+1,0,+1). Based on these results, it is possible that that the phosphorous concentration can influence trace elemental absorption by *G. sulphuraria.* Such an impact would affect cellular growth and functioning.

5.1.1 Growth Modeling And Equations

In this thesis, the Monod equation was not used to establish a relationship between specific growth rate and the substrate concentration due to the lack of equipment to measure nitrogen and phosphorous concentration during the experiment. Instead, a regression model was used to analyse the results and establish a correlation between the biomass or amino acid production and the three variables.

Equations are built to show the importance, relationship, and interaction among the three variables. These equations also show the association and impact of the three macronutrients

variables (carbon, nitrogen, and phosphorous) on the daily growth of the algae. These numbers and results, Table 25, lead to the conclusion that both carbon and nitrogen factors have the dominate control on regulating and influencing *G. sulphuraria* biomass production; however, three levels of a factor are not sufficient to elucidate interactions between nitrogen and carbon. Since the importance of nitrogen can be clearly seen in the early stages of the growth curve, luxurious nitrogen uptake is most likely dominant during the early stages of growth. However, nitrogen effects disappearing toward the end of the experiment, and carbon becomes the dominate nutrient limiting growth. These two behaviors illustrate the uniqueness of the different growth stages. In the beginning, adaptation to the environment, enzymatic activities, and preparation for exponential phase are essential for *G. sulphuraria* to reproduce. Nitrogen plays a key role in all of these activities. This type of algae also contains high nitrogen content mostly in its cell wall. Nonetheless, cells almost at the end of the growth curve and at the beginnings of the stationary phase focus on maintaining their ability to survive successfully without the need to reproduce. Furthermore, this process requires huge amount of energy, carbon source, due to the need to maintain cell viability and the large number of cells present in the culture. With these equations, R-square values drops slightly as the time progress because the measurement of carbon, nitrogen, and phosphorous is only known at the initial time of the experiment. Lack of measurement is a result of equipment and resources limitations, especially in measuring nitrogen and phosphorous. Additionally, the initial concentration is changing as these three elements are used up by algae cells to grow.

5.2 Quantifying Amino Acid Standard from High Performance Liquid Chromatography (HPLC)

To quantify amino acids using HPLC for this research, the standards must undergo derivatization, the pre-column, and chromatographic separation using two mobile phases. FMOC and OPA are the two reagents that are used for amino acids pre-column derivatization. The result of the standard deviation comes from taking the average of five replications for each concentration of the amino acid standard. As the concentration of the amino acids increases, the standard deviation value increases (Appendix A in Table 68). This growth in error is due to the integration of peaks in the software system. The integration can be done by the software automatically or by the operator manually. While amino acids concentrations were increased in the sample, the peak areas in the HPLC were getting bigger compared to small concentrations of the same amino acid type (Figure 10). With these standard deviation results, the R-squared assessment for all amino acids has a value of 99.2 and above. This high value reveals that the model can justify all variability of the response data around its mean. Furthermore, both results, standard deviation and R-squared, signify the importance of taking multiple sample measurements of the same concentration to minimize the error of integration.

5.3 Amino Acids Extraction Method

In these days, many analytical methods and tools are used to quantify amino acid amounts in the biome. For amino acids analysis, there are not a single standard method to quantify all proteins and amino acids in a given sample. Amino acids are produced by protein hydrolysis, breaking protein to its building blocks chemically. A common and current extraction procedure uses a

dilute acid (0.1 M hydrochloric acid) to break amine bonds without affecting the concentration of amino acids. These amino acids are the most damaged or affected are threonine, methionine, tryptophan, and cysteine. The results obtained in this work indicate that the method of using 0.1 M hydrochloric acid to the homogenized samples are reproducible with minimal standard deviation. The standard deviation is within the range and agreeable to a published paper by Perez-Palacios (Perez-Palacios, et al. 2015). A comparison in the results between 6M and 0.1M hydrochloric acid of experiment number one (-1,-1,0) can be seen (Appendix A in Table 69). The standard deviation of the two methods are similar to the standard deviation within 6M method (Appendix A in Table 70). 6M hydrochloric acid method has been performed by the agriculture department of University of Alberta. Proline is not included in the comparison between the two methods due to the cost of the analysis. There are no published data on the amino acids profile for *G. sulphuraria* to form a basis for comparison. Furthermore, most reported amino acid profiles for other microorganisms do not report growth phase of the collected sample. As it can be seen later in this research, amino acid profile is changing rapidly with time as the growth phase is changing.

5.4 Amino Acids Production for *Galdieria sulphuraria*

For *G. sulphuraria*, no studies have been reported on the algal amino acids content, so this research is the first to look at the amino acids content for this strain. Twenty amino acids can show an organisms' genetic code and are very important for understanding the functioning of the cells. Due to limitation in resources, seventeen amino acids are examined for this algal strain instead of the full twenty. Every amino acid has unique properties distinguish itself from other types. Furthermore, amino acids can be categorized in various modes and types, and these seventeen amino acids have been characterised per their chemical properties. All amino acids

have a highly dynamic expression in the cell that is changing rapidly from day to day due to changes in cell requirements. Amino acids production is influenced predominantly by the concentration of two elements, carbon and nitrogen. Each factor has different degree of influence on the regulatory system in *G. sulphuraria*. Phosphorous concentration did not show a major impact on the amino acid production. Amino acids biosynthesis is a complex process and can affect the production of each other as regulatory mechanisms inside the cell. Each one of these seventeen amino acids is employed differently to perform a variety of functions within the cell or others released outside of the cell to help algae to endure the surrounding ecosystem.

5.4.1 Amino Acids with Electrically Charged Side Chains (Acidic)

In this group, there are two amino acids, Aspartic acid and glutamic acid, acting as an acid in pH 7 with their terminal carboxylic site. These two amino acids initiate and control the production of other amino acids. Usage and conversion of aspartic acid and glutamic acid to produce other amino acids impact their concentration in algae. Both amino acids cannot be correlated directly to the concentration of carbon, nitrogen, or phosphorous.

Aspartic acid, which is also known as aspartate, is not considered an essential amino acid and is used in protein synthesis. Aspartate can be considered an acid with a pK_a of 3.5, containing an α-amino group and α-carboxylic acid group (Lundblad 2014). Production results of aspartic acid for *G. sulphuraria* can be seen in Figure 19. In aspartic acid production, experiments number five (-1,0,-1) and three (-1,+1,0) have produced the highest value of this amino acid, which they

have 4.17 g and 4.01 g per 100 g of sample respectively. However; high carbon and nitrogen produced the lowest aspartic acid production in experiments number four (+1,+1,0) and fourteen (0,0,0) with a result of 0.09 g and 0.11 g correspondingly. Within each experiment, the concentration was changing rapidly and hardly remained constant. These dynamic changes can be clearly seen for each experiment when the average production calculation is used for total days. Experiments that have the following numbers produced the highest average concentration one (-1,-1,0), twelve (0,+1,+1), three (-1,+1,0), and seven (-1,0,+1) respectively.

In microorganism in general, this amino acid has shown more factors such as other amino acids (glutamate) regulating the production of aspartic acid (Azevedo, Lancien and Lea 2006). Furthermore, aspartic acid has two forms that can be found inside the cell, aspartate and asparagine. Aspartate also plays a key role in synthesizing lysine, methionine, threonine, and isoleucine.

Glutamic acid is not considered an essential amino acid and is used in protein synthesis. Glutamic acid can be considered an acid with a pK_a of 4.25, containing an α-amino group, α-carboxylic acid group, and a side chain of another carboxylic acid (Dewick 2006).

The production results of this amino acid, glutamic acid, for *Galdieria sulphuraria* can be seen in figure 20. For glutamic acid production, experiments number one (-1,-1,0), two (+1,-1,0), and three (-1,+1,0) have produced the highest value of this amino acid, which they have 1.02 g, 1.32 g, and 0.97 g per 100 g of sample respectively. Furthermore, these results indicate the need for very low carbon and nitrogen concentration or alternating very high nitrogen and very low carbon concentration or vice versa. However, the lowest glutamic acid production have totally different behavior as can be seen in experiments number six (+1,0,-1), eight (+1,0,+1), and four (+1,+1,0) with a result of 0.13 g, 0.17, and 0.16 g per 100 g of sample correspondingly. In

average for total days, experiments that have the following numbers produced the highest concentrations two (+1,-1,0), five (-1,0,-1), three (+1,-1,0), and eleven (0,-1,+1) respectively.

In microorganisms, glutamic acid has shown to control almost all factors such as other amino acids production and their intermediates (Azevedo, Lancien and Lea 2006). Moreover, glutamic acid has also two forms that can be found inside the cell, glutamine and glutamate. Glutamine can control the production of asparagine or be converted to glutamate. This glutamate can be used to regulate the production of aspartate, which has the ability to synthesize lysine, methionine, threonine, and isoleucine. In the other hand, glutamine is formed by catalyzing the condensation of glutamate and ammonia. It is the most abundant free amino acid and occupies a central position in amino acid metabolism. Glutamine forms the basis for synthesizing arginine and proline.

5.4.2 Amino Acids with Polar Neutral Side Chains

While this group contains four amino acids similar in their chemical properties, they act and are synthesized in different ways. Two amino acids, cysteine and methionine, contain sulfur bonds while the other two, serine and threonine, do not contain sulfur. In addition, the relationship between the concentration of the three elements and the production of those amino group is not alike. Serine production is directly linked to the concentration of carbon and nitrogen whereas the other three amino acids do not have a straightforward connection with the three factors (carbon, nitrogen, and phosphorous).

Serine is classified as a polar-uncharged amino acid due to the R group, having a hydroxyl group, giving serine the ability to form a hydrogen bonds with water (Blachier, Wu and Yin 2013). Serine is also considered nonessential amino acid and is used for protein synthesis in organisms.

Due to the concentration of phosphorous being low, the production results for serine in experiments number nine (0,-1,-1), two (+1,-1,0), five (-1,0,-1), and three (-1,+1,0) have produced the highest value of this amino acid, which they have 1.36 g, 0.97 g, 0.82 g, and 0.81 g per 100 g of sample respectively. In addition, the lowest serine production is for experiments number eight (+1,0,+1), fifteen (0,0,0), four (+1,+1,0), and fourteen (0,0,0) with a result of 0.15 g, 0.16 g, 0.17g and 0.18 g per 100 g of sample correspondingly with high concentration of phosphorous. On average, experiments that have the following experimental numbers produced the highest concentrations: nine (0,-1,-1), five (-1,0,-1), three (-1,+1,0), and seven (-1,0,+1) respectively.

Serine biosynthesis occurs in two different pathways, photorespiration and non-photorespiration. The photorespiration uses glycine amino acid to form serine. One molecule of glycine is decarboxylated and deaminated by glycine decarboxylase complex (Ros, Munoz-Bertomeu and Krueger 2014). The methylene from the broken glycine is transferred to tetrahydrofolate to form methylene tetrahydrofolate, which reacts with another glycine to form serine (Ros, Munoz-Bertomeu and Krueger 2014). The non-photorespiration uses phosphorylated pathway that involves Calvin cycle or uses cytosolic glycolysis to produce serine (Ros, Munoz-Bertomeu and Krueger 2014). Alanine and glycine are involved in producing and synthesizing serine amino acid. Due to the production complexity of serine, this amino acid is highly and closely regulated as seen in from the result.

Threonine is a polar-uncharged amino acid that is considered an essential amino acid. Threonine has α-amino group and a carboxylic acid with alcohol as a side chain (Blachier, Wu and Yin 2013).

For threonine production the following patterns was observed, experiments number two (+1,-1,0), five (-1,0,-1), and seven (-1,0,+1) have produced the highest concentration value of this amino acid, which they have 0.93 g, 0.87 g, and 0.86 g per 100 g of sample respectively. Moreover, the lowest threonine production is for experiments number eight (+1,0,+1), fourteen (0,0,0), and fifteen (0,0,0) with a result of 0.11 g and 0.14 g, and 0.15 g per 100 g of sample correspondingly. For the average concentration for total days for each trial, experiments that have the following numbers produced the highest concentration five (-1,0,-1), eleven (0,-1,+1), and seven (-1,0,+1), respectively.

One of the amino acid in the aspartate group is threonine because its biosynthesis pathway begins with the formation of aspartic acid. In this pathway, aspartic acid undergoes numerous steps or intermediates to convert to threonine. One of these steps is the production of homoserine, an amino acid that does not participate in protein synthesis. This homoserine amino acid is phosphorylated and transformed to threonine.

Cysteine is the only one in the seventeen amino acids that contain a thiol group (Fleck and Petrosyan 2014). This functional group is giving properties no other amino acids can have such as increasing mechanical and thermal stability for protein structure (Fleck and Petrosyan 2014). The production dynamic for cysteine, experiments number two (+1,-1,0), ten (0,+1,-1), nine (0,-1,-1), and eleven (0,-1,+1) had produced the highest value of concentration for this amino acid, which they had 1.24 g, 1.24 g, 1.11 g, and 1.11 g per 100 g of sample respectively. Moreover, the lowest cysteine concentration production was for experiments number eight (+1,0,+1) and six (+1,0,-1) with a result of 0.17 g and 0.25 g correspondingly. Based on the average production for all days, experiments that had the following experimental numbers produced the highest concentrations eleven (0,-1,+1), nine (0,-1,-1), and ten (0,+1,-1) respectively.

The inorganic sulfur that is available in the media is used for cysteine biosynthesis. This amino acid is used as a donor to generate methionine. Cysteine biosynthesis can be simplified to three steps. The first step is to reduce sulfate to sulfide. The second step is to use the combined serine and acetyl-CoA, which they formed o-acetylserine. The third step is to add the sulfide to o-acetylserine to form cysteine.

Methionine is another amino acid that contains sulfur group (S-methyl thioether) as a side chain, α-amino group, and a carboxylic acid (Brosnan and Brosnan 2006). Methionine is considered one of the most hydrophobic amino acid and classified as a non-polar and aliphatic amino acid (Fasman 1989).

For methionine production behavior, experiments number three (-1,+1,0), seven (-1,0,+1), and two (+1,-1,0) had produced the highest value of this amino acid, which they had 0.78 g, 0.63 g, and 0.61 g per 100 g of sample respectively. Moreover, the lowest methionine production was for experiments number eight (+1,0,+1), six (+1,0,-1), and four (+1,+1,0) with a result of 0.10 g, 0.12 g, and 0.11 g correspondingly. Based on the average productions for total days, experiments that had the following numbers produced the highest concentration seven (-1,0,+1), three (-1,+1,0), and two (+1,-1,0) respectively.

Methionine is considered one of the amino acid in aspartate group because its biosynthesis pathway begins with formation of aspartic acid. In this pathway, aspartic acid undergoes numerous steps or intermediates similar to threonine production. One of these steps is the production of homoserine, an amino acid does not participate in protein synthesis. This homoserine amino acid is phosphorylated, o-phosphohomoserine, to be used in either in threonine or methionine production. However, the presence of cysteine causes the process to shift for methionine biosynthesis.

5.4.3 Amino Acids with Electrically Charged Side Chains (Basic)

For the basic group, there are three amino acids histidine, lysine, and arginine acting as a base with their terminal amino group at pH 7. These three amino acids have similar chemical properties; however, they perform and are synthesized in different ways. In addition, the association between the concentration of the three factors tested in this study and the production of the amino acids in this group are not identical. Histidine and arginine production are directly associated to the concentration of carbon and nitrogen; but, lysine does not have direct correlation with the three factors (carbon, nitrogen, and phosphorous).

Histidine is another amino acid in the list that is considered essential for protein synthesis and has an α-amino group and a carboxylic acid with imidazole as the side chain (Lundblad 2014). Histidine is positively charged amino acid and is needed for growth and development for microorganisms, plant, and animal (Ingle 2011).

Histidine production had the following expression that was similar to the amino acids mentioned above. Experiments number two (+1,-1,0), one (-1,-1,0), and eleven (0,-1,+1) produced the highest value of this amino acid, which they had 1.07 g, 1.01 g, and 1.0 g per 100 g of sample respectively. On the other hand, the lowest concentrations were with the following numbers eight (+1,0,+1), fourteen (0,0,0), and fifteen (0,0,0) with a result of 0.12 g, 0.16 g, and 0.16 g correspondingly. For average production for total days, experiments that had the following numbers produced the highest concentrations one (-1,-1,0), eleven (0,-1,+1), and two (+1,-1,0) respectively.

Histidine biosynthesis is closely regulated to nucleotide metabolism, which is a process for synthesizing and degrading nucleic acids (DNA and RNA). Biosynthesis of histidine requires an

intermediate that is supplied by the pentose phosphate pathway and a glutamate as an amino group donor. This amino acid biosynthetic process, excluding tryptophan biosynthesis, is different and isolated from other amino acids biosynthesis routes.

Arginine contains α-amino group and a carboxylic acid with three carbon aliphatic straight chain as a side chain for this amino acid (Watson and Zibadi 2013). Arginine is considered positively charged amino acid due to the presence of guanidine group (Blachier, Wu and Yin 2013).

In arginine production, the activity was high in experiments number one (-1,-1,0), three (-1,+1,0), and seven (-1,0,+1) because they had produced the highest value of this amino acid, 0.83 g, 0.81 g, and 0.69 g per 100 g of sample respectively. Moreover, the lowest arginine production was for experiments number six (+1,0,-1), fourteen (0,0,0), fifteen (0,0,0), and four (+1,+1,0) with a result of 0.09 g, 0.14 g, 0.16 g, 0.16 g correspondingly. On average, experiments that had the following numbers produced the highest concentrations eleven (0,-1,+1), three (-1,+1,0), and two (+1,-1,0) respectively.

Arginine synthesis is a process that requires eight steps from the beginning to the end production. Initially, glutamate amino acid is converted to ornithine amino acid, which transformed to arginine through enzymatic reactions.

Lysine has lysyl, amino group attaching to a long hydrocarbon chain ($(CH_2)_4NH_2$), as a side chain, α-amino group, and a carboxylic acid (Craig, et al. 2014). Lysine is an essential amino acid that is classified as a positively charged and aliphatic amino acid (Blachier, Wu and Yin 2013). Lysine production is dynamic, experiments number two (+1,-1,0), four (+1,+1,0), and three (-1,+1,0) had produced the highest concentration value for this amino acid, which they had 0.98 g, 0.84 g, and 0.82 g per 100 g of sample respectively. Moreover, the lowest lysine production was for experiments number six (+1,0,-1), eight (+1,0,+1), and fifteen (0,0,0) with a

result of 0.16 g, 0.16 g, and 0.19 g correspondingly. Based on the average production, experiments that had the following numbers produced the highest concentration three (-1,+1,0), two (+1,-1,0), and eleven (0,-1,+1) respectively.

Lysine is one of the amino acids that can be put under the aspartate group because its biosynthesis pathway starts with the formation of aspartic acid. In this pathway, aspartic acid undergoes numerous steps or intermediates to be converted it to aspartate semialdehyde. This molecule, aspartate semialdehyde, undergoes multiple steps to have or produce lysine amino acid.

5.4.4 Amino Acids with Hydrophobic Side Chain (Aliphatic)

Aliphatic group includes four amino acids that are related in their chemical properties. These amino acids are alanine, valine, isoleucine, and leucine. The link between these amino acid productions and the concentration of the three elements studied in this work is not similar. Alanine production is not directly connected with the three factors (carbon, nitrogen, and phosphorous). The other three amino acids were directly connected to the concentration of carbon and nitrogen.

Alanine contains an α-amino group and a carboxylic acid with a methyl group as a side chain (D'Mello 2015). Alanine is considered an aliphatic and nonpolar and is considered as a non-essential amino acid (Blachier, Wu and Yin 2013). For this amino acid production, experiments with low carbon concentrations, number one (-1,-1,0), three (-1,+1,0), and seven (-1,0,+1), had produced the highest value of alanine, which they had 0.83 g, 0.81 g, and 0.69 g per 100 g of sample respectively. Nevertheless, the lowest concentration productions were with the following experimental number six (+1,0,-1), fourteen (0,0,0), fifteen (0,0,0), and four (+1,+1,0) with a result of 0.09 g, 0.14 g, 0.16 g, and 0.16 g per 100 g of sample correspondingly. On average,

experiments that had the following experimental numbers produced the highest concentrations eleven (0,-1,+1), three (-1,+1,0), and two (+1,-1,0) respectively.

Alanine amino acids is biosynthesized by converting either glutamate or valine by transamination of one molecule of pyruvate, which is made from glucose through glycolysis.

Valine is non-polar amino acid that has α-amino group and a carboxylic acid with isopropyl group as a side chain (Hirs and Timasheff 1986). This amino acid is one of the essential amino acid and can be considered hydrophobic.

In valine production, experiments number two (+1,-1,0), eleven (0,-1,+1), and one (-1,-1,0) had showed the highest value of this amino acid, which they had 1.10 g, 0.91 g, and 0.84 g per 100 g of sample respectively. Moreover, the lowest valine production was for experiments number fourteen (0,0,0), four (+1,+1,0), and six (+1,0,-1) with a result of 0.08 g, 0.12 g, and 0.13 correspondingly. On average, experiments that had the following numbers produced the highest concentrations two (+1,-1,0), ten (0,+1,-1), and five (-1,0,-1), respectively.

The pathway of valine biosynthesis has four enzymatic steps, which begins with reacting two pyruvate molecules. These two molecules undergo two more reactions to give α-ketoisovalerate, which is converted to valine by the presence of alanine or glutamate.

Isoleucine is another isomer for leucine, where one methyl group bonds to C4 instead of C5 (Fleck and Petrosyan 2014). Isoleucine is an essential amino acid that has a hydrocarbon side chain, an amino group, and a carboxylic acid (Barnes 2007). Isoleucine is considered aliphatic, non-polar, and hydrophobic amino acid (Blachier, Wu and Yin 2013). With low nitrogen concentration, isoleucine production for these experiments number two (+1,-1,0), one (-1,-1,0), and eleven (0,-1,+1) had produced the highest concentration value for this amino acid, which they had 0.56 g, 0.51 g, and 0.47 g per 100 g of sample respectively. On the other hand, the

lowest isoleucine production was for experiments number six (+1,0,-1), eight (+1,0,+1), and four (+1,+1,0) with high carbon concentration, 0.07 g, 0.07 g, and 0.08 g correspondingly. On average, experiments that had the following numbers produced the highest concentration two (+1,-1,0), eleven (0,-1,+1), and three (-1,+1,0) respectively.

Another amino acid in the aspartate group is isoleucine because its biosynthesis pathway starts with the formation of aspartic acid. In this pathway, aspartic acid undergoes numerous steps or intermediates to be converted it to threonine. One of these steps is the production of homoserine, an amino acid does not participate in protein synthesis. This homoserine amino acid is phosphorylated and transformed to threonine, which interacts further in a parallel way with the production process of valine amino acid to produce isoleucine.

Leucine is an essential amino acid that has an isobutyl side chain, an amino group, and a carboxylic acid (Fleck and Petrosyan 2014). Leucine is an essential amino acid that is classified as non-polar and hydrophobic (Blachier, Wu and Yin 2013). Leucine production had a similar pattern to isoleucine because the following experimental numbers three (-1,+1,0), seven (-1,0,+1), and one (-1,-1,0) had produced the highest concentration value for this amino acid by having 0.87 g, 0.79 g, and 0.67 g per 100 g of sample respectively. Moreover, the lowest leucine production was for experiments number eight (+1,0,+1), six (+1,0,-1), and fifteen (0,0,0) with a result of 0.16 g, 0.16 g, and 0.18 g correspondingly. On average, experiments that had the following numbers produced the highest concentration three (-1,+1,0), seven (-1,0,+1), and five (-1,0,-1) respectively. Leucine biosynthesis uses the same pathway that produces valine amino acid. The pathway of valine biosynthesis has four enzymatic steps, which begins with reacting two pyruvate molecules. These two molecules undergo two more reactions to give α-ketoisovalerate, which is converted to valine by the presence of alanine or glutamate. In the case

of leucine biosynthesis, the existence of Acetyl-CoA in the pathway adds acetyl group to α-ketoisovalerate, which undergoes multiple steps to form α-ketoisocaproate. This α-ketoisocaproate molecule is converted to leucine in the presence of glutamate.

5.4.5 Amino Acids with Hydrophobic Side Chains (Aromatic)

Although the aromatic group of amino acids contains only two amino acids with identical chemical properties, they are also synthesized in nearly same pathway with only small differences. The two amino acids, tyrosine and phenylalanine, contain benzene rings with tyrosine's benzene ring having an additional bond to a hydroxyl group. However, the relationship between the concentration of the three factors studied in this work and the production of this group of amino acids is not alike. Phenylalanine production is directly linked to the concentration of carbon and nitrogen where tyrosine does not have direct correlation with any of the three factors (carbon, nitrogen, and phosphorous).

Tyrosine is non-essential amino acid that has a polar side chain. Tyrosine has α-amino group and a carboxylic acid with a phenol group as a side chain (Lemke, et al. 2013). For tyrosine, experiments number one (-1,-1,0), nine (0,-1,-1), and eleven (0,-1,+1) with low concentration of nitrogen had produced the highest concentration, in which the production was 2.62 g and 1.24 g, and 1.36 g per 100 g of sample respectively. Moreover, the lowest tyrosine production was for experiments number eight (+1,0,+1) and six (+1,0,-1) with a result of 0.18 g and 0.18 g corresponding to the high concentration of carbon. On average, experiments that had the following experimental numbers produced the highest concentration concentrations of tyrosine: eleven (0,-1,+1), one (-1,-1,0), and nine (0,-1,-1) respectively.

Tyrosine is biosynthesized through the shikimate pathway in which this metabolic process is used in algae to synthesize aromatic amino acids. This process has seven steps to biosynthesize tyrosine by using an intermediate, prephenate, which is oxidatively decarboxylated. In the presence of glutamate, p-hydroxyphenylpyruvate is transformed to amino acid of tyrosine.

Phenylalanine is considered an essential amino acid that occurs in almost all proteins (Gurskaya 1968). Phenylalanine has an amino group, a carboxylic acid, and a phenyl group as a side chain (Gurskaya 1968). Because of the aromatic side chain, phenylalanine is relatively nonpolar with hydrophobic property (Blachier, Wu and Yin 2013). With phenylalanine production, experiments with low nitrogen concentration, number two (+1,-1,0), one (-1,-1,0), and eleven (0,-1,+1), had produced the highest concentration of this amino acid, which they had 0.75 g, 0.74 g, and 0.71 g per 100 g of sample, respectively. Moreover, the lowest phenylalanine production was for experiments number six (+1,0,-1), eight (+1,0,+1), and four (+1,+1,0) with a result of 0.11 g, 0.12 g, and 0.13 g corresponding to high carbon and medium nitrogen concentrations. On average, experiments that had the following numbers produced the highest concentration two (+1,-1,0), eleven (0,-1,+1), and nine (0,-1,-1). As in tyrosine amino acid, Phenylalanine is biosynthesized through the shikimate pathway. This aromatic amino acid uses chorismate, an intermediate to be used for synthesizing phenylpyruvate. With the availability of glutamate, phenylpyruvate is transformed to phenylalanine.

5.4.6 Unique Amino Acids

For this group, there are only two amino acids with different chemical properties, they are also synthesized via different pathways. The two amino acids, glycine and proline, are not similar to any other amino acids or to each other. Proline contains tetrahydropyrrole, a cyclic secondary amine, as a side chain. For glycine, the chemical structure is the simplest form among all amino

acids mentioned in this thesis. The relationship between the concentration of the three elements and proline or glycine production is not comparable. Proline production is directly linked to the concentration of carbon and nitrogen where glycine does not have direct correlation with the three factors (carbon, nitrogen, and phosphorous).

Glycine is the smallest amino acid that is known today and is considered a non-essential amino acid (Sapse 2000). Glycine is nonpolar and hydrophobic amino acid that has amino group and a carboxylic acid with a side chain consisting of one hydrogen (Blachier, Wu and Yin 2013). Glycine production was the highest with the following experimental number eleven (0,-1,+1) and one (-1,-1,0), which they had 0.96 g and 0.67 g per 100 g of sample, respectively. Both experiments had low concentration of nitrogen. Moreover, the lowest glycine production was for experiments number six (+1,0,-1), four (+1,+1,0), fifteen (0,0,0) with a result of 0.11 g and 0.22 g, and 0.22 g correspondingly. On average, experiments that had the following numbers produced the highest concentrations of glycine: eleven (0,-1,+1), nine (0,-1,-1), and ten (0,+1,-1). Biosynthesis of glycine requires the formation of serine amino acid that is synthesized in photorespiratory glycolate pathway. Both amino acids, serine and glycine, share the same metabolic pathway, which play a major and central role in the formation of other amino acids, nucleic acids and phospholipids. Glycine biosynthesis comes from removing the methyl group that is found in amino acid of serine.

Proline has α-amino group, α-carboxylic acid group, and pyrrolidine. Pyrrolidine is a cyclic secondary amine with hydrocarbon ($(CH_2)_4NH$) as a side chain (Silver and Christiansen 1999). Proline is non-essential amino acid that is classified as non-polar and hydrophobic (Blachier, Wu and Yin 2013). With this amino acid, experiments eleven (0,-1,+1), two (+1,-1,0), and three (-1,+1,0) produced the highest concentration value of proline, which they had 0.64 g, 0.48, and

0.42 g per 100 g of sample, respectively. On the other hand, the lowest proline production was for experiments number fourteen (0,0,0), fifteen (0,0,0), eight (+1,0,+1), six (+1,0,-1), and four (+1,+1,0) with a result of 0.06 g, 0.06 g, 0.06 g, 0.06 g, and 0.06 g, respectively. On average, experiments that had the following numbers produced the highest concentration eleven (0,-1,+1), two (+1,-1,0), and three (-1,+1,0). As in arginine biosynthesis, proline production starts with glutamate. Glutamate is converted to ornithine amino acid, which is transformed to proline through enzymatic reactions.

5.4.7 The Relationship Between Amino Acids Production and Concentrations of Carbon, Nitrogen, and Phosphorous

As mentioned above, amino acids can be simply arranged into those that are directly correlated to the concentration of two factors, carbon and nitrogen, or those that are indirectly related to carbon, nitrogen, and phosphorous by having other unmeasured stimuli that controls their production. These relationships have been determined by using the Box-Behnken experimental design method that was implemented in this thesis. The amino acids that are directly correlated to the concentration of these macronutrients (glucose, nitrogen, and phosphorous) are serine, histidine, arginine, valine, phenylalanine, isoleucine, leucine, and proline. All these amino acids have relatively high R-square values and low p-values. Furthermore, these amino acids' equations show similar patterns in the relationship and interaction between the initial value of the three variables and amino acid production by starting with nitrogen depletion and ending in carbon consumption in the last day of the experiment. The associations and impacts of the three variables on the daily production of amino acids are very dynamic and rapidly changing. R-

square values declines slightly as the time progress as the quantity of carbon, nitrogen, and phosphorous is only taken in the initial period of the experiment. Lack of measurement is a consequence of shortage in equipment and resources particularly in measuring nitrogen and phosphorous. Moreover, the initial concentration is changing as these three elements are used up by algae cells to grow.

Remarkably, a poor correlation was observed between the concentration of the manipulated macronutrients (glucose, nitrogen, and phosphorous) and the amino acids of aspartic acid, glycine, threonine, alanine, tyrosine, cysteine, methionine, and lysine. The same analysis has been carried for these amino acids as those amino acids that have direct correlation with carbon, nitrogen, and phosphorous concentrations. Low R-square and high p-values values have been observed for all these types of amino acids, which indicate the low regulation of the three factors over the production system of this algae. However, fast dynamic and rapid changes were observed on the daily production of these amino acids. All amino acids' equations for both classifications produce the highest power of a given factor that is tested during the experiment. The usage of the highest power in any equation indicates the need to use more levels to see the effect and remove any bias in the equation.

Both amino acids groupings, direct and indirect correlations to the three factors, show a trend where other factors can influence the association between carbon, nitrogen, or phosphorous and amino acids synthesis. For instance, proline and serine production is highly associated with the concentration of tested factors; but; proline production in the second day does not show any linkage to carbon, nitrogen, or phosphorous concentration. Proline is the only amino acids which demonstrates this type of display. A behavioral change is a solid indication to the presence of other factors or key players to regulate the amino acid production such as oxygen concentration.

On the other hand, the indirect amino acids can show a pattern change in their relationship with the tested factors. All these types of amino acids exhibit a form of low relationship with nitrogen and carbon. For example, aspartic acid is not impacted by the concentration of the three tested factors except for the second day where carbon was significant. The same behavior also can be observed with threonine production at the third day. A similar behavior is detected for glycine and tyrosine production with nitrogen concentration instead of carbon concentration at the first day. Alanine production is linked to the concentration of nitrogen in the first day while the production is related to carbon and nitrogen in the third day. For lysine, glutamic acid, cysteine, and methionine, the relationship between the concentration of three factors and amino acids production can be seen in their equations, but they have low R-squares in most of their days.

5.5 Amino Acids production by Changing Sugar Types

After examining the effects of the three factors, a single variation in the carbon source was tested. By changing the carbon source in the medium, amino acids production are highly influenced in *Galdieria sulphuraria* even though all other parameters are kept at the same concentrations. Experiment number three (-1,+1,0) was used for these experiments. The dry weight for these algae feeding on different sugars had almost the same weight at the end of the experiment except for the arabinose (Figure 28). The sugar effect on the production of the different amino acids can be seen clearly on *Galdieria sulphuraria* (Figure 29 - 31). These data shows that each sugar type undergoes different metabolic pathways which is reflected in the different amino acid concentrations. The culture that was supplied with xylose has the highest amino acid concentration, but these concentrations have changed very fast in the third day compared to the other sugar types (Table 30 - 31). Xylose has produced more amino acid concentration than the other three sugar types excluding aspartic acid in glucose medium, which

is decreased by 178%. The production of amino acids from a xylose containing medium has a rising value of 70% for serine, 61% for histidine, 75% for arginine, 78% for valine, 71% for phenylalanine, 71% for isoleucine, 70% for leucine, 80% for proline, 48% for glutamic acid, 95% for glycine, 69% for threonine, 76% for alanine, 84 % for tyrosine, 74% for cysteine, 46% for methionine, and 80% for lysine. Even though arabinose has the same molecular weight of xylose, this sugar does not have the same effect or match the influence of xylose in amino acid production behavior. The same effect can be seen between mannitol and glucose where mannitol has lower amino acid concentrations than glucose although both are hexose sugars with six carbons. Furthermore, mannitol has the lowest values of all three sugar types for the average of three days. The results of amino acid concentrations for different sugars suggests that amino acid production is irrelevant to the number of carbons in sugars.

CHAPTER SIX: RECOMMENDATION AND CONCLUSION

The experimental results support the idea that the amino acid production in *G. sulphuraria* can be controlled without the need for genetic modification. In the case of this research, a small change on the system, three factors, have impacted the production of amino acids of the whole system. As food supplement industries requires specific amino acids to be amplified and used in a product, algae, such as *G. sulphuraria,* can be used to meet the required amount. For instance; experiment number three (-1,+1,0) can be used with the addition of xylose as a carbon source to have an amino supplement containing eight essential amino acids for certain type of animal feeds. Furthermore, there are five from those eight essential amino acids can be controlled by changing the concentration of carbon and nitrogen along with the type of carbon. All these changes in the system can be done without genetic modification and can be easily scaled to industrial production.

G. sulphuraria has exceptional characteristics among other algae. It can grow photoautotrophically, mixotrophically, and heterotrophically. These algae can be fed many forms of carbon source in any given condition such as carbon dioxide in photoautotrophic growth conditions, glucose for mixotrophic growth, and xylose for heterotrophic growth. This species requires extreme conditions such as low pH and high temperature to thrive, which a very small number of competitors can tolerate and live. This strain is not only resilient to low pH and high heat, but it also can tolerate high salt, toxic metals, and other abiotic stressors. Since difficult and extreme conditions subjected to *G. sulphuraria,* amino acid production is the most essential process inside this type of algae. These amino acids are required and used to play a crucial role to keep and regulate cell structures and functions in these extreme conditions. The amino acids production depends on the concentration of different components in the media. Carbon, nitrogen, and phosphorous are used for testing to observe the effect on amino acid production. By employing Box-Behnken experimental design, the change in amino acid production can be seen clearly with different levels of concentrations for each factor. However, the three factors are not in equal strength to alter the amino acid production. The nitrogen is the main factor to determine the concentration of the amino acid. Box-Behnken experimental design showed amino acids can be broadly put in two categories. The first category is that amino acids are directly correlated to the concentration of these macronutrients such as serine, histidine, arginine, valine, phenylalanine, isoleucine, leucine, and proline. The second category is that amino acids are weakly correlated to the concentration of the manipulated macronutrients and the amino acids such as aspartic acid, glutamic acid, glycine, threonine, alanine, tyrosine, cysteine, methionine, and lysine. These experiments suggest that there are more factors in the media to control in addition to the concentration level of three factors.

Many different pathways can be explored further for future experiments. One of these pathways is switching from batch flask to continuous reactor experiments to keep the concentrations of the components in the media constant. In addition, the air flow to the reactor should be controlled to see the effect of carbon dioxide and oxygen on the system. The sampling can be taken in shorter periods to see the fluctuation in the amino acid concentrations. The subsequent experiments should include the relationship of the growth phases with the concentration of the amino acids during each growth phase. The types of sugar can be an influential and important factor to be considered since *G. sulphuraria* feeds on fifty types of carbon source under heterotrophic conductions.

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APPENDIX A

APPENDIX A1: DRY BIOMASS PRODUCTION

TABLE 66: The Growth of *Galdieria sulphuraria* with Different Concentrations of Sugar (Glucose), Nitrogen, And Phosphorous

Time (h)	*Dry Weight EXP 1*	*STD*	*Dry Weight EXP 2*	*STD*	*Dry Weight EXP 3*	*STD*	*Dry Weight EXP 4*	*STD*	*Dry Weight EXP 5*	*STD*	*Dry Weight EXP 6*	*STD*	*Dry Weight EXP 7*	*STD*
0	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**
24	**0.38**	**0.0000**	**0.33**	**0.0001**	**0.77**	**0.0001**	**0.58**	**0.0000**	**0.89**	**0.0001**	**1.00**	**0.0000**	**0.80**	**0.0000**
48	**0.48**	**0.0000**	**0.45**	**0.0001**	**0.66**	**0.0002**	**1.50**	**0.0000**	**0.58**	**0.0000**	**2.06**	**0.0003**	**0.67**	**0.0001**
72	**0.44**	**0.0000**	**0.41**	**0.0001**	**0.59**	**0.0002**	**2.31**	**0.0005**	**0.63**	**0.0001**	**2.59**	**0.0003**	**0.63**	**0.0001**
96	**0.74**	**0.0000**	**0.63**	**0.0001**	**0.60**	**0.0001**	**1.80**	**0.0000**	**0.56**	**0.0000**	**1.86**	**0.0005**	**0.59**	**0.0002**

TABLE 67: The Growth of *Galdieria sulphuraria* with Different Concentrations of Sugar (Glucose), Nitrogen, And Phosphorous

Time (h)	*Dry Weight EXP 8*	*STD*	*Dry Weight EXP 9*	*STD*	*Dry Weight EXP 10*	*STD*	*Dry Weight EXP 11*	*STD*	*Dry Weight EXP 12*	*STD*	*Dry Weight EXP 13*	*STD*	*Dry Weight EXP 14*	*STD*	*Dry Weight EXP 15*	*STD*
0	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**	**0.02**	**0.0000**
24	**0.85**	**0.0003**	**0.38**	**0.0003**	**0.62**	**0.0004**	**0.39**	**0.0005**	**0.79**	**0.0001**	**0.86**	**0.0005**	**0.91**	**0.0001**	**0.99**	**0.0001**
48	**1.90**	**0.0001**	**0.39**	**0.0004**	**1.60**	**0.0003**	**0.41**	**0.0001**	**1.72**	**0.0002**	**1.93**	**0.0002**	**2.24**	**0.0003**	**2.38**	**0.0000**
72	**2.86**	**0.0001**	**0.49**	**0.0002**	**1.23**	**0.0001**	**0.45**	**0.0001**	**1.53**	**0.0001**	**1.54**	**0.0000**	**2.88**	**0.0000**	**2.66**	**0.0002**
96	**2.21**	**0.0002**	**0.56**	**0.0003**	**1.13**	**0.0001**	**0.56**	**0.0002**	**1.22**	**0.0000**	**1.38**	**0.0003**	**2.08**	**0.0002**	**2.16**	**0.0000**

APPENDIX A2: AMINO ACIDS ANALYZATION

TABLE 68: HPLC Results for Amino Acids Standards

Amino Acids	*1000 pmol*	*STD*	*250 pmol*	*STD*	*100 pmol*	*STD*	*25 pmol*	*STD*	*10 pmol*	*STD*	*Graph R^2*
Aspartic Acid	**363.78**	**11.29**	**84.98**	**3.31**	**32.58**	**0.65**	**7.13**	**0.17**	**3.15**	**0.50**	**0.99977**
Glutamic Acid	**353.33**	**15.70**	**77.07**	**1.71**	**27.03**	**2.64**	**5.77**	**0.96**	**3.16**	**1.00**	**0.99852**
Serine	**392.89**	**19.59**	**93.63**	**4.31**	**36.33**	**1.11**	**9.25**	**0.27**	**4.45**	**0.39**	**0.9996**
Histidine	**255.34**	**34.14**	**60.25**	**7.36**	**22.64**	**3.47**	**5.17**	**1.12**	**1.89**	**0.66**	**0.99961**
Glycine	**210.95**	**68.77**	**64.40**	**9.01**	**28.40**	**10.89**	**7.41**	**1.89**	**4.77**	**1.26**	**0.997774**
Threonine	**570.19**	**24.72**	**128.94**	**1.14**	**46.86**	**6.51**	**12.22**	**0.21**	**5.30**	**0.07**	**0.99902**
Arginine	**403.09**	**10.05**	**98.58**	**1.70**	**36.66**	**1.53**	**7.88**	**1.17**	**3.15**	**0.67**	**0.99978**

Alanine	**448.27**	**27.65**	**123.40**	**10.93**	**35.81**	**2.10**	**11.86**	**2.47**	**6.46**	**0.09**	**0.9985**
Tyrosine	**410.62**	**15.70**	**101.36**	**3.92**	**36.30**	**2.46**	**14.46**	**1.75**	**7.80**	**0.21**	**0.99928**
Cystine	**765.46**	**87.15**	**196.07**	**18.06**	**77.69**	**3.95**	**16.74**	**6.58**	**8.37**	**0.45**	**0.99986**
Valine	**333.15**	**103.05**	**91.98**	**19.18**	**35.27**	**6.71**	**8.66**	**1.69**	**3.37**	**1.41**	**0.99939**
Methionine	**624.89**	**90.54**	**141.12**	**24.32**	**55.04**	**7.79**	**12.84**	**1.57**	**4.99**	**0.37**	**0.99919**
Phenylalanine	**311.76**	**53.33**	**75.69**	**16.73**	**30.81**	**6.57**	**6.55**	**2.83**	**3.54**	**1.15**	**0.99993**
Isoleucine	**533.56**	**20.32**	**136.36**	**21.47**	**53.97**	**6.40**	**10.36**	**5.43**	**5.37**	**0.44**	**0.99982**
Leucine	**436.52**	**24.60**	**109.19**	**6.59**	**42.76**	**1.54**	**11.62**	**1.56**	**3.90**	**0.22**	**0.99993**
Lysine	**638.99**	**55.62**	**147.67**	**14.78**	**57.07**	**2.99**	**14.15**	**3.49**	**6.90**	**0.64**	**0.99961**
Proline	**134.67**	**13.72**	**44.08**	**0.85**	**19.04**	**2.73**	**2.51**	**1.03**	**1.40**	**0.20**	**0.9925**

TABLE 69: Results Difference Between the Two Methods of 6M HCl and 0.1M HCl to Analysis Amino Acids Concentrations (gram per 100 grams of sample) for *Galdieria sulphuraria*

AMINO ACIDS	6M HCL**	STD	0.1M HCL	STD	STD*
SERINE	0.39	0.01	0.27	0.01	0.08
THREONINE	0.4	0.01	0.32	0.01	0.06
ASPARTIC ACID	0.4	0.01	0.47	0.03	0.05
GLUTAMIC	1.09	0.02	0.46	0.01	0.45
HISTIDINE	0.11	0.00	0.43	0.07	0.22
GLYCINE	0.23	0.01	0.24	0.05	0.01
ARGININE	0.24	0.01	0.38	0.07	0.10
ALANINE	0.29	0.01	0.21	0.04	0.06
TYROSINE	0.47	0.01	0.39	0.01	0.06
VALINE	0.41	0.01	0.35	0.04	0.04
PHENYLALANINE	0.25	0.01	0.37	0.01	0.08
ISOLEUCINE	0.30	0.01	0.24	0.01	0.04
LEUCINE	0.38	0.01	0.27	0.01	0.08
METHIONINE	0.17	0.01	0.27	0.01	0.07
LYSINE	0.33	0.01	0.31	0.03	0.01
CYSTEINE	0.30	0.01	0.53	0.01	0.16

*Standard Deviation Between the Two Methods of Amino Acids Concentrations

** Average of three replicate

TABLE 70: Results Variations for 6M HCl Method in Analyzing Amino Acids Concentrations (gram per 100 grams of sample) for *Galdieria sulphuraria*

AMINO ACIDS	6M HCL	STD
SERINE	0.53	0.03
THREONINE	0.53	0.02
ASPARTIC ACID	0.53	0.04
GLUTAMIC	1.49	0.08
HISTIDINE	0.14	0.01
GLYCINE	0.29	0.02
ARGININE	0.34	0.01

ALANINE	0.38	0.03
TYROSINE	0.62	0.03
VALINE	0.58	0.03
PHENYLALANINE	0.33	0.02
ISOLEUCINE	0.38	0.02
LEUCINE	0.51	0.03
METHIONINE	0.17	0.02
LYSINE	0.52	0.03
CYSTEINE	0.30	0.01

TABLE 71: ASPARTIC ACID PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	1.98	0.40	2.33	0.40	0.36	0.02	0.32	0.05	2.24	0.66	2.80	0.00	1.85	0.51
48	0.49	0.09	1.38	0.32	4.01	0.95	0.13	0.01	3.60	0.62	1.59	0.34	3.21	0.59
72	0.53	0.09	0.91	0.09	3.35	0.84	0.09	0.01	2.92	0.06	0.43	0.03	1.24	0.79
96	0.47	0.03	0.69	0.06	2.57	0.51	0.13	0.01	4.17	0.22	2.01	0.83	2.73	0.55

TABLE 72: ASPARTIC ACID PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*

(h)	*8*		*9*		*10*		*11*		*12*		*13*		*14*		*15*	
24	0.81	0.02	0.71	0.12	0.25	0.02	2.24	0.93	2.85	0.52	1.11	0.57	0.18	0.05	0.77	0.14
48	0.41	0.03	0.38	0.02	0.13	0.01	1.93	0.18	2.05	0.52	1.21	0.09	0.11	0.01	0.32	0.02
72	0.36	0.02	0.25	0.03	0.17	0.02	1.46	0.13	3.27	0.69	2.28	0.66	0.12	0.03	2.07	0.40
96	1.02	0.69	0.81	0.15	0.18	0.03	0.96	0.14	3.36	0.68	0.98	0.01	0.11	0.04	1.19	0.20

TABLE 73: GLUTAMIC ACID PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*
(h)	*1*		*2*		*3*		*4*		*5*		*6*		*7*	
24	1.02	0.01	1.32	0.35	0.28	0.04	0.49	0.06	0.57	0.01	0.55	0.05	0.46	0.03
48	0.58	0.04	0.66	0.10	0.97	0.11	0.22	0.03	0.83	0.08	0.25	0.01	0.73	0.05
72	0.62	0.01	0.71	0.02	0.90	0.10	0.17	0.01	0.64	0.03	0.13	0.01	0.46	0.06
96	0.46	0.01	0.38	0.00	0.61	0.08	0.26	0.02	0.81	0.02	0.29	0.01	0.62	0.09

TABLE 74: GLUTAMIC ACID PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.33	0.01	0.64	0.03	0.49	0.03	0.84	0.09	0.60	0.01	0.47	0.06	0.38	0.02	0.39	0.02
48	0.19	0.01	0.56	0.01	0.27	0.02	0.71	0.05	0.35	0.02	0.33	0.01	0.21	0.01	0.19	0.01
72	0.16	0.01	0.43	0.01	0.38	0.04	0.69	0.02	0.51	0.04	0.56	0.04	0.29	0.01	0.41	0.01
96	0.25	0.02	0.50	0.02	0.43	0.07	0.50	0.08	0.61	0.05	0.46	0.02	0.29	0.01	0.47	0.04

TABLE 75: SERINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.73	0.01	0.97	0.03	0.31	0.01	0.48	0.01	0.42	0.06	0.40	0.02	0.45	0.02
48	0.35	0.02	0.54	0.09	0.81	0.02	0.22	0.02	0.82	0.08	0.25	0.01	0.79	0.06
72	0.41	0.06	0.50	0.01	0.79	0.04	0.17	0.02	0.65	0.01	0.14	0.01	0.51	0.04
96	0.27	0.01	0.32	0.04	0.67	0.09	0.29	0.01	0.93	0.02	0.39	0.03	0.80	0.07

TABLE 76: SERINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.33	0.01	0.94	0.06	0.46	0.01	0.73	0.02	0.44	0.01	0.35	0.02	0.32	0.06	0.30	0.05
48	0.17	0.02	1.36	0.04	0.20	0.01	0.58	0.01	0.25	0.05	0.22	0.01	0.18	0.05	0.16	0.01
72	0.15	0.03	0.65	0.05	0.35	0.01	0.62	0.01	0.36	0.01	0.38	0.03	0.25	0.05	0.26	0.01
96	0.20	0.02	0.84	0.07	0.40	0.09	0.45	0.02	0.42	0.01	0.31	0.02	0.25	0.05	0.34	0.03

TABLE 77: HISTIDINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	1.01	0.02	1.07	0.02	0.39	0.01	0.55	0.04	0.44	0.08	0.43	0.08	0.46	0.01
48	0.78	0.01	0.65	0.02	0.65	0.01	0.22	0.03	0.71	0.03	0.21	0.06	0.62	0.02
72	0.64	0.01	0.75	0.03	0.71	0.01	0.15	0.01	0.64	0.02	0.14	0.07	0.53	0.08
96	0.43	0.07	0.47	0.04	0.68	0.03	0.20	0.02	0.89	0.02	0.31	0.01	0.68	0.05

TABLE 78: HISTIDINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.37	0.01	0.81	0.01	0.53	0.01	1.00	0.06	0.54	0.07	0.40	0.04	0.36	0.04	0.35	0.01
48	0.17	0.01	0.74	0.01	0.23	0.01	0.92	0.01	0.26	0.02	0.21	0.01	0.16	0.01	0.16	0.01
72	0.12	0.01	0.57	0.01	0.31	0.01	0.80	0.02	0.32	0.03	0.31	0.02	0.17	0.01	0.24	0.01
96	0.17	0.02	0.65	0.01	0.34	0.02	0.62	0.06	0.40	0.01	0.28	0.01	0.19	0.01	0.23	0.05

TABLE 79: GLYCINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.67	0.07	0.54	0.03	0.33	0.04	0.55	0.08	0.31	0.01	0.28	0.04	0.35	0.03
48	0.34	0.03	0.48	0.02	0.41	0.09	0.30	0.04	0.47	0.08	0.16	0.07	0.45	0.05
72	0.37	0.05	0.48	0.01	0.42	0.03	0.22	0.03	0.38	0.01	0.11	0.05	0.43	0.05
96	0.24	0.05	0.27	0.08	0.43	0.03	0.30	0.06	0.45	0.01	0.16	0.07	0.56	0.04

TABLE 80: GLYCINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.43	0.01	0.61	0.09	0.65	0.08	0.69	0.05	0.43	0.02	0.43	0.08	0.49	0.07	0.39	0.07
48	0.26	0.06	0.54	0.09	0.36	0.06	0.63	0.06	0.23	0.07	0.28	0.01	0.29	0.09	0.23	0.08
72	0.25	0.04	0.43	0.05	0.44	0.01	0.55	0.06	0.28	0.01	0.39	0.01	0.36	0.02	0.22	0.02
96	0.23	0.03	0.45	0.07	0.50	0.09	0.40	0.05	0.33	0.01	0.35	0.08	0.29	0.07	0.29	0.03

TABLE 81: THREONINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.56	0.05	0.93	0.07	0.34	0.03	0.74	0.05	0.60	0.02	0.62	0.02	0.58	0.05
48	0.38	0.01	0.54	0.07	0.68	0.06	0.34	0.04	0.87	0.09	0.29	0.01	0.86	0.08
72	0.46	0.05	0.41	0.01	0.67	0.05	0.28	0.05	0.72	0.01	0.16	0.09	0.52	0.06
96	0.32	0.01	0.31	0.07	0.53	0.02	0.42	0.04	0.98	0.04	0.32	0.01	0.63	0.08

TABLE 82: THREONINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.28	0.09	0.54	0.01	0.48	0.04	0.80	0.04	0.56	0.04	0.34	0.07	0.27	0.07	0.29	0.07
48	0.11	0.07	0.48	0.09	0.28	0.01	0.76	0.02	0.29	0.04	0.18	0.01	0.14	0.08	0.15	0.08
72	0.11	0.08	0.33	0.01	0.37	0.05	0.64	0.09	0.32	0.01	0.31	0.03	0.28	0.07	0.24	0.01
96	0.15	0.09	0.47	0.08	0.42	0.04	0.50	0.07	0.39	0.01	0.25	0.01	0.28	0.05	0.28	0.02

TABLE 83: ARGININE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.83	0.01	0.92	0.01	0.42	0.05	0.66	0.02	0.41	0.01	0.38	0.06	0.44	0.03
48	0.62	0.03	0.69	0.05	0.64	0.05	0.38	0.02	0.58	0.04	0.18	0.06	0.54	0.01
72	0.65	0.06	0.69	0.03	0.63	0.04	0.25	0.02	0.50	0.01	0.13	0.05	0.52	0.04
96	0.38	0.07	0.40	0.01	0.54	0.01	0.36	0.01	0.59	0.01	0.20	0.06	0.64	0.06

TABLE 84: ARGININE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.44	0.02	0.77	0.05	0.65	0.01	0.78	0.02	0.47	0.08	0.45	0.07	0.46	0.08	0.42	0.07
48	0.20	0.01	0.70	0.01	0.32	0.01	0.79	0.09	0.22	0.04	0.22	0.01	0.25	0.04	0.21	0.02
72	0.20	0.01	0.54	0.01	0.40	0.01	0.64	0.01	0.27	0.06	0.30	0.05	0.31	0.03	0.19	0.01
96	0.18	0.04	0.52	0.02	0.44	0.05	0.49	0.01	0.31	0.01	0.29	0.01	0.30	0.02	0.29	0.01

TABLE 85: ALANINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.83	0.02	0.64	0.02	0.23	0.05	0.28	0.02	0.27	0.01	0.44	0.03	0.29	0.03
48	0.25	0.00	0.48	0.05	0.81	0.07	0.28	0.03	0.50	0.06	0.19	0.01	0.50	0.03
72	0.27	0.00	0.49	0.02	0.61	0.04	0.16	0.08	0.36	0.02	0.09	0.01	0.35	0.07
96	0.21	0.04	0.27	0.01	0.38	0.01	0.29	0.02	0.42	0.03	0.27	0.01	0.69	0.01

TABLE 86: ALANINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.30	0.01	0.49	0.07	0.33	0.01	0.59	0.08	0.45	0.01	0.38	0.04	0.26	0.05	0.30	0.03
48	0.19	0.07	0.43	0.05	0.21	0.01	0.55	0.01	0.28	0.05	0.22	0.01	0.14	0.02	0.16	0.02
72	0.21	0.06	0.36	0.01	0.25	0.05	0.52	0.06	0.38	0.03	0.38	0.02	0.22	0.04	0.27	0.01
96	0.20	0.08	0.42	0.09	0.29	0.03	0.41	0.09	0.43	0.01	0.39	0.02	0.23	0.05	0.36	0.04

TABLE 87: TYROSINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	2.62	0.01	0.76	0.02	0.32	0.01	0.51	0.01	0.37	0.01	0.28	0.01	0.47	0.02

48	0.51	0.01	0.74	0.01	0.54	0.03	0.51	0.03	0.63	0.04	0.20	0.04	0.90	0.05
72	0.55	0.01	0.59	0.01	0.87	0.03	0.28	0.08	0.44	0.01	0.18	0.02	0.41	0.03
96	0.39	0.01	0.39	0.01	0.49	0.01	0.56	0.02	0.65	0.01	0.36	0.05	0.86	0.08

TABLE 88: TYROSINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.39	0.02	1.42	0.10	0.61	0.01	1.36	0.10	0.60	0.01	0.76	0.05	0.46	0.01	0.50	0.03
48	0.18	0.01	1.00	0.09	0.37	0.01	1.06	0.09	0.30	0.02	0.31	0.01	0.23	0.01	0.20	0.02
72	0.25	0.01	0.58	0.01	0.45	0.03	1.09	0.09	0.38	0.02	0.35	0.01	0.28	0.02	0.25	0.01
96	0.22	0.01	0.60	0.01	0.50	0.03	0.68	0.01	0.63	0.02	0.49	0.01	0.34	0.02	0.30	0.02

TABLE 89: CYSTINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*

(h)	*1*		*2*		*3*		*4*		*5*		*6*		*7*	
24	1.08	0.02	1.24	0.02	0.65	0.04	1.03	0.08	0.53	0.01	0.47	0.05	0.65	0.01
48	0.81	0.01	0.90	0.01	0.86	0.04	0.49	0.09	0.85	0.01	0.30	0.08	0.76	0.01
72	0.90	0.02	0.95	0.01	0.84	0.03	0.31	0.01	0.69	0.01	0.25	0.08	0.77	0.04
96	0.53	0.01	0.60	0.04	0.73	0.02	0.49	0.06	0.81	0.02	0.35	0.01	0.87	0.05

TABLE 90: CYSTINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*	*EXP*	*STD*
(h)	*8*		*9*		*10*		*11*		*12*		*13*		*14*		*15*	
24	0.65	0.09	1.11	0.01	1.24	0.01	1.11	0.03	0.63	0.09	0.63	0.05	0.89	0.02	0.64	0.02
48	0.29	0.03	1.05	0.01	0.64	0.01	1.05	0.02	0.33	0.05	0.32	0.02	0.50	0.05	0.35	0.06
72	0.27	0.07	0.81	0.02	0.91	0.04	0.95	0.02	0.35	0.04	0.73	0.06	0.55	0.03	0.28	0.01
96	0.17	0.01	0.85	0.03	1.02	0.07	0.75	0.03	0.44	0.05	0.42	0.01	0.65	0.01	0.52	0.02

TABLE 91: VALINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.84	0.04	1.10	0.06	0.37	0.07	0.43	0.04	0.53	0.09	0.42	0.09	0.39	0.03
48	0.53	0.07	0.74	0.08	0.60	0.05	0.17	0.01	0.79	0.07	0.19	0.01	0.64	0.05
72	0.55	0.05	0.66	0.06	0.78	0.07	0.12	0.01	0.57	0.07	0.13	0.01	0.54	0.04
96	0.35	0.04	0.41	0.03	0.68	0.09	0.17	0.03	0.78	0.08	0.27	0.03	0.60	0.05

TABLE 92: VALINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.37	0.02	0.71	0.07	0.43	0.01	0.91	0.09	0.50	0.03	0.42	0.05	0.20	0.04	0.38	0.02
48	0.23	0.01	0.61	0.07	0.16	0.01	0.83	0.03	0.28	0.01	0.27	0.01	0.11	0.01	0.17	0.01
72	0.17	0.01	0.43	0.03	0.25	0.04	0.66	0.04	0.35	0.02	0.59	0.06	0.08	0.01	0.31	0.03
96	0.20	0.02	0.49	0.02	0.29	0.05	0.50	0.06	0.38	0.03	0.40	0.03	0.10	0.01	0.31	0.02

TABLE 93: METHIONINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.53	0.05	0.61	0.07	0.37	0.02	0.44	0.06	0.27	0.01	0.37	0.05	0.55	0.05
48	0.42	0.02	0.45	0.03	0.78	0.06	0.18	0.01	0.38	0.03	0.19	0.01	0.59	0.06
72	0.45	0.02	0.48	0.03	0.55	0.06	0.12	0.01	0.45	0.06	0.12	0.01	0.47	0.03
96	0.27	0.01	0.35	0.01	0.48	0.05	0.16	0.01	0.55	0.08	0.25	0.01	0.63	0.05

TABLE 94: METHIONINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.24	0.01	0.54	0.07	0.36	0.02	0.51	0.04	0.38	0.03	0.33	0.2	0.44	0.05	0.28	0.01
48	0.12	0.01	0.52	0.04	0.18	0.01	0.48	0.03	0.18	0.01	0.16	0.01	0.18	0.01	0.13	0.01
72	0.10	0.01	0.41	0.01	0.25	0.02	0.44	0.04	0.23	0.02	0.17	0.01	0.25	0.01	0.14	0.01
96	0.13	0.02	0.36	0.02	0.28	0.03	0.36	0.01	0.30	0.02	0.19	0.01	0.33	0.01	0.19	0.02

TABLE 95: PHENYLALANINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.74	0.01	0.75	0.02	0.35	0.03	0.48	0.01	0.34	0.01	0.33	0.04	0.33	0.01
48	0.52	0.02	0.75	0.02	0.51	0.04	0.20	0.01	0.48	0.06	0.18	0.01	0.43	0.01
72	0.55	0.02	0.64	0.01	0.51	0.06	0.13	0.01	0.48	0.01	0.11	0.01	0.48	0.01
96	0.37	0.01	0.42	0.01	0.49	0.04	0.17	0.01	0.60	0.01	0.20	.02	0.60	0.02

TABLE 96: PHENYLALANINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.34	0.02	0.69	0.01	0.47	0.04	0.72	0.03	0.41	0.07	0.34	0.03	0.31	0.02	0.30	0.02
48	0.16	0.01	0.63	0.03	0.21	0.01	0.67	0.06	0.20	0.02	0.20	0.01	0.15	0.02	0.14	0.01
72	0.12	0.01	0.51	0.03	0.28	0.02	0.61	0.04	0.28	0.01	0.33	0.03	0.15	0.01	0.20	0.02
96	0.16	0.03	0.47	0.02	0.32	0.03	0.46	0.01	0.33	0.03	0.27	0.02	0.18	0.02	0.22	0.03

TABLE 97: ISOLEUCINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.51	0.04	0.56	0.04	0.25	0.02	0.34	0.05	0.21	0.01	0.25	0.05	0.16	0.01
48	0.37	0.01	0.39	0.01	0.42	0.03	0.13	0.01	0.38	0.03	0.13	0.01	0.29	0.01
72	0.40	0.01	0.43	0.01	0.43	0.06	0.08	0.01	0.36	0.01	0.07	0.01	0.32	0.05
96	0.24	0.01	0.30	0.04	0.46	0.02	0.11	0.01	0.46	0.01	0.14	0.01	0.38	0.03

TABLE 98: ISOLEUCINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.23	0.01	0.37	0.01	0.31	0.01	0.38	0.03	0.29	0.04	0.19	0.01	0.21	0.01	0.16	0.01
48	0.10	0.01	0.46	0.01	0.14	0.01	0.47	0.05	0.14	0.02	0.13	0.01	0.11	0.01	0.09	0.00
72	0.07	0.01	0.36	0.01	0.18	0.01	0.43	0.01	0.17	0.01	0.22	0.01	0.09	0.00	0.13	0.01
96	0.10	0.02	0.34	0.01	0.20	0.01	0.33	0.02	0.21	0.01	0.18	0.01	0.11	0.01	0.14	0.01

TABLE 99: LEUCINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.67	0.03	0.73	0.03	0.34	0.04	0.53	0.02	0.45	0.01	0.43	0.03	0.46	0.04
48	0.43	0.01	0.52	0.04	0.84	0.03	0.23	0.03	0.63	0.02	0.27	0.06	0.69	0.01
72	0.46	0.03	0.52	0.01	0.87	0.04	0.19	0.01	0.55	0.04	0.16	0.01	0.54	0.01
96	0.27	0.01	0.34	0.05	0.62	0.03	0.22	0.01	0.73	0.02	0.36	0.03	0.79	0.03

TABLE 100: LEUCINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.39	0.01	0.59	0.03	0.54	0.04	0.68	0.07	0.44	0.02	0.41	0.02	0.41	0.01	0.40	0.06
48	0.16	0.01	0.50	0.04	0.29	0.01	0.60	0.08	0.20	0.01	0.23	0.01	0.24	0.01	0.18	0.01
72	0.17	0.02	0.39	0.02	0.38	0.03	0.54	0.05	0.31	0.02	0.45	0.03	0.27	0.01	0.31	0.01
96	0.19	0.02	0.43	0.01	0.43	0.03	0.40	0.03	0.40	0.03	0.33	0.02	0.27	0.02	0.36	0.06

TABLE 101: LYSINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.73	0.02	0.98	0.04	0.39	0.01	0.84	0.05	0.40	0.03	0.41	0.04	0.48	0.02
48	0.47	0.01	0.68	0.02	0.71	0.03	0.35	0.02	0.68	0.06	0.23	0.01	0.68	0.02
72	0.48	0.03	0.57	0.04	0.82	0.03	0.30	0.01	0.57	0.04	0.16	0.01	0.59	0.00
96	0.31	0.03	0.37	0.04	0.71	0.06	0.38	0.01	0.73	0.01	0.28	0.01	0.71	0.03

TABLE 102: LYSINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.46	0.00	0.66	0.03	0.78	0.06	0.77	0.03	0.49	0.03	0.43	0.05	0.55	0.03	0.42	0.05
48	0.16	0.02	0.60	0.07	0.35	0.01	0.72	0.01	0.27	0.02	0.26	0.01	0.28	0.03	0.19	0.00
72	0.18	0.02	0.51	0.01	0.51	0.04	0.61	0.03	0.39	0.03	0.44	0.02	0.37	0.02	0.27	0.01
96	0.23	0.03	0.57	0.01	0.57	0.04	0.47	0.03	0.42	0.05	0.36	0.02	0.37	0.02	0.38	0.02

TABLE 103: PROLINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 1*	*STD*	*EXP 2*	*STD*	*EXP 3*	*STD*	*EXP 4*	*STD*	*EXP 5*	*STD*	*EXP 6*	*STD*	*EXP 7*	*STD*
24	0.35	0.00	0.48	0.05	0.16	0.01	0.21	0.00	0.22	0.01	0.26	0.00	0.17	0.05
48	0.23	0.00	0.32	0.03	0.37	0.04	0.08	0.00	0.35	0.01	0.14	0.07	0.40	0.00
72	0.25	0.00	0.29	0.00	0.42	0.03	0.06	0.00	0.24	0.01	0.06	0.04	0.28	0.00
96	0.20	0.01	0.19	0.00	0.32	0.03	0.07	0.00	0.38	0.01	0.16	0.08	0.38	0.00

TABLE 104: PROLINE PRODUCTION GRAM IN 100 GRAMS OF SAMPLE

Time (h)	*EXP 8*	*STD*	*EXP 9*	*STD*	*EXP 10*	*STD*	*EXP 11*	*STD*	*EXP 12*	*STD*	*EXP 13*	*STD*	*EXP 14*	*STD*	*EXP 15*	*STD*
24	0.20	0.01	0.34	0.02	0.21	0.01	0.39	0.02	0.25	0.02	0.20	0.01	0.15	0.00	0.17	0.00
48	0.08	0.00	0.32	0.03	0.08	0.00	0.64	0.07	0.14	0.01	0.08	0.00	0.06	0.00	0.06	0.00
72	0.06	0.00	0.23	0.02	0.11	0.00	0.32	0.03	0.14	0.01	0.21	0.02	0.05	0.00	0.23	0.01
96	0.09	0.00	0.32	0.02	0.13	0.01	0.26	0.02	0.19	0.01	0.19	0.01	0.07	0.00	0.14	0.00

TABLE 105: DRY WEIGHT OF GALDIERIA SULPHURARIA FEEDING ON DIFFERENT SUGARS

Time (h)	*Dry Weight Average Xylose*	*STD*	*Dry Weight Average Arabinose*	*STD*	*Dry Weight Average Glucose*	*STD*	*Dry Weight Average Mannitol*	*STD*
0	**0.01**	**0.00**	**0.01**	**0.00**	**0.02**	**0.00**	**0.01**	**0.00**
24	**0.01**	**0.00**	**0.49**	**0.00**	**0.77**	**0.00**	**0.23**	**0.00**
48	**0.06**	**0.00**	**0.46**	**0.00**	**0.66**	**0.00**	**0.43**	**0.00**
72	**0.62**	**0.00**	**0.42**	**0.00**	**0.59**	**0.00**	**0.60**	**0.00**

TABLE 106: AMINO ACIDS PRODUCTION IN 100 GRAMS of SAMPLE FOR DIFFERENT SUGARS AFTER 24 HOURS

Amino Acids	*Average 24 h*	*STD*	*Average 24 h*	*STD*	*Average 24 h*	*STD*	*Average 24 h*	*STD*

	(Mannitol)		*(Xylose)*		*(Arabinose)*		*(Glucose)*	
Aspartic Acid	**4.56749E-07**	**0.00**	**0.00**	**0.00**	**0.11**	**0.05**	**0.36**	**0.22**
Glutamic Acid	**5.89641E-07**	**0.00**	**0.00**	**0.00**	**0.32**	**0.02**	**0.28**	**0.04**
Serine	**7.8244E-07**	**0.00**	**0.00**	**0.00**	**0.58**	**0.03**	**0.31**	**0.01**
Histidine	**5.43855E-07**	**0.00**	**0.00**	**0.00**	**0.25**	**0.01**	**0.39**	**0.01**
Glycine	**1.33492E-06**	**0.00**	**0.00**	**0.00**	**2.10**	**0.10**	**0.33**	**0.04**
Threonine	**7.13064E-07**	**0.00**	**0.00**	**0.00**	**0.38**	**0.03**	**0.34**	**0.03**
Arginine	**7.30E-07**	**0.00**	**0.00**	**0.00**	**0.47**	**0.02**	**0.42**	**0.05**
Alanine	**1.14E-06**	**0.00**	**0.00**	**0.00**	**0.99**	**0.03**	**0.23**	**0.05**
Tyrosine	**1.05E-06**	**0.00**	**0.00**	**0.00**	**0.50**	**0.02**	**0.32**	**0.01**

Cystine	**9.36E-07**	**0.00**	**0.00**	**0.00**	**0.78**	**0.05**	**0.65**	**0.04**
Valine	**7.24E-07**	**0.00**	**0.00**	**0.00**	**0.61**	**0.03**	**0.37**	**0.07**
Methionine	**4.83E-07**	**0.00**	**0.00**	**0.00**	**0.18**	**0.00**	**0.37**	**0.02**
Phenylalanine	**6.05E-07**	**0.00**	**0.00**	**0.00**	**0.36**	**0.02**	**0.35**	**0.03**
Isoleucine	**4.83E-07**	**0.00**	**0.00**	**0.00**	**0.18**	**0.00**	**0.25**	**0.02**
Leucine	**7.54E-07**	**0.00**	**0.00**	**0.00**	**0.60**	**0.06**	**0.34**	**0.04**
Lysine	**8.30E-07**	**0.00**	**0.00**	**0.00**	**0.59**	**0.05**	**0.39**	**0.01**
Proline	**4.83E-07**	**0.00**	**0.00**	**0.00**	**0.27**	**0.01**	**0.16**	**0.01**

TABLE 107: AMINO ACIDS PRODUCTION IN 100 GRAMS of SAMPLE FOR DIFFERENT SUGARS AFTER 48 HOURS

Amino Acids	***Average 48 h (Mannitol)***	***STD***	***Average 48 h (Xylose)***	***STD***	***Average 48 h (Arabinose)***	***STD***	***Average 48 h (Glucose)***	***STD***
Aspartic Acid	**0.21**	**0.01**	**1.44**	**0.02**	**0.14**	**0.01**	**4.01**	**0.95**
Glutamic Acid	**0.31**	**0.01**	**1.85**	**0.07**	**0.33**	**0.02**	**0.97**	**0.69**

Serine	**0.51**	**0.02**	**2.70**	**0.02**	**0.60**	**0.02**	**0.81**	**0.02**
Histidine	**0.26**	**0.01**	**1.68**	**0.02**	**0.27**	**0.01**	**0.65**	**0.01**
Glycine	**1.55**	**0.05**	**7.92**	**0.26**	**2.10**	**0.07**	**0.41**	**0.09**
Threonine	**0.42**	**0.01**	**2.21**	**0.10**	**0.39**	**0.01**	**0.68**	**0.06**
Arginine	**0.41**	**0.01**	**2.53**	**0.20**	**0.48**	**0.02**	**0.64**	**0.05**
Alanine	**0.55**	**0.02**	**3.40**	**0.30**	**0.96**	**0.03**	**0.81**	**0.07**
Tyrosine	**0.49**	**0.01**	**3.48**	**0.16**	**0.54**	**0.01**	**0.54**	**0.03**
Cystine	**0.62**	**0.03**	**3.31**	**0.07**	**0.76**	**0.05**	**0.86**	**0.04**
Valine	**0.23**	**0.01**	**2.67**	**0.20**	**0.59**	**0.02**	**0.60**	**0.05**
Methionine	**0.47**	**0.01**	**1.43**	**0.00**	**0.19**	**0.00**	**0.78**	**0.06**
Phenylalanine	**0.29**	**0.01**	**1.74**	**0.08**	**0.39**	**0.01**	**0.51**	**0.04**
Isoleucine	**0.20**	**0.00**	**1.43**	**0.00**	**0.19**	**0.00**	**0.42**	**0.03**
Leucine	**0.50**	**0.02**	**2.83**	**0.10**	**0.66**	**0.05**	**0.84**	**0.03**
Lysine	**0.55**	**0.02**	**3.52**	**0.30**	**0.78**	**0.05**	**0.71**	**0.03**
Proline	**0.25**	**0.01**	**1.84**	**0.10**	**0.30**	**0.01**	**0.37**	**0.04**

TABLE 108: AMINO ACIDS PRODUCTION IN 100 GRAMS of SAMPLE FOR DIFFERENT SUGARS AFTER 72 HOURS

Amino Acids	*Average 72 h (Mannitol)*	*STD*	*Average 72 h (Xylose)*	*STD*	*Average 72 h (Arabinose)*	*STD*	*Average 72 h (Glucose)*	*STD*
Aspartic Acid	**0.15**	**0.01**	**0.16**	**0.01**	**0.13**	**0.01**	**3.35**	**0.84**
Glutamic Acid	**0.25**	**0.01**	**0.24**	**0.02**	**0.36**	**0.01**	**0.90**	**0.10**
Serine	**0.43**	**0.04**	**0.42**	**0.02**	**0.68**	**0.02**	**0.79**	**0.04**
Histidine	**0.19**	**0.01**	**0.20**	**0.01**	**0.30**	**0.01**	**0.71**	**0.01**
Glycine	**1.41**	**0.09**	**1.49**	**0.04**	**2.23**	**0.06**	**0.42**	**0.03**
Threonine	**0.34**	**0.03**	**0.29**	**0.02**	**0.43**	**0.02**	**0.67**	**0.05**
Arginine	**0.34**	**0.02**	**0.37**	**0.02**	**0.51**	**0.01**	**0.63**	**0.04**
Alanine	**0.42**	**0.02**	**0.45**	**0.02**	**1.02**	**0.03**	**0.61**	**0.04**
Tyrosine	**0.32**	**0.03**	**0.35**	**0.02**	**0.58**	**0.05**	**0.87**	**0.03**
Cystine	**0.56**	**0.04**	**0.58**	**0.03**	**0.82**	**0.02**	**0.84**	**0.03**
Valine	**0.18**	**0.01**	**0.45**	**0.02**	**0.64**	**0.01**	**0.78**	**0.07**

Methionine	**0.43**	**0.03**	**0.14**	**0.00**	**0.21**	**0.00**	**0.55**	**0.06**
Phenylalanine	**0.25**	**0.03**	**0.29**	**0.01**	**0.41**	**0.05**	**0.51**	**0.06**
Isoleucine	**0.14**	**0.00**	**0.14**	**0.00**	**0.21**	**0.00**	**0.43**	**0.06**
Leucine	**0.45**	**0.04**	**0.47**	**0.03**	**0.68**	**0.02**	**0.87**	**0.04**
Lysine	**0.51**	**0.07**	**0.57**	**0.04**	**0.76**	**0.08**	**0.82**	**0.03**
Proline	**0.21**	**0.01**	**0.21**	**0.01**	**0.32**	**0.01**	**0.42**	**0.03**

TABLE 109: Cell Lysis Techniques Comparison Between HCl and Polyton Homogonizer

AMINO ACIDS	***LYSIS WITH HCL SOLUTION ONLY***	***LYSIS WITH WATER AND POLYTON HOMOGONIZER***
ASPARTIC ACID	**58.42**	**30.53**
GLUTAMIC ACID	**51.43**	**7.76**
SERINE	**51.69**	**7.73**
HISTIDINE	**31.36**	**2.98**

GLYCINE	**55.12**	**12.29**
THREONINE	**83.96**	**2.63**
ARGININE	**36.39**	**2.77**
ALANINE	**64.65**	**13.08**
TYROSINE	**27.10**	**24.90**
CYSTINE	**32.27**	**2.23**
VALINE	**43.60**	**8.31**
METHIONINE	**29.76**	**0.00**
PHENYLALANINE	**32.31**	**2.13**
ISOLEUCINE	**39.94**	**0.85**
LEUCINE	**62.21**	**3.63**
LYSINE	**70.70**	**3.23**
PROLINE	**43.34**	**1.16**